

Guidance at the construction site

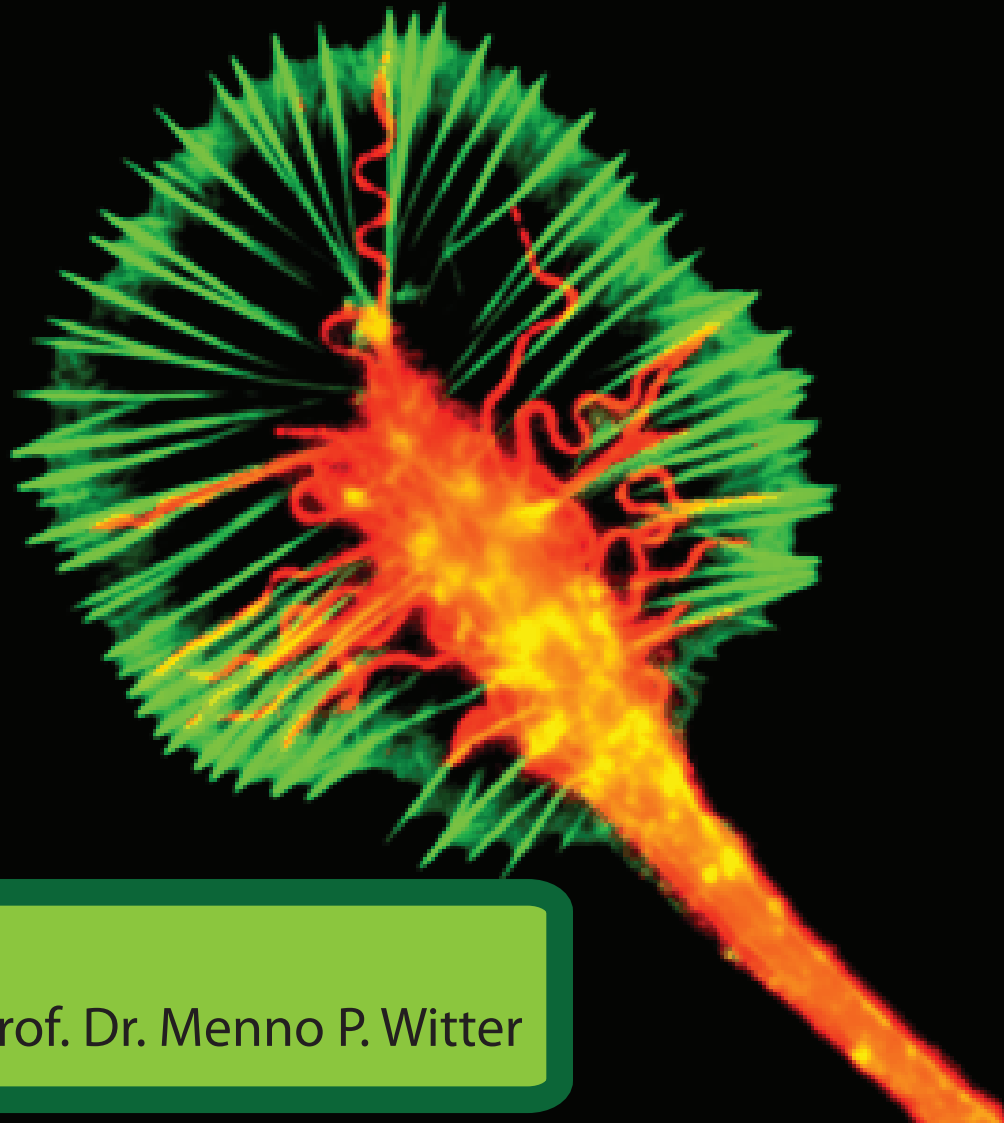
The role of axon guidance molecules in postnatal development of the hippocampus

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Frontpage: The axonal growth cone, actin bundles (green) provide a scaffold for the survival of protruding microtubules (red), axon guidance molecules modulate the behaviour of the growth cone in order to guide the axon towards its target. Cover picture from the Journal of Cell Biology, 2002; 157 (5), copyright by The Rockefeller University Press.

Abbreviations

3T3 cells:	Fibroblast cells line. 3 -day transfer, inoculum 3 x 10 ⁵ cells
Cos cells:	Immortalized cell line from African Green monkey kidney. CV -1 from o origin and carrying the SV -40 genetic material
DiI:	1,1',di-octadecyl-3,3,3'3'-tetramethylindocarbo cyanine perchlorate (tracer)
E(e.g.E19):	Embryonic day (e.g. embryonic day 19)
Fc-tag:	Fragment crystallisable region protein-tag
GABA:	gamma-aminobutyric acid (neurotransmitter)
GFAP:	Glial fibrillary acidic protein
GPI:	Glycosyl-phosphatidyl-inositol (membrane-anchor)
P(e.g. P19):	Postnatal day (e.g. postnatal day 19)
mRNA:	Messenger ribonucleic acid
siRNA:	Small-interfering ribonucleic acid

Anatomical names

CA 1-3:	Cornu Ammonis (subfield 1-3)
Cx:	Neocortex
DG:	Dentate gyrus
DLS:	Dorsal lateral septum
EC:	Entorhinal cortex
IML:	Inner-third of stratum moleculare within the dentate gyrus
MML:	Medial layer of stratum moleculare within the dentate gyrus ²
MSDBC:	Medial septal-diagonal band complex
OML:	Outer two-thirds of stratum moleculare within the dentate gyrus
PSD:	Postsynaptic density
SG:	Stratum granulosum
SL:	Stratum lucidum
SLM:	Stratum lacunosum-moleculare
SO:	Stratum oriens
SP:	Stratum pyramidale
SR:	Stratum radiatum

Axon guidance molecules

AGM:	Axon guidance molecule
BDNF:	Brain-derived neurotrophic factor
DCC:	Deleted in colon cancer
LAR:	Leukocyte common antigen-related receptor (RPTP)
NPN:	Neuropilin
NGF:	Nerve growth factor
NGL-3:	Netrin-G ligand-3
NT-3:	Neurotrophin-3
p75NTR:	p75 neurotrophin receptor
RGM:	Repulsive guidance molecule
Robo:	Roundabout receptor
RPTP:	Receptor protein tyrosine phosphatase
Sema:	Semaphorin
TrkB/C:	Tyrosine receptor kinase B/C

Abstract

Precise wiring of neuronal networks during development is of utmost importance regarding the function of the network. This becomes apparent if one studies the etiology of various neurological diseases such as autism, epilepsy, or schizophrenia, in which alteration in hippocampal network wiring is related to the development of these pathologies (Eastwood et al., 2003; Gant et al., 2008; Holtmaat et al., 2003). The objective of the present work is to provide an overview on the molecular mechanisms that support the development of the hippocampus. Specialized families of proteins, called axon guidance molecules (AGMs), provide these molecular mechanisms and have been studied in the past decades in relation to network wiring. Interestingly the development of the hippocampus as well as the activity of these AGMs continues postnatally (Nadel and Willner, 1989; Skutella and Nitsch, 2001). For these reasons the scope of the present work is the involvement of AGMs in hippocampal development from postnatal day 0 to 28 (P0-28). Three major conclusions can be drawn from the reviewed work; 1) Several AGMs are important for the laminar organization of the hippocampus. 2) Support from, in particular, Eph-ephrin complexes and semaphorins are needed to convey the process of synaptogenesis and maturation. 3) The specific temporo-spatial expression of AGMs correlates with the establishment of innervation from specific input regions. Alteration in expression of these AGMs impacts the timing and spatial distribution of specific projections.

Keywords:

Postnatal development, axon guidance molecules, hippocampus, laminar organization, synaptogenesis

Table of contents

Abbreviations	3
Abstract	4
Table of contents	5
Introduction	6
Anatomy of the hippocampus	6
Axon guidance molecules lead the way	7
Line of research	10
From embryonic development to the hippocampus in the neonate	12
Entorhino-hippocampal connections	12
Septo-hippocampal fibers	15
Intra- and interhippocampal pathways	16
Schaffer-collaterals	16
Commissural-associative tracts	17
Mossy fibers	18
Molecules from the past: Axon guidance molecules support hippocampal maturation.	18
Maturation of the entorhino-hippocampal pathway	19
Maturation of the septo-hippocampal pathway	22
Maturation of the intra- and interhippocampal pathways	24
Schaffer-collaterals	24
Commissural-associative tracts	25
Mossy fibers	27
AGMs in synaptogenesis and synaptic maturation	29
Conclusions	32
Literature research	38
Acknowledgements	38
References	39
Appendix A	45

Introduction

As progress in science emerges, it becomes clearer that the hippocampus is an important brain area involved in cognitive behaviour like spatial navigation, learning and memory (Mulder et al., 2004; Whitlock et al., 2006). Current hypotheses state that mis-wiring within the hippocampal network affects these behaviours and is related to several pathologies such as autism, epilepsy, or schizophrenia (Eastwood et al., 2003; Gant et al., 2008; Holtmaat et al., 2003). It is therefore important to investigate and understand the development of this brain area. The architecture of the hippocampus consists of specific neuronal connections between subfields in which the innervation follows a high degree of lamina-specificity (Frotscher et al., 1997; Supèr et al., 1998; Witter and Amaral, 2004). Specialized mechanisms are involved in the regulation of neuronal network wiring in order to attain such a complex organization. Behavioural and anatomical data indicate that the hippocampus is not fully matured after birth, pinpointing the importance of post-natal development of the network (Angevine, 1965; Gage and Buzsáki, 1989; Li et al., 2009; Nadel and Willner, 1989). The scope of this paper is the involvement of these specialized mechanisms from postnatal day 0 until 28 (P0-28), development of the hippocampus in rodents.

Anatomy of the hippocampus

The hippocampus¹ can be subdivided into the dentate gyrus (DG) and the hippocampal proper, which consists of subfields called the Cornu Ammonis 1-3 (CA1-3) (Figure 1). The principal cells of the hippocampal proper and DG, respectively pyramidal and granular neurons, are aligned within the stratum pyramidale and granulosum, respectively. Interestingly the hippocampal proper and DG can be divided into lamina, based upon the distribution of the afferents they receive. For the hippocampal proper, basal dendrites of the pyramidal cells extend to the stratum oriens, whereas the apical dendrites cross the stratum radiatum, lacunosum, and moleculare. An additional lamina is found in the CA3, stratum lucidum, which receives input from the DG. Within the DG the C-shaped cell layer, stratum granulosum, has a suprapyramidal blade at the apical side of the CA3 pyramidal cells, and an infrapyramidal blade that is localized at the height of the basal side of pyramidal cells. Apical dendrites of the granular cells extend into the stratum moleculare, which can be divided into three layers: the inner-third of the stratum moleculare (IML), medial layer of the stratum moleculare (MML), and the outer two-thirds of the stratum moleculare (OML)². Basal dendrites and axons extend to the hilus, a

¹ For an elaborate review on the anatomy of the hippocampus, see Witter and Amaral (2004)

² Often the OML is referred to the outer two-thirds of the stratum moleculare, which also includes the MML, in most cases one refers only to the IML and OML. However in some studies (Martínez et al. (2005), Otal et al. (2006)) more detail is given about the lamina-specific

polymorphic area. The border between the suprapyramidal blade of the DG and the CA1 is called the fissure. At the fissure, axons of the perforant pathway from the entorhinal cortex (EC) cross to innervate the OML. Ventro-lateral to the CA3, the fimbria is situated. Major afferents from the septal region enter the hippocampus through the fimbria whereas commissural projections towards the contralateral hippocampus transverse the brain via the fimbria. Adjacent to the CA1 is the subiculum followed by the EC. The latter is one of the major brain areas that have afferents to the hippocampus. The EC can be subdivided into separate layers based on histological properties, such as the presence of a specific types of neurons. In the present work the subdivision of the entorhinal cortex is based upon the Ramón y Cajal scheme adapted by Insausti (1997). This scheme describes six layers, from most superficial molecular/plexiform layer I, to the innermost/deep layer VI.

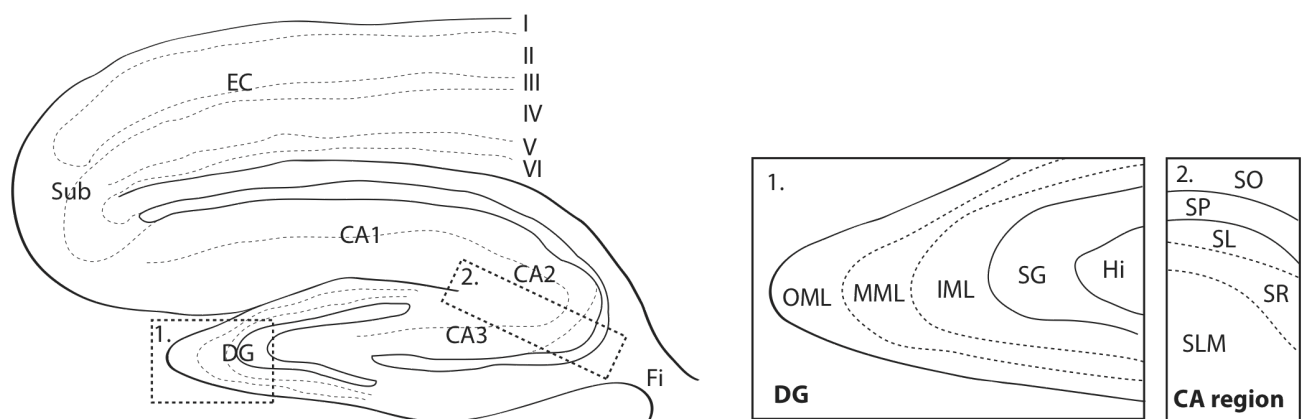


Figure 1, Schematic overview of the hippocampus. The hippocampus includes the dentate gyrus (DG), and hippocampal proper, which consists of the Cornu Ammonis subfields 1-3 (CA1-3). Adjacent to the hippocampal proper are the subiculum (Sub), and the entorhinal cortex (EC); the latter is subdivided into layers I-VI. Several projections enter and leave the hippocampus via the fimbria (Fi). The border between the CA1 and the suprapyramidal blade of the DG is called the fissure. Enlargement 1 represents the layers of the dentate gyrus (DG); outer one-third of stratum moleculare (OML), middle third of stratum moleculare (MML), inner-third of stratum moleculare (IML), stratum granulosum (SG), and the polymorphic area or hilus (Hi). Enlargement 2. Represents the layers within the CA3 subfield; stratum oriens (SO), stratum pyramidale (SP), stratum lucidum (SL), stratum radiatum (SR), stratum lacunosum-moleculare (SLM).

Axon guidance molecules lead the way

As mentioned-above, a general question related to the wiring of neuronal networks is which and how molecular mechanisms support the wiring and bring about its specificity regarding synaptic contact. Various proteins families have been identified, called axon guidance molecules (AGMs), which are key players in the guidance of protruding axons towards their target. In general³,

innervation or distribution of proteins expressed. For these reasons the MML, which is the inner third of the OML, is used to divide the OML into two separate layers.

³ Some members of the AGMs, for example Sema7A, have been characterized to provide a growth-inducible effect on the growth cone rather than inducing attraction or repulsion.

these proteins either attract or repel the leading edge of the protruding axon, called the growth cone. It is important to emphasize that both the AGM and the receptor determine the behaviour of the growth cone. For example, the presence of a receptor from a specific class, and in some cases a second receptor from another class (co-receptor), can change the response to the AGM from attractant to repulsive or *vice versa* (Dickson, 2002). In general, AGM-receptor binding results in a downstream signalling that induces remodelling of the growth cone cytoskeleton, by which the growth behaviour changes. The downstream components of the AGM-receptor signalling pathways will not be discussed here.

