Université de Montréal

Étude ultrastructurale et développementale du récepteur EphA4 dans l'hippocampe du rat

par
Marie-Ève Tremblay

Département de pathologie et biologie cellulaire
Faculté de médecine

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Cette thèse intitulée :

Étude ultrastructurale et développementale du récepteur EphA4 dans l'hippocampe du rat

présentée par :
Marie-Ève Tremblay

a été évaluée par un jury composé des personnes suivantes :

Dr Trevor Drew, président-rapporteur
Dr Guy Doucet, directeur de recherche
Dr Mustapha Riad, co-directeur
Dre Adriana DiPolo, membre du jury
Dr Armen Saghatelyan, examinateur externe
Dr John Francis Kalaska, représentant du doyen de la FÉSP

Résumé

Afin de mieux comprendre l'évolution des fonctions du récepteur EphA4 pendant le développement du système nerveux central (SNC), nous avons étudié sa localisation cellulaire et subcellulaire dans l'hippocampe du rat, d'abord chez l'adulte, puis pendant le développement postnatal, ainsi que ses rôles potentiels dans la genèse, la migration ou la maturation des cellules granulaires dans l'hippocampe adulte. Pour ce faire, nous avons utilisé la méthode d'immunocytochimie en microscopie photonique, électronique et confocale.

En microscopie photonique, une forte immunoréactivité (peroxydase/DAB) pour EphA4 est observée aux jours 1 et 7 suivant la naissance (P1 et P7) dans les couches de corps cellulaires, avec un marquage notamment associé à la surface des corps cellulaires des cellules granulaires et pyramidales, ainsi que dans les couches de neuropile du gyrus dentelé et des secteurs CA3 et CA1. L'intensité du marquage diminue progressivement dans les couches de corps cellulaires, entre P7 et P14, pour devenir faible à P21 et chez l'adulte, tandis qu'elle persiste dans les couches de neuropile, sauf celles qui reçoivent des afférences du cortex entorhinal. En microscopie électronique, après marquage à la peroxydase/DAB, EphA4 décore toute la surface des cellules pyramidales et granulaires, du corps cellulaire jusqu'aux extrémités distales, entre P1 et P14, pour devenir confiné aux extrémités synaptiques, c'est-à-dire les terminaisons axonales et les épines dendritiques, à P21 et chez l'adulte. À la membrane plasmique des astrocytes, EphA4 est redistribué comme dans les neurones, marquant le corps cellulaire et ses prolongements proximaux à distaux, à P1 et P7, pour devenir restreint aux prolongements périsynaptiques distaux, à partir de P14. D'autre part, des axones en cours de myélinisation présentent souvent une forte immunoréactivité punctiforme à leur membrane plasmique, à P14 et P21. En outre, dans les neurones et les astrocytes, le réticulum endoplasmique, l'appareil de Golgi et les vésicules de transport, organelles impliquées dans la synthèse, la modification post-traductionnelle et le transport des protéines glycosylées, sont aussi marqués, et plus intensément chez les jeunes animaux. Enfin, EphA4 est aussi localisé dans le corps cellulaire et les dendrites des cellules granulaires générées chez l'adulte, au stade de

maturation où elles expriment la doublecortine (DCX). De plus, des souris adultes knockouts pour EphA4 présentent des cellules granulaires DCX-positives ectopiques, c'est-à-dire positionnées en dehors de la zone sous-granulaire, ce qui suggère un rôle d'EphA4 dans la régulation de leur migration.

Ces travaux révèlent ainsi une redistribution d'EphA4 dans les cellules neuronales et gliales en maturation, suivant les sites cellulaires où un remodelage morphologique s'effectue : les corps cellulaires lorsqu'ils s'organisent en couches, les prolongements dendritiques et axonaux pendant leur croissance, guidage et maturation, puis les épines dendritiques, les terminaisons axonales et les prolongements astrocytaires distaux associés aux synapses excitatrices, jusque chez l'adulte, où la formation de nouvelles synapses et le renforcement des connexions synaptiques existantes sont exercés. Ces localisations pourraient ainsi correspondre à différents rôles d'EphA4, par lesquels il contribuerait à la régulation des capacités plastiques du SNC, selon le stade développemental, la région, l'état de santé, ou l'expérience comportementale de l'animal.

Mots-clés : migration cellulaire, guidage axonal, synaptogenèse, plasticité synaptique, neurogenèse, myélinisation, interactions neurone-glie, immunocytochimie, microscopie électronique, microscopie confocale

Abstract

To gain more insight into the various functions of EphA4 receptor during the development of the central nervous system (CNS), we have characterized its cellular and subcellular localization in the rat hippocampus, first in the adult, and second during the postnatal development. We have also examined its potential roles in the genesis, migration, or maturation of the granule cells in the adult hippocampus. For that purpose, we have used immunocytochemistry in light, electron, and confocal microscopy.

At the light microsocpic level, a strong EphA4 immunoreactivity (peroxidase/DAB) is observed at postnatal days 1 and 7 (P1 and P7) in the cell body layers, with a labeling notably associated with the surface of pyramidal and granule cell bodies, as well as in the neuropil layers of CA3, CA1, and dentate gyrus regions. The intensity of the labeling diminishes progressively in the cell body layers, between P7 and P14, to become weak at P21 and in the adult, while it persists in the neuropil layers, except in those receiving inputs from the entorhinal cortex. At the electron microscopic level, after peroxidase/DAB labeling, EphA4 covers the entire surface of pyramidal and granule cells, from the cell body to the distal extremities, between P1 and P14, but becomes restricted to the synaptic extremities, i.e. the axon terminals and dendritic spines, at P21 and in the adult. At the plasma membrane of astrocytes, EphA4 is redistributed as in neurons, from the cell body and proximal to distal processes, at P1 and P7, to the distal perisynaptic processes, at P14 and older ages. In addition, axons in the process of myelination present strong punctiform immunoreactivity at their plasma membrane, at P14 and P21. Moreover, in neurons and astrocytes, the endoplamic reticulum, Golgi apparatus, and transport vesicles, organelles involved in the synthesis, post-translational modifications, and transport of glycosylated proteins, are also labeled, and also more intensely in younger animals. Lastly, EphA4 is located in the cell body and dendrites of adult-generated granule cells, at the stage of maturation where they express doublecortin (DCX). In addition, EphA4 adult knockout mice display DCX-positive granule cells in an ectopic position, outside of the subgranular zone, suggesting a role for EphA4 in the regulation of their migration.

This work thus reveals a redistribution of EphA4 in neuronal and glial cells, in the cellular sites where cellular motility occurs during their maturation: the cell bodies when they position and organize themselves into layers, the dendritic and axonal processes during their growth, guidance, and maturation, and the dendritic spines, axon terminals, and distal astrocytic processes when synapses are formed or strengthened. These locations could thus reflect different roles for EphA4, similarly associated with the regulation of plasticity in the CNS, according to the stage of development, the region, the CNS integrity, or the behavioural experience of an animal.

Keywords: cell migration, axon guidance, synaptogenesis, synaptic plasticity, neurogenesis, myelination, neuron-glia interactions, immunocytochemistry, electron microscopy, confocal microscopy

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Liste des sigles et des abréviations

ADN : acide désoxyribonucléique

AMPA : acide alpha-amino-3-hydroxy-5-méthyl-4-isoxazolépropionique

AMPc : adénosine monophosphate cyclique

ARN : acide ribonucléique

ARNm : ARN messager

BrdU : 5-bromo-2-désoxyuridine

C : carboxy

CA (CA1, CA3) : *cornu ammonis*

CaM kinase II : calcium-calmoduline kinase-II

cdk5 : kinase cycline-dépendante-5 (*cyclin-dependant kinase-5*)

CPG : générateur central de motifs locomoteurs (*central pattern generator*)

CREB : élément de liaison à l'ADN en réponse à l'AMPc *(cAMP response element binding)*

CSPG : protéoglycanes à chondroïtine sulfate *(chondroitin sulfate proteoglycan)*

DAB : 3,3'-Diaminobenzidine tétrahydrochloride

DCX : doublecortine

E : jour embryonnaire

Eph : dérivé de cellules de carcinome hépatocellulaire productrices d'érythropoïétine (*erythropoietin-producing hepatocellular carcinoma cell-derived)*

Éphrine : protéines interactrices des récepteurs Eph (*Eph receptor interacting proteins)*

FRET : transfert d'énergie entre molécules fluorescentes (*Fluorescence* ou *Förster Resonnance Energy Transfer)*

GABA : acide γ-aminobutyrique

GAPs : protéines activatrices des GTPases (*GTPase activating protein*)

GEFs : facteurs d'échange de nucléotides guanine *(guanine nucleotide exchange factors)*

GFAP : protéine gliale fibrillaire acide *(glial fibrillary acidic protein)*

GFP : protéine fluorescente verte *(green fluorescent protein)*

GPI : glycosylphosphatidylinositol

GTP : guanine triphosphate

IgG : immunoglobulines-G1

INFγ : interféron γ

JM : juxtamembranaire

LIF : facteur inhibiteur de la leucémie *(leukemia inhibitory factor)*

LTD : dépression à long terme *(long-term depression)*

LTP : potentialisation à long terme *(long-term potentiation)*

MAG : glycoprotéine associée à la myéline *(myelin-associated glycoprotein)*

MAP kinases : protéines kinases activatrices de la mitose *(mitogen-activated protein kinases)*

NMDA : N-méthyl-D-aspartate

N : amino

NO : oxyde d'azote *(nitric oxide)*

OMgp : glycoprotéine de la myéline oligodendrocytaire *(oligodendrocyte myelin glycoprotein)*

P : jour postnatal

P75NTR : récepteur à basse affinité des neurotrophines p75

PCR : réaction de polymérisation en chaîne *(polymerase chain reaction)*

PDZ : PSD-95/ *disc-large*/ *zonula occludens-1*

PI-PLC : phospholipase-C spécifique aux groupements phosphatidylinositol *(phosphatidylinositol-specific phospholipase C)*

PLCγ1 : phospholipase Cγ-1

PSD-95 : protéine densité postsynaptique 95 *(postsynaptic density protein 95)*

RMS : courant de migration rostral *(rostral migratory stream)*

RTKs : récepteurs tyrosine kinase

RT-PCR : PCR avec transcriptase inverse *(reverse transcription PCR)*

SAM : motif α-stérile *(sterile α motif)*

SH2 : domaine d'homologie pour Src *(Src homology 2)*

SNC : système nerveux central

SON : noyau supraoptique *(supraoptic nucleus)*

TUNEL : *Terminal deoxynucleotidyl transferase dUTP nick end labeling*

Tyr : résidu tyrosine

VGCCs : canaux calciques régulés par le voltage *(voltage-gated calcium channels)*

Vglut1 : transporteur vésiculaire du glutamate de type 1

ZSG : zone sous-granulaire

ZSV : zone sous-ventriculaire

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Introduction générale

1. Mise en contexte

La plasticité est l'habileté du système nerveux central (SNC) à changer, par des remodelages morphologiques ou fonctionnels de la circuiterie neuronale. Elle est particulièrement grande au cours du développement embryonnaire et postnatal, pendant l'établissement initial des connexions entre neurones. Les axones qui croissent pour atteindre leurs cibles cellulaires possèdent alors à leur extrémité distale un cône de croissance qui perçoit les indices moléculaires dans l'environnement. Ces molécules sont solubles et diffusibles, liées à la surface cellulaire ou à la matrice extracellulaire. Plusieurs familles de ces molécules de guidage axonal dirigent les axones en élongation, en induisant l'attraction ou la répulsion, notamment les sémaphorines, les slits, les nétrines, les Eph et les éphrines (Efn), mais aussi les morphogènes Wnt, sonic hedgehog ou protéines morphogéniques des os, et les molécules d'adhérence cellulaire de types immunoglobulines ou cadhérines (voir Charron and Tessier-Lavigne, 2005; Maness and Schachner, 2007; Manitt and Kennedy, 2002; Tessier-Lavigne, 1995). Le motif des connexions neuronales est ensuite raffiné par l'expérience sensorielle, surtout pendant la période critique du développement postnatal. Des perturbations de l'expérience sensorielle induisent alors une réorganisation de la circuiterie, rendant possible l'adaptation de l'animal au nouvel environnement. La sécrétion de molécules de la matrice extracellulaire par les astrocytes, notamment les protéoglycanes à chondroïtine sulfate (CSPG), ainsi que la myélinisation des axones par les oligodendrocytes, coïncideraient pour leur part avec la diminution des capacités plastiques brutes à la fin de cette période (voir Harel and Strittmatter, 2006).

En fonction de l'expérience sensorielle, une plasticité fine continue à s'exercer tout au long de la vie, principalement par la formation de nouvelles connexions synaptiques et le renforcement ou l'affaiblissement des synapses existantes. Dans l'hippocampe et le bulbe olfactif, de nouveaux neurones sont aussi continuellement intégrés à la circuiterie (voir Kempermann, 2006). Plusieurs molécules de guidage axonal sont encore exprimées chez l'adulte, mais leurs fonctions sont peu connues. Elles seraient pour la plupart inhibitrices de

la croissance et du guidage des axones, tel qu'observé à la suite de lésions de la moelle épinière (voir Goldshmit et al., 2006b; Harel and Strittmatter, 2006), et elles réguleraient plutôt la formation et la plasticité des synapses excitatrices, la neurogenèse et la myélinisation axonale (Arikkath and Reichardt, 2008; Colon-Ramos et al., 2007; Fitzgerald et al., 2006; Jarjour et al., 2008; Kiss and Muller, 2001; Low et al., 2008; Marques, 2005; Pasquale, 2005; Williams et al., 2007; Yamashita et al., 2007). Les rôles des soit disant molécules de guidage axonal pourraient ainsi changer selon le stade développemental, la région du SNC, l'expérience ou l'état de santé de l'animal. Selon le contexte, elles réguleraient positivement ou négativement les capacités plastiques du SNC.

Plus particulièrement, les Eph et les Efn sont abondamment exprimés dans le SNC tout au long du développement, de l'embryon jusque chez l'adulte, principalement dans l'hippocampe, où leur fonction est très peu connue. Dans cette perspective, mes travaux de doctorat ont porté sur la localisation subcellulaire du récepteur EphA4 dans les cellules neuronales et gliales de l'hippocampe, chez le rat néonatal, juvénile et adulte, en vue d'une meilleure compréhension de l'évolution de ses fonctions pendant le développement postnatal et chez l'adulte (incluant lors de la neurogenèse; voir section 5. Hypothèses et objectifs).

2. Les Eph et les Efn

2.1. Historique

Les récepteurs Eph et leurs ligands Efn sont phylogénétiquement conservés chez les porifères, les cnidaires, les nématodes, les insectes, les échinodermes, les céphalochordés, les urochordés et les vertébrés, chez lesquels ils réguleraient notamment le dynamisme du cytosquelette d'actine, requis pour le mouvement des corps cellulaires ou le remodelage morphologique de leurs prolongements, ainsi que l'attraction et la répulsion entre cellules ou avec la matrice extracellulaire (voir sections 2.5. et 2.7.; Mellott and Burke, 2008; Pasquale, 2005; 2008).

2.1.1. Découverte des Eph

Le premier récepteur Eph fut identifié par Hirai et al. (1987) dans le cadre de travaux visant à découvrir de nouveaux récepteurs tyrosine kinase (RTKs) potentiellement impliqués dans le cancer. Une sonde comprenant la séquence du domaine kinase des RTKs a servi au criblage d'une banque d'ADN complémentaire générée à partir de carcinomes hépatocellulaires sécrétant l'hormone érythropoïétine. L'hybridation croisée a alors permis d'identifier, puis de cloner, un nouveau RTK qui fut nommé Eph (*erythropoietin-producing hepatocellular carcinoma cell-derived*). L'hybridation croisée et la réaction de polymérisation en chaîne (PCR) de la séquence du domaine kinase de ce récepteur ont ensuite servi à identifier plusieurs autres Eph (Chan and Watt, 1991; Flanagan and Vanderhaeghen, 1998; Gilardi-Hebenstreit et al., 1992; Lai and Lemke, 1991; Lindberg and Hunter, 1990). D'autres Eph ont aussi été isolés à partir de l'activité catalytique de leur domaine kinase, lors du criblage d'une banque d'expression protéique avec des anticorps dirigés contre les résidus tyrosine phosphorylés (Henkemeyer et al., 1994; Letwin et al., 1988; Pasquale, 1991; Pasquale and Singer, 1989; Sajjadi et al., 1991; Zhou et al., 1994).

2.1.2. Découverte des Efn

Les Eph furent considérés comme des récepteurs orphelins pendant sept ans. Les premières Efn (*Eph receptor interacting proteins*) furent identifiées par une approche basée sur l'affinité entre ligands et récepteurs solubles (Bartley et al., 1994; Beckmann et al., 1994; Cheng and Flanagan, 1994; Davis et al., 1994; Shao et al., 1994). Selon cette approche, une protéine de fusion comprenant la séquence extracellulaire d'un Eph ainsi qu'un rapporteur moléculaire, soit la phosphatase alcaline ou le fragment Fcγ des immunoglobulines-G1 (IgG) humaines, sert de sonde soluble pour la détection des ligands s'y liant avec une haute affinité, en culture de cellules ou de tranches de tissu. D'autres Efn ont ensuite été identifiées par l'hybridation croisée d'acides nucléiques (Bennett et al., 1995; Kozlosky et al., 1995; Winslow et al., 1995) ou par leur homologie avec des séquences retrouvées dans les bases de données (Bergemann et al., 1995; Cerretti et al., 1995; Gale et al., 1996a).

2.2. Nomenclature

Une analyse phylogénétique récente a démontré que les Eph et les Efn se seraient diversifiés à différents moments de l'histoire évolutive, à partir d'un seul Eph et de deux Efn chez un ancêtre virtuel commun des urochordés et des vertébrés (Mellott and Burke, 2008). Chez les vertébrés, les Eph constituent le plus grand sous-ensemble de RTKs. Au nombre de 16, les Eph sont subdivisés en sous-classes A (EphA1 à EphA10) et B (EphB1 à EphB6), selon leur séquence extracellulaire et leur affinité respective pour les EfnA (EfnA1 à EfnA5) ou B (EfnB1 à EfnB3) (Eph Nomenclature Committee, 1997; voir Tableau 1). À la différence des ligands habituels des autres RTKs, les Efn sont des protéines membranaires de la surface cellulaire : les EfnA sont ancrées dans la membrane plasmique par un groupement glycosylphosphatidylinositol (GPI) et les EfnB par un domaine transmembranaire. Deux récepteurs font exception à cette sélectivité de liaison intraclasse: EphA4, qui a la propriété unique de lier toutes les EfnA et EfnB, ainsi qu'EphB2 qui se lie à toutes les EfnB en plus de l'EfnA5 (Himanen et al., 2007; Pasquale, 2005; voir Figure 1).

Autre particularité des Eph et Efn, la nature membranaire des « ligands » autant que des « récepteurs » rend possible une signalisation bidirectionnelle pouvant s'exercer non seulement dans la cellule qui exprime l'Eph, mais aussi dans celle exprimant l'Efn, comme nous le verrons dans la section 2.5. En conséquence, les Eph et les Efn peuvent agir comme récepteurs ou ligands, dépendamment du contexte. Afin de tenir compte de cette particularité, nous utiliserons désormais les appellations Eph et Efn, sans les qualifier de récepteurs ou ligands.

Eph

Noms actuels	Noms précédents	Références
EphA1	Eph, Esk	(Hirai et al., 1987; Lickliter et al., 1996)
EphA2	Eck, Myk2, Sek2	(Andres et al., 1994; Becker et al., 1994; Ganju et al., 1994; Lindberg and Hunter, 1990)
EphA3	Cek4, Mek4, Hek, Tyro4, Hek4	(Boyd et al., 1992; Fox et al., 1995; Lai and Lemke, 1991; Sajjadi et al., 1991; Wicks et al., 1992)
EphA4	Sek, Sek1, Cek8, Hek8, Tyro1	(Becker et al., 1995; Fox et al., 1995; Gilardi-Hebenstreit et al., 1992; Lai and Lemke, 1991; Sajjadi and Pasquale, 1993)
EphA5	Ehk1, Bsk, Cek7, Hek7, Rek7	(Fox et al., 1995; Maisonpierre et al., 1993; Siever and Verderame, 1994; Zhou et al., 1994)
EphA6	Ehk2, Hek12	(Maisonpierre et al., 1993)
EphA7	Mdk1, Hek11, Ehk3, Ebk, Cek11	(Araujo and Nieto, 1996; Ciossek et al., 1995; Ellis et al., 1995)
EphA8	Eek, Hek3	(Chan and Watt, 1991; Park and Sanchez, 1997)
EphA9		(Sasaki et al., 2003)
EphA10		(Aasheim et al., 2005)
EphB1	Elk, Cek6, Net, Hek6	(Letwin et al., 1988; Sajjadi and Pasquale, 1993; Tang et al., 1995)
EphB2	Cek5, Nuk, Erk, Qek5, Tyro5, Sek3, Hek5, Drt	(Becker et al., 1994; Chan and Watt, 1991; Fox et al., 1995; Henkemeyer et al., 1994; Kiyokawa et al., 1994; Lai and Lemke, 1991; Pasquale et al., 1992)
EphB3	Cek10, Hek2, Mdk5, Tyro6, Sek4	(Becker et al., 1994; Bohme et al., 1993; Ciossek et al., 1995; Lai and Lemke, 1991; Sajjadi and Pasquale, 1993)
EphB4	Htk, Myk1, Tyro11, Mdk2	(Andres et al., 1994; Bennett et al., 1995; Ciossek et al., 1995; Lai and Lemke, 1991)
EphB5	Cek9, Hek9	(Sajjadi and Pasquale, 1993)
EphB6	Mep	(Gurniak and Berg, 1996)

Efn

Noms actuels	Noms précédents	Références
EfnA1	B62, LERK-1, EFL-1	(Bartley et al., 1994; Beckmann et al., 1994; Davis et al., 1994; Holzman et al., 1990)
EfnA2	ELF-1, Cek7-L, LERK-6	(Cerretti et al., 1995; Cheng and Flanagan, 1994; Shao et al., 1994)
EfnA3	Ehk1-L, EFL-2, LERK-3	(Davis et al., 1994; Kozlosky et al., 1995)
EfnA4	LERK-4, EFL-4	(Kozlosky et al., 1995)
EfnA5	AL-1, RAGS, LERK-7, EFL-5	(Drescher et al., 1995; Kozlosky et al., 1995; Winslow et al., 1995)
EfnA6		(Menzel et al., 2001)
EfnB1	LERK-2, Elk-L, EFL-3, Cek5-L, STRA-1	(Beckmann et al., 1994; Bouillet et al., 1995; Davis et al., 1994; Sefton and Nieto, 1996; Shao et al., 1994)
EfnB2	Htk-L, ELF-2, LERK-5, NLERK-1	(Bennett et al., 1995; Bergemann et al., 1995; Cerretti et al., 1995; Nicola et al., 1996)
EfnB3	NLERK-2, Elk-L3, EFL-6, ELF-3, LERK-8	(Bergemann et al., 1995; Gale et al., 1996a; Nicola et al., 1996; Tang et al., 1997)

