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Synapse formation on adult-born hippocampal neurons

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Abstract

It is now widely accepted that adult neurogenesis plays a fundamental role in hippocampal function. Neurons born in the adult dentate gyrus of the hippocampus undergo a series of events before they fully integrate in the network and eventually become undistinguishable from neurons born during embryogenesis. Adult hippocampal neurogenesis is strongly regulated by neuronal activity and neurotransmitters, and the synaptic integration of adult-born neurons occurs in discrete steps, some of which are very different from perinatal synaptogenesis. Here, we review the current knowledge on the development of the synaptic input and output of newborn neurons, from the stem/progenitor cell to the fully mature neuron. We also provide insight on the regulation of adult neurogenesis by some neurotransmitters and discuss some specificities of the integration of new neurons in an adult environment.

The understanding of the mechanisms regulating the synaptic integration of adult-born neurons is not only crucial for our understanding of brain plasticity, but also provides a framework for the manipulation and monitoring of endogenous adult neurogenesis as well as grafted cells, for potential therapeutic applications.

Introduction

The generation of neurons in the adult mammalian dentate gyrus was detected as early as 1965 (Altman & Das, 1965) and shortly thereafter was it demonstrated that these cells receive mature synaptic input (Kaplan & Hinds, 1977). Yet, it took another 30 years and the advent of genetic-based labeling techniques to confirm these observations and to demonstrate that adult-born hippocampal neurons differentiate, mature, migrate and integrate into the hippocampal network and finally become morphologically and functionally undistinguishable from all other granule neurons (See also the reviews from Schinder et al. ; Aasebo et al., in this issue).

Relatively little is known on how adult-born neurons connect to the mature network and the rules of the game for a newcomer in the adult brain can be drastically different than those governing synaptogenesis in the fetal brain. Indeed, contrary to neurons born during prenatal development, adult-born neurons develop in a mature and functioning environment and therefore these cells can potentially be shaped by various factors including: Neighbor cells, brain activity and experience, hormonal variations, stress and disease. Conversely, the addition of neurons can remodel the connectivity of the pre-existing network, which may in turn result in behavioral modifications, the formation of new connectivity or the repair of damaged networks. Therefore, a better understanding of the mechanisms regulating the integration of new neurons will not only shed light on the plastic properties of the adult brain and on the function of adult neurogenesis, but it will also provide a framework for the development and evaluation of cell-replacement therapies.

Here, we review the current knowledge of the synaptic integration of neurons born in the adult hippocampus and explore such questions as: Are adult-born neurons similar to neurons born during embryogenesis? Does synaptogenesis on adult-born neurons follow the same rules as prenatal synaptogenesis? Does the insertion of newborn neurons interfere with the mature network? What is the interplay between neuronal activity and the integration of new neurons?

Main text

Connectivity of the dentate gyrus

The dentate gyrus is formed by three layers. The closest to the cortical surface is the molecular layer, which comprises mainly dendrites and axons. Below it, lays the granule cell layer which contains the bodies of the granule neurons and forms a V-shaped structure. Below the granule cell layer lays the hilus, formed by the axons of the granule cells and interneurons.

The entorhinal cortex provides the hippocampus with its major excitatory, glutamatergic input through perforant path axons which project to the dentate gyrus and contact the granule neurons in the molecular layer. Most of these synapses are found on dendritic spines in the distal two-thirds of the dendrites of the granule neurons. Another glutamatergic input originates from hilar mossy cells, mainly on the proximal third of the dendrites of granule neurons. The granule neurons then project mossy fiber axons to the CA3 pyramidal cells, which project Schaffer collaterals to the CA1. In turn, the CA1 pyramidal neurons project back to the entorhinal cortex, through the subiculum. The mossy fibers of the granule neurons also establish collaterals in the hilus and synapse with the proximal dendrites of the mossy cells, the basal dendrites of the basket cells and other unidentified cells.

The main GABAergic input to the granule neurons is situated mainly in the inner third portion of the dendrites and on the cell body and originates mainly from hilar basket cells. Another inhibitory input comes from chandelier-type cells situated in the molecular layer and which contact the axon initial segment of granule neurons.