“Classical” AGM families, which genuinely have been identified to exert their role in axon guidance, are Semaphorins (Sema), ephrins, repulsive guidance molecules (RGMs), netrins and Slits. In addition, neurotrophins are important for the guidance of the growth cone but are not classified as “classical” chemotrophic AGMs (Table 1)

Semaphorins are the largest family among the AGMs, with nineteen members (Semaphorin Nomenclature Committee, 1999). Based upon their homologous sema-domain there are eight classes, of which two invertebrate (Sema1-2), five vertebrate (Sema3-7) and one viral (SemaV) are being identified. Apart from Sema7A most of the semaphorins have a repulsive effect towards the growth cone (see footnote 3). All semaphorins contain a class-specific C-terminus with additional signalling sequences. Regarding their membrane anchorage, semaphorins differ per class, which is either transmembrane (Sema1, 4-6), secreted (Sema2-3, V) or Glycosylphosphatidyl-inositol anchored (GPI) (Sema7). Semaphorins conduct their signalling via neuropilin (Npn), cell adhesion molecules (CAMs), integrin, or plexin receptors (Pasterkamp and Giger, 2009).

Ephrins are the second largest family of AGMs, which consists of two classes: ephrin-A and -B. Ephrin-A class ligands are GPI-anchored to the membrane, whereas ephrin-B ligands are transmembrane proteins. Ephrin-receptors, called EphA and B, conduct forward signalling that, in general, results in a repulsive response by the growth cone. In most cases A-class ephrins bind EphA receptors while B-class ephrins bind EphB receptors, however some ephrin ligands are capable of binding Ephs of the opposite class (e.g. ephrin-B2/-B3-EphA4, ephrin-A5-EphB2). Interestingly ephrin-Eph complexes exert bi-directional signalling pathways through both receptor and ligand. This means that both ephrins and Eph receptors do have their own downstream signalling. Ephrins therefore are not solely ligands that affect the Eph-receptor signalling but also conduct a downstream signalling at their side upon binding to their receptor, called reverse signalling (Murai and Pasquale, 2003). For the GPI-anchored ephrin-A class it has been proposed that recruitment of other transmembrane proteins enables the reverse signalling (Hattori et al., 2000).

Repulsive guidance molecules (RGMs) are the most recently discovered AGM-family, which consist of 3 members (A-C) (Oldekamp et al., 2004). All members are GPI-anchored to the membrane and include an RGD-motif and a von Willebrand-domain. RGMa/b are expressed throughout the brain, but only RGMa has been functionally described regarding hippocampal development. RGMa has a repulsive effect on the growth cone, presumably via neogenin that recognizes the RGD-motif present at the RGMa (Severyn et al., 2009).

Netrins are among the most conserved AGMs regarding their role in axon guidance and have the earliest appearance of AGMs in species with bilateral symmetry (Moore et al., 2007). Five members of the netrin-family, netrin (-1, -3, -4,) and netrin related GPI-anchored (netrin-G1, -G2), are expressed by mammals. Notably, netrin function has been extensively investigated regarding its role, as chemoattractant in midline crossing by commissural axons (Kennedy et al., 1994). Netrin's effect on the growth cone behaviour, either repellent or attractant, is dependent upon the presence of the receptor/co-receptor availability. Receptors/co-receptors include: Neogenin, deleted in colon cancer (DCC), UNC5, and netrin-G ligand (NGL) availability.

The **Slit** family expressed by mammals is comprised of three members, Slit-1-3 (Chédotal, 2007). Slits exert repulsive action on the growth cone, which is mediated by binding to the Roundabout receptor 1-3 (Robo1-3⁴)(Nguyen Ba-Charvet et al., 1999). Slits have an important contribution in the regulation of midline crossing by commissural axons. Once commissural axons have crossed the midline (due to the attractant netrin-1 signalling) they need to be repelled away from the midline. The repulsive signalling at the midline is mediated by Slit-Robo interaction. Interestingly the expression of Robo by commissural projections changes with respect to the position of the growth cone to the midline. Low levels of Robo are detected before the crossing and are increased after the crossing. This transient in receptor availability is necessary to allow axons to cross the midline in the absence of the repulsive Slit-Robo interaction, and later on to prevent the growth cone from remaining at the midline by the netrin-1 attractant cue (Nguyen Ba-Charvet et al., 1999; Stein, 2001).

Aside from the presence of "classical" AGMs, other factors, such as **neurotrophins**, are important to support the axonal outgrowth. Neurotrophins are a family of the neurotrophic factors comprised of the brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophin (NT-3, -4, -5). All members have equal affinity for the p75 neurotrophin receptor (p75NTR). Interestingly, p75NTR activation has both positive and negative effects, like

⁴ A fourth member of the Robo-family in mammals has been found. However the ability to bind this receptor by Slit is still being debated (Chédotal, 2007).

growth promoting and apoptosis. Binding to a second receptor, tyrosine kinase (TrkA,B,C) has positive effects such as growth promoting and cell survival. The downstream signalling upon activation of p75NTR and/or one of the Trk receptors converge in order to enhance or suppress the consequences of receptor activation (Gillespie, 2003).

In addition a group of **Receptor protein tyrosine phosphatases (RPTPs)** are involved in regulation of axon guidance. To date there are no characterized ligands of the RPTPs that are involved in axon guidance. However, lateral binding with contactin and neural cell adhesion molecules (NCAMs) within the same membrane, have been proposed to be involved in axon guidance signal transduction (Johnson and Van Vactor, 2003; Kostic et al., 2007). The transmembrane receptors are subdivided into eight subfamilies, of which two members, Leukocyte common antigen-related receptor (LAR) and RPTP α , are most widely studied. These two receptors are implicated in the process of synapse formation and axonal outgrowth (Kostic et al., 2007; Petrone et al., 2003; Woo et al., 2009; Yeo et al., 1997). For this reason the role of RPTPs in axon guidance will be discussed in this paper.

Line of research

The major objective of the present work is to review the role of axon guidance molecules in the postnatal development of the rodent hippocampus. In order to put the contribution of AGMs during development into a broader prospective, a brief overview is given on the embryonic phase of development, followed by an elaborate description of the postnatal P0-P28 development. This will illustrate the dynamic contribution of the AGMs in neuronal network wiring.

Family	Member	Receptor	Cue	Function	Temporal expression	References
Axon guidance molecules	Sema3A	Npn-1		repulsive	E16-P15	Chedotal et al. (1998), Skallora et al. (1998), Pozas et al. (2001), Pascual et al. (2005)
	Sema3C	Npn-2		repulsive	P0-adult	Steup et al. (2000), Pascual et al. (2005)
	Sema3E	Npn-1		repulsive	P0-adult	Pascual et al. (2005)
	Sema3F	Npn-2		repulsive	E16-P5	Chedotal et al. (1998), Pascual et al. (2005)
	Sema5B	n.a.		n.a.	P0-adult	O'Conner et al. (2009)
	Sema6A	plexin-A2/A4		non/repulsive (resp.)	P1-?	Suto et al. (2007)
	ephrin-A3	EphA2-A8		repulsive	E17-P2-?	Stein et al. (1999), Matinéz and Soriano (2005)
	ephrin-A5	EphA2-A8, EphB2		repulsive	P2-15	Stein et al. (1999), Matinéz and Soriano (2005), Ojal et al. (2006)
	ephrin-B1	EphB1-4/6		repulsive	P5-adult	Leibl et al. (2003), Matinéz and Soriano (2005)
	ephrin-B2	EphB1-4/6		repulsive	P5-adult	Leibl et al. (2003), Matinéz and Soriano (2005)
	ephrin-B3	EphB1-4/6, EphA4		repulsive	P5-adult	Leibl et al. (2003), Matinéz and Soriano (2005)
	RGMs	n.a.		repulsive	E14.5-P7	Brinks et al. (2004), Oldenkamp et al. (2004)
	Netrins	netrin-1	DCC	attractant	E16-P8-?	Barallobre et al. (2000), Steup et al. (2000)
	Silts	Sil1/1	Robo1	n.a.	E18-P3 +	Nguyen Ba-Charvet et al. (1999)
	Sil2/2	Robo1/2	repulsive	E18-P3 +	Nguyen Ba-Charvet et al. (1999)	
Axon guidance molecule-receptors	Npn	Npn-1		CgC, subiculum, CA1-3, MSDBC	E14-adult	Pascual et al. (2005), Chauvet et al. (2007)
		Npn-2		EC, CA1-3, DG, MSDBC, DLS	E14-adult	Chedotal et al. (1998), Pascual et al. (2005)
	plexin-A2	plexin-A2		CA1-3, hilus, DG	P1-?	Suto et al. (2007)
	plexin-A4	plexin-A4		CA1-3, hilus, DG	P1-?	Suto et al. (2007)
	EphA3	EphA3		EC, CA1-3, hilus, DG	P0-P21	Ojal et al. (2006)
	EphA4	EphA4		Cx, CA1-3, DG	P5-adult	Leibl et al. (2003)
	EphA5	EphA5		EC, CA1-3	E17-P1	Stein et al. (1999)
	EphB1	EphB1		Cx, CA3, hilus, DG	P5-adult	Leibl et al. (2003)
	EphB2	EphB2		Cx, CA1-3, DG	P5-adult	Leibl et al. (2003)
	EphB3	EphB3		CA1, hilus, DG	P5-adult	Leibl et al. (2003)
	DCC	DCC		CA1-3, DG, MSDBC, DLS	E16-P8-?	Barallobre et al. (2000), Steup et al. (2000)
	NGL	NGL-3		Cx, CA1-3, DG	E18-adult +	Kim et al. (2003)
	Robo	Robo1		Subiculum, CA1-3, hilus, DG	?-E20-P8 +	Nguyen Ba-Charvet et al. (1999)
		Robo2		Subiculum, CA1-3, hilus, CR cells in DG	?-E20-P8 +	Nguyen Ba-Charvet et al. (1999)
Axon guidance related molecules	NT-3	TrkB, p75NTR	attractant/growth promoting	CA1-2, DG	E17-adult +	Maisonpierre et al. (1990), Collazo et al. (1992), Das et al. (2001), Gillespie et al. (2003)
	BDNF	TrkB, p75NTR	attractant/growth promoting	CA2-3, hilus, DG	P0-adult +	Maisonpierre et al. (1990), Collazo et al. (1992), Das et al. (2001), Gillespie et al. (2003)
	NGF	TrkA, p75NTR	attractant/growth promoting	DG, SO	P0-adult +	Maisonpierre et al. (1990), Collazo et al. (1992), Das et al. (2001), Gillespie et al. (2003)
	TrkB			EC, subiculum, CA1-3, DG	P0-adult	Martinéz et al. (1998), Luikart et al. (2005)
	alpha			CA1-3, SO, DG	n.a.	Petrone et al. (2003)
	LAR			Cx, hippocampus	?-P7-adult +	Dunah et al. (2005)

Table 1, Overview of axon guidance molecules and axon guidance related molecules with their receptors as discussed in the present work. Spatial and temporal expression is based on mRNA expression analysis in mice, except for some studies (+) the analysis was performed in rat brains. In several cases the referred studies restricted the examination of expression levels within a particular brain region or time-window. Especially the data about temporal expression is not conclusive, in the sense that earlier or later expression of the mRNA is likely a question mark is denoted. The table should be used as a case functional experiments suggest that earlier or later expression of the mRNA is likely a question mark is denoted. The table should be used as a reference not as a complete overview. Neocortex (Cx), cingulate cortex (CgC), entorhinal cortex (EC), Cornus Ammonis (CA), stratum oriens (SO), stratum lacunosum-moleculare (SLM), dentate gyrus (DG), medial septum (MS), medial septum-diagonal band complex (MSDBC), dorsal lateral septum (DLS), striatum (STR).

From embryonic development to the hippocampus in the neonate

The first signs of the hippocampal formation arise, in mice and rats, as early as embryonic day 10 (E10) and day 14/16 (E14/16) respectively (Angevine, 1965; Bayer and Altman, 2004; Schlessinger et al., 1978). At this stage pyramidal cells of the CA3 and CA1 subfield are born. Progenitor cells from the ventricular germinal layer supply the hippocampus with newborn neurons. As time progresses the number of neurons increase and neurites start to develop. As mentioned in the introduction, axon guidance molecules play a key role in the navigation of outgrowing axons to make projections to their target cells. In this chapter the embryonic development of the hippocampus is discussed. The focus will be on the major afferents (entorhino-hippocampal and septo-hippocampal projections) and the major intra- and interhippocampal projections (Schaffer collaterals, commissural-associative projections and mossy fibers).

Entorhino-hippocampal connections

All subfields of the hippocampus, CA1-3/DG, receive input from the entorhinal cortex. The majority of entorhinal fibers cross the pyramidal layer of the subiculum or CA1 towards the hippocampal fissure. Here axons originating predominantly from layer II will cross the fissure in order to terminate in the OML (Figure 2) (Witter, 1989). Afferents from the same layer II innervate the pyramidal neurons of the CA3 and CA2 in the stratum lacunosum-moleculare, however they do not cross the fissure. Afferents from layer III innervate CA1 pyramidal neurons in the stratum lacunosum-moleculare (Deng et al., 2007; Witter and Amaral, 2004)⁵. The hippocampal afferents, innervating the DG and the CA1/CA3 in the stratum lacunosum-moleculare, are often referred as the perforant pathway. A separate group of entorhinal afferents, the alvear pathway, show a distinct route by following along the alveus. These fibers innervate the stratum oriens, and lacunosum-moleculare of CA1. The alvear fibers transverse via the fimbria to travel along side of commissural fibers and innervate the

⁵ In the tracing-studies performed by Deng, Yu, et al. (2007) the characterized origin of entorhinal fibers, layer II and IV, differs from the layers, II and III proposed by Witter and Amaral (2004). There are two possible reasons, 1) tracing during embryonic stage may differ with tracing experiments in adult brain, 2) histological determination of the entorhinal cortical layers may be differently performed by Deng, Yu, et al. (2007). Nonetheless, regarding the age at which the experiment is conducted the results from the tracing experiments are still informative.

contralateral hippocampus. In rats, tracing experiments that determine the fiber development show that the alvear pathway can already be visualized at E15 (Deng et al., 2007).

The perforant fibers towards the CA1/CA3 are visible in the stratum lacunosum-moleculare around E17 (Figure 2). EphA5 is expressed by EC axons as early as E17, in mice, whereas its ligand, **ephrin-A3**, is expressed in the pyramidal and granule cell layer of the hippocampus (Stein et al., 1999). Exposure of EC explant cultures, from E18 rats, to ephrin-A3 and ephrin-A5 in a stripe assay⁶ shows the repulsive cue of ephrin-A3 towards EC neurites. This suggest that expression of ephrin-A3 by the pyramidal and granular cell layer prevents the growth of entorhinal axons into the stratum pyramidale and granulosum, respectively. In addition, experiments placing the anterograde tracer DiI (1,1',di-octadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate) into the EC of P0 **netrin-1**^{-/-} mice showed that projections towards the hippocampus terminated within the stratum radiatum of the CA1/CA3 subfield instead of the stratum lacunosum-moleculare (Barallobre et al., 2000). This indicates that netrin-1 is also involved in establishing the layer-specificity for the entorhino-hippocampal projections during embryonic development.

