Neuroscience Area

PhD course in Functional and Structural Genomics

Foxg1 promotes neuron allocation to engrams and facilitates fear memorization

Thesis submitted for the degree of “Doctor Philosophiae”

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Be the change you wish to see in the world — Mahatma Gandhi

Notes

This work is available on bioRxiv: Foxg1 promotes neuron allocation to engrams and facilitates fear memorization.

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Abstract

In addition to orchestrate telencephalic development, the transcription factor Foxg1 is involved in mutual positive feedback with neuronal activity. Based on that, we hypothesized an involvement of it in learning and engram dynamics. We observed that its sparse and mild neuronal upregulation improved learning abilities as evaluated by a CFC assay. This effect was specifically pronounced upon early memory retrieval and disappeared when Foxg1-GOF neurons were silenced by a Gi DREADD effector. A prevailing positive relationship was detectable between Foxg1 expression level and the probability of a neuron getting recruited into training and recall engrams, in structures involved in both short- and long-term memory formation. Moreover, Foxg1 upregulation elicited a generalized shrinkage of engrams and increased the fraction of late recall engram cells already active at the time of training. Together, these findings establish Foxg1 as a key effector linking neuronal excitability to engram allocation and memory recall.

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1. Introduction

1.1. Building the Forebrain: Principles of Patterning, Histogenesis, and Plasticity

Human behavior largely emerges from the coordinated activity of neuronal circuits within the forebrain, particularly, neocortex, the most structurally and functionally complex part of the mammalian brain. The neocortex is involved in high-order information processing and cognitive functions such as sensory perception, language, consciousness, emotion, and memory. Expansion of the neocortex is regarded as a key evolutionary modification underlying the emergence of mammalian intellectual abilities[1]. Its remarkable diversity of neuronal cell types, layered organization, and long-range connectivity emerge through a tightly regulated sequence of developmental events that transform a simple neuroepithelial sheet into a highly organized and functionally specialized structure. A defining feature of the neocortex is its six-layered cytoarchitecture, a structure that arose relatively late in the evolution of vertebrates and is specific to mammals[2,3] (**Figure. 1**). The establishment of this laminar organization takes place during neurogenesis, which in placental mammals is mostly prenatal. Neocortical laminar formation is a highly orchestrated process governed by genetic programs during early embryonic development. Understanding how this complexity arises during development has been a central goal of developmental neurobiology for decades, as perturbation at any stage of forebrain development can lead to a wide spectrum of neurological dysfunctions, ranging from cognitive impairment to epilepsy and neuropsychiatric disorders.

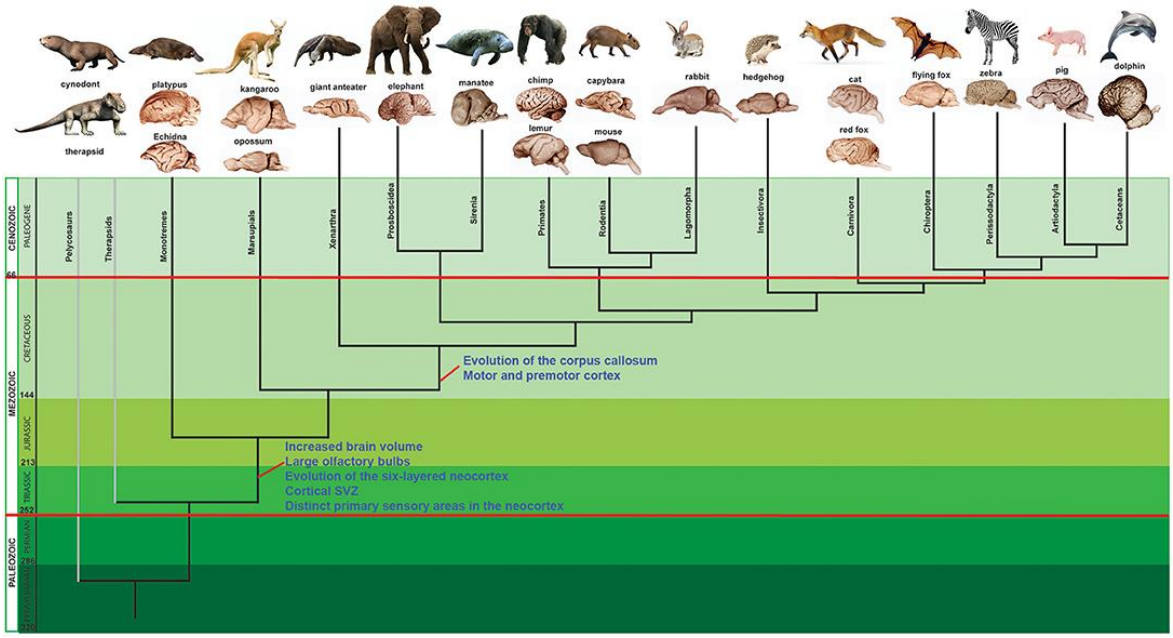


Figure. 1: Phylogenetic tree of mammalian evolution, highlighting emergence of cortex at each point[3].

At the end of gastrulation, the embryonic dorsal leaflet, named neuroectoderm, enters neurulation phase, a morphogenetic program that gives rise to the central nervous system (CNS). The CNS is organized along the rostral axis into three primary encephalic structures known as Forebrain (Prosencephalon), Midbrain (Mesencephalon), and Hindbrain (Rhombencephalon), and caudally by the spinal cord. While the mesencephalon stays undivided, the prosencephalon splits into telencephalon (anterior most part of forebrain) and diencephalon, whereas the rhombencephalon gives rise to the metencephalon and myelencephalon. Development of the telencephalon proceeds through three main sequential events: 1) rostro-caudal and dorso-ventral specification; 2) control of cellular proliferation and differentiation rate; 3) patterning and cerebral layering. Through these processes the telencephalon generates the olfactory bulbs, cerebral cortex, and hippocampal formation, as well as a number of subcortical structures, including striatum, pallidum, and amygdala, which together form the anatomical substrate for higher-order cognitive, emotional, and mnemonic functions[4–6] (**Figure. 2**).

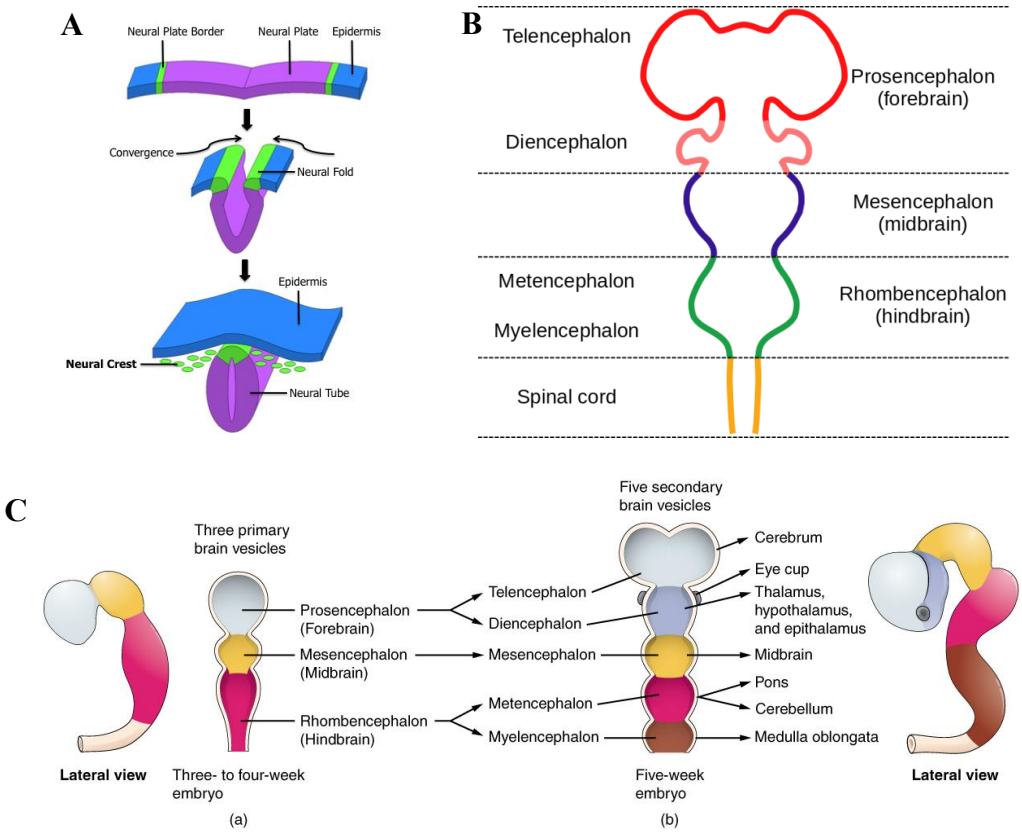


Figure. 2: A. Formation of neural tube. **B**. Three primary encephalic structures. **C**. Primary and secondary vesicle stage development, which give rise to several cortical, subcortical structures, midbrain, and hindbrain.

Early studies of cortical development gave rise to two influential and seemingly opposing models of cortical arealization, a process by which cortical areas acquire their distinct identities. The “protomap” model, proposes that cortical progenitor cells are pre-patterned prior to the arrival of thalamic afferences, giving rise to neurons with region-specific features. While the “protocortex” theory, suggests that the cortex is initially homogeneous and develops area-specific features only in response to extrinsic signals. Contemporary evidence supports a more integrative view in which early intrinsic patterning establishes a protomap that is later refined and elaborated by extrinsic signals and activity-dependent mechanisms. This synthesis highlights the importance of early developmental events in constraining later circuit formation and functional specialization[7–9].

Central to forebrain patterning is the action of numerous extrinsic and intrinsic factors that orchestrate this process in a tightly spatiotemporal manner. Key extrinsic factors, also called morphogens, include members of the fibroblast growth factor (FGF) family, most notably Fgf8, as well as bone morphogenetic proteins (BMPs), Wingless (Wg) and Int-1 (WNT) proteins, and Sonic hedgehog (SHH). These morphogens are secreted from discrete signaling centers and form concentration gradients that convey positional information to neuroepithelial progenitors. Specifically, SHH is secreted ventrally by the prechordal plate underlying the telencephalon, Fgf8 is produced rostrally by the anterior neural ridge (ANR), BMPs and WNTs are secreted caudomedially from the dorsal telencephalic roof plate, and SFRP1 (secreted frizzled-related proteins 1) and TGF-β (transforming growth factor β) from the ventral pallium[10–12]. Neuroepithelial cells interpret these gradients through intracellular signaling cascades and transcriptional responses, resulting in the spatially restricted expression of key transcription factors (TFs) that define distinct telencephalic domains and guide subsequent cortical development[13].

For example: members of FGF family (Fgf8, 15, 17, 18) promote rostral telencephalic identity by binding their specific membrane receptors on telencephalic progenitors and activating the MAPK and PI3K-Akt signaling cascades[14–16]. SHH is essential for the development of ventral

telencephalic structures[17], whereas BMPs (BMP 2, 4, 5, 6, 7) functionally cooperate with WNTs to induce dorsomedial patterning and promote neuronal differentiation in the telencephalon[18]. SFRP1 has been shown to regulate dorso-ventral telencephalic development, by modulating WNT and Notch signaling, and further contributes to patterning of anterior hippocampus[19,20]. TGF-β superfamily also plays important roles in the subpallium development, acting through functional interactions with Dlx and Smad proteins[21]. The morphogen gradients subsequently induce spatially restricted expression of intrinsic TFs, which drive region-specific neurodevelopmental programs and cell-fate decision within the telencephalon. At least seven TFs critical for cortical arealization are differentially expressed including Emx1, Emx2, Pax6, Lhx2, Foxg1, COUP-TF1 and Sp8[12,19]. Their declining, tangential expression gradients are oriented in distinct directions: from caudomedial to rostro-lateral (Lhx2, Emx2, Emx1); rostro-lateral to caudomedial (Pax6, Foxg1); caudolateral to rostro-medial (COUP-TF1); and rostro-medial to caudolateral (Sp8)[19] (**Figure. 3**).

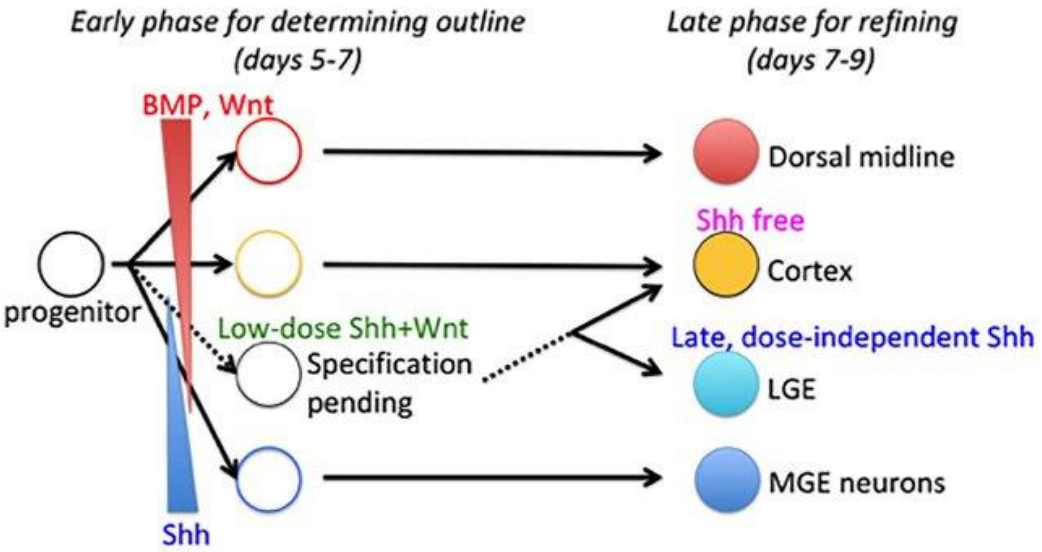


Figure. 3: Two-phase specification model for telencephalon patterning. In this model, the fate of dorsal midline telencephalic cells is primarily determined by BMP/WNT signaling, whereas ventral-most cells of the telencephalon, such as those in the medial ganglionic eminence (MGE), are specified by high levels of SHH signaling during the early phase (illustrated by solid lines). The fate of intermediate subregions is conditional, influenced by both dorsal and ventral signals (illustrated by dashed lines). During the late phase, the lateral ganglionic eminence (LGE) is specified by a dose-independent SHH signal, mediated through a temporal shift in progenitor competence to respond to SHH[22].

Notably, prior to patterning the cortical domain, some of these factors (e.g., Emx2, Pax6, Foxg1) contribute to subdivision of the telencephalon into dorsal domain (pallium) and the ventral domain (subpallium)[23,24]. The pallium gives rise to excitatory projection neurons of the cerebral cortex and hippocampus, while the subpallium generates inhibitory GABAergic interneurons and basal ganglia structures. Within the pallium, further regional specialization leads to the formation of the neocortex (isocortex), paleocortex, and archicortex, each characterized by distinct cytoarchitecture. The archicortex consists of hippocampal formation, located ventromedially relative to the neocortex and is essential in learning and memory. The paleocortex includes olfactory bulbs, part of amygdala, piriform cortex, and secondary olfactory cortex, positioned ventrolaterally relative to the neocortex. The neocortex has a central location within the pallial domain and represents the largest region of the telencephalon. The subpallium consists of three ganglionic eminences: lateral (giving rise to caudatus and putamen), medial (giving rise to globus palidus), and caudal (ancestor of part of amygdala). Within these eminences GABAergic interneurons are generated and subsequently migrate into the developing cortex[22,25,26].

1.2. Cortical Histogenesis

Following regional patterning, forebrain undergoes histogenesis, a developmental process by which neurons and glial cells are generated, migrate to their appropriate locations, and assemble into layered and interconnected structures. The establishment of cytoarchitecture occurs through three consecutive and partially overlapping waves of cell generation, with neurons produced first, followed by astrocytes, and, subsequently, oligodendrocytes[27]. Importantly, not all the cortical cell types originate within the cortex itself. Cortical progenitors primarily give rise to excitatory glutamatergic projection neurons of layers II-VI, astrocytes, and postnatal oligodendrocytes[28,29]. Conversely, several key cell populations, including glutamatergic Cajal-Retzius cells (CRs) of layer I, inhibitory GABAergic interneurons, and prenatal oligodendrocytes are generated outside the pallium or extracortical regions. These cells reach the developing cortex via tangential migration across the telencephalon, where they integrate into emerging cortical circuits[30–32].

In mouse, cortical histogenesis begins around embryonic day 9.5-10 (E9.5-10), when neural progenitors are generated within the ventricular zone (VZ), a highly proliferative layer of the dorso-lateral telencephalic neuroepithelium. During this early phase, apical progenitors of VZ

start to proliferate by repeated symmetric cell divisions, leading to increase in the VZ thickness. At E.10.5, neuroepithelial cells progressively transform into radial glial cells (RGCs), making the pool of principal neural stem cells of developing cortex. RGCs express glial markers (Glast, Blbp, Rc2) and are highly polarized cells, with their cell bodies residing in the VZ and radial marginal protrusions extending from the ventricular surface to the pial surface, providing both structural support and migratory scaffolds for newly generated neurons[33,34]. RGCs undergo two different cell divisions i.e., symmetric or asymmetric. They divide primarily through asymmetric cell divisions, resulting in either post-mitotic neurons directly (direct neurogenesis) or basal progenitors, also known as intermediate progenitor cells (IPCs), which delaminate from the ventricular surface and populate the subventricular zone (SVZ)[35]. IPCs then further undergo a limited number of symmetric divisions before differentiating into neurons, thus amplifying the neuronal output. Interestingly, the abundance and proliferative capacity of IPCs vary markedly across mammalian species. In the mouse cortex, most IPCs undergo symmetric divisions to generate two neurons, contributing modestly to the cortical expansion[36].

From E11.5 to E17.5, corresponding to the neuronogenic window, RGCs and IPCs continue to proliferate and sequentially generate projection neurons of distinct cortical layers in a tightly controlled spatio-temporal order. Early born neurons initially exit the proliferative zone or VZ, and settle superficial to it, resulting in preplate (PP) formation. This structure is then split into two distinct compartments, a superficial marginal zone (MZ), which later becomes layer I of the postnatal cortex and a deeper subplate (SP) located beneath layer VI. MZ is largely populated by CR neurons, which serve as a primary source of Reelin, a large extracellular glycoprotein essential for proper radial migration of newborn neurons and cortical lamination. The SP is a transient developmental structure fated to disappear either through programmed cell death or becoming incorporated into the deepest portion of layer VI. In contrast, later-born neurons, after leaving the germinal zones, migrate radially along the scaffold of radial glial fibers, traverse the intermediate zone (IZ), and populate the expanding cortical plate (CP), which forms between the MZ and SP. Once in the CP, these neurons are arranged according to the characteristic inside-out pattern of cortical lamination, whereby early-born neurons occupy deeper neocortical layers (initially layer VI, followed by layer V), while later-born neurons migrate past them to populate progressively more superficial layers (layer IV and subsequently layers II/III)[12,37–39] (**Figure. 4**).

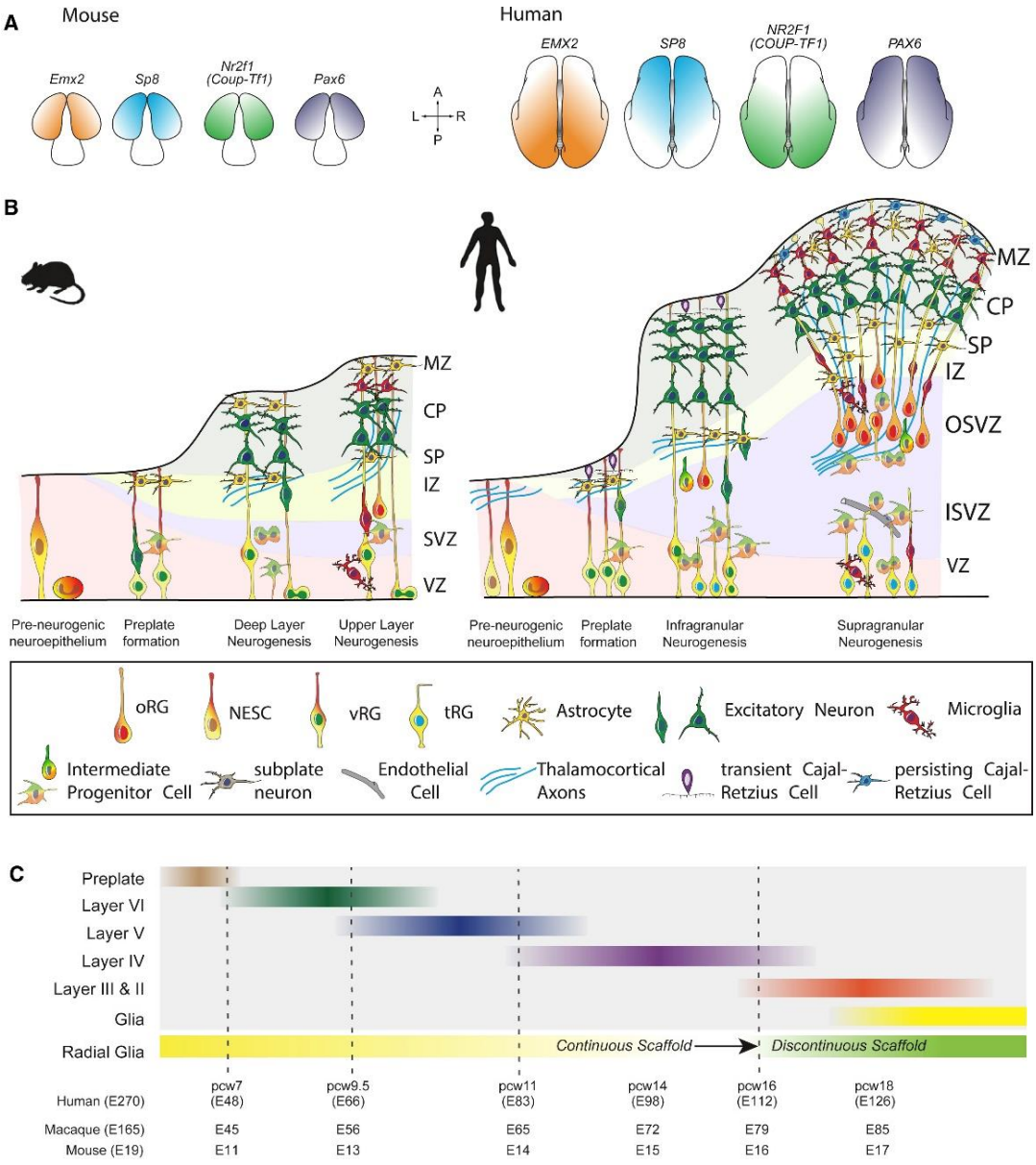


Figure. 4: **Species-Specific Dynamics of Cerebral Cortical Neurogenesis**. **A**. Spatial establishment of key patterning gradients of morphogens and TFs across telencephalic neuroepithelium. **B**. Schematic comparison of the mouse and human cerebral cortex at peak neurogenic stages highlights that, although core developmental processes are evolutionary conserved, several species-specific differences can be observed. Including pronounced expansion of the outer subventricular zone (OSVZ) and of the outer radial glia (oRG) population that populates this compartment; substantial enlargement of the SP, associated with the expansion of the axonal plexus, rather than increased cell numbers; increase in the proportion of upper-layer excitatory neurons; the discontinuation of the radial glial scaffold; the persistence

of CRs, and the enlargement of axonal plexuses in the OSVZ, leading to the formation of multi-laminar axonal-cellular compartments; and early arrival of thalamocortical plexus in the cortical anlage. **C**. A broader cross-species comparison of neurogenesis timing. Embryonic days (E) shown next to species names indicate approximate gestational duration[12].

Neurogenesis in the cortex is largely completed before birth, at which point radial glial progenitors undergo a developmental switch from neurogenesis to gliogenesis, giving rise to astrocytes, oligodendrocytes, and ependymal cells[39]. Notably, a small proportion of neuronal production persists into adulthood in specific brain regions, including the generation of interneurons in the olfactory bulbs and granule cells in the dentate gyrus of the hippocampus. This process, known as adult neurogenesis, contributes to structural and functional plasticity in the mature brain[40] (**Figure. 5**).

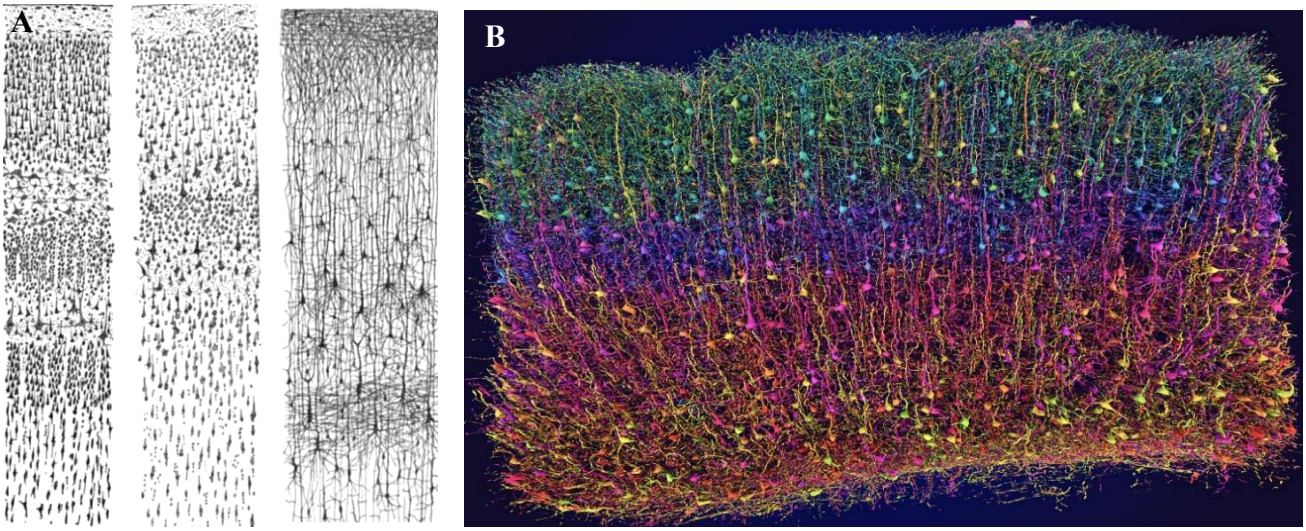


Figure. 5: **A**. First drawings of the neocortical layers in human, by Ramón y Cajal, 1911. Left. Nissl staining of the adult visual cortex. Middle: Nissl staining of the adult motor cortex. Right: Golgi staining of the infant neocortex. **B**. 3D reconstructions of cortical circuitry from mouse visual cortex, Virtual Observatory of the Cortex (VORTEX) project, MICrONS.

Structural development of the mammalian CNS requires precise spatiotemporal regulation of neural progenitor proliferation, differentiation, motility, and cell fate specification. The crosstalk between extrinsic signals and intrinsic transcriptional programs is essential to initiate region-specific histogenetic programs. These programs, in turn, guide the genome toward producing mRNAs and proteins required for dedicated cellular functions. Among the key intrinsic

regulators, TFs play a central role by directly interacting with the genome and translating external cues into defined developmental outputs. One of the largest TFs families is represented by the evolutionary conserved Forkhead Box (FOX) genes, which often exhibit pioneering role in transcriptional regulation.

FOX genes are broadly distributed across eukaryotes, from unicellular organisms to mammals, although they appear to be absent in plants. The name FOX originates from the Drosophila melanogaster gene fork head (fkh), in which mutations result in defective head fold involution during embryogenesis, leading to a characteristic “spike-head” phenotype in the adult fly[41]. Members of the FOX family participate in a wide array of physiological and developmental processes, including organogenesis, cell cycle regulation, metabolic control, stem cell maintenance, and signal transduction[42,43]. While many Fox genes are most prominently active during embryonic development, a substantial subset continues to be expressed postnatally, indicating that these transcription factors also contribute to physiological functions in adulthood.

1.3. The FOX Gene Family: Molecular Features and Functional Roles

In the human genome, 50 forkhead genes have been identified and classified into 19 subfamilies (FOXA-FOXS) based on sequence similarity[44]. Among these, FOXG1, formerly known as Brain Factor 1 (BF-1) is predominantly expressed in the cerebrum, where it exerts non-redundant functions in cortical development[45,46]. It is a winged-helix transcription factor gene located on chromosome 14q12 in humans and 12qB3 in mouse, and, like other FOX proteins, contains ~110 amino acid Forkhead DNA-binding domain (FHD)[41,47]. Foxg1 contains a single open reading frame which surrounds the FHD, and the amino acid sequence from FHD to the C-terminal domain is highly conserved (96%) across the species, thus rendering it the name of evolutionary conserved transcription factor. On contrary the N-terminal domain of Foxg1 is quite variable. Though the first 32 amino acids and successive histidine (H) repeats are well-conserved, small insertions (six amino acids) and successive proline (P)-glutamine (Q) repeats (HPQ rich domain) are observed in the mammals particularly in primates (**Figure. 6**). These evolutionary changes in the Foxg1 sequence are important not only for its canonical roles, but also for acquisition of novel regulatory interactions with other proteins that are responsible for transmitting diverse downstream events.

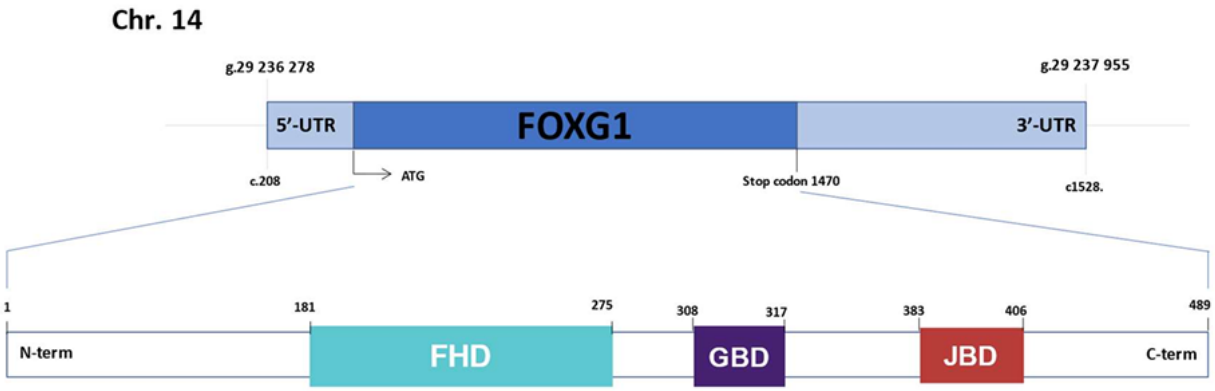


Figure. 6: **Structure of FOXG1 gene**. FOXG1 gene and the three main domains[48].