Additionally, granule neurons receive various subcortical inputs which include cholinergic and GABAergic input from the medial septal nucleus and the diagonal band of Broca, glutamatergic input from the supramammilary area, noradrenergic input from the locus coeruleus, serotoninergic input from the raphe nucleus and dopaminergic input from the ventral tegmental area (Amaral *et al.*, 2007).

Maturation of adult-born neurons and their synaptic connectivity

During embryonic development, granule neurons migrate from the basal polymorph layer and appear in the granule cell layer at embryonic day E20 in the rat (Crain *et al.*, 1973; Altman & Bayer, 1990). The first synapses are observed at 4 days after birth, but most of these synapses are symmetric and axo-dendritic, pointing to a GABAergic input (Crain *et al.*, 1973). Postnatal synaptogenesis is most active between 4 and 11 days and is complete at about 28 days. Glutamatergic dendritic spines first appear at 4 days closest to the granule cell body and gradually occupy the entire dendritic field (Crain *et al.*, 1973). This developmental stage coincides with the appearance of the first perforant path axons in the dentate gyrus (Singh, 1977b). Synaptogenesis is complete at about 28 days, although dendritic spines still undergo morphological maturation thereafter.

In contrast, adult-born neurons develop from stem/progenitor cells located in the subgranular zone lining the granule cell layer (Figure 1) (Kriegstein & Alvarez-Buylla, 2009). The primary stem cells, also named type 1 cells, are slowly dividing, are assumed to have an unlimited self-renewal capacity and express stem cell markers such as nestin or Sox2. They project a single process which crosses the granule cell layer and intensely ramifies into the proximal part of the molecular layer. Upon division, type 1 cells give rise to intermediate neuronal progenitor cells, type 2 cells, which are more proliferative and have only short processes. Type 2 cells are subdivided in type 2a cells, which also express stem cell markers, and type 2b cells which start to express immature neuronal markers such as doublecortin and PSA-NCAM (the polysialylated form of the Neuronal Cell Adhesion Molecule). Type 2b cells then lead to type 3 cells, which also have a proliferative potential but no longer express stem cell markers and give rise to neurons (Filippov *et al.*, 2003; Fukuda *et al.*, 2003; Kronenberg *et al.*, 2003; Kempermann *et al.*, 2004; Zhao *et al.*, 2008).

1. Type 1/stem cells

As mentioned above, neuronal activity regulates cell proliferation. Indeed, increased activity such as epilepsy promotes proliferation (Parent *et al.*, 1997; Jessberger *et al.*, 2007), and voluntary exercise is the most efficient physiological way to enhance hippocampal neurogenesis (van Praag *et al.*, 1999b). This effect is likely mediated by glutamate since NMDA (N-methyl-D-aspartate) receptor activity regulates cell proliferation *in vivo* (Cameron *et al.*, 1995). However, on type 1 cells, no glutamatergic or GABAergic synapses have been detected, and the presence of receptors on these cells is debated: Using a transgenic mouse expressing the green fluorescent protein under the control of the stem cell marker nestin and electrophysiological recordings, Wang et al. reported the presence of glutamate and GABA receptors on cells with a type-1 morphology (Wang *et al.*, 2005) whereas Tozuka et al. found no evidence of such receptors using the same mouse model and the same techniques (Tozuka *et al.*, 2005). Using immunohistochemistry, a third study has found NMDA receptors at the surface of GFAP-expressing, putative type 1 cells (Nacher *et al.*, 2007). The discrepancy between these observations can be attributed to the heterogeneity in the maturation stage of the identified cells. These results nonetheless support the view that type-1 cells may be regulated by neuronal activity, although indirectly.