Tableau 1. Nomenclature pour les Eph et les Efn

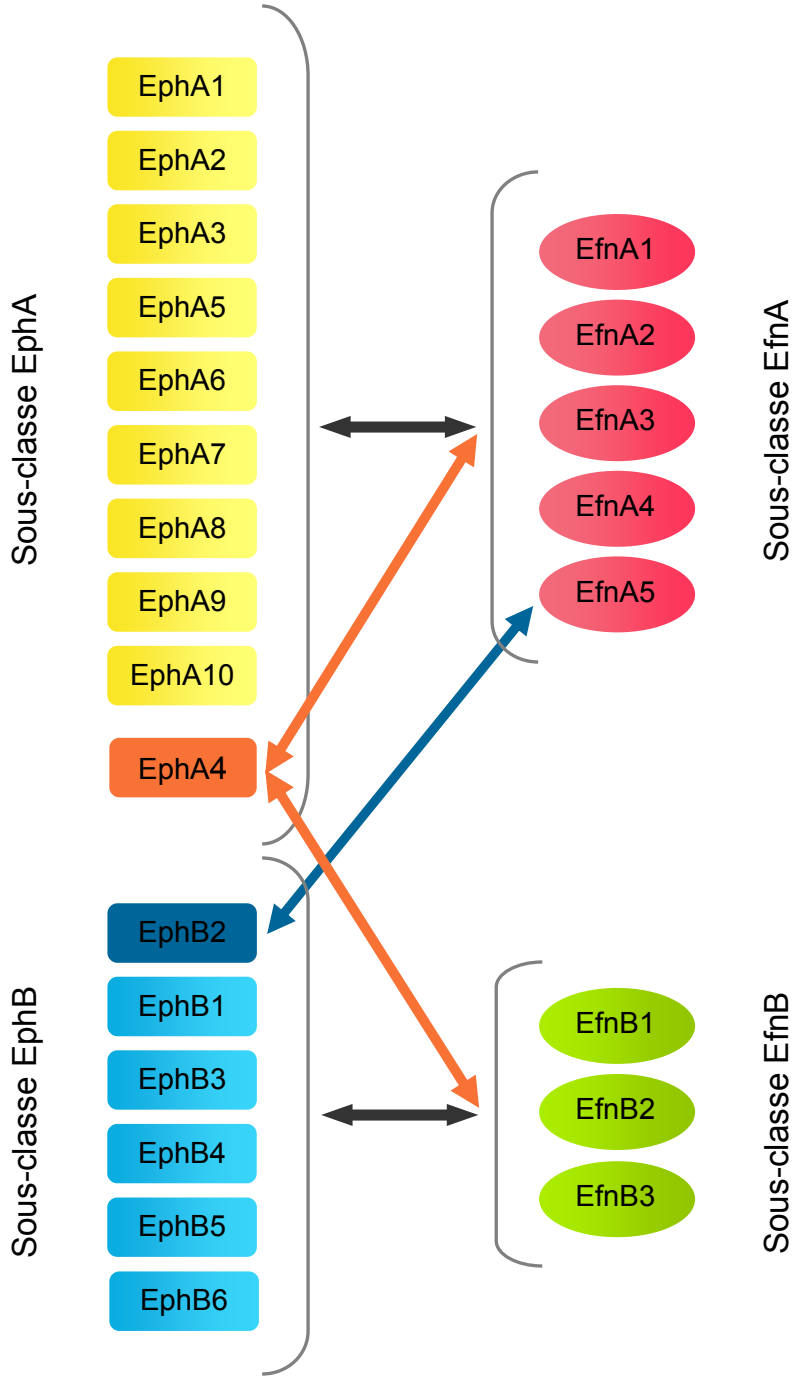

Figure 1. Sélectivité de liaison des Eph et des Efn

2.3. Structure protéique des Eph et des Efn

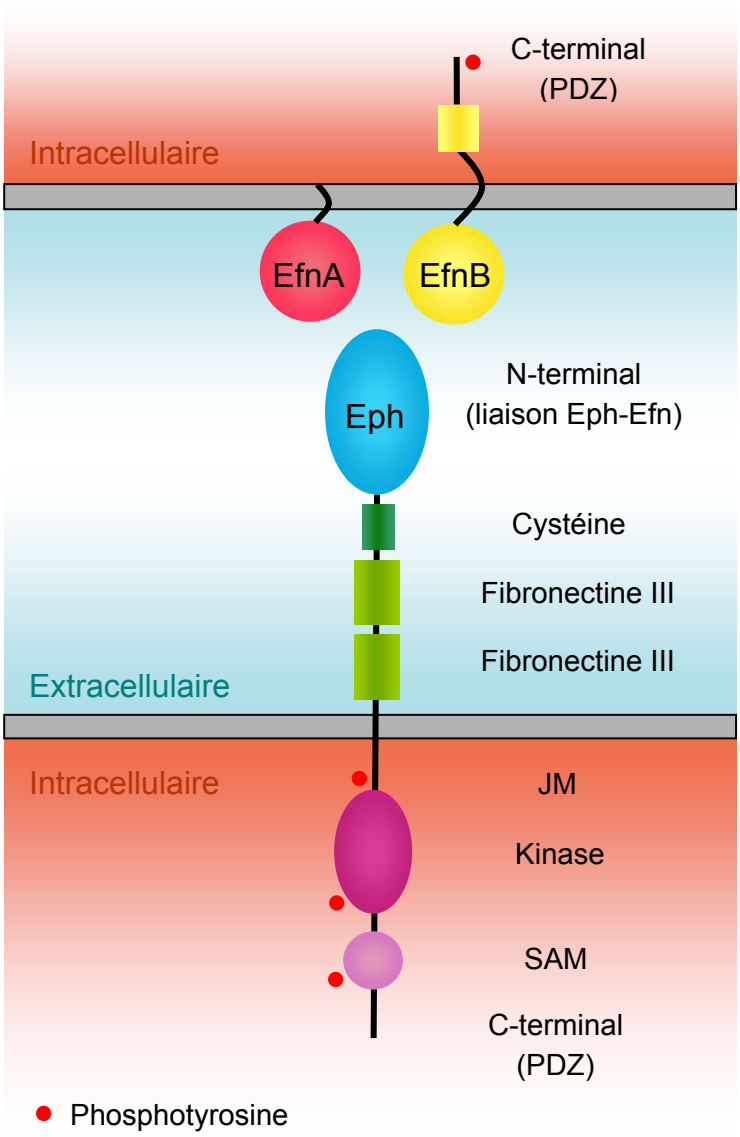

Figure 2. Structure protéique des Eph et des Efn

2.3.1. Structure des Eph

Les Eph sont des protéines transmembranaires de type I, typiquement constituées d'une chaîne polypeptidique traversant une seule fois la membrane plasmique (voir Figure 2). Leur segment extracellulaire contient un domaine amino (N) -terminal hautement conservé qui sert à la reconnaissance et à la liaison des Efn. Ce domaine est suivi d'une

région riche en résidus cystéine, ainsi que de deux répétitions fibronectine de type III (voir Himanen and Nikolov, 2003; Himanen et al., 2007; Pasquale, 2005). Les résidus cystéine forment des ponts disulfure, requis pour stabiliser la structure protéique. Les répétitions fibronectine de type III sont des modules de 90 acides aminés, lesquels sont répétés 15 à 17 fois dans la protéine fibronectine (voir Ruoslahti, 1988). Elles servent aux interactions latérales entre molécules Eph ou avec d'autres types de récepteurs, incluant les récepteurs glutamatergiques ionotropes de type N-méthyl-D-aspartate (NMDA) (Dalva et al., 2000; Lackmann et al., 1998). Le segment intracellulaire des Eph contient un domaine juxtamembranaire (JM), un domaine tyrosine kinase hautement conservé, un domaine à motif α-stérile (SAM), ainsi qu'un domaine carboxy (C) -terminal à motif PDZ (*Postsynaptic density-95* (PSD-95) / *disc-large* / *zonula occludens-1*) (voir Himanen and Nikolov, 2003; Himanen et al., 2007; Pasquale, 2005). Le domaine JM sert à réguler la phosphorylation des résidus tyrosine du domaine kinase. En effet, lorsqu'un Eph est activé par la liaison d'une Efn, deux résidus tyrosine du segment JM sont phosphorylés (Y596 et Y602 pour EphA4; Davis et al., 1994; Ellis et al., 1996); ce qui induit un changement conformationnel perturbant l'association structurelle entre le domaine JM et le domaine kinase, permettant la phosphorylation des résidus tyrosine du domaine kinase. Ceux-ci constituent alors des sites pour le recrutement de diverses protéines, notamment des facteurs d'échange de nucléotides guanine (GEFs) qui activent la voie de signalisation des petites GTPases Rho (voir Egea and Klein, 2007), comme nous le verrons plus loin dans la section 2.5. Enfin, les domaines à motifs SAM (10 à 80 acides aminés) et PDZ (120 acides aminés) servent au recrutement de protéines intracellulaires à motifs similaires.

Mentionnons que l'anticorps anti-EphA4 utilisé dans le cadre de mes travaux de doctorat est précisément dirigé contre les 11 derniers acides aminés du domaine C-terminal de la protéine EphA4 (voir Figure 3 et l'Annexe III pour la désignation des acides aminés), lesquels sont identiques chez le poulet, la souris, le rat et l'humain (Soans et al., 1994). La protéine EphA4 du rat comprend 986 acides aminés. Son poids moléculaire théorique est de 109 729 Da (www.ensembl.org et www.ncbi.nlm.nih.gov/protein), alors que son poids moléculaire apparent peut atteindre 120 000 Da (Martone et al., 1997).

MAGIFYFILFSFLFGICDAVTGSRVYPANEVTLLDSRSVQGELGWIASPLEGGWEEVSIMDEKNTPIRTYQVC
NVMEASQNNWLRTDWITREGAQRVYIEIKFTLRDCNSLPGVMGTCKETFNLYYYESDNDKERFIRESQFGKID
TIAADESFTQVDIGDRIMKLNTEIRDVGPLSKKGFYLAFQDVGACIALVSVRVFYKKCPLTVRNLAQFPDTIT
GADTSSLVEVRGSCVNNSEEKDVPKMYCGADGEWLVPIGNCLCNAGHEEQNGECQACKIGYYKALSTDATCAK
CPPHSYSVWEGATSCTCDRGFFRADNDAASMPCTRPPSAPLNLISNVNETSVNLEWSSPQNTGGRQDISYNVV
CKKCGAGDPSKCRPCGSGVHYTPQQNGLKTTRVSITDLLAHTNYTFEIWAVNGVSKYNPSPDQSVSVTVTTNQ
AAPSSIALVQAKEVTRYSVALAWLEPDRPNGVILEYEVKYYEKDQNERSYRIVRTAARNTDIKGLNPLTSYVF
HVRARTAAGYGDFSEPLEVTTNTVPSRIIGDGANSTVLLVSVSGSVVLVVILIAAFVISRRRSKYSQAKQEAD
EEKHLNQGVRTYVDPFTYEDPNQAVREFAKEIDASCIKIEKVIGVGEFGEVCSGRLKVPGKREICVAIKTLKA
GYTDKQRRDFLSEASIMGQFDHPNIIHLEGVVTKCKPVMIITEYMENGSLDAFLRKNDGRFTVIQLVGMLRGI
GSGMKYLSDMSYVHRDLAARNILVNSNLVCKVSDFGMSRVLEDDPEAAYTTRGGKIPIRWTAPEAIAYRKFTS
ASDVWSYGIVMWEVMSYGERPYWDMSNQDVIKAIEEGYRLPPPMDCPIALHQLMLDCWQKERSDRPKFGQIVN
MLDKLIRNPNSLKRTGPESSRPNTALLDPSSPEFSAVVSVGDWLQAIKMDRYKDNFTAAGYTTLEAVVHMSQD
DLARIGITAITHQNKILSSVQAMRTQ**MQQMHGRMVPV** = épitope reconnu par l'anti-EphA4

Domaines dont la séquence est connue: N-terminal (bleu pâle), Fibronectine III (vert pâle), Kinase (mauve foncé), SAM (mauve pâle), C-terminal (rouge)

Acides aminés qui diffèrent chez la souris : soulignés

Figure 3. Séquence protéique d'EphA4 chez le rat et la souris

2.3.2. Structure des Efn

Le domaine N-terminal, extracellulaire, des Efn est constitué d'une séquence relativement conservée d'environ 125 acides aminés et sert à la liaison des Eph (voir Figure 2). Les EfnA sont ancrées dans la membrane plasmique par un groupement GPI, tandis que les EfnB possèdent un segment transmembranaire et un court segment intracellulaire hautement conservé qui inclut un domaine C-terminal à motif PDZ (voir Himanen and Nikolov, 2003; Himanen et al., 2007; Pasquale, 2005).

2.3.3. Structure des regroupements entre Eph et Efn

Afin d'induire une signalisation physiologique, la liaison entre Eph et Efn requiert la dimérisation entre Eph, comme pour la plupart des RTKs, mais aussi la formation de regroupements multimériques (*higher-order clusters*) (voir Egea and Klein, 2007; Himanen and Nikolov, 2003). Par cristallographie aux rayons X, Himanen et al. (2001) ont dévoilé la structure de l'association entre les domaines d'interaction d'EphB2 et de l'EfnB2. Cette étude a suggéré que les Eph et les Efn sont d'abord liés entre eux (1 :1) par une première

interface fort étendue, puis agrégés en un dimère (2 :2) avec une affinité moindre et une interface plus restreinte. En outre, Wimmer-Kleikamp et al. (2004) ont montré, par transfert d'énergie de résonance entre molécules fluorescentes (FRET) en microscopie confocale, que le nombre d'EphA3 recrutés dans un même regroupement multimérique peut excéder plusieurs fois celui d'EfnA5, alors que des isoformes d'EphA3 pourvues de mutations empêchant la liaison aux Efn sont aussi recrutées puis phosphorylées par des interactions latérales (en *cis*) avec d'autres EphA3. Cependant, il n'est pas connu si des Eph distincts peuvent se retrouver à l'intérieur d'un même regroupement multimérique.

2.5. Signalisation bidirectionnelle des Eph et des Efn

La liaison entre Eph et Efn induit une signalisation bidirectionnelle, pouvant s'exercer dans la cellule qui exprime l'Eph (signalisation antérograde; *forward signaling*) aussi bien que dans celle qui exprime l'Efn (signalisation inverse; *reverse signaling*). Les deux types de signalisation pourraient se produire dans les mêmes types de cellules, où elles induiraient des réponses différentes. En effet, Marquardt et al. (2005) ont démontré *in vitro* que l'activation de la signalisation antérograde en aval des EphA par des protéines de fusion EfnA1-Fc, lesquelles comprennent le domaine extracellulaire de l'EfnA1 ainsi que le fragment Fcγ des IgG humaines, induit une répulsion et un affaissement des cônes de croissance, alors que la signalisation inverse en aval des EfnA, stimulée par EphA3-Fc ou EphA7-Fc, entraîne une attraction et une croissance axonale des motoneurones du poulet.

2.5.1. Protéines de fusion Efn-Fc et Eph-Fc

Les protéines de fusion Efn-Fc et Eph-Fc sont abondamment utilisées *in vivo* ou *in vitro* pour activer ou bloquer les voies signalétiques en aval des interactions entre Eph et Efn. Les Efn-Fc peuvent être infusées soit sous forme dimérique, lorsqu'elles sont utilisées telles quelles, soit sous forme multimérique, lorsqu'elles sont agglomérées pendant une incubation préalable avec des anticorps dirigés contre le fragment Fcγ des IgG humaines (Stein et al., 1998). Aussi bien les Efn-Fc agglomérées que non agglomérées peuvent induire la phosphorylation des Eph auxquels elles se lient, tel que montré par transfert western avec des anticorps dirigés contre les résidus tyrosine phosphorylés (Stein et al.,

1998). Leurs effets pourraient se ressembler, Murai et al. (2003a) ayant observé une rétraction des épines dendritiques similaire suite à l'infusion d'EfnA3-Fc agglomérées et non agglomérées en culture organotypique de tranches d'hippocampe, ou différer, puisque seules les EfnB1-Fc agglomérées peuvent induire la formation des capillaires à partir de cellules endothéliales *in vitro* (Stein et al., 1998). En outre, par la liaison d'Eph endogènes, les Efn-Fc activeraient la signalisation antérograde en aval de ces Eph, en plus de bloquer la signalisation inverse en aval des Efn auxquelles ces Eph se lient normalement. Par exemple, l'EfnA5-Fc activerait présumément la signalisation antérograde en aval des EphA et d'EphB2 et elle bloquerait la signalisation inverse en aval de toutes les EfnA et EfnB qui interagissent avec les EphA et EphB2. L'EfnA3-Fc activerait plutôt les voies signalétiques antérogrades en aval des EphA et bloquerait les voies signalétiques inverses en aval des EfnA (qui lient tous les EphA) et EfnB (qui lient EphA4 uniquement) (voir Figure 1).

Quant aux protéines de fusion Eph-Fc, elles peuvent aussi être employées sous forme agglomérée ou non agglomérée (Conover et al., 2000). Ces deux formes pourraient agir comme des antagonistes fonctionnels, puisque leur liaison aux Efn bloquerait la signalisation antérograde en aval des Eph endogènes (Murai et al., 2003a; Winslow et al., 1995). Toutefois, elles activeraient aussi présumément la signalisation inverse en aval des Efn endogènes (Marquardt et al., 2005). Par exemple, EphA4-Fc stimulerait la signalisation inverse des EfnA et EfnB, mais bloquerait également la signalisation antérograde en aval des EphA et EphB auxquels elles se lient. Enfin, EphB2-Fc activerait des voies signalétiques inverses en aval des EfnB et de l'EfnA5, en plus de bloquer des voies signalétiques antérogrades en aval des EphB et EphA avec lesquels elles peuvent interagir (voir Figure 1).

2.5.2. Signalisation antérograde des Eph

Les voies de signalisation activées par les Eph sont résumées dans la Figure 4. D'abord, elles influencent la morphologie et le comportement cellulaires en régulant le remodelage du cytosquelette d'actine, par l'activation des petites GTPases de la famille Rho (RhoA, Rac et Cdc42) (voir Egea and Klein, 2007; Himanen et al., 2007; Murai and Pasquale, 2003). Ces dernières fonctionnent comme des interrupteurs moléculaires

permettant d'alterner entre des conformations inactive (Rho lié au GDP) et active (Rho lié au GTP). Dans les cônes de croissance, l'activation de RhoA cause la rétraction de l'axone et l'affaissement du cône de croissance (Dickson, 2001; Luo, 2000; Yuan et al., 2003), alors que Rac et Cdc42 favorisent la formation des lamellipodes et l'extension des filopodes en périphérie du cône de croissance (Kozma et al., 1997; Yuan et al., 2003). Les petites GTPases Rho sont activées par le recrutement de GEFs, qui catalysent le remplacement du GDP par du GTP (voir Egea and Klein, 2007). Différents GEFs sont recrutés en aval du domaine kinase des EphA ou EphB, dans des cônes de croissance ou des épines dendritiques. Dans les cônes de croissance, les GEFs éphexine-1 (*Eph-interacting exchange protein-1*), Vav2 et α2-chimérine sont spécifiquement recrutés par les EphA pour activer RhoA ou Rac (Cowan et al., 2005; Sahin et al., 2005; Shamah et al., 2001; Wegmeyer et al., 2007). Dans les prolongements dendritiques, les GEFs Tiam1, intersectine et kalirine sont recrutés par les EphB afin d'activer Rac ou Cdc42, ce qui induit la morphogenèse des épines dendritiques (Irie and Yamaguchi, 2002; Penzes et al., 2003; Tolias et al., 2007). En outre, une étude récente a démontré que l'éphexine-1 est activé en aval des EphA, indirectement par l'intermédiaire de la kinase cycline-dépendante-5 (cdk5), aussi bien dans les cônes de croissance que dans les prolongements dendritiques, où il inhibe la formation des épines dendritiques (Fu et al., 2007a).

Les Eph régulent aussi négativement l'activité des petites GTPases de la famille Ras (H-Ras, R-Ras, Rap1, Rap2). Le membre le mieux caractérisé de cette famille est H-Ras, qui active la cascade signalétique des protéines kinases activatrices de la mitose (MAP kinases) (voir Chang and Karin, 2001; Johnson and Lapadat, 2002). Les effets les plus connus de la voie Ras-MAP kinases concernent la régulation transcriptionnelle et l'accroissement de la prolifération cellulaire. Cependant, elle influence aussi la migration cellulaire, la croissance et le guidage axonaux (voir Borasio et al., 1989; Forcet et al., 2002). R-Ras régule notamment l'adhérence et la migration cellulaires, ainsi que la croissance des neurites des neurones de la rétine, du cortex et de l'hippocampe en culture primaire (Ivins et al., 2000; Keely et al., 1999; Kinbara et al., 2003; Zhang et al., 1996). En outre, Rap1 et Rap2 sont impliquées dans la régulation de la plasticité synaptique et de la morphologie des épines dendritiques (Fu et al., 2007b; Pak et al., 2001; Zhu et al., 2002).

Différentes protéines activatrices des GTPases (GAPs) seraient recrutées en aval du domaine JM des EphA et EphB pour inactiver des petites GTPases de la famille Ras. Dail et al. (2006) et Richter et al. (2007) ont récemment démontré que les EphA activent la Ras-GAP p120 et la Rap-GAP SPAR, afin d'induire l'affaissement des cônes de croissance des neurones hippocampiques en culture primaire.

Enfin, les Eph recrutent différentes protéines à motif SH2, par exemple la phospholipase-Cγ-1 (PLCγ1) en aval d'EphA4 dans les cellules COS-7 et les neurones de l'hippocampe en culture primaire (Zhou et al., 2007). Cette interaction est notamment requise pour la régulation du remodelage morphologique des épines dendritiques. D'autres protéines à motif SH2 pourraient servir à inhiber R-Ras ou H-Ras, comme SHEP1, Grb2 et Fyn (Choi and Park, 1999; Dodelet et al., 1999; Tong et al., 2003).

2.5.3. Signalisation inverse des Efn

Holland et al. (1996) et Bruckner et al. (1997) ont démontré que la partie extracellulaire d'EphB2 suffit pour induire la phosphorylation de l'EfnB1, dans les cellules de la lignée neuronale NG108-15, ce qui suggère l'activation de voies signalétiques inverses en aval des EfnB. Des kinases de la famille Src phosphoryleraient les EfnB (Holland et al., 1996; Kalo et al., 2001; Palmer et al., 2002), qui transmettraient alors des signaux par l'entremise de protéines à domaine SH2, comme la protéine adaptatrice Grb4 (Cowan and Henkemeyer, 2001; Segura et al., 2007), ou à domaine PDZ, comme la synténine, PICK1, GRIP1 et GRIP2 (Bruckner et al., 1999; Lin et al., 1999; Torres et al., 1998).

Les EfnA véhiculeraient aussi des signaux inverses par des protéines à domaine SH2, comme Fyn (Davy et al., 1999; voir Murai and Pasquale, 2003). Récemment, Lim et al. (2008) ont démontré que les EfnA peuvent induire de tels signaux par l'entremise du récepteur à basse affinité des neurotrophines p75 (p75NTR). Dans les axones des cellules ganglionnaires de la rétine *in vitro*, l'interaction directe entre les EfnA et le récepteur p75NTR est requise pour la phosphorylation de Fyn en aval de la liaison entre Eph et EfnA, ainsi que pour l'activation d'une voie signalétique causant une répulsion axonale.

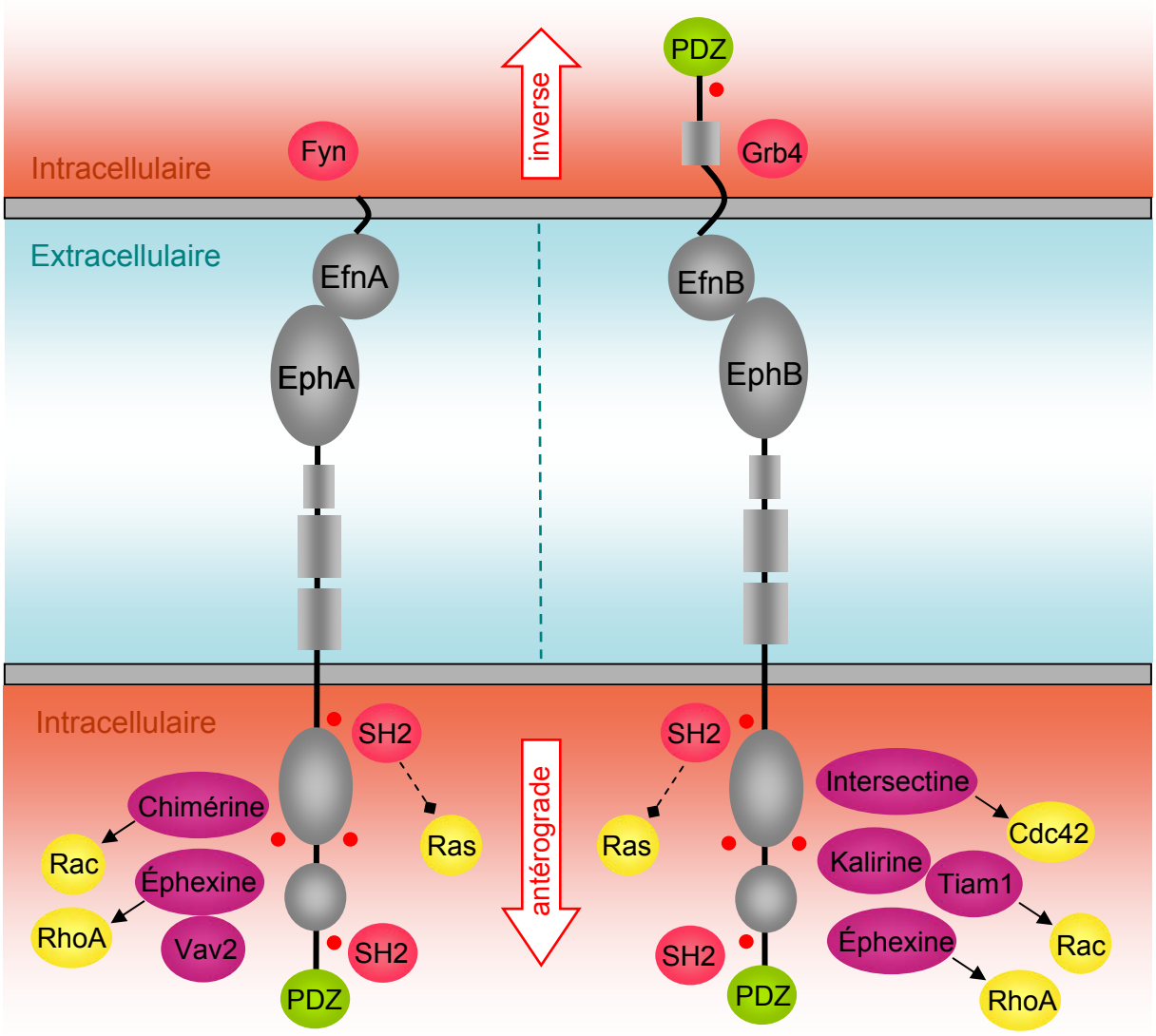

Figure 4. Voies de signalisation des Eph et des Efn

2.6. Distribution anatomique des Eph et des Efn dans le SNC

Des études d'hybridation *in situ* ou de transfert western ont montré l'abondance de l'ARNm des Eph et des Efn, ou des protéines elles-mêmes, dans le cerveau et la moelle épinière du rat, de la souris ou du poulet, embryonnaires ou adultes (voir Flanagan and Vanderhaeghen, 1998; Tuzi and Gullick, 1994). Plusieurs études ont subséquemment montré la distribution régionale étendue de plusieurs Eph et Efn dans le SNC du rat ou de la

souris, pendant le développement embryonnaire, postnatal ou même chez l'adulte (Gao et al., 1998; Liebl et al., 2003; Mackarehtschian et al., 1999; Stein et al., 1999; Vanderhaeghen et al., 2000; Yun et al., 2003). Leurs niveaux d'expression sont toutefois progressivement atténués pendant le développement postnatal, dans la plupart des régions du SNC (Liebl et al., 2003).