FOXG1 expression is detected in multiple neural tissues and cell types in mammals, including the cerebral cortex, subpallial telencephalon, inner ear, retina, and olfactory epithelium, where it primarily functions as a transcriptional repressor[49]. FOXG1 is among the earliest transcription factors specifically expressed within the telencephalic primordium and is essential for the initiation and expansion of telencephalic structurs[50]. Consistent with this role, Foxg1 knockout mice exhibit severe telencephalic hypoplasia[45], while in humans, haploinsufficiency of FOXG1 results in postnatal microcephaly, global developmental delay, and marked structural abnormalities, including cerebral atrophy, gyral simplification, hypomyelination, and corpus callosum defects[51]. Studies have also reported that aberrant FOXG1 expressions result in tumorigenesis, indicating its effect on cellular proliferation and survival pathways[52,53].

Foxg1 is highly conserved both at the mRNA and protein levels in mammals[54,55]. In mouse, Foxg1 produces two transcript variants: a major mono-exonic isoform (3,985 nt) and a secondary di-exonic isoform (3,861 nt), while in humans only a single FOXG1 transcript (3,491 nt) has been reported. Despite these structural differences in exon, all transcripts encode a single open reading frame, producing a 481 amino acid protein in mouse and a 489 amino acid protein in humans. Recent structural work by Dai and colleagues provided high-resolution characterization of the human FOXG1-FHD and its interaction with the target DNA (DBD). The FHD adopts a conserved winged-helix fold, consisting of four α-helices (three major helices H1, H2, H3, and a shorter 3_{10}-helix H4) packed against an antiparallel four-stranded β-sheet (S1-S4), flanked by two wing-like loops (wing 1 and wing 2). FOXG1 binds DNA as a monomer, recognizing a canonical 5′-RYAAAYA-3′ Forkhead consensus sequence (R = purine, Y =

pyrimidine); in FOXG1, this corresponds to 5′-GTAAACA-3, found in promoter regions of its target genes. Wing 2 is the most variable and least conserved region of the FHD across FOX family members, yet it can adopt multiple conformations, and this structural plasticity contributes substantially to DNA-binding specificity among FOX transcription factors[56,57] (**Figure. 7**).

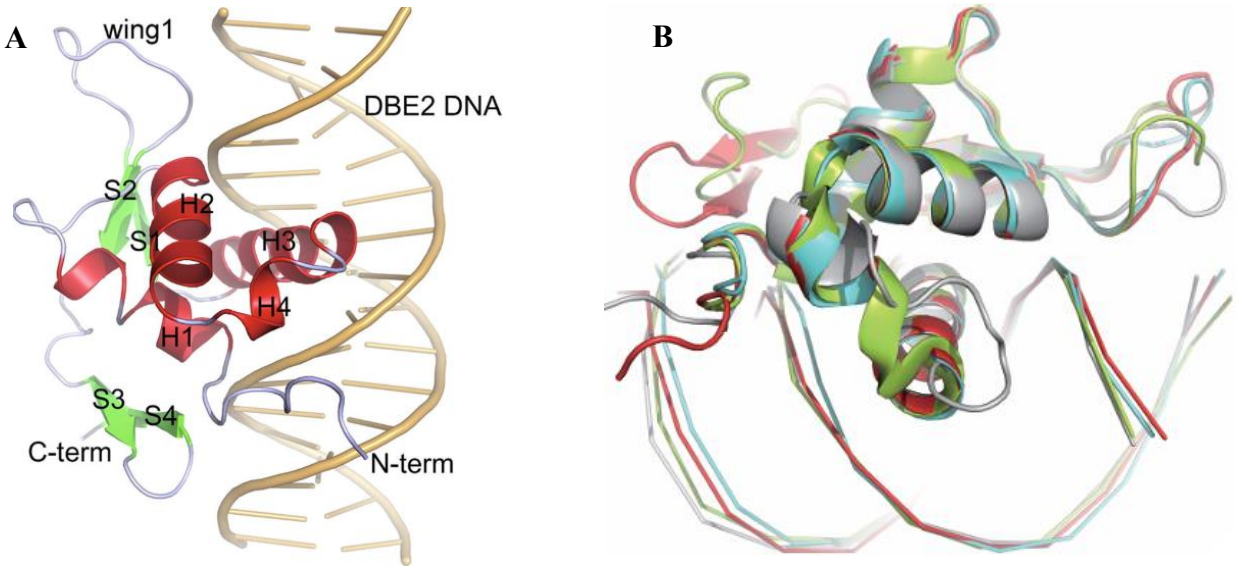


Figure. 7: **Structure of the FOXG1/DBE2 complex and detailed protein-DNA** interactions. **A**. Cartoon representation of FOXG1 in complex with DBE2 DNA element. Secondary structural elements are indicated, with helixes colored red, β-strands colored green, and loops colored light blue. DNA recognition by the FOXG1-DBD H3 helix. **B**. Structural superposition of the FOXG1/DBE2 structure (red) with the previously determined FOXO1/DBE2 (PDB: 3CO7, gray), FOXA2/DBE2 (PDB: 5XO7, cyan) and FOXC2/DBE2 (PDB: 6AKO, gray) structures[56].

1.4. Decoding FOXG1 Function in the Developing and Mature Cortex

In vertebrates, FOXG1 exerts non-redundant and pleiotropic functions during brain development. It is essential for telencephalon formation, and specification, and patterning of both rostro-caudal and dorsal (pallial)-ventral (subpallial). The first functional studies on FOXG1 date back to the early 1990s, when Xuan et al. generated Foxg1 knock-out mice (then referred to as BF-1). They demonstrated that BF1-null homozygous mutants exhibit severe microcephaly, with a marked reduction of the ventral telencephalic neuroepithelium and reduced pre-neurogenic proliferation of dorsal progenitors[45]. Moreover, FOXG1 also regulates

neural progenitor proliferation, neuronal differentiation and migration, and cerebral cortex patterning and layering. Collectively, these functions position FOXG1 as a central regulator of telencephalic development, providing a mechanistic explanation for why even modest perturbations in its expression levels can profoundly affect neurodevelopmental processes and higher cognitive functions, resulting in neurological and neurodevelopmental disorders [50,51,58] (**Figure. 8**).

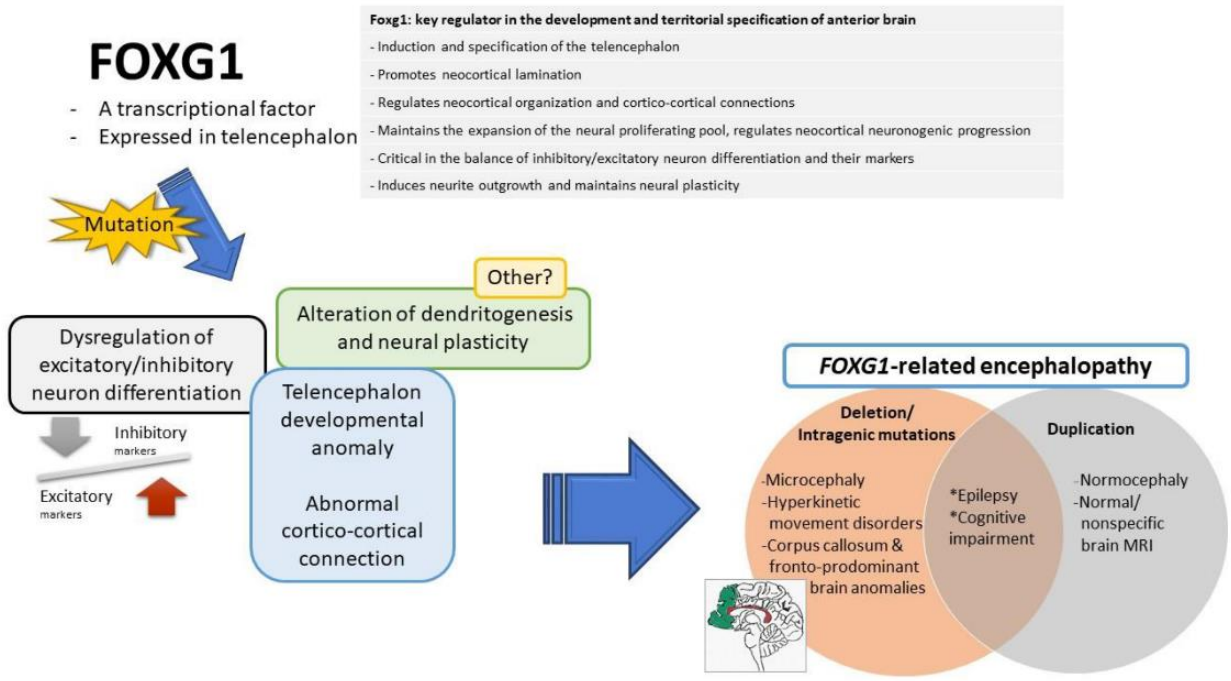


Figure. 8: FOXG1 Pleiotropic Functions and Disease Outcomes[58].

1.5. Telencephalic Development, Specification, and Patterning

The earliest cues, responsible for regulating antero-posterior patterning also regulate Foxg1 induction within the anterior neural plate. In the mouse, Foxg1 expression starts at embryonic day E8.0-8.5 in the rostral neural plate, adjacent to the anterior neural ridge (ANR). At this stage, Foxg1 activation is primarily driven by Fgf8, whose secretion from the ANR is permitted because of Wnt signals buffering by sFRPs produced by anterior neural border (ANB). SHH secreted from underlying prechordal plate also induce high Foxg1 levels in the medial-anterior domain, the prospective ventral telencephalon. This coordinated action of Fgf8 and SHH establishes an antero-ventral-high to postero-dorsal-low gradient of Foxg1 expression[50,59]. Foxg1 and SHH subsequently cooperate in patterning the nascent telencephalon via

modulation of Fgf8. Foxg1 directly stimulates Fgf8 transcription[60], whereas SHH promotes Fgf8 indirectly by preventing Gli3 repressor (Gli3R) formation[61]. Together, these interactions help maintain a signaling environment permissive for ventral telencephalic identity (**Figure. 9**).

In addition to its ventralizing functions, the dorso-ventral gradient of Foxg1 expression restricts excessive dorsalization of the telencephalon mediated by the roof plate signaling center. Specifically, Foxg1 limits the expression of BMP and Wnt ligands to the roof plate and its immediate surroundings[62,63], thereby preventing corresponding pathways driving exaggerated derealization. This restriction confines the archicortical program and the generation of caudo-medial CR cells to the dorsal-most pallial anlage, while allowing space for the activation of the more lateral neocortical program[63].

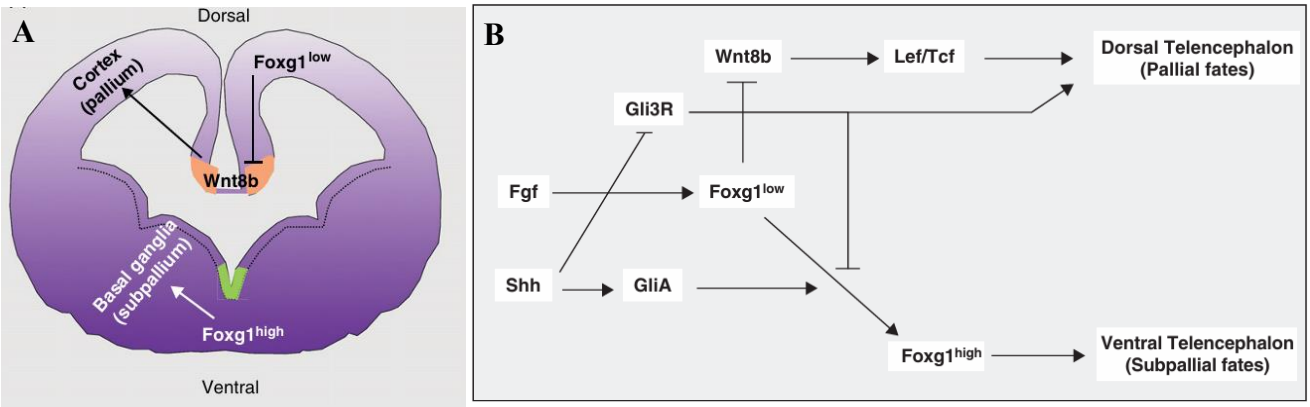


Figure. 9: **Schematic of Foxg1-dependent telencephalic patterning**. **A**. Transverse section of the mouse embryonic telencephalon illustrating Foxg1 expression gradients and their impact on dorsoventral fate specification. **B**. FOXG1 functions as a central integrator of multiple signaling pathways during telencephalic development[50].

Finally, Foxg1-null mice fail to express characteristic markers of the neocortical field. More specifically, the subpallium is not developed, while the dorsal telencephalon, although reduced in size, adopts molecular features of the archicortex and cortical hem, transforming ventral identities into more dorsal fates (**Figure. 10**). These morphological and molecular defects are accompanied by perturbations in key signaling centers: dorsalizing signals, such as BMP4, expand ventrally, whereas ventralizing ligands, including SHH, are lost[45,62,63].

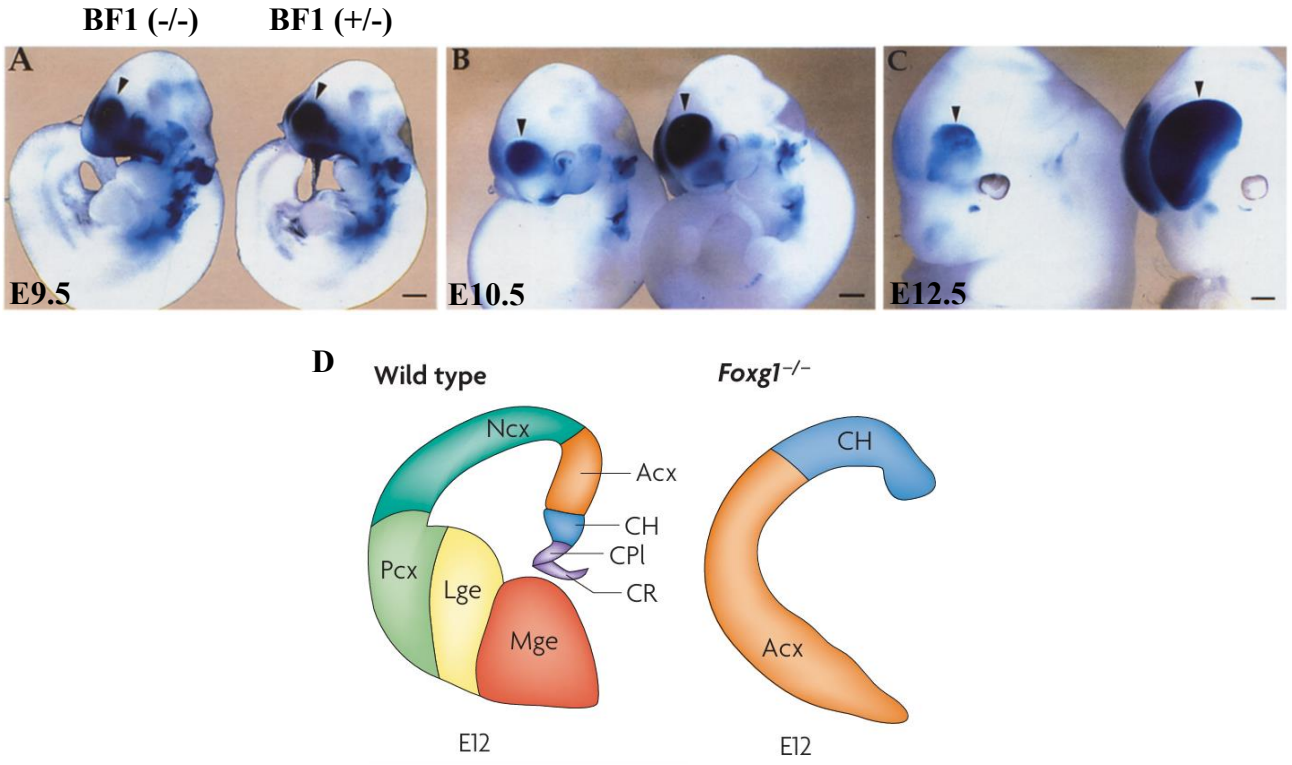


Figure. 10: **Telencephalic defects in Foxg1 loss-of-function (LOF) mice**. **A-C**. During E9.5 to E12.5, the telencephalon is markedly reduced in size in BF1 (-/-) compared to BF1 (+/-)[45]. **D**. At E12, the subpallium fails to form, and the majority of the telencephalon acquires archicortical identity, indicating a shift from ventral to dorsal fates[38].

Together, these findings underscore the central role of Foxg1 in establishing ventral and dorsal telencephalic compartments: high levels, downstream of SHH/Fgf8 signaling, promote ventral telencephalic fate, whereas lower levels restrain the dorsal signaling center, thereby controlling the size of the future cerebral cortex.

1.6. Foxg1 as a Key Regulator of Neural Progenitor Dynamics and Neuronal Differentiation

Following telencephalic specification, cerebral expansion requires a boost in the production of neural cells. Neural stem cells (NSCs) initially undergo a period of rapid proliferation followed by a gradual deceleration of the cell cycle, due to a fourfold lengthening of the G1 phase of the cycle[64]. This deceleration of the cell cycle facilitates the exit of NSCs from proliferation and prompts their differentiation into post-mitotic neurons. Cell division shifts progressively from symmetric self-renewal to asymmetric neurogenic differentiation, ensuring a tightly regulated

generation of diverse neuronal populations[65,66]. This second phase, termed neurogenesis, produces the majority of neurons that will populate the postnatal telencephalon and therefore must be tightly regulated to ensure correct neuronal output in time and space. In this highly monitored context, Foxg1 plays a pivotal role in balancing proliferation and differentiation by regulating cell cycle. Research reports that, partial or full loss of Foxg1 expression, results in premature cell cycle exit, reflected in an increased proportion of NSCs in G1/G0 phase[45,67,68]. Consistently, manipulation of FOXG1 levels in human iPSC-derived NSCs demonstrated that partial depletion increases the fraction of cells in G1 and favors differentiation toward GABAergic interneurons, while elevated FOXG1 expression enhances NSC proliferation and inhibits their differentiation into post-mitotic neurons[69–71].

This pro-proliferative and differentiation effect of Foxg1 on cells is mediated mainly through either DNA-binding-dependent or DNA-binding-independent mechanisms. Fog1 mainly enhances cell proliferation by suppressing anti-proliferative TGF-β signaling pathway via DNA-binding-independent mechanisms i.e., Foxg1 lacking DNA-binding activity ($Foxg1^{NHAA}$; mutations from N165 and H169 to alanines) can still increase NSC proliferation but fails to repress differentiation[67,72]. Foxg1 blocks TGF-β signaling by interfering with FOXO/SMAD complex, either by competing for DNA binding sites or via direct physical interaction. These antagonistic mechanisms result in the reduction of transcription of the cell cycle inhibitor $p21^{Cip1}$ (also known as Cdk1a), thereby promoting cell cycle progression[73,74]. Foxg1 also interacts with Foxh2 (FAST-2) and blocks the formation of FAST-Smad complex, thus repressing TGF-β mediated transcription and sustaining proliferation. Additionally, Foxg1 phosphorylation-induced nuclear localization or recruitment of Sfn21/ISWI to the Foxg1 locus during mid-neurogenesis, mechanisms, further suppresses p21 expression and cell cycle regulation[75]. On the contrary, repression of pro-differentiative BMP and Wnt genes requires Foxg1 DNA-binding activity, demonstrating a direct involvement of Foxg1 through the FHD domain[18,76].

Most patients carrying mutations in FOXG1 exhibit congenital microcephaly during infancy. A study conducted on $Foxg1^{+/-}$ mice demonstrated a reduction in Eomesodermin (Eomes/Tbr2-expressing intermediate progenitors), a key driver of neurogenesis in the SVZ[77] and Ki67-positive proliferating cells, accompanied by upregulated Cdkn1a expression and lengthening of the cell cycle[78]. On contrary, Foxg1 overexpression represses both Cdkn1a and cyclin B1 expression and overall proportion of cells in G2 phase of the cell cycle[79]. As Cdkn1a is a

transcriptional target of TGFβ pathway, Hou and colleagues reported that Foxg1 haploinsufficiency might interfere with antagonistic effect of Foxg1 on TGFβ signaling, reducing the pool of Eomes/Tbr2-positive intermediate progenitors[80]. Finally, as anticipated above, the co-repressor interaction between Foxg1 and Gro/TLEs proteins is a key component of its anti-neurogenic activity, keeping NPCs in proliferative state[81–83] (**Figure. 11**).

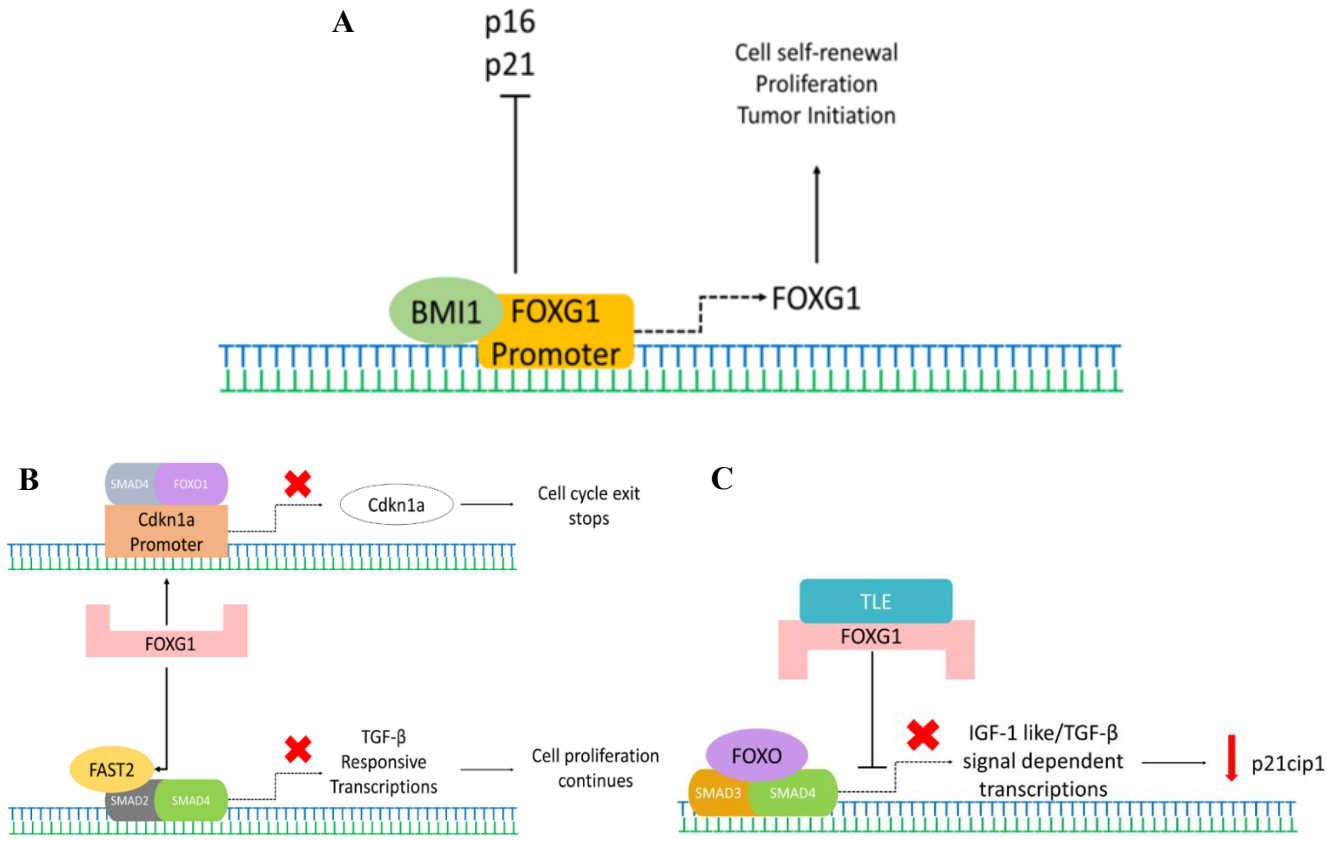


Figure. 11: **Potential pathways through which FOXG1 controls distinct cellular mechanisms**. **A**. BMI1 (B-cell specific moloneymurine leukemiavirus insertion site 1) interacts with FOXG1 promoter initiating FOXG1 transcription, which results in cell self-renewal, proliferation, and tumor growth, while repressing cell-cycle inhibitors p21 and p16. **B**. FOXG1 acts at Cdkn1a promoter by associating with SMAD4 and FOXO1 and represses Cdkn1a transcription thus preventing cell-cycle exit and differentiation. FOXG1 inhibits formation of the FAST2-SMAD2-SMAD4 complex by interacting with FAST2, blocking TGF-β responsive transcription and supporting proliferation. Red crosses indicate loss of transcriptional activity. **C**. FOXG1-TLE complex inhibits apoptosis while promoting cell growth and proliferation by

repressing FOXO-SMAD3-SMAD4 mediated p21-Cip1 transcription downstream of IGF-1-like/TGF-β signaling[58].

These findings indicate that Foxg1 employs both DNA-binding-dependent and independent mechanisms to coordinate proliferation and differentiation during cortical development; the pro-proliferative function of Foxg1 is based on DNA-binding-independent mechanisms, via protein-protein interactions and the neuronal anti-differentiative function requires the DNA-binding activity of Foxg1.

1.7. Foxg1-Mediated Control of Neuron Generation and Cortical Layer Formation

Following telencephalic specification, Foxg1 not only promotes expansion of cerebral vesicles by controlling NSCs proliferation but also orchestrates the spatial-temporal progression of corticogenesis, including laminar identity acquisition and radial neuronal migration. These events occur in a highly stereotyped sequence during neocortical development. First step in this process is the switch in the production of CR cells to deep-layer (DL) and upper layer (UL) neurons. Rodent studies demonstrate that Foxg1 along with Lhx2 (LIM homeobox 2) is critical and sufficient for the production of DL projection neurons from early born CR cells[84,85]. Mechanistically, Foxg1 interacts with layer-specifying transcriptional networks i.e., it promotes Fezf2 and Ctip2 transcription which in turn drive deep layer specification and it does so by repressing TBR1[86]. In addition, rostro-caudal gradients of Foxg1 expression modulate TFs expressed in early progenitors such as Ebf2/3, Dmrt and Eya2 to regulate the CR→DL switch in a spatially ordered manner[85]. Competence then shifts from DL to UL projection neuron production, and Foxg1 drives this transition primarily through derepression of DL neuronal identity. For instance, Hou and co-workers demonstrated that a crosstalk among Egr2, Foxg1 and COUP-TFI leads to layer 4 (L4) neuron specification. Specifically, Egr2, activated in early postmitotic neurons, suppresses Foxg1 in the lower IZ and this in turn derepresses COUP-TFI to specify L4 short-range projection neurons, highlighting that Foxg1 levels act as a temporal cue for laminar subtype specification[80] (**Figure. 12**).

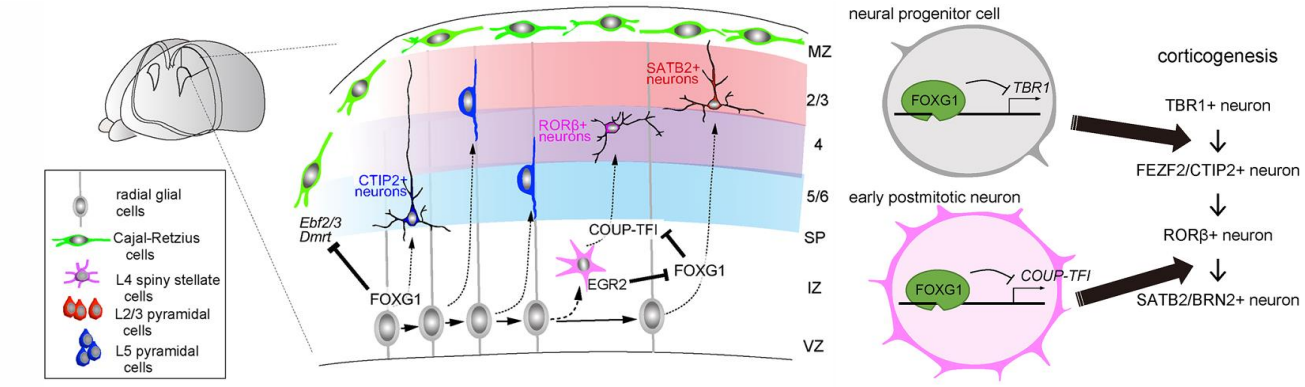


Figure. 12: **FOXG1 orchestrates laminar fate decisions during corticogenesis**. **Left**. FOXG1 regulates the sequential production, migration, and layer integration of cortical neurons. **Right**. In NPCs, FOXG1 onset suppresses the generation of L1/6 TBR1-expressing neurons and promotes the production of L5 FEZF2/CTIP2 expressing neurons. In early postmitotic neurons, FOXG1 inhibits COUP-TFI, enabling the switch from L4 RORβ-expressing neurons to L2/3 SATB2/BRN2-expressing neurons[80].

Foxg1 is also critical for the radial migration and axonal outgrowth of the neurons. Once cell cycle ends, pyramidal neurons undergo a characteristic multipolar phase in the IZ, during which axons elongate, and neurons transiently detach from radial glia. Foxg1 expression is dynamically regulated during this period: brief downregulation of Foxg1 levels initiates NeuroD1 and Unc5D expression (a receptor for transmembrane proteins, involved in tangential migration) facilitating the early-to-late multipolar processes. Failure in the downregulation of Foxg1 expression at this stage leads to permanent arrest of multipolar neurons in the IZ and superficial defects in lamination[87]. Subsequent re-expression of Foxg1 is essential for neurons to exit their multipolar state and enter the CP. And after migration, Foxg1 remains expressed in post-migratory glutamatergic neurons, where it contributes to callosal formation and correct laminar architecture[88]. A similar dynamic regulation of Foxg1 is observed in telencephalic GABAergic interneurons, where a transient downregulation of Foxg1 occurs during tangential migration and re-expression happens upon CP integration[87]. Several studies report that Foxg1 dosage affect migration, differentiation and maturation of cortical interneurons[87,89,90]. Studies done on organoids derived from autism spectrum disorder (ASD) patients demonstrate that elevated FOXG1 levels bias progenitors toward GABAergic fate with altered DLX1/2 and GAD1

expression[91]. A recent study using ASD mouse models also underlines the role of Foxg1 in establishing juvenile cortical GABAergic circuits[92] (**Figure. 13**).