2. Type 2/progenitor cells

Type 2b cells, identified by the expression of nestin and PSA-NCAM, express GABA receptors and some appear to receive GABAergic synaptic inputs, but lack glutamate receptor. The activation of GABA receptors induces the expression of the proneuronal transcription factor NeuroD and promotes neuronal differentiation (Tozuka *et al.*, 2005; Wang *et al.*, 2005). However, when their date of birth is identified by retrovirus labeling, electrophysiological recordings of new cells of 1 week of age or younger, indicate the expression of receptors for the major neurotransmitters GABA and glutamate, but not for afferent synaptic connections (Esposito *et al.*, 2005; Ge *et al.*, 2006). Again, the discrepancy between these observations can be attributed to the heterogeneity of the type 2 cell

population and the differences in the identification methods. However, these results also point to a regulation of type 2-cell proliferation and function by neuronal activity, although non-synaptically.

3. Immature neurons

During the second week after cell division, newborn neurons start to extend their axonal processes in the hilus and their dendritic processes towards the molecular layer. At this stage, electrophysiological recordings show that they receive direct slow GABAergic synaptic input, suggesting a dendritic localization (Esposito *et al.*, 2005; Ge *et al.*, 2006). This GABAergic input plays a great role in the maturation of the nascent neuron. Indeed, similarly to embryonic neurogenesis, immature neurons born in the adult hippocampus have a high intracellular concentration of chloride, due to the expression of NKCC1, a chloride importer. Therefore, a stimulation of GABA receptors results in cell membrane depolarization. This depolarization is crucial for the future maturation of the new neuron, since an experimental conversion of the GABA-induced excitation into an inhibition in these cells, impairs their development and synaptic integration *in vivo* (Ge *et al.*, 2006). At the end of the second week after birth, electrophysiological and morphological evidence shows that new neurons receive their first glutamatergic input on dendritic spines (Esposito *et al.*, 2005; Ge *et al.*, 2006; Zhao *et al.*, 2006). At the same period of time, the same approaches show that they project their first glutamatergic output on CA3 pyramidal cells (Faulkner *et al.*, 2008; Toni *et al.*, 2008).

During the third week, new neurons continue to extend their dendritic processes further into the molecular player and their axonal processes more distally into the CA3. The cells start to express the chloride exporter KCC2, which produces the GABA input to switch from depolarizing to hyperpolarizing (Ge *et al.*, 2006). Dendritic spine growth and motility is maximal during this period (Zhao *et al.*, 2006; Toni *et al.*, 2007).

Between 3 and 6 weeks of age, adult-born neurons show an increased synaptic plasticity and long-term potentiation expression at perforant path synapses (Schmidt-Hieber *et al.*, 2004; Ge *et al.*, 2007; Deng *et al.*, 2009; Mongiat *et al.*, 2009), supporting the view that these cells may be involved in mechanisms of learning (see also the review of Aasebo et al. in this issue). The final step of synaptic

integration is reached by the formation of fast, perisomatic GABAergic synapses (Esposito *et al.*, 2005). After this stage, although morphological maturation is reached, microscopy shows that the dendritic spines and mossy fiber boutons continue to mature up to 8 weeks after birth (Zhao *et al.*, 2006; Toni *et al.*, 2007; Faulkner *et al.*, 2008; Toni *et al.*, 2008). At the end of this period, neurons born in the adult hippocampus are functionally and morphologically similar to neurons born during embryogenesis. They receive afferences and project efferences with similar synaptic partners, of similar morphology and function (van Praag *et al.*, 2002; Laplagne *et al.*, 2006; Toni *et al.*, 2008).

As mentioned above, adult neurogenesis is also modulated by other neurotransmitters such as Dopamine or Serotonin, but their role and their connectivity has been much less studied so far. Acetylcholine promotes neuronal proliferation *in vivo* (Ide *et al.*, 2008; Itou *et al.*, 2010), possibly by direct synaptic afferences from the medial septum, innervating adult-born neurons as early as 7 days after cell division (Ide *et al.*, 2008). Cocaine inhibits cell proliferation, suggesting an effect of Dopamine on adult neurogenesis (Dominguez-Escriba *et al.*, 2006). Norepinephrine promotes neurogenesis (Jhaveri *et al.*, 2010), so does Serotonin (Brezun & Daszuta, 2000; Santarelli *et al.*, 2003). It is however unknown whether these neurotransmitters act through primary or secondary synapses or extrasynaptic release.