At the same age ephrin-A3 is expressed, E17, **Sema3A** mRNA expression is found throughout the EC, DG and subiculum, establishing a barrier between CA1 and subiculum (Skaliora et al., 1998; Steup et al., 1999). Neurite outgrowth experiments (BOX 1) in which either CA1 or CA3 explants,

Box 1. Neurite outgrowth assays are used to determine the behaviour of outgrowing neurites in response to a specific AGM or tissue explant. Tissue explant of interest is cultured from which the neurite outgrowth behaviour is studied. In close proximity to the former an AGM-source is cultured, such as an island of AGM expressing cell line or a tissue explant from another brain area. After an appropriate time in culture, outgrowth of the neurites is quantified in length and direction of growth. The latter is expressed in percentage of neurites growing radial, proximal or distal to the AGM-source. Exposure to a repulsive cue results in a significant amount of neurites to preferentially grow distal from the source. Conversely, attractant cues induce a significant amount of neurites to grow towards to the source.

Since it is difficult to differentiate between dendrites and axons in these assays all processes are referred neurites.

⁶ Tissue from the entorhinal cortex is cultured on a surface of alternating lanes of ephrin-A3/-A5 expressing 3T3-cells. Cultures are stained with a fluorescent dye to monitor the neurite outgrowth. The 3T3-cells is a Fibroblast cell-line, the name is an acronym for **3**-day **t**ransfer, inoculum **3** x 10⁵ cells.

from E17 mouse embryos, are opposed to EC explants, reveals the repulsive action of EC towards CA pyramidal cells (Chédotal et al., 1998; Steup et al., 2000). Placing *Sema3A* expressing COS cells in proximity to CA1/CA3 explants resulted in a repulsion of growing neurites. In line, the expression of *Sema3A* by entorhinal axons at E16 repels the CA1 pyramidal neurons, which express the *Sema3A*-responsive Npn-1 receptor (Chauvet et al., 2007). This will avoid the growth of CA1 pyramidal neurons into the EC⁷. Blockade of Npn-1 by antibodies abolished the *Sema3A* mediated repulsion (Chédotal et al., 1998; Pozas et al., 2001). Interestingly, exposure of EC explants to *Sema3A* expressing COS cells also induced repulsion of the neurites, indicating that *Sema3A* expressed within the EC forces axons to grow away from their origin (Steup et al., 1999).

The development of entorhinal afferents into the DG starts later, around E19. These afferents penetrate the fissure towards the OML from P2 on (Figure 2) (Supèr and Soriano, 1994). Around that age, E18 and later in rats, entorhinal axons express **Slit2**, whereas *Robo1* and *2* are both expressed a bit later at E20 by hilar mossy fibers residing in the DG hilus (Nguyen Ba-Charvet et al., 1999). DG explants exposed to *Slit2* expressing COS cells⁸ are repelled such that hilar mossy fibers are incapable of entering the stratum moleculare. This indicates that *Slit2*, expressed by innervating EC projections, acts as a repellent cue towards hilar mossy fibers, preventing them to form synapses on their own dendrites within the stratum moleculare.

All together, the entorhinal cortex projects from layer II and III to the hippocampal formation. The lamina specific innervation by the perforant pathway is supported by several identified AGMs, such as ephrins, Slits and semaphorins. Whether AGMs are involved in the development of the alvear pathway is unclear. Last, the age-dependent tracing of the entorhino-hippocampal tracts underlines that, at least not in the DG, the outgrowth of axons is completed before birth.

⁷ As suggested by Witter (1989) the entorhino-hippocampal circuitry is composed of a more complex network in which the EC receives direct input from the CA1. The above-proposed model for which *Sema3A* acts as a barrier against CA1 pyramidal axons growing towards the EC is not directly in contrast to the entorhino-hippocampal circuitry. Since the expression of *Sema3A* decreases in later stages of the hippocampal development, CA1 projections toward the EC can be formed at later stages.

⁸ COS-cell line was derived from the kidney of the African Green Monkey and was immortalized. The name is an acronym for **CV-1** from **o**rigin and carrying the **SV-40** genetic material.

Septo-hippocampal fibers

Cholinergic and GABAergic neurons from the septal region, predominantly the medial septal-diagonal band complex (MSDBC), innervate the hippocampal formation (Figure 3)(Makuch et al., 2001). As discussed in the entorhino-hippocampal pathway section, **Sema3A** provides repulsive cues for protruding axons. Several papers indicate that this AGM together with **Sema3F** is important for the guidance of protruding septal fibers within the hippocampus (Pascual et al., 2004). Both Sema3A and Sema3F are expressed within the cingulate cortex and striatum and provide a repellent gradient that results in a tunnel through which the protruding septal axons are forced to grow towards the hippocampus. In the neurite outgrowth assay, explant cultures from E16 mouse embryos, show the repulsive effect of cingulate cortex and striatum towards the MSDBC neurites, which is mediated by binding to the receptors of Sema3A and Sema3F, Npn-1 and -2, respectively (Pascual et al., 2004).

At E16 **netrin-1** is expressed by the fimbria, which is the gateway for septo-hippocampal fibers to enter the hippocampal formation (Figure 3) (Steup et al., 2000). At the same time DCC, the netrin-1 receptor, is expressed by the MSDBC and dorsal lateral septum (DLS). Using neurite outgrowth assay, co-culturing of mouse E16 explant of MSDBC tissue with fimbria tissue induces MSDBC neurite outgrowth towards the fimbria. This response is abolished when the MSDBC is exposed to fimbria tissue from *netrin-1^{-/-}* mice. Tracing of septo-hippocampal fibers in *netrin^{-/-}* mice showed an overall reduction of septo-hippocampal innervation of the hippocampal formation (Barallobre et al., 2000; Pascual et al., 2004). Therefore netrin-1 expression in the fimbria is proposed to provide attractant cues for protruding septal fibers towards the hippocampus around E17 (Supèr and Soriano, 1994).

Interestingly hippocampo-septal fibers innervating the medial septum are observed earlier, at E15 (Supèr and Soriano, 1994). These fibers probably provide a scaffolding structure for the ascending septo-hippocampal fibers. Septo-hippocampal fibers form terminals all over the hippocampus, from which the majority can be found in the IML and hilus of the DG (Pascual et al., 2005). The presence of RPTP-member leukocyte antigen-related receptor (LAR) has been shown to be important during the targeting of axons of cholinergic neurons towards the DG. In *LAR^{-/-}* transgenic mice⁹, reduction of innervation by

⁹ The *LAR^{-/-}* transgenic mouse is a genetically modified strain in which a gene trap cassette is inserted into the LAR-gene. This results in a truncated non-functional

cholinergic neurons in the IML and granular cell layer of the DG supragranular blade is observed. Together with a decrease in cell-body size, this points towards an important role of this RPTP, LAR, in the development of the septo-hippocampal pathway (Yeo et al., 1997).

To summarize, the septal fibers originating from the MSDBC enter the hippocampus via the fimbria around E17. Semaphorins and netrins guide the protruding septal fibers during this process, whereas LAR is important for the arrival of cholinergic neurons in the DG.

Intra- and interhippocampal pathways

Neuronal tracts within and between the hippocampi can be divided into the Schaffer-collaterals (CA3 to CA1), commissural tracts (contralateral projections), associative tracts (projections within the same field), mossy fibers (DG to CA3), and intrahippocampal tracts (between CA1 and subiculum). In addition, a circuitry of interneurons is present within the hippocampal formation, which will not be described further. In view of the focus on the postnatal development of Schaffer-collaterals, commissural/associative tracts, and mossy fibers in the next chapter, only these pathways will be described in detail.

Schaffer-collaterals

Originating from the CA3 pyramidal neurons, collateralized axons travel towards the CA1 subfield in a column specific way. The CA3-fibers that provide the massive innervation of the CA1 field are called the Schaffer-collaterals. Projections arising from the CA3 proximal part, near the DG, terminate in the distal part of the CA1 field, whereas the distal positioned CA3 pyramidal neurons project to the proximal part, close to CA2, within the CA1. Within the CA1 field, Schaffer-collaterals terminate at dendrites located within the stratum oriens and radiatum/lacunosum (Ishizuka et al., 1990; Pokorný and Yamamoto, 1981a; Witter and Amaral, 2004).

Little is known about the time-related development of the Schaffer-collaterals and the involvement of AGMs in the guidance of the protruding CA3 fibers. **Netrin-1**, however, is likely to be involved in the lamina-specific targeting of Schaffer-collaterals towards the stratum radiatum/lacunosum. Anterograde tracing

expression of the LAR-gene. Note that the transcript of the LAR-gene is still present, however not functional any more.

experiments within the CA3, using DiI in P0 mice, showed that Schaffer-collaterals in *netrin-1*^{-/-} mice protrude to the stratum radiatum and, in contrast to wild-type mice, also the stratum lacunosum/moleculare (Barallobre et al., 2000). Regarding the postnatal development of dendrites it is likely that Schaffer collaterals are not fully developed before birth (Pokorný and Yamamoto, 1981a).

Commissural-associative tracts

Commissural-associative tracts refer to the group of projections that target their axons within the same subfield of the ipsi- and/or contralateral hippocampus (respectively associative and commissural-associative tracts). The CA3 commissural tracts are one exception on the rule, which also have projections to the contralateral CA2 and CA1 (Witter and Amaral, 2004).

In general, the commissural-associative fibers terminate in the IML and hilus of the DG and within the stratum oriens/radiatum of the hippocampus proper (Figure 4) (Pokorný and Yamamoto, 1981a; Supèr and Soriano, 1994). In rats the commissural-associative fibers enter the contralateral fimbria and dorsal part of the hippocampus (white matter) around E18. From there axons start to innervate the stratum oriens and stratum radiatum of the hippocampus proper and the hilus of the DG around the first postnatal day. Projections become denser in these regions around P2-5. Additionally the commissural-associative projections towards the IML start to enter the superior blade at P2 and later on, at P5, the inferior blade of the DG (Supèr and Soriano, 1994).

Not much is known regarding the role of axon guidance molecules during the early, prenatal process, of commissural-associative tract formation. However, projections towards the contralateral hippocampus have been shown to be dependent on the presence of the chemoattractant **netrin-1** (Barallobre et al., 2000). Knockout of this protein results in an abnormal outgrowth of commissural tracts (Barallobre et al., 2000). The majority of the projections are incapable of crossing the midline and alternatively grow more ventral towards the septal region. The DCC-receptor, which is responsible for the downstream signalling of the attractant cue, is expressed by the neurons of the various hippocampal subfields that give rise to the commissural-associative tracts (Barallobre et al., 2000). The importance of netrin-1 is illustrated by the fact that commissural projections from several brain subfields in the netrin-1 knock-out mouse are incapable of crossing the midline of the brain (Kennedy et al., 1994; Serafini et al., 1996).

Mossy fibers

A proportion of the granular cells situated in DG project their axons, the mossy fibers, to the CA3 field. A particular characteristic of mossy fibers is the innervation of a rather low number of unique pyramidal cells. Each single fiber connects with, on estimation, 7-12 unique pyramidal cells (Witter and Amaral, 2004). The mossy fibers run throughout the whole length of the CA3 field and make both *en passant* synaptic contacts with GABAergic interneurons and giant mossy fiber boutons complexes with the target CA3 pyramidal cell. The postsynaptic sites can be identified as structures called thorny excrescence. Several bundles of mossy fibers can be distinguished based on the route by which they travel (for instance suprapyramidal and infrapyramidal bundle), however most innervate the stratum lucidum within the CA3 field and do not travel any further than the CA3/CA2 border (Figure 5)(Witter and Amaral, 2004).

The majority of the mossy fibers presumably develops postnatally, given the fact that the time of birth for most granular cells in the DG is at peri-/postnatal ages (Angevine, 1965). The constant addition of newborn granular cells in the DG makes it difficult to trace the development of mossy fibers (Jones et al., 2003).

Molecules from the past: Axon guidance molecules support hippocampal maturation.

During postnatal development of the hippocampus axons from several pathways still need to be guided towards target regions and form synapses. How is the postnatal development, maturation of the hippocampal formation and its afferents supported, and most important which mechanisms regulate these processes? Several classes of axon guidance molecules are expressed in the postnatal hippocampus according to a spatiotemporal specific pattern (Maisonpierre et al., 1990; Migani et al., 2009). Provided with the knowledge that these molecules are important for the guidance of axons and formation of neuronal tracts during embryonic stages, the hypothesis arises that AGMs support the development of projections, synaptogenesis and synaptic maturation synapses in the hippocampal formation at postnatal age. Synaptogenesis refers to the formation of synapses, whereas synaptic maturation involves the process of establishing synaptic contact and specialisation of contact. The next sections will discuss the involvement of AGMs in the postnatal development of several hippocampal pathways, which will contribute to the process of answering the above-stated question (Table 1).

Maturation of the entorhino-hippocampal pathway

As mentioned earlier, the outgrowth of entorhino-hippocampal axons is not completed before birth. For instance, the perforant tract continues to innervate the DG during postnatal development. Second the maturation of neurons in the CA1/CA3 and DG takes place at later phases (Jones et al., 2003; Pokorný and Yamamoto, 1981a, b). Support is needed during the establishment of lamina-specific connections and formation of synapses. Regarding the entorhino-hippocampal pathway, this includes formation of synapses in the stratum lacunosum-moleculare of the CA1 and CA3 as well in the OML of DG (Figure 2).

The perforant pathway originating from the EC is subjected to **Sema3A** expressed in the subiculum-CA1 boundary. Binding activity of Sema3A in the stratum lacunosum-moleculare and oriens of the CA1 suggests that Neuropilin-1 expressing EC axons within this layer are forced to grow away from the stratum radiatum and pyramidale (Pozas et al., 2001; Steup et al., 2000). It would seem that ablation of this repellent cue, Sema3A, lead to incorrect innervation of the CA1 subfield. However, knockout of Sema3A in mice results in a rather normal lamina-specific innervation of the entorhinal tract into the CA1 stratum lacunosum-moleculare and oriens, except for a low number of ectopic axon terminals in the stratum radiatum. In addition, compared to embryonic stages, Sema3A expression becomes less pronounced postnatally. It has been proposed that, around P0-2, the presence of Sema3A at the border between the CA1 and subiculum prevents commissural projections from innervating the contralateral EC (Pozas et al., 2001). On the other hand, the reduced expression of Sema3A after P2 might result in reciprocal innervation from CA1 to the ipsilateral EC. In neurite outgrowth assays in which CA1 explant cultures from P0 mice are exposed to either deep or outer-layers of the EC shows that CA1 neurites are attracted towards the deep layer of the EC, whereas the outer-layer is neutral regarding neurite outgrowth. The transition of cues, from chemorepulsion to -attraction, provided by EC towards axons originating from the CA1 through the developmental stages support the above stated observation in which CA1 pyramidal neurons innervate the EC at later postnatal stages. How this switch is mediated remains unclear.

As expression of Sema3A within the CA1 becomes less pronounced away from the subiculum, the high expression of **Sema3F** within the hippocampal proper probably takes over its repellent action. At P0-2, EC neurites are repelled by the presence of Sema3F (Pozas et al., 2001). This repellent cue is mediated by the

presence of Neuropilin-2. The expression of *Sema3F* mRNA at P2 is present in the CA1/CA3 regions, whereas the receptor Neuropilin-2 is expressed by EC neurons. As the tracing observations in *Sema3A*^{-/-} mice suggest, other chemorepellent cues are provided to prevent entorhinal innervation in the stratum radiatum. *Sema3F* is likely to exert this function at postnatal phases.