L'hybridation *in situ* a révélé l'expression des Eph et des Efn dans plusieurs populations de neurones, de cellules gliales et de cellules souches ou progénitrices (Liebl et al., 2003; www.allenbrainatlas.org). Le marquage X-gal chez des souris knockins pour la β-galactosidase, sous le contrôle du promoteur d'Eph ou d'Efn spécifiques a aussi permis de préciser les types cellulaires exprimant ces molécules. Cependant, ces deux techniques ne permettent pas de déterminer la localisation subcellulaire des protéines elles-mêmes, puisque l'ARNm est restreint aux corps cellulaires, d'une part, et que la β-galactosidase ne se distribue pas nécessairement comme un Eph ou une Efn dans la cellule, d'autre part, étant dépourvue des domaines de liaison adéquats. Pour documenter la localisation subcellulaire des Eph ou Efn, l'immunocytochimie avec des anticorps spécifiques pour ces molécules reste encore la meilleure méthode. Selon nos propres observations, la spécificité des anticorps utilisés dans certaines études pour localiser EphA4, EphB2, l'EfnB2, ou l'EfnB3 (Buchert et al., 1999; Greferath et al., 2002; Grunwald et al., 2004; Martone et al., 1997; Murai et al., 2003a) ne fait pas de doute puisqu'elle a été démontrée par une absence de marquage sur des coupes du cerveau de souris knockouts (voir les Chapitres II et III, Bouvier et al. (2008) et des résultats récents non publiés de notre laboratoire). Celle des anticorps utilisés dans d'autres études pour EphA3 (Kudo et al., 2005; Otal et al., 2006), EphA7 (Buchert et al., 1999), EphB3 (Buchert et al., 1999) ou l'EfnA3 (Murai et al., 2003a) reste à démontrer. Dans certains cas, nos propres tests, sur coupes de cerveau de souris knockouts nous font douter de leur spécificité puisque le marquage, obtenu dans nos conditions de préparation, était semblable chez les souris knockouts et les types sauvages respectifs. Certains de ces anticorps sont tout de même convenables pour des techniques biochimiques, comme le transfert western. Cette rareté d'anticorps spécifiques pour l'immunocytochimie de ces molécules explique que la localisation subcellulaire des Eph et des Efn soit actuellement très peu connue, malgré l'intérêt qu'elle suscite dans le domaine.

Concernant plus particulièrement EphA4, qui fait l'objet de cette thèse, quelques études ont décrit sa localisation régionale dans le SNC de la souris ou du rat, pendant le développement embryonnaire et postnatal. Par hybridation *in situ* et immunocytochimie, l'ARNm et la protéine ont été rapportés comme localisés, à partir de E10,5, dans le cortex cérébral, l'hippocampe, le thalamus, la moelle épinière, les noyaux gris centraux, le bulbe olfactif et le cervelet, ainsi que dans de nombreux faisceaux de matière blanche (Greferath et al., 2002; Liebl et al., 2003; Mackarehtschian et al., 1999; www.allenbrainatlas.org). Greferath et al. (2002) ont montré que l'intensité du marquage immunocytochimique diminue progressivement pendant le développement postnatal. Chez le rat adulte, l'ARNm ou la protéine sont plus concentrés dans l'hippocampe, mais se retrouvent aussi dans d'autres régions du SNC reconnues comme demeurant plus plastiques, comme le cortex cérébral, le bulbe olfactif et le cervelet (Liebl et al., 2003; Martone et al., 1997). Martone et al. (1997) ont aussi examiné la localisation subcellulaire de la protéine EphA4 dans quelques régions du SNC du rat adulte, par immunocytochimie en microscopie électronique. Cette étude a révélé la présence d'EphA4 dans les corps cellulaires, les axones et les épines dendritiques des neurones épineux du néostriatum, de même que dans les axones myélinisés du fornix et de la matière blanche de la moelle épinière. Des épines dendritiques immunopositives ont également été observées dans l'hippocampe, mais le secteur et la couche cellulaire examinés n'étaient pas précisés.

2.7. Principales fonctions des Eph et des Efn dans le SNC

Chez les vertébrés, les Eph et les Efn contribuent par leurs activités variées au développement, notamment du SNC comme nous le verrons dans les sections suivantes, mais aussi à la physiologie de nombreux organes matures (voir Egea and Klein, 2007; Himanen et al., 2007; Pasquale, 2005; 2008). Par exemple, des interactions entre Eph et Efn réguleraient la sécrétion d'insuline par les cellules β-pancréatiques, le renouvellement des cellules de l'épithélium intestinal, la formation des os, des vaisseaux sanguins et des vaisseaux lymphatiques, ainsi que la maturation des thymocytes en cellules immunitaires de type T (voir Kuijper et al., 2007; Pasquale, 2008).

2.7.1. Fonction des Eph et des Efn dans la migration cellulaire et la formation de motifs cellulaires complexes

Le SNC déploie une grande diversité de motifs et de formes, par son organisation en régions, secteurs, couches, noyaux et segments. Or, les Eph et les Efn sont impliqués dans la migration et la ségrégation cellulaires requises pour générer des motifs cellulaires complexes. Dans le rhombencéphale, Gilardi-Hebenstreit et al. (1992) ont d'abord observé par hybridation *in situ* l'expression d'EphA4 dans les rhombomères trois et cinq (r3 et r5) chez la souris embryonnaire âgée de 9,5 jours (E9,5). Subséquemment, l'expression selon divers motifs a été décrite pour quelques autres Eph (Becker et al., 1994; Ellis et al., 1995; Ganju et al., 1994; Henkemeyer et al., 1994; Irving et al., 1996; Ruiz and Robertson, 1994; Taneja et al., 1996; Winning and Sargent, 1994) et pour certaines Efn (Bergemann et al., 1995; Cheng and Flanagan, 1994; Flenniken et al., 1996; Gale et al., 1996a). Ces motifs d'expression suggéraient un rôle pour les interactions entre Eph et Efn dans la segmentation des rhombomères. Xu et al. (1995) ont effectivement démontré que la surexpression de formes dominantes-négatives d'EphA4 entraîne l'expression ectopique de marqueurs spécifiques à r3 et r5 dans les segments pairs adjacents, chez le xénope et le poisson zébré. Par ailleurs, dans le prosencéphale, la matrice et les striosomes du striatum sont générés séquentiellement pendant le développement embryonnaire, pour former une mosaïque de compartiments morphologiquement et fonctionnellement distincts. Passante et al. (2008) ont récemment révélé un rôle d'EphA4 et des Efn, notamment l'EfnA5, dans la régulation de la compartimentation entre matrice et striosomes. En effet, EphA4 et différentes Efn (EfnA2, EfnA3, EfnA5, EfnB1, EfnB2, et EfnB3) présentent des motifs d'expression spatialement et temporellement distincts, tel que révélé par hybridation *in situ*, alors que les souris knockouts pour EphA4 ou l'EfnA5 présentent un défaut de ségrégation entre les cellules de la matrice et des striosomes.

2.7.2. Fonction des Eph et des Efn dans le guidage axonal et la formation de cartes de projection

Les premiers indices d'une fonction axonale exercée par les Eph proviennent d'études d'immunolocalisation montrant la présence d'EphA4 ou EphB2 dans des

faisceaux d'axones et leurs cônes de croissance, dans le cervelet et la moelle épinière du poulet embryonnaire et postnatal (Flanagan and Vanderhaeghen, 1998; Henkemeyer et al., 1994; Pasquale et al., 1992; Soans et al., 1994). Dans plusieurs systèmes, notamment moteur, somatosensoriel, visuel, auditif et olfactif, les projections axonales adoptent une organisation précise et ordonnée, formant une topographie qui reflète celle des récepteurs sensoriels ou des muscles sur le corps, à chaque relais. Le rôle des interactions entre Eph et Efn dans la formation de cartes de projection a ensuite été montré par des gradients complémentaires d'expression des Eph et des Efn dans les structures de projection et leurs cibles (Cheng et al., 1995; Drescher et al., 1995; Flanagan and Vanderhaeghen, 1998), des effets topographiquement spécifiques sur la répulsion axonale et l'affaissement des cônes de croissance axonaux *in vitro* ou *in vivo* (Drescher et al., 1995; Monschau et al., 1997), ainsi que des malformations de projections topographiques chez des souris knockouts ou knockins (Brown et al., 2000; Cang et al., 2008; McLaughlin and O'Leary, 2005). Par exemple, des souris knockins pour EphA3, chez lesquelles EphA3 est anormalement exprimé dans une grande proportion de cellules ganglionnaires de la rétine, ont deux cartes de projection plutôt qu'une dans les collicules supérieurs (Brown et al., 2000).

Par ces approches, plusieurs études ont mis à jour des interactions répulsives ou attractives entre Eph et Efn qui participent à l'établissement d'une variété de cartes de projection, incluant celles entre la rétine et les collicules supérieurs (mammifères) ou le tectum optique (poulet, oiseaux, poissons), entre la rétine et le corps genouillé latéral du thalamus, entre l'hippocampe et le septum, ainsi qu'entre le thalamus et le cortex somatosensoriel (voir Flanagan and Vanderhaeghen, 1998; Frisen et al., 1999; Knoll and Drescher, 2002; Pasquale, 2005; Wilkinson, 2001). Certains Eph seraient aussi essentiels au guidage de projections axonales qui traversent la ligne médiane du SNC, incluant la commissure antérieure, le corps calleux et la voie corticospinale. Des souris knockouts pour EphA4, EphA8, EphB2 ou EphB3 présentent effectivement des malformations au niveau de ces projections (voir Frisen et al., 1999). Par ailleurs, des souris knockouts pour EphA4 et l'EfnB3, ainsi que des souris knockins qui expriment des isoformes d'EphA4 dépourvues de domaine kinase, ont une démarche anormale dénuée de mouvements alternes des membres postérieurs (Dottori et al., 1998; Kullander et al., 2001; Yokoyama et al., 2001).

En plus des malformations de la voie corticospinale, le guidage axonal des interneurones excitateurs du générateur central de motifs locomoteurs (CPG) serait impliqué. En effet, Kullander et al. (2003) ont révélé que les segments lombaires de moelle épinière isolée de souris nouveau-née, knockout pour EphA4 ou pour l'EfnB3, ont des motifs rythmiques synchrones au niveau des racines ventrales, plutôt qu'une alternance gauche/droite telle qu'observée avec des souris de type sauvage. De plus, chez des souris knockouts pour EphA4, plusieurs interneurones excitateurs croisent anormalement la ligne médiane de la moelle épinière, dans sa portion ventromédiane où se trouve le CPG. Enfin, l'application de sarcosine, un bloqueur de la recapture de la glycine et de l'acide γ-aminobutyrique (GABA), restaure l'alternance gauche/droite chez ces souris, suggérant qu'un renforcement de l'inhibition puisse contrebalancer l'innervation excitatrice aberrante. Ainsi, des interactions entre EphA4, dans les interneurones excitateurs, et l'EfnB3, située à la ligne médiane, empêcheraient la traversée de la ligne médiane par les interneurones excitateurs, ce qui est requis pour l'exercice d'un motif locomoteur normal.

2.7.3. Fonction des Eph et des Efn à l'inhibition de la régénération axonale après une lésion de la moelle épinière

L'environnement extracellulaire du SNC mature joue un rôle important dans l'inhibition de la régénération axonale à la suite d'une lésion. La présentation de molécules inhibitrices par la myéline, notamment Nogo-66, la glycoprotéine associée à la myéline (MAG) et la glycoprotéine de la myéline oligodendrocytaire (OMgp), serait impliquée dans ce blocage de la régénération (Bandtlow and Schwab, 2000; Caroni and Schwab, 1988; McKerracher et al., 1994; Mukhopadhyay et al., 1994; Wang et al., 2002), tout comme la prolifération et l'activation des astrocytes, qui conduit à la formation d'une cicatrice gliale au site de lésion et change la composition de la matrice extracellulaire, notamment par la sécrétion de CSPG, de ténascines et de collagène de type IV (Goldshmit et al., 2006b; McKeon et al., 1991; Silver and Miller, 2004; Stichel et al., 1999).

L'expression de nombreux Eph et Efn est augmentée après une lésion de la moelle épinière chez l'adulte, surtout dans les astrocytes réactifs et les axones corticospinaux ou rubrospinaux lésés (Bundesen et al., 2003; Fabes et al., 2007; Fabes et al., 2006; Goldshmit

et al., 2004; Miranda et al., 1999; Willson et al., 2002). Toutefois, ces molécules de guidage axonal auraient un effet inhibiteur sur la régénération axonale (voir Goldshmit et al., 2006b; Harel and Strittmatter, 2006). En effet, Goldshmit et al. (2004) ont observé chez des souris knockouts pour EphA4 une régénération des axones corticospinaux et rubrospinaux six semaines après une hémisection gauche de la moelle épinière, en plus d'un recouvrement des habiletés sensorielles et motrices. Or, la gliose astrocytaire est aussi absente chez ces souris knockouts pour EphA4, tel que révélé par une diminution significative du nombre d'astrocytes immunopositifs pour la protéine gliale fibrillaire acide (GFAP). *In vitro*, les neurones corticaux cultivés sur des astrocytes de souris EphA4 knockouts ont une meilleure croissance neuritique, comparativement à des astrocytes témoins. En outre, les astrocytes de ces souris ne prolifèrent pas lorsque des cytokines inflammatoires sont appliquées, soient le facteur inhibiteur de la leucémie (LIF) et l'interféron gamma (INFγ). L'amélioration de la régénération axonale chez les souris knockouts EphA4 pourrait ainsi résulter, du moins partiellement, d'une incapacité des astrocytes qui expriment normalement cette molécule de réagir à la lésion par la formation d'une cicatrice gliale.

Plus récemment, Fabes et al. (2007) ont observé la croissance et le bourgeonnement d'axones corticospinaux lésés chez la souris, 28 jours après une hémisection dorsale de la moelle épinière et suite à l'infusion locale d'un peptide antagoniste d'EphA4 (Murai et al., 2003b), malgré que le site de lésion était alors envahi par des prolongements astrocytaires GFAP-positifs. De plus, ils ont rapporté une amélioration significative des habiletés sensorielles et motrices de ces souris, comparativement à des souris lésées non infusées. Il semble ainsi que le rôle d'EphA4 dans l'inhibition de la régénération axonale ne soit pas seulement d'induire la réactivité gliale, mais qu'il agisse aussi comme ligand ou récepteur glial ou axonal, dans les interactions cellulaires qui inhibent la croissance des axones.

2.7.4. Fonction des Eph et des Efn dans la formation, le maintien et la plasticité des synapses excitatrices

Puisque cette fonction a surtout été examinée dans l'hippocampe, elle sera détaillée dans la section 4.2.

3. L'hippocampe

Avant d'aborder les rôles des Eph et des Efn dans l'hippocampe, nous présenterons une courte revue de son organisation laminaire et de son développement, chez le rat, ainsi que de sa plasticité synaptique à long terme (basée sur Andersen, 2007; Paxinos, 2004; sauf lorsque d'autres références sont précisées).

3.1. Cellules neuronales principales et circuiterie excitatrice

L'hippocampe est une structure corticale impliquée dans l'apprentissage et la mémoire. Sa circuiterie excitatrice comprend une boucle véhiculant de l'information sensorielle polymodale entre les couches II/III du cortex entorhinal, le gyrus dentelé, le secteur CA3, le secteur CA1 et la couche V du cortex entorhinal (voir Figure 4).

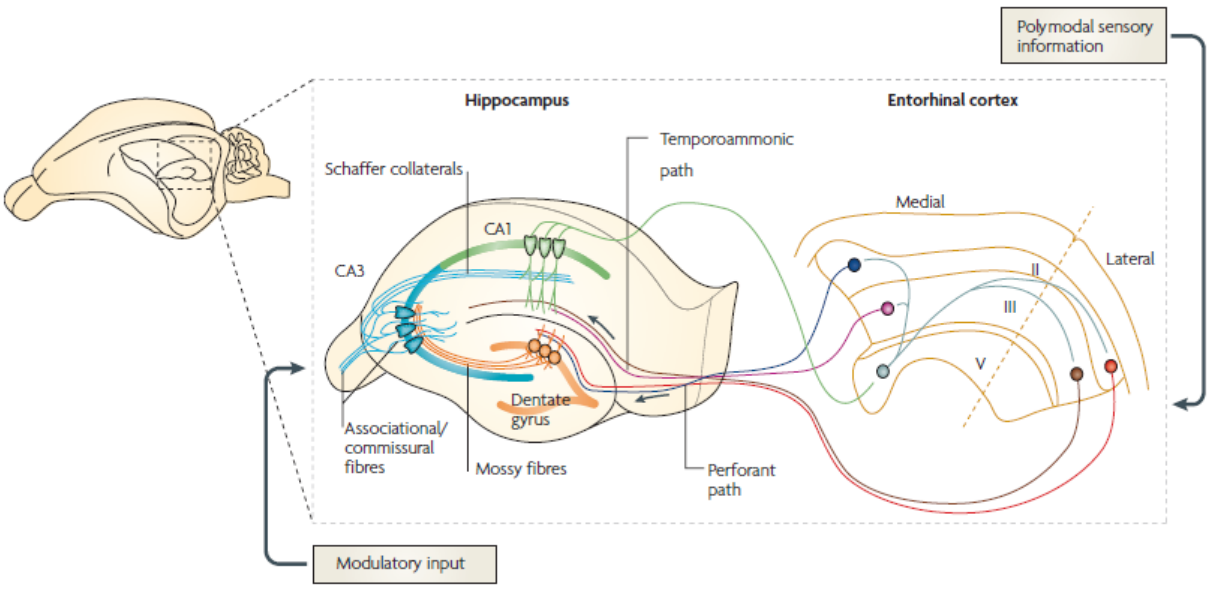

Figure 5. Circuiterie excitatrice de l'hippocampe (Neves et al., 2008)

3.1.1. Gyrus dentelé

Le gyrus dentelé comprend trois couches : moléculaire, granulaire et polymorphe. Ses cellules principales sont les cellules granulaires. Elles se dénombrent à environ 1,2 million dans un seul gyrus dentelé chez le rat, nombre qui varie tout au long de la vie et qui

augmente particulièrement lorsque l'animal est élevé dans un environnement enrichi permettant l'activité physique et la socialisation (voir Kempermann, 2006). Les cellules granulaires possèdent une arborisation dendritique apicale, située dans la couche moléculaire, avec une portion proximale dans la couche moléculaire interne et une portion distale dans la couche moléculaire externe. Les épines dendritiques de la portion distale reçoivent la plus importante afférence excitatrice de l'hippocampe, soit la voie perforante en provenance de la couche II du cortex entorhinal. Les axones des cellules granulaires, dénommés fibres moussues, projettent aux cellules pyramidales et aux interneurones du CA3, ainsi qu'aux cellules moussues du gyrus dentelé dont le corps cellulaire se trouve dans la couche polymorphe. Les axones des cellules moussues projettent aux épines dendritiques des cellules granulaires, dans la couche moléculaire interne.

3.1.2. Secteurs CA3 et CA1

Les cellules pyramidales sont les cellules principales des secteurs CA3 et CA1. Leur arborisation dendritique basale est située dans la couche oriens et leur arborisation dendritique apicale s'étend dans les couches lucidum (CA3 uniquement), radiatum, puis lacunosum-moleculare. La couche lucidum du CA3, principalement, mais aussi les couches pyramidale et oriens, reçoivent les terminaisons des fibres moussues qui y forment des contacts synaptiques morphologiquement complexes avec les épines dendritiques des cellules pyramidales, lesquelles sont appelées excroissances épineuses (*thorny excrescences*). Les cellules pyramidales du CA3 sont aussi fortement innervées par des collatérales d'axones du même secteur (projections associatives) et de ceux du CA3 contralatéral (projections commissurales), dans les trois couches. Des collatérales des cellules pyramidales du CA3 projettent également aux cellules moussues dans la couche polymorphe du gyrus dentelé et aux cellules pyramidales du CA1, dans les couches oriens et radiatum. Ces projetions du CA3 aux CA1 se nomment collatérales de Schaffer. Dans le CA1, les axones qui voyagent dans l'alveus et dans la couche oriens en direction du subiculum envoient des collatérales aux cellules pyramidales du CA1, dans les couches oriens et des cellules pyramidales. En outre, la partie septale du CA1 reçoit des afférences de la couche III du cortex entorhinal, la dénommée voie perforante qui emprunte l'alveus et

perfore la couche de cellules pyramidales pour rejoindre la couche lacunosum-moleculare. En retour, les cellules pyramidales du CA1 projettent à la couche V du cortex entorhinal.

L'hippocampe reçoit de nombreuses afférences sous-corticales, représentant toutefois une faible proportion de ses connexions synaptiques. Brièvement, le gyrus dentelé reçoit des entrées du noyau septal de la région supramammillaire de l'hypothalamus postérieur, ainsi que d'un grand nombre de noyaux monoaminergiques du tronc cérébral, principalement du locus coeruleus et du raphé. La projection septale, cholinergique, dérive des cellules du noyau septal médian et du noyau de la bande diagonale de Broca. Elle se termine principalement dans la couche polymorphe du gyrus dentelé. Des afférences noradrénergiques et sérotoninergiques, respectivement en provenance du noyau pontin du locus coeruleus et de plusieurs subdivisions du noyau du raphé, se terminent aussi dans la couche polymorphe du gyrus dentelé. De plus, des fibres de l'aire tegmentaire ventrale, dont certaines sont dopaminergiques, sont distribuées diffusément dans toutes les couches du gyrus dentelé. Dans le CA3, la projection septale cholinergique innerve majoritairement la couche oriens, tandis que la couche lucidum et la portion la plus superficielle de la couche lacunosum-moleculare reçoivent des terminaisons noradrénergiques. Des afférences sérotoninergiques et quelques fibres dopaminergiques projettent diffusément sur des interneurones, dans toutes les couches. Enfin, le CA1 reçoit des projections cholinergiques, dopaminergiques, noradrénergiques et sérotoninergiques diffuses, dans toutes ses couches.

3.2. Interneurones

Une population variée d'interneurones dont le neurotransmetteur principal est le GABA se trouve aussi dans toutes les couches précédemment décrites de l'hippocampe. La diversité concerne les aspects morphologiques, électrophysiologiques et neurochimiques de leur phénotype (voir Freund, 2003; Kullmann and Lamsa, 2007). Brièvement, des cellules en panier sont positionnées dans la couche granulaire du gyrus dentelé et dans les couches de cellules pyramidales des secteurs CA3 et CA1. Leurs terminaisons GABAergiques forment des contacts inhibiteurs, avec des jonctions de types symétriques, sur les corps cellulaires et les branches des dendrites apicales des cellules granulaires ou pyramidales. Des interneurones nommés cellules axo-axoniques sont localisés dans la couche

moléculaire du gyrus dentelé, ainsi que dans la couche radiatum du CA3 et du CA1. Leur plexus axonal forme des contacts symétriques, uniquement sur le segment axonal initial des cellules granulaires ou pyramidales. Des interneurones immunopositifs pour la somatostatine se trouvent aussi dans la couche polymorphe du gyrus dentelé et la couche oriens des secteurs CA3 et CA1. Leurs axones s'étendent jusqu'aux couches moléculaire externe et lacunosum-moleculare pour établir des contacts symétriques avec les rares épines des dendrites distales des cellules granulaires ou pyramidales, lesquelles reçoivent également des synapses asymétriques des afférences enthorhinales. Enfin, dans le CA1 et le CA3, les neurones bistratifiés distribuent leurs axones dans les couches radiatum et oriens, où se terminent la plupart des projections excitatrices de l'hippocampe.

3.3. Développement de l'hippocampe

3.3.1. Neurogenèse et migration cellulaire

Les cellules pyramidales sont générées dans l'épithélium germinatif de la zone ventriculaire du télencéphale dorsal entre E16 et E21. Elles migrent le long de la glie radiaire pour atteindre leur secteur cible, soit le CA3 ou le CA1. La migration radiaire est courte et, donc, brève pour les cellules pyramidales du CA1, puisque la zone germinative borde ce secteur. Celles du CA3 nécessitent trois à quatre jours de migration supplémentaires. Quant aux cellules granulaires, elles sont aussi générées dans l'épithélium germinatif de la zone ventriculaire du télencéphale dorsal, à partir de E16. Cependant, seulement 15% des cellules granulaires sont générées avant la naissance. Elles migrent le long de la glie radiaire, du ventricule jusqu'au gyrus dentelé, au travers l'étroite zone intermédiaire entre l'alveus et la future couche oriens du CA1 (Altman and Bayer, 1990). Entre P10 et P25, la couche polymorphe du gyrus dentelé constitue une zone germinative secondaire (Bayer, 1980). La neurogenèse se poursuit ensuite, et ce tout au long de la vie, dans une troisième zone germinative nommée la zone sous-granulaire (ZSG).

Plusieurs études ont décrit la genèse, la migration, la maturation et l'intégration fonctionnelle des nouvelles cellules granulaires générées dans l'hippocampe adulte (voir Duan et al., 2008; Kempermann, 2006; Lledo et al., 2006; Ming and Song, 2005; Figure 6).

Toni et al. (2008) ont récemment démontré que les nouvelles cellules granulaires établissent des synapses glutamatergiques fonctionnelles, aussi bien avec les interneurones, les cellules moussues du gyrus dentelé, qu'avec les cellules pyramidales du CA3. Afin de démontrer le rôle de cette neurogenèse chez l'adulte, Imayoshi et al. (2008) ont créé des souris chez lesquelles les cellules progénitrices qui débutent leur différenciation neuronale chez l'adulte sont sélectivement éliminées par apoptose, par l'expression inductible du fragment A de la toxine diphtérique sous le promoteur de la nestine. Comparativement à des souris de type sauvage, ces souris transgéniques présentent une diminution significative du nombre de nouvelles cellules granulaires, identifiées par l'intégration de 5-bromo-2-désoxyuridine (BrdU) dans leur ADN, en plus de déficits comportementaux, révélés par leur performance à des tests de mémoire spatiale et de conditionnement contextuel à la peur. Ainsi, l'ajout et l'intégration de nouvelles cellules granulaires dans la circuiterie hippocampique participeraient de façon importante dans l'apprentissage et la mémoire.