These observations on the role of neurotransmitters on the synaptic integration of adult-born neurons raise the question as to whether the activity-regulated synapse formation and the subsequent survival of neurons could be a selection mechanism for the insertion of neurons at the appropriate time and place. Indeed, besides regulating new neurons' integration, neuronal input seems to also regulate the survival of these cells: A large fraction of new neurons die within the third week after cell division (Kempermann *et al.*, 2003) and their survival is regulated by activity (Kempermann *et al.*, 1997; Gould *et al.*, 1999; van Praag *et al.*, 1999a). This control may involve glutamate and NMDA receptor activity, since a cell-specific knock-out of the NR1 subunit in adult-born neurons dramatically increases death during the third week after cell division (Tashiro *et al.*, 2006). Thus, adult-born neurons undergo a critical time window for their survival and it seems that

the activity of their NMDA receptors is a survival signal. Since most dendritic spines are formed at the beginning of this time window, it is likely that synapse formation and glutamatergic neurotransmission are required for cell survival. Furthermore, the synaptic competition occurring during the early phases of synaptogenesis may lead to a Darwinian selection of neurons. Finally, this selection mechanism may be underlying the reduced neurogenesis in Alzheimer's disease. Indeed, Amyloid beta reduces NMDA receptor expression (Snyder *et al.*, 2005) and spine formation (Spires *et al.*, 2005) *in vivo*, which may in turn reduce the survival of adult-born neurons which is observed in this disease (Li *et al.*, 2009) (Sun *et al.*, 2009).

Thus, adult-born neurons appear to integrate into the hippocampal network similarly to neurons generated during embryonic development. They follow similar milestones (dendritic and axonal development, GABAergic input followed by glutamatergic input, dendritic spine development, GABA switch from depolarizing to hyperpolarizing, etc…) and, according to the current state of our knowledge, eventually become indistinguishable. However, since adult-born neurons integrate in a mature environment, they are likely influenced by factors which do not exist in the embryo. These factors may result in differences in the course of the development of these cells. For example, neurons born in the adult develop slower than neurons born during embryogenesis (Overstreet-Wadiche *et al.*, 2006; Faulkner *et al.*, 2008). In the following paragraph, we will give an overview of some of these differences and emphasize the potential role of these specificities on the function of adult neurogenesis.

Specificity of synaptogenesis on neurons born in the adult brain

1. Maturation of the connectivity of adult-born neurons

With the concomitant extension of their axons and dendrites, it is likely that adult-born neurons switch synaptic network during their maturation. Indeed, during a short period of time, dendrites are restricted to the inner molecular layer and, at the same period of time, axons are restricted to the hilus. The hilus is densely populated with GABAergic interneurons such as basket cells (Amaral *et al.*,

2007) and the proximal portion of the dendrites receive mostly afferences from mossy cells. Thus, the first synapses (both afferent and efferent) formed by adult-born neurons involve mainly interneurons. At this stage, newborn neurons, which also show an increased excitability (Schmidt-Hieber *et al.*, 2004; Mongiat *et al.*, 2009) are therefore expected to modulate the activity of interneurons and neighboring granule neurons which are interconnected by the same interneurons. This transient state may have a potential effect on information coding by the dentate gyrus (Aimone *et al.*, 2006). As they mature, the axonal and dendritic processes of these cells extend and form synapses with more diverse partners including glutamatergic input from the entorhinal cortex and output to pyramidal cells in the CA3. This shift in connectivity is unique to neurons born during adulthood and may induce modifications in their physiology along the course of their maturation, with possible functional implications.

2. Interaction with pre-existing synapses

While adult-born neurons develop dendritic spines, perforant path afferences adapt to this change by remodeling their axon terminals to innervate the new neurons. In a similar manner, the projection of mossy fibers from adult-born neurons towards the CA3 remodels the fine anatomy of this area. To which extent are the synaptic partners of adult-born neurons remodeled by their integration? Does the adult brain generate new pre- and post-synaptic partners to accommodate the new neurons, or do new neurons connect to pre-existing partners?