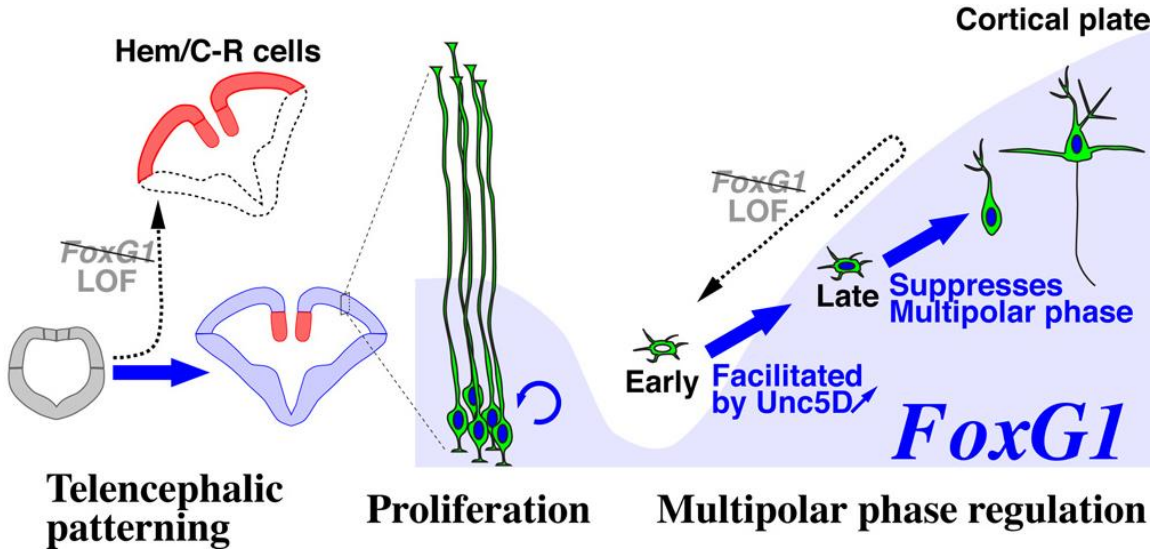


Figure. 13: **Iterative roles of FoxG1 in neuronal migration and laminar specification**. **Left**. During telencephalon development, FoxG1 suppresses cortical hem and CR cell fate to establish proper pallial patterning. **Middle**. FoxG1 promotes expansion of neocortical progenitors. **Right**. Temporary downregulation of FoxG1 in early postmitotic neurons activates Unc5D expression, initiating the transition from early to late multipolar phases and proper entry into the cortical plate[87].

Together, these findings highlight that well-regulated Foxg1 expression is a critical determinant of cortical neuron output, subtype specification, migration dynamics, and axonal circuit assembly, linking its developmental function to later cognitive and behavioral outcomes.

1.8. Foxg1-Dependent Steps of Neuronal Maturation

Neuronal maturation is a process of molecular, structural, and functional changes in post-mitotic neurons through which these cells acquire their definitive identity and become integrated into neural circuits. Neuronal maturation is comprised of a sequence of coordinated steps: migration to their appropriate cortical or subcortical locations, neuritogenesis, neuronal polarization, synaptogenesis, and finally activity-dependent maturation[93–97]. Through these tightly regulated steps, newly generated neurons develop appropriate morphology, electrophysiology, and circuit-level properties.

1.8.1. Neuritogenesis

Neuritogenesis refers to morphogenetic changes that newly differentiated neurons undergo, as a result of which these cells extend immature processes called neurites, that will later differentiate into a single axon and multiple dendrites. This process involves cytoskeletal remodeling, membrane trafficking, and extracellular signaling, and constitutes the structural basis for subsequent neuronal polarization, dendritic arborization, axon pathfinding, and synaptic integration[96,98]. Among several critical moderators, Foxg1 also plays a critical role in neuritogenesis. Multiple studies have highlighted its role in shaping dendritic architecture of projecting neurons. Brancaccio and colleagues first demonstrated *in vitro* that high Foxg1 levels in intermitotic progenitors can dramatically result in neurite outgrowth[69]. Conversely, Foxg1 ablation leads to a significant reduction in dendritic complexity and spin density, reported in hippocampal slices and primary neural cultures[99]. A recent study employing Foxg1 gain- and loss-of-function strategies *in vitro* and *in vivo* identified Hes1, Syt, Ndr1, and pCreb1 as key molecular effectors mediating Foxg1 control of neuronal morphology. Specifically, Foxg1 transactivates the Hes Family bHLH Transcription Factor 1 (Hes1) gene, which in turn upregulates the well-established pro-dendritogenic effector pCreb1. Moreover, Foxg1 downregulates the BAF-subunit-encoding gene Syt and non-race-specific disease resistance 1 (Ndr1), two known antagonists of dendrite elongation. Finally, PKA and AKT signaling are also implicated in Foxg1-dependent pCreb1 overexpression, which is associated with decreased PP1/PP2A phosphatase activity[100].

Regarding Foxg1 impact on axonal length, studies report contrasting results. For instance, Foxg1 did not affect both axonal growth and elongation in neocortical cultures[100]. However, in hippocampal neuronal cultures, Foxg1 deletion significantly reduced axonal length[99]. Furthermore, in callosal neocortical projection neurons, Foxg1 forms a complex with Rp58/Zbtb18 to regulate axon projection[88]. Interestingly, Foxg1 influence on neuronal architecture is not restricted to excitatory projection neuron, it also regulates dendritic outgrowth in other classes of neurons too, such as inhibitory interneurons. In the postnatal cortex, Foxg1 represses dendritic growth in interneurons through the Dlx1-Pak3 signaling pathway[89]. Altogether, these findings indicate that FOXG1 expression critically shapes the neuritic tree of developing neurons. Thus, abnormal FOXG1 expression levels, as observed in FOXG1 syndrome, are likely to contribute to neuropathological outcomes.

1.8.2. Activity-Dependent Neuronal Maturation and Foxg1

The active role of Foxg1 in shaping the neuritic tree (described above) suggests a broader contribution of it in sustaining neuronal electrical activity and plasticity. A study from our group demonstrated that post-mitotic neocortical cultures overexpressing Foxg1 display hyperactivity and altered expression of ion channels and neurotransmitter receptors such as voltage-gated Na^+ and Ca^{2+} channels and glutamate/GABA receptor subunits. Interestingly, Foxg1 transcription itself responds to changes in neuronal electrical activity, and a set of immediate-early genes (IEGs) linking neuronal activity to Foxg1 mRNA levels has been identified, suggesting a bidirectional relationship between Foxg1 expression and neuronal excitability[101]. Furthermore, evidence from *in vivo* models supports the role of Foxg1 in synaptic transmission and plasticity. Conditional deletion of Foxg1 by tamoxifen-inducible Cre recombinase in Calcium/Calmodulin-dependent Protein Kinase II alpha (Camk2α) adult neurons, lead to deficits in learning, memory, and social behavior. These phenotypic changes were accompanied with significant alterations of evoked post-synaptic currents (eEPSCs) in CA1 pyramidal neurons, reduction of NMDA receptor activity, and the impairment of long-term potentiation (LTP). These functional changes correlate with reduced dendritic arborization, spine density, and axonal maintenance, indicating that Foxg1 supports both the structural and physiological bases of synaptic plasticity[99].

Foxg1 expression in dorsal (low) and ventral (high) telencephalic progenitors is crucial for the establishment and maintenance of excitatory/inhibitory (E/I) balance within the cortical circuits. Well-regulated Foxg1 levels are essential for balancing the distinctive neurotransmitter subtypes which are required for optimized cortical circuit transmission, as minute alterations in E/I connectivity result in neuropathological conditions ranging from epileptic seizure to ASD[91,102,103]. For instance, dysregulation of Foxg1 in both excitatory and inhibitory circuits has been reported to result in permanent neurocircuital changes as indicated by altered cortical EEG patterns and ASD-related social behavioral phenotype[104]. Multiple studies demonstrated crucial role of Foxg1 in GABAergic interneuron development and inhibitory circuitry. Interneuron precursors derived from MGE cultures develop shorter neurites and fewer branches upon Foxg1 depletion while *in vivo* ablation of Foxg1 expression impairs interneuron migration and increases seizure susceptibility[89,90].

Human iPSC-derived cortical organoids from ASD patients show FOXG1 overexpression-driven expansion of GABAergic interneurons, consistent with a proliferative effect of Foxg1 on ventral telencephalic progenitors[91]. On contrary, controlled reduction of endogenous FOXG1 by CRISPR/Cas9 and SMASh6 (small molecule-assisted shut-off) technology to ~30%, suppresses MGE induction and interneuron development, indicating that precise FOXG1 dosage is required for interneuron specification[71]. Interestingly, neurons derived from iPSCs originating from patients with FOXG1 mutations, displayed increased levels of inhibitory synaptic markers and decreased levels of excitatory ones. Among these altered markers is Grid1 (orphan glutamate receptor δ 1 subunit), encoding for a synaptic cell adhesion protein which shifts the balance excitatory/inhibitory synapses towards the inhibitory one[105].

Altogether these findings highlight that FOXG1 functions beyond early neurogenesis to regulate dendritic development, neuronal excitability, synaptic plasticity, and cortical E/I homeostasis. Alterations in its expression perturb these interconnected processes, that can explain the spectrum of cognitive, epileptic, and behavioral phenotypes associated with FOXG1-related disorders (**Figure. 14**).

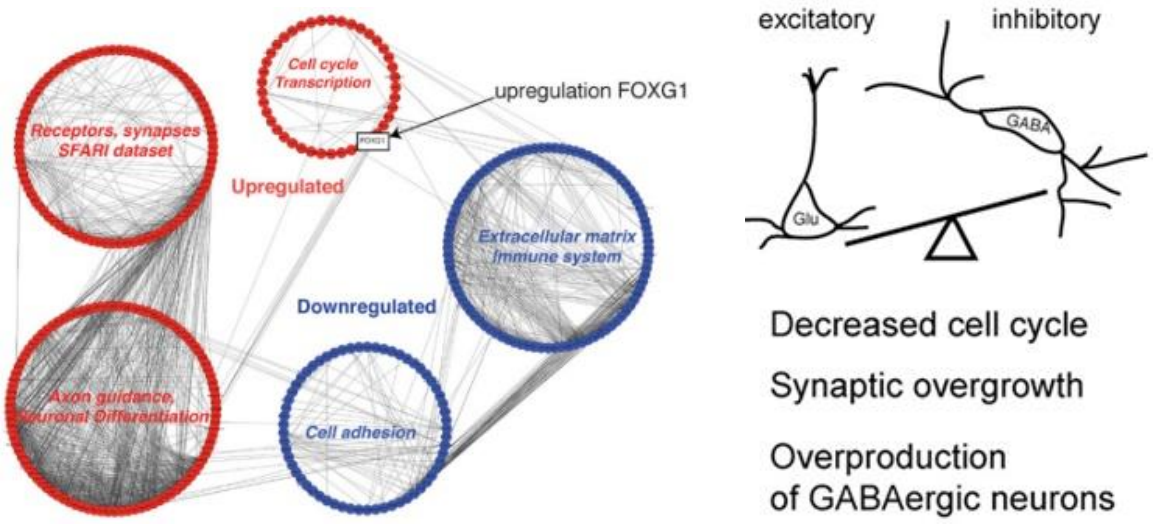


Figure. 14: **FOXG1-driven GABAergic fate shift in ASD cortical organoids**. RNA interference identifies FOXG1 overexpression as the driver of reduced cell cycling, overproduction of GABAergic interneurons, and synaptic overgrowth in cortical organoids generated from iPSCs of individuals with idiopathic ASD[92].

1.9. FOXG1 Controls the Neurogenic-to-Gliogenic Switch in the Developing Cortex

While in the midst of neuronogenesis, neural stem and progenitor cells generate the principal glial cell lineages: astrocytes and oligodendrocytes, a process called gliogenesis[106]. In the mammalian forebrain, gliogenesis is driven by both a myriad of intrinsic and extrinsic factors such as Notch, BMP, and JAK/STAT, that progressively shift the progenitor's competence from neurogenesis to glial fates[107,108]. Astrocytes are the first cells generated followed by oligodendrocytes, the former plays crucial roles in synaptic plasticity, metabolic support for neurons, and is a component of blood-brain barrier, while the latter is responsible for myelin production[109,110]. Perturbation in gliogenesis can affect proper circuit maturation and have been implicated in diverse neurodevelopmental disorders, highlighting the importance of identifying the mechanisms that initiate and regulate this process. Foxg1, classically recognized for sustaining NSC proliferation and neurogenesis, has also emerged as a key regulator of this neurogenic-gliogenic switch.

1.9.1. Foxg1 Mediated Control of NSC-to-Astrocyte Lineage Progression

Two pivotal studies demonstrated that elevated Foxg1 levels maintain NSCs in a neuronogenic state thus preventing their progression to astrocyte fate. Brancaccio et al. and Falcone et al. showed both *in vitro* and *in vivo,* Foxg1 promotes NSC self-renewal while suppressing gliogenesis, thereby maintaining NSCs in neurogenic state[69,111]. On contrary, the natural progressive decline in Foxg1 expression in these progenitors initiate the switch from neurogenic to gliogenic phase, indicating instrumental role of Foxg1 for the temporal articulation of pallial lineage decisions. Falcone et al. provided mechanistic details of Foxg1's anti-astrogenic activity and identified three major processes:

1. Repression of astroglial fate determinant: Foxg1 suppresses the transcription factors necessary for astrocytic lineage commitment such as COUP-TF1, Sox9, Zbtb20, and Nfia, which are essential drivers of the NSC-to-astrocyte progenitor transition[112–115].
2. Direct repression of astroglial promoter targets: Foxg1 directly binds to canonical astrocytic gene promoters (GFAP, S100β, AQP4) and likely represses their transcription.
3. Perturbation of pro-glial signaling pathways: Foxg1 perturbs key pathways that normally induce astroglial gene expression, including IL6/Jak2/Stat1,3; BMP/SMAD1,5,8;

Nrg1/ErbB4ICD-NCoR; and Dll1/Notch1ICD signaling, collectively dampening gliogenic signals[111].

More recently, Santo et al. demonstrated that Foxg1 is also involved in the spatial control of astrogenesis within the pallium. This work revealed that lower Foxg1 levels in caudo-medial progenitors facilitate astrocyte commitment via reduced repression of *Zbtb20*. Whereas elevated Foxg1 in astrocyte committed progenitors enhance their self-renewal capacity, thereby increasing the proliferative astroblast fraction ($Ki67^{+}$/$GFAP^{+}$ cells). These results highlight that Foxg1 not only acts by preventing premature NSCs gliogenesis but also promotes proliferation within the committed astroglial compartment[116]. Consistent with these findings, overexpression of Foxg1 and Sox2 in post-mitotic astrocytes lead to de-differentiation toward a proliferative NSC-like state, via coordinated effects on cell-cycle regulators and epigenetic modifiers such as *Foxo3*, *Dnmt1/3b* and *Tet3*[117]. This dual ability of Foxg1 to inhibit astrocytic differentiation while sustaining proliferative potential has placed it as a key contributor to glioblastoma multiforme biology[118]. A recent *in vivo* study identified a novel FOXG1 target, Fgfr3, a pro-gliogenic component of the FGF pathway. FOXG1 directly binds to Fgfr3 and suppresses its expression in progenitors, thus limiting their sensitivity to FGF signalling and delaying the onset of gliogenesis. Apart from this cell autonomous role, FOXG1 also regulates gliogenesis non-autonomously by controlling the expression of FGF ligands (e.g., Fgf18) in postmitotic upper-layer neurons. This study further demonstrated that loss of FOXG1 in these neurons enhanced the FGF-ERK signaling in progenitors and increased gliogenesis[119] (**Figure. 15**).

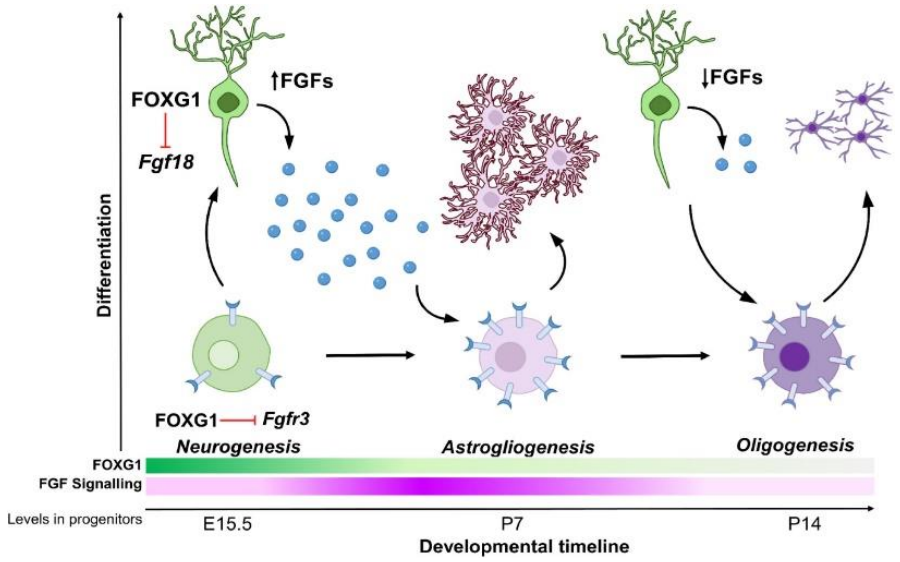


Figure. 15: **FOXG1-dependent control of gliogenic timing**. Elevated FOXG1 suppresses Fgfr3 thus maintaining neurogenesis. As FOXG1 levels decline Fgfr3 increases, thereby promoting astrogliogenesis. Later, low FOXG1 and FGF permit oligogenesis[119].

In conclusion, Foxg1 orchestrates spatio-temporal articulation of pallial astrogenesis by (i) preventing NSC progression to astrocyte progenitors, (ii) repressing astroglial differentiation programs, and (iii) supporting the self-renewal of astrocyte-committed progenitors. Through dosage-dependent and region-specific mechanisms, Foxg1 serves as a central determinant of lineage allocation in the developing telencephalon.

1.9.2. FOXG1 in Oligodendrogenesis and Myelination

FOXG1, in addition to its regulatory roles in neurogenesis and astrogenesis, has also been reported to impact the oligodendrocyte lineage and myelination, although its functions in this context are complex and sometimes contradictory. Earlier works suggest that FOXG1 negatively influences the oligodendrocyte output, consistent with its role promoting neural progenitor self-renewal and neurogenic fate[69]. Studies manipulating endogenous FOXG via CRISPR/Cas9 and SMASh technology, in hPSC-derived organoids, demonstrated that reduced FOXG1 levels, lower the expression of oligodendrocyte precursor cell (OPC) markers such as PDGFRα, MYT1, SOX10, and OLIG2 indicating a deficit in oligodendroglial differentiation[71]. A study done in a conditional Foxg1 knockout (KO) mice treated with cuprizone toxin to induce demyelination, exhibited reduced OPCs proliferation and accelerated differentiation into mature oligodendrocytes, both *in vivo* and *in vitro*. This effect was lined to repression of the canonical Wnt signaling pathway which is known to regulate oligodendrocyte development and myelination[120]. In this work, FOXG1 loss alleviated toxin-induced demyelination while facilitating remyelination of the damaged corpus callosum. Further work focused on early postnatal mice revealed that conditional deletion of Foxg1 via tamoxifen in neural progenitors using *NestinCreER; Foxg1$^{fl/fl}$* mice at postnatal day 0 (P0) resulted in a transient *decrease* in myelin formation within the first two weeks after birth but ultimately recovered to the control levels by P30. This effect appeared due to attenuation of PDGFRα signaling and reduced OPC cell-cycle exit, resulting in excessive OPC proliferation and delayed differentiation. Foxg1 deletion also affected the expression of factors such as Hes5 (a myelin inhibitor), Olig2, and Sox10, pointing to dysregulated transcriptional control of the oligodendrocyte lineage[121].

Human neuropathological analyses further support disruptions in oligodendrocyte development associated with FOXG1 deficiency. Clinically, delayed or abnormal myelination is commonly observed in children with FOXG1 syndrome which may improve with age, consistent with a developmental delay rather than a complete block in oligodendrogenesis[48]. A study done on fetal brains with premature stop codon FOXG1 mutations revealed an increase in PDGFRα-positive OPCs but reduced Olig2-positive pre-oligodendrocytes in the cortical plate relative to controls, consistent with defective lineage progression and delayed oligodendrocyte maturation[122].

These apparently contradictory findings i.e., reduced OPCs differentiation in organoid and human fetal studies vs accelerated differentiation and remyelination in adult demyelination models may suggest the complex and context dependent regulation by FOXG1. Such as, FOXG1 appears to be necessary for the proper timing of OPC maturation and myelin formation, likely through modulation of cell-cycle dynamics and pathway interactions (e.g., PDGFRα, Notch/Wnt) during early development. While in adulthood or injury contexts, FOXG1 loss may relieve progenitors of their inhibitory constraints on differentiation, thus promoting remyelination. Overall, these studies demonstrate FOXG1 role in oligodendrocyte lineage progression and myelination in a context- and stage-dependent manner, with dysregulation leading to developmental delays in oligodendrocyte maturation and myelin formation observed in FOXG1 syndrome.

1.10. Beyond a Classical Transcription Factor

Apart from a classical nuclear transcription factor that regulates telencephalic development via direct DNA-binding mechanisms or indirect chromatin-based actions, Foxg1 has also been recently reported to have non-nuclear and post-transcriptional roles, revealing a broader role in neuronal differentiation, synaptic maturation, and neuroplasticity.

1.10.1. Cytoplasmic Foxg1 and Post-Translational Regulation

The first evidence of non-nuclear Foxg1 localization was reported in frog and mouse forebrain i.e., Foxg1 is nuclear in progenitor cells but becomes cytoplasmic in differentiating neurons (e.g. CR cells and layer VI neurons). This compartmental switch is post-translationally regulated by two reciprocal signaling pathways; Casein kinase I (CKI) which phosphorylates Ser19 and

promotes FOXG1 nuclear import, and FGF/PI3K-AKT signaling that phosphorylates Thr226 within the FHD and drives nuclear export and enhances neuronal differentiation. Pharmacological inhibition of AKT signaling prevents FGF-mediated Foxg1 nuclear export, while CK1 inhibition results in cytoplasmic accumulation of Foxg1. Together, these findings indicate that nuclear export attenuates FOXG1-dependent proliferative transcriptional functions while favoring differentiation[123] (**Figure. 16**).

1.10.2. Mitochondrial Foxg1

FOXG1 is also found in mitochondria of post-mitotic neurons, suggesting additional non-nuclear roles. Specifically, it has been reported that, proteolytic cleavage of Foxg1 at N- terminal generates three main forms: full-length protein (FL-Foxg1, 481 aa), a mitochondrial form (mt-Foxg1, 272-481 aa), and a small cytosolic form (cyt-Foxg1, 315-481 aa). Foxg1 mitochondrial import is energy dependent, and 277-302 aa motif plays a key role in this transport. Functional studies revealed that overexpression of FL-Foxg1 promotes mitochondrial fission, increases membrane potential, and neuronal proliferation, whereas mt-Foxg1 overexpression drives mitochondrial fusion and supports neuronal differentiation (**Figure. 16**). Given that mitochondria play a critical role in brain development and plasticity, this study establishes a mechanistic link between Foxg1, mitochondrial energetics, neuronal maturation, and plasticity[53,124,125,126].

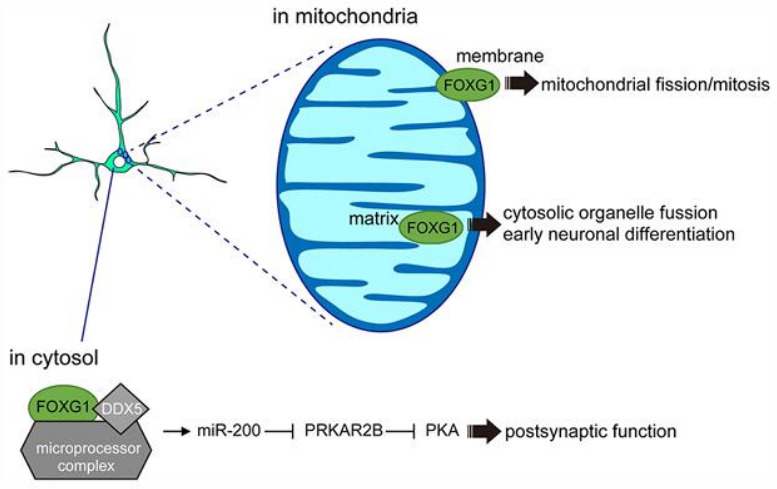


Figure. 16: **Non-nuclear FOXG1**. FOXG1 cytosolic and mitochondrial functions[126].

1.10.3. Post-Transcriptional Regulation and RNA Processing

Some recent studies report that Foxg1 also impacts gene expression post-transcriptionally. Proteomic analysis in N2a cells revealed that FOXG1 interacts with RNA helicase DDX5 and is recruited to the DROSHA complex. This interaction facilitates miRNA biogenesis, especially for the miR-200 family[127]. Moreover, Foxg1 itself is a target of miR200, suggesting a feedback loop in the regulation of Foxg1 expression[128]. *In vivo* studies of Foxg1 heterozygous mice reported that miR-200 members are dysregulated in hippocampal neurons of these mice along with synaptic genes including Prkar2b (protein kinase cAMP-dependent regulatory type II beta), whose upregulation may contribute to impaired synaptic transmission via reduced PKA activity[127]. Additionally, mass-spectrometry datasets and protein interaction databases (e.g., BioGRID) indicate that FOXG1 is directly involved in pre-mRNA splicing, mRNA transport and RNA degradation, ribosome biogenesis, and translation (e.g., SNRNP200, NUP205, PUM1, LARP1, EEF1D). Amino acid sequence analysis of human FOXG1 (as well as of murine Foxg1, and many other orthologues) further reveals a putative EIF4E-binding motif (374–380 aa), similar to domains found in translational regulators and homeoproteins, suggesting a potential role in translational control[129].

A recent study reported that cytosolic FOXG1 acts as a translational regulator in murine neocortical neurons. Authors of this study found that FOXG1 interacts with EIF4E and enhances ribosomal recruitment and translational efficiency of target mRNAs, particularly Grin1 (which encodes for the main subunit of NMDA receptor). Moreover, they found that within murine neocortical cultures, Grin1 de novo synthesis is tightly regulated by FOXG1. Omics analysis revealed that FOXG1 modulates translation of hundreds of neuronal genes and it does so by regulating ribosomal engagement and progression. This study expands our understanding of cytosolic FOXG1 and suggests its potential role in cortical plasticity and FOXG1-related neurodevelopmental disorders[130].

Collectively, these findings place Foxg1 at the interface of both post-transcriptional and translational regulation of neural cells, highlighting its multifunctional roles within nucleus, cytosol, mitochondria, and RNA processing. Its subcellular localization and expression are modulated by microRNAs, kinase signaling pathways, and organelle-specific mechanisms, linking Foxg1 to neuronal differentiation, synaptic function, and neuroplasticity.

1.11. FOXG1 Syndrome

Aforementioned findings demonstrated that FOXG1 expression is finely regulated in each step of telencephalic development and neuronal differentiation. In humans, FOXG1 copy number variations as well as any structural mutations on chromosome 14 have been identified, which result in a wide spectrum of rare neuropathological conditions collectively named as FOXG1 syndrome[48] (OMIM #613454). Hemizygosity for FOXG1 results in a variant of classical Rett syndrome, characterized by microcephaly, myelination anomalies, ASD-like symptoms (including limited speech, stereotypies, sleep disturbances, and social deficits), and epilepsy. On contrary, FOXG1 duplication may lead to West syndrome (WS), which includes symptoms like infantile spasms, hypsarrhythmic EEG patterns, epilepsy, and severe cognitive impairment. Besides copy-number changes, missense, nonsense, and frameshift structural mutations have been reported. To date, the SFARI database lists 92 FOXG1 variants implicated in ASD-linked neurodevelopmental phenotypes, whereas ClinVar reports approximately 248 FOXG1 variants in total, with around 100 linked to FOXG1-related disorders and 7 classified as pathogenic.

The resulting phenotypes range from benign to very severe and have overlapping symptom subsets relative to gene deletions and duplications. More severe outcomes of FOXG1 syndrome mainly result from mutations upstream of the FHD, predominantly nonsense and frameshift, while missense mutations within FHD show diverse severity. Apart from these, less frequent mutations in COOH-terminal half, including those leading to loss of GBD and/or the JBD, can also result in diverse neurological phenotypes[58,131]. These findings suggest that FOXG1 protein domains mediating DNA recognition, GBD repression, and JARID1b interactions may contribute differentially to specific molecular functions governed by FOXG1 (**Figure. 17**). Considering this, FOXG1 mutations can consequently disrupt distinctive neurodevelopmental processes resulting in an array of pathologies.

Given this complexity, dissecting underlying mechanisms in homotypic human neural tissue is highly time-demanding owing to prolonged duration of histogenesis and maturation, both *in vivo* and in neuro-organoids, thus limiting rapid translational application for newly identified mutations. The use of primary rodent neural cultures may provide a practical alternative, as neurodevelopmental processes underlying rodent brain morphogenesis take place in much shorter times compared to primates (<1/10 of primates) and fundamental Foxg1/FOXG1-dependent mechanisms of neocortical development are evolutionarily conserved[78,111,117,132–134].

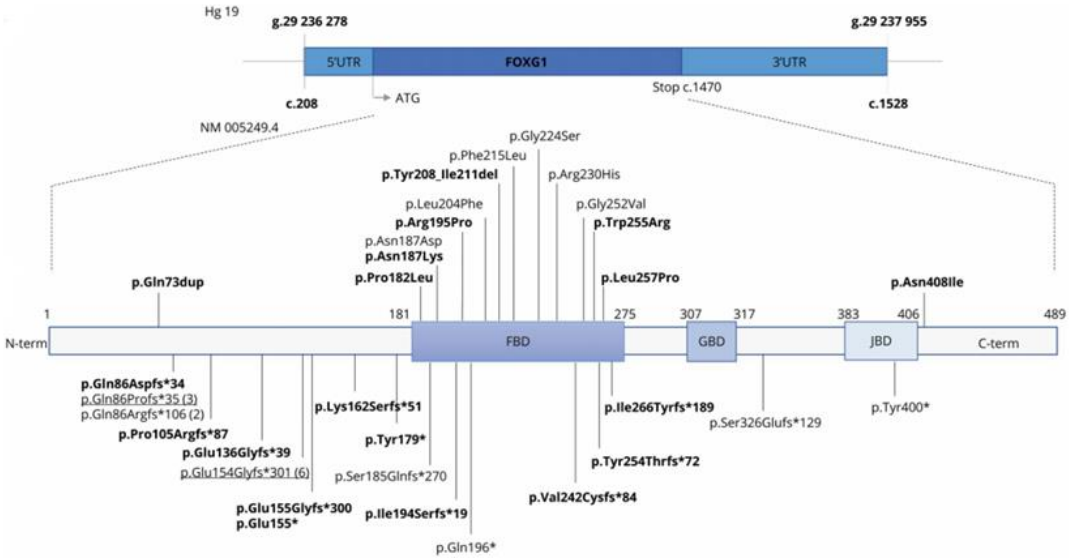


Figure. 17: Schematic representation of the FOXG1 gene and protein domain structure with positions of the identified pathogenic variants[48].