As the semaphorin family contribute to the organization of perforant pathway innervation into the CA1-3, neurotrophin receptors TrkB and TrkC are likely to support the axonal arborization and synaptogenesis within the subfields. Knock-outs of both receptors resulted in a similar innervation pattern to wild-type animals but showed reduced axon arborization and synapse density (Martínez et al., 1998).

Regarding the arrival of entorhino-hippocampal projections within the DG around P0-2, **Ephrin-A3** and **RGMa** are proposed to support the laminar organization (Figure 2). The first AGM is expressed in the Cx, CA1, CA3 and DG at P2 (Stein et al., 1999). This ligand binds to EphA5 receptor, exerting a repulsive effect on the growth cone. Postnatally, receptor EphA5 is expressed by the protruding entorhinal axons that terminate at the OML. Binding assays with exogenous truncated EphA5-Fc showed pronounced binding of EphA5 to the IML. Most likely, granule neurons express the ephrin-A3 at their dendrites in the IML to which binding of EphA5-Fc is observed. These data suggest that entorhinal fibers expressing the EphA5 are forced to terminate in the OML since the presence of chemorepellent, ephrin-A3, is expressed in the adjacent IML.

The second AGM that support the lamina specific innervation by entorhino-hippocampal projections is **RGMa** which is highly expressed in mouse P7 hippocampus (Oldekamp et al., 2004). RGMa mRNA expression is found throughout the whole hippocampus whereas its binding is restricted to CA3 stratum radiatum and the IML. Stripe-assays with EC explant cultures on lanes with/without RGMa present indicate the repulsive response of EC neurites towards RGMa (Brinks et al., 2004). Blockade of RGMa-receptor binding by RGMa-specific antibodies in organotypic cultures disturbs the normal OML restricted innervation by EC axons. This advocates the importance of RGMa presence in the DG regarding the lamina-specific innervation by entorhino-hippocampal projections.

In summary, the postnatal development of the entorhino-hippocampal pathway is partly mediated by the above-mentioned AGMs: ephrin-A3, *Sema3A/3F*, and

RGMa. Most of them have a repulsive effect on the EC axonal growth cone, providing a boundary for protruding axons. In particular the role of ephrin-A3 and RGMa in the laminar organization of the molecular layer of the DG has been studied extensively.

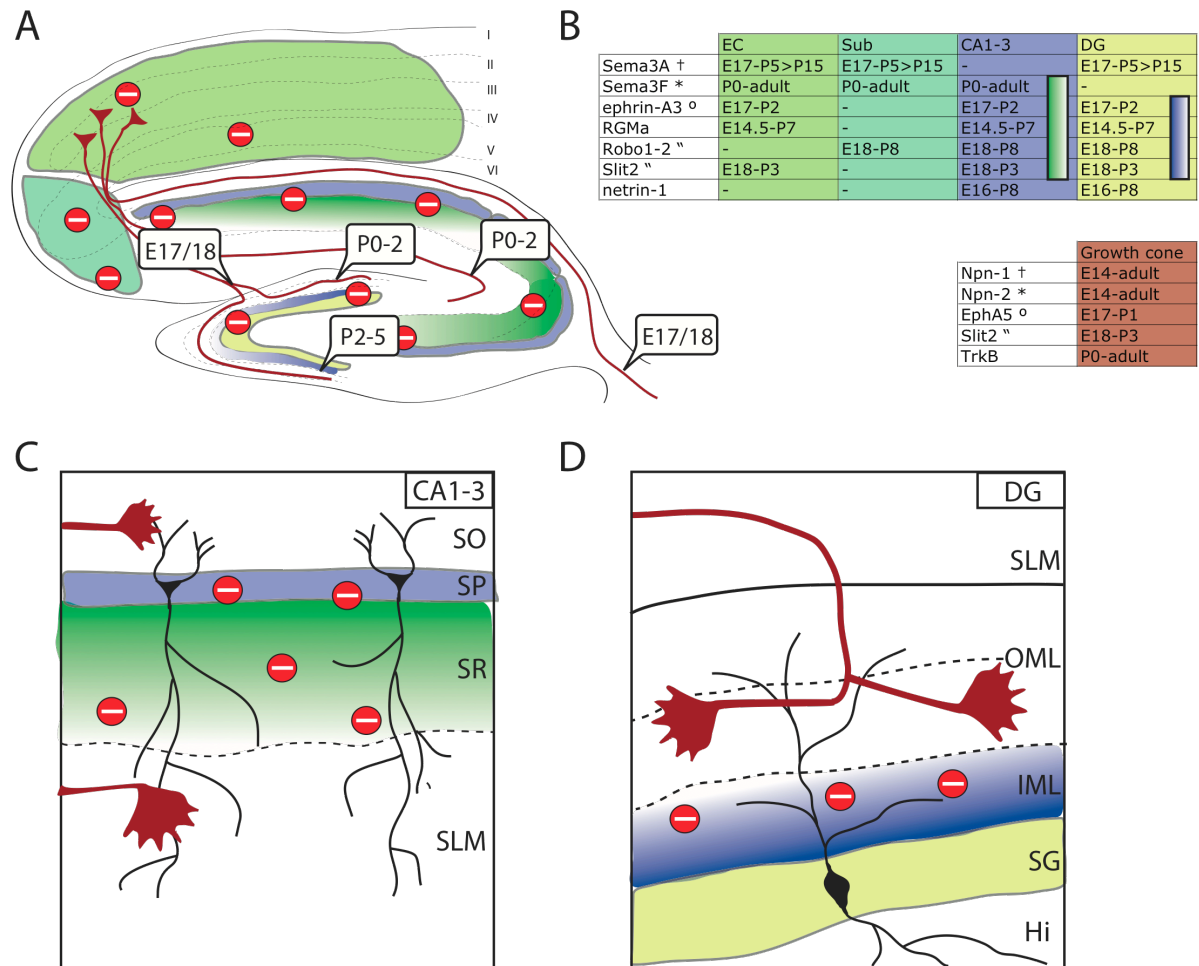


Figure 2, Overview of the developing entorhino-hippocampal projections. **A**. Schematic overview of the hippocampus. At E17-18 entorhinal axons arrive at the hippocampal proper [perforant pathway] and fimbria [alvear pathway]. The EC and subiculum force the axons to move away due to expression of repellents (B). Around P0-2 entorhinal axons protrude the stratum lacunosum-moleculare (SLM) and the OML at the suprapyramidal blade. Repellents expressed by the stratum pyramidale (SP) and stratum granulosum (SG) avoid ingrowth of the entorhinal axons. Around P2-5 the OML of the infrapyramidal blade receive afferents from the EC. **B**. Overview of the AGM and receptor expression in the subfields, brain areas and protruding growth cone. [entorhinal cortex (EC), subiculum (Sub), Cornu Ammonis (CA), dentate gyrus (DG)]. Several pairs of AGMs and receptors are expressed during the development: †Sema3A-Npn1, *Sema3F-Npn2, °ephrin-A3-EphA5, "Slit2-Robo1/2. Along the stratum radiatum (SR) within the CA1-3 [green gradient bar] and the IML within the DG [blue gradient bar] gradients of AGMs are expressed. **C**. Protruding entorhinal axons are repelled from the stratum pyramidale and stratum radiatum. **D**. Arrival of entorhinal axons within the OML is supported by gradients of repulsive cues are present within the IML. Abbreviations: stratum oriens (SO), hilus (Hi).

Maturation of the septo-hippocampal pathway

Tracing septo-hippocampal projections with the anterograde tracer DiI showed that development of the pathway continues at postnatal stages (Makuch et al., 2001; Nguyen Ba-Charvet et al., 1999). At P0, **Sema3E** is highly expressed in septal areas, interneurons within the stratum lacunosum-moleculare and CA1 pyramidal neurons. **Sema3C** transcript levels are high in the MSDBC from P0-15. The expression of repellents Sema3C and 3E in the septal area suggest that these AGMs force septal fibers away from their origin (Figure 3). As mentioned earlier, septo-hippocampal fibers can be divided into cholinergic and GABAergic projections. It has been suggested that cholinergic neurons innervate Sema3E and Sema3A positive pyramidal/granular neurons while GABAergic-septal neurons prefer Sema3C expressing interneurons (Pascual et al., 2005)¹⁰.

Postnatal mRNA expression of **Sema3A, 3F** and their receptors, Neuropilin-1 and -2, indicate that these AGMs play a role in the guidance of septal fibers (Figure 3)(Pascual et al., 2004). Expression of Neuropilin-1 mRNA, at P0 in mice, is high in the DLS and moderate in the MSDBC, whereas Neuropilin-2 mRNA is strongly expressed in the entire septal area. At the same age, Sema3A mRNA is expressed in the pyramidal and granular neurons but Sema3A expression is reduced at later ages (P15). Sema3F follows a similar expression pattern, but at an earlier age of P5 protein expression declines. Interestingly both neuron populations, those residing in the MSDBC and DLS, express neuropilin-1 and -2. However, only MSDBC neurons innervate the hippocampus (Pascual et al., 2005). Divergence in semaphorin-mediated growth cone response is likely to occur which allows selective innervation of the hippocampus by MSDBC originating axons rather those from the DLS. This might be supported by presence of co-receptors, for example plexins, and/or differentiation in downstream signalling pathways.

At P2 the first cholinergic fibers arrive in the IML, starting at the suprapyramidal blade and continues to grow towards the infrapyramidal blade of the DG at later ages (P4-11) (Figure 3). Interestingly, the development of the granular cell layer

¹⁰ Interpretation of the data provided by Pascual, Pozas et al. (2005) has to be taken with care. BDA tracer injections were made in the medial septum and diagonal band, which according to the authors display a high concentration of GABAergic neurons. BDA tracer staining on it self is not selective for GABAergic/cholinergic fibers. An additional selection parameter for GABAergic innervation from septal areas in the hippocampus was the presence of basket-like synapse formation at interneurons, a characteristic phenotype of GABAergic innervation. With this approach, it remains possible that false-positive GABAergic innervated interneurons are selected in the analysis of innervation preferences.

of the DG follows the same pattern, starting in the suprapyramidal blade and extending towards the infrapyramidal blade at later ages (Angevine, 1965; Brinks et al., 2004). Synchronic to this development, **BDNF** and **NT-3** mRNA expression activity follows the same spatiotemporal pattern (Makuch et al., 2001). Within the hippocampus BDNF and NT-3 are likely to function as attractants for the protruding septal axons towards the IML and other hippocampal regions innervated by septo-hippocampal projections.

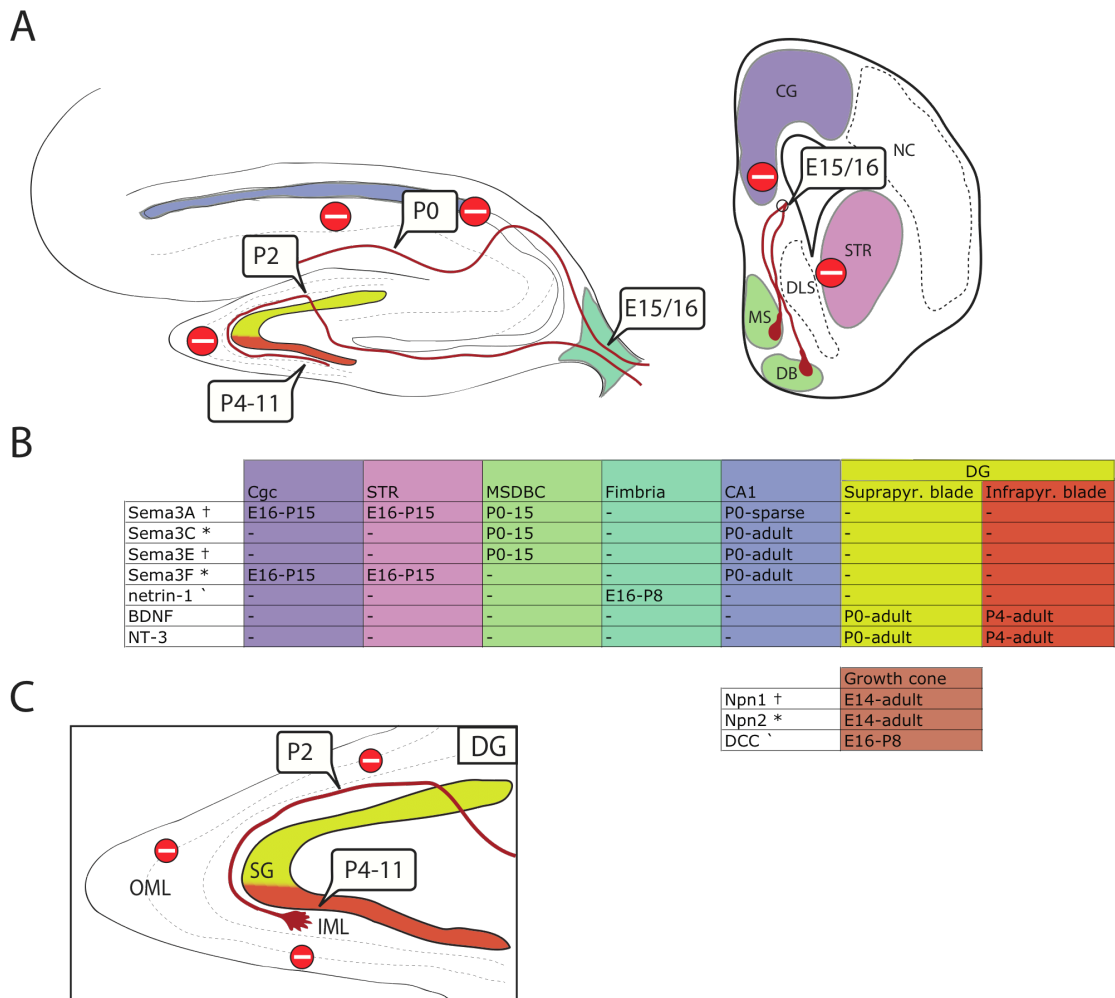


Figure 3, Overview of the developing septo-hippocampal projections. **A**. Schematic overview of the hippocampus (*left*) and septal area (*right*). Septal axons originating from the medial septum (MS) and diagonal band (DB) grow towards the fimbria around E15/16 and are guided by the repulsive cues present in the cingular cortex (Cg) and striatum (STR). The first septal axons within the stratum lacunosum-moleculare arrive around P0. Innervation of the IML at the suprapyramidal blade can be observed at P2 subsequently the infrapyramidal blade is protruded at P4-11. Repulsive cues present in the OML and stratum pyramidale avoid the protrusion of septal axons within the latter two layers. **B**. Overview of the expressed AGMs and receptor within the subfields, brain areas and the protruding growth cone. [medial septum and diagonal band complex (MSDBC), Cornu Ammonis 1 (CA1), dentate gyrus (DG), Suprapyramidal blade (Suprpyr. blade), Infrapyramidal blade (Infrapyr. blade)]. AGM and receptor pairs are expressed during the development of the septo-hippocampal projections: †Sema3A/3E-Npn1, *Sema3C/3F-Npn2, `netrin-1-DCC. **C**. Protrusion of the septal axons within the IML start at the suprapyramidal blade (P2) and moves towards the infrapyramidal blade (P4-11). Abbreviations: neocortex (NC), stratum granulosum (SG)

Maturation of the intra- and interhippocampal pathways

Schaffer-collaterals

The embryonic development of the Schaffer-collaterals has not been studied in great detail, however, more is known about postnatal development. Specifically, synapse formation at the CA1 pyramidal dendrites has been studied in detail using *in vitro* neuronal cultures (Henkemeyer et al., 2003; O'Connor et al., 2009; Paradis et al., 2007). Two AGMs families have been shown to be involved in the development of Schaffer collaterals.