Electron microscopy observations support the latter hypothesis (Figure 2, upper panel). Filopodia and dendritic spines from adult-born neurons were analyzed in three dimensions using serial-section electron microscopy (Toni *et al.*, 2007). Filopodia are immature and very motile dendritic protrusions which probe between potential synaptic partners and eventually stabilize into dendritic spines and form synapses (Marrs *et al.*, 2001; Yuste & Bonhoeffer, 2004; Knott *et al.*, 2006; Lohmann & Bonhoeffer, 2008). On newborn neurons as well as on more mature neurons, the tip of filopodia is always found very close (within 100 nanometers) to pre-existing synapses. This distance is smaller

than the distance predicted if filopodia grew randomly, suggesting that these structures are attracted by pre-existing synapses. This preference for pre-existing synapses is supported by the observation of the more mature dendritic spines: When dendritic spines are three-dimensionally reconstructed, they are found to form multiple synapse boutons, i.e. contact with axon terminals already involved in a synapse with another neuron. One month after cell division, about two-thirds of their dendritic spines contact multiple-synapse boutons (i.e. axonal boutons synapsing with at least two postsynaptic partners), whereas the other third of the spines contacts axonal boutons devoid of other synapse. This observation suggests that the dendritic spines of newborn neurons have a preference for pre-existing synapses. Although the mechanism for this preference is currently unknown, several experiments from developmental synaptogenesis support the idea that glutamate may attract nascent filopodia. Indeed, although they do not make a mature synaptic contact, filopodia express NMDA receptors (Takumi *et al.*, 1999) and focal glutamate application induces filopodia growth (Portera-Cailliau *et al.*, 2003; Konur & Yuste, 2004). Thus, glutamate spillover resulting from synaptic activity (Kullmann & Asztely, 1998) may induce filopodia growth towards active synapses, and their stabilization into spines contacting multiple-synapse boutons.

Intriguingly, during the course of neurons' maturation, their dendritic spines contacted multiple-synapse boutons in a smaller proportion (about one third), whereas their filopodia continued to be very close to pre-existing synapses, suggesting a transformation of multiple-synapse boutons into single-synapse boutons over time (Toni *et al.*, 2007). This supports the hypothesis that dendritic spines from adult-born neurons compete with dendritic spines from older neurons and eventually replace them (Figure 2, upper panel). Such a mechanism is reminiscent of the synaptic competition occurring during the development of the neuromuscular junction, where several axons innervate several muscle fibers and compete until a single axon remains for each fiber (Buffelli *et al.*, 2003).

Interestingly, the development of mossy terminals from adult-born neurons follows a similar mechanism: The first axon terminals contact the dendritic shafts from CA3 pyramidal cells. Then, mossy terminals contact individual small thorny excrescences protruding from these dendrites, or

share mature thorny excrescences with other granule neurons. And it is only after 2 months of maturation that mossy terminals contact individual and mature thorny excrescences (Toni *et al.*, 2008), Figure 2 lower panel). Together, these observations suggest that both the synaptic output and the synaptic input of adult-born neurons have a time window during which synaptic partners are shared with pre-existing neurons and that competition may occur at both the input and output levels of a nascent neuron.

The formation of multiple synapse boutons is not restricted to the context of adult neurogenesis. Rather, these structures are formed by most, if not all, manipulations which induce synaptogenesis in the adult brain such as: lesion-induced synaptogenesis (McWilliams & Lynch, 1978; Ito *et al.*, 2006), Estrogen (Yankova *et al.*, 2001), or even associative learning (Geinisman *et al.*, 2001) and LTP induction (Desmond & Levy, 1990; Toni *et al.*, 1999). However, an increase of multiple synapse boutons has not been reported during perinatal development in the hippocampus. This difference between postnatal and adult synaptogenesis may be due to the rate of presynaptic development. Indeed, during developmental neurogenesis, perforant path axons develop concomitantly to the dendrites of granule neurons (Singh, 1977a) and it is therefore likely that boutons are connected by individual nascent spines. Thus, multiple-synapse bouton formation and the subsequent competition which may occur on these structures may be a mechanism of synaptogenesis specific for the adult brain, which is particularly active in adult-born neurons.