1.11.1. FOXG1 and the Congenital Rett Phenotype

Rett syndrome (RTT, OMIM #312750), first described by Andreas Rett in the 1960s[135], is a severe neurodevelopmental disorder affecting approximately 1 in 10,000-15,000 live-born females. Typical RTT features include neurodevelopmental regression beginning around 12 months of age, loss of acquired speech and hand movements, emergence of stereotypic hand wringing, and gait abnormalities. Patients who do not meet all diagnostic criteria are diagnosed with “atypical RTT”, which can be split in three subtypes: congenital variant, early seizure variant, and preserved speech variant[136]. 95% of classical RTT and 40-50% of atypical RTT cases result from mutations in methyl-CpG binding protein 2 (MECP2), though reported frequencies vary across ethnic backgrounds[137]. Additionally, cyclin-dependent kinase-like 5 (CDKL5) mutations have also been identified in both female and male patients with infantile seizures variant of RTT[138–140]. Because of the rarity of RTT in males and the localization of MECP2 and CDKL5 on the X chromosome, RTT was historically classified as an X-linked dominant disorder.

In 2005, FOXG1 was identified as the causal gene in some cases of congenital RTT. The first FOXG1-related mutation was reported in a 7-year-old boy carrying a balanced de novo translocation t(2;14)(p22;q12)[134], followed by cases of FOXG1-null mutations in two unrelated girls[141], establishing FOXG1 as a cause of congenital RTT. To note, FOXG1 mutations occur at

comparable frequencies in females and males. The known FOXG1-associated phenotype spectrum includes atypical RTT, congenital RTT, and craniosynostosis[51,142,143]. Most patients carrying point mutations or deletions of FOXG1 present distinct clinical features such as generalized hypotonia, severe postnatal microcephaly, severe intellectual disability with an absence of speech, stereotypic movements, corpus callosum abnormalities, and seizures[51,144,145] (**Figure. 18**). Larger 14q12 deletions affecting FOXG1 are additionally associated with craniofacial dysmorphisms[146]. Maternal somatic and germline mosaicism for FOXG1 mutations have also been documented, highlighting the complexity of hereditary patterns[144]. Despite the partial overlapping with MECP2-related RTT, FOXG1 associated disorders exhibit various distinct phenotypic characteristics that "FOXG1 syndrome" is now recognized as a separate clinical entity[51].

1.11.2. West Syndrome and Neurodevelopmental Outcomes in FOXG1 Duplication

Patients with FOXG1 duplications, compared to loss-of-function mutations, display less severe neurological defects i.e., they have normal brain size, near or near-normal head-circumference, no non-specific MRI findings, and their motor and language skills less profoundly affected. Typical symptoms include global developmental delay and autistic behaviors, often accompanied by absent or delayed speech. The recent literature reports approximately 21 individuals with 14q duplications involving the FOXG1 locus, with duplication sizes ranging from ~3.1 to ~33.9 Mb and potentially encompassing multiple regulatory elements and neighboring genes. A key clinical distinction is emergence of epileptic seizures; in FOXG1 duplication carriers, seizures emerge earlier (around 3-7 months), and frequently present as infantile spams. EEG findings typically display hypsarrhythmia or focal/multifocal epileptiform discharges, in contrast to the broader age range and seizure types observed in deletion syndromes[58,147].

WS occurs approximately in 1/1,650-20,000 individuals, affecting both sexes but has a higher incidence in males[148]. Despite etiological heterogeneity, WS can result from chromosomal anomalies such as Down syndrome, del.1p36 or single-gene mutations like ARX and FOXG1. Microdeletions of 14q12 involving FOXG1 account for up to ~5% of WS cases[149]. Early reports of WS include a female patient carrying a small supernumerary marker chromosome (SMC) which spans 14q11.2-14q12 and encompass FOXG1[150], followed by several other cases of WS

with 14q12 microduplications usually displaying infantile spasms and abnormal EEG patterns[151,152].

Typical FOXG1 deletions occur de novo. Their phenotypic expressivity is variable, and incomplete penetrance is documented. Several cases with 14q12 duplications including FOXG1 have been described with normal cognitive capacities, normal development, and no epilepsy[153,154]. This phenotypic variability is generally attributed to larger duplication size which involves additional genes and other regulatory elements, incomplete penetrance, and genetic mosaicism (**Figure. 18**).

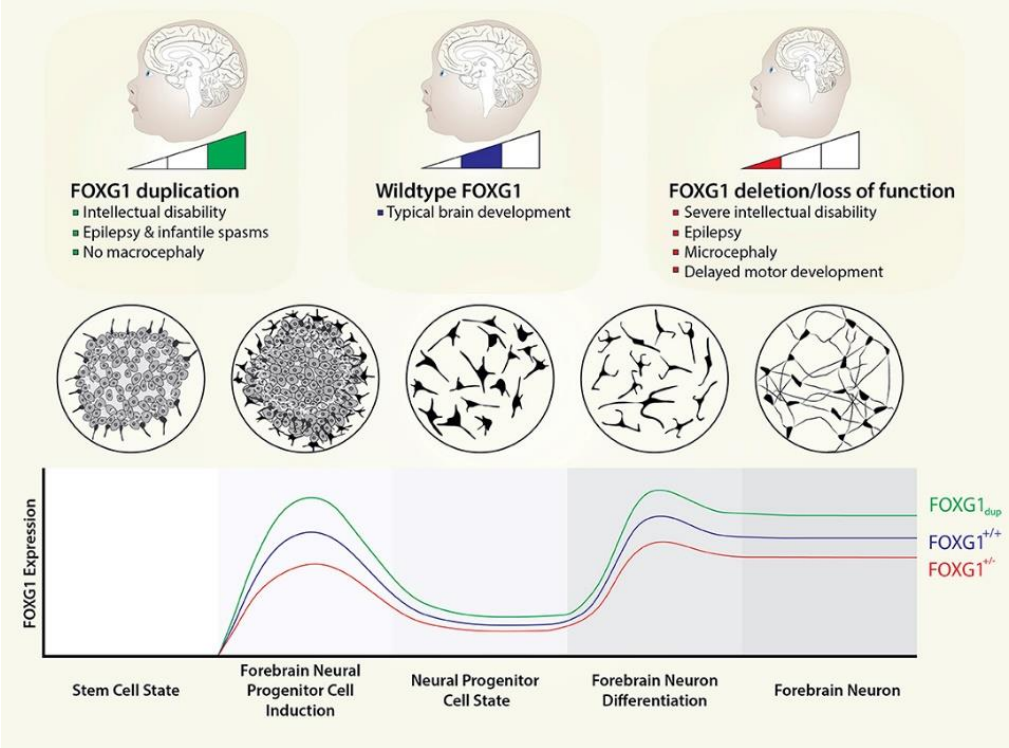


Figure. 18: Foxg1 dosage dynamics during normal telencephalic development and related neurodevelopmental pathologies[58].

1.12. Engram Biology: How Memory is Stored and Retrieved

The ability of the brain to acquire, store, and retrieve information underlies the core principles of memory formation, a fundamental process essential for adaptive behavior and survival of an organism. The fact that memory persists even after the initial experience suggests that an internal representation of this acquired information is stored in the brain and can be retrieved when needed. Human memory comprises of multiple distinct systems that differ in their function, duration, content, and neurobiological substrates. The earliest system to come into action is sensory memory, which acts a brief buffer towards incoming perceptual information (e.g., audio or visual cues), and it lasts from milliseconds to seconds. This system allows the brain to integrate immediate sensory experience before it decays or is transferred for further processing. Afterwards, the intended information can enter short-term memory (STM) and working memory (WM), which temporarily store and manipulate information and support ongoing cognitive tasks such as reasoning, language, and decision-making[155]. On the contrary, long-term memory (LTM) retains information that lasts from hours to a lifetime and is divided into explicit (declarative) and implicit (non-declarative) forms. Explicit memory is conscious recollection of the information and includes episodic and semantic memory. Implicit memory includes procedural memory, priming, and associative learning, and is mainly regulated without conscious awareness and relies on distinct neural circuits such as the basal ganglia and cerebellum[156–159].

The concept of a memory cell/engram, i.e., an enduring physical substrate of memory, has evolved from early theoretical formulations to modern circuit-level and molecular definitions. Previously, engram was defined as hypothetical unit linking sensory experience to a lasting behavioral change. Classical behavioral experiments demonstrated that memory could persist even after a significant neural disruption, highlighting that mnemonic information is neither centralized nor easily abolished. These earlier studies laid the foundation of viewing the memory as a distributed, robust, and partially redundant system. Technological advancements in the field of cell tagging and selective single-cell manipulations have made it possible to pinpoint these memory storage units across distinct behavioral phases (encoding, consolidation, retrieval). Recent studies have redefined an engram stating that it refers to a population of neurons activated by a learning experience, which are physically or chemically modified during this experience and capable of driving memory recall when reactivated either

naturally or artificially[160–162]. These findings demonstrate that the behavioral manifestation of memory correlates with the selective reactivation of tagged neuronal populations, experimentally supporting the engram hypothesis at cellular resolution.

Beyond single brain regions, recent work emphasizes that memories are embedded within distributed, brain-wide networks. Mounting evidence suggests that a single memory is supported by multiple engram ensembles collectively called engram complex, which are distributed brain-wide and is interconnected via an engram cell pathway[163,164] (**Figure. 19**). For example, contextual fear memories are modulated via coordinated communication of engram populations in the hippocampus, amygdala, and neocortex[165,166]. Each region encodes complementary features such as hippocampus is responsible for spatial-contextual information, amygdala for affective valance, and cortex for multimodal sensory details. During learning, engram cells emerge in hippocampus, amygdala, thalamus, and neocortex, linked by functional connectivity patterns that evolve over time. In consolidation phase, there is a gradual shift of memory retrieval from hippocampal engrams to neocortical circuits[163,167]. Reactivation during sleep or offline states enhances cortical-cortical connectivity and promotes hippocampal disengagement[168]. To note, hippocampal engrams are not erased entirely but serve as indexing nodes for remote memory storage[169].

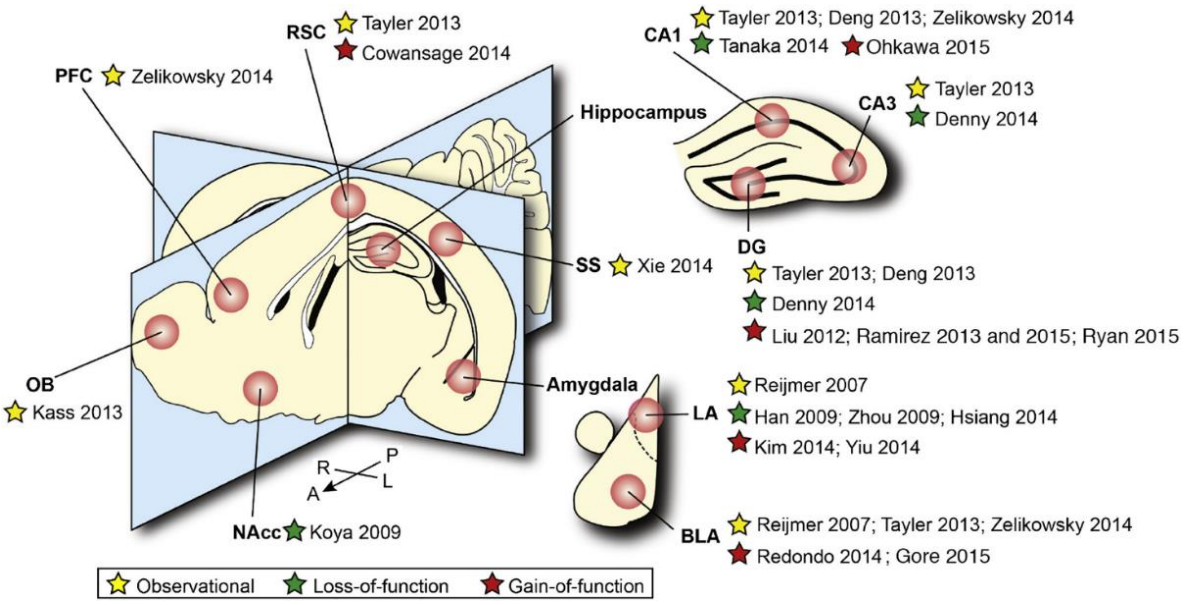


Figure. 19: **Regional localization of memory engram cell populations across brain**. Memory engram cell populations in multiple brain regions based on different lines of evidence

(observational, loss-of-function, and gain-of-function), supported by representative published studies. Abbreviations: OB, olfactory bulb; PFC, prefrontal cortex; RSC, retrosplenial cortex; NAcc, nucleus accumbens; SC, sensory cortex; LA, lateral amygdala; BLA, basolateral amygdala[170].

This dynamic restructuring highlights memory as a fluid and adaptive network property rather than a static anatomical state and explains why localized lesions generally result in degradation rather than complete loss of specific memories. Having said this, memory disorders may result from faulty engram allocation, their connectivity, stabilization, or their incomplete retrieval. For example, in Alzheimer's disease, engrams may form but remain inaccessible due to synaptic disruption[171]. Traumatic brain injury, neurodevelopmental disorders or ageing may similarly disturb engram-supporting circuitry[172]. The distributed engram framework therefore bridges molecular plasticity with cognitive symptoms, offering mechanistic explanations for memory loss, intrusion, and fragmentation across neurological disease.

Collectively these behavioral, cellular, and network-level studies converge on a multidimensional view of the engram i.e., a widely distributed assembly of neurons linked by stable yet plastic connections, whose coordinated activity mediates the storage and expression of memory (**Figure. 19**). This framework provides a conceptual bridge between internal mechanisms and observable memory behaviors, and it underscores how memory research now spans molecular biology, systems neuroscience, and cognitive science. Continued advances in circuit mapping, genetic tagging, and whole-brain imaging will further refine our understanding of underlying processes of how engrams are formed, maintained, and altered across the lifespan and in disease.

1.13. What Drives Memory Formation: Transcriptional and Epigenetic Programs within Engram Cells

While classical theories have emphasized synaptic plasticity as the primary substrate for memory, recent advances point toward a more integrated perspective in which neuron ensembles, otherwise called engram cells, undergo coordinated changes at the transcriptional and epigenetic levels that persist beyond the transient activity patterns. These molecular programs convert short-lived neuronal firing into long-lasting changes in cellular identity, synaptic connectivity, and responsiveness to future stimuli. A defining feature of engram

neurons is their elevated intrinsic excitability compared to neighboring neurons making them likely to be recruited during learning[173,174]. In addition, engram neurons have increased synaptic plasticity e.g., larger spine density and more effective connectivity to other downstream engram cells[175,176]. These key characteristics of engram cells are regulated by molecular drivers such as TFs, epigenetic modifiers, and synaptic proteins[177,178] that persist from encoding to remote recall. These characteristics help differentiate engram neurons from non-engram neighbors and are responsible for their selective recruitment into memory traces. Interestingly, engram networks are not static but rather dynamic, they undergo reorganization, strengthening, or attenuation depending on behavioral relevance, new learning, or pathological conditions.

1.13.1. Molecular Basis of Memory Encoding

The first step of engram formation is rapid transcriptional activation, mainly through IEGs such as Fos, Arc (Activity-regulated cytoskeleton-associated protein), Egr1 (early growth response 1), and Npas4 (Neuronal PAS domain protein 4)[179,180]. These genes are activated within minutes of neuronal depolarization via calcium-dependent signaling cascades such as CaMKII, MAPK/ERK, and CaMKIV pathways, resulting in CREB (Cyclic AMP Response Element Binding Protein) phosphorylation and transcriptional activation[181]. Some of these IEGs encode transcription factors that serve multiple roles essential for early engram function. For example, Fos and Egr1 drive secondary gene expression waves, others such as Arc, Homer1a encode structural or synaptic fold proteins that modify AMPA receptor trafficking and cytoskeletal reorganization[182]. This temporal layering of IEG induction is critical to prime engram neurons for subsequent state changes by enhancing their excitability, synaptic connectivity, and recruitment probability[174].

Activity-dependent allocation of neurons into engram ensembles is not entirely stochastic; rather, studies report that neurons with elevated intrinsic excitability or enhanced CREB levels are preferentially selected at the time of learning, highlighting a causal link between transcriptional regulation and engram formation[173,183]. Interestingly, ablating the activity of engrams can result in impaired memory retrieval while reactivating these neurons can elicit memory recall even in the absence of any cue (**Figure. 20**). These findings demonstrate that transcription is not merely a downstream consequence of firing, but rather an active determinant of memory storage architecture.

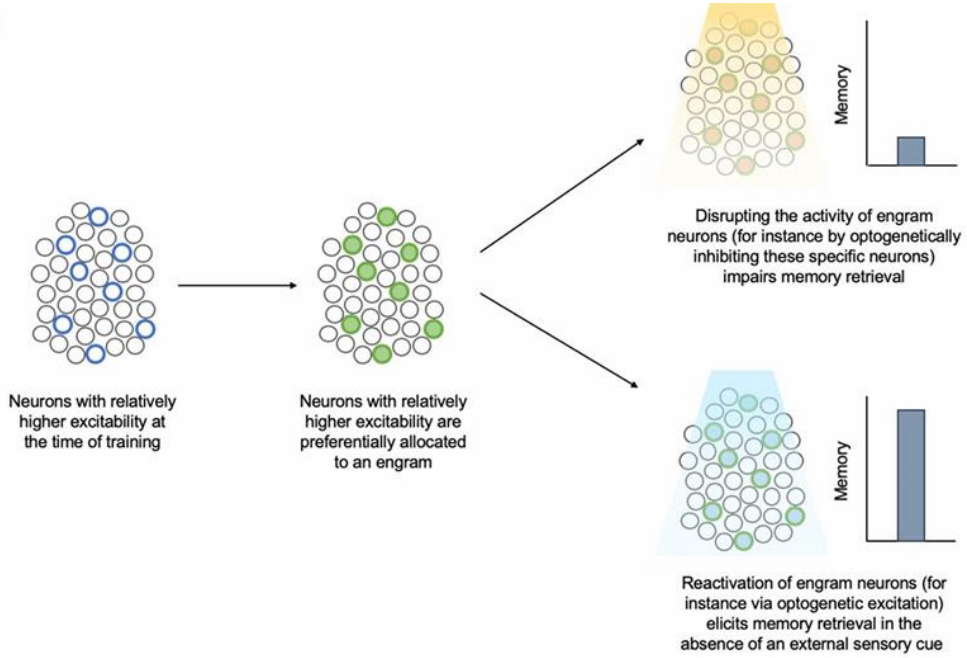


Figure. 20: Allocation of neurons into engram ensembles[164].

1.13.2. Transcriptional Programs and Memory Consolidation

The transition from encoding to consolidation depends on sustained waves of gene expression that unfold over hours to days and not on initial transient spikes. Though IEGs initiate the encoding response, delayed transcriptional programs are the ones that regulate memory consolidation via modulating synaptogenesis, spine morphology, protein synthesis, and metabolic support. Single-cell RNA sequencing of engram neurons has identified upregulation of genes related to:

- synaptic adhesion (e.g., *Neuroligin*, *SynCAM*)
- cytoskeletal remodeling (e.g., *Cofilin*, *Actb*)
- mitochondrial biogenesis and metabolism (e.g., *PGC-1α*)
- miniature synaptic event regulation (e.g., *Syngap1*, *Dlg4*)

Such genes are responsible for structural remodeling including dendritic spine stabilization and excitatory synapse maturation that is essential for long-term memory[164,184].

Apart from changes at transcriptional level, studies also report that translational control contributes to another layer of consolidation-related regulation. RNA-binding proteins such as

FMRP (Fragile X Mental Retardation Protein), CPEB (Cytoplasmic Polyadenylation Element Binding Protein), and TIA-1 (Cytotoxic Granule Associated RNA Binding Protein) modulate activity-dependent mRNA translation at dendritic sites to support synaptic tagging and capture[185]. This transcriptional and translational coupling ensures synaptic modification is precisely regulated, a requirement for memory specificity (**Figure. 21**). In addition to neurons, glial cells and neurovascular system also plays a crucial role in memory consolidation process. Astrocytes regulate metabolic coupling and glutamate uptake, and microglia contribute to synaptic pruning and spine refinement through complement signaling pathways[186], highlighting that memory stabilization is a distributed process and involves multiple cell types.

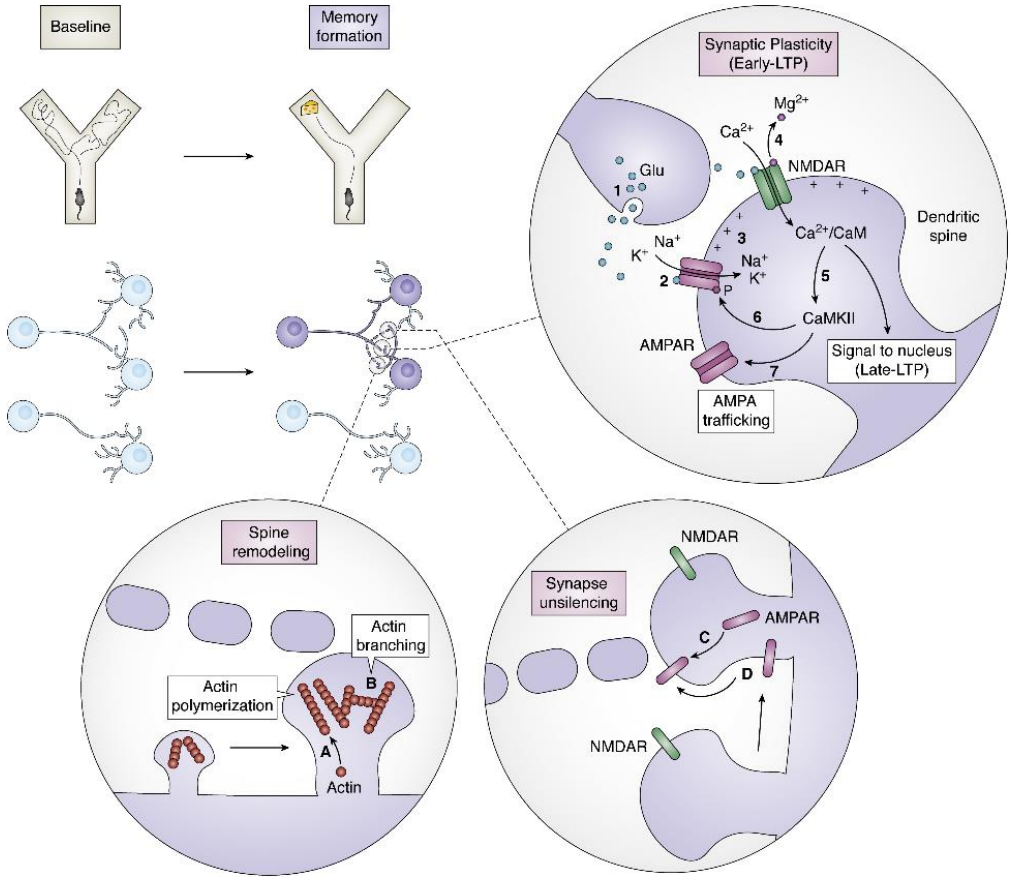


Figure. 21: **Molecular mechanisms of engram formation**. During memory encoding, a subset of neurons forms engram ensemble and their synapses undergo long-term potentiation. (1-7) Glutamate (Glu) is released from presynaptic terminals and binds to AMPARs in the postsynaptic membrane and initiate cascades of depolarization. (A-D) Dendritic spines are structurally remodeled via actin polymerization and branching, and AMPAR enrichment occurs through vesicular insertion and lateral membrane diffusion[188].

1.13.3. Epigenetic Programs and Chromatin Remodeling in Engram Stabilization

Learning induces changes in epigenetics programs such as histone acetylation, histone methylation, DNA methylation, and chromatin accessibility. These epigenetic modifications provide a mechanism for memory persistence over long timescales and collectively shape the molecular landscape of engram cells.

Histone acetylation is strongly associated with transcriptional activation and is mediated by HATs (Histone Acetyltransferases) such as CBP and opposed by HDACs (Histone Deacetylases). During memory formation, increase in H3K27ac (Histone H3 Lysine 27 Acetylation) and H3K9ac (Histone H3 Lysine 9 acetylation) at promoters and enhancers is observed, which facilitates IEGs expression and enhance plasticity gene accessibility[187]. On contrary, histone methylation has both activating (e.g., H3K4me3) and repressive (e.g., H3K9me3, H3K27me3) functions, enabling combinatorial fine-tuning of gene expression[189]. Literature reports that manipulating these pathways can alter the memory: CBP haploinsufficiency impairs LTP and memory, while inhibiting HDAC enhances memory consolidation and rescue cognitive deficits in neurodegenerative disorders models[190,191].

During memory formation, neurons exhibit dynamic DNA methylation changes such as DNA methyltransferases (DNMTs) silence plasticity-inhibiting genes activity while promoting the demethylation at plasticity-promoting loci via TET enzymes mediated hydroxymethylation[192]. These modifications persist for long time periods, suggesting an underlying mechanism for memory maintenance even in the absence of ongoing protein turnover. Recent ATAC-seq and ChIP-seq studies demonstrate that learning induces persistent increase in the chromatin accessibility at enhancers associated with neuronal plasticity genes[193]. Such enhancers become primed, meaning they remain accessible long after the transcription ceases and biases the engram neurons toward preferential reactivation upon presentation of retrieval cues. These epigenetic priming forms a molecular scaffold supporting memory recall (**Figure. 22**).

The discovery of transcriptional and epigenetic engram mechanisms has transformed our understanding of memory-related disorders. Alterations in engram encoding or stabilization processes may contribute to: neurodegeneration triggered memory loss (e.g., Alzheimer's disease), intrusive memory retrieval (PTSD), hallucinatory or delusional memory traces (schizophrenia), and intellectual disability and autism spectrum symptoms (genes affecting

synaptic transcription programs). Targeting chromatic regulators (such as HDAC inhibitors, BET protein modulators, CBP active) has shown promise in rescuing cognitive function in preclinical models. Moreover, manipulating CREB, IEGs or epigenetic enzymes may offer potential strategies for reshaping memory traces therapeutically, either strengthening, weakening, or erasing maladaptive engrams.

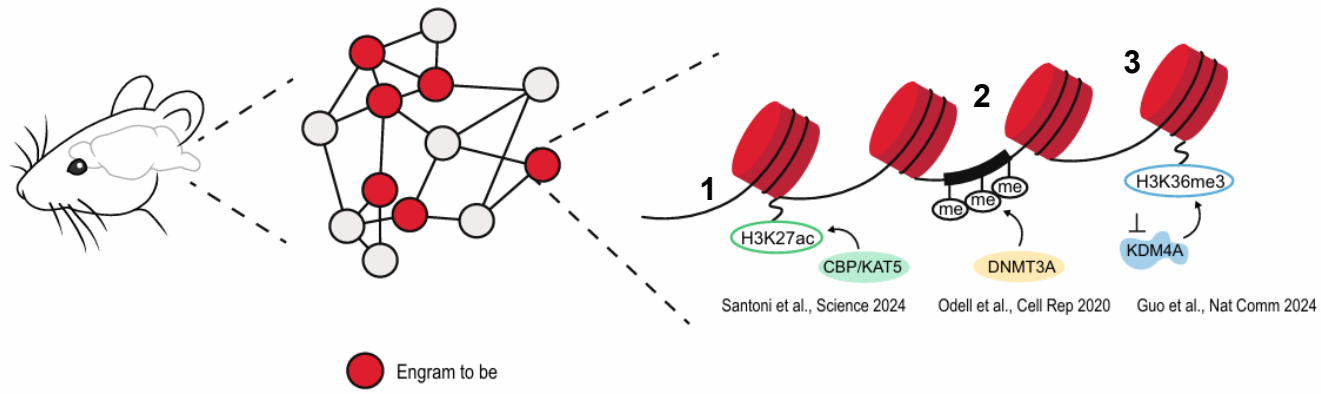


Figure. 22: **Multiple layers of epigenetic regulation accompany engram allocation process**. **1**. Histone acetyltransferases such as CBP/KAT5 increase HEK27ac which in turn enhance chromatin accessibility, transcription of synapse related genes and neuronal excitability. **2**. The DNMT3A (DNA methyltransferase) elevates CpG methylation, promoting transcription and excitability. **3**. Removal of KDM4A (histone demethylase) lifts repression, allowing H3K36me3 accumulation and transcription of memory encoding genes[194].

Transcriptional and epigenetic regulation within engram cells forms a multi-layered molecular framework that allows memories to be encoded rapidly, consolidated structurally, and stored persistently. By integrating rapid IEG induction, delayed transcriptional programs, translation at activated synapses, and long-lasting chromatin remodeling, the brain generates a molecular memory code that preserves experiences across time. Insights into these mechanisms have begun to bridge molecular biology, systems neuroscience, and clinical research, offering new conceptual and therapeutic avenues for disorders in which memory is impaired, fragmented, or pathologically persistent.

1.14. Classical IEGs and their Role in Engram and Memory Formation

Classical IEGs such as cFos, Arc, and Egr1 have long served as molecular markers for engram labeling[160,195]. Upregulation of their upstream transcriptional regulators such as CREB have

been shown to enhance neuronal excitability and neuronal allocation to engrams, whereas reducing CREB impairs memory recall[173]. Other TFs such as Npas4, an activity dependent TF, regulates excitatory-inhibitory neuronal balance in hippocampus and contributes to contextual discrimination[196], KLF family members and SRF have also been reported to stabilize memory associated circuits[197–199]. Together, these findings highlight TFs as critical molecular switches that regulate activity of neurons, their allocation to engram, and their role in memory storage and retrieval.

1.14.1. From Proto-Oncogene to Engram Marker: The Evolution of Fos

Fos, also known as c-Fos, a proto-oncogene, belongs to activator protein 1 (AP-1) transcription factor family. FOS forms a heterodimer with one of the JUN family members such as c-Jun and regulates gene expression. The very first *in vitro* studies from 1980s found that rapid and transient Fos transcription was observed following depolarization induced calcium influx in neuronally differentiated PC12 cells[200,201]. *In vivo* evidence for Fos activation was first demonstrated by its robust induction in the CNS following seizure activity[202]. Later studies reported elevated Fos expression in specific regions following physiological stimulation and behavioral training[203–206]. Since these pioneering studies, Fos has been used as a neuronal activity marker and its roles in learning and memory have been explored extensively. Studies have found that selective ablation of Fos impairs hippocampus dependent spatial and associative learning, a phenomenon linked to reduction of LTP at the CA3-CA1 hippocampal synapses[207]. These early findings suggested that Fos may be an essential component of LTP driven hippocampus dependent learning, whether learning causes synaptic changes on the Fos ensembles specifically was not tested until recently.