First, **Sema5B** is expressed postnatally (P1-60) in the CA1, CA3 and DG of the hippocampus (O'Connor et al., 2009). Immuno-labelling with Sema5B-specific antibodies shows protein expression along the dendrites of the pyramidal neurons, predominantly within the stratum radiatum. Overexpression of Sema5B in *in vitro* hippocampal cultures results in a reduction of presynaptic markers apposed to postsynaptic density (PSD) labelling. Due to the low transfection-efficiency of the Sema5B expression-vector, discrimination could be made upon the effect of Sema5B overexpression at the presynaptic or postsynaptic membrane. Sema5B overexpression in the postsynaptic neuron resulted in a reduction of synapses contacts whereas overexpression of Sema5B in the presynaptic neuron did not affect the number of synapse contacts. Therefore elimination of synapses by Sema5B has been attributed to the expression of Sema5B at the postsynaptic membrane (O'Connor et al., 2009).

The **BDNF** receptor, TrkB, has been implicated to play an important role in synaptogenesis. The role of TrkB in postnatal development of the Schaffer collaterals has been investigated by using a conditional knockout mouse¹¹ (Luikart et al., 2005). In general, the ablation of TrkB under the control of the glial fibrillary acidic protein (GFAP) or synapsin promoter showed normal Schaffer-collateral projections within the hippocampus. However, a more detailed analysis of the dendritic spine density, axon varicosites and apposition of

¹¹ Conditional knockouts are of particular interest because they allow knockout of a particular gene at a desired time-point in a specific brain area. Mice expressing the enzyme Cre-recombinase under control of spatiotemporal specific promoters (synapsin, GFAP, or CaMKII) are crossed with floxed-TrkB transgenic mice. Offspring mice have TrkB knocked out at various time-point (E12.5, E17 or P18 respectively) and within specific structures. Synapsin driven knockout of TrkB is restricted to the presynaptic neurons (CA3), whereas both GFAP and CaMKII driven knockout of TrkB results in a ablation of the gene in both pre- and postsynaptic neurons (CA1/3).

pre/postsynapse markers revealed that GFAP-promoter driven ablation of TrkB at both pre- and postsynaptic sites led to a pronounced reduction in synapse formation. Presynapse restricted ablation of TrkB by synapsin-driven Cre-recombinase expression did not have major effects on the synapse formation. By use of *in vitro* cultures postsynapse specific knockout of TrkB resulted in reduction of PSD-95 labelling and synaptic contact formation. Therefore TrkB has a cell-autonomous function in the Schaffer collateral synaptogenesis (Luikart et al., 2005).

All together, the role of AGMs during postnatal development of Schaffer collaterals has thus far been characterized at the level of synaptogenesis and synapse elimination.

Commissural-associative tracts

Commissural projections towards the contralateral hippocampus start to innervate the IML around P2-5 (Supèr and Soriano, 1994). Less is known about the postnatal development of associational tracts within the ipsilateral hippocampus. Two AGMs, netrin-1 and ephrin-A5, are both expressed in the hippocampus postnatally and have been shown to support the postnatal development of the commissural-associative tracts (Figure 4).

Expression profiles of Netrin-1 in P1-8 rat hippocampi indicate that this AGM is expressed in CA1 and CA3 (Steup et al., 2000). The DCC-receptor is expressed in the DG and CA1-3. Taken together, these data suggests that netrin-1 might be involved in the postnatal development of commissural-associative tracts. However, it becomes difficult to investigate this question with the traditional netrin-1 knockout strain, since netrin-1^{-/-} already influences the prenatal development of the commissural-associative tracts (see previous chapter).

The targeting of commissural-associative tracts is also mediated by EphA-ephrinA interaction as work by Otal et al. (2006) indicated. **Ephrin-A5** has been shown to bind EphA3 (Himanen et al., 2004) and both are expressed in a dynamic pattern during postnatal development. Ephrin-A5 transcript is abundantly expressed in all subfields of the hippocampus and DG until P5 when the expression starts to decline (Figure 4). For EphA3, protein expression is observed within both stratum lacunosum/moleculare and the MML between early postnatal days (P0-5). This pattern changes after P5, when the IML and the stratum radiatum express EphA3 protein. The entorhinal protrusions, which arrive first within the hippocampus,

probably express EphA3 first within the stratum lacunosum-moleculare, followed by the commissural-associative fibers within the stratum radiatum. Anterograde tracing, using DiI, within the contralateral hippocampus of ephrin-A5^{-/-} mice reveals that commissural-associative projections are misdirected towards the stratum lacunosum/moleculare (CA1-3) and the stratum granulosum (DG) around P5. A proposed mechanism is that repulsive cues provided by ephrin-A5 within the DG inhibit commissural axons from protruding into the stratum granulosum. This same mechanism could apply to the specific termination of the projections within the stratum radiatum for which EphA3 receptors receive the ephrin-A5 cues from the pyramidal cells in the hippocampal proper. The presence of ephrin-A5 is important for the lamina-specific targeting of commissural projections. The fact that ephrin-A5 and EphA3 are expressed during a specific time-window (\pm P0-15) suggests that the formation of contralateral projections during this period is dependent upon these AGMs.

All together, two AGMs, netrin-1 and ephrin-A5, have been discussed in relation to their role during axon guidance. In addition, as discussed in the entorhino-hippocampal pathway-section, Sema3A might establish a boundary at the CA1-subiculum to avoid commissural axons from entering the subiculum and EC.

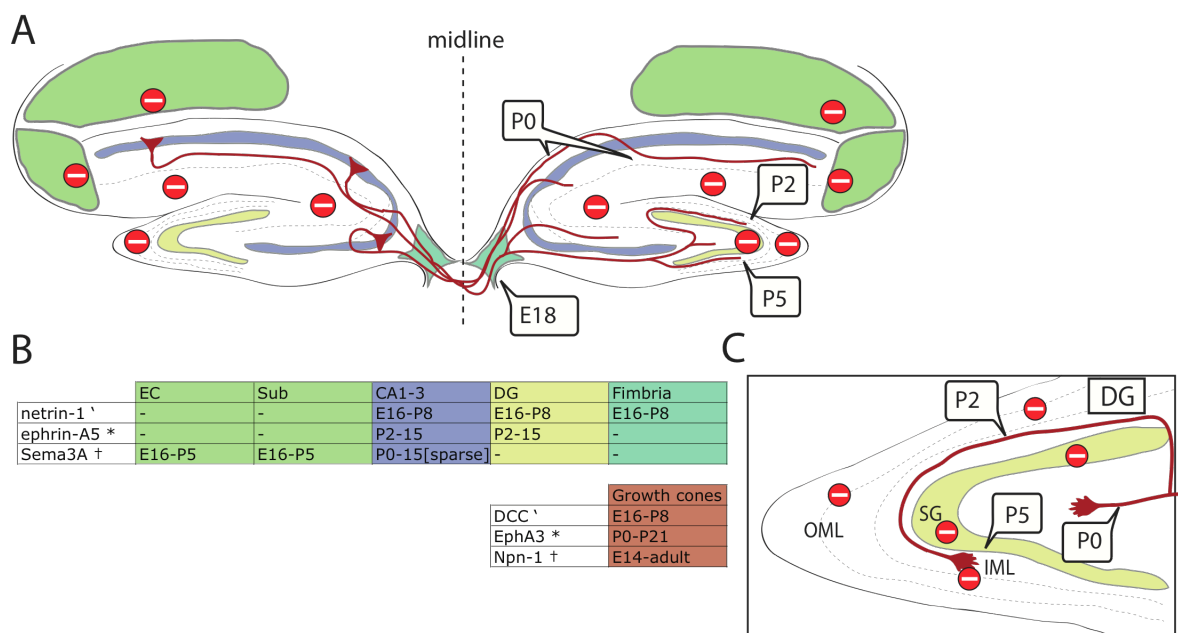


Figure 4, Overview of the developing commissural-associative projections. A. Schematic overview of the hippocampus. Commissural projections cross the midline and arrive around E18 at the fimbria of the contralateral hippocampus. At P0 the first projections terminate within the stratum oriens and radiatum and the hilus (C.). As described in the entorhino-hippocampal section, Sema3A expression within the subiculum and EC prevent commissural axons to protrude these areas. Commissural projections arrive at the suprapyramidal blade within the IML around P2 and subsequently, P5, grow towards the infrapyramidal blade of the same layer. B. Overview of the AGMs and receptors expressed by the subfields, brain areas and the protruding growth cone during the development of

this pathway. Various pairs of AGM and receptor are identified: †Sema3A-Npn-1, *ephrin-A5-EphA3, `netrin-1-DCC. C. Arrival of the commissural projections within the contralateral DG. Repulsive cues provided within the stratum granulosum (SG) avoid protrusion of the commissural axons. Presumably the early arrival of entorhinal axons within the OML provides repulsive cues to restrict commissural axons to the IML.

Mossy fibers

As suggested previously, mossy fibers presumably develop postnatally given that most of the granular cells appear in the DG during the peri- and postnatal periods (Angevine, 1965). Several AGMs, such as Slit1 and 2, Sema6A, NT-3, and BDNF are related to the process of postnatal mossy fiber development. These AGMs are involved in the lamina-specific innervation of mossy fibers via predominantly suprapyramidal or infrapyramidal bundles (Figure 5).

Slit1 and **Slit2** are expressed in the postnatal (P3) hippocampus (Nguyen Bacharvet et al., 1999). In particular Slit2 has a repulsive action on the growth of mossy fiber towards the OML. Entorhinal axons in the OML express Slit2, whereas hilar mossy fibers are able to respond to Slit2 by their expression of both Robo1 and -2 (Figure 2). In this way Slit2 expression in the OML prevent mossy fibers to protrude this layer. It is currently unknown if Slit2 specifically binds to either Robo1 or -2. The Cajal-Retzius cells present in the DG express Robo2 as well. The key role of Cajal-Retzius cells is to guide the protruding axons into the appropriate area or lamina (Del Río et al., 1997). Ablation of Cajal-Retzius cells disrupts the outgrowth of the perforant pathway within the stratum lacunosum-moleculare and OML. Protruding EC axons, expressing Slit2, might therefore affect the Cajal Retzius cells via the repulsive Slit2/Robo-2 complex. This might result in a re-localization of the Cajal Retzius cells, which influences the distribution of newly arriving projections.

Sema6A and its receptors, plexin-A2/A4, are lamina-specifically expressed in the postnatal hippocampus (Figure 5). CA3 pyramidal cells express Sema6A along their apical and basal dendrites. Sema6A has a repulsive action on the growth cone via plexin-A4, whereas plexin-A2 attenuates this repulsive action (Suto et al., 2007). Plexin-A4 is predominantly expressed by granular cells of the DG, and stimulates the mossy fibers to move away from the Sema6A expressing lamina. The knockout of plexin-A4, in mice, therefore gives rise to a broadly distributed innervation of all CA3 lamina by mossy fibers. Plexin-A2^{-/-} knock-outs produce an innervation of the CA3 stratum pyramidale, since Sema6A expression is relatively low at the cell body. The Sema6A receptor, plexin-A2 is particularly expressed at the proximal, apical dendrites of CA3 pyramidal cells at the height of stratum

lucidum, so both plexin-A2 and Semaphorin 6A are expressed at the same membrane. The specific innervation of CA3 stratum lucidum by mossy fibers could therefore be explained by the selective attenuation of the Semaphorin 6A-plexin-A4 repulsive complex via plexin-A2 within stratum lucidum. How plexin-A2 attenuates the Semaphorin 6A-plexin-A4 repulsive complex remains unknown. The authors, Suto et al. (2007), suggest that plexin-A2 disables the binding of Semaphorin 6A to plexin-A4 *in cis*, meaning at the same membrane Semaphorin 6A is expressed at, by masking Semaphorin 6A. Interestingly, Semaphorin 6A knock-outs, as well as double knockouts of Semaphorin 6A and plexin-A2, have a similar phenotype, regarding mossy fiber-CA3 pathway, as wild-type animals. This suggests that other, presumably attractant, cues are present to guide mossy fibers towards the stratum lucidum in the absence of Semaphorin 6A-plexin-A4/A2 signalling. Semaphorin 6A and plexin-A2 are predominantly expressed between P0-10, this is the time-window in which the first mossy fibers innervate the CA3 (Suto et al., 2007). New protruding mossy fibers at a later age presumably follow the established tracts to innervate the appropriate CA3 lamina.

Candidates for the attractant cue to support the mossy fiber growth towards CA3 stratum lucidum are **BDNF** and **NT-3** (Figure 5). Both neuroattractants are expressed in the CA3 stratum lucidum from P7 onwards (Das et al., 2001). A recent study, however, showed that BDNF overexpression by CA3 pyramidal cells did not result in an increase of innervation of these pyramidal cells by mossy fibers (Tamura et al., 2009). Instead, once passed pyramidal cells with increased BDNF expression axons start to group and form bundles. This shows that BDNF induces the fasciculation of the mossy fibers rather than attract the growth cones. The prolonged expression of BDNF from P7 onwards, within the CA stratum lucidum, might therefore contribute to the guidance of new mossy fiber protrusions by fasciculation of the mossy fiber bundle. A functional role for NT-3 expressed in the CA3 stratum lucidum is not yet investigated.

In conclusion, mossy fibers find their way to CA3 stratum lucidum with the help of Semaphorin 6A and BDNF expression. In addition, Slit2 prevents the formation of synaptic contact between mossy fibers and the apical dendrites originating from the same granular neuron within the IML.

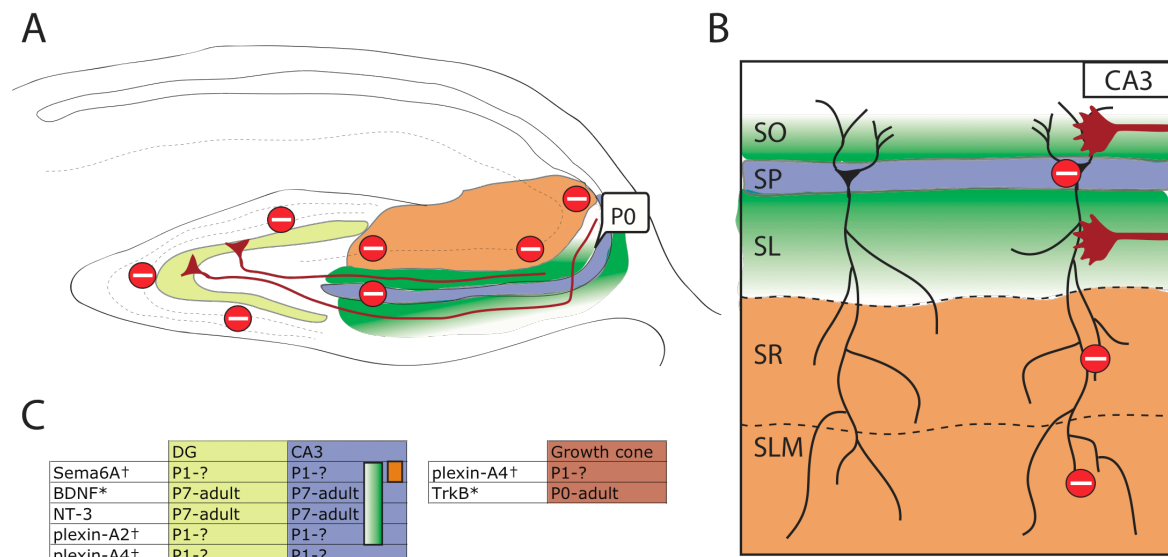


Figure 5, Overview of the developing mossy fibers. A. Schematic overview of the hippocampus. Granular cells projects mossy fibers towards the CA3 via suprapyramidal or infrapyramidal bundles from P0 onwards. As discussed earlier, entorhino-hippocampal projections present in the OML express Slit2, whereas granular neurons express Robo1 and -2. Slit2-Robo1/2 complexes prevent mossy fibers to form synaptic contacts with the apical dendrites originating from the same granular neuron within the IML. B. Arrival of projections within the CA3. Growth is restricted to the stratum lucidum (SL) and stratum oriens (SO) by the expression of repellents within the stratum pyramidale, radiatum and lacunosum-moleculare (SP, SR and SLM). C. Overview of the AGMs and receptors expressed within the subfields and growth cone: †Sema6A-plexin-A2/A4, *BDNF-TrkB. Gradients of plexin-A2 [green gradient bar] are proposed to attenuate the repulsive action of Sema6A-plexin-A4 complex [orange bar and green gradient bar].