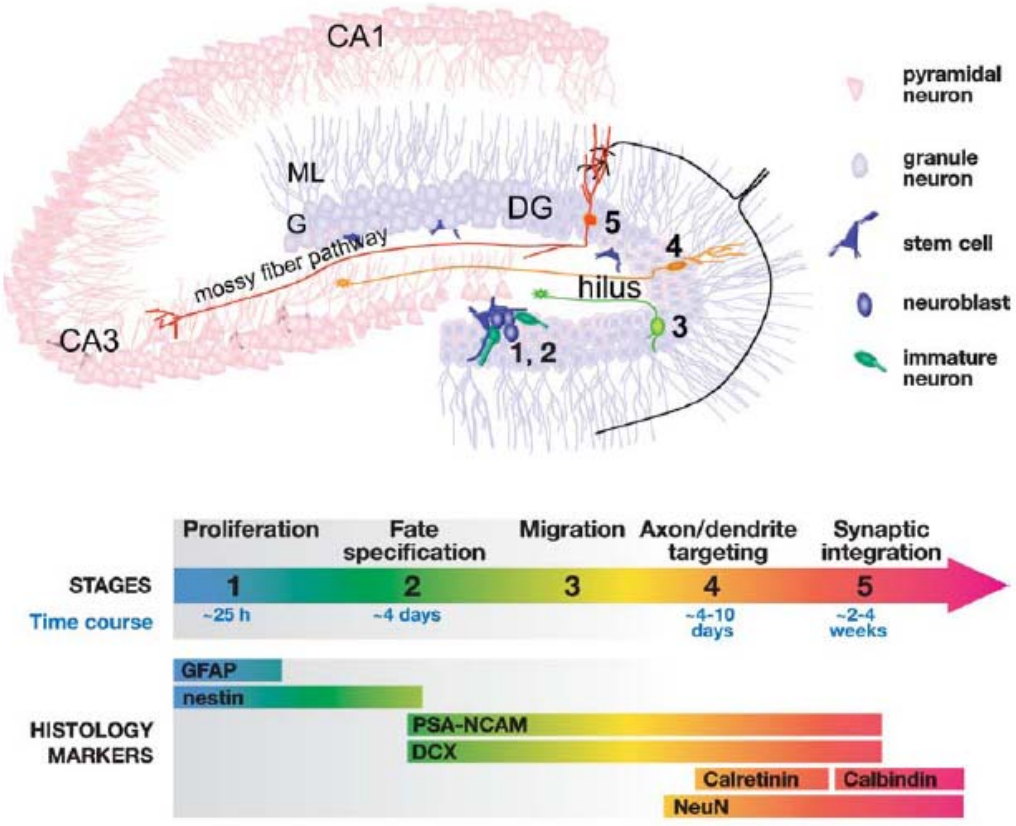

Figure 6. Neurogenèse dans l'hippocampe adulte (Ming and Song, 2005)

En outre, les interneurones sont aussi générés avant la naissance, entre E13 et E18 (voir Danglot et al., 2006), principalement dans le sous-pallium télencéphalique à partir duquel ils migrent avec des orientations tangentielles et radiaires, pour atteindre plusieurs régions cibles, soient le cortex, le striatum, l'amygdale, le bulbe olfactif, le noyau accumbens, ainsi que l'hippocampe (Pleasure et al., 2000). Ceux de l'hippocampe sont spécifiquement générés dans les zones prolifératives du télencéphale ventral, nommées éminences ganglionnaires médiane et caudale (Nery et al., 2002).

Par ailleurs, les couches de cellules pyramidales subissent un réarrangement spatial pendant le développement postnatal. Le nombre de rangées de corps cellulaires neuronaux diminue, de six à dix couches à la naissance, jusqu'a deux à trois couches chez l'adulte. Les mécanismes moléculaires qui régulent cette réorganisation sont peu connus. L'apoptose ne serait pas impliquée, au contraire des interactions entre cellules gliales et cellules pyramidales. En effet, l'irradiation aux rayons-X de l'hippocampe de rats néonataux, laquelle affecte alors principalement la prolifération les cellules gliales et granulaires du gyrus dentelé, perturbe cette maturation de la couche de cellules pyramidales dans le secteur CA1 de l'hippocampe (Czurko et al., 1997).

3.3.2. Guidage axonal, développement dendritique et établissement de la circuiterie excitatrice

Pendant le développement de l'hippocampe, les projections excitatrices sont guidées vers des couches spécifiques pour établir des connexions synaptiques avec l'arborisation dendritique épineuse des cellules pyramidales. Les afférences du cortex entorhinal innervent l'hippocampe vers E15 et le gyrus dentelé entre E18 et E19 (Ceranik et al., 1999; Super and Soriano, 1994). À ce stade, les cellules pyramidales et granulaires sont dépourvues d'arborisation dendritique distale. Puisque la plupart des cellules granulaires ne sont pas encore générées, les cellules de Cajal-Retzius de la zone marginale, soit la future couche moléculaire externe du gyrus dentelé, servent de cibles dendritiques provisoires aux afférences du cortex entorhinal (Del Rio et al., 1996). Les cellules de Cajal-Retzius sont éventuellement éliminées par apoptose, autour de P10. Les connexions entorhinales se réorganisent et forment alors de nouvelles synapses sur les épines dendritiques des cellules

granulaires (Ceranik et al., 1999). Il est à mentionner que l'établissement de la voie perforante est toutefois requis pour la maturation et le maintien de l'arborisation épineuse distale des cellules granulaires (Drakew et al., 1999; Frotscher et al., 2000). Au contraire, l'arborisation dendritique proximale est déjà développée lorsque les premiers axones commissuraux arrivent dans les secteurs CA1 et CA3 contralatéraux, vers E18, et dans le gyrus dentelé, vers P2, chez la souris (Deller et al., 1999). Enfin, les connexions des fibres moussues sont établies encore plus tardivement (Amaral and Dent, 1981). À P0, de petites terminaisons immatures forment des contacts synaptiques sur les branches dendritiques des cellules pyramidales du CA3, les excroissances épineuses n'apparaissant que vers P9.

Par ailleurs, il est à mentionner que l'arbre dendritique des cellules granulaires gagne en volume dans la couche moléculaire du gyrus dentelé jusqu'à P14. Par la suite, certaines branches dendritiques individuelles continuent à croître alors que d'autres sont éliminées, sans changement global du volume de l'arbre dendritique (Rahimi and Claiborne, 2007). Quant aux cellules pyramidales, leurs branches dendritiques s'allongent et se ramifient dans la couche radiatum du CA1 jusqu'à P24 (Pokorny and Yamamoto, 1981). Pendant leur maturation postnatale, ces arbres dendritiques sont progressivement décorés d'épines dendritiques, lesquelles sont formées à partir de filopodes dendritiques qui se rétractent ou de branches dendritiques qui s'allongent (Fiala et al., 1998).

3.4. Plasticité synaptique à long terme

La potentialisation à long terme (LTP) et la dépression à long terme (LTD) sont les phénomènes expérimentaux de la plasticité synaptique les plus étudiés, particulièrement dans l'hippocampe (voir Alvarez and Sabatini, 2007; Kandel, 2000; Malenka and Bear, 2004; Neves et al., 2008; Squire, 2002; sauf lorsque d'autres références sont précisées).

La LTP est induite par des stimulations brèves à haute fréquence, par exemple quatre trains de stimuli à 100 Hz. Toutes les formes de la LTP seraient exercées par l'augmentation de la concentration intracellulaire en ions calciques dans les structures postsynaptiques. À la synapse des collatérales de Schaffer, dans le secteur CA1, elle présente trois propriétés classiques, soient la coopérativité, l'associativité et la spécificité

des entrées, toutes expliquées par le postulat formulé par Donald Hebb, en 1949, selon lequel une activité synchrone dans deux neurones connectés par des synapses induit un renforcement de la transmission à ces synapses. À la synapse des collatérales de Schaffer, le calcium entre nécessairement par les récepteurs NMDA, lesquels sont activés par la liaison du glutamate lorsque le niveau de dépolarisation atteint un seuil critique (coopérativité). Lorsque deux entrées axonales distinctes convergent sur une même cible postsynaptique, l'activité de l'entrée faible, qui comprend le plus petit nombre d'afférences stimulées, ne parvient pas à dépolariser suffisamment la cellule postsynaptique, sauf lorsqu'elle coïncide avec l'activité d'une entrée plus forte, qui enrôle un plus grand nombre d'afférences stimulées (associativité et spécificité des entrées). En outre, l'entrée de calcium au travers des canaux calciques régulés par le voltage (VGCCs), en plus de la libération de calcium des réserves intracellulaires, peut contribuer à augmenter la concentration calcique intracellulaire, par exemple à la synapse des fibres moussues dans le secteur CA3 où l'induction de la LTP ne dépend pas des récepteurs NMDA. À la plupart des synapses, l'influx calcique entraîne l'activation de plusieurs enzymes qui dépendent du calcium, comme la kinase dépendante de la calmoduline II (CaM kinase II), ainsi que l'activation de la voie des MAP kinases qui converge vers la phosphorylation de l'élément de liaison à l'ADN en réponse à l'adénosine monophosphate cyclique (AMPc) (CREB) et l'induction de gènes qui encodent des protéines responsables des changements morphologiques et fonctionnels à la synapse, notamment l'augmentation du volume et la diminution de la motilité des épines dendritiques, ainsi que l'accroissement du nombre de récepteurs AMPA insérés à la membrane postsynaptique (voir Hayashi and Majewska, 2005). Des messagers rétrogrades sont aussi libérés, notamment l'oxyde d'azote (NO), afin d'augmenter la libération de glutamate par les terminaisons présynaptiques (Arancio et al., 1996; O'Dell et al., 1994).

En ce qui concerne la LTD, elle est induite par une stimulation prolongée de faible fréquence, par exemple pendant dix minutes à 1 Hz. Elle est exprimée sous plusieurs formes qui dépendent ou pas des récepteurs NMDA. Brièvement, elle prend la forme d'une diminution persistante de la concentration calcique intracellulaire dans les structures postsynaptiques, ce qui induit la déphosphorylation de plusieurs enzymes qui dépendent du

calcium, lesquelles sont au contraire phosphorylées pendant l'expression de la LTP, afin de diminuer la libération de glutamate par les terminaisons présynaptiques.

4. Les Eph et les Efn dans l'hippocampe

4.1. Localisation des Eph et des Efn dans l'hippocampe

Plusieurs études fonctionnelles ont révélé des rôles importants pour les Eph et les Efn dans l'hippocampe (section 4.2.), mais l'identité exacte des molécules impliquées et les sites précis où elles agissent restent à préciser. À cette fin, il sera nécessaire d'examiner leurs localisations subcellulaires respectives dans les types cellulaires de l'hippocampe, notamment aux différentes synapses de sa circuiterie.

Au niveau cellulaire, certaines études ont déjà montré, par hybridation *in situ,* que les ARNm d'EphA3, EphA4 et EphA5 sont exprimés dans les cellules principales du gyrus dentelé, du CA3 et du CA1, chez le rat et la souris néonatals ou adultes (Liebl et al., 2003; Mackarehtschian et al., 1999; Stein et al., 1999; www.allenbrainatlas.org). Les ARNm d'EphB1, EphB2 et EphB3 sont aussi exprimés par les cellules principales de l'hippocampe chez la souris embryonnaire, néonatale ou adulte : EphB1 dans le CA3 et le gyrus dentelé, EphB2 dans le CA3, le CA1 et le gyrus dentelé et EphB3 dans le CA1 (Grunwald et al., 2004; Liebl et al., 2003). Pour tous ces Eph, le niveau d'expression diminue progressivement pendant le développement postnatal. Chez l'adulte, EphA4 est le plus abondamment exprimé (voir Pasquale, 2005). Plusieurs Efn sont aussi exprimées par les cellules principales de l'hippocampe (voir Martinez and Soriano, 2005). Des expériences de liaison sur coupe avec la protéine de fusion EphA4-phosphatase alcaline ont montré que les EfnA sont fortement exprimées dans l'hippocampe postnatal (Stein et al., 1999), avec une distribution laminaire ressemblant à celle d'EphA4 (Greferath et al., 2002; Martone et al., 1997; Murai et al., 2003a). Selon les observations en hybridation *in situ*, les ARNm de l'EfnA3 et de l'EfnA5, mais pas celui de l'EfnA4, sont exprimés dans l'hippocampe du rat embryonnaire (E17, E19), néonatal (P1, P2) et adulte. L'EfnA3 est exprimée dans le CA1, le CA3 et le gyrus dentelé et l'EfnA5 seulement dans le CA1 et le CA3 (Mackarehtschian et al., 1999; Stein et al., 1999). Leur expression est maintenue chez l'adulte (Stein et al.,

1999). D'autre part, les ARNm des EfnB1, EfnB2 et EfnB3 étaient faiblement détectés dans l'hippocampe néonatal. Par contre, chez l'adulte, les EfnB2 et EfnB3 deviennent abondantes, respectivement, dans le CA1 et le gyrus dentelé, et dans le CA1 uniquement (Grunwald et al., 2001; Liebl et al., 2003). En outre, les ARNm d'EphA1, EphA3, EphA4 et EphA8, ainsi que des EfnA2, EfnA3 et EfnB2 seraient aussi exprimés par les astrocytes protoplasmiques de l'hippocampe, selon des expériences de RT-PCR sur des astrocytes isolés en aigu de la couche radiatum du CA1 de souris adultes (Murai et al., 2003a; Nestor et al., 2007).

Puisque la localisation subcellulaire des Eph et des Efn peut uniquement être déterminée par immunocytochimie, elle est demeurée essentiellement obscure malgré le grand nombre d'études fonctionnelles réalisées ces dix dernières années, en raison de la rareté des anticorps spécifiques dirigés contre ces molécules. Buchert et al. (1999), Martone et al. (1997) et Grunwald et al. (2004) ont rapporté la localisation d'EphA4, EphB2 et de l'EfnB2, dans les épines dendritiques du secteur CA1 de l'hippocampe du rat adulte, après des examens plutôt superficiels par immunocytochimie en microscopie électronique. Ces études n'excluent pas que ces molécules puissent aussi être localisées dans des terminaisons axonales, où elles seraient bien positionnées pour interagir en *trans* avec les Efn ou les Eph localisés dans les épines dendritiques. En effet, Murai et al. (2003a) ont observé, par immunocytochimie en microscopie confocale, la présence d'EphA4 dans les axones et les terminaisons axonales des fibres moussues. De plus, Grunwald et al. (2004) ont suggéré une localisation présynaptique pour EphA4 sur la base d'un fractionnement subcellulaire montrant la présence de cette protéine dans les fractions dépourvues de densités postsynaptiques aussi bien que dans celles qui en étaient enrichies. L'expression d'ARNm de plusieurs Eph et Efn par les astrocytes protoplasmiques est aussi en faveur de leur localisation dans les petits prolongements astrocytaires périsynaptiques, tel que suggéré pour l'EfnA3 par Murai et al. (2003a). En dehors des synapses, les rôles des Eph et des Efn dans la migration cellulaire et le guidage axonal pendant le développement postnatal (voir 4.2.1.), ainsi que lors de la neurogenèse postnatale et adulte (voir section 4.2.4.), suggèrent d'autres localisations subcellulaires qui devront être examinées.

4.2. Rôles fonctionnels des Eph et des Efn dans l'hippocampe

4.2.1. Rôles des Eph et des Efn dans l'établissement de la circuiterie hippocampique

Des Eph et des Efn participeraient au guidage axonal des projections commissurales et associatives, ainsi que des fibres moussues. En effet, Otal et al. (2006) ont observé, chez des souris knockouts pour l'EfnA5, des terminaisons de la projection commissurale qui envahissent la portion de la couche moléculaire interne recevant normalement celles de la projection associative, ainsi que des terminaisons de la projection associative qui sont anormalement distribuées dans la couche moléculaire externe, laquelle reçoit normalement les afférences du cortex entorhinal. Une analyse en microscopie électronique a aussi mis en lumière une diminution de la densité des terminaisons axonales des projections commissurales et associatives, ainsi que des fibres moussues, dans leurs couches respectives. En outre, l'aire, le périmètre et le nombre de contacts synaptiques des terminaisons des fibres moussues sont significativement diminués.

Quelques études *in vitro* ont aussi impliqué différentes Efn dans la croissance, le branchement et l'élagage des axones hippocampiques, ainsi que l'affaissement de leurs cônes de croissance. En effet, Gao et al. (1999) et Brownlee et al. (2000) ont montré une augmentation de la croissance et de l'embranchement des axones, en plus du nombre de neurites, de neurones hippocampiques de souris E18-19 en culture sur une monocouche de cellules NIH3T3 exprimant l'EfnA2, l'EfnA3 ou l'EfnA5, comparativement à des cultures de cellules non transfectées. Enfin, Dail et al. (2006) et Richter et al. (2007) ont observé un affaissement des cônes de croissance suivant l'application d'EfnA1-Fc ou EfnB1-Fc agglomérées, à des cellules pyramidales de l'hippocampe ou à des cellules ganglionnaires de la rétine de rats embryonnaires. Mentionnons que ces protéines de fusion pourraient notamment activer les voies signalétiques antérogrades en aval des EphA ou EphB auxquels elles se lient (voir section 2.5.).

4.2.2. Rôles des Eph et des Efn dans la morphogenèse des épines dendritiques et la formation des synapses excitatrices

Plusieurs études soutiennent un rôle pour EphB1, EphB2 et EphB3 dans la morphogenèse des épines dendritiques et la formation des synapses excitatrices pendant le développement postnatal, notamment par une interaction directe avec la sous-unité NR1 des récepteurs NMDA qui induit une augmentation de l'influx calcique (Buchert et al., 1999; Ethell et al., 2001; Henkemeyer et al., 2003; Kayser et al., 2006; Kayser et al., 2008; Penzes et al., 2003; Torres et al., 1998). Henkemeyer et al. (2003) ont aussi montré l'absence d'épines dendritiques, la diminution de la densité des récepteurs NMDA et alpha-amino-3-hydroxy-5-méthyl-4-isoxazolépropionique acide (AMPA), ainsi que la diminution du nombre de synapses excitatrices, dans des cultures primaires de neurones hippocampiques de souris triplement knockouts pour EphB1, EphB2 et EphB3, comparativement à des cultures témoins de neurones de souris de type sauvage. Les souris triplement knockouts ont aussi moins d'épines dendritiques, tel qu'observé *in vivo* dans le secteur CA3. De plus, les épines dendritiques restantes sont dépourvues de têtes ou pourvues de petites têtes immatures, en plus de posséder une densité postsynaptique de moindre étendue.

Par ailleurs, Martinez et al. (2005) ont décrit le phénotype des souris knockins qui expriment EphA5-Fc sous le contrôle du promoteur de la GFAP, perturbant présumément les interactions endogènes entre les Efn auxquelles EphA5 peut se lier, soient toutes les EfnA, et les EphA (voir section 2.5.). Ces souris présentent une densité significativement réduite de synapses excitatrices et d'épines dendritiques, dans les couches recevant normalement les terminaisons des projections commissurales, associatives et entorhinales. Les terminaisons des fibres moussues sont aussi plus fréquentes, surtout dans la couche oriens où leur surface, périmètre et nombre de contacts synaptiques sont plus élevés, tout comme la densité des excroissances épineuses recevant ces terminaisons.

4.2.3. Rôles des Eph et des Efn dans la plasticité à long terme des synapses excitatrices

4.2.3.1. Plasticité fonctionnelle

Deux études pionnières ont d'abord suggéré des rôles importants pour les Eph et les Efn dans la plasticité synaptique, la mémoire et l'apprentissage (Gerlai and McNamara, 2000; Gerlai et al., 1999). À la suite de l'infusion chronique *in vivo* d'EphA5-Fc dans l'hippocampe de souris adultes, une réduction significative de leur performance à deux tâches d'apprentissage associées à l'hippocampe est observée. De plus, l'application d'EphA5-Fc diminue la durée de la LTP à la synapse des collatérales de Schaffer, dans le secteur CA1 de tranches d'hippocampe. Au contraire, l'infusion chronique d'EfnA5-Fc améliore la performance comportementale et l'amnésie rétrograde suivant l'anesthésie *in vivo*, en plus d'augmenter la durée de la LTP *in vitro*. Or, l'infusion chronique d'EphA5-Fc peut perturber les interactions endogènes entre les EfnA et n'importe quel EphA, et la signalisation antérograde qui en résulte, alors que celle d'EfnA5-Fc peut activer la signalisation antérograde en aval de tous les EphA et EphB2 (voir section 2.5.). Ces études ne permettent donc pas de préciser quels Eph et Efn sont spécifiquement impliqués dans la LTP, la mémoire et l'apprentissage. Pour ce faire, il sera nécessaire de connaître les Eph et les Efn qui sont localisés au niveau des divers types de synapses de l'hippocampe, ainsi que leurs localisations subcellulaires relatives, dans les épines dendritiques, les terminaisons axonales ou les petits prolongements astrocytaires périsynaptiques.

Quelques études *in vitro* ont aussi suggéré la participation des Eph et des Efn dans la plasticité synaptique à long terme. Grunwald et al. (2004; 2001) ont effectivement montré des déficits dans l'induction de la LTP et de la LTD chez des souris knockouts pour EphA4, l'EfnB2 ou l'EfnB3, au niveau des synapses des collatérales de Schaffer, dans le CA1. En revanche, des souris transgéniques knockins pour une isoforme d'EphA4 dont la portion cytoplasmique était remplacée par la séquence codante de la protéine fluorescente verte (GFP) ne présentaient pas de déficits de LTP ou de LTD; suggérant que ces phénomènes fassent intervenir la signalisation inverse qui résulte des interactions entre EphA4 et les EfnB2 et EfnB3. Cependant, les sites sub-synaptiques (terminaisons axonales, épines dendritiques, petits prolongements astrocytaires périsynaptiques) où ces molécules

exercent cette fonction ne sont pas connus, d'où l'importance de caractériser la localisation subcellulaire précise de chacune de ces molécules au niveau de ces synapses.

En outre, des Eph et des Efn seraient impliqués dans l'induction de la LTP aux synapses des fibres moussues, dans le secteur CA3. En effet, Contractor et al. (2002) ont observé une potentialisation diminuée à la suite de l'infusion d'EphB2-Fc ou d'EfnB1-Fc non agglomérés, mais pas après l'infusion d'EphA7-Fc non agglomeré (voir section 2.5. pour l'effet des protéines de fusion). Des déficits de LTP ont également été observés chez les souris knockins pour une isoforme de l'EfnB3 dont le domaine C-terminal avait été remplacé par la β–galactosidase; indiquant que cette Efn joue un rôle de ligand dans ce phénomène (Armstrong et al., 2006). En conséquence, bien que ces études démontrent un rôle de la signalisation antérograde en aval des interactions entre EphB (ou EphA4) et EfnB dans l'induction de la LTP à la synapse des fibres moussues, la difficulté d'en tirer des conclusions plus précises réitère donc l'importance d'examiner les localisations subcellulaires relatives des Eph et des EfnB à cette synapse, pour mieux comprendre leurs interactions fonctionnelles.

4.2.3.2. Plasticité structurelle

Par ailleurs, il semble que des interactions entre EphA4 et des EfnA ou EfnB puissent réguler la morphologie des épines dendritiques, tel que démontré *in vitro* en culture organotypique de tranches d'hippocampe de jeunes souris (P19 à P21) (Murai et al., 2003a). L'application d'EfnA3-Fc, agglomérée ou non agglomérée, induit notamment une rétraction des épines dendritiques et une diminution du volume de leurs têtes, au site présumé des synapses des collatérales de Schaffer dans le secteur CA1. L'application d'EphA4-Fc perturbe aussi la morphologie des épines dendritiques, réduisant notamment le volume de leurs têtes. De telles irrégularités au niveau des épines dendritiques ont aussi été décelées chez des souris knockouts pour EphA4. L'EfnA3-Fc peut activer EphA4, mais les autres EfnA et EfnB le peuvent aussi, de sorte que ces expériences ne renseignent pas sur les Efn spécifiques impliquées dans cette fonction. Au contraire, la participation d'EphA4 à cette fonction est fort probable, puisque l'application d'EfnA3-Fc chez des souris knockouts pour EphA4 a peu d'effets sur la morphologie des épines dendritiques, tout

comme l'application d'EfnB1-Fc chez des souris de type sauvage, laquelle activerait surtout la signalisation en aval des EphB auxquels elle se lie avec une plus grande affinité (Gale et al., 1996b; Mellitzer et al., 1999; North et al., 2007). En somme, cette étude démontre un rôle important d'EphA4, mais ne précise pas quelles sont les Efn impliquées dans la régulation de la morphologie des épines dendritiques. Pour ce faire, il sera nécessaire d'examiner la localisation subcellulaire relative d'EphA4 et des EfnA ou EfnB particulières, aux synapses des collatérales de Schaffer.