The earliest study on identifying the functional roles of Fos ensemble neurons in memory expression demonstrated that activating these engram cells in DG of Fos-tTA transgenic mice via optogenetics results in memory recall in neutral context i.e., in non-conditioning context[160,161,208]. This was further confirmed in some recent studies as well which highlights that though these DG Fos ensembles are not necessary for memory recall but have been shown to promote memory generalization[178,209]. Other studies also explored the role of DG Fos ensembles to modulate contextual fear memory expression. One such finding demonstrated that creating false memories by optogenetic manipulations, i.e., Fos neurons captured in a neutral context (without any foot shock), can elicit a fear response when activated during fear

conditioning in a different context[161]. This phenomenon of memory inception may arise from preferential allocation of contextual fear memory to the Fos ensembles representing the neutral context, driven by optogenetic activation that biases these neurons toward engram recruitment[210]. This finding differs from an earlier study, where a similar behavioral paradigm led to formation of a synthetic contextual memory that combined features of both contexts rather than a simple implanted memory selectively linked to neutral context[208]. Moreover, these findings were also in contrast with a study by Liu et al., as optogenetic activation of Fos ensembles alone was insufficient for memory retrieval[160].

Apart from DG, Fos ensembles in other brain regions have also been reported to drive learned behaviors. Fos engram neurons of retrosplenial cortex that encode fear memory when activated optogentically can drive memory recall in a neutral context[211]. Fos ensemble neurons can also encode positive memories, as shown in DG and basolateral amygdala[212–214]. Recently, a study also reported role of Fos neuronal ensembles in driving social memory: using TRAP mouse model, they captured social memory engrams in medial prefrontal cortex and reported that upon optogenetic inhibition of these ensembles social memory is impaired[215]. Roy et al. demonstrated that simultaneous activation of multiple Fos ensembles in different brain regions result in enhanced memory recall than the activation of a single Fos ensemble, highlighting importance of distributed engram complex[216].

Experience driven modifications followed by physiological remodeling of synapses has been believed to underlie the memory expression. Recently researchers have started to examine the learning induced synaptic changes on Fos engram neurons and their role in driving memory encoding and recall. This has been best explored in contextual fear conditioning (CFC) experiments and specifically in hippocampal formation, where literature reports that NMDA receptor mediated LTP occurs in hippocampus Fos ensemble neurons. In DG, Fos ensemble neurons have been observed to have higher AMPA/NMDA ratio and receive stronger excitatory inputs from entorhinal cortex compared to non-engram neurons. Additionally, these Fos neurons had increased dendritic spines, which were lost on protein synthesis inhibitor anisomycin administration resulting impaired memory retrieval[217]. A recent study also reported similar synaptic changes in DG Fos ensemble neurons i.e., higher excitatory inputs from medial entorhinal cortex (MEC) and AMPA/NMDA ratio relative to neighboring non-engram neurons. Moreover, they also demonstrated that MEC input on the DG Fos ensemble might support the

memory generalization driven by these engram neurons[209]. CA1 Fos ensembles also undergo similar synaptic changes following CFC visualized by the dual-eGRASP (Green Fluorescent Protein Reconstitution Across Synaptic Partners) technique. Apart from enhanced synaptic plasticity, these CA1 neurons also receive stronger inputs from CA3 Fos ensemble neurons, strengthening enpassage synapses, which was further confirmed by electrophysiological recordings[218]. Outside hippocampal formation, learning induced synaptic changes on Fos ensemble neurons have also been observed in basal amygdala and nucleus accumbens[219,220].

Apart from changes at synaptic level, a transient elevation in intrinsic excitability of Fos neurons in the hours following a learning experience in CA1 and retrieval in DG has also been observed. This temporary experience-triggered alterations in neuronal excitability appears to be crucial not only for linking memories encoded in close temporal proximity but also for enhanced retrieval precision[178,221]. Post-transcriptional and translational mechanisms are likely to drive these experience- dependent neuronal excitability changes (**Figure. 23**).

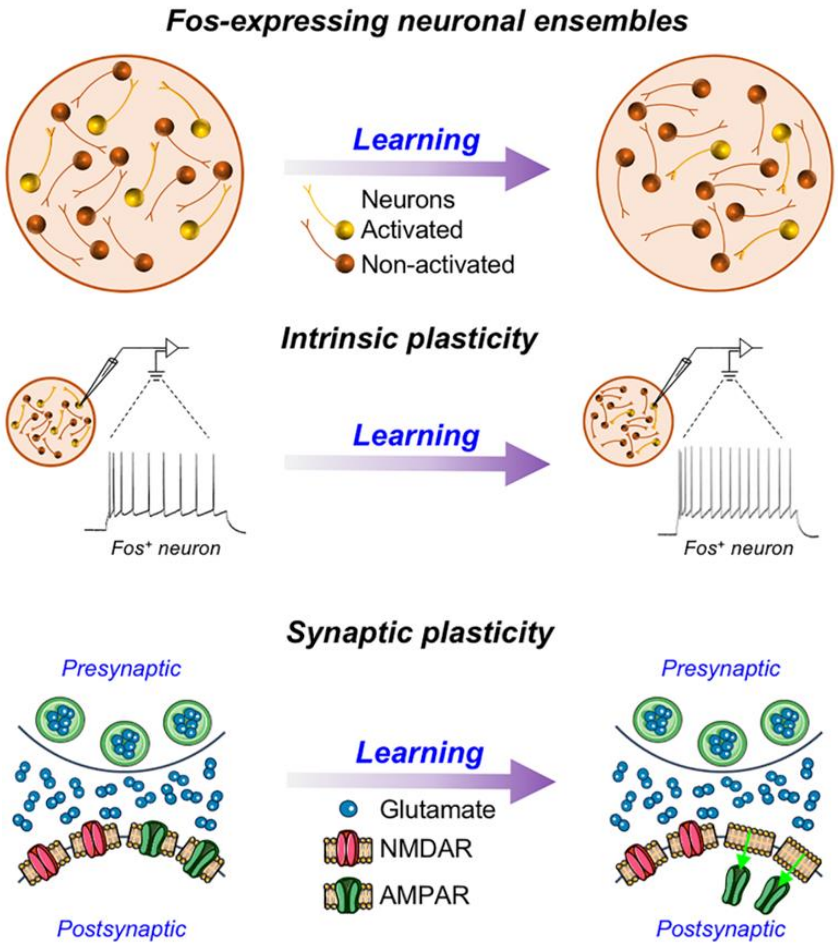


Figure. 23: (**Top**). The Fos-expressing neurons becomes more selective after learning, resulting in reduced but more specialized neuronal population. (**Middle**). After initial learning phase, Fos-tagged neurons exhibit increased intrinsic excitibility and higher spike activity. (**Bottom**). Learning also induces synpatic remodeling in Fos neurons such as rise in NMDA receptor only silent synapses[224].

1.14.2. Arc-Defined Engram Ensembles: Molecular, Synaptic, and Functional Properties

Activity-regulated cytoskeleton-associated protein (Arc)[222], also named as activity-regulated gene (Arg3.1)[223], is a non-transcription factor IEG and a cytosolic signaling protein, that has been extensively used in engram labeling and visualization. In physiological conditions, Arc is mainly expressed in excitatory neurons, its expression is sensory and behavioral experiences driven[225–227], and in inhibitory neurons it can be induced upon strong stimuli[228]. Arc plays critical roles in synaptic plasticity processes such as LTP and LTD and its deletion in mice result in circuit dysfunction and impaired learning and memory[229–232]. Arc mRNA can be translated locally at active synapses, where it regulates excitatory synaptic plasticity[229,233–235] and Arc protein is capable of self-assembling into viral-like capsids that mediate inter-neuronal transfer of genetic material[236]. ArcCreERT2 BAC transgenic mouse studies revealed that optogenetically inhibiting CA3 or DG Arc ensembles significantly impair memory recall during CFC[225,226]. Interestingly, inhibition of the entire DG or of Fos-expressing ensembles does not affect memory recall[209,237], whereas selective silencing of Arc engram neurons in DG, significantly disrupts retrieval. These findings suggest that DG Arc ensembles might be functionally different from Fos ensembles.

Similar to learning induced synaptic changes on other IEGs ensembles, Arc neurons also exhibit increased mEPSC frequency relative to non-engram neurons in BLA due to enhanced excitatory inputs from cortical pathway[238]. In lateral amygdala, Arc expressing neurons display heightened baseline excitability and are selectively incorporated into newly formed memory ensembles and have increased AMPA/NMDA ratio[183]. Moreover, following motor learning, Arc expressing neurons in frontal cortex are hyperactive compared to Arc negative neurons, and this hyperactivity is only observable in trained animals[239]. Arc-positive neurons have higher spontaneous firing rates in somatosensory neurons, even in untrained mice[240]. Approximately 80% of Arc expressing neurons also express Fos, behavioral evidence suggests that these ensembles may have distinct functional roles[225].

1.14.3. Egr1 in Synaptic Plasticity and Memory Formation

Egr1 also known as NGFI-A[241], Krox-24[242], and TIS8[243,244] is one of the earliest IEGs identified. It is a zinc finger protein thus also named Zif268[245] and is a member of EGR family of transcription factors. It was initially reported to be induced by growth factors like nerve growth factors (NGF)[241], but later *in vivo* studies found that Egr1 expression is also initiated by neuronal activity[246]. Moreover, EGR1 was also identified to act upstream of Arc i.e., Arc is one of the potential direct targets of this IEG[247]. Earlier studies have explored various roles of Egr1 in both short- and long-term memory formation as well as its retrieval. A complete Egr1 knockout mouse model exhibits defective late LTP and long-term memory despite having intact early LTP and short-term memory[248,249]. This knockout mouse model also has both consolidation and reconsolidation of contextual fear memory impaired. Egr1 heterozygous mice display deficits in memory reconsolidation and not in consolidation, similar to its knockdown in dorsal hippocampus[250].

Specific knockdown of Egr1 in entire amygdala or only in lateral amygdala results in defective contextual fear memory in the former and impaired auditory fear memory in the latter[251,252]. Interestingly, reducing the Egr1 expressing engram neurons specifically in CA1 region of hippocampus improves motor learning while chemogenetically activating these neurons result in defective motor performance[253]. Stimulus specific Egr1 ensembles have been observed in layer II of several cortical regions such as motor, visual, somatosensory, and retrosplenial cortices[254].

1.14.4. Npas4-Dependent Regulation of Synaptic Plasticity and Memory

Npas4 a rather new TF compared to other classical IEGs, was first reported to play a role in inhibitory synapses development on excitatory neurons[255]. It is expressed only in neurons, both excitatory and inhibitory, and its expression is activity dependent. Npas4 not only recruits inhibitory synapses on excitatory neurons but also strengthens excitatory synapses on inhibitory GABAergic neurons during development. Specifically, it limits the excitatory mossy fiber input from DG granule cells and is essential in driving contextual fear memory expression in CA3[255–259]. Moreover, in lateral amygdala, it mediates both fear and safety memory formation and consolidation[260,261] and in primary motor cortex it drives motor learning[262]. Npas4 engram neurons are different from Fos ensembles i.e., Npas4 ensembles play a critical role in

contextual discrimination, which is lost upon inhibition by DREADDs. Specifically, in DG, Npas4 neurons are required for discrimination of different contexts while DG Fos engrams promote contextual memory generalization[209]. Another study demonstrated similar findings but in stress induced memory generalization model, where they demonstrated that inhibition of Npas4 ensembles not Fos neurons captured at training can reverse the stress triggered fear generalization during social defeat stress treatment[263].

At synaptic level, changes in DG Npas4 engram neurons also differ from Fos ensembles such as Npas4 neurons recruit inhibitory GABAergic inputs from cholecystokinin-expressing (CCK) interneurons, while keeping excitatory inputs unaltered during CFC. The CCK inhibition increases with foot shock intensity during CFC training session and is responsible for the memory discrimination i.e., upon abolishing the CCK interneurons activity, precise memory recall is lost[209]. Apart from contextual memory formation, Npas4 is also crucial for motor learning, and it drives this mostly by somatostatin (SST)-expressing GABAergic interneurons (SST-IN) activity in motor cortex. Over the course of multiday training sessions, SST-IN Npas4 ensemble neurons activity decreases, a process essential for proper motor learning. Moreover, if the activity of these SST-IN Npas4 ensemble neurons is elevated during learning phase, it blocks the motor learning completely. This study also reported that if Npas4 SST-IN ensemble activity is kept elevated during training sessions, synaptic organization such as spine density and turnover is lost completely[262].

1.15. Technologies for Tagging and Visualizing Memory Engrams

Engram tagging relies on the principle that a sparse subset of neurons is activated within a broader neural circuit during memory encoding. The key features of these neurons as mentioned before are elevated excitability, activity-dependent gene expression, and enduring synaptic and molecular modifications. Cell tagging technologies exploit these characteristics by coupling neuronal activity to permanent genetic or molecular markers to label neurons that are active during a defined temporal window corresponding to learning. For a tagging system to be efficient, it must occur within the defined temporal window corresponding to memory encoding, rather than labeling general activity or arousal. This temporal specificity distinguishes engram labeling from broader activity mapping. Another key feature of engrams is their reactivation fidelity: engram neurons are preferentially reactivated during memory retrieval, sleep-

dependent consolidation, and memory updating. Therefore, an effective tagging system allows longitudinal tracking of the same neuronal ensemble across behavioral states and time.

1.15.1. IEGs based Tagging Systems: IEGs as Activity Sensors

IEGs based tagging systems lay the foundation of modern engram research. These approaches utilize the rapid and transient induction of IEGs in neurons in response to synaptic activity and depolarization, allowing to selectively label neurons engaged during specific behavioral experiences. Since IEGs transcription is tightly coupled to neuronal activation, they serve as endogenous activity sensors, converting transient physiological events into durable molecular tags. Their activation is driven by calcium influx via NMDA receptors and voltage-gated calcium channels, resulting in the initiation of downstream signaling pathways such as CaMKII, MAPK/ERK, and CREB. The discovery that IEG promoters could be utilized to drive genetic expression in active neuronal populations represented a major breakthrough for engram research. Unlike immunohistochemistry, genetic tagging provides a means to permanently label, visualize, manipulate, and molecularly characterize engram neurons across time. Some of the canonical neuronal IEGs include Fos (a component of the AP-1 transcription factor complex), Arc (involved in synaptic plasticity and AMPAR trafficking), Egr1 (associated with long-term potentiation and memory consolidation), and Npas4 (that regulates excitatory-inhibitory balance). Each IEG therefore reflects a distinct aspect of neuronal activation, and the choice of promoter can influence tagging toward selective neuronal populations or functional states.

The **TetTag** system was the first widely adopted IEG-based genetic tagging strategy. In this system, an IEG promoter (commonly Fos) drives expression of tetracycline- controlled transactivator (tTA). In the absence of doxycycline, tTa binds to tetracycline response elements (TREs), which leads to activation of downstream reporter or effector genes such as GFP, channelrhodopsins, or DREADDs. This system allows activity-dependent labeling of the neurons restricted to a defined time window and controlled by doxycycline. Using TetTag system, seminal studies demonstrated that neurons tagged (active) during fear conditioning are preferentially reactivated upon memory retrieval, providing some of the first direct experimental support for the engram hypothesis. Despite its historical significance, TetTag system has some limitations as well, such as relatively slow labeling kinetics, leaky expression, and incomplete temporal precision.

Targeted Recombination in Active Populations (**TRAP**) and its recently improved version TRAP2 systems represent a refined second-generation IEG-based tagging. In TRAP mice, an IEG promotor (mostly Fos) drives the expression of a tamoxifen-inducible Cre recombinase (CreER). This tamoxifen mediated Cre-recombination takes place only in neurons that are transcriptionally active, resulting in permanent expression of a reporter or effector gene. This dual requirement offers high temporal specificity, allowing precise labeling coupled with a distinct learning or experience. TRAP system allows long-term tracking of engram neurons, cell-type-specific targeting via Cre-dependent alleles, and integration with optogenetics, chemogenetics, and molecular profiling. Both TRAP and TRAP2 have been extensively used to tag and track engram neurons across multiple brain regions including hippocampal formation, amygdala, neocortex and many others.

These findings highlight how IEG-based tagging systems have fundamentally reshaped the study of memory by enabling direct access to the neurons that encode experience. By exploiting transient neuronal activity these strategies have transformed the visualization, manipulation, and molecular dissection of engram cells with unprecedented precision.

1.15.2. Experimental Approaches to Label Fos Engram Ensembles

The earliest developed system that employed Fos based genetic labeling harbored a piece of murine Fos genomic DNA to generate various transgenic models for Fos ensemble studies. It encodes a fusion protein containing first 315 amino acids of FOS protein and LACZ. The resulting Fos-LacZ transgenic mice and rats were then used to study recently activated neurons in fixed tissues[264,265]. Moreover, these animals were also used to inhibit Fos ensemble neurons, taking advantage of LacZ product beta-galactosidase to convert the prodrug Daun02 into Daunorubicin, which leads to inactivation of neurons via apoptosis, or blockade of voltage gated calcium channels[266,267] (**Figure. 24**). Later the same Fos genomic DNA was fused with an EGFP encoding sequence to generate Fos-EGFP transgenic mouse strain, which allowed the visualization of the Fos ensemble neurons that were activated by an experience 4-6hours previously. These Fos-EGFP mice have been used both for ex-vivo electrophysiological studies[268,269] and in vivo imaging[270]. One of the limitations of Fos-LacZ and Fos-EGFP mice is the potential overexpression of functional part of the FOS protein, an aspect that has not been examined thoroughly.

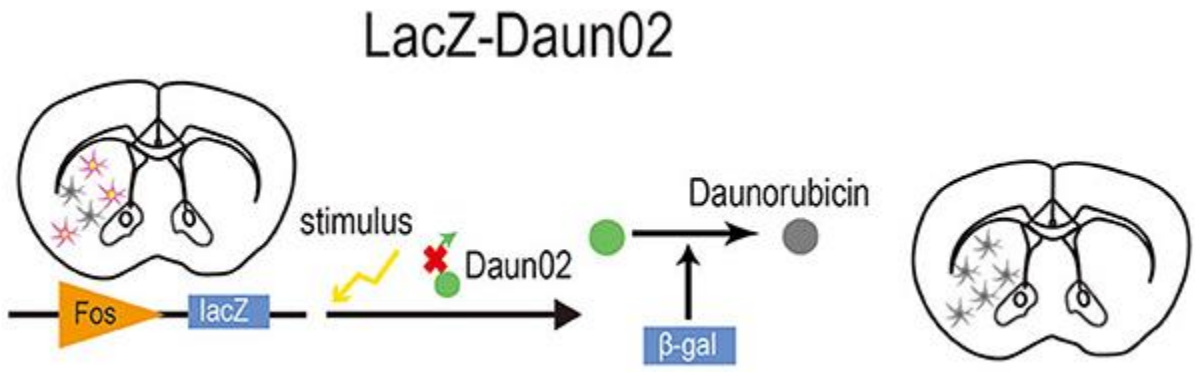


Figure. 24: **LacZ-Daun02 labelling strategy**. Upon local infusion of the prodrug Daun02, β-gal converts it into the active compound daunorubicin, which selectively silences Fos expressing activated neurons. This method allows targeted inactivation of neuronal ensembles to test their role in memory and behavior[271].

The next Fos based tagging strategy i.e., Fos-tTA transgenic mouse is also probably the most widely used system for engram research. This mouse strain consists of a Fos promoter-driven tTA and a Fos promoter-driven short half-life (~2 hours) GFP (shGFP). Fos-tTA is half of the TetTag mouse and it has TetO-lacz as other second half, lacZ expression is TREs dependent and is suppressed in the presence of doxycycline[272]. This system not only provides doxycycline-based temporal windows for labeling Fos ensembles but also the flexibility to express other reporter molecules such as opsins, chemogenetic actuators or genetically encoded calcium sensors (**Figure. 25**).

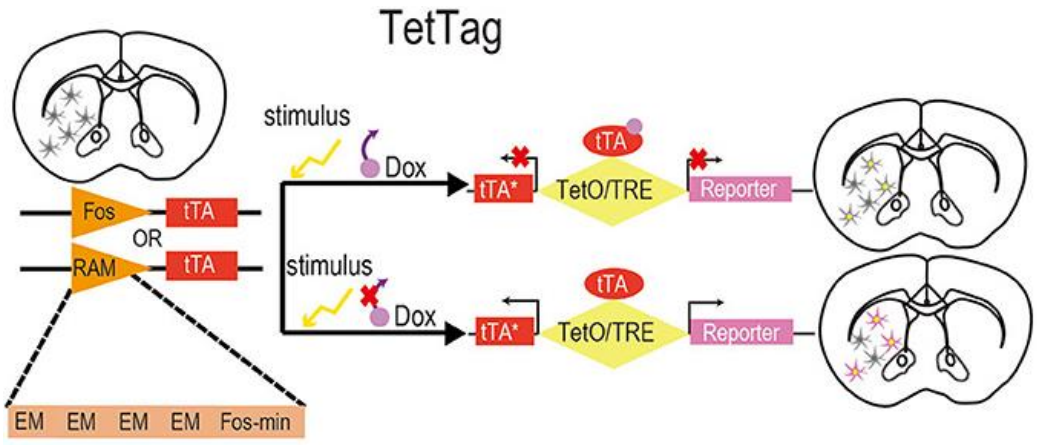
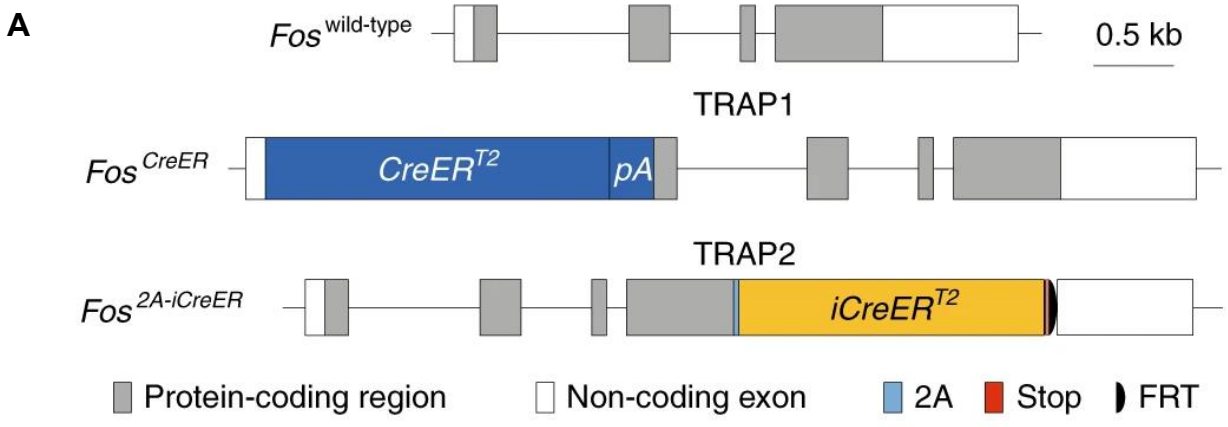


Figure. 25: **Fos/RAM-tTA transgenic models**. In Fos-tTA mouse model, absence of doxycycline result in activity induced Fos expression driven tTA production, which then binds to

TetO elements to activate reporter genes, thus enabling temporally controlled labelling of activated neurons. RAM system uses a synthetic activity-dependent promoter which destabilizes tTA to label activated neurons. This strategy provides better temporal precisions, tagging efficiency and signal-to-noise ratio[271].

Next in line is Fos-TRAP mouse model, which uses a truncated Fos promoter and has Fos open reading frame replaced with tamoxifen dependent Cre recombinase iCreERT2[273,274]. This model is used together with a Cre-dependent recombinase reporter, either transgenic or viral, to permanently label the Fos engram neurons. This system like Fos-tTA also provides temporal flexibility thanks to tamoxifen-dependent translocation of iCreERT2 protein from the cytosol to the nucleus. New and improved version has been recently developed named TRAP2, which has in-frame insertion of a 2A-iCreERT2 cassette at the end of the Fos open reading frame, thereby preserving endogenous Fos expression[275]. Despite the fact that TRAP strategy overcame some of the technical limitations associated with previous Fos tagging systems, nevertheless a few restrictions still exist. For instance, Cre-dependent reporter protein takes time to accumulate and TRAP system has lower tagging efficiency compared to Fos-tTA mouse strain (**Figure. 26**).



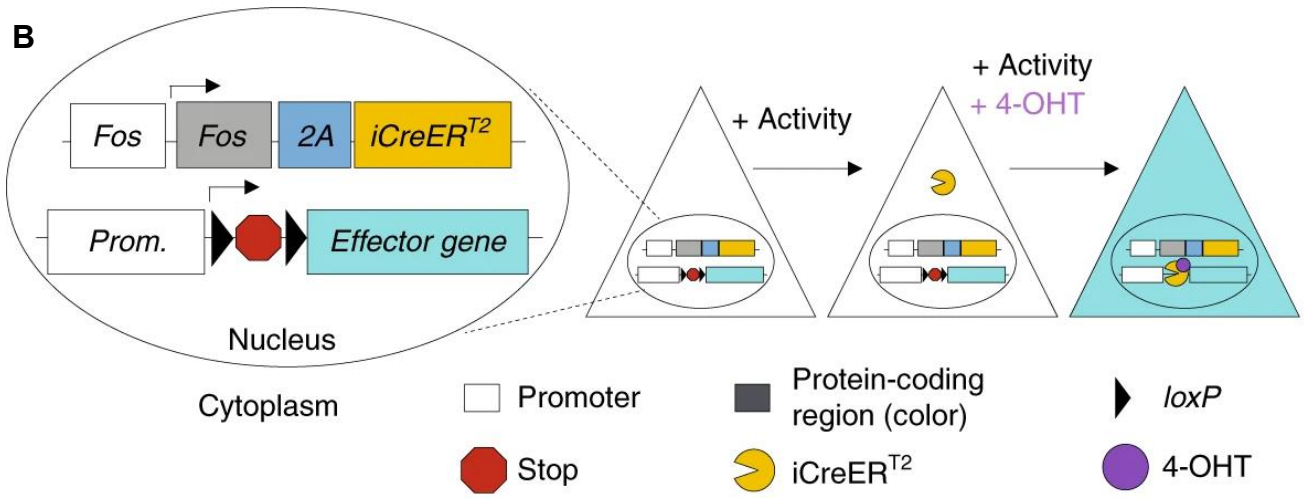


Figure. 26: **TRAP mouse models**. **A**: TRAP1 and TRAP2 comparison. **B**: TRAP2 mouse model schematics[166].

The most recent engram capturing strategy is based on Robust Activity Marking (RAM) system. This system has improved Dox-off strategy which allows destabilization of tTA or d2-tTA thus reducing the accumulation of tTA from prior irrelevant neuronal activation, resulting in improved signal-to-noise ratio of engram cells capturing[276]. Moreover, RAM reporters can be packaged into AAV particles thus bypassing the need for transgenic animals and this viral-based RAM strategy can be applied to species other than mice[276,277]. F-RAM (FOS-dependent Robust Activity Marking) is the first one to be developed to tag the Fos ensembles and it uses AP-1 DNA binding element as a reporter which is activated by endogenous Fos protein[209]. It offers multiple advantages over the previous strategies, such as it recapitulates the endogenous activation dynamics of Fos, selectively labels neurons in which Fos activation leads to transcriptional, structural, and functional outcomes, and lastly, it provides high noise-to-signal ratio and is compact enough to be packaged into AAVs.

1.15.3. Arc Ensembles Labeling Strategies

The first mouse model developed to label active Arc positive neurons was knock-in mouse strain which harbored a destabilized d2EGFP sequence replacing entire Arc open reading frame. d2EGFP allowed *in vivo* monitoring of Arc activation in cortical regions during various behavioral paradigms[239,278–280]. Arc-d2EGFP mice contain only a single copy of endogenous Arc allele, which leads to reduced reporter expression and consequently weaker imaging

signals. More recent BAC transgenic mouse lines, such as Arc-dVenus[281] and TgArc/Arg3.1-d4EGFP[282] have substantially improved reporter signal intensity due to multiple transgene copies and are widely employed for *in vivo* 2 photon imaging[278,282,283]. Additional models include GENSAT BAC transgenic Arc mouse line and the Arc-luciferase BAC transgenic line[284,285]. The former has primarily been used for *in vitro* studies[240], whereas the latter for *in vivo* monitoring of Arc expression[285].

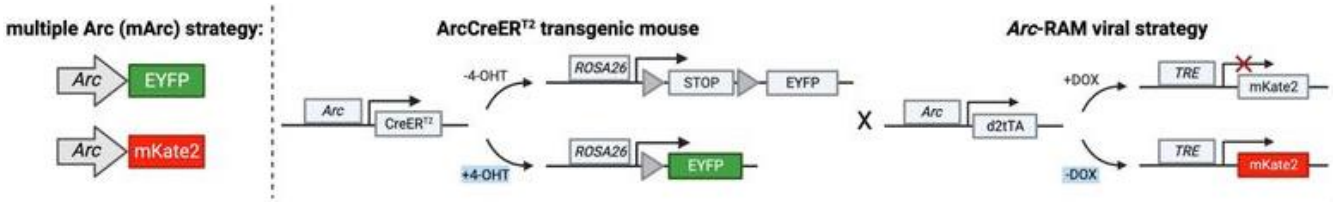


Figure. 27: Arc ensembles labeling strategies[286].

1.15.4. Egr1 based Engram Labeling Strategies

Despite being one of the earliest IEGs identified in nervous system, relatively few genetic strategies and mouse models have been developed to tag and visualize Egr1 engram neurons. The Egr1-EGFP BAC transgenic mouse, first generated by the GENSAT project[284,287], was the earliest model developed to visualize Egr1-expressing neurons, and subsequent studies have shown that EGFP expression faithfully recapitulates endogenous Egr1 transcription[254,288]. A more recent line, Egr1-CreERT2 enables permanent labeling of Egr1 expressing active neurons[289] and has been successfully used to tag neuronal populations activated by seizures and cocaine exposure[253] (**Figure. 28**).