AGMs in synaptogenesis and synaptic maturation

So far, AGMs have been discussed that are implicated in the guidance of growth cones during postnatal development of various anatomical pathways. However, once an axon has reached a dendrite, whether the axon stay and forms a connection or is retracted depends on cues such as AGMs. Several AGMs play a role in synaptogenesis or synaptic maturation without being related to a specific anatomical pathway. As mentioned earlier, synaptogenesis refers to the formation of synapses, whereas synaptic maturation involves the process of establishing synaptic contact and specialisation of contact. The fact that these AGMs are not related to a specific pathway might be due to the use of the model in which it has been studied. For example, several of the studies used mainly *in vitro* cell cultures with neurons from different subfields and did not test them in *in vivo* models (O'Connor et al., 2009; Paradis et al., 2007; Woo et al., 2009). This makes it difficult to relate the AGM of interest to the process of synaptogenesis within a specific pathway. Further studies should provide information about which pathway, or pathways, the function of these AGMs is restricted. This section will discuss these AGMs that are found to play a role in synaptogenesis or synaptic maturation during the postnatal development.

Both **NGL-3** and **LAR** receptors are involved in the formation of pre- and postsynapses, respectively (Dunah et al., 2005; Kim et al., 2006; Woo et al., 2009). Functional blockade of the interaction between NGL-3 and LAR with exogenous soluble LAR resulted in a reduction of presynaptic markers. In contrast, neuronal cultures formed postsynaptic structures with LAR expressing non-neuronal cells, indicating that LAR is involved in the formation of the postsynapse (Woo et al., 2009). This is supported by observations in LAR knock-out animals, in which cholinergic septo-hippocampal projections had reduced synaptic contacts (Yeo et al., 1997).

Ephrins have been proposed to play a key role in synaptogenesis and maturation (Martínez and Soriano, 2005). A study conducted by Martínez et al. (2005) aimed to investigate the role of Eph-ephrin complexes in postnatal development by disrupting these complexes with soluble Eph molecules. Using an *in vivo* system, transgenic mice that express soluble EphA5-Fc have a remarkable reduction in synapse density in nearly all lamina of the CA1-CA3 subfields (Martínez et al., 2005). This reduction can be observed in both pre- and postsynaptic densities, which implies that disruption of the EphA-ephrinA complex by EphA5-Fc reduces synaptogenesis. Interestingly, the same study shows that overexpression of EphA5-Fc has a reverse effect regarding the formation of synapses by mossy fibers within the CA3. Specifically, the mossy fibers that originate from the infra-pyramidal blade of the DG show an increase in synapse density and size and number of synaptic contacts. It remains unclear how these contradicting effects of EphA-ephrinA complex disruption are mediated. In addition it is unclear if EphA5 contribution to the process of synaptogenesis is restricted to a specific pathway, since the effect of EphA5-Fc overexpression results in a reduction of synapse density in nearly all lamina.

Ephrin receptor EphA4 has been found to be important for the dendritic spine structure (Murai et al., 2003; Tremblay et al., 2007; Tremblay et al., 2009). Interestingly, its ligand, **ephrin-A3**, is expressed by astrocytes. Most of these astrocytes have their processes wrapped around the dendritic spines. As suggested by other studies, astrocytes provide a supportive structure for the formation and maintenance of synaptic contacts, which possibly is partly provided by EphA4 and ephrin-A3 interaction (Goldshmit et al., 2006; Nishida and Okabe, 2007). This is a relatively new function for astrocytes in the developing brain. Knock-out of EphA4 results in a disruption of dendritic spine structure and organization, whereas activation of the receptor by exogenous ephrin-A3 induces

similar structural changes. These observations seem to contradict each other, however one could argue that exogenous ephrin-A3 induced activation of EphA4 does not mimic the interaction as between dendritic spine and astrocyte. Bidirectional signalling between the two structures via EphA4 and the GPI-anchored ephrin-A3 might result in the maintenance of the spine structure, whereas unidirectional signalling as a consequence of EphA4 activation by exogenous ephrin-A3 induces dendritic spine remodelling. In any case, EphA4 and ephrin-A3 is important in the spine morphology and further research will need to be done to provide insights into the signalling related to spine morphology. Apparently, EphA-ephrinA complexes do have an overall role in synaptogenesis but their exact function may differ per tract.

Like class-A Eph-ephrin complexes the class-B complexes are also implicated in the synaptogenesis and maturation (Ethell et al., 2001; Henkemeyer et al., 2003). Interestingly, knock-out of at least two, but most efficiently three, EphB receptors resulted in a reduction of mature spines and synaptic contacts. On the other hand the knock-out of one EphB receptor did not alter spine morphology or number of synaptic contacts. This indicates that the process of synaptogenesis and maturation requires a combined effort from several EphB-ephrin complexes.

In addition to ephrins, several semaphorins have been identified to play a major role in this process. One example, **Sema5B** (O'Connor et al., 2009), has already been described in the present work. Other semaphorins have been identified in an elaborate knock-down screening with small-interfering RNA (siRNA)¹², Paradis et al. (2007) showed that in *in vitro* neuronal cultures, both **Sema4B** and **Sema4D** contribute to synapse formation within the hippocampus (Paradis et al., 2007). It remains unclear as to which hippocampal pathway these two AGMs contribute to the synaptogenesis. However the same study showed that Sema 4D specifically regulates the postsynaptic density in GABAergic contacts. Sema4B seems to be involved in formation of postsynapses among GABAergic and glutamatergic contacts.

¹² RNAi-based screening utilizes a technique that blocks the translation of specific mRNA with small-interfering RNA molecules (siRNAs). These siRNAs bind complementary to the mRNA of interest, whereby it forms double-stranded RNA. Endogenous mechanisms are known to degrade double-stranded RNA, disrupting the translation of the targeted mRNA. Efficiency of functional gene ablation is typically low, therefore the term knock-down of a gene is most appropriate.

Conclusions

In general AGMs provide attractant or repellent cues for the protruding axon. Apart from the “classical” AGMs, several other proteins, such as neurotrophins and RPTP receptors, are important in the guidance of axonal outgrowth. This becomes, in particular, apparent when one studies the hippocampal development in genetically modified mice strains which have a deficiency in expression of a particular AGM, neurotrophin, RPTP or one of the AGM/neurotrophin receptors. The major objective of the present work is to provide an overview on the involvement of AGMs during the postnatal development of the hippocampus in rodents. A general concept of AGM involvement during hippocampal development is to provide boundaries along which axons can grow. The laminar organization within the hippocampus can be attributed to the spatial expression of the reviewed AGMs and neurotrophins. During postnatal development, the emphasis is put on establishing this laminar organization together with the formation of synapses, called synaptogenesis, and subsequent synapse maturation. The reviewed work proposes that in all three processes, AGMs are involved and have a major impact on the organization of the hippocampal network (Table 2).

Laminar organization of the afferents within the hippocampus is largely supported and regulated by AGMs. For most of the reviewed projections it becomes apparent that, adjacent to the protruded layers, repulsive cues are expressed. Depending on the presence of receptors that are receptive for these repulsive cues, the protruding growth cone arrives at the right layer (Barallobre et al., 2000; Otal et al., 2006). So the divergence is not only made by the specific distribution of the AGMs/neurotrophins within the layers, but also by the particular set of receptors at the growth cones. To understand and support this, more knowledge is required about the precise distribution of the proteins related to the protruding axons rather than solely the mRNA expression patterns.

Synaptogenesis within several subfields has been studied. In particular, ephrin-Eph complexes and semaphorins are implicated to support synaptogenesis. It is interesting to see that astrocytes are proposed to play a role in the maintenance and genesis of spine morphology via ephrin-Eph complexes since it adds an additional function for the presence of astrocytes in the developing brain (Goldshmit et al., 2006; Murai et al., 2003; Nishida and Okabe, 2007). In some cases, it remains difficult to relate the involvement of AGMs to synaptogenesis within a specific pathway. The question therefore remains as to whether the

knowledge provided by these studies reflects general mechanisms of synaptogenesis.

Synaptic maturation is not elaborately discussed in the present work. Nonetheless, reduction of mature synapses in knock-outs of EphB receptors or TrkB indicate that these receptors with, presumably, their complementary AGMs/neurotrophin are involved in the maturation of synapses (Henkemeyer et al., 2003; Luikart et al., 2005). In addition, one study performed by O'Conner et al. (2009) showed that Sema5B is involved in the elimination of synaptic contact once it has been formed. This is an essential step in the fine-tuning of neuronal networks for which the "right" partner selection regulates what input the postsynaptic neuron receives and therefore the information processing at later ages.

As mentioned earlier it becomes apparent that most of the studied AGMs provide boundaries along which axons can grow. For several projections it is unclear if there are also attractant or growth inducing factors involved in the development of the pathway. For example, it remains unclear how mossy fibers are "encouraged" to grow towards the stratum lucidum within the CA3. The role of BDNF should not be assigned as a pure attractive function, which leaves the question as to whether or not NT-3 is involved (Tamura et al., 2009). Future work could address the question about whether NT-3 acts as an attractant to the mossy fibers. In addition, the driving force for EC axons to protrude the hippocampus remains unclear¹³. Either the presence of attractant cues along the route, or the pushing force of repellent cues from already protruded subfields, such as CA1 or subiculum, might force the growth cone to move away at later age. In other words, there could be a critical time-window in which protruding entorhinal projections need to pass the CA1 and subiculum. After this time-point, the recently protruded areas repel the entorhinal projections. This suggests that the precise regulation of protrusion timing and spatial expression of AGMs is crucial in the establishment of pathways. Additionally, this underlines the importance of better knowledge about the spatiotemporal expression pattern of AGMs in relation to the time-dependent axon protrusion. As the first steps are

¹³ As Barallobre et al. (2000) has shown that lamina-specificity of protruding entorhinal-hippocampal fibers is lost in netrin-1^{-/-} mice it remains unknown what attractant is responsible for the targeting of these fibers to the hippocampus at all. In other words, the loss of netrin-1 does not abandon the ability of entorhinal axons to grow towards the hippocampus.

already made by elaborate studies investigating the spatiotemporal expression pattern of AGMs, one could try to narrow down the exact time-window in which axons are able to protrude specific areas. One study by Skutella et al. (1999) addressed whether or not there is a time-related preference for protrusion of EC tracts into the hippocampus. Using rat brain tissue, exposure of postnatal EC explants (P0) to hippocampal cell membranes from different ages (E15-P60) showed preferential neurite outgrowth towards hippocampal preparation from early postnatal age (P0) over early embryonic ages (E15-E18) and late postnatal ages (P15-<P60). This advocates that transitions in time of cues present within the hippocampus determines a specific time-window in which EC projections innervate the hippocampus (E19-P10). A similar approach using hippocampal cell membranes from AGM knockout mice of different ages could be used to address the question which AGM(s) determine this time-window specific innervation of the hippocampus by EC projections. Alternatively, EC explant cultures from transgenic mice in which a specific AGM-receptor is knocked out would be useful to address the question of what receptors are required for the time-related protrusion.

When comparing the spatio-temporal order of hippocampal innervation by the entorhinal, septo-hippocampal and commissural-associative tracts, the sequential innervation of the same area by these different projections becomes apparent (Supèr and Soriano, 1994). For example, the early innervation of OML by the entorhinal tract is followed by the septo-hippocampal and commissural-associative tracts protruding into the IML (E19-P5). This early innervation by the entorhinal tract might establish a barrier for the protruding septo-hippocampal and commissural-associative tracts. The latter two tracts seem to innervate the DG simultaneously, possibly providing a 'supportive structure' to establish more projections at later ages (P2-5). A function for axon guidance molecules, regarding this 'supportive structure', could be to establish a barrier of chemorepulsive cues at the OML, and/or establish the fasciculation of the protruding septo-hippocampal and commissural-associative tracts. This concept, however, is a matter of controversy (Frotscher et al., 1997). Frotscher et al. (1997) reviews several studies which observed either no or less pronounced alteration in laminar termination by commissural projections as a result of lesioning the entorhino-hippocampal afferents. On the other hand, ablation of Cajal-Retzius cells in the DG has a more pronounced effect on the laminar innervation. The authors therefore propose that the presence of Cajal-Retzius cells in the DG, rather than the temporal sequence of innervation, is important for the

lamina-specific pattern. The former, involvement of the Cajal-Retzius, are likely to be involved but could be affected by the protruding EC axons as suggested earlier, leaving it unclear if the temporal sequence of innervation has a functional role at all.

This observation might also introduce a problem towards the way one interprets anatomical abnormalities in various knockout mice. The loss of a certain AGM may not always have a direct consequence on each pathway apart, but might affect the protrusion of one pathway, leading to subsequent consequences for another pathway. For example, the knockout of an AGM (e.g. RGMa or ephrin-A3) leads to a disruption of the lamina specificity for the entorhinal fibers projecting towards the OML. At a later age, also the innervation of the DG by septo-hippocampal or commissural-associative tracts within the IML may show loss of lamina-specificity. One likely explanation would be that deficiency of the AGM resulted in loss of lamina-specificity for all three pathways. However, these consequences do not necessarily rely upon the direct effect of the AGM towards each of the three different projections. It could be that changes in the innervation pattern of the entorhinal axons results in the disruption of a 'supportive environment' normally provided by the presence of the entorhinal axons. This 'supportive environment' includes the presence of various AGMs (e.g. Slit2) expressed by entorhinal axons present in the DG. The changed organization of EC axons in the DG, and therefore the loss of the 'supportive environment' a priori could therefore result in the loss of laminar organization of the septo-hippocampal and commissural-associative projections arriving in the DG at the later age. Ultimately, this possible cascade of events adds a layer of complexity to the interpretation of tracing experiments in knockout mice. In particular the use of traditional knockout mice makes it difficult to address such a dilemma.

One way to approach this problem is by using conditional knockout strains that enable us to specifically knockout the receptor that bind the AGM of interest (Luikart et al., 2005). The conditional knockout of the receptor could be restricted to the EC during the period the entorhino-hippocampal projections enter the DG (E18-P2) and therefore disrupt the targeting of the entorhinal projections. As a result, the presence of the AGM is not altered and only the entorhinal projections are incapable of innervating the target lamina. The entorhinal projections would then not provide the 'supportive environment' in the DG. With this approach the cause and effect could be determined. If the septo-hippocampal and the commissural-associative pathways still appear normal, the previous disruption

was due to the loss of the AGM. If, on the other hand, the septo-hippocampal and the commissural-associative pathways are also altered, it is more likely that the effect is caused by a disruption of the EC fibers. Such conclusion, however, should be made with caution. In case knockout of the receptor is not restricted to the EC alterations in the septo-hippocampal and the commissural-associative pathways could still be due to lack of the receptor instead of the disruption of the 'supportive environment' provided by entorhino-hippocampal projections within the DG.