D'autre part, Nestor et al. (2007) ont récemment démontré un rôle similaire des interactions entre EphA4 et EfnA ou EfnB dans le remodelage morphologique des prolongements astrocytaires, en culture organotypique de tranches d'hippocampe de souris ou rats postnatals (P6) visualisées par fluorescence en vidéomicroscopie. L'infusion d'EfnA3-Fc ou de phospholipase-C spécifique aux groupements phosphatidylinositol (PI-PLC), laquelle coupe les liens GPI des EfnA, induit notamment une croissance rapide de filopodes sur des astrocytes témoins, mais pas sur des astrocytes exprimant une forme d'EphA4 dépourvue de domaine catalytique, capable de perturber les interactions endogènes entre EfnA ou EfnB et les Eph auxquels elles se lient normalement (voir section 2.5.), tout autant que d'agir comme une forme dominante-négative et perturber la signalisation des EphA4 endogènes en s'introduisant dans leurs regroupements multimériques (Murai et al., 2003a). Ces données démontrent ainsi l'implication d'EphA4 dans le remodelage astrocytaire. Enfin, par vidéomicroscopie en fluorescence de cultures organotypiques de tranches d'hippocampe de souris (P4-P5), Nishida et Okabe (2007) ont récemment dévoilé des contacts directs entre prolongements astrocytaires et dendritiques, dont la durée est corrélée avec la stabilisation éventuelle des prolongements dendritiques et leur maturation subséquente en épines dendritiques. Puisque l'application d'EphA4-Fc ou d'EfnA3-Fc diminue la durée des contacts ainsi que la tendance des prolongements dendritiques à se stabiliser et former des épines dendritiques, la signalisation entre EphA ou EphB et EfnA ou EfnB semble impliquée (voir section 2.5.). Encore une fois, l'analyse de la localisation subcellulaire relative des Eph (particulièrement d'EphA4) et des Efn est requise pour révéler les Eph et les Efn qui participent spécifiquement dans ces fonctions.

4.2.4. Rôles des Eph et des Efn lors de la neurogenèse chez l'adulte

Quelques études ont mis en lumière un rôle des interactions entre Eph et Efn dans la neurogenèse chez l'adulte. Chumley et al. (2007) ont examiné les cellules nouvellement générées dans la ZSG de l'hippocampe, chez des souris à la fois knockouts pour EphB1 et knockins pour le transgène nestine-GFP. Chez ces souris, le nombre de cellules progénitrices, GFP-positives et marquées au BrdU, est diminué de 50% dans le gyrus dentelé, comparativement à des souris de types sauvages. Des cellules progénitrices, prolifératives (Ki-67-positives) ou granulaires immatures (DCX-positives) sont aussi observées en position ectopique, c'est-à-dire en dehors de la ZSG. Les cellules ectopiques DCX-positives ont souvent une arborisation dendritique basale anormale dans les couches de cellules granulaires et polymorphe. En outre, des souris doublement knockouts pour EphB1 et EphB2 présentent des déficits encore plus prononcés, alors que des souris knockouts pour l'EfnB3 ont des déficits similaires de positionnement cellulaire et de développement dendritique. Ces données suggèrent donc que des interactions entre EphB1 ou EphB2 et l'EfnB3 régulent la migration et le branchement dendritique des cellules progénitrices ou granulaires immatures, lors de la neurogenèse chez l'adulte.

Plus récemment, Jiao et al. (2008) ont démontré une implication des EfnA2 et EfnA3 dans la régulation négative de la prolifération des cellules progénitrices. Ces auteurs ont cultivé des cellules dissociées de régions non-neurogéniques du SNC de souris adultes, incluant le cortex cérébral (sauf la zone sous-ventriculaire; ZSV), la moelle épinière ou le cervelet, avec des astrocytes isolés du cortex de souris néonatales ou adultes, de même que de la ZSV de souris adultes. La formation de neurosphères, soit le regroupement de cellules souche neurales en culture, est cinq à dix fois plus élevée lorsque les astrocytes sont isolés du cortex de souris néonatales ou de la ZSG de souris adultes, comparativement au cortex de souris adultes. De plus, les cellules souches neurales se différencient en neurones, oligodendrocytes et astrocytes, tel que révélé par des marquages immunocytochimiques. Ces observations suggèrent donc que des cellules progénitrices multipotentes soient présentes dans les régions non-neurogéniques du SNC, mais que les astrocytes de ces régions régulent négativement leur prolifération. Or, les astrocytes isolés du cortex (région

non-neurogénique) de souris adultes, mais pas ceux isolés de la ZSG (région neurogénique) de ces mêmes souris, expriment fortement les EfnA2 et EfnA3, tel que notamment révélé par PCR après transcription inverse (RT-PCR) et transfert western. En outre, l'application des protéines de fusion EfnA2-Fc et EfnA3-Fc agglomérées diminue significativement la formation de neurosphères, alors que les souris doublement knockouts pour les EfnA2 et EfnA3 présentaient *in vivo* des cellules prolifératives, identifiées par la colocalisation du BrdU et du marqueur de cellules en division mitotique Ki67, partout dans le cortex cérébral. Ces observations soutiennent ainsi un rôle des EfnA2 et EfnA3 astrocytaires, ainsi que des Eph avec lesquels elles interagissent (possiblement EphA4), dans une régulation négative de la prolifération des cellules progénitrices en dehors des niches neurogéniques chez l'adulte.

5. Hypothèses et objectifs

Les objectifs généraux dans lesquels s'inscrit mon travail de doctorat étaient de caractériser les changements de localisation cellulaire et subcellulaire des Eph et Efn en fonction du stade développemental, de la région du SNC, de l'expérience ou de l'état de santé de l'animal, en vue d'une meilleure compréhension de leurs fonctions potentielles dans la régulation, positive ou négative selon le contexte, des capacités plastiques du SNC. L'objectif spécifique de ce travail de thèse était de vérifier l'hypothèse selon laquelle la localisation subcellulaire d'EphA4 évoluerait en fonction du stade de maturation des cellules neuronales ou gliales qui l'expriment, dans l'hippocampe du rat néonatal, juvénile et adulte, afin de mieux comprendre ses rôles fonctionnels pendant le développement postnatal et chez l'adulte (incluant lors de la neurogenèse).

Nous avons choisi d'examiner EphA4, en particulier, pour ses propriétés uniques qui le rendent très intéressant. Il s'agit notamment du seul Eph capable d'interagir avec les Efn des deux sous-classes. Par ses interactions avec de nombreuses EfnA ou EfnB, EphA4 participe ainsi aux principales fonctions attribuées aux Eph pendant le développement embryonnaire et postnatal, ainsi que chez l'adulte. Par cette particularité, il serait similaire au Eph ancestral des chordés primitifs, tel que récemment suggéré par une étude de phylogénétique moléculaire (Mellott and Burke, 2008). Par ailleurs, l'hippocampe constitue

un excellent modèle pour examiner l'évolution de la localisation et de la fonction d'EphA4 de la naissance jusqu'à l'âge adulte. En effet, EphA4 y est fortement exprimé dès la migration des cellules principales chez l'embryon, jusque chez l'adulte. Puisque l'organisation laminaire et l'ultrastructure de l'hippocampe sont bien documentées, ce modèle facilite aussi l'identification des structures EphA4-positives ainsi que de leur appartenance aux différents types cellulaires, par exemple les cellules granulaires et moussues du gyrus dentelé, les cellules pyramidales du CA3 ou du CA1, les interneurones, les astrocytes protoplasmiques ou les autres types de cellules gliales, ainsi qu'aux projections excitatrices, les projections commissurale et associative, la voie perforante, les fibres moussues ou les collatérales de Schaffer, ou bien inhibitrices, entre interneurones et cellules granulaires, moussues ou pyramidales.

Localization of EphA4 in Axon terminals and Dendritic Spines of Adult Rat Hippocampus

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Localization of EphA4 in Axon terminals and Dendritic Spines of Adult Rat Hippocampus

Marie-Ève Tremblay[1], Mustapha Riad[1], David Bouvier[1], Keith K. Murai[2], Elena B. Pasquale[3, 4], Laurent Descarries[1] and Guy Doucet[1*]

[1] Département de pathologie et biologie cellulaire and Groupe de recherche sur le système nerveux central, Université de Montréal, Montréal, Québec, Canada H3C 3J7

[2] Centre for Research in Neuroscience, Department of Neurology and Neurosurgery, McGill University Health Centre, Montreal General Hospital, Montréal, Québec, Canada H3G 1A4

[3] The Burnham Institute for Medical Research, La Jolla, California 92037

[4] Pathology Department, University of California, San Diego, La Jolla, California 92037

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*Correspondence to: E-mail: [redacted], Telephone: (514) 343-6255, Fax: (514) 343-5755.

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ABSTRACT

Eph receptors and their ephrin ligands assume various roles during central nervous system development. Several of these proteins are also expressed in the mature brain, and notably in the hippocampus, where EphA4 and ephrins have been shown to influence dendritic spine morphology and long-term potentiation (LTP). To examine the cellular and subcellular localization of EphA4 in adult rat ventral hippocampus, we used light and electron microscopic immunocytochemistry with a specific polyclonal antibody against EphA4. After immunoperoxidase labeling, EphA4 immunoreactivity was found to be enriched in the neuropil layers of CA1, CA3 and dentate gyrus. In all examined layers of these regions, myelinated axons, small astrocytic leaflets, unmyelinated axons, dendritic spines and axon terminals were immunolabeled in increasing order of frequency. Neuronal cell bodies and dendritic branches were immunonegative. EphA4-labeled dendritic spines and axon terminals corresponded to 9-19% and 25-40% of the total number of spines and axon terminals, respectively. Most labeled spines were innervated by unlabeled terminals, but synaptic contacts between two labeled elements were seen. The vast majority of synaptic junctions made by labeled elements was asymmetrical and displayed features of excitatory synapses. Immunogold labeling of EphA4 was mostly located on the plasma membrane of axons, dendritic spines and axon terminals, supporting its availability for surface interactions with ephrins. The dual preferential labeling of EphA4 on pre- or postsynaptic specializations of excitatory synapses in adult rat hippocampus is consistent with roles for this receptor in synaptic plasticity and LTP.

INTRODUCTION

Ephrins and Eph tyrosine kinase receptors are membrane proteins playing key roles during central nervous system (CNS) development (reviewed in Wilkinson, 2001; Palmer and Klein, 2003; Klein, 2004). In mammals, these proteins, comprising eight ligands and 14 receptors, are grouped into subclasses A and B, according to their sequence homology and reciprocal binding affinities. Ephrin-A ligands are attached to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor, and ephrin-Bs are transmembrane proteins. EphA receptors (EphA1-8, and -10) bind the ephrin-As (ephrin-A1-5), and EphB receptors (EphB1-4, and -6) the ephrin-Bs (ephrin-B1-3). EphA4, which can bind ephrin-B2 and -B3 in addition to ephrin-As (Gale et al., 1996a; b), and EphB2 which recognizes ephrin-A5 in addition to ephrin-Bs (Himanen et al., 2004), are exceptions to this subclass specificity (reviewed in Flanagan and Vanderhaeghen, 1998). The cell surface localization of both ephrins and Eph receptors enables these proteins to signal bidirectionally, at sites of cell-to-cell contact such as synapses.

During CNS development, ephrin-Eph interactions exert both repulsive and attractive/adhesive activities in the establishment of segmental boundaries in somites and rhombomeres, the maintenance and stabilization of tissue patterns, the regulation of cell migration, the closure of the neural tube, the regulation of axon guidance and remodeling, and the formation of topographic maps, including the retino-tectal, retino-thalamic, hippocampo-septal, thalamo-cortical and olfactory projections (see Palmer and Klein, 2003; Klein, 2004).

In line with their ability to partake in many activities during CNS development, Eph receptors and ephrins have been implicated in neurogenesis in adult CNS (Holmberg et al., 2005; Ricard et al., 2006), as well as in synaptogenesis, synaptic plasticity, memory and learning in maturing and adult CNS, notably in the hippocampus (for review see Murai and Pasquale, 2004; Yamaguchi and Pasquale, 2004; Martinez and Soriano, 2005). Understanding the role of each of these molecules in any of these processes would greatly

benefit from detailed analyses of their discrete subcellular localization within the hippocampal circuitry. For example, postsynaptic EphA4 has been implicated in the regulation of dendritic spine morphology in hippocampus, possibly through interactions with ephrin-A3, expressed in astrocytes (Murai et al., 2003). A presynaptic role of EphA4 was also suggested in CA1 long-term potentiation (LTP), through interactions with ephrin-Bs (Grunwald et al., 2004).

To date, there has been only one electron microscopic immunocytochemical study of EphA4 localization in the adult CNS. Using a polyclonal antibody raised against the 11 carboxy-terminal amino acids of EphA4 (Soans et al., 1994), Martone et al. (1997) have mapped the distribution of EphA4 in the adult rat brain and spinal cord. At the light microscopic level, these authors described intense immunoreactivity, notably in the cerebral cortex and hippocampus, as well as in several white matter tracts. At the electron microscopic level, EphA4 immunoreactivity was detected in dendritic spines of the hippocampus, and in myelinated axons (in the fornix and spinal cord).

In the present study, we used a purified EphA4 polyclonal antibody raised against the same carboxyterminal peptide, to further investigate the regional, layer-specific, and subcellular distribution of EphA4 in CA1, CA3 and dentate gyrus (DG) of adult rat ventral hippocampus. The results provide insights into how EphA4 may play a multifunctional role in the hippocampus through its localization on distinct subcellular elements that are known to undergo plasticity in the adult CNS.

MATERIALS AND METHODS

Animals

This study was carried out on hippocampal tissue from five adult male Sprague-Dawley rats (250 ± 25 g; Charles River, St-Constant, Québec, Canada). Two EphA4 KO mice (EphA4 $^{-/-}$) and two wild-type littermates (EphA4 $^{+/+}$) provided by Mirella Dottori (Center for Reproduction and Development, Monash Institute of Medical Research,

Clayton, Australia; Dottori et al., 1998) were also used as controls, to assess the specificity of several anti-EphA4 antibodies from various sources. The animals were housed at constant temperature (21°C) and humidity (60%), under a fixed 12 h light/dark cycle and with free access to food and water. All procedures were conducted in strict accordance with the *Guide to the Care and Use of Experimental Animals* (Ed2) of the Canadian Council on Animal Care. The experimental protocols were approved by the Animal Care Committee of the Université de Montréal.

Antibodies

The production and characterization of the anti-EphA4 antibody (Ab11) raised in rabbit against the last 11 carboxyterminal amino acids of EphA4, which are identical in rat, mouse, chicken, and human EphA4, have been fully described previously (Soans et al., 1994). This antibody has been shown to recognize EphA4 in rat, mouse, and human (Soans et al., 1994; Martone et al., 1997; Murai et al., 2003). Commercial anti-EphA4 antibodies [goat anti-mouse EphA4 extracellular domain (R&D, Mineapolis, MN), AF641; rabbit anti-human EphA4 near C-terminal region (Santa Cruz, Santa Cruz, CA), sc-921; rabbit anti-human EphA4 C-terminal region (Zymed, South San Francisco, CA), 34-7900] were also tested.

Immunocytochemical specificity controls included omission of the primary antibody and pre-adsorption of the primary antibody with blocking peptide immunogen, for rat brain sections, as well as processing of sections from EphA4 $^{-/-}$ and EphA4 $^{+/+}$ mice. Of all tested anti-EphA4 antibodies, only the Ab11 was specific, without staining in EphA4 $^{-/-}$ mice. The other antibodies were not used further in our analysis.

Tissue preparation

The animals were deeply anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and perfused through the aortic arch with a solution of 3.5% acrolein, followed by 4.0% paraformaldehyde (PFA), both in 0.1 M sodium phosphate buffer (PB), pH 7.4. The brain

was removed, further fixed in 4% PFA for 1 hour at 4°C, and washed in sodium phosphate-buffered saline (PBS; 0.9% NaCl in 50 mM PB, pH 7.4). Transverse sections, 50 µm thick, were cut in cooled PBS with a vibratome, immersed in 0.1% sodium borohydride in PBS for 30 min at room temperature (RT), and washed in PBS before immunocytochemical processing.

Immunocytochemistry

The sections were processed free-floating for immunocytochemistry, following a pre-embedding immunoperoxidase protocol previously described in detail (Riad et al., 2000; 2001). Briefly, sections were rinsed in PBS, followed by a 1 hour pre-incubation at RT in a blocking solution of PBS containing 5% normal goat serum and 0.5% gelatin. Sections were then incubated for 48 hours at RT in 1 µg/ml of the rabbit anti-EphA4 antibody diluted in blocking solution, and rinsed in PBS. After incubation for 2 hours at RT in goat anti-rabbit IgGs conjugated to biotin (Jackson Immunoresearch, West Grove, PA), and with streptavidine-horseradish-peroxidase (Jackson Immunoresearch) for 1 hour in blocking solution. Labeling was revealed with 0.05% diaminobenzidine (DAB) and 0.01% hydrogen peroxide in Tris/HCl-buffered saline (50 mM, pH 7.4). For immunogold labeling, sections were incubated overnight at room temperature in goat anti-rabbit IgGs conjugated to 1 nm colloidal gold particles (AuroProbe One, Amersham Biosciences, Oakville, Ontario, Canada) in blocking solution. The diameter of the immunogold particles was increased using a silver enhancement kit (IntenSE, Amersham Biosciences) for 12 to 15 minutes at RT.

Light and electron microscopy

Sections intended for light microscopy were mounted on microscope slides, dehydrated in increasing concentrations of ethanol, cleared in toluene, and coverslipped with DPX (Fluka, Sigma-Aldrich, Oakville, ON).

Sections intended for electron microscopy were rinsed in PB, postfixed flat in 1% osmium tetroxide for 30 minutes, and dehydrated in ascending concentrations of ethanol. They were then treated with propylene oxide, impregnated with resin overnight (Durcupan ACM; Sigma), mounted on aclar embedding film (EMS, Hatfield, PA), and cured at 55°C for 48 hours. Areas of interest (CA1, CA3 and DG) from ventral hippocampal formation, at a level approximating transverse planes A + 3.48 to A + 3.84 in the Stereotaxic Atlas of Paxinos and Watson (2005), were excised from the slides. After re-embedding at the tip of resin blocks, ultrathin (70-80 nm) sections were cut with an ultramicrotome (Reichart Ultracut S, Leica Canada, St- Laurent, QC), collected on bare square-mesh copper grids, stained with lead citrate, and examined at 60 kV with a Philips CM100 electron microscope.

Data analysis

EphA4-DAB-immunoperoxidase-labeled sections derived from the ventral hippocampal formation from three rats, and EphA4 $^{-/-}$ and EphA4 $^{+/+}$ mice were examined by light microscopy (Leitz Diaplan). Photomicrographs were taken with a SpotRT color digital camera, using the Spot 4.0.5 software for Windows (Diagnostic Instruments, Sterling Heights, MI). Images were adjusted for brightness and contrast with the Adobe Photoshop (CS) software.

Electron microscopic examination was carried out on material from five rats after DAB-immunoperoxidase (n=3), or immunogold labeling (n=2), as well as from the EphA4 $^{-/-}$ and EphA4 $^{+/+}$ mice processed in parallel, under the same conditions. Data was collected from three layers in each of CA1 (stratum oriens, pyramidal cell layer and stratum radiatum), CA3 (stratum oriens, pyramidal cell layer and strata lucidum/radiatum), and DG (molecular, granule cell and polymorphic layers; nomenclature by Witter and Amaral, 2004). Cellular profiles were identified according to criteria defined by Peters et al. (1991). Small processes of protoplasmic astrocytes were recognized by their irregular and angular shapes, which distinguished them from the characteristic smooth or rounded shape of

neuronal profiles. Unmyelinated axons were identified as such, rather than small dendritic branches, when found within fascicles of similar profiles. Dendritic spines displayed their characteristic fluffy content, with or without a spiny apparatus, and frequent synaptic contacts with axon terminals. Small dendritic branches contained microtubules, neurofilaments and occasional mitochondria. Axon terminals or varicosities corresponded to enlarged portions of axons containing aggregated synaptic vesicles, often containing one or more mitochondria or showing a junctional synaptic specialization.

For immunogold labeling, only dendritic spines, axon terminals or astrocytic processes decorated by two or more silver-intensified gold particles were considered labeled. Nevertheless, given that very few particles were found in structures that were unlabeled by immunoperoxidase (cell bodies, dendrites, blood vessels), small unmyelinated axons found in fascicles were also considered labeled when decorated by a single particle, because individual axon fascicles usually contained several such unmyelinated axons labeled with a single particle.

The frequency of EphA4-labeled dendritic spines and axon terminals was determined in the immunoperoxidase-stained material from three rats. Twenty micrographs per rat were captured in every examined layer at a magnification of X7,900. The film negatives were scanned (Epson Perfection 3200) and converted into positive pictures at a final magnification of X28,500. Dendritic spines (n=4,198) and axon terminals (n=7,403), labeled and unlabeled, were counted in all pictures, for a total tissue area of 900 μm^2 per layer/hippocampal region/animal. The total neuropil area was calculated (NIH image) in every layer, after exclusion of the areas covered by perikarya or blood vessels, and the results were expressed in number of immunostained profiles per 10^3 μm^2 of neuropil. Labeling indices were also calculated for dendritic spines and axon terminals, in percentage of labeled profiles over the total number of corresponding profiles. The morphology (symmetric vs. asymmetric) of junctional complexes including labeled pre- or postsynaptic element(s) was also determined. Only synaptic profiles presenting an unequivocal postsynaptic density were considered as asymmetric. Presynaptic partners (unlabeled or

labeled axon terminals) of labeled dendritic spines, as well as postsynaptic partners (unlabeled or labeled dendritic spines or dendrites) of labeled axon terminals were also counted, and the results expressed as percentages of the total number of labeled dendritic spines or axon terminals presenting a synapse.

RESULTS

Specificity of EphA4 immunolabeling

The specificity of the EphA4 immunolabeling with Ab11 was unequivocal. As shown in Figure 1, the laminar distribution of the DAB labeling in ventral hippocampus was very similar in rats and wild-type mice and there was no immunostaining in light microscopic sections from EphA4 $^{-/-}$ mice. In contrast, all commercial antibodies tested stained the hippocampal cell body layers in rat sections and those derived from wild-type and EphA4 $^{-/-}$ mice; although pre-adsorption of some of the latter antibodies with the respective immunizing EphA4 peptides was able to abolish the labeling.

Laminar distribution

Following immunoperoxidase/DAB labeling, all cell poor (neuropil) layers of rat and mouse ventral hippocampus displayed relatively strong EphA4 immunoreactivity, except the stratum lacunosum-moleculare of CA1 and CA3, which showed weaker intensity labeling (Fig. 1A,B). In CA1, the densest labeling was that of strata oriens and radiatum, and in CA3, that of stratum lucidum. In the DG, both the molecular and polymorphic layers were immunopositive, with denser labeling of the inner, compared to outer part of the molecular layer. The main cellular layers, containing the perikarya of pyramidal and granule cells, appeared very weakly labeled (Fig. 1A,B). The intensity of the EphA4 immunoreaction was generally stronger in rat than mouse. A similar laminar distribution of EphA4 at the light microscopic level was previously reported for adult mouse and rhesus monkey (Martone et al., 1997; Murai et al., 2003; Xiao et al., 2006).

Cellular distribution

EphA4 immunoreactivity was found on multiple structural elements within various regions of the hippocampus. Immunoperoxidase/DAB-labeled profiles of myelinated axons, small astrocytic processes, unmyelinated axons, dendritic spines and axon terminals (Figs. 2-5) were found in this increasing order of frequency in every layer of CA1, CA3, and DG examined by electron microscopy. Neuronal cell bodies and dendritic branches, even of small caliber, were immunonegative. The few immunopositive myelinated axons (Fig. 2C) were seen mostly in stratum lucidum, pyramidal cell layer and stratum radiatum of CA3, and the polymorphic layer of DG. Astrocytic leaflets, often perisynaptic, showed immunoprecipitate in their cytoplasm and beneath their plasma membrane (Fig. 2B), whereas astrocytic cell bodies were always immunonegative. EphA4-positive unmyelinated axons were found within fascicles, intermingled with immunonegative axons, mostly in the pyramidal cell layers of CA1 and CA3.

The subcellular profiles of highest immunoreactivity included dendritic spines and axon terminals. In dendritic spines, the peroxidase reaction product always stained the postsynaptic density, and generally extended to the spiny apparatus (Fig. 3). In axon terminals, the labeling always concerned only a subset of the varicosities (Fig. 4). The labeling was associated with the membranes of medium to large spherical synaptic vesicles (Fig. 4A,D), usually found away from (Fig. 4A), and only sometimes near (Fig. 4B), synaptic junctions. The presynaptic active zone was also occasionally labeled (Fig. 4B,C).

Labeled dendritic spines were most often contacted by an unlabeled axon terminal (Fig. 3A). In some cases, two dendritic spines, one labeled and one unlabeled, were simultaneously contacted by the same unlabeled terminal (Fig. 3B). On rare occasions, both pre- and postsynaptic partners were immunopositive (Fig. 3C). In the polymorphic layer of DG, immunolabeled pedunculated spines, which are contacted by mossy fiber terminals (Witter and Amaral, 2004), were seen (Fig. 3D). In the stratum lucidum of CA3 (Fig. 4D) as well as in polymorphic layer of DG, labeled mossy fiber terminals, typically containing

densely packed vesicles, were also observed in multiple synaptic contacts with dendritic spines.

Because the peroxidase/DAB product is known to diffuse to the nearest membranous material, we performed silver-intensified immunogold labeling to reveal further the precise subcellular distribution of EphA4. We then found immunogold labeling with an obvious predilection for the plasma membrane of small unmyelinated axons (Fig. 5A), dendritic spines (Fig. 5B), axon terminals (Fig. 5B,C), and astrocytic processes (not shown). In dendritic spines and axon terminals, the immunogold particles were never found directly on the synaptic junction (Fig. 5 B,C), but this might be due to the poor penetration of colloidal gold-labeled antibodies.