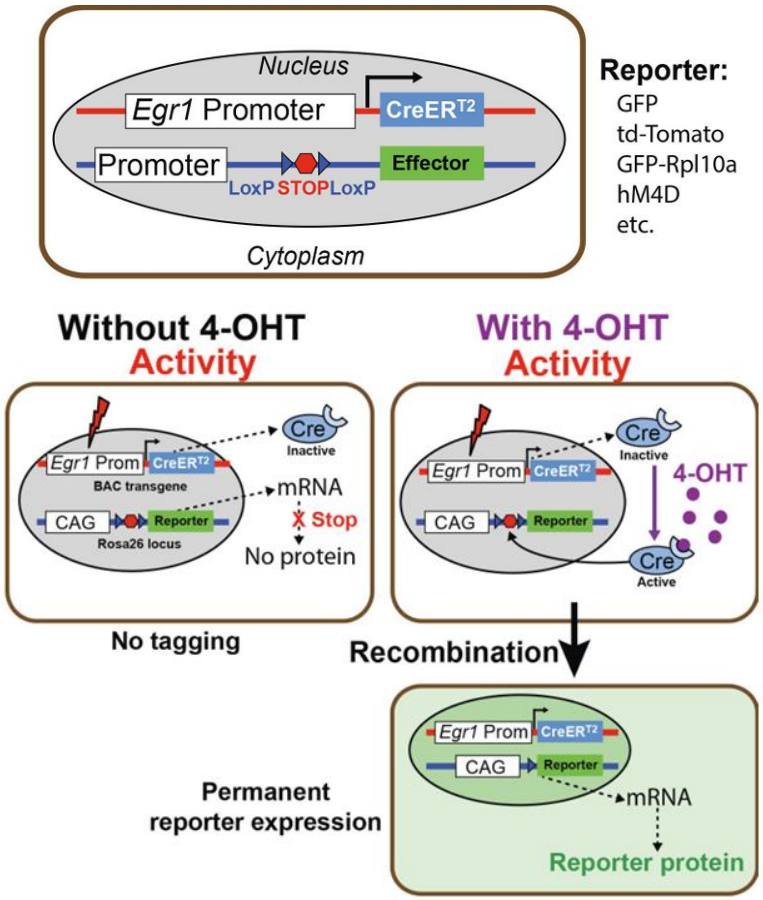


Figure. 28: **Activity-Dependent Permanent Labeling of Egr1 expressing neurons.** In Egr1-CreERT2 reporter mice, neuronal activation drives Egr1-dependent expression of CreERT2, but recombination occurs only in the presence of tamoxifen or 4-OHT, which enables nuclear translocation of CreERT2. Activated Cre removes a STOP cassette in Cre-dependent reporter alleles (e.g., EGFP, td-Tomato, TRAP, or hM4D), allowing effector expression. Once recombination occurs, the labeled neurons are permanently marked, enabling long-term tracking or manipulation of activity-defined ensembles[289].

1.15.5. Npas4-Dependent Neuronal Ensemble Labeling

Labeling strategies to tag Npas4 engram neurons are still in development but a few that have already been used include activity dependent NPAS4-RAM reporter system. This system consists of DNA-binding elements of NPAS4 and reflects the transcriptional output of Npas4 protein rather than gene and has an improved Dox-off system for temporal control[209,276]. Npas4-CreERT2 or Npas4TRAP mouse lines have also been generated, however, they have not yet been employed for investigating memory engrams[290,291] (**Figure. 29**).

Figure. 29: NPAS4-TRAP mouse model[291].

2. Aims

Previous findings from our lab demonstrated that neocortical pyramidal neurons with elevated Foxg1 expression exhibit increased spike frequency in electroencephalography recordings and are more susceptible to kainic acid-induced seizures. These Foxg1 gain-of-function neurons were observed to be hyperactive and hypersynchronized. Interestingly, artificially increasing the electrical activity of neurons resulted in upregulation of Foxg1 at both mRNA and protein levels. Since a neuron's intrinsic excitability strongly influences its likelihood of being recruited into a memory engram, we hypothesized that Foxg1 may influence the recruitment of neurons into engram ensembles and the expression of contextual fear memory. To test this hypothesis, we designed the following experimental objectives:

I. Determine the role of Foxg1 in driving short- and long-term contextual fear memory expression.
II. Assess whether Foxg1 influences the allocation and engagement of neurons into engram ensembles.
III. Explore the activity-dependent crosstalk between Foxg1 expression and neuronal excitability, a potential mechanism that may drive memory formation.

To address this, we combined adeno-associated viral manipulation of Foxg1 expression via artificial micro-RNAs (miRNAs), chemogenetic control of neuronal activity, contextual fear conditioning (CFC) assays, activity-dependent genetic labeling (TRAP2), and immunoprofiling of immediate early gene (IEG)-expressing engram cells.

3. Materials and methods

3.1. Mouse genetics

Homozygous Fos2A-iCreER/2A-iCreER (TRAP2^{+}/$^{+}$) mice on C57Bl/6N genetic background were bought from Jackson Labs and were bred and raised on site. TRAP2^{+}/$^{+}$ male mice were then mated with homozygous B6.Cg-Gt(ROSA)26Sortm9(CAG-tdTomato)Hze (Ai9^{+}/$^{+}$) females, kept on C57Bl/6J genetic background, generated starting from the corresponding pairs of founders available to us (a kind gift by prof. Paul Heppenstall, SISSA, Trieste). TRAP2^{+}/$^{-}$;Ai9^{+}/$^{-}$ mice were group-housed on a 12h light/dark cycle with food and water ad libitum. All experiments took place during the light phase and included mice from both sexes. Each experimental treatment included mice derived from at least 2 separate litters, each providing an identical or closely similar number of animals to each experimental group. At postnatal day 21 TRAP2^{+}/$^{-}$;Ai9^{+}/$^{-}$ mice were weaned from the breading cages and group-housed with same sex-littermates in standard mouse housing cages (3-5 mice per cage) till postnatal day 30. These animals were specifically employed for short-term fear conditioning assays.

C57BL/6 wild-type mice, males and females, were conversely used for long-term memory and chemogenetics experiments. They underwent same maintenance, light/dark parameters and experimental grouping procedures mentioned above.

CD1 wild-type mice, males and females, were used for KA experiments and underwent same maintenance and light/dark parameters mentioned above. On postnatal day 30, they were treated with KA for different time periods and were then subjected to sacrifice for further analysis.

3.2. Drugs

Tamoxifen

Tamoxifen (Sigma, T5648) was dissolved in corn oil at a concentration of 20 mg/ml by shaking at 65°C for one hour (tube protected from light with tin foil). The stock was kept at 4°C for up to a week. Before injecting, tamoxifen was warmed at 65°C for 10min. A 27-gauge needle was used to do a single intra-peritoneal injection of tamoxifen, 100mg per kg of body weight.

DREADD agonist Deschloroclozapine (DCZ)

DCZ dosage was determined based on previous studies. Specifically, 100mg DCZ (Sigma, cat# SML3651) were administered per kg of animal body weight. To this aim, DCZ was dissolved in 2% dimethyl sulfoxide (DMSO) in saline, to achieve a working solution to be injected at 0.1ml/animal.

Kainic acid (KA)

KA (Sigma, cat# K0250) was prepared in normal saline and was administered via intra-peritoneal route at a dosage of 20mg/kg of mouse body. This dosage was enough to induce sufficient increase in neuronal activity without any seizures, for which mice were monitored throughout the experimental procedure.

3.3. AAVs constructs and production

We used AAVs for mildly increasing or decreasing neuronal Foxg1 expression levels, as well as for altering neuronal excitability.

AAVs employed in this study include:

scAAV_hSyn1p-EmGFP-miR(-);

scAAV_hSyn1p-EmGFP-miR.aFoxg1.1690;

scAAV_hSyn1p-EmGFP-miR.aFoxg1.1694;

ssAAV_hSyn1p-dALPP.P2A.EmGFP-miR(-);

ssAAV_hSyn1p-hM3D(Gq).P2A.EmGFP-miR(-);

ssAAV_hSyn1p-hM4D(Gi).P2A.EmGFP-miR(-);

ssAAV_hSyn1p-hM3D(Gq).P2A.EmGFP-miR.aFoxg1.1690;

ssAAV_hSyn1p-hM4D(Gi).P2A.EmGFP-miR.aFoxg1.1694.

Full sequences of their cargo plasmids are reported in Supplementary Table 1.

These AAVs were pseudotyped as BBB-permeant, CPP21 particles. They were generated and titrated in house as described.

3.3. Surgery/AAVs injections

All surgical procedures took place at P30 unless otherwise specified. Mice were anesthetized by intraperitoneal administration of xylazine (20 μg/gram of body mass) and ketamine (80 μg/gram of body mass) and were transferred to a heated surface (at 37°C) to prevent hypothermia. Afterwards, a single AAV injection was done into the retro-ocular sinus, carried by a 300μl zero dead volume insulin syringe, equipped with 31-gauge needle. To ease the access of the retro-orbital vein, slight pressure was applied on both sides of the eye in order to make the eyeball protrude, paying attention not to damage its walls. The required number of recombinant AAVs (10^8 viral genomes/mouse) was resuspended in a PBS volume not exceeding 100ul and then injected into the vein. The injection was performed slowly, in approximately 10 seconds, and the needle was left in the venous sinus for another 5 seconds and then gently withdrawn. Once AAV administration was completed, mice received a subcutaneous injection of non-steroidal anti-inflammatory Carprofen (5μg per gram of body mass) and an intraperitoneal injection of antibiotic Baytril (5μg per gram of body mass) to prevent any discomfort/pain or infections. Immediately afterwards, mice were transferred to a hot plate set at 37°C and monitored for the recovery. About 30 minutes after they woke up, mice were transferred back to their respective home cages and monitored regularly for infection symptoms (hypomobility or hyperactivity) till the day of behavioral testing.

3.4. Fear conditioning

Contextual fear conditioning (Figure 1) was performed in test chambers (UgoBasile 46000-596) equipped with an electrified grid floor and dual video camera (for visible and infrared light) connected with AnyMaze software for the automated quantification of time of immobilization of the animal. Mice were trained at P49, 16-20 hrs after tamoxifen administration, in the contextual chamber A (grid-floor with black and white square walls). During the training session, mice were kept in the chamber for 2min, then they received three 0.5mA foot-shocks, each lasting 2sec and spaced by 1min intervals, and finally, 1min after the last foot-shock, they were removed from the chamber. The next day, mice were placed in the training chamber (context A) and (a) total freezing time (cessation of movement, except for breathing, with threshold set at 2sec), (b) number of freezing episodes, and (c) latency to freeze, were scored during the entire testing session, automatically by the Anymaze software. One day later, mice were also tested in a similar but novel chamber (Context B), which was the same size as context A, equipped with

white plastic floor and plain gray walls, where they were scored for the same parameters described above.

A similar contextual fear conditioning paradigm (Figure 3) was followed for the long-term memory retrieval. In this case, mice were trained at P49 in the context A and were tested in the same chamber at P56 for the memory recall, scoring them for total freezing time, number of freezing episodes, and latency to freeze.

As for the chemogenetics experiments (Figure 4), a similar contextual fear conditioning paradigm was also followed. In this case, all groups were trained on P56, and one day later, 20min before the memory retrieval session, DCZ was administered to either activate or inhibit neuronal activity of transduced cells. Saline was given to control animals. Mice were then tested in the training context for memory retrieval.

3.5. Brain dissection

For engram studies, mice subjected to contextual fear conditioning were anesthetized one hour after the last retrieval step via intraperitoneal administration of xylazine (60 μg/gram of body mass) and ketamine (240 μg/gram of body mass). Subsequently, they were perfused transcardially with cold (4°C) phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. Immediately afterwards, they were decapitated. Brains were extracted, kept in 4% PFA at 4°C for 24 hours and transferred to 30% sucrose in PBS solution till sectioning.

For KA experiments, KA administered mice were anesthetized as above, perfused with cold PBS and decapitated. Half the brain from each animal was used to dissect neocortex, and hippocampal formation, for RNA extraction. The other half was kept in 4% PFA overnight and then transferred to 30% sucrose solution, till sectioning for immunofluorescence.

3.6. Total mRNA profiling

Total RNA was extracted from neocortex and hippocampal formation by Trizol reagent (Thermofisher) according to manufacturer’s instructions. RNA was precipitated by isopropanol overnight at −80°C, washed with 75% ethanol twice and resuspended in 20μl sterile nuclease-free deionized water. Spectrophotometric measurements (NanoDrop ND-1000) and agarose gel electrophoresis were used to estimate RNA concentration, quality and purity. Before retro-

transcription, all RNA samples were treated with TURBO DNA-freeTMKit (ThermoFisher Scientific) to remove any residual DNA. For each experimental session at least 500ng of purified RNA was retro-transcribed by SuperScript-IIITM (Invitrogen) in the presence of random hexamers, according to the manufacturer's instructions. 1/100 of the resulting cDNA was used as a substrate of any subsequent qPCR reaction. Negative controls were run on RT (-) cDNA preparations and included in each PCR session. qPCR reactions were done using SsoAdvanced SYBR Green SupermixTM platform (Biorad) according to the manufacturer's instructions. Three technical replicates were run per each cDNA sample, analyzed against absolute standards and averaged. These averages were then normalized against Gapdh and further normalized against controls.

qPCR oligonucleotides are listed below:

GAPDH-Forward: 5' ATC TTC TTG TGC AGT GCC AGC CTC GTC 3'

GAPDH-Reverse: 5' GAA CAT GTA GAC CAT GTA GTT GAG GTC AAT GAA GG 3'

Foxg1-midF- Forward: 5' GAC AAG AAG AAC GGC AAG TAC GAG AAG C 3'

Foxg1-midF- Reverse: 5' GAA CTC ATA GAT GCC ATT GAG CGT CAG G 3'

mFos/F1N- Forward: 5' CTG ACA GAT ACA CTC CAA GCG GAG ACA G 3'

mFos/R1N- Reverse: 5' ACA TCT CCT CTG GGA AGC CAA GGT CAT C 3'

ARC-Forward: 5' CCC TCA TCT GTC TGC CCT GG 3'

ARC-Reverse: 5' ACC CAA AGA GCC CTG GAC AC 3'

3.7. Histology and Immunostaining

Brains were sectioned using a microtome into 50μm thick coronal sections (for engram studies) and 20μm thick sagittal sections (for kainic acid treatment experiments). These sections were collected in 0.01% sodium azide in PBS for long-term storage at 4°C. For Foxg1, cFos and GFP immunostaining, sections were incubated in permeabilization buffer (0.1% Triton-X100 in PBS) for 1hour at RT, then in blocking mix (5% bovine serum albumin in PBS) for 1hour at RT and finally stained with rabbit polyclonal anti-Foxg1 (a gift from G.Corte, 1:200), mouse anti-cFos (Abcam 208942, 1:1000), and chicken anti-GFP (GeneTex GTX13970, 1:4000) antibodies in

blocking mix overnight at 4°C. All sections were then washed 3 times for 10min with washing solution (0.05% Triton-X100 in PBS) and then incubated with secondary antibodies, anti-mouse Alexa 647 (Invitrogen, 1:600) and anti-chicken Alexa 488 (Invitrogen, 1:600) in blocking mix, along with DAPI, for 2hours at room temperature. Sections were then washed 3 times for 10min with washing solution, mounted on Superfrost Plus slides, coverslipped by Vectashield mounting medium (Vector), and kept at 4°C till imaging. Confocal images were finally obtained with a Nikon A1 confocal microscope.

3.8. Image processing and analysis

Cell counts were calculated by an operator blind to experimental conditions. At least 8-9 images were analyzed from each mouse brain and for accurate regional analysis these images were aligned to mouse Allen Brain Atlas. Cells stained for DAPI, EGFP, cFos and tdTomato (in Ai9 mice) were quantified using the quPath software, with cell detection parameters optimized and validated against negative controls.

Quantification of Foxg1 expression in active ($cFos^+$) vs non-active ($cFos^-$) neuronal populations upon KA administration was done by using Volocity software. Per each animal, absolute Foxg1 signal intensity was calculated in both populations and $cFos^+$ cells Foxg1 intensity was then normalized against cFos- cells.

3.9. Statistical analysis

Primary data originating from each experimental session were pre-normalized against the control average of such session and subsequently merged with data from other sessions, for further analysis. All these data are shown in Supplemental Table 2. Statistical significance of differences among behavioral (freezing time, number of freezing episodes and latency) and engram parameters (ROR and absolute percentages of $cFos^+$ and $TdTomato^+$ cells) was calculated by one-tailed t-test (paired and unpaired in case of intra- and inter-group comparisons). In any case the number of statistical replicates equaled the number of animals analyzed.

4. Results

4.1. Foxg1 promotes neuron recruitment into short-term memory engrams and facilitates fear memory retrieval

As mentioned, the probability of a neuron being recruited into an engram is strongly influenced by its intrinsic excitability[172,173]. Since Foxg1 promotes neuronal activity and excitability[101], we asked whether manipulating its expression levels within a random subset of neurons accordingly modulates their recruitment into an engram. To achieve sparse (and gentle) modulation of neuronal Foxg1 expression levels, we employed adeno-associated viruses (AAVs) encoding for artificial, Foxg1-activating or -inhibiting microRNAs. These AAVs and their control also encoded for Emerald Green Fluorescent Protein, EmGFP, allowing the identification of transduced cells.

To track neurons engaged in the training engram, we utilized the tamoxifen-activatable TRAP2:Ai9 reporter system[166,273]. Specifically, on post-natal day 28 (P28), TRAP2:Ai9 reporter mice received retro-orbital injections of Foxg1-modulating AAVs. Next, on P56, they were intraperitoneally injected with 100mg tamoxifen per kg of body weight and, 16-20 hours later, they underwent a CFC assay, to permanently label neurons activated during memory encoding (**Figure. 1A, B**).

Interestingly, at this time all treatment groups displayed comparable freezing times, suggesting similar efficiency in encoding fear experience (**Figure. 1C** and Supplementary Figure. 1A, B). One day later, trained mice were interrogated for memory retrieval in the original A training context. Animals made sparsely gain-of-function (“GOF”) for Foxg1 exhibited longer freezing times compared to both controls (Ctrl) and sparsely loss-of-function (“LOF”) models [GOF: (168.7±12.9)s vs Ctrl: (130.4±12.6)s, with $p<0.03$, and vs LOF: (130.8±14.9)s, with $p<0.03$], indicating a positive impact of Foxg1 on memory recall (**Figure. 1D**). One more day later, the same animals were further interrogated for memory retrieval in a novel B context, with distinct tactile and visual cues. Here, GOF mice showed an appreciable trend to outperform LOF models in contextual discrimination ($p<0.09$) (**Figure. 1E**). This suggests that sparsely elevated Foxg1 not only strengthens memory retrieval but may also improve its precision.

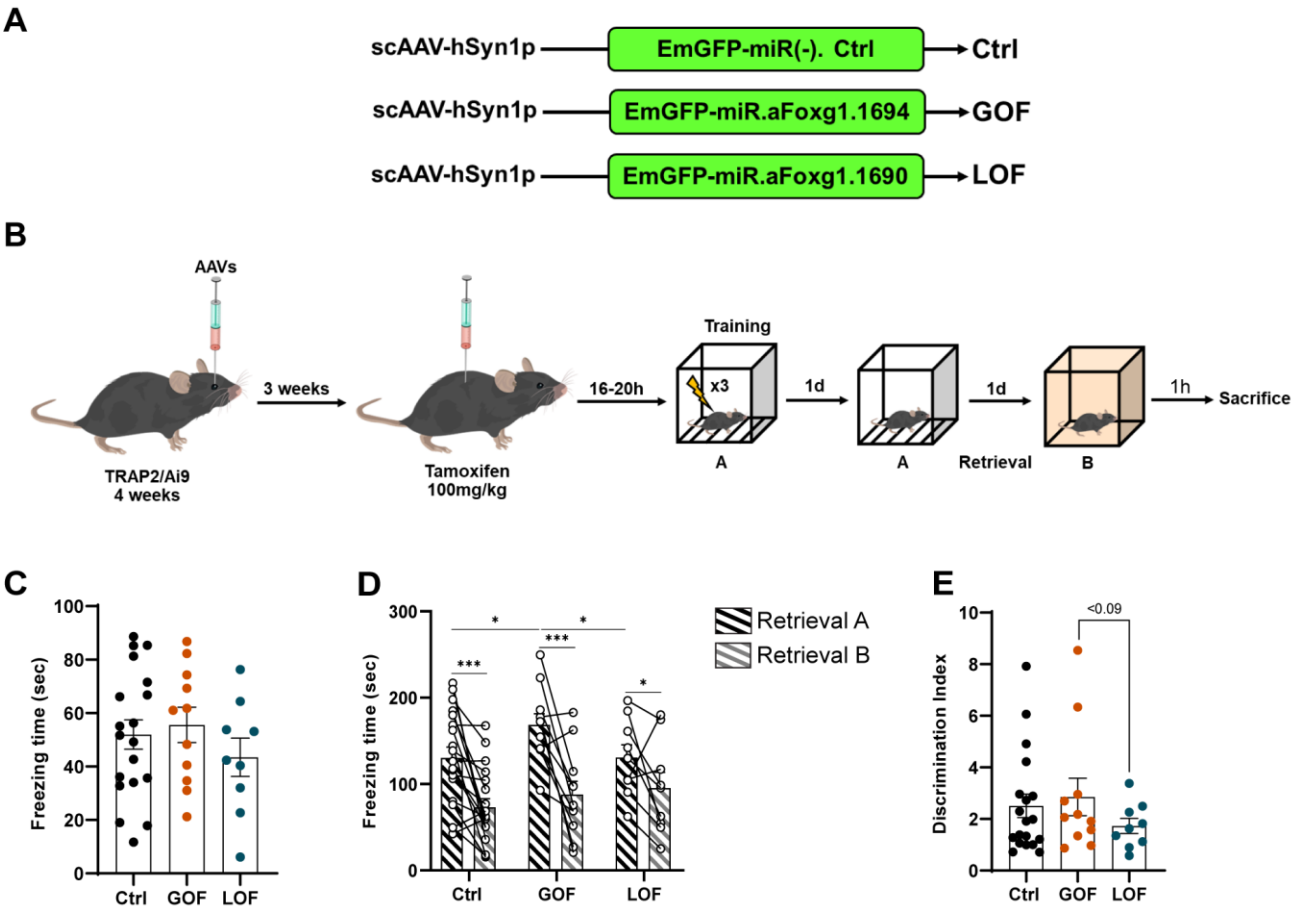
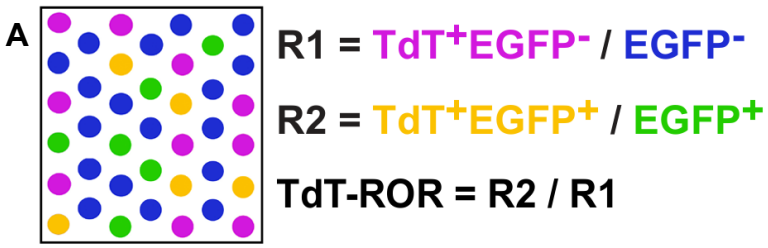
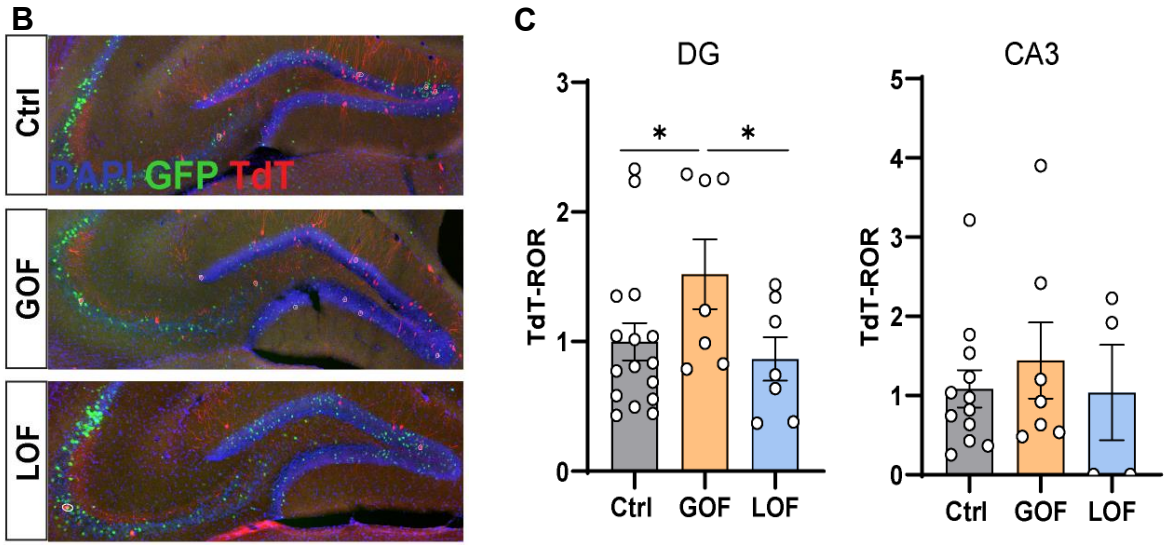


Figure. 1: **Foxg1 facilitates fear memory retrieval**. **A**: Schematics of adeno associated viruses (AAVs), control (Ctrl), gain-of function (GOF), and loss of function (LOF) for Foxg1, delivered retro-orbitally into TRAP2/Ai9 mice. **B**: Experimental timeline. **C**: Distribution of total freezing times (sec) during training session of contextual fear conditioning (CFC), in Ctrl, GOF, and LOF groups. **D**: Distribution of total freezing time (sec) during retrieval sessions, in the training context (Retrieval A) and a novel context (Retrieval B). **E**: Context discrimination ratio (Retrieval A vs Retrieval B) in Ctrl, GOF, and LOF groups. C, D, E: n = 19 (Ctrl), 11 (GOF), 9 (LOF). Error bars indicate standard error of the mean (SEM). Statistical significance of results was evaluated by t-test assay (unpaired between groups, paired within groups, one-tailed). * p<0.05, ** p< 0.01, *** p< 0.001.

Finally, two hours after the latter retrieval session, to quantify the impact of distinctive Foxg1 manipulations on neuron recruitment into engrams underlying training and retrieval processes, the animals were perfused and analyzed (**Figure. 1B**). As for the training engram, the

prevalence of TRAPed cells (TdTomato$^+$ cells) among transduced (EGFP$^+$) and non-transduced neurons (EGFP$^-$) was evaluated, and the ratio between the former and the latter (ratio of ratios, ROR) was employed as an index of this impact (**Figure. 2A**). This analysis was primarily performed across key short-term memory-related regions, such as the hippocampal formation, including dentate gyrus (DG) and Cornu Ammonis 3 field (CA3), and the amygdala, including basolateral (BLA) and central amygdala (CeA) (**Figure. 2B, D**). Compared to controls, TdTomato-ROR (TdT-ROR) was increased in DG [+(52.01±26.87)%, $p<0.03$] and CeA [+(104.64±50.45)%, $p<0.01$] upon Foxg1 upregulation and decreased in BLA [-(47.06±13.92)%, $p<0.03$] and CeA [-(34.97±12.02)%, $p<0.05$] upon Foxg1 downregulation (**Figure. 2C, E**), as expected. Additional memory-associated regions were further profiled. Mirroring the pattern observed in DG and amygdala, Foxg1 upregulation increased TdT-ROR in medial prefrontal cortex, including prelimbic (PL) and infralimbic (IL) cortices, as well as in the hypothalamus (Hypo), while the opposite manipulation reduced TdT-ROR in Hypo (Supplementary Figure. 2B, C). Curiously, Foxg1 downregulation increased neuronal engagement in motor and somatosensory cortex (MC, SC) (Supplementary Figure. 2A).





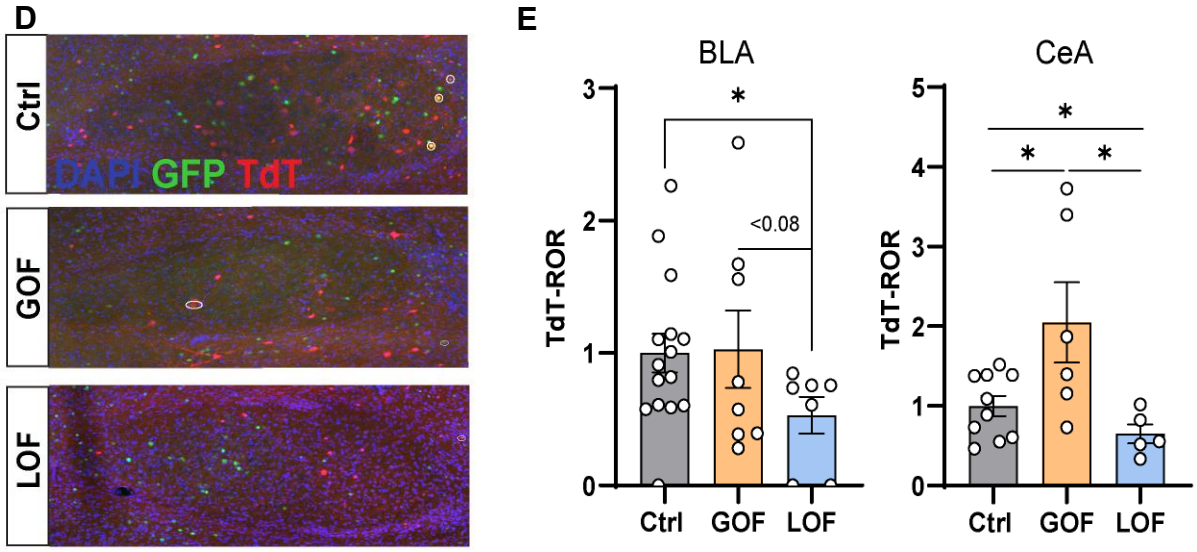


Figure. 2: **Foxg1 promotes neuron recruitment into training engrams**. **A**. Engram analysis procedure. **B, C**: Representative images of DG, CA3 and quantification of TdT-RORs in these regions of brains treated with Ctrl, GOF, and LOF AAVs. **D, E**: Representative images of BLA, CeA and their respective quantified TdT-RORs. C,E: n = 10-16 (Ctrl), 6-7 (GOF), 4-7 (LOF). ROR, ratio of ratios; DG, dentate gyrus; CA3, hippocampal CA3 field; BLA, basolateral amygdala; CeA, central amygdala. Error bars indicate standard error of the mean (SEM). Statistical significance of results was evaluated by t-test assay (unpaired between groups, paired within groups, one-tailed). * $p<0.05$.

These results indicate that, rather than generally promoting neuronal recruitment into the training engram, Foxg1 exerts a region-specific bidirectional effect, whereby higher Foxg1 promotes neuronal recruitment into hippocampal, amygdalar, and prefrontal ensembles, and reduces this process within sensory and motor cortices.