3. Astrocytes

During embryonic development, neurogenesis precedes gliogenesis (Nixdorf-Bergweiler *et al.*, 1994; Catalani *et al.*, 2002). But neurons born in the adult brain immediately come in contact with mature astrocytes. Astroglia forms the stem cell niche and influence the proliferation, differentiation and maturation of neuroblasts (Doetsch *et al.*, 1999; Lim & Alvarez-Buylla, 1999; Seri *et al.*, 2004; Shapiro *et al.*, 2005; Lledo *et al.*, 2006). The regulation mechanisms are still unclear, but astrocytes express different molecules such as cytokines, chemokines (Barkho *et al.*, 2006), IL-6 (Oh *et al.*, 2010), or

Wnt3 (Lie *et al.*, 2005), some of which participate to the regulation of stem cell differentiation. A clear demonstration of the importance of astrocytes in regulating neurogenesis came from *in vitro* experiments showing that astrocytes from the subgranular zone enable progenitor cells to differentiate into neurons (Song *et al.*, 2002a; Song *et al.*, 2002b), thereby demonstrating that astrocytes actively regulate stem cell differentiation. More recent results indicate that these cells can also control the maturation and survival of adult born neurons. In the subventricular zone/rostro-migratory stream, glutamate released by astrocytes activates NMDA receptors in neuroblasts and enables the maturation and survival of newborn neurons in the olfactory bulb (Platel *et al.*, 2010). Another mechanism by which astrocytes may regulate adult neurogenesis is by the formation of perisynaptic processes. Indeed, astrocytic perisynaptic processes are present around mature synapses in the cortex (Volterra & Meldolesi, 2005; Faissner *et al.*, 2010) where they form tripartite synapses composed of the presynaptic terminal, the postsynaptic membrane and the astrocytic perisynaptic process, based on the demonstration of the existence of a bidirectional communication between astrocytes and neurons (Araque *et al.*, 1999a; Araque *et al.*, 1999b; Haydon, 2001). This communication plays a crucial role during synaptogenesis (Ullian *et al.*, 2001) and enhances synapse maturation and efficacy (Mazzanti & Haydon, 2003; Murai *et al.*, 2003) and is also very likely regulating the synaptic integration of adult-born neurons.

Conclusion

The constant addition of several hundreds of granule neurons in the dentate gyrus throughout the whole life of the animal generates major network remodeling, which may be greatly involved in learning and memory. Adult-born neurons fully integrate in the hippocampal network and eventually become undistinguishable from neurons born during embryogenesis. However, the specificity of adult-born neurons lies in the mechanisms of their integration, the existence of a critical time-window for enhanced excitability and plasticity, the possible competition with synapses from more mature cells and the activity-dependent survival and insertion of these cells. These specificities

indicate that adult neurogenesis is a major player in hippocampal function and plasticity. Furthermore, the mechanisms involved in the synaptic integration of adult-born neurons may provide a promising therapeutic target for interventions aiming at improving adult neurogenesis for cognitive enhancement, or brain repair.

Figure legends

Figure 1: Timeline showing the morphological development of adult-born hippocampal neurons as well as the development of GABA and glutamate receptor expression and connectivity.

Figure 2: Schematics illustrating the hypothetical sequence of events involved in the synaptic integration of adult-born neurons into the glutamatergic network. Upper panel (input): A filopodia of an adult-born neuron (green) is attracted by a pre-existing synapse between an axonal bouton (blue) and another neuron (red). When the filopodia stabilizes and matures into a dendritic spine, a multiple-synapse bouton is formed. Progressively, the spine from the adult-born neuron increases in size and the spine from the other neuron decreases in size until it retracts, transforming the multiple-synapse bouton in a single-synapse bouton. Lower panel (output): Upon reaching the CA3 area, mossy fiber terminals contact dendrites of pyramidal cells. At about one month, mossy terminals contact thorny excrescences, some of which are shared with pre-existing neurons and it is only after 2 months that individual contacts are made between pre- and post-synaptic partners.