Adjacent to this problem, influence of AGM deficiency earlier in development might mask the involvement of the same AGM at later ages. Since netrin-1 has been shown to affect the outgrowth of several commissural-associative fibers during embryonic stages it is difficult to show with use of classical netrin-1 knockout, that netrin-1 is directly involved in the postnatal development of the same pathway. In order to show the latter the early development should be intact. This problem could be tackled with the use of conditional knock-out targeting the netrin-1 gene at different ages and within specific brain areas/subfields, as proposed above. In this way, the contribution of netrin-1 in guidance of a specific pathway can be investigated within the desired time-window. As mentioned above, it will be difficult to discriminate between the effects due directly to netrin-1 loss and secondary effects on a specific pathway.

In conclusion, AGMs and axon guidance related molecules (neurotrophins and RPTPs) play an important role during the development of the hippocampus. These proteins support the process of **laminar organization, synaptogenesis** and **synaptic maturation**, in particular, during the postnatal development. Future research should provide more in-depth knowledge about the causal relationship between temporal specific protrusion of axons and the expression pattern of AGMs, neurotrophins and RPTPs. In addition, the hypothesis that the dynamic regulation of AGM/neurotrophin receptor presence on growth cones attributes to a great extent to the specificity of growth behaviour could be studied more elaborately. A third direction of research might be investigating the role of astrocytes in synaptogenesis via AGMs or axon guidance related molecules. The knowledge obtained from the present discussed studies, and those performed in the future, contribute to a better understanding of mechanisms underlying hippocampal development and pathologies related to mis-wiring of the hippocampal neuronal network.

Family	Member	Network	Process	References	
Axon guidance molecules	Sema3A	Entorh-hip (E/P), Septo-hip (E/P), Comm-ass (E)	Laminar org.	Chedotal et al. (1998), Skallora et al. (1998), Steup et al. (2000), Pozas et al. (2001), Pascual et al. (2005)	
	Sema3C	Septo-hip (P)	outgrowth, laminar org.	Steup et al. (2000), Pascual et al. (2005)	
	Sema3E	Septo-hip (P)	outgrowth, laminar org.	Pascual et al. (2005)	
	Sema3F	Entorh-hip (P), Septo-hip (E/P)	Laminar org.	Chedotal et al. (1998), Steup et al. (2000), Pozas et al. (2001), Pascual et al. (2004), Pascual et al. (2005)	
	Sema5B	Schaffer-co (P)	synaptogenesis	O'Conner et al. (2009)	
	Sema6A	Mossy fiber (P)	Laminar org.	Suto et al. (2007)	
	ephrin-A3	Entorh-hip (E/P)	Laminar org.	Stein et al. (1999), Matinéz and Soriano (2005)	
	ephrin-A5	Comm-ass (P)	Laminar org.	Stein et al. (1999), Matinéz and Soriano (2005), Otal et al. (2006)	
	ephrin-B3	n.a.	synaptogenesis, maturation	Henkemeyer et al. (2003), Liebl et al. (2003), Matinéz and Soriano (2005)	
	RGMa	Entorh-hip (P)	Laminar org.	Brinks et al. (2004), Oldenkamp et al. (2004)	
	Netrin-1	Entorh-hip (E), Schaffer-co (E), Comm-ass (E/P)	Laminar org.	Barralobre et al. (2000), Steup et al. (2000), Pascual et al. (2004)	
	Silts	Entorh-hip (E/P), Mossy fiber (P)	Laminar org.	Nguyen Ba-Charvet et al. (1999)	
	Axon guidance molecule-receptors	Npn-1	Entorh-hip (E), Septo-hip (E)	Laminar org.	Steup et al. (2000), Pascual et al. (2005), Chauvet et al. (2007)
		Npn-2	Entorh-hip (P), Septo-hip (E/P)	Laminar org.	Pozas et al. (2001), Chedotal et al. (1998), Pascual et al. (2005)
plexin-A2		Mossy fiber (P)	Laminar org.	Suto et al. (2007)	
plexin-A4		Mossy fiber (P)	Laminar org.	Suto et al. (2007)	
EphA3		Comm-ass (P)	Laminar org.	Otal et al. (2006)	
EphA4		n.a.	synaptogenesis	Liebl et al. (2003)	
EphA5		Entorh-hip (P)	Laminar org.	Stein et al. (1999), Otal et al. (2006)	
EphB1		n.a.	synaptogenesis, maturation	Henkemeyer et al. (2003), Liebl et al. (2003)	
EphB2		n.a.	synaptogenesis, maturation	Henkemeyer et al. (2003), Liebl et al. (2003)	
EphB3		n.a.	synaptogenesis, maturation	Henkemeyer et al. (2003), Liebl et al. (2003)	
DCC		Comm-ass (P)	Laminar org.	Barralobre et al. (2000), Steup et al. (2000)	
NGL		n.a.	synaptogenesis	Kim et al. (2003)	
Robo1		Entorh-hip (E/P), Mossy fiber (P)	Laminar org.	Nguyen Ba-Charvet et al. (1999)	
Robo2		Entorh-hip (E/P), Mossy fiber (P)	Laminar org.	Nguyen Ba-Charvet et al. (1999)	
Axon guidance related molecules	NT-3	Septo-hip (P), mossy fiber (P)	outgrowth, laminar org.	Maisonpierre et al. (1990), Collazo et al. (1992), Das et al. (2001), Makuch et al. (2001), Gillespie et al. (2003)	
	BDNF	Septo-hip (P), mossy fiber (P)	outgrowth, fasciculation	Maisonpierre et al. (1990), Collazo et al. (1992), Das et al. (2001), Makuch et al. (2001), Gillespie et al. (2003)	
	TrkB	Entorh-hip (P), Schaffer-co (P)	outgrowth, synaptogenesis	Martínéz et al. (1998), Luikart et al. (2005)	
	alpha	n.a.	radial migration neuron	Petrone et al. (2003), Kostic et al. (2007)	
	LAR	Septo-hip (E)	outgrowth	Yeo et al. (1997), Dunah et al. (2005)	

Table 2, Overview of AGM involvement during hippocampal development. AGMs can be involved in specific networks/projections: Entorhino-hippocampal (Entorh-hip), Septo-hippocampal (Septo-hip), Schaffer-collaterals (Schaffer-co), Commissural-associative (Comm-ass), Mossy fiber (mossy fiber), or unknown networks (n.a.) during embryonic (E) and/or postnatal (P) phases.

Literature research

A select amount of review articles and books were used to attain a general overview of studies that have been conducted regarding hippocampal anatomy and development (Witter, 1989; Witter and Amaral, 2004), AGMs (Dickson, 2002; Huber et al., 2003) and the involvement of AGMs during hippocampal development (Heimrich et al., 2002; Skutella and Nitsch, 2001). Key papers were selected from these reviews and recent published studies were subsequently added in order to provide a complete as possible representation of data known to date. Some papers were excluded from being reviewed in the present work due to lack of specificity regarding the role of the AGM within the hippocampus or the time it is involved in development, for example after P28.

In general, the online PubMed scientific library provided by National Center for Biotechnology Information (NCBI) and the U.S. National Library of Medicine (NLM) (NCBI, 2009) was used for the acquisition of papers. All known synonyms for the various AGMs were used as search terms, in order to cover all relevant data about the AGMs.

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Appendix A

All used figures will be displayed in full-page format.

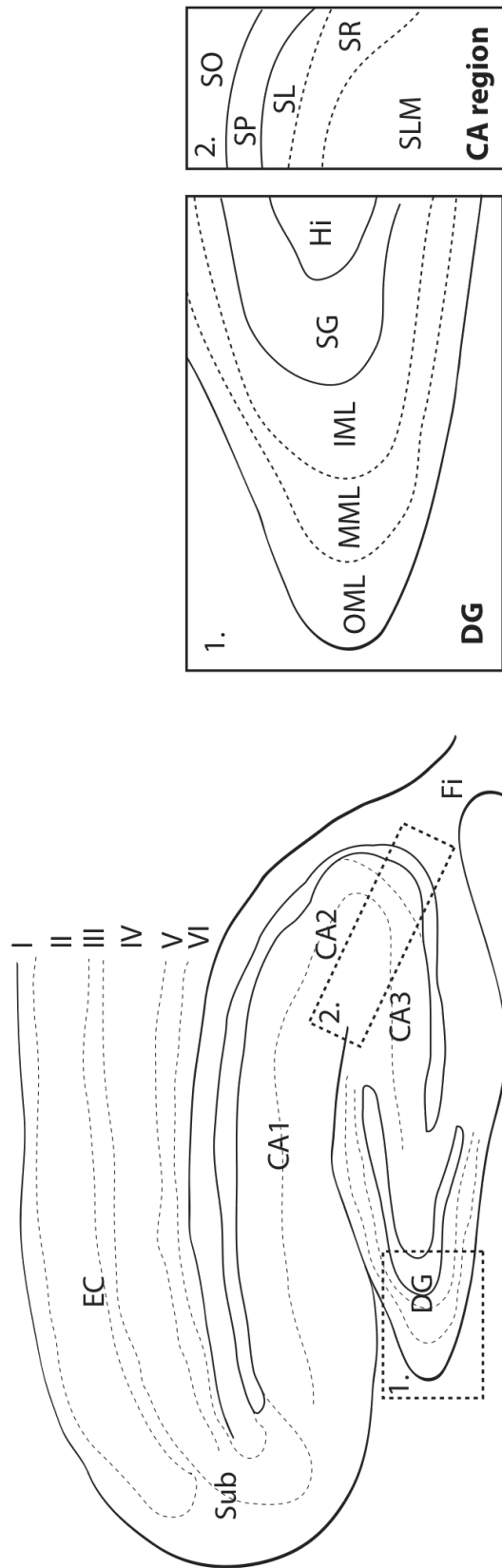


Figure 1, Schematic overview of the hippocampus. The hippocampus includes the dentate gyrus (DG), and hippocampal proper, which consists of the Cornu Ammonis subfields 1-3 (CA1-3). Adjacent to the hippocampal proper are the subiculum (Sub), and the entorhinal cortex (EC); the latter is subdivided into layers I-VI. Several projections enter and leave the hippocampus via the fimbria (Fi). The border between the CA1 and the suprapyramidal blade of the DG is called the fissure. Enlargement 1 represents the layers of the dentate gyrus (DG); outer one-third of stratum moleculare (OML), middle third of stratum moleculare (MML), inner-third of stratum moleculare (IML), stratum granulosum (SG), and the polymorphic area or hilus (Hi). Enlargement 2. Represents the layers within the CA3 subfield; stratum oriens (SO), stratum pyramidale (SP), stratum lucidum (SL), stratum radiatum (SR), stratum lacunosum-moleculare (SLM).

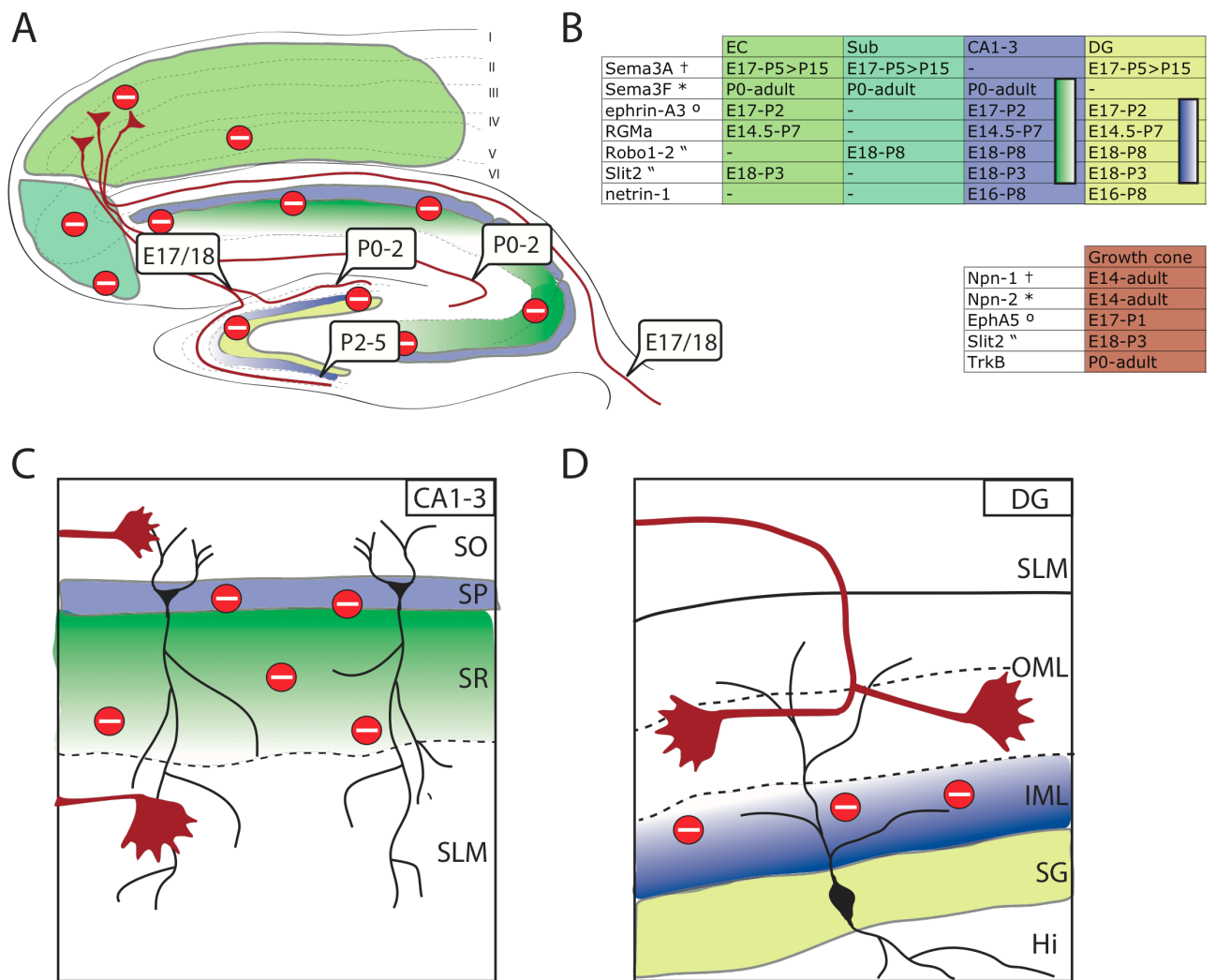


Figure 2, Overview of the developing entorhino-hippocampal projections. **A**. Schematic overview of the hippocampus. At E17-18 entorhinal axons arrive at the hippocampal proper [perforant pathway] and fimbria [alvear pathway]. The EC and subiculum force the axons to move away due to expression of repellents (B). Around P0-2 entorhinal axons protrude the stratum lacunosum-moleculare (SLM) and the OML at the suprapyramidal blade. Repellents expressed by the stratum pyramidale (SP) and stratum granulosum (SG) avoid ingrowth of the entorhinal axons. Around P2-5 the OML of the infrapyramidal blade receive afferents from the EC. **B**. Overview of the AGM and receptor expression in the subfields, brain areas and protruding growth cone. [entorhinal cortex (EC), subiculum (Sub), Cornu Ammonis (CA), dentate gyrus (DG)]. Several pairs of AGMs and receptors are expressed during the development: †Sema3A-Npn1, *Sema3F-Npn2, °ephrin-A3-EphA5, "Slit2-Robo1/2. Along the stratum radiatum (SR) within the CA1-3 [green gradient bar] and the IML within the DG [blue gradient bar] gradients of AGMs are expressed. **C**. Protruding entorhinal axons are repelled from the stratum pyramidale and stratum radiatum. **D**. Arrival of entorhinal axons within the OML is supported by gradients of repulsive cues are present within the IML. Abbreviations: stratum oriens (SO), hilus (Hi).