Concerning Foxg1 impact on neuronal engagement in the retrieval engram, the prevalence of $cFos^+$ cells among $EGFP^+$ and $EGFP^-$ neurons was evaluated, and the corresponding ROR (cFos-ROR) was employed as an index of this impact (**Figure. 3A**). Compared to controls, cFos-ROR was increased in DG [+(47.29±17.88)%, $p<0.02$], CA3 [+(196.60±67.80)%, $p<10^{-4}$], BLA [+(152.65±61.90)%, $p<0.002$], and CeA [+(52.03±46.77)%, $p<0.08$] upon Foxg1 upregulation, and decreased in CeA [-(42.95±4.70)%, $p<0.03$] upon Foxg1 downregulation (**Figure. 3B, C**). Interestingly, Foxg1 also positively affected cFos-ROR in PL, IL, Hypo, MC

and piriform cortex (PC) (Supplementary Figure 2D-F). Altogether, these results further suggest that at the time of memory retrieval, elevated Foxg1 generally favors neuronal engagement in the engram.

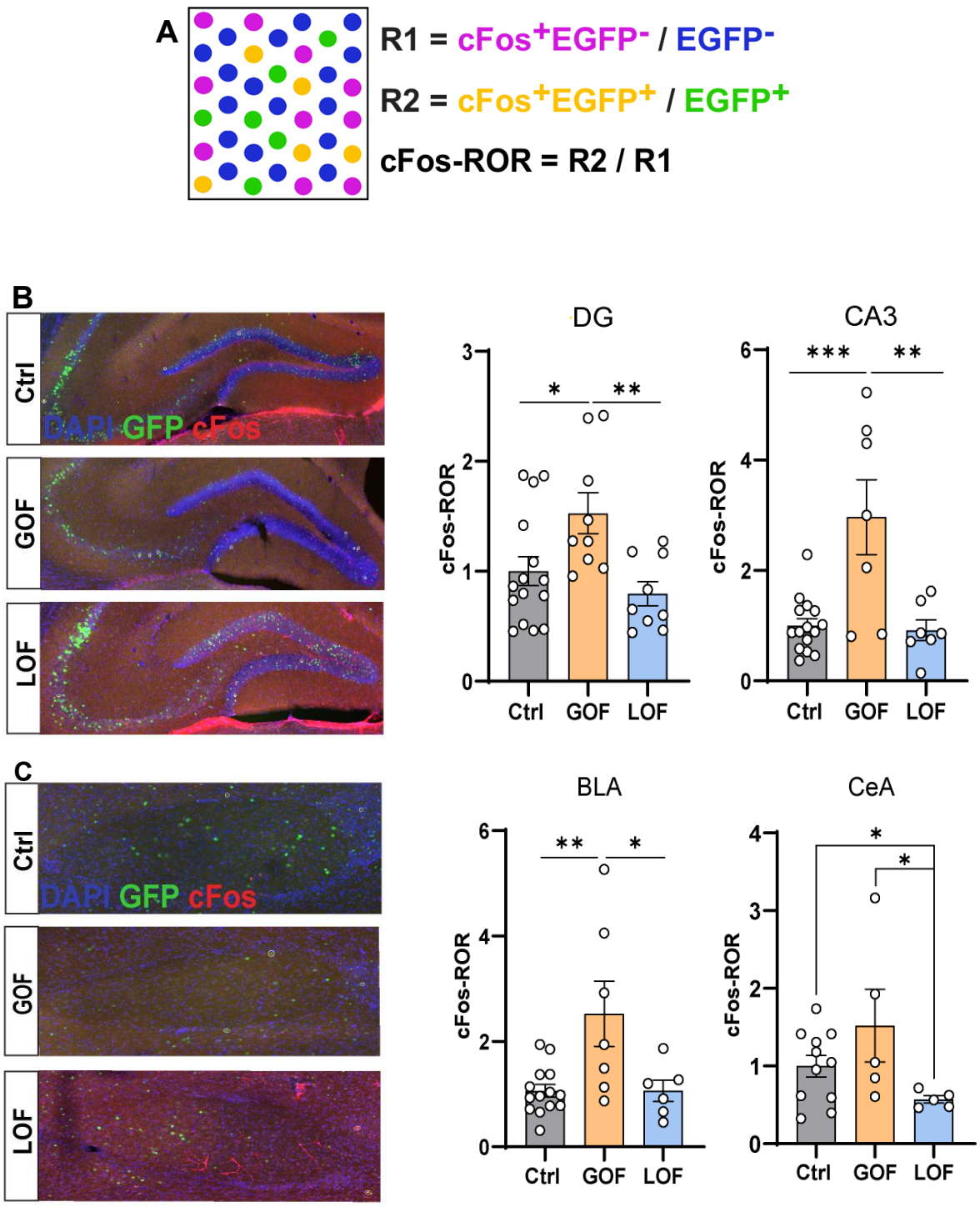


Figure. 3: **Foxg1 promotes neuron recruitment into short term memory retrieval engrams**. **A**. Engram analysis procedure. **B**: Representative images of DG, CA3 and quantification of cFos-RORs in these regions of brains treated with Ctrl, GOF, and LOF AAVs. **C**: Representative images of BLA, CeA and their respective quantified cFos-RORs. C,B: n = 11-15 (Ctrl), 5-9 (GOF), 5-9 (LOF). ROR, ratio of ratios; DG, dentate gyrus; CA3, hippocampal CA3 field; BLA, basolateral amygdala; CeA, central amygdala. Error bars indicate standard error of the mean (SEM). Statistical significance of results was evaluated by t-test assay (unpaired between groups, paired within groups, one-tailed). * p<0.05, ** *p*< 0.01, *** *p*< 0.001.

4.2. Foxg1 reduces engram size at the time of memory encoding and short-term retrieval

A key feature of memory traces is the dynamic regulation of engram size, i.e., the proportion of neurons recruited into an engram during encoding and active during recall[292–294]. To test whether Foxg1 has any effect on this engram dynamics, first we analyzed engram size during memory encoding (TdTomato$^+$/DAPI$^+$) and retrieval (cFos$^+$/DAPI$^+$) across hippocampal, amygdalar, cortical, and hypothalamic regions.

Foxg1 manipulations produced consistent effects on engram size. Its upregulation resulted into a shrinkage of the training engram, in DG [GOF: (0.62±0.08)% vs. Ctrl: (1.40±0.23)%, *p*<0.02], CA3 [GOF: (0.24±0.06)% vs. Ctrl: 0.33±0.03%, *p*<0.08], and CeA [GOF: (1.11±0.25)% vs. Ctrl: (2.42±0.30)%, *p*<0.004] (**Figure. 4A-D**), and the recall engram, in DG [GOF: (2.26±0.25)% vs. Ctrl: (2.86±0.29)%, *p*<0.07] and CA3 [GOF: (0.63±0.09)% vs. Ctrl: (1.27±0.26)%, *p*<0.05] (**Figure. 4E, F**). Similar phenomena were observed in neocortex, PC, prefrontal cortices and Hypo (Supplementary Figure. 3A-F) Surprisingly, a size reduction of training engram was observed in CA3 upon Foxg1 downregulation [LOF: (0.20±0.01)% vs. Ctrl: (0.33±0.03)%, *p*<0.02] (**Figure. 4B**). Considering the small fraction of AAV-transduced cells, these results indicate that the effects of Foxg1 manipulations are also largely not-cell autonomous.

Next, we evaluated the fraction of recall engram neurons that were also part of the training engram (TdTomato$^+$cFos$^+$/cFos$^+$). We found that this parameter was positively correlated to Foxg1 expression level, in most of the regions analyzed (CA3, BLA, CeA, MC, SC, AC, PC, PL, IL, and Hypo), displaying statistically significant changes in same cases (**Figure. 4I-L** and Supplementary Figure. 3G-I).

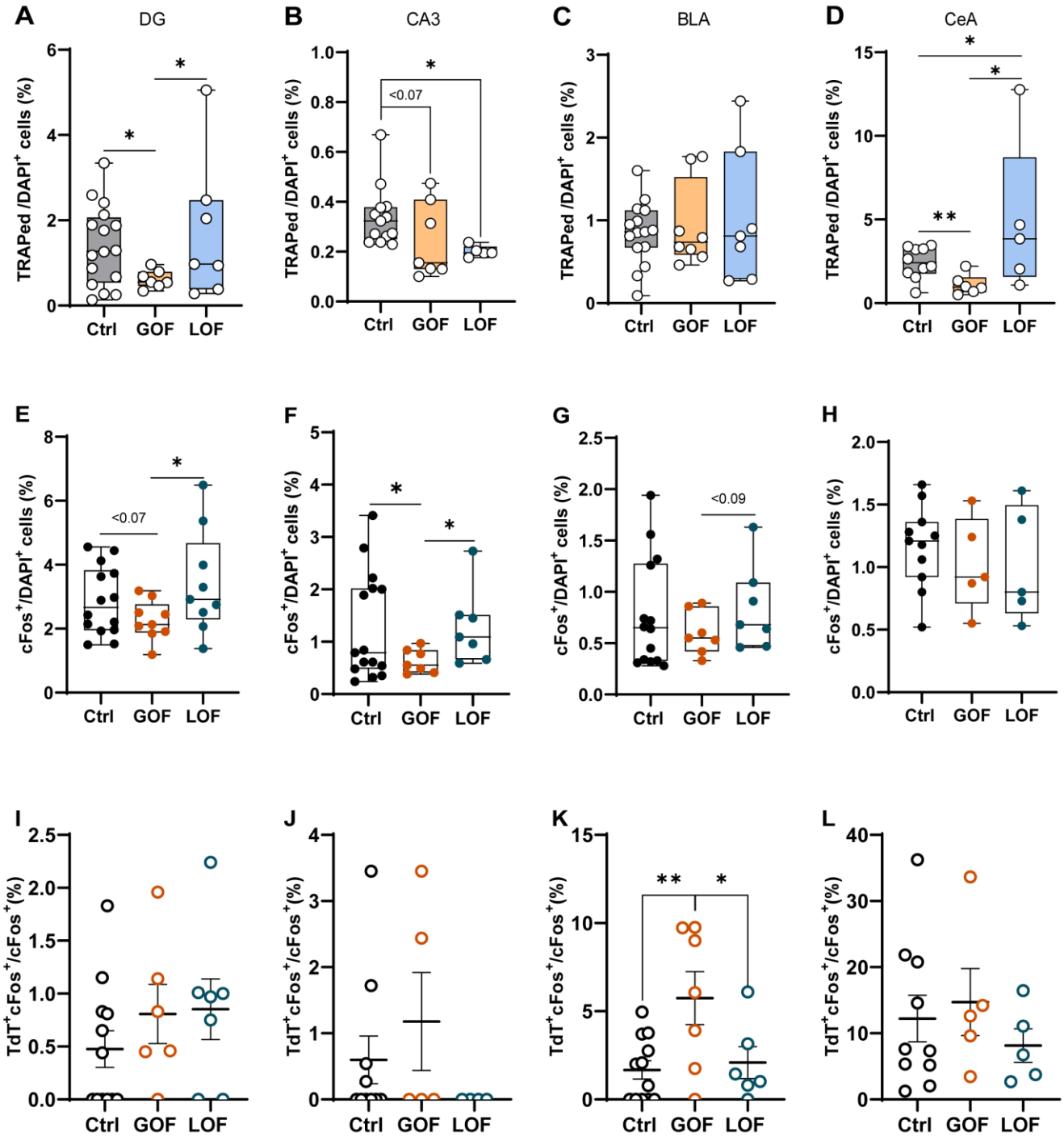


Figure. 4: **Foxg1 reduces engram size at the time of memory encoding and short-term retrieval. A-L**: Quantification of TRAPed cells (TdTomato⁺ cells/DAPI⁺ cells, **A-D**), cFos⁺ cells (cFos⁺ cells/DAPI⁺ cells, **E-H**), and double positive cells (TdTomato⁺ cFos⁺ cells/cFos+ cells, **I-L**), all displayed as absolute percentages. n = 10-17 (Ctrl), 4-10 (GOF), 5-9 (LOF). Error bars indicate standard error of the mean (SEM). Statistical significance of results was evaluated by

t-test assay (unpaired, one-tailed). * p<0.05, ** p<0.01. DG, dentate gyrus; CA3, hippocampal CA3 field; BLA, basolateral amygdala; CeA, central amygdala.

In synthesis, the engram downsizing and stabilizing effects elicited by Foxg1 upregulation apply to both training and retrieval engrams.

4.3. Foxg1 promotes neuron engagement in late memory engrams

Long-term memory recall requires the persistence of engram ensembles across time[295–297]. Previous work has shown that transcriptional regulators such as CREB, NFAT, and Npas4 contribute to the stabilization of memory traces during remote recall[173,258,298]. However, whether Foxg1 manipulations exert persisting effects on long-term memory recall and engram allocation remains elusive.

To test this, AAV-treated wild-type mice were trained in CFC training chamber on P49 and tested for recall in the same training context on P56 (**Figure. 5a: A, B**). All groups displayed robust memory retention, indicated by strong freezing behavior during recall. Although differences did not reach statistical significance, GOF mice exhibited the longest freezing time, (173.93±18.45)s, followed by the Ctrl group with (168.76±24.16)s, whereas LOF mice showed the shortest, (147.34±27.72)s (**Figure. 5a: C**). Consistent differences were also detectable in number of freezing episodes and freezing latency (Supplementary Figure. 4A, B).

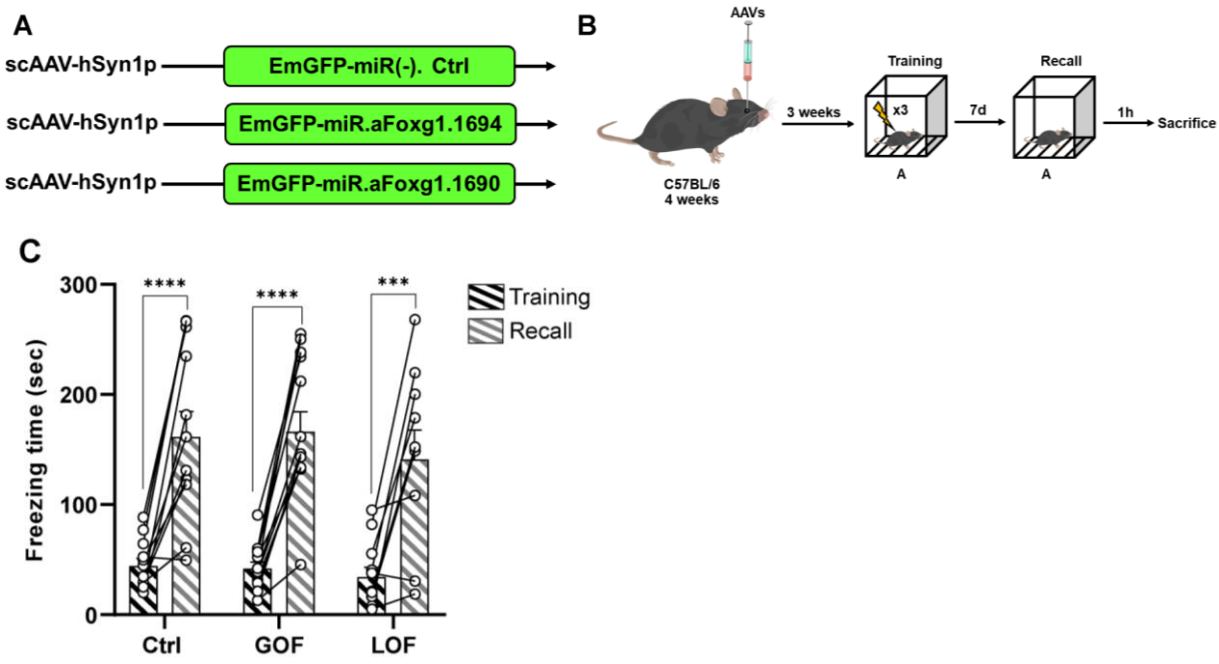
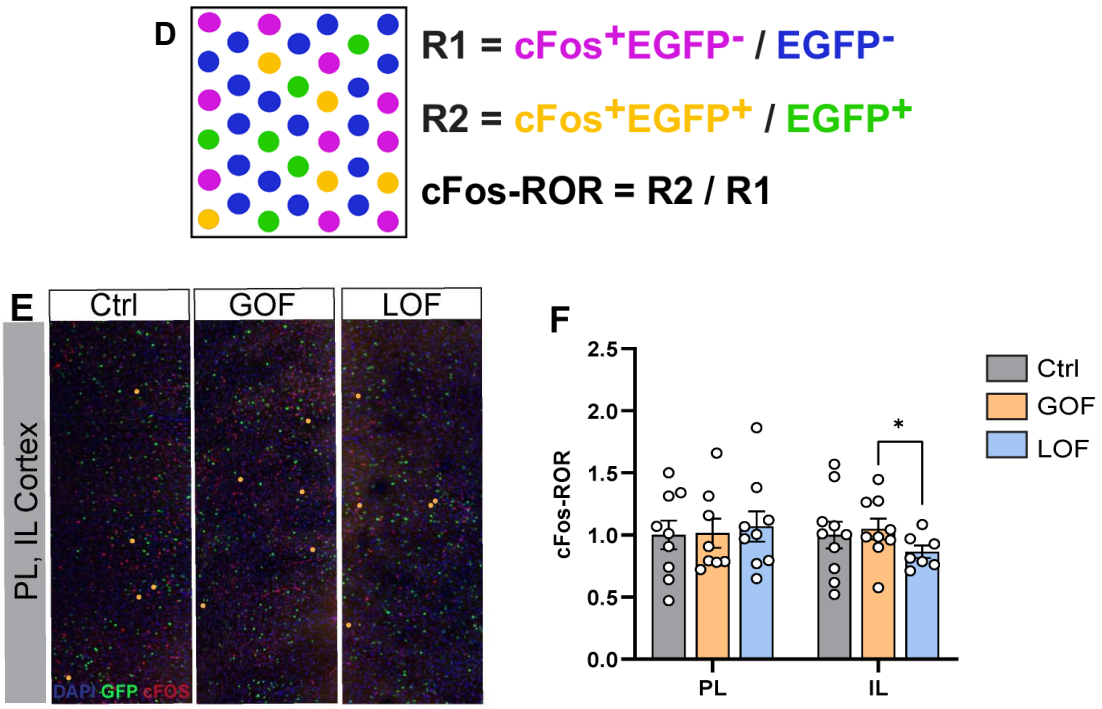


Figure. 5a: **Foxg1 modulates late fear memory recall**. **A**. Schematics of adeno associated viruses (AAVs), control (Ctrl), gain-of function (GOF), and loss of function (LOF) for Foxg1, delivered retro-orbitally into C57Bl/6 mice. **B**. Experimental timeline. **C**. Distribution of total freezing times (sec) during training and late recall sessions of CFC assay, in Ctrl, GOF, and LOF groups. n = 11 (Ctrl), 12 (GOF), 9 (LOF). Error bars indicate standard error of the mean (SEM). Statistical significance of results was evaluated by t-test assay (unpaired between groups, paired within groups, one-tailed). *** p<0.001.

To assess whether Foxg1 influences underlying neuron allocation to engrams, we quantified cFos-ROR (**Figure. 5b: D**) in cortical regions, preferentially involved in long-term memory formation. Here, "GOF mice" showed higher cFos-ROR compared to the "LOF group", with pronounced differences in IL [GOF: (104.83±8.48)% vs. LOF: (86.53±5.04)%, p<0.05)], MC [GOF: (124.05±15.06)% vs LOF: (47.09±7.89)%, p<0.001], SC [GOF: (137.17±20.74)% vs LOF: (82.63±12.75)%, p<0.05], AC [GOF: (118.25±16.96)% vs LOF: (76.15±11.86)%, p<0.04], and PC [GOF: (124.20±19.53)% vs LOF: (83.13±6.62)%, p<0.05]. Ctrl cFos-RORs generally were between GOF and LOF values (**Figure. 5b: E,F,H,I**). Consistently, Foxg1 down-regulation reduced the recall engram cFos-ROR in CA3, BLA, CeA and Hypo. Curiously, a reduction of this index was also achieved upon Foxg1 upregulation in Hypo (Supplementary Figure. 4C-E).



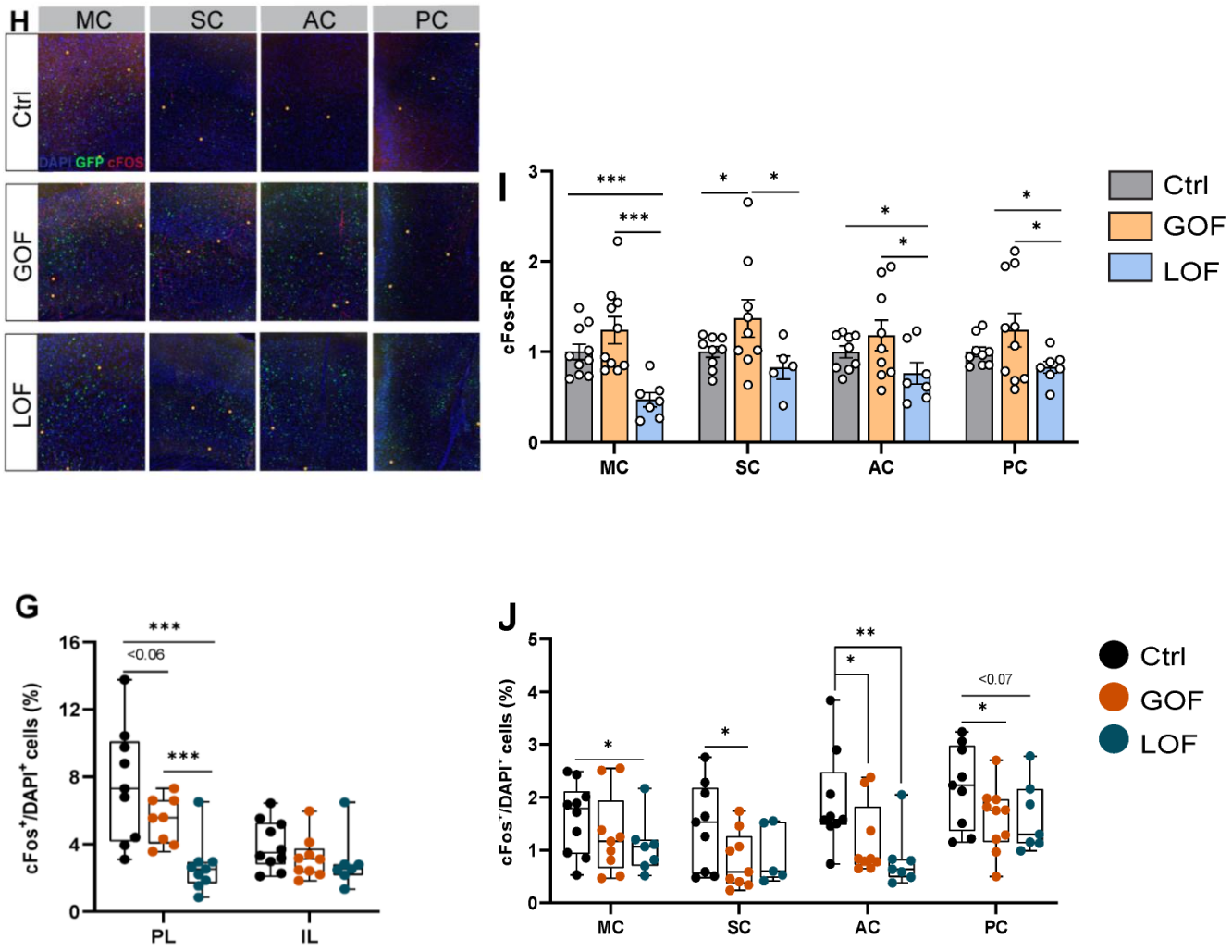


Figure. 5b: **Foxg1 promotes neuron engagement in late memory engrams**. **D**. Quantification analysis procedure. **E,H**. Representative immunofluorescence images employed for this analysis. **F,G,I,J**. Quantification of Ctrl-normalized cFos-ROR (F,I) and absolute cFos$^+$/DAPI$^+$ cell ratio (G,J) in selected regions of brains treated with Ctrl, GOF, and LOF AAVs. F,G,I,J: n = 9-11 (Ctrl), 8-11 (GOF), 5-9 (LOF). Error bars indicate standard error of the mean (SEM). Statistical significance of results was evaluated by t-test assay (unpaired between groups, paired within groups, one-tailed). * p<0.05, ** p<0.01, *** p<0.001, ROR, ratio of ratios; PL, pre-limbic cortex; IL, infra-limbic cortex; MC, motor cortex; SC, somatosensory cortex; AC, auditory cortex; PC, piriform cortex.

We next examined whether Foxg1 manipulations altered overall engram size, quantified as the percentage of cFos$^+$ cells relative to total (DAPI$^+$) cells. Compared to Ctrl, even at this stage, Foxg1 upregulation led to a shrinkage of engram in all analyzed regions except for Hypo.

Differently from what we saw at short-term memory formation, a reduced engram size was also generally observed upon Foxg1 down-regulation (**Figure. 5b: G,J** and Supplementary Figure. 4F-H).

Together, these results demonstrate that Foxg1 continues to regulate engram dynamics even during long-term recall, i.e., 7 days after initial training, however according to a non-monotonic pattern. Specifically, GOF enhanced preferential recruitment of neurons while reducing engram size, whereas LOF diminished both parameters.

4.4. Foxg1 regulates memory retrieval partly through activity-dependent mechanisms

Previous work from our laboratory demonstrated that increasing Foxg1 expression in primary neocortical neurons enhances their electrical activity, whereas reducing Foxg1 diminishes it[101]. Given that a key feature of engram neurons is their higher intrinsic excitability, we hypothesized that Foxg1 may regulate engram allocation and memory formation through activity-dependent mechanisms. To answer this question, we tested whether suppressing neuronal activity by inhibitory (hM4Di, Gi) Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) could counteract the effects of Foxg1 gain-of-function (GOF) manipulation on memory retrieval. The experimental session included five groups: control without DREADDs (Ctrl), control with inhibitory DREADD (Ctrl-Gi), Foxg1 GOF with Gi (GOF-Gi), and, as specificity controls, control with excitatory DREADD (Ctrl-Gq), Foxg1 LOF with Gq (LOF-Gq) (**Figure. 6A**). All mice were trained in the CFC chamber and, the following day, tested for memory recall in the same context (**Figure. 6B**). Fifteen to thirty minutes prior to recall, Deschloroclozapine (DCZ) was administered to either inhibit (Gi) or activate (Gq) transduced neurons. Memory recall was quantified using three parameters, i.e., total freezing time, number of freezing episodes, and freezing latency.

Ctrl-Gi mice displayed impaired memory recall, with the lowest total freezing and fewer freezing episodes, indicating that suppressing neuronal activity impairs memory expression[299,300] (LOF-Gq mice conversely exhibited freezing behavior comparable to Ctrl). GOF-Gi mice also displayed reduced freezing relative to Ctrl (**Figure. 6C**), further indicating that suppressing activity can counteract the effects of elevated Foxg1 (**Figure. 1D**). Consistently, changes in freezing episodes and latency evoked by Foxg1-GOF alone (Supplementary Figure. 1) were also abolished upon GOF-Gi manipulation (**Figure. 6D,E**).

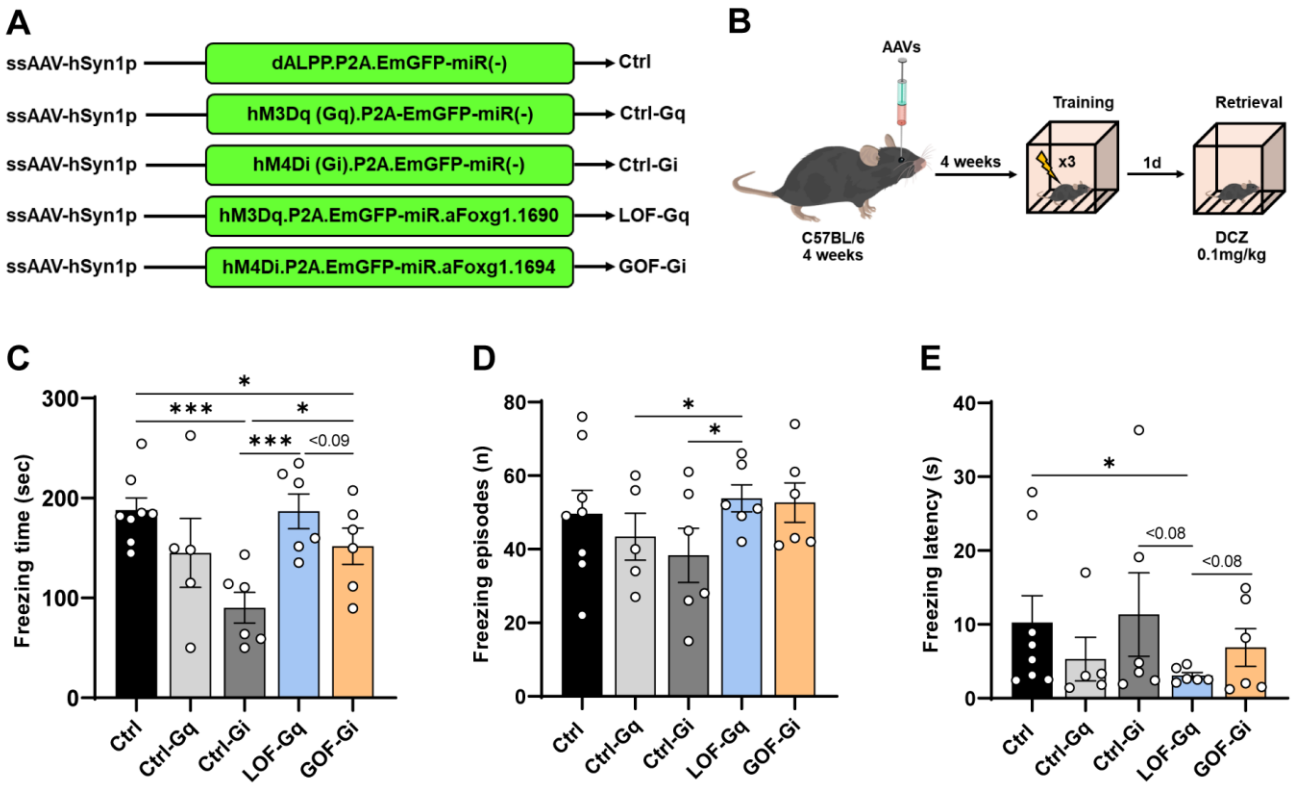


Figure. 6: **Foxg1 regulates memory retrieval partly through activity-dependent mechanisms**. **A**. Schematics of adeno-associated viruses (AAVs), control (Ctrl), Foxg1-Ctrl coupled with excitatory DREADD (Ctrl-Gq), Foxg1-Ctrl coupled with inhibitory DREADD (Ctrl-Gi), Foxg1 LOF with excitatory DREADD (LOF-Gq), and Foxg1-GOF with inhibitory DREADD (GOF-Gi), delivered retro-orbitally into C57Bl/6 mice. **B**. Experimental timeline. **C-E**. Distribution of total freezing time, freezing episodes, and freezing latency in Ctrl, Ctrl-Gq, Ctrl-Gi, LOF-Gq, and GOF-Gi AAVs treated mice, during memory retrieval in the training context. n = 8 (Ctrl), 5 (Ctrl-Gq), 6 (Ctrl-Gi), 6 (LOF Gq), and 6 (GOF-Gi). Error bars indicate the standard error of the mean (SEM). Statistical significance of results was evaluated by t-test assay (unpaired, one-tailed). * p<0.05, ** p<0.01, *** p<0.001.