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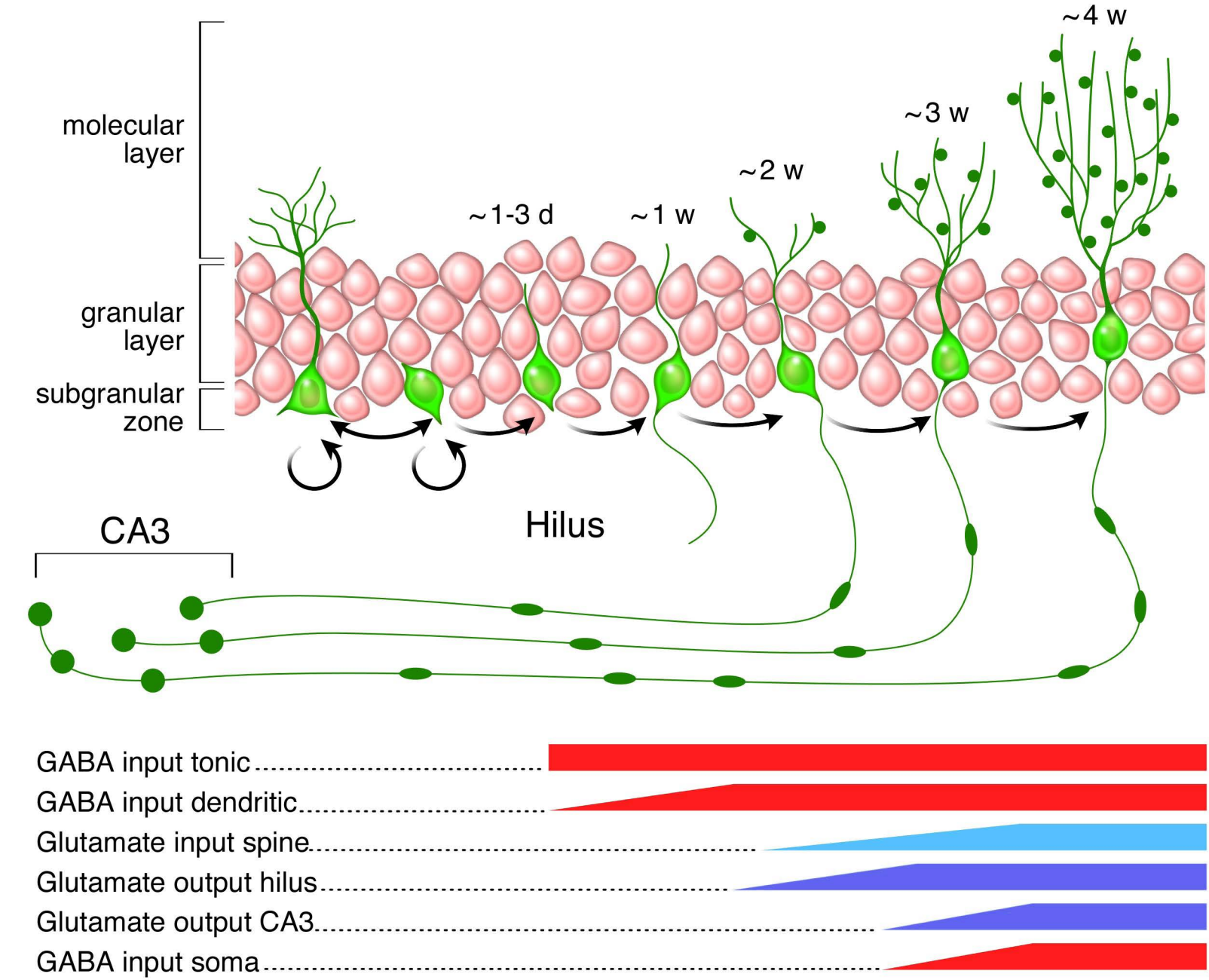