Quantified distribution of EphA4-labeled dendritic spines and axon terminals

Immunolabeled dendritic spines and axon terminals were more numerous in CA1 than CA3 and DG and much more frequent in the neuropil rather than cell body layers (Fig. 6A,D). As counted in number of sectional profiles per 10^3 μm^2 of neuropil, labeled dendritic spines were found, with increasing frequency, in the polymorphic layer of DG (17), strata lucidum/radiatum (23) and oriens (26) of CA3, molecular layer of DG (33), and strata oriens (36) and radiatum (49) of CA1 (Fig. 6A,B). In the cellular layers, they were rare in the granule cell layer of DG (2) and pyramidal cell layer of CA3 (4), but more frequent in the pyramidal cell layer of CA1 (23). Overall, the proportion of dendritic spines that were labeled relative to total (labeled+unlabeled) varied between 9-19% (average of 13%), with the highest values in the stratum radiatum and the pyramidal cell layer of CA1. The vast majority (89%) of labeled dendritic spine profiles was seen in synaptic contact with axon terminals. As shown in Table 1A, on average, 78% of the synaptic labeled spines were contacted by unlabeled and 22% by labeled axon terminals. Thus, fewer labeled axon terminals were seen in synaptic contact with labeled spines than expected from their relative proportion in tissue (36%).

Labeled axon terminals were much more numerous, and more uniformly distributed between regions and layers (Fig. 6C,D). Similar to the labeling of spines, EphA4 on terminals was more frequent in the neuropil rather than cell body layers (119-163 as opposed to 32-83 per 10^3 μm^2). Their relative proportion was rather similar among regions and layers (33-40% in cell poor vs. 25-30% in cellular layers), averaging 36% throughout.

About half (52%) of the labeled axon terminal profiles were seen in synaptic contact with unlabeled spines, unlabeled dendrites, or labeled spines, in decreasing order of frequency (Table 1B). Visualization of such a high proportion of synaptic contacts in single thin sections is indicative of one or more synaptic contacts per labeled terminal (Umbriaco et al., 1995). Labeled terminals in synaptic contact with unlabeled spines were numerous in all examined layers (34-68 per 10^3 μm^2), except in the cellular layers of CA3 and DG (7 in the pyramidal, and 6 in the granule cell layers). Unlabeled spines represented 62 to 83% of the synaptic targets of labeled terminals, except in the pyramidal cell layer of CA3 (40%) and granule cell layer of DG (30%). In these layers, containing mostly neuronal cell bodies, unlabeled dendrites were the most frequent synaptic targets for labeled terminals (60% and 70% respectively). In contrast, only 12-23% of synaptic labeled terminals had unlabeled dendrites as synaptic target in the other examined layers. Overall, labeled spines represented 8% of all synaptic targets and 10% of the spine targets of labeled terminals, a figure near the calculated spine labeling index (13%) for the whole hippocampus.

Among the synaptic contacts with one or both labeled partners, a vast majority in all layers (86-95%) could be classified as asymmetrical in single thin sections (see Figs. 3A,C and 4A,B and D). Labeled terminals also displayed other features of excitatory terminals (Peters et al., 1991), such as their spherical synaptic vesicles and their large proportion (80%) of dendritic spines as targets (Matsuda et al., 2004). Neuronal perikarya and proximal dendrites, which receive most inhibitory synapses, were never seen as synaptic targets.

DISCUSSION

Axon terminals and dendritic spines were the most frequent sites of EphA4 receptor immunoreactivity, throughout adult rat ventral hippocampus. EphA4 was also detected in a small percentage of myelinated and unmyelinated axons, as well as in astrocytic processes, but not in neuronal cell bodies and dendritic branches, nor in glial somata. The frequent localization of EphA4 receptor on either side of excitatory synaptic junctions, as well as its preferential distribution on the plasma membrane of both axon terminals and dendritic spines, provided further support for its implication in various hippocampal functions through surface interactions with different ephrins.

Transport of the EphA4 protein in axons and dendrites?

The presence of EphA4 immunoreactivity in myelinated as well as unmyelinated axons in addition to axon terminals was consistent with an axonal transport of EphA4 receptor from its site of synthesis, in neuronal cell bodies, to axon terminals. However, the plasma membrane localization of the immunogold labeling of unmyelinated axons also favoured a local role along these processes. EphA4 might indeed be implicated in the regulation of axon fasciculation, as already suggested for peripheral axons during their sorting and correct dorso-ventral distribution in nerve branches of peripheral nerve plexuses (Eberhart et al., 2000).

EphA4 was frequently found in dendritic spines, but not in dendritic shafts/branches, even though pyramidal as well as granule cells in rodent hippocampus have been shown to express EphA4 by in situ hybridization (Liebl et al., 2003, see also www.genepaint.org). EphA4 might be transported within dendrites at a concentration too low for immunodetection under the present stringent conditions of tissue fixation, devised for compatibility of immunodetection with adequate ultrastructural preservation. An alternative is that of a local synthesis of the protein in dendritic spines following dendritic transport of its mRNA (Duchaine et al., 2002; Dugre-Brisson et al., 2005; Goetze et al., 2006).

Glial processing of EphA4 following neuronal-glial interaction

The small number of astrocytic processes displaying EphA4 immunoreactivity often ensheathed synapses, whereas immunolabeled astrocytic perikarya or proximal processes remained immunonegative. Immunodetection or expression of EphA4 in astrocytes has already been reported in the intact or injured spinal cord (Goldshmit et al., 2004; Fabes et al., 2006) and in the retina (Petros et al., 2006). The selective localization of EphA4 in distal processes of hippocampal astrocytes might result from trans-endocytosis as a complex with ephrin ligands expressed in astrocytes (Marston et al., 2003; Zimmer et al., 2003; Cowan et al., 2005). Murai et al. (2003) have indeed detected ephrin-A3 in perisynaptic astrocytes of adult mouse hippocampal slices, and suggested its interaction with EphA4 receptor localized in dendritic spines. Other ephrin ligands might also be involved, as expression of ephrins in astrocytes has been reported to follow spinal cord injury or transection of entorhinal afferents (Bundesen et al., 2003; Wang et al., 2005).

EphA4 in the hippocampal circuitry

As previously reported, EphA4 was located in dendritic spines (Martone et al., 1997; Murai et al., 2003). However, we found that this receptor is more prominent in axon terminals, which had not been shown before. On confocal microscopy, EphA4 was not found to co-localize with synaptophysin (Murai et al., 2003). Nevertheless, at the electron microscopic level, the DAB immunoprecipitate generally concerned only subpopulations of the vesicles in labeled axon terminals, which may represent transport rather than synaptic vesicles. This may explain why EphA4 and synaptophysin immunofluorescence rarely overlapped.

The laminar distribution of EphA4-labeled dendritic spines and axon terminals provided clues as to the respective anatomical origin and transmitter identity of both these elements. The labeled spines most likely belonged to pyramidal cells in CA1 and CA3 and to granule and mossy cells in DG, which have previously been shown to express EphA4 receptor (Liebl et al., 2003, see also www.genepaint.org).

The commissural/associative axons arising from the contra- and ipsilateral CA3 and DG could represent a major source of EphA4-labeled axon terminals. Indeed, together with the perforant path axons from the entorhinal cortex, these axons constitute the main excitatory inputs of the hippocampal formation. Even though both these inputs give rise to asymmetrical synaptic terminals (Nafstad, 1967; Gottlieb and Cowan, 1972; Witter et al., 1992; Desmond et al., 1994), they are distributed in a complementary fashion in the hippocampal layers (see Witter and Amaral, 2004). The commissural/associative axons terminate on pyramidal cell dendritic spines in stratum oriens, pyramidal cell layer and stratum radiatum of CA1 and CA3, in addition to granule cells in the inner part of the DG molecular layer, which are all layers displaying intense EphA4 immunoreactivity. The perforant path axons innervate the stratum lacunosum-moleculare of CA1 and CA3, as well as the outer part of the DG molecular layer, which are only faintly labeled for EphA4.

Ephrin-A5 has recently been shown to modulate the topographic mapping and connectivity of commissural axons in mouse hippocampus (Otal et al., 2006). Tracing experiments in ephrin-A5 $^{-/-}$ mice have revealed a mistargeting of commissural projections. Alterations in the density of synapses and dendritic spines in commissural/associative layers, but not in entorhinal layers, were observed via electron microscopy. In view of its abundance in the commissural/associative layers, EphA4 receptor could well participate in these processes.

In the stratum lucidum of CA3 and the polymorphic layer of DG, there were mossy fiber terminals exhibiting EphA4 immunoreactivity. Mossy fibers originate from granule cells and terminate on the very large and complex spines, called *thorny excrescences*, of the CA3 pyramidal cells and DG mossy cells (see Witter and Amaral, 2004). Schaffer collaterals from CA3 pyramidal cells similarly terminate on the dendritic spines of pyramidal cells, in strata oriens and radiatum of CA1, and probably accounted for labeled terminals in CA1, even if they could not be positively identified as such because of their less typical morphology.

Distinct roles for EphA4 depending on its cellular location

EphA4 was thus detected in dendritic spines as well as axon terminals of excitatory synapses, but generally in only one of the two synaptic partners. The proportion of synapses with both pre- and postsynaptic partners containing EphA4 might be higher than detected (5-38%), considering that the present observations were made in tissue prepared for electron microscopy. Indeed, if a proportion of the processes containing EphA4 went undetected in these conditions, the probability of detecting the protein in both partners of a given synapse would be decreased. In any case, the dual localization of EphA4 in axon terminals and dendritic spines throughout hippocampus suggests an involvement of this receptor in various functions dependent on synaptic plasticity, such as learning and LTP, in addition to the morphological shaping of dendritic spines and formation of synapses.

The evidence for an involvement of EphA4 receptor in learning remains indirect. Learning has been shown to be disrupted or improved, respectively, by *in vivo* infusion into adult rat hippocampus of EphA5-Fc or ephrin-A5-Fc (extracellular domain of the receptor or ligand fused to the Fc fragment of human IgG; Gerlai et al., 1998; Gerlai et al., 1999; Gerlai and McNamara, 2000). Both these immunoadhesins are likely to interfere with or to activate EphA4 signaling, since EphA5-Fc and ephrin-A5-Fc are not specific for single ephrins or Eph receptors.

EphA4 might be argued to participate in mossy fiber synapse LTP in CA3. Mossy fiber synapse LTP is known to involve presynaptic mechanisms (see Nicoll and Schmitz, 2005), and intracellular injection of peptides or antibodies blocking specifically the PDZ-binding domain of EphB2, into CA3 pyramidal cells, as well as extracellular perfusion of EphB2-Fc or of ephrinB1-Fc, have been shown to interfere with LTP in neonate hippocampal slices (Contractor et al., 2002), suggesting that postsynaptic EphB2 was signaling in reverse through ephrin-Bs expressed presynaptically. Similar defects in LTP were also observed in mice expressing ephrin-B3 deleted of its intracellular domain, while ephrin-B3 could be immunolocalized to mossy fibers by light microscopy (Armstrong et

al., 2006). Many of these observations could be interpreted as involving also EphA4, since it has a high affinity for ephrin-B2 and -B3 (see Flanagan and Vanderhaeghen, 1998), and is present on both sides of mossy fiber synapses.

Recent studies have demonstrated that EphA4 ligands, ephrin-B2 and -B3, regulate LTP in the Schaffer collateral to CA1 synapses (Grunwald et al., 2001; 2004). The profound deficits in CA1 LTP disclosed in ephrin-B2 $^{-/-}$ and -B3 $^{-/-}$ mice were similar to those observed in EphA4 $^{-/-}$ mice, whereas LTP defects in EphB2 $^{-/-}$ mice were only mild, and restricted to the late phase of LTP. Ephrin-B2 was also immunodetected by electron microscopy at postsynaptic densities of CA1 (Grunwald et al., 2004). Grunwald et al. concluded that LTP depended on the activation of postsynaptic ephrin-Bs by presynaptic EphA4, and the existence of EphA4 in presynaptic Schaffer terminals was hypothesized (Grunwald et al., 2004). The present observations demonstrate this presynaptic localization of EphA4. On the other hand, EphA4 being also present on the postsynaptic side of these synapses, must there be interacting with other ephrins expressed either in presynaptic terminals or in astrocytes. Interactions in *cis* are also possible, and it will be interesting to know whether coexpressed ephrins and Eph receptors are segregated into distinct lipid raft microdomains (Marquardt et al., 2005; Carvalho et al., 2006).

EphA4 and EphB2 appear to function in parallel also in regulating dendritic spine morphology. Indeed, activation of EphA4, possibly by astrocytic ephrin-A3, in the CA1 region of hippocampal slices from P21 mice, was shown to regulate dendritic spine morphology (Murai et al., 2003). Deficient retraction of filopodia, or distortion of spine shape and organization, was disclosed in slices from EphA4 $^{-/-}$ mice or in slices transfected with a kinase-inactive EphA4. Experiments on dissociated hippocampal neurons have also suggested a role for EphB2, via syndecan-2 phosphorylation, in the regulation of spine maturation (Ethell et al., 2001). In such neurons expressing a kinase-dead EphB2, syndecan-2 was not phosphorylated, and dendritic spine maturation was compromised.

Conclusions

The present immunocytochemical results indicate that EphA4 is strategically located to play several roles in adult rat hippocampus, through effects on synaptic plasticity primarily exerted at excitatory synapses. These various roles may require different combinations of ligands expressed on distinct subcellular compartments (i.e. presynaptic, postsynaptic, or glial-localized ephrins) to modify synaptic function. Further insight into these functions will be obtained from dual-labeling experiments with other Eph receptors and ephrin-A or -B ligands, in order to determine the extent of their colocalization or their potential intercellular relationships with EphA4, depending on its pre- or postsynaptic localization.

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Table.

A

Labelled spines

		Synaptic partners	
		Unlabelled terminals	Labelled terminals
CA1	o	29 ± 13 (84 %)	4 ± 1 (16 %)
	p	14 ± 4 (86 %)	2 ± 2 (14 %)
	r	34 ± 8 (74 %)	17 ± 2 (26 %)
CA3	o	22 ± 5 (87 %)	4 ± 2 (13 %)
	p	5 ± 5 (100 %)	0
	l/r	16 ± 3 (71 %)	7 ± 5 (29 %)
DG	m	26 ± 8 (80 %)	7 ± 3 (20 %)
	g	7 ± 2 (100 %)	0
	po	8 ± 7 (65 %)	5 ± 3 (35 %)

B

Labelled terminals

		Synaptic partners		
		Unlabelled spines	Unlabelled dendrites	Labelled spines
CA1	o	64 ± 24 (78 %)	10 ± 5 (17 %)	4 ± 1 (5 %)
	p	34 ± 11 (81 %)	5 ± 1 (12 %)	2 ± 2 (5 %)
	r	57 ± 22 (66 %)	12 ± 5 (20 %)	17 ± 2 (14 %)
CA3	o	56 ± 31 (78 %)	8 ± 3 (16 %)	4 ± 2 (16 %)
	p	7 ± 4 (42 %)	11 ± 5 (58 %)	0
	l/r	68 ± 31 (75 %)	12 ± 7 (18 %)	7 ± 5 (7 %)
DG	m	64 ± 21 (68 %)	21 ± 6 (21 %)	7 ± 3 (7 %)
	g	6 ± 6 (18 %)	14 ± 8 (82 %)	0
	po	34 ± 24 (47 %)	17 ± 2 (40 %)	5 ± 3 (8 %)

Table 1. Synaptic partners of EphA4-labeled dendritic spines and axon terminals

Figures

Figure 1. Low magnification photomicrographs of EphA4-immunolabeled sections of the hippocampus from adult rat (**A**), wild-type mouse (**B**) and EphA4 $^{-/-}$ mouse (**C**). Note the absence of immunostaining on the section from the EphA4 $^{-/-}$ mouse, demonstrating the specificity of the anti-EphA4 antibody (Ab11). Cell poor layers containing the pyramidal and granule cell processes are strongly labeled, whereas cell body layers are very weakly labeled. Stratum oriens (o), pyramidal cell layer (p), and stratum radiatum (r) of CA1; stratum oriens (o), pyramidal cell layer (p) and strata lucidum/radiatum (l/r) of CA3; and molecular (m), granule cell (g) and polymorphic (po) layers of DG. DG, dentate gyrus. Scale bars = 500 μm.

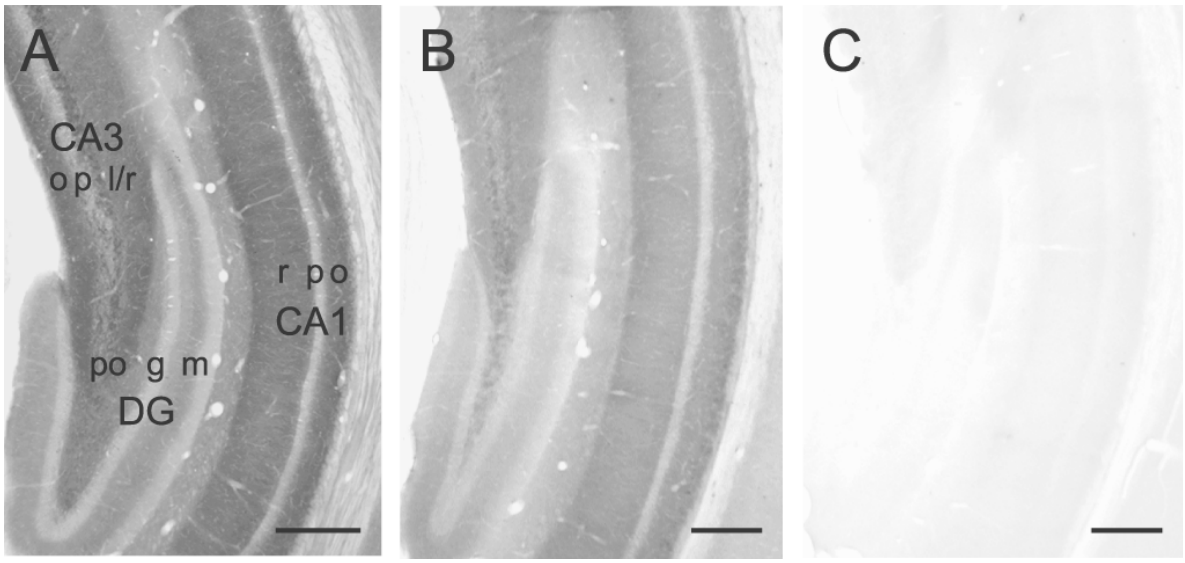

Figure 1. Low magnification photomicrographs of EphA4-immunolabeled sections of the hippocampus from adult rat, wild-type mouse and EphA4 knockout mouse

Figure 2. Electron micrographs showing EphA4 immunolabeling in small unmyelinated axons (A), a small perisynaptic astrocytic process (B), and a myelinated axon (C). In A, a small fascicle from CA3 pyramidal cell layer displays unmyelinated EphA4-positive axons (ua+) intermingled with unlabeled axons (ua-). B: A labeled perisynaptic astrocytic process (asterisk) in the DG polymorphic layer. Note the strong staining beneath the plasma membrane. ma-, immunonegative myelinated axon. C: One of the few immunopositive myelinated axons (ma+) observed in the DG polymorphic layer. Scale bars = 250 nm.

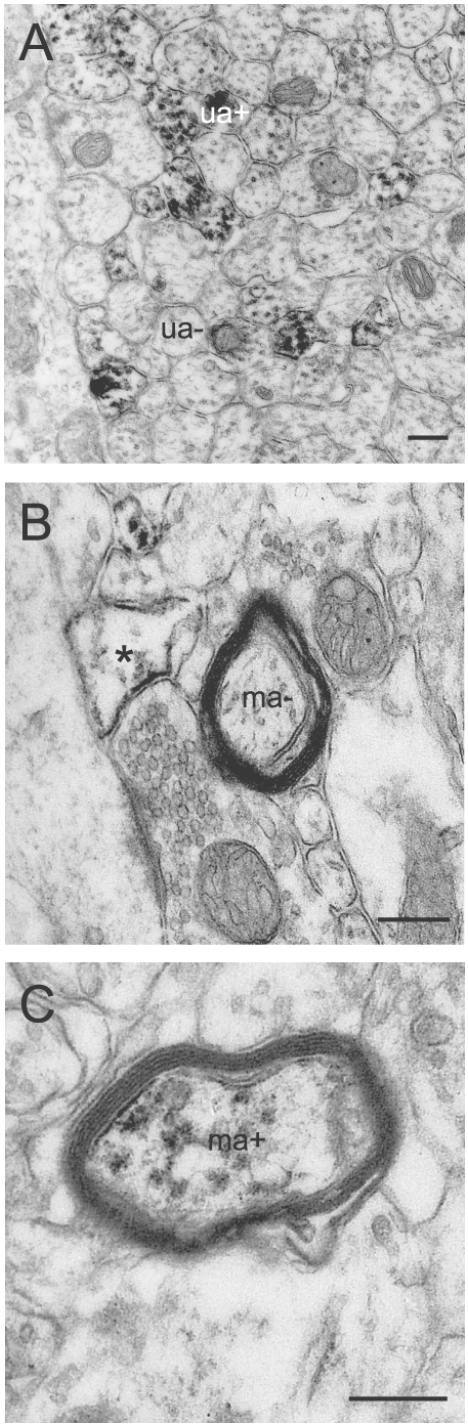

Figure 2. Electron micrographs showing EphA4 immunolabeling in small unmyelinated axons, a small perisynaptic astrocytic process, and a myelinated axon

Figure 3. EphA4-immunoreactive dendritic spines. In **A**, an unlabeled axon varicosity (t-) forms an asymmetrical synapse (between arrowheads) with the larger of two labeled dendritic spines (s+) in the polymorphic layer of DG. **B**: The labeled spine (s+) is contacted by an unlabeled axon terminal (t-) which also makes a synaptic contact (between arrowheads) with another, unlabeled spine (s-), (CA3, pyramidal cell layer). **C**: Axospinous synapse (between arrowheads) between two labeled partners (s+, labeled dendritic spine; t+, labeled axon terminal in the DG molecular layer). **D**: Single unlabeled mossy fiber terminal (t-) contacting one labeled (s+) and two unlabeled (s-) thorny excrescences in the DG polymorphic layer. Scale bars = 250 nm.

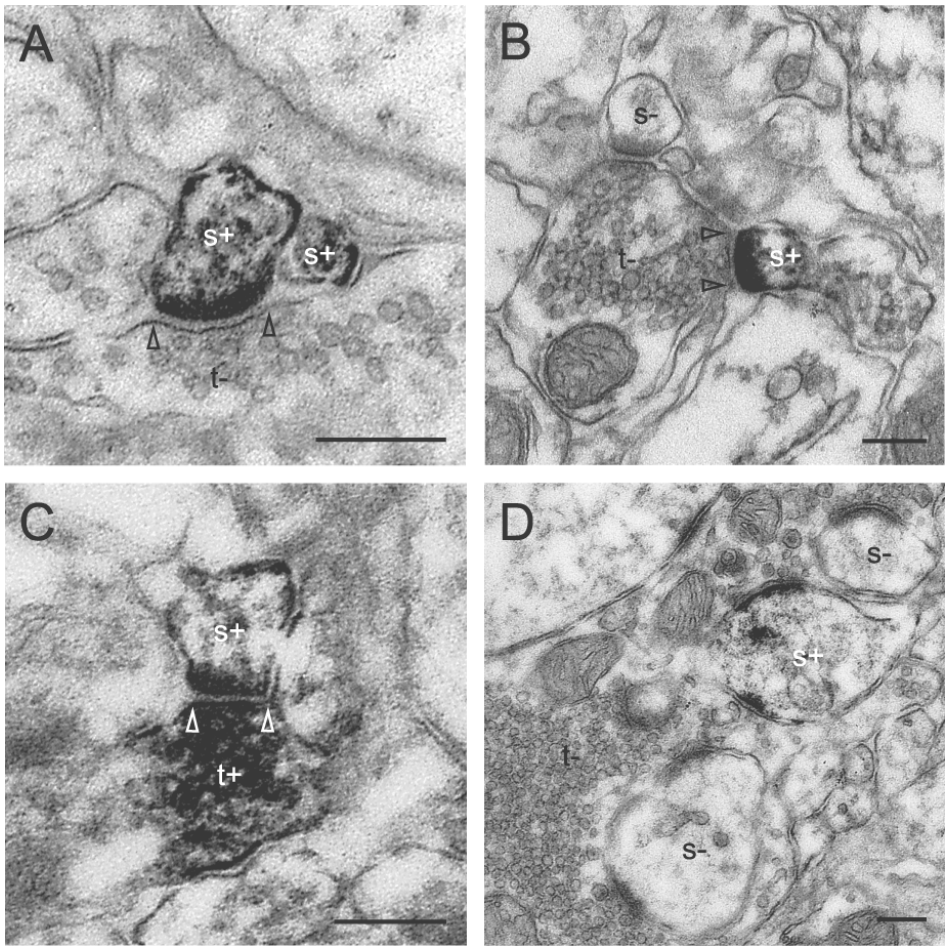

Figure 3. EphA4-immunoreactive dendritic spines

Figure 4. EphA4-immunolabeled axon terminals. In **A** and **B**, the labeled terminals (t+, -arrows) make asymmetric synaptic contacts (between arrowheads) with unlabeled dendritic spines (s-) in strata oriens and lucidum/radiatum of CA3, respectively (t-, unlabeled axon terminal). **C**: This labeled axon terminal (t+) makes a synaptic contact that looks symmetrical (between black arrowheads) on an unlabeled dendritic branch (d ; DG, polymorphic layer). **D**: Labeled mossy fiber axon terminal (t+, -arrow) in contact with several unlabeled thorny excrescences (s-) in stratum lucidum of CA3. Scale bars = 250 nm.