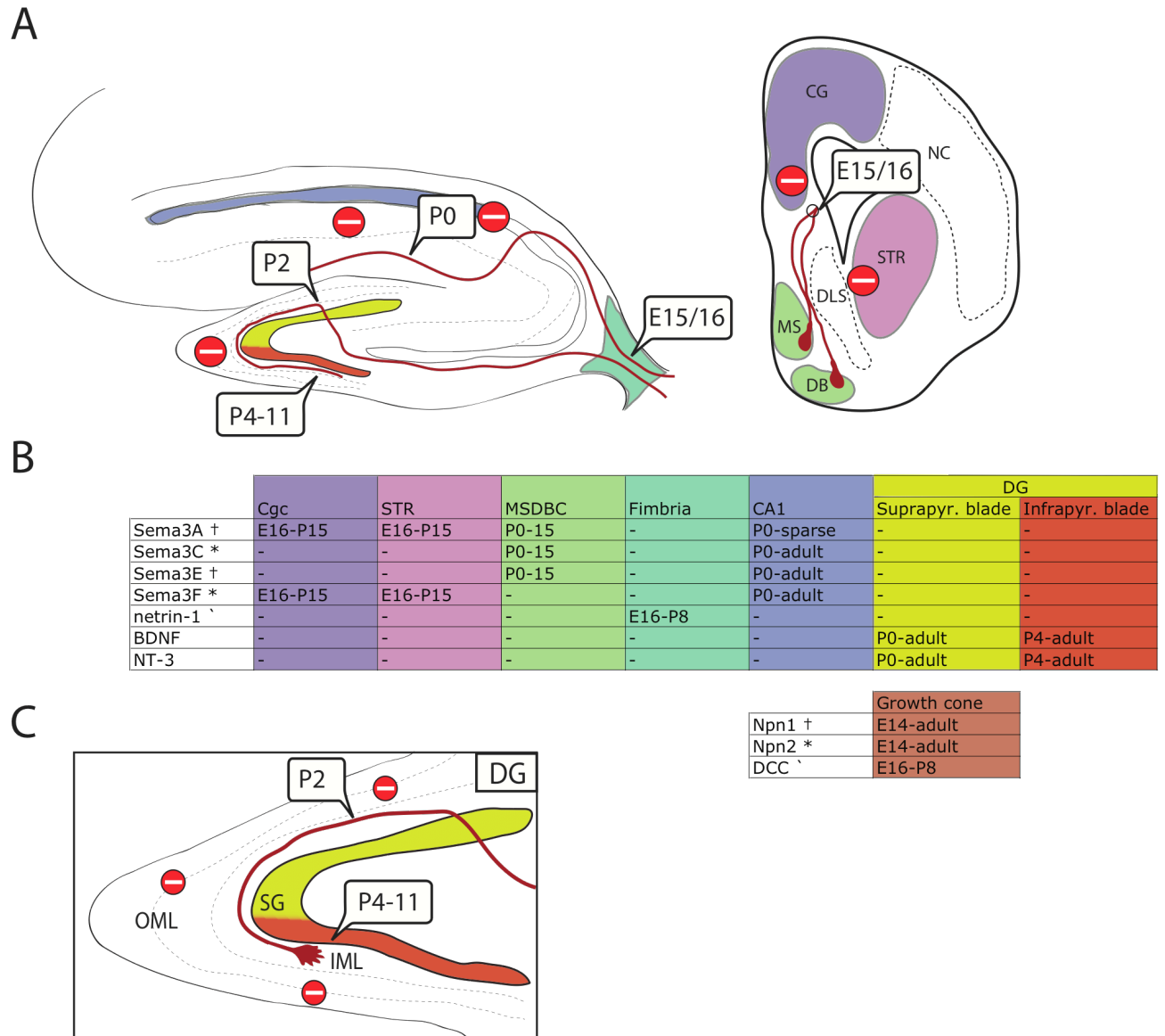


Figure 3, Overview of the developing septo-hippocampal projections. **A**. Schematic overview of the hippocampus (*left*) and septal area (*right*). Septal axons originating from the medial septum (MS) and diagonal band (DB) grow towards the fimbria around E15/16 and are guided by the repulsive cues present in the cingular cortex (Cg) and striatum (STR). The first septal axons within the stratum lacunosum-moleculare arrive around P0. Innervation of the IML at the suprapyramidal blade can be observed at P2 subsequently the infrapyramidal blade is protruded at P4-11. Repulsive cues present in the OML and stratum pyramidale avoid the protrusion of septal axons within the latter two layers. **B**. Overview of the expressed AGMs and receptor within the subfields, brain areas and the protruding growth cone. [medial septum and diagonal band complex (MSDBC), Cornu Ammonis 1 (CA1), dentate gyrus (DG), Suprapyramidal blade (Suprpyr. blade), Infrapyramidal blade (Infrapyr. blade)]. AGM and receptor pairs are expressed during the development of the septo-hippocampal projections: †Sema3A/3E-Npn1, *Sema3C/3F-Npn2, `netrin-1-DCC. **C**. Protrusion of the septal axons within the IML start at the suprapyramidal blade (P2) and moves towards the infrapyramidal blade (P4-11). Abbreviations: neocortex (NC), stratum granulosum (SG)

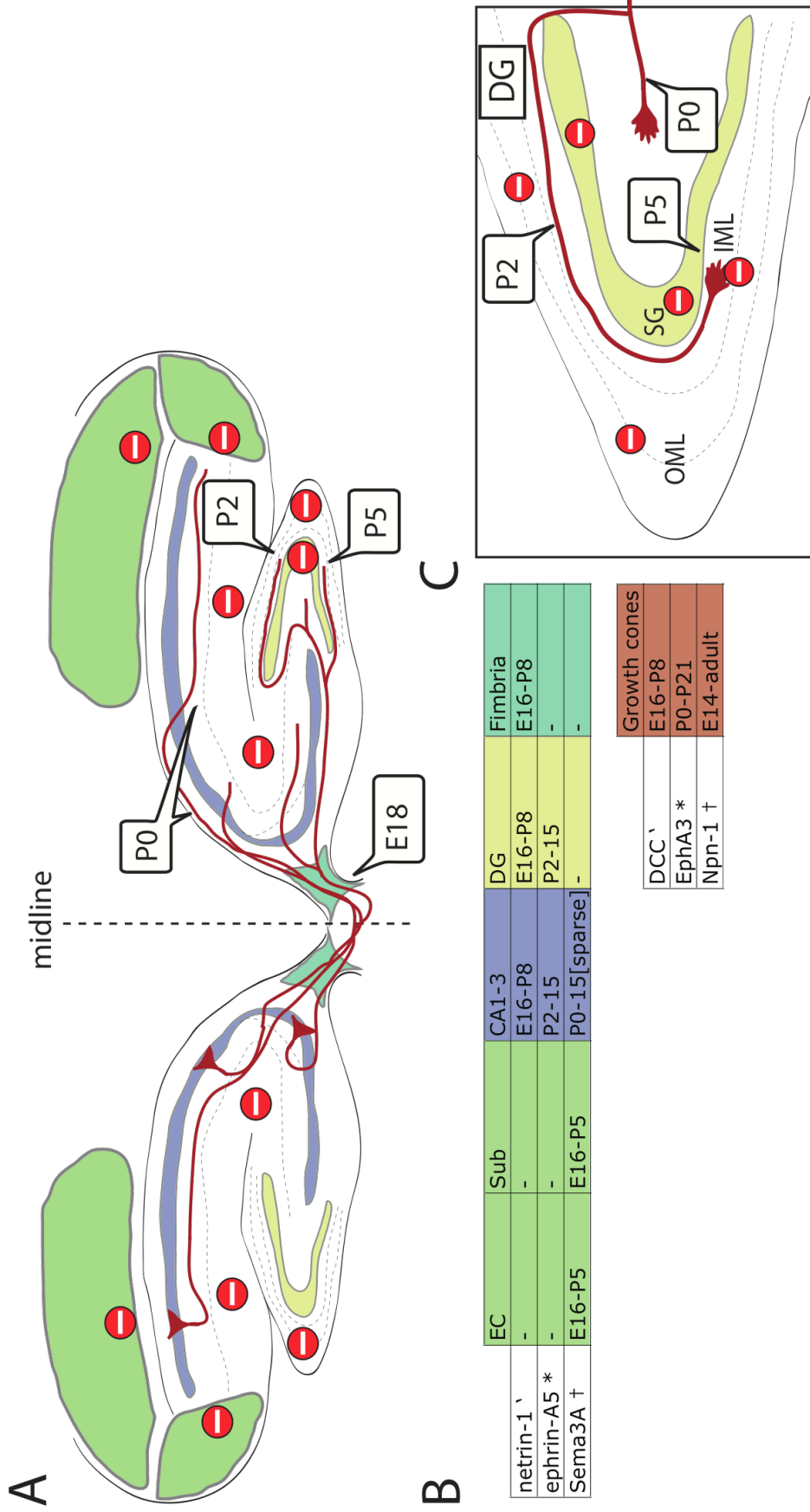


Figure 4, Overview of the developing commissural-associative projections. A. Schematic overview of the hippocampus. Commissural projections cross the midline and arrive around E18 at the fimbria of the contralateral hippocampus. At P0 the first projections terminate within the stratum oriens and the radiatum and the hilus (C.). As described in the entorhino-hippocampal section, Sema3A expression within the subiculum and EC prevent commissural axons to protrude these areas. Commissural projections arrive at the suprapyramidal blade within the IML around P2 and subsequently, P5, grow towards the infrapyramidal blade of the same layer. B. Overview of the AGMs and receptors expressed by the subfields, brain areas and the protruding growth cone during the development of this pathway. Various pairs of AGM and receptor are identified: †Sema3A-Npn-1, *ephrin-A5-EphA3, `netrin-1-DCC. C. Arrival of the commissural projections within the contralateral DG. Repulsive cues provided within the stratum granulosum (SG) avoid protrusion of the commissural axons. Presumably the early arrival of entorhinal axons within the OML provides repulsive cues to restrict commissural axons to the IML.

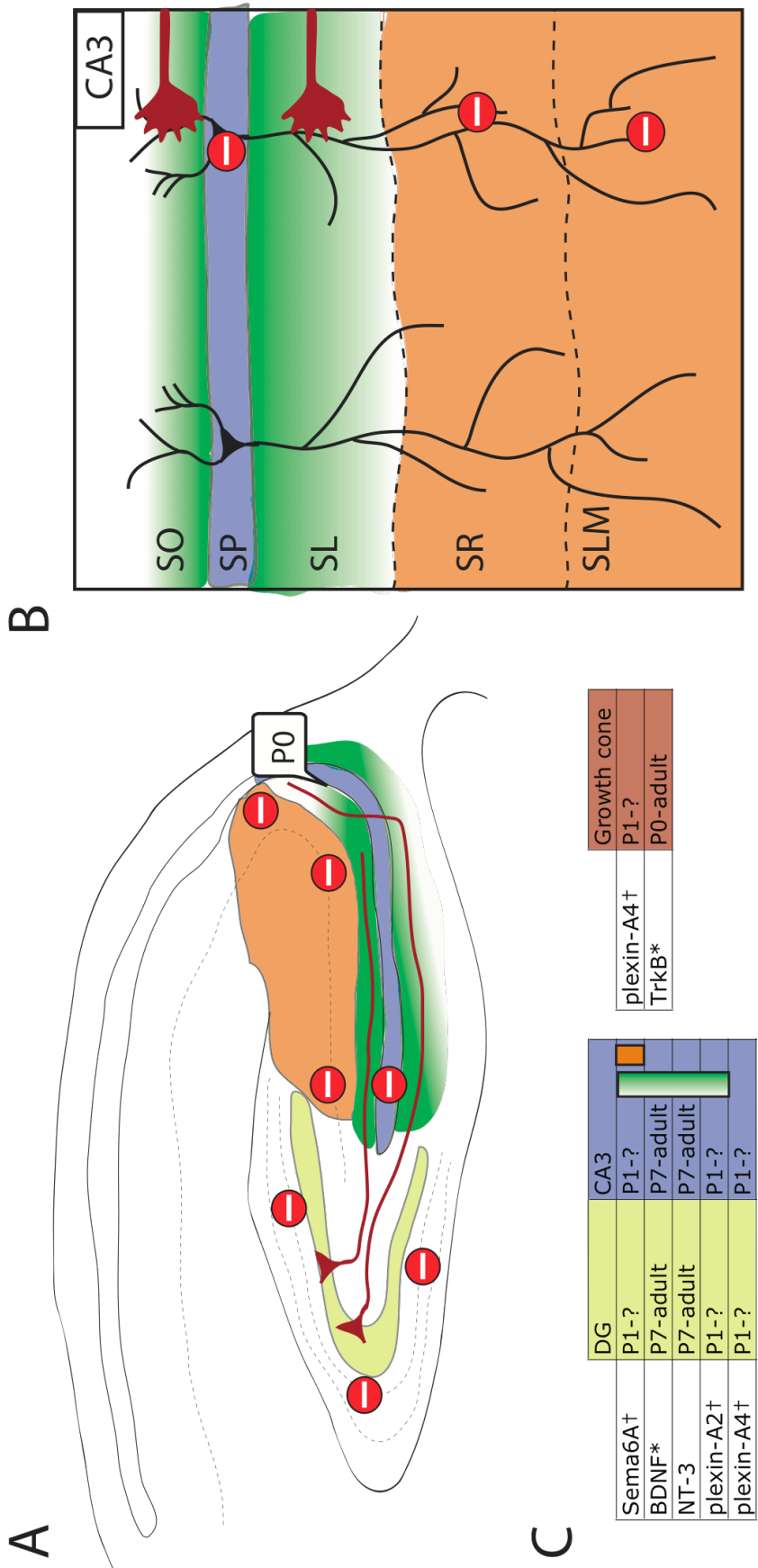


Figure 5, Overview of the developing mossy fibers. A. Schematic overview of the hippocampus. Granular cells projects mossy fibers towards the CA3 via suprapyramidal or infrapyramidal bundles from P0 onwards. As discussed earlier, entorhino-hippocampal projections present in the OML express Slit2, whereas granular neurons express Robo1 and -2. Slit2-Robo1/2 complexes prevent mossy fibers to form synaptic contacts with the apical dendrites originating from the same granular neuron within the IML. B. Arrival of projections within the CA3. Growth is restricted to the stratum lucidum (SL) and stratum oriens (SO) by the expression of repellents within the stratum pyramidale, radiatum and lacunosum-moleculare (SP, SR and SLM). C. Overview of the AGMs and receptors expressed within the subfields and growth cone: †Sema6A-plexin-A2/A4, *BDNF-TrkB. Gradients of plexin-A2 [green gradient bar] are proposed to attenuate the repulsive action of Sema6A-plexin-A4 complex [orange bar and green gradient bar].