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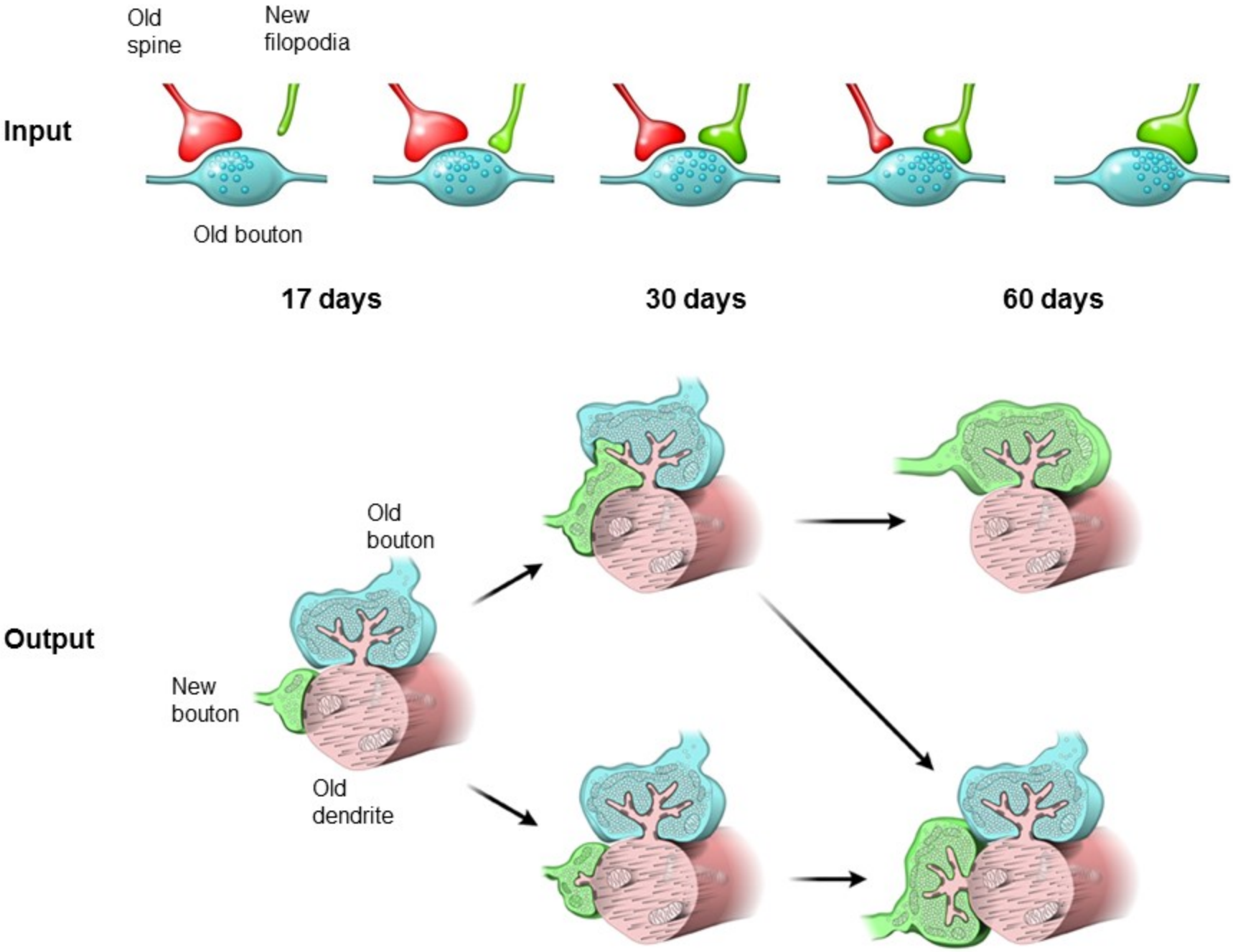
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