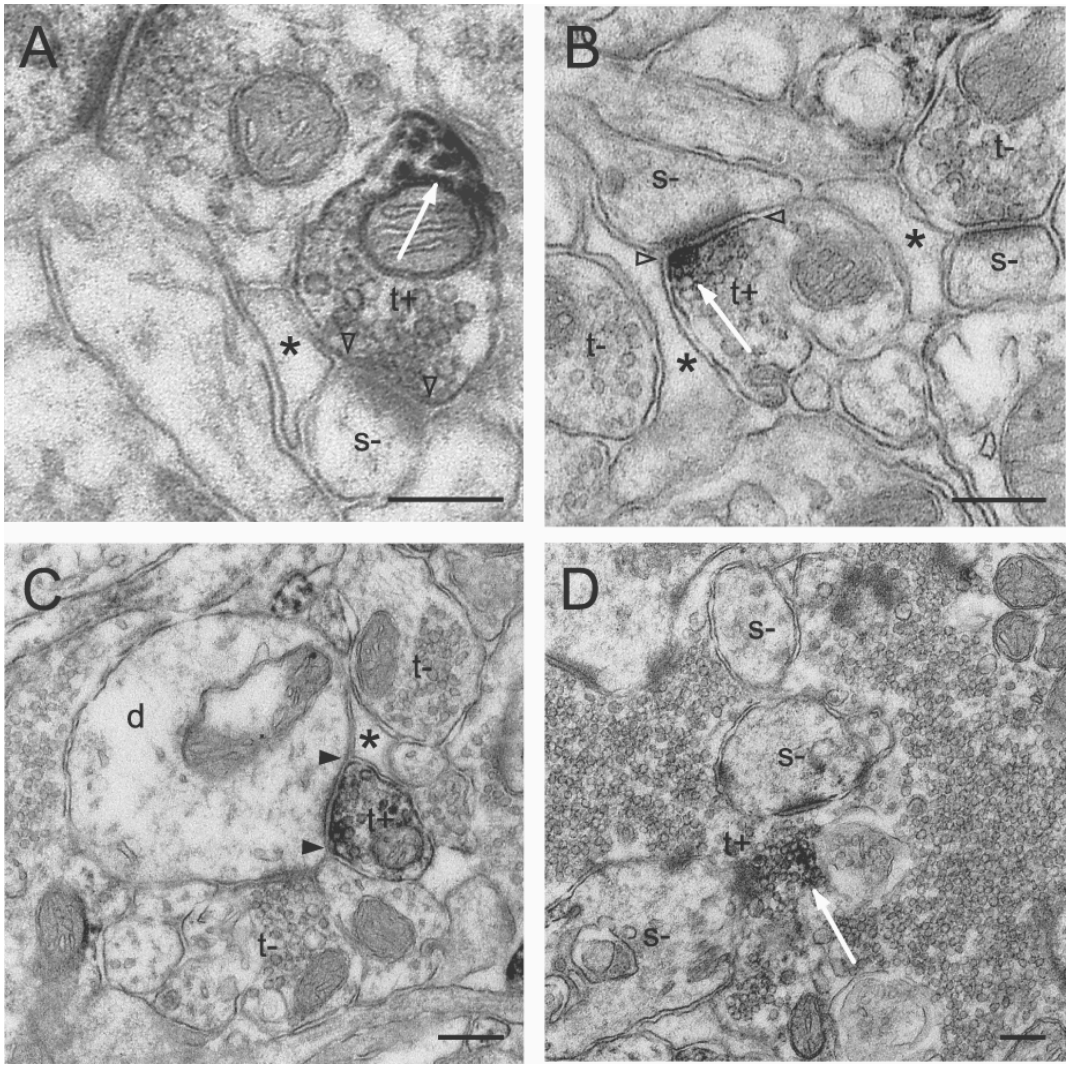

Figure 4. EphA4-immunolabled axon terminals

Figure 5. Immunogold labeling of EphA4 on neuronal elements from strata lucidum and radiatum of CA3. **A**: Several small unmyelinated axons display immunogold particles on their plasma membrane (ua-, unlabeled unmyelinated axons; ua+, labeled unmyelinated axons). In **B**, an immunogold-labeled axon terminal (t+), also showing plasma membrane labeling, forms an asymmetrical synaptic contact with an unlabeled spine (s-; t-, unlabeled axon terminal). In **C**, an axon terminal (t+) and a dendritic spine (s+) in synaptic contact (between arrowheads) with one another, as well as a nearby axon terminal (lower right corner), exhibit EphA4 labeling on their extrajunctional plasma membrane. Scale bars = 250 nm.

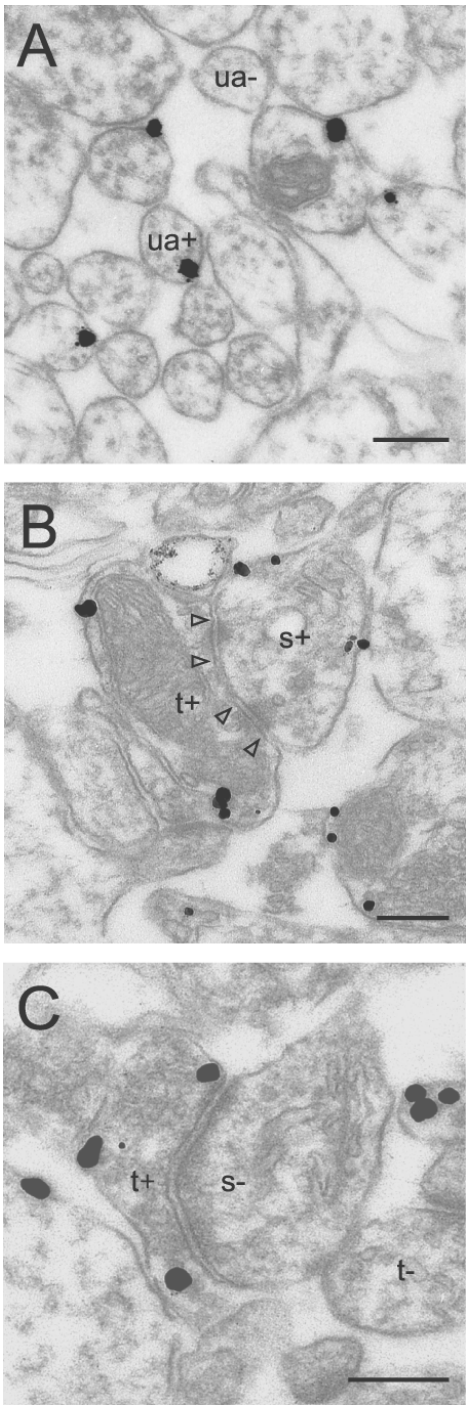

Figure 5. Immunogold labeling of EphA4 on neuronal elements

Figure 6. Number of labeled dendritic spines (**A**) and axon terminals (**B**) per 10^3 μm^2 of neuropil surface (± SEM), in stratum oriens (o), pyramidal cell layer (p) and stratum radiatum (r) of CA1; stratum oriens (o), pyramidal cell layer (p) and strata lucidum/radiatum (l/r) of CA3; and molecular (m), granule cell (g) and polymorphic (po) layers of DG. Dendritic spine and axon terminal labeling indices (± SEM; **C** and **D** respectively), in percentages of labeled dendritic spines or axon terminals over total numbers of labeled + unlabeled dendritic spines or axon terminals, in every examined layer.

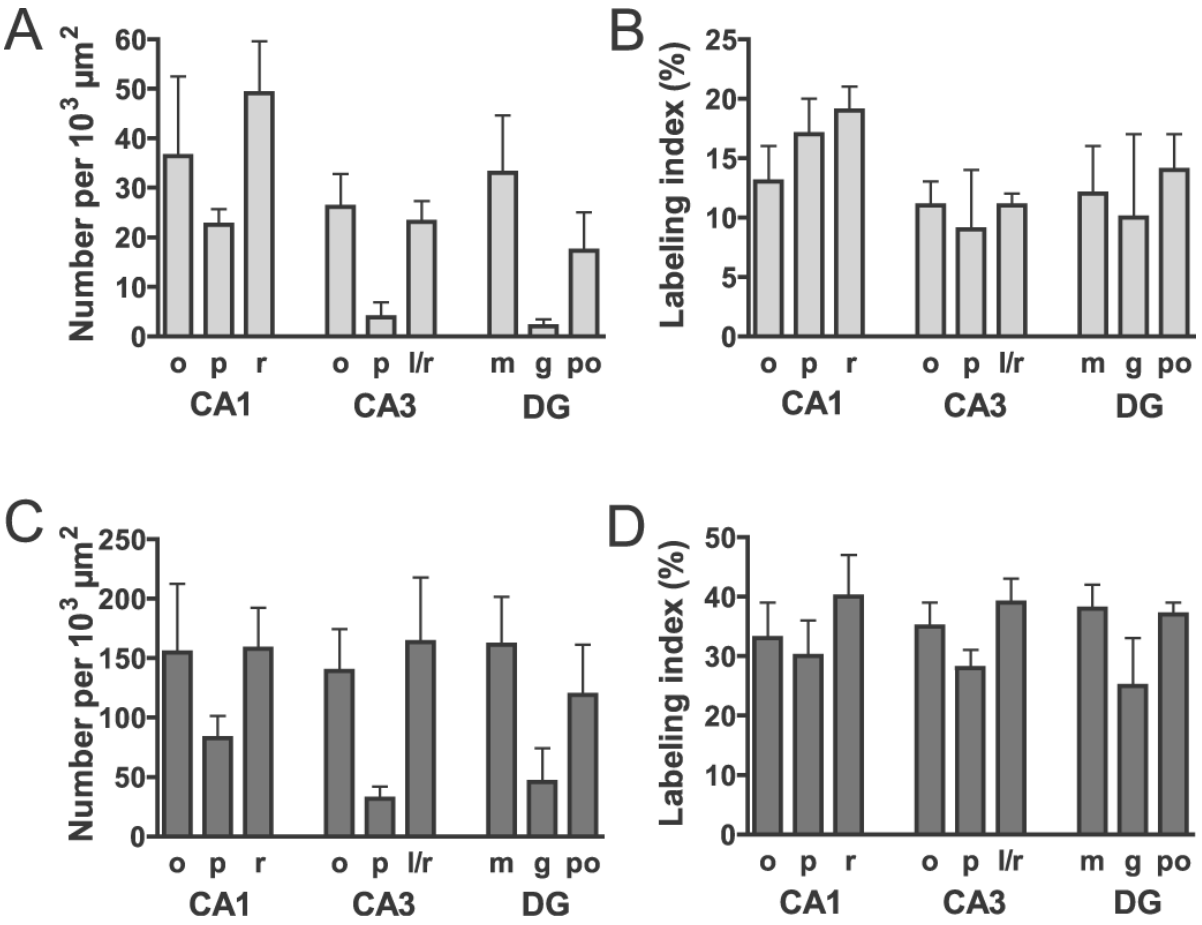

Figure 6. Number of labeled dendritic spines and axon terminals per 10^3 µm^2 of neuropil surface

Developmental Course of EphA4 Cellular and Subcellular Localization in the Postnatal Rat Hippocampus

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Comme première auteure, j'ai contribué à cette étude par la réalisation des expériences, l'analyse des résultats, la préparation des figures, la rédaction de la première version du manuscrit, ainsi que par la collaboration aux versions subséquentes.

Developmental Course of EphA4 Cellular and Subcellular Localization in the Postnatal Rat Hippocampus

Marie-Ève Tremblay[1], Mustapha Riad[1], Sabrina Chierzi[2], Keith K. Murai[2], Elena B. Pasquale[3], and Guy Doucet[1*]

[1] Département de pathologie et biologie cellulaire and Groupe de recherche sur le système nerveux central (GRSNC), Université de Montréal, Montréal, Québec, Canada H3C 3J7

[2] Centre for Research in Neuroscience, Department of Neurology and Neurosurgery, McGill University Health Centre, Montreal General Hospital, Montréal, Québec, Canada H3G 1A4

[3] The Burnham Institute for Medical Research, La Jolla, and Department of Pathology, University of California, San Diego, California 92037

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*Correspondence to: E-mail: [redacted], Telephone: (514)343-6255, Fax: (514) 343-5755.

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ABSTRACT

From embryonic development to adulthood, the EphA4 receptor and several of its ephrin-A or –B ligands are expressed in the hippocampus, where they presumably play distinct roles at different developmental stages. To help clarify these diverse roles in the assembly and function of the hippocampus, we examined the cellular and subcellular localization of EphA4 in postnatal rat hippocampus by light and electron microscopic immunocytochemistry. On postnatal day (P) 1, the EphA4 immunostaining was robust in most layers of CA1, CA3 and the dentate gyrus, and then decreased gradually, until P21, especially in the cell body layers. At the ultrastructural level, focal spots of EphA4 immunoreactivity were detected all over the plasma membrane of pyramidal and granule cells, between P1 and P14, from the perikarya to the dendritic and axonal extremities, including growth cones and filopodia. This cell surface immunoreactivity then became restricted to the synapse-associated dendritic spines and axon terminals by P21. In astrocytes, the EphA4 immunolabeling showed a similar cell surface redistribution, from the perikarya and large processes at P1-P7, to small perisynaptic processes at P14-P21. In both cell types, spots of EphA4 immunoreactivity were also detected, with an incidence decreasing with maturation, on the endoplasmic reticulum, Golgi apparatus, and vesicles, organelles involved in protein synthesis, post-translational modifications and transport. The cell surface evolution of EphA4 localization in neuronal and glial cells is consistent with successive involvements in the developmental movements of cell bodies, first, followed by process outgrowth and guidance, synaptogenesis, and, finally, synaptic maintenance and plasticity.

INTRODUCTION

Eph receptors constitute the largest subfamily of receptor tyrosine kinases, influencing cell morphology and behavior by regulating the actin and microtubule dynamics (Egea and Klein, 2007; Himanen et al., 2007; Pasquale, 2005; Suh et al., 2004). In mammals, Eph receptors are divided into EphA (EphA1-A8 and EphA10) and EphB (EphB1-B4 and EphB6) subclasses, based on sequence similarities and binding preferences for the ephrin-As (ephrin-A1-6) or ephrin-Bs (ephrin-B1-3), respectively. Ephrin-As are attached to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor, whereas ephrin-Bs are transmembrane proteins. EphA4 uniquely binds all ephrin-As and ephrin-Bs (see Pasquale, 2005). Interestingly, a phylogenetic analysis suggests that the ancestral vertebrate Eph receptor also interacted with both subclasses of ephrins (Mellott and Burke, 2008).

At points of cell-cell contact, interactions between cell surface Eph receptors and ephrins elicit bi-directional signaling events into both the Eph- and ephrin-expressing cells. These signals may induce repulsion and cell separation or promote attraction and cell adhesion (reviewed in Egea and Klein, 2007; Pasquale, 2005; 2008). The repulsion or attraction mediated by EphA4, interacting with either ephrin-As or –Bs, are involved in important steps of embryonic development (reviewed in Klein, 2004; Palmer and Klein, 2003; Wilkinson, 2001), notably in somite (Barrios et al., 2003) and rhombomere morphogenesis (Cramer et al., 2000), as well as neural crest cell migration (McLennan and Krull, 2002). In the central nervous system (CNS), they are particularly important for axon guidance events (Coonan et al., 2001; Eberhart et al., 2002; Egea et al., 2005; Helmbacher et al., 2000), including midline crossing (Mendes et al., 2006), somatosensory and motor circuit assembly (Gallarda et al., 2008; Oury et al., 2006), as well as the formation of topographic maps in the retino-tectal (Marin et al., 2001), hippocampo-septal (Yue et al., 2002), thalamo-cortical body representation (Vanderhaeghen et al., 2000), and auditory frequency systems (Huffman and Cramer, 2007). In the mature hippocampus, EphA4 participates in the morphological shaping of dendritic spines and the induction of long-term potentiation and long-term depression (Bourgin et al., 2007; Fu et al., 2007; Grunwald et al., 2004; Murai et al., 2003; Richter et al., 2007; Zhou et al., 2007). Thus, EphA4 has diverse roles, particularly in the development and function of neural connections.

During embryonic and early postnatal development, the EphA4 mRNA or protein has been localized in the cerebral cortex, hippocampus, dentate gyrus (DG), thalamus, basal ganglia, olfactory bulb and cerebellum, as well as in numerous white matter tracts (Greferath et al., 2002; Liebl et al., 2003; Mackarehtschian et al., 1999). Upon maturation, the immunoreactivity was reported to decrease in these brain regions (Greferath et al., 2002). In the adult, EphA4 is prominent in the hippocampus and DG, but is also present in other regions such as the cerebral cortex, olfactory bulb, and cerebellum (Liebl et al., 2003; Martone et al., 1997).

Various ephrin ligands of EphA4 are also expressed in the hippocampus (reviewed in Martinez and Soriano, 2005). Cytochemical binding assays with the EphA5-AP fusion protein have shown that ephrin-As are strongly expressed in the postnatal hippocampus (Stein et al., 1999), with a laminar distribution resembling that of EphA4 (Greferath et al., 2002; Martone et al., 1997; Murai et al., 2003; Tremblay et al., 2007; Xiao et al., 2006). Ephrin-A3 and ephrin-A5, but not ephrin-A4 mRNAs have been detected in the hippocampus at postnatal stages: ephrin-A3 in CA1, CA3, and DG regions, and ephrin-A5 only in CA1 and CA3 (Mackarehtschian et al., 1999; Stein et al., 1999). Their expression is maintained in the adult (Stein et al., 1999). In contrast, the early postnatal hippocampus shows only low levels of ephrin-B1-B3 mRNAs, whereas in the adult, ephrin-B2 becomes more abundant in CA1, and ephrin-B3 increases in CA1 and the DG (Grunwald et al., 2001; Liebl et al., 2003). Thus, EphA4 could potentially interact with several ephrins during the postnatal development of hippocampus: first mainly with ephrin-As, and later with both ephrin-As and ephrin-Bs.

To gain more insight into the potential contributions of EphA4 at the various stages of the assembly and function of the hippocampus, a detailed analysis of its discrete cellular and subcellular distribution is required. Since EphA4 and several ephrins are expressed in the hippocampus throughout the postnatal development and at maturity, an analysis of the localization pattern of EphA4 also provides a good model for how the distribution of a remodeling-associated cell surface receptor evolves to meet the needs of neuronal maturation and circuitry formation.

In this study, we took advantage of the availability of a specific antibody to examine the localization of EphA4 in CA1, CA3 and DG regions of the P1, P7, P14, and P21 rat

hippocampal formation, using immunocytochemistry in light and electron microscopy (EM). The results support the hypothesis of various successive roles for EphA4, as its localization changes from neuronal and glial cell bodies to distal processes in correlation with perikarya migration, process elongation, and synaptic formation and plasticity.

MATERIALS AND METHODS

Animals

Hippocampal tissue was obtained from five postnatal day (P) 1, five P7, five P14, and two P21 male Sprague-Dawley rats (Charles River, St-Constant, Québec, Canada). Two pairs of P7 and P14 EphA4 knockout ($EphA4^{-/-}$) and wild-type littermate mice ($EphA4^{+/+}$) provided by Dr. Mirella Dottori (Center for Reproduction and Development, Monash Institute of Medical Research, Clayton, Australia; Dottori et al., 1998) were also used as controls, to confirm the specificity of the immunocytochemical staining. The animals were housed at constant temperature (21°C) and humidity (60%) under a fixed 12h light/dark cycle and with free access to food and water. The experimental protocols were approved by the Animal Care Committee of the Université de Montréal. All procedures were conducted in strict accordance with the *Guide to the care and use of experimental animals* (Ed2) of the Canadian Council on Animal Care. The animals were deeply anaesthetized with sodium pentobarbital (80 mg/kg, i.p.) and perfused through the aortic arch with a solution of 3.5% acrolein, followed by 4.0% paraformaldehyde (PFA), both in 0.1 M sodium phosphate buffer (PB), pH 7.4. The brain was removed, post-fixed in 4% PFA for 2 to 3 h at 4°C, and washed in sodium phosphate-buffered saline (PBS; 0.9% NaCl in 50 mM PB, pH 7.4).

Antibody

The production and characterization of the anti-EphA4 antibody (Ab11), raised in rabbit against the last 11 carboxy-terminal amino acids of EphA4, which are identical in rat, mouse, chicken, and human EphA4, have been fully described previously (Soans et al., 1994). This antibody was shown to recognize chicken EphA4, but also the mouse, rat, and human homologs. Indeed, its specificity was tested by immunoprecipitation followed by immunoblotting in rat, mouse, and human cells lines (Soans et al., 1994), as well as in adult rat cerebellum and cerebrum (Martone et al., 1997). We also demonstrated the specificity of

the immunocytochemical staining obtained with this antibody by showing an absence of staining on brain sections from adult EphA4$^{-/-}$ mice processed in parallel with corresponding sections from EphA4$^{+/+}$ mice (Tremblay et al., 2007). In the present study, the specificity of the immunocytochemical staining with this antibody was again confirmed in light microscopy and EM, since there was no staining on brain sections from P7 and P14 EphA4$^{-/-}$ mice (not shown).

Immunocytochemistry

Transverse sections of the brain (50 μm thick) were produced with a vibratome in ice-cold PBS, at rostrocaudal levels including the ventral part of the hippocampus. They were treated with 0.1% sodium borohydride in PBS for 30 minutes, at room temperature (RT), washed in PBS, and processed freely floating following a pre-embedding immunoperoxidase protocol, as described in Riad et al. (2000). Briefly, sections were incubated during 1h, at RT, in a blocking solution of PBS containing 5% normal goat serum and 0.5% gelatine, and then during 48 hours at RT in 1.5 μg/ml of the rabbit anti-EphA4 antibody (1:500) in blocking solution. A goat anti-rabbit IgG conjugated to biotin (1:1000; 2h at RT; Jackson Immunoresearch, West Grove, PA) and a streptavidin-horseradish peroxidase complex (1:1000; 1h at RT; Jackson Immunoresearch) were used as secondary reagents and labeling was revealed with 0.05% diaminobenzidine (DAB) and 0.01% hydrogen peroxide in Tris/HCl-buffered saline (50 mM, pH 7.4). For this study, we used the immunoperoxidase method, because our previous experience with pre-embedding or post-embedding immunogold approaches (Bouvier et al., 2008; Tremblay et al., 2007; and unpublished results from the present study) showed that they were not sufficiently sensitive for analysis of the distribution of a protein that is not as abundant as some neurotransmitters or neurotransmitter receptors (e.g., Riad et al., 2000). Indeed, the EphA4 immunoreactivity was not detectable following embedding, and the post-embedding approach did not provide good enough ultrastructural preservation for a detailed analysis, whereas the pre-embedding immunogold technique yielded only very few silver-intensified immunogold particles on most labeled profiles (see Bouvier et al., 2008).

Sections for light microscopy were mounted onto microscope slides, dehydrated in ethanol, cleared in toluene, and coverslipped with DPX (Fluka, Sigma-Aldrich, Oakville, Ontario, Canada). For EM, sections were rinsed in PB, post-fixed flat in 1% osmium

tetroxide for 30 minutes, dehydrated in ethanol and impregnated with resin (Durcupan ACM; Sigma). They were mounted between aclar embedding films (Electron Microscopy Sciences, Hatfield, PA), and cured at 55°C for 48 hours. Areas of interest were excised from the slides, glued at the tip of resin blocks, and sectioned (70-80 nm thin) with an ultramicrotome (Reichart Ultracut S; Leica, Wetzlar, Germany). The ultrathin sections were collected on bare square-mesh copper grids, stained with lead citrate, and examined at 60 kV with a Philips CM100 electron microscope.

Sampling and analysis

Light microscope pictures were taken with a SpotRT color digital camera (Diagnostic Instruments, Sterling Heights, MI). Images were adjusted for brightness and contrast with Adobe Photoshop (CS).

Electron microscopic examination was carried out on materials from three P1, three P7, three P14, and two P21 rats. In every rat, 20 micrographs (covering ~1,800 μm^2) were taken at X7,900, in each of CA1 stratum radiatum, CA3 stratum lucidum/radiatum, and DG polymorphic layer, and 10 micrographs (covering ~900 μm^2) in each of CA1 and CA3 pyramidal cell layers, and DG granule cell layer. In one P14 and one P21 rats, 10 micrographs sampling axon fascicles were also taken in CA1 stratum oriens and the alveus fiber tract. The EM film negatives were scanned (800 dpi, Epson Perfection 3200) and converted into positive pictures for analysis. To facilitate their visualization, some cellular profiles displayed in the figures were coloured in light blue, green, lavender, pink, or yellow, using the Layer tool and the Difference Blending option in Adobe Photoshop (CS).

Cellular profiles were identified in EM according to criteria defined by Peters et al. (1991), Skoff and Hamburger (1974) and Deitch and Banker (1993). Processes of protoplasmic astrocytes were recognized by their irregular and angular shapes, which distinguished them from neuronal profiles having a characteristic rounded shape. They were discerned from processes of oligodendrocytes by their lucent cytoplasm, which contrasts with the moderately dense cytoplasm of oligodendrocytes (Peters et al., 1991). Mitochondria and endoplasmic reticulum, although present in large astrocytic processes, were absent from smaller ones. Lastly, bundles of fibrils were sometimes seen in the larger processes. Dendritic branches were distinguished from unmyelinated axons by their more

irregular contours, less regularly spaced microtubules, frequent protuberances (spines, filopodia, small branches), and usual synaptic contacts with axon terminals. Dendritic spines displayed their characteristic fluffy content, with or without a spiny apparatus, and frequent synaptic contacts with axon terminals. Dendritic filopodia, identified when protruding from dendritic branches, were distinguished from spines by their greater length or pointed, rather than bulbous head (Fiala et al., 1998). Unmyelinated axons were positively identified only when found within fascicles of similar profiles (isolated profiles of similar size were left as unidentified). Axons becoming myelinated were also observed, either wrapped by oligodendrocytic processes filled by a moderately dark cytoplasm or ensheathed by just a few turns of compact myelin and loose outer sheets. Oligodendrocytic processes were positively identified only when seen encircling unmyelinated axons. Axon terminals corresponded to enlarged portions of axons containing aggregated synaptic vesicles and often showing a synaptic specialization. Axonal growth cones were identified as considerable enlargements of axons, presenting a dark cytoplasm filled with large amounts of smooth endoplasmic reticulum and pleomorphic vesicles (Skoff and Hamburger, 1974; Peters et al., 1991; Deitch and Banker, 1993). They were distinguished from dendritic growth cones by their more frequent filopodial extensions, which were often contacting dendritic branches rather than axon terminals. Occasionally, such profiles were in continuity with axon terminals. Axonal filopodia were positively identified when extending from axonal growth cones. Nevertheless, distinction between axonal and dendritic growth cones or filopodia was not always obvious, and some of those identified as axonal might have been dendritic.