5. Discussion

Our study identifies the transcription factor Foxg1 as a critical regulator of neuronal engram allocation, memory retrieval, and, possibly, context discrimination. To address these aspects, we employed a combination of approaches including TRAP2-based genetic labeling, viral vector-mediated Foxg1 modulation, and chemogenetic control of neuronal activity. We observed that sparse and mild Foxg1 upregulation improved learning abilities as evaluated by a CFC assay (**Figure. 1**). This effect was pronounced when 1 day elapsed between conditioning and retrieval sessions and lost statistical significance 7 days after conditioning (**Figures. 1, 5a**). Interestingly, this effect also disappeared when neurons gain-of-function for Foxg1 were silenced by a Gi DREADD effector (**Figure. 6**). Analysis of TRAPed and cFos^{+} cells displayed an overt positive relationship between Foxg1 expression level and the probability of a neuron getting recruited into training, retrieval and recall engrams. This phenomenon was detectable in DG, CA3, BLA and CeA, regions mainly involved in CFC. Here, it was observable at time of training and early retrieval upon Foxg1 upregulation, and at the time of delayed recall upon the opposite manipulation (**Figures. 2,3** and Supplementary Figure. 4). Moreover, a similar phenomenon was observed at the time of delayed recall in MC, SC, AC and PC, brain structures reported to be involved in late memory retrieval (**Figure. 5b**). Interestingly, Foxg1 upregulation also elicited a generalized shrinkage of engrams, at time of training, early retrieval and delayed recall (**Figures. 4,5b**). This shrinkage might account for better context discrimination displayed by Foxg1-GOF mice at time of early retrieval (**Figure. 1**). Lastly, Foxg1 upregulation also increased the fraction of recall engram cells already active at the time of training (**Figure. 4** and Supplementary Figure. 3).

5.1. Foxg1 promotes neuron allocation to engrams in an activity-dependent way

Engram neurons exhibit elevated excitability and enhanced plasticity that bias their allocation during learning and recall[173,178,300]. Prior studies have identified TFs such as CREB[173], Npas4[258], and SRF[198] as positive regulators of this process. Our work extends this framework by positioning Foxg1 among TFs involved in engram allocation and memory expression.

To note, artificial modulation of Foxg1 leads to anticorrelated fluctuations of Npas4 and SRF (Ref[101] and our unpublished results). This suggests that Foxg1 impact on neuronal engram allocation is not mediated by Npas4 and SRF. On the contrary, pCreb1 protein is upregulated

upon Foxg1 overexpression and its functional shut down by a dominant-negative effector decreases Foxg1-mRNA[100]. This conversely suggests that a mutual positive feedback between Foxg1 and pCreb1 may favor this allocation.

These dynamics were observed to be region-, recall time- and Foxg1 expression level-specific. In particular, a positive relationship between Foxg1 levels and neuronal recruitability into engrams could be observed in hippocampus and amygdala at the time of training and short-term memory retrieval (**Figures. 2,3** and Supplementary Figure. 4), and in cortical regions upon late memory recall (**Figure. 5b** and Supplementary Figure. 2). Depending on cases, this effect was detectable upon Foxg1 upregulation, downregulation or both (**Figures. 2,3,5b**). These findings resonate with recent work suggesting that neuronal engram allocation is circuit-specific and dynamically regulated[164,216]. In this context, the ability of Foxg1 to bias neuronal allocation over time suggests it may act as a key gatekeeper of engram selectivity.

Region/time-specific transcription cofactors and epigenetic programs are likely to underlie differential effects elicited by Foxg1 on neuronal recruitment into the engram. Future studies focusing on these cofactors and programs will be needed to clarify how this takes place.

Our chemogenetic experiments also showed that Foxg1 may drive memory dynamics by promoting neuronal activity. In fact, suppressing this activity reversed the enhanced memory performance elicited upon Foxg1 upregulation (**Figures. 1 and 6**). Previous work from our lab reported that Foxg1 enhances neuronal activity both in vitro and in vivo[101]. Instrumental in these phenomena can be changes in expression levels of genes encoding ion channels, receptors, plasticity and dendritic development effectors, dependent on Foxg1[100,101,301]. Their relative contribution to Foxg1-driven memory dynamics waits to be investigated.

5.2. Activity-dependent promotion of Foxg1 might stabilize engrams

Previous in vitro studies from our lab showed that artificial neuronal activation results in upregulation of Foxg1 mRNA and protein levels[101]. To validate these findings in vivo, we administered the established proconvulsant kainic acid[302,303] (KA) to juvenile wild-type mice and we evaluated these levels in neocortex and hippocampal formation. As expected, we found a timed and transient upregulation of both Foxg1 mRNA and protein (Supplementary Figure. 5B-J). Remarkably, in the same structures of mock-injected, control mice, Foxg1 protein expression was enriched in cFos$^+$ neurons compared to cFos$^-$ cells (Supplementary Figure. 5K), pointing

to a physiological regulatory role of neuronal activity in promoting Foxg1 expression. This basal enrichment in active neurons and activity-dependent induction suggest that Foxg1 participates in a positive feedback loop: neurons with higher Foxg1 levels are more excitable and more likely to be reactivated during learning, leading to further Foxg1 upregulation and stabilization of engram identity (**Figure. 4J,K** and Supplementary Figure. 3G-I). Such feedback loops have been proposed for pCreb1 and other transcriptional regulators that integrate excitability and plasticity to define memory allocation[164,177]. In this context, the timed non-monotonic progression of Foxg1 products evoked by neuronal hyperactivity could have evolved for proper dynamical replacement of a subset of early engram neurons with new ones.

5.3. Foxg1 tunes cumulative engram size

An intriguing aspect of the phenotype evoked by sparse Foxg1 manipulation was the modulation of the total frequency of neurons belonging to the engram. In general, Foxg1 upregulation reduced this prevalence, regardless of the structure taken into account and the time of the evaluation, whereas Foxg1 down-regulation gave rise to a more variegated scenario (**Figure. 4** and Supplementary Figure. 3). These phenomena likely originated from the not-cell autonomous effects exerted by manipulated cells on excitability of surrounding ones, including glutamatergic stimulation and GABAergic inhibition. In this respect, the variable outcome of Foxg1 manipulation on engram size could reflect the relative weights of these two processes. Moreover, until now the capability of Foxg1 to promote neuronal activity has been documented in bulk neuronal populations, likely reflecting its actual impact on largely prevailing glutamatergic neurons. An evaluation of Foxg1 activity specifically restricted to GABAergic cells will be needed to further clarify this point.

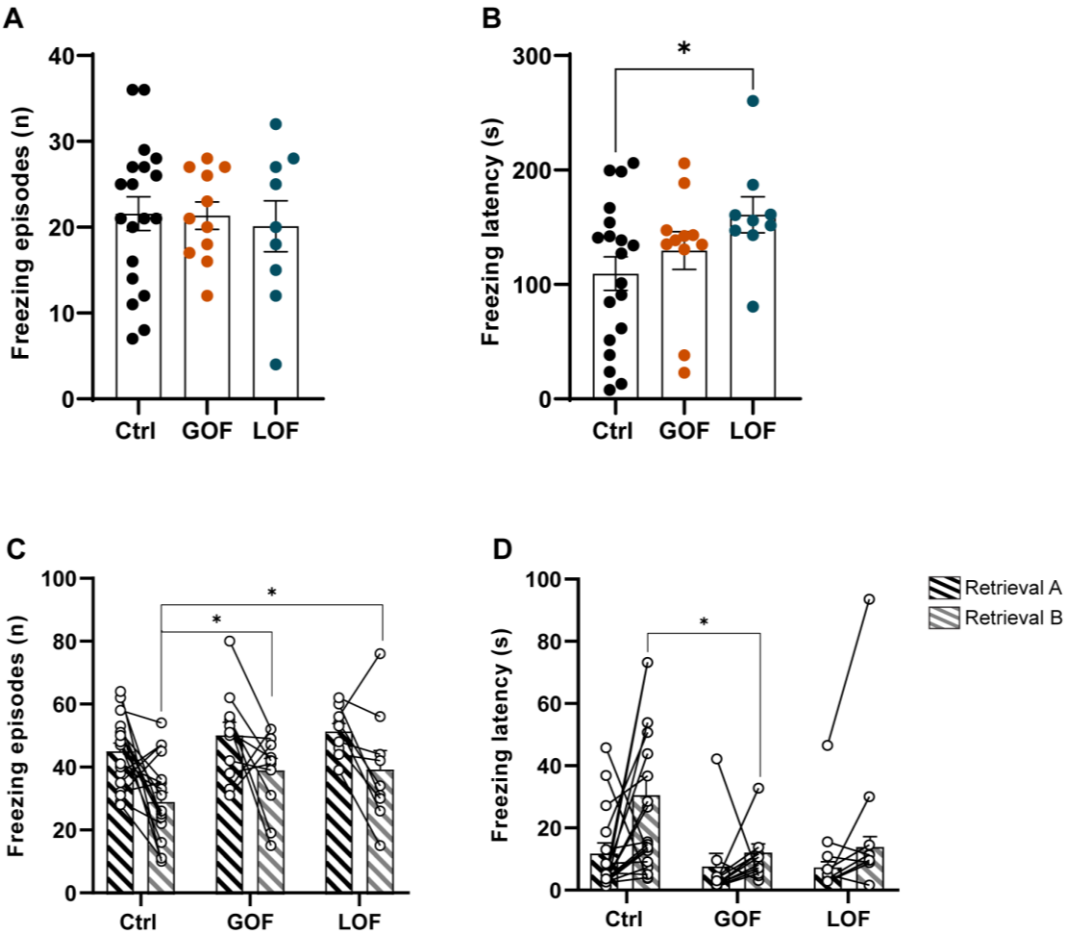
6. Conclusion

In summary, we identified Foxg1 as a key transcriptional regulator of neuron engram allocation and memory recall. As such, Foxg1 provides a mechanistic bridge between transcriptional control and memory dynamics. This expands our understanding of the molecular programs governing memory traces. It further suggests a special caution in precision treatment of neuropathogenic Foxg1 haploinsufficiencies and highlights Foxg1 as a potential therapeutic target for disorders of memory and cognition.

7. Limitations and future directions

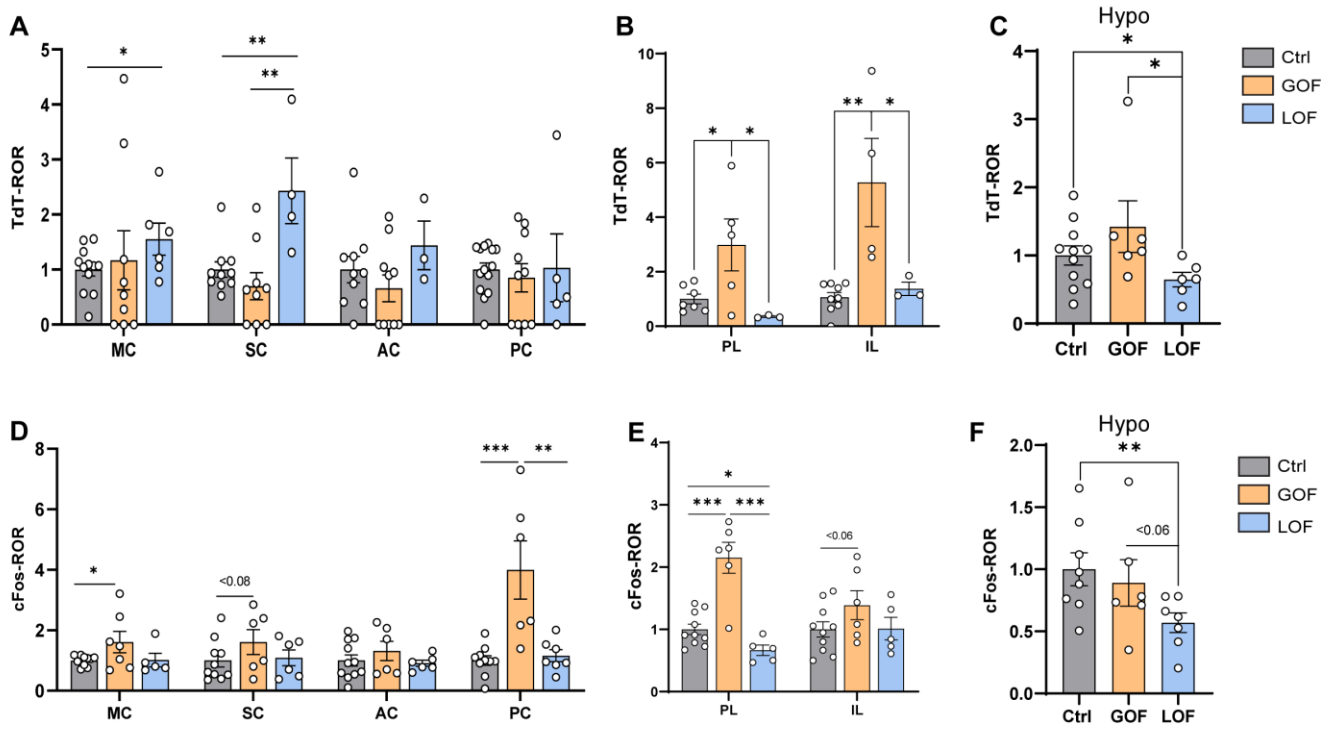
First, our study primarily relied on CFC; whether Foxg1 exerts similar effects in other behavioral paradigms, such as hippocampal-dependent spatial navigation or amygdala-driven emotional memories remains to be determined. Second, while our chemogenetic manipulations support an activity-dependent mechanism, the precise downstream effectors of Foxg1 (e.g., ion channel genes, synaptic proteins, or epigenetic regulators) require direct investigation. Finally, the region-specific effects observed suggest that Foxg1 may interact with local transcriptional landscapes or network states, an avenue for future single-cell and circuit-level studies.

8. Supplementary Figures



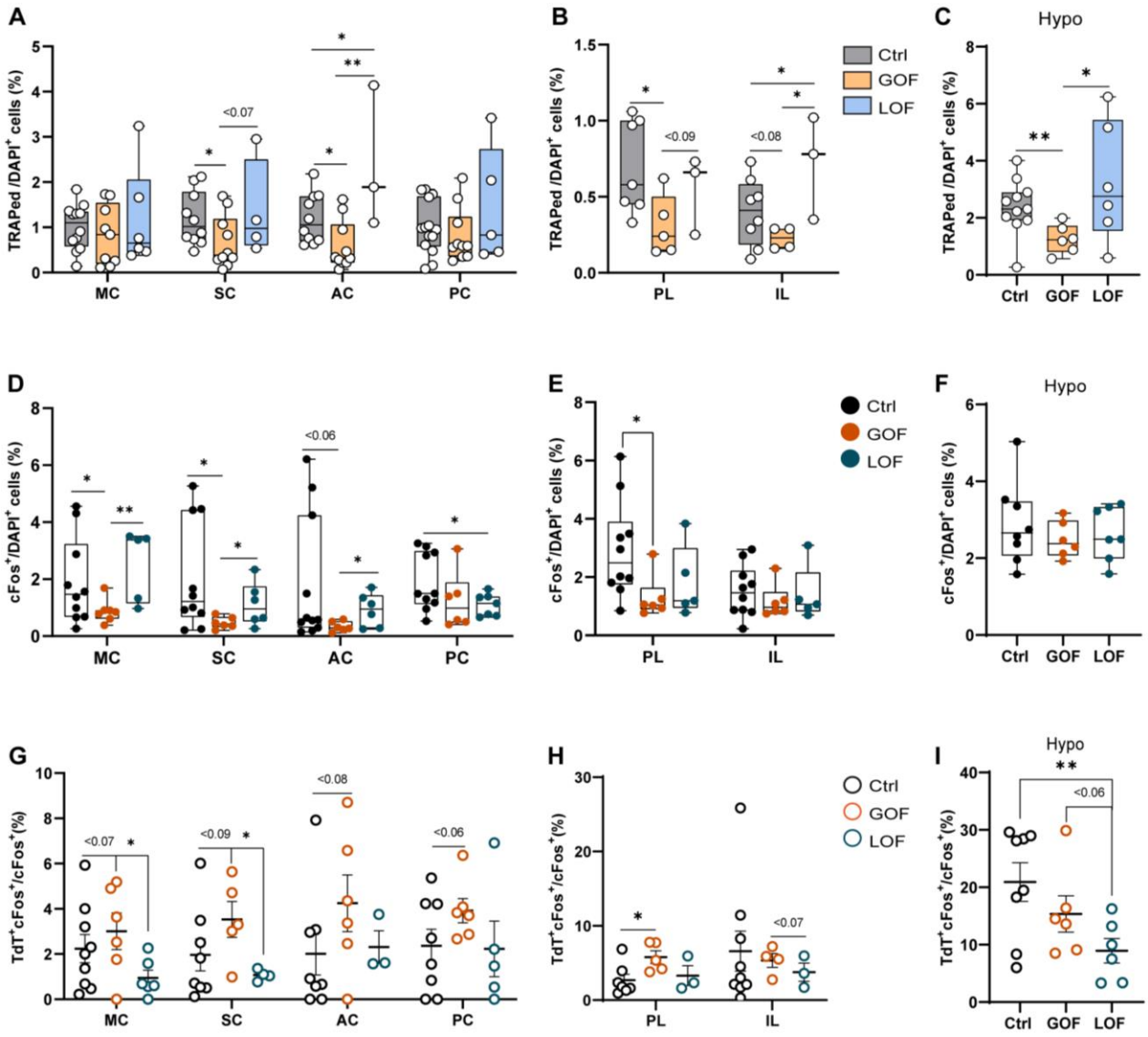
Supplementary Figure. 1: Supplementary data linked to Figure. 1A-E.

A-B. Total freezing episodes and freezing latency during training session of contextual fear conditioning (CFC) in Ctrl, GOF, and LOF groups. **C-D**. Total freezing episodes and freezing latency during retrieval session, in the training context (Retrieval A) and a novel context (Retrieval B). A-D. Error bars indicate standard error of the mean (SEM), n = 19 (Ctrl), 11 (GOF), 9 (LOF). Statistical significance of results was evaluated by t-test assay (unpaired between groups, paired within groups, one-tailed). * $p<0.05$.



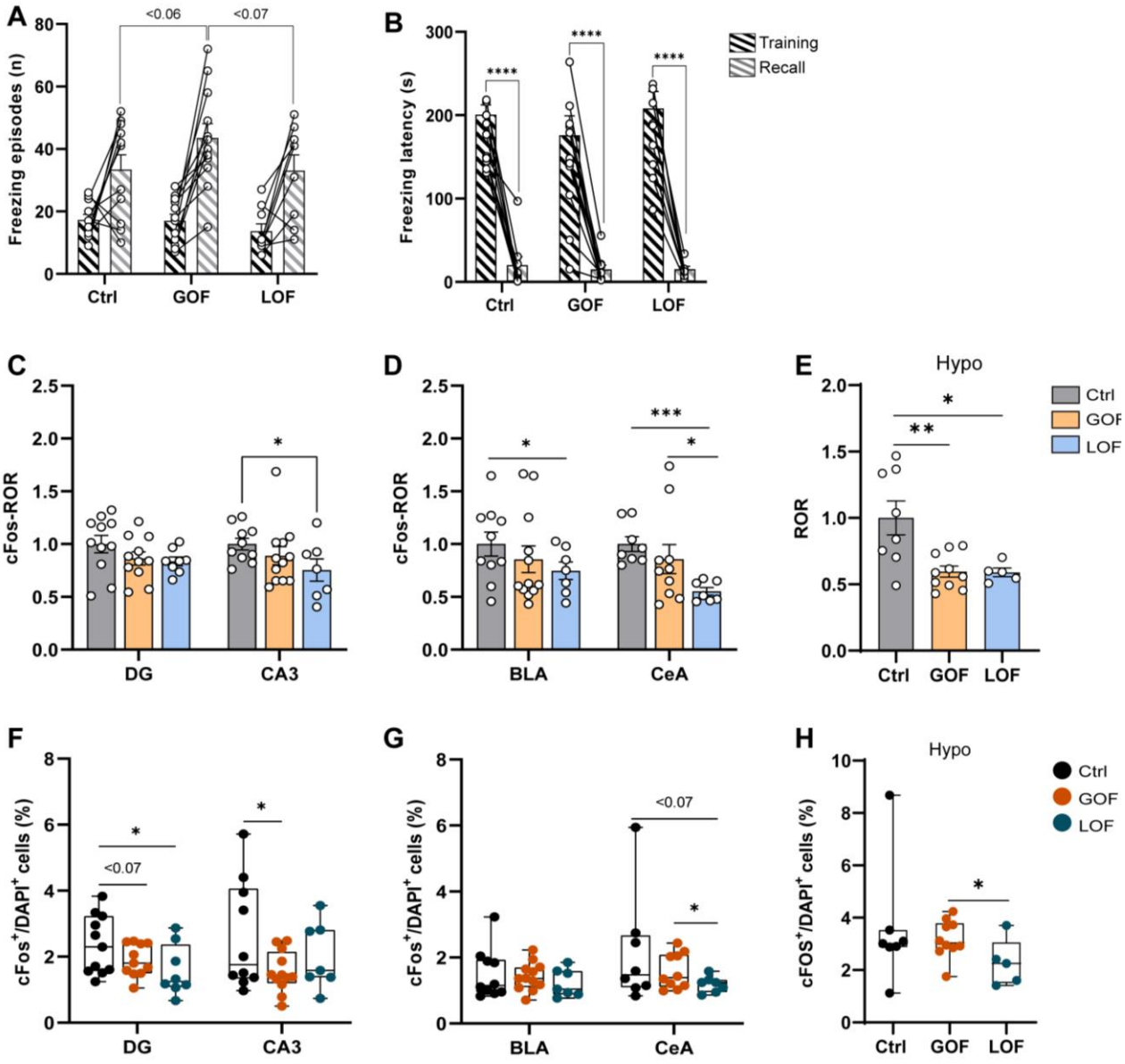
Supplementary Figure. 2: Supplementary data linked to Figure. 2 and 3.

A-F. Quantification of Ctrl-normalized TdT-ROR (A-C) and cFos-ROR (D-F) in selected regions of brains treated with Ctrl, GOF, and LOF AAVs. n = 7-14 mice per Ctrl group, 4-10 mice per GOF group, 3-7 mice per LOF group. Error bars indicate standard error of the mean (SEM). Statistical significance of results was evaluated by t-test assay (unpaired, one-tailed). * $p<0.05$, ** $p<0.01$, *** $p<0.001$. ROR, ratio of ratios; MC, motor cortex; SC, somatosensory cortex; AC, auditory cortex; PC, piriform cortex; PL, pre-limbic cortex; IL, infra-limbic cortex; Hypo, hypothalamus.



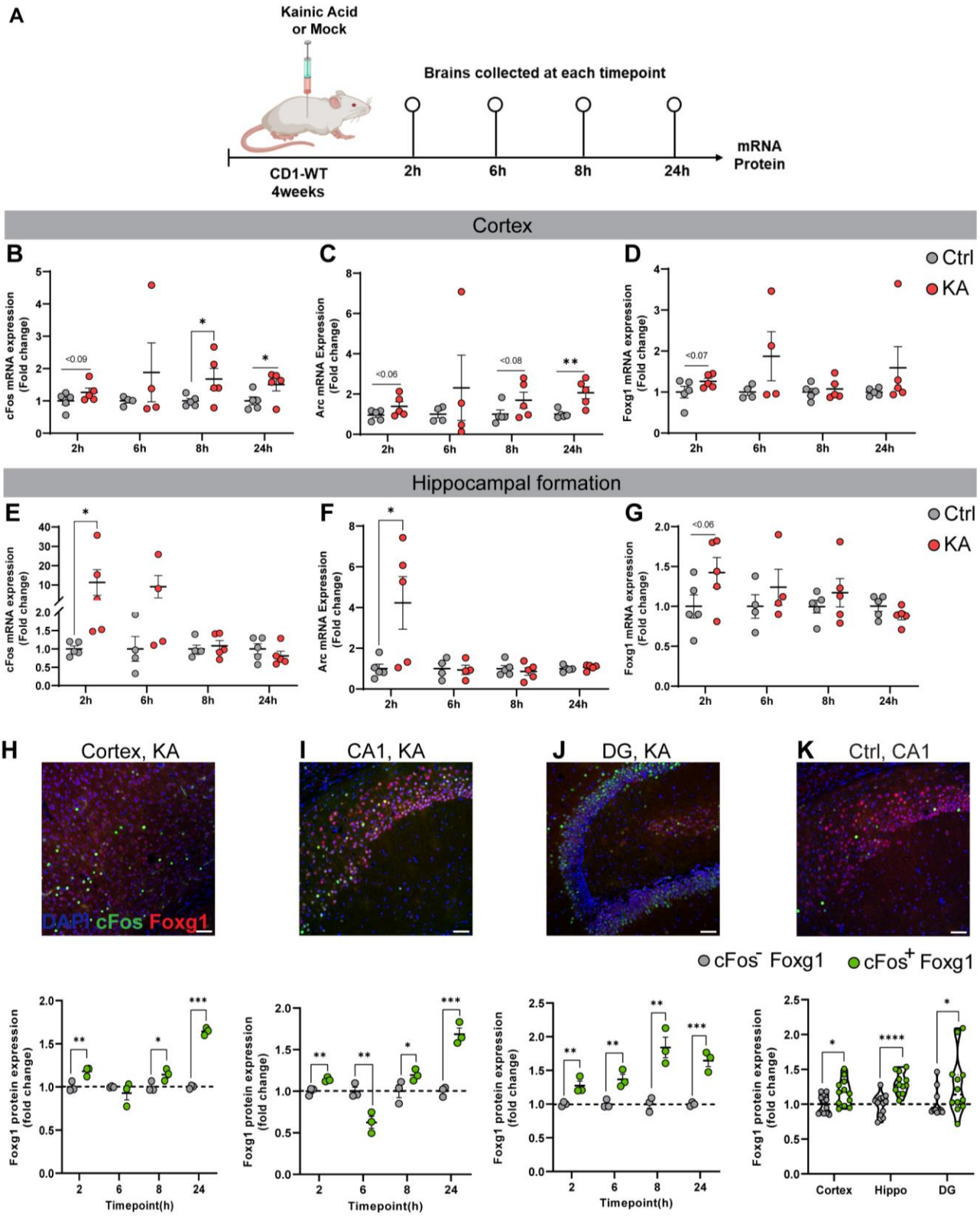
Supplementary Figure. 3: Supplementary data linked to Figure. 4.

A-I. Quantification of TRAPed cells (TdTomato$^+$ cells/DAPI$^+$ cells, **A-C**), cFos$^+$ cells (cFos$^+$ cells/DAPI$^+$ cells, **D-F**), and double positive cells (TdTomato$^+$ cFos$^+$ cells/cFos$^+$ cells, **G-I**), all displayed as absolute percentages. n = 7-12 (Ctrl), 5-8 (GOF), 4-7 (LOF). Error bars indicate standard error of the mean (SEM). Statistical significance of results was evaluated by t-test assay (unpaired, one-tailed). * p<0.05, ** p<0.01, *** p<0.001. MC, motor cortex; SC, somatosensory cortex; AC, auditory cortex; PC, piriform cortex; PL, pre-limbic cortex; IL, infra-limbic cortex; Hypo, hypothalamus.



Supplementary Figure. 4: Supplementary data linked to Figure. 5a,b.

A-B. Distribution of total freezing episodes and freezing latency in Ctrl, GOF and LOF groups during training and late memory recall in training context. **C-H**. Quantification of Ctrl-normalized cFos-ROR and cFos$^+$/DAPI$^+$ cell ratio in brains of Ctrl, GOF, and LOF groups. A-B. n = 11 (Ctrl), 12 (GOF), 9 (LOF). C-H. n = 8-11 (Ctr), 9-12 (GOF), 5-8 (LOF). Error bars indicate standard error of the mean (SEM). Statistical significance of results was evaluated by t-test assay (unpaired between groups, paired within groups, one-tailed). * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. ROR, ratio of ratios; DG, dentate gyrus; CA3, hippocampal CA3 field; BLA, basolateral amygdala; CeA, central amygdala.


A
Kainic Acid
or Mock
Brains collected at each timepoint
CD1-WT
4weeks
2h
6h
8h
24h
mRNA
Protein
Cortex
B
cFos mRNA expression
(Fold change)
C
Arc mRNA Expression
(Fold change)
D
Foxg1 mRNA expression
(Fold change)
Ctrl
KA
Hippocampal formation
E
F
G
H
Cortex, KA
I
CA1, KA
J
DG, KA
K
Ctrl, CA1
DAPI cFos Foxg1
cFos- Foxg1
cFos+ Foxg1
Foxg1 protein expression
(fold change)
Timepoint(h)
Cortex
Hippo
DG

Supplementary Figure. 5: *In vivo*, activity-dependent Foxg1 modulation.

A. Experimental design. Mice administered intraperitoneally with 20mg/kg of kainic acid (KA) and their time-matched mock-injected controls (Ctrl) were sacrificed 2h, 6h, 8h, and 24h after drug administration, and profiled for gene expression. **B-G**. Immediate early genes (IEGs) and Foxg1 mRNA levels in cortex and hippocampal formation of KA and Ctrl mice. **H-J**. Representative images of cFos and Foxg1 immunofluorescence, in Cortex, Hippocampus (CA1), and Dentate Gyrus (DG) from KA groups, scale bar 100μm (upper lane). Foxg1 protein expression levels in the above-mentioned structures, specifically within $cFos^+$ and $cFos^-$ cells, shown as $cFos^-$ normalized values (lower lane). **K**: Representative image of cFos and Foxg1 immunofluorescence, in Hippocampus (CA1) from Ctrl mice, scale bar 100μm (upper lane). Foxg1 protein expression levels from Ctrl groups, specifically within $cFos^+$ and $cFos^-$ cells, shown as $cFos^-$ normalized values. B-G. n = 5 (2h,Ctrl), 4 (6h,Ctrl), 5 (8h,Ctrl), 5 (24h,Ctrl), 5 (2h,KA), 4 (6h,KA), 4 (8h,KA), 5 (24h,KA). H-J: n = 3 (per each group). K. n = 12 (Ctrl). Error bars indicate standard error of the mean (SEM), Statistical significance of results was evaluated by t-test assay (unpaired between groups, paired within groups, one-tailed). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

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