Lastly, the morphology of synaptic complexes (symmetric vs. asymmetric), including labelled pre- or postsynaptic elements, was also noted. Only synaptic profiles presenting an unequivocal postsynaptic density were considered asymmetric.

RESULTS

Laminar Distribution of EphA4 (Light Microscopy)

We first examined the regional and layer-specific distribution of EphA4 immunoreactivity in the developing hippocampus. The overall intensity of the EphA4 immunoperoxidase-DAB staining diminished gradually, but differentially among layers,

between P1 and P21, in CA1, CA3 and DG regions, particularly in the main cellular layers (Fig. 1). On the other hand, the intensity increased in the alveus and in the mossy fibers, which became the most intensely stained zones at P14 and P21 (Fig. 1C,D,G,H).

The main cellular layers of CA1, CA3, and DG showed maximum immunoreactivity at P1 and P7 (Fig. 1A,B,E,F,I,J,M,N), which was associated with the perikarya of pyramidal and granule cells. There was no evidence of interneuron immunostaining in these or other layers. The intensity of immunolabeling decreased in these cellular layers at P14 (Fig. 1C,G,K,O) and became very weak at P21 (Fig. 1D,H,L,P), as in the adult rat, mouse and rhesus monkey (Martone et al., 1997; Murai et al., 2003; Tremblay et al., 2007; Xiao et al., 2006).

The neuropil layers were immunoreactive at all ages, with the exception of the CA1 and CA3 stratum lacunosum-moleculare, which receives the perforant path input from the entorhinal cortex (see Andersen et al., 2007). In CA1 stratum radiatum, there was, at P1, a steep decreasing gradient of immunoreactivity, between the pyramidal layer and the hippocampal fissure (Fig. 1A,E), which flattened out gradually between P7 and P21 (Fig. 1B-D, F-H). A similar gradient of labeling was apparent at P1 in CA3 stratum lucidum/radiatum (Fig. 1A,I). Labeling was less intense, but more uniform, in CA1 and CA3 stratum oriens. In DG, the molecular and polymorphic layers were of similar intensity, at each examined age (Fig. 1M-P).

In summary, from P1 to P21, a robust EphA4-immunoreactivity persisted in the neuropil layers, whereas it decreased considerably in the main cellular layers.

Cellular and Subcellular distribution of EphA4 (Electron Microscopy)

With EM, we examined the cellular and subcellular distribution of EphA4 in the pyramidal and granule cell layers as well as in stratum radiatum in CA1, stratum lucidum/radiatum in CA3, and the polymorphic layer in the DG. The latter neuropil layers were chosen because they contain commissural-associative fibers in the 3 regions (Schaffer collaterals in CA1 and the mossy fibers in CA3 and DG). These projections were identified previously as EphA4-positive (Tremblay et al., 2007).

The EphA4-immunolabeling was observed on the cell surface as focal spots on the plasma membrane (Figs. 2,3,5-9). It also decorated the postsynaptic density of some synapses. It was additionally found intracellularly as spots decorating the endoplasmic reticulum (ER) and Golgi apparatus, as well as on vesicles of various sizes. Collectively, labeling of these structures is consistent with EphA4 being a glycosylated transmembrane protein (Soans et al., 1994). The external membrane of some mitochondria, as well as some microtubules were also occasionally labeled, often near spots of plasma membrane or vesicle labeling (e.g., Figs. 3C,D, 6C, 7C). Although this labeling was also absent in EphA4-/- mice, it was considered as artifactual, and due to the local diffusion of the DAB reaction product from nearby organelles (Brown, 2001). Indeed, the avidity of oxidized DAB for mitochondria and microtubules has also been reported following immunostaining of substance P (Kakudo et al., 1981) and membrane receptors for angiotensin, dopamine, glutamate or ATP (Carlton et al., 2001; Kharazia et al., 1996; Le et al., 1998; Paspalas and Goldman-Rakic, 2005; Thomas et al., 2003). Nevertheless, we preferred this immunoperoxidase approach, because it provided discrete labeling on the plasma membrane as well as intracellular vesicular or tubular organelles with a much higher sensitivity than immunogold procedures (see Materials and Methods).

Neuronal Cell Bodies

Between P1 and P14, cell surface and intracellular (ER, Golgi and vesicles) immunolabeling was found easily in the pyramidal and granule cell perikarya (Fig. 2) and proximal dendrites. Detection of such EphA4-positive perikarya became more difficult at P21 (not shown), as in the adult (Tremblay et al., 2007). The spots of immunostaining on the plasma membrane were often situated at sites of contact with unlabeled axon terminals (Fig. 2A), especially at P1-P7, as well as with other cell bodies (Fig. 2B), or astrocytic processes (not shown), at all examined ages.

Neuropil Labeling

In the neuropil of the 3 regions, the immunostaining was associated with profiles of dendritic branches and filopodia (Fig. 3), dendritic spines (Fig. 4), axonal growth cones and filopodia (Fig. 5), axons and axon terminals (Fig. 6-9), as well as processes of protoplasmic

astrocytes (Fig. 8). We describe below the localization of EphA4 in these subcellular compartments.

Dendritic Elements

In dendrites, spots of labeling were found on the plasma membrane, often located at sites of contact with unlabeled axon terminals, as well as on the ER and various vesicles (Fig. 3). This labeling was stronger at P1-P14 and became weaker at P21 (not shown). At P1 and P7, synaptic contacts between labeled dendritic branches and unlabeled axon terminals were frequent (Fig. 3A). These synaptic junctions were typically symmetrical. At P7, filopodia frequently protruded from dendritic branches, displaying labeling at their pointed extremities, notably at sites of contact with labeled axonal filopodia (Fig. 3B). At P14, the plasma membrane labeling of dendrites mostly occurred on small protrusions, which juxtaposed labeled axonal filopodia (Fig. 3C) or unlabeled axon terminals (Fig. 3D). Such labeled dendritic protrusions were still observed at P21, but much less frequently. To sum up, the labeling of dendrites (excluding the spines) concerned mostly their branches at P1; followed by filopodia and small protrusions between P7 and P21.

Labeled dendritic spines were first observed at P7, but became numerous at P14-P21, mostly in synaptic contact with unlabeled axon terminals (Fig. 4). At these synapses, in contrast to those between dendritic branches and axon terminals at P1-P7, the synaptic junctions were usually asymmetrical, suggesting they were excitatory (Peters et al., 1991). The labeling was particularly strong at P14 (Fig. A,B). At all ages, the spine labeling was always associated with the post-synaptic density (Fig. 4A-C), and often extended to the spine apparatus, when present, and plasma membrane (Fig. 4B). A similar labeling pattern was previously described in the adult (Tremblay et al., 2007). In summary, dendritic spines were intensely labeled already at P7, and gradually became the most frequently labeled dendritic element by P21.

Axonal Elements

Immunoreactive axonal growth cones and filopodia were frequent at P1 and P7 (Fig. 5), and only occasional at P14 and P21 (not shown). These growth cones were identified as axonal enlargements with a dark cytoplasm, containing numerous vesicles of various sizes and shapes (Peters et al., 1991). They were distinguished from dendritic

growth cones by their more frequent filopodial extensions, and their contacts with dendritic branches. In axonal growth cones (Fig. 5A,B) and filopodia (Fig. 5C), the labeling was associated with both the plasma membrane and vesicles. The plasma membrane of growth cones was immunoreactive at sites near unlabeled dendritic branches (Fig. 5A,B). EphA4-positive axonal filopodia extending along the length of unlabeled dendritic filopodia were also frequently seen at P7 (Fig. 5C). Thus, immunoreactive axonal growth cones and filopodia were numerous at P1 and P7, nearby generally unlabeled dendritic branches or filopodia.

Axons and axon terminals were also labeled at their surface or on intracellular vesicles (Fig. 6), from P1 to P21. Already at P1-P7, axon terminals made synaptic contacts with small dendritic branches (Fig. 6A,C) and filopodia (Fig. 6B), although the spots of labeling did not always face the synaptic partner (Fig. 6C). By P14-P21, labeled nerve terminals became numerous, as in the adult (Tremblay et al., 2007). These contacted small dendritic branches (Fig. 6E) and filopodia (not shown), but also dendritic spines (Fig. 6D-F). In axon terminals, the labeling usually concerned spots of the plasma membrane or subsets of vesicles found either near (Fig. 6A,B,F) or away from (Fig. 6C,D) synaptic junctions. Labeled axons were also encountered, in continuity with unlabeled nerve terminals (Fig. 6E,F). The synapses between labeled axon terminals and unlabeled dendritic spines displayed asymmetric junctions (Fig. 6D–F).

Among axons and axon terminals, the DG-derived mossy fibers were particularly identifiable by their location and peculiar morphology. By P14, the small unmyelinated axons and large axon terminals of mossy fibers were immunoreactive, both in CA3 (Fig. 7) and DG (not shown). Within axon fascicles, immunolabeled axonal profiles were intermingled with unlabeled profiles (Fig. 7A). The labeling concerned the plasma membrane or occasional vesicles, but also microtubules, presumably by diffusion of the reaction product from nearby spots of specific labeling (see above). In mossy fiber axon terminals, the immunoreactivity was associated with subsets of vesicles (and nearby mitochondria) (Fig. 7B,C). These terminals made synaptic contacts with dendritic branches (Fig. 7B) and pedunculated dendritic spines, so-called *thorny excrescences* (Fig. 7C).

Astrocytes

At all ages, numerous processes of protoplasmic astrocytes were EphA4-positive (Fig. 8). At P1 and P7 (Fig. 8A-D), the perikarya and large-caliber processes were labeled on their plasma membrane, ER, Golgi apparatus, and vesicles, as described above for neurons. Some of the astrocytic processes surrounded capillaries, with spots of labeling contacting the basal lamina (Fig. 8A,C), near endothelial cells or pericytes, while others were apposed to axon terminals (Fig. 8B) or synapses between dendritic branches and axon terminals (Fig. 8D). Astrocytic perikarya or proximal processes were rarely labeled after P7. By P14-P21, the EphA4 labeling specifically concerned the plasma membrane and occasional vesicles of small caliber astrocytic processes (Fig. 8E,F). At P14, astrocytic processes ensheathing blood capillaries were still labeled (Fig. 8E), but no such labeling was found at P21. At both P14 and P21, these thin astrocytic processes were apposed to axon terminals (Fig. 8F) or synapses, as in the adult (Tremblay et al., 2007). In brief, the astrocytic localization of EphA4 followed a developmental time course similar to that in neurons, being first observed all over the plasma membrane of perikarya and large processes, to become restricted to the small distal processes by P21.

Myelinated Axons

At P14-P21, EphA4 was also found on myelinating axons, as observed in the CA1 stratum oriens and alveus fiber tract (Fig. 9). Axons in the myelination process often displayed strong focal immunoreactivity at their plasma membrane, at sites of contact with the processes of oligodendrocytes encircling or spiraling around them (Fig. 9A-C,E,F). Labeling of microtubules was also detected in some myelinated axons (Fig. 9D), as in the adult (Tremblay et al., 2007), suggesting diffusion of the oxidized DAB from nearby spots of specific labeling, presumably on the plasma membrane or vesicles (in the same profiles or in adjacent sections).

DISCUSSION

We have examined the cellular and subcellular localization of EphA4 at several ages in the postnatal rat hippocampus. To our knowledge, this is the first detailed analysis of the ultrastructural distribution of any Eph receptor or ephrin in the hippocampus during

neuronal maturation and circuitry formation. When observed at low magnification, a strong EphA4-immunoreactivity persisted between P1 and P21 in the neuropil layers of CA1, CA3, and DG, in contrast with the cell body layers, where immunoreactivity decreased considerably with maturation. At the ultrastructural level, focal spots of EphA4 immunoreactivity decorated all the plasma membrane of the principal neuronal cells, from the perikarya to the distal extremities, between P1 and P14. This labeling became restricted to the synaptic extremities of the cells by P21. In particular, EphA4-positive distal extremities gradually developed from small dendritic branches, dendritic filopodia, axonal growth cones, and axonal filopodia, into synapse-associated dendritic spines and axon terminals. At the plasma membrane of protoplasmic astrocytes, EphA4 was similarly redistributed, decorating cell bodies and large-caliber processes at P1-P7 to gradually become restricted to small distal processes, including perisynaptic structures, at P14-P21. Lastly, in both cell types it was found that organelles involved in the synthesis, post-translational modification and transport of glycosylated proteins, such as EphA4, (ER, Golgi and vesicles) were also labeled, and more abundantly in younger than adult animals, reflecting a higher synthesis of the protein during neural development.

Interpretation of Immunoperoxidase Labeling

This discrete localization of EphA4 was visualized in EM with a pre-embedding immunoperoxidase-DAB protocol. This approach provides an excellent preservation of morphology and ultrastructure with a high sensitivity of immunodetection (Brown, 2001). However, the DAB reaction product can diffuse locally, at some distance from the site of antigen-antibody binding (Brown, 2001). Messing et al. (1985) have examined the lateral spread of the DAB reaction product using plasma membrane insertion of ferritin; a protein that is detectable directly in EM by its 7 nm-large electron-dense core (Dykstra, 1992), in cultured ciliary ganglion neurons. Using immunoperoxidase-DAB co-detection of ferritin, they estimated the lateral spread of the reaction product to an average of 54-81 nm. In the present study, consequently, the intracellular membranes were considered as EphA4-positive when they were separated by more than 100 nm from one another or from a plasma membrane spot of labeling. Thus, in cases when the DAB reaction product was found on small groups of vesicles or on the plasma membrane as well as on nearby vesicles, it was not possible to determine which organelles contained the EphA4 protein. Nevertheless,

there were many cases where only the plasma membrane was labeled or where labeled vesicles were found far enough from the plasma membrane to confirm their labeling. Moreover, our previous work with the pre-embedding immunogold procedure confirmed the localization of EphA4 on the plasma membrane as well as on vesicles of neuronal and astrocytic elements, at P1, P7, P14 (unpublished results), and in the adult (Bouvier et al., 2008; Tremblay et al., 2007).

Synthesis, Maturation and Transport of the EphA4 Protein

At all ages, EphA4 was associated with the ER, Golgi apparatus, and vesicles, in pyramidal, granule and astrocytic cells. This is consistent with the synthesis, maturation and transport of the EphA4 protein, prior to its plasma membrane insertion. The intensity of the immunostaining greatly diminished between P14 and P21, suggesting lower rates of synthesis. In the adult, we previously did not detect EphA4 on neuronal and astrocytic perikarya (Tremblay et al., 2007). However, upon further examination of the pictures taken for that study, we found that some neuronal and astrocytic cell bodies did display weak reactivity. Indeed, EphA4 mRNA has been detected in both principal neurons (Liebl et al., 2003; Mackarehtschian et al., 1999) and astrocytes (Nestor et al., 2007) in the P0 or adult hippocampus.

Localization at the Plasma Membrane of Neuronal Cell Bodies

Between P1 and P14, EphA4 was often located at the plasma membrane of principal neuronal cell bodies, at sites of contact with neighboring neuronal cell bodies and astrocytic processes. As the hippocampus gets larger postnatally, the pyramidal cell layers undergo spatial rearrangement: the numbers of rows of neuronal somata shows thinning down from 6-10 layers at birth, to 2-3 layers in the adult (see Andersen et al., 2007). Postnatally generated glial cells might contribute to this reorganization, because neonatal X-ray irradiation, which affects proliferation of glial cells and newly generated DG granule cells, prevents this maturation of the CA1 pyramidal cell layer (Czurko et al., 1997). Interactions between EphA4 and ephrins, either on neuronal cell bodies or astrocytic processes, could be involved, in line with the known roles of this receptor in regulating the migration of neural crest cells (Smith et al., 1997), cell shape changes and relationships between cells

during somite morphogenesis (Barrios et al., 2003), and rhombomere pattern formation (Cramer et al., 2000).

Particularly at P1-P7, EphA4 was also found on the plasma membrane, juxtaposing unlabeled axon terminals. At axo-somatic synapses, released GABA provides an excitatory drive for neuronal cell outgrowth (reviewed in Ben-Ari, 2002), at least until P7 in male rats (Nunez and McCarthy, 2007). The role(s) of EphA4 at these synapses would be worth examining.

Localization in Distal Dendritic, Axonal and Astrocytic extremities

Between P1 and P14, EphA4 immunoreactivity decorated the surface of neuronal cells, from the perikarya to the dendritic and axonal extremities, whereas by P21 it became restricted to the synapse-associated extremities. During growth, maturation and plasticity, these distal extremities similarly sculpt their morphologies via changes in actin and microtubule dynamics.

Functional Maturation of Dendritic Spines

At the light microscopic level, the intensity of EphA4-immunostaining in CA1 stratum radiatum (and CA3 stratum lucidum/radiatum) at P1 followed a decreasing gradient from the pyramidal cell layers to the hippocampal fissure. This gradient progressively dissolved between P7 and P21. This progression of the immunolabeling follows the developmental time course of CA1 pyramidal cell apical dendritic trees, which elongate and expand their ramification into the stratum radiatum until P24 in the rat (Pokorny and Yamamoto, 1981).

Through development, these dendritic trees become increasingly decorated by dendritic spines, which emerge as protrusions on dendritic branches or from retracting filopodia (Fiala et al., 1998). During their maturation, dendritic spines acquire a distinctive mushroom-like shape, which is determined by their actin cytoskeleton (see Pasquale, 2005). The presence of EphA4 in dendritic branches, filopodia, and spines provides evidence for a role in the postnatal development of these structures. Indeed, EphA4 has known functions in the morphological shaping of dendritic spines, in the mature hippocampus (Bourgin et al., 2007; Fu et al., 2007; Murai et al., 2003; Richter et al., 2007; Zhou et al., 2007).

Moreover, other Eph receptors (EphB1, EphB2 and EphB3) regulate dendritic spine morphogenesis and functional maturation, in postnatal hippocampal slices or primary neuron cultures (Ethell et al., 2001; Henkemeyer et al., 2003; Kayser et al., 2006; Penzes et al., 2003). In vivo, the CA3 region of EphB1-B2-B3 triple knockout mice has fewer spines, with headless morphologies or small and immature bulbous heads (Henkemeyer et al., 2003). Together with the present observations, these data support an important role for EphA4 in spine formation.

Growth and Guidance of Axonal Projections

The excitatory mossy fiber, commissural, and associative pathways (including the Schaffer collaterals) terminate on dendritic spines of the principal neuronal cells, particularly in the stratum radiatum of CA1, the stratum lucidum/radiatum of CA3, and the polymorphic layer of the DG (see Andersen et al., 2007), which were here examined by EM. These likely represent the major EphA4-labeled axonal elements described at the ultrastructural level. The role of Eph receptors in axon guidance was initially suggested by their immunocytochemical detection in axons (see Pasquale, 2005). In the present study, the localization of EphA4 in axons, growth cones, and filopodia suggests its involvement in growth and guidance, notably of the commissural and mossy fiber projections which reach their targets shortly after birth (Amaral and Dent, 1981; Super and Soriano, 1994).

In line with this hypothesis, specific defects in the guidance of the commissural axons were observed in ephrin-A5$^{-/-}$ mice, together with a diminution of axon terminal density from both commissural and mossy fiber projections (Otal et al., 2006). Tracing experiments in EphA5-Fc over-expressing mice, in which EphA/ephrin-A signaling is disrupted, notably revealed a reduced density of commissural axon terminals, and longer mossy fiber axons with larger terminals and greater number of often ectopic synaptic contacts (Martinez et al., 2005). These observations could also be interpreted as involving EphA4, a receptor that interacts with all ephrin-As. Moreover, ephrin-As are involved in the outgrowth, branching, and pruning of hippocampal axons (Brownlee et al., 2000; Gao et al., 1999), as well as collapse of hippocampal growth cones (Dail et al., 2006; Richter et al., 2007), and EphA4 is known to participate in multiple axon guidance events throughout the CNS (Coonan et al., 2001; Dufour et al., 2006; Eberhart et al., 2002; Egea et al., 2005;

Helmbacher et al., 2000; Huffman and Cramer, 2007; Kullander et al., 2003; Marin et al., 2001; St John et al., 2002; Vanderhaeghen et al., 2000).

Assembly, Maturation and Plasticity of Axon Terminals

Especially from P14 onwards, EphA4 was frequently localized to axon terminals contacting small dendritic branches, filopodia or spines. There, it was usually associated with groups of vesicles and sometimes with the synaptic plasma membrane. This is in agreement with the results of Bouvier et al. (2008), following subcellular fractionation of adult mouse forebrain, showing the presence of EphA4 and EphB2 in the pre-synaptic active zone fractions. At the active zone, EphA4 could induce the assembly and maturation of axon terminals. Indeed, EphB2 was shown to promote the assembly of the pre-synaptic specialization in primary cortical neurons (Kayser et al., 2006). Moreover, EphA4 was often localized on the extrasynaptic plasma membrane. From this site, it could participate in the structural remodeling of axon terminals, in line with its equivalent role in dendritic spines (Bourgin et al., 2007; Fu et al., 2007; Murai et al., 2003; Richter et al., 2007; Zhou et al., 2007). Indeed, to different extents, axon terminals and dendritic spines perform similar structural changes (Deng and Dunaevsky, 2005; Holtmaat et al., 2008; Konur and Yuste, 2004; Umeda et al., 2005).

Association of Astrocytic Processes with Cerebral Vasculature and Excitatory Synapses

Up to P14, EphA4 was detected essentially on the perikarya and on relatively large processes of astrocytes. EphA4 then became gradually noticeable in processes of smaller sizes and by P21 was restricted to the most distal processes. This spatio-temporal immunolabeling pattern partly reflects the morphological maturation of protoplasmic astrocytes, which only have their main processes and a dozen secondary processes devoid of ramification at P7, but progressively develop a fine, dense and elaborate arborization by P21 (Bushong et al., 2004). Nevertheless, as in neurons, EphA4 disappears from the surface of astrocytic perikarya and proximal processes and becomes restricted to the distal portions of the processes at maturity, i.e. near synapses and endothelial cells. Indeed, we found that EphA4-labeled astrocytic processes initially contact the basal lamina of blood vessels as well as axon terminals, dendritic branches and dendritic spines, between P1 and P14,

whereas, by P21, they become essentially associated with excitatory synapses, and no longer with blood vessels.

Close interactions of endothelial cells, constituting the wall of cerebral microvessels, with neurons, pericytes, and astrocytes contribute to the formation and function of the blood brain barrier (see Banerjee and Bhat, 2007). After spinal cord injury in wild-type mice, astrocytes also tightly associate with blood vessels, whereas in EphA4$^{-/-}$ mice, the much shorter astrocytic processes fail to create such dense perivascular networks (Goldshmit et al., 2006). This suggests a role for astrocytic EphA4 in the induction of the blood brain barrier, during neural development as well as during repair. The observation of the EphA4-labeled plasma membrane of astrocytes in contact with the basal lamina suggests an interaction with releasable ligands present in the latter extracellular matrix structure. Such releasable ligands could be ephrin-As (Nestor et al., 2007) or other types of molecules as reported recently (Tsuda et al., 2008).

At maturity, the localization of EphA4 in pre- and postsynaptic elements, as well as in perisynaptic astrocytic processes, is consistent with its roles in the dynamic and cooperative association between dendritic spines and astrocytic processes (Haber et al., 2006). In hippocampal slice cultures, the respective activation of astrocytic or dendritic EphA4 receptors can induce a coordinated extension of astrocytic processes and retraction of dendritic spines (Murai et al., 2003; Nestor et al., 2007). These opposing changes suggest that EphA4 is coupled to different downstream signaling cascades, in neurons and astrocytes, to differently rearrange their respective actin cytoskeleton (Nestor et al., 2007).

Myelination of Axons

Lastly, EphA4 immunoreactivity was also found as intense spots on the plasma membrane of axons undergoing myelination. This suggests interactions between axonal EphA4 and oligodendrocytic ephrins, possibly ephrin-B3 which is expressed by myelinating oligodendrocytes, at least in the mouse spinal cord (Benson et al., 2005). These could be involved in myelination. The reduced EphA4 immunoreactivity in the alveus fiber tract, after P14, may be due to a decreasing rate of myelination at the later stages of development. Moreover, it will be important to examine the presence of EphA4 in other

developing myelinated fascicles of the CNS. A role for EphA4 in myelination would make this protein an interesting target to consider in myelination diseases.

Conclusion

During the postnatal development of the rat hippocampus, the cell surface EphA4 redistributes between the perikarya and its processes, according to the local needs of maturing neuronal and astrocytic cells for dynamic remodeling of their actin cytoskeleton. Indeed, EphA4 is localized on neuronal cell bodies when they migrate and position themselves within the cellular layers; on dendritic branches undergoing elongation and ramification; on small dendritic branches, filopodia and spines during morphogenesis of the dendritic spines; on axons, axonal growth cones and filopodia during the growth and guidance of hippocampal projections; and finally on synapse-associated dendritic spines, axon terminals and astrocytic processes near and at maturity, when excitatory synapses form and require plasticity or maintenance. Consequently, the multiple subcellular locations of EphA4 correlate with the cellular sites where remodeling occurs during growth, functional maturation and plasticity.

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Figures

Figure 1. Light photomicrographs of EphA4-immunostained sections of the hippocampus from P1 (**A**,**E**,**I**,**M**), P7 (**B**,**F**,**J**,**N**), P14 (**C**,**G**,**K**,**O**) and P21 (**D**,**H**,**L**,**P**) rats. Overall, the intensity of the EphA4 immunoperoxidase staining diminished gradually between P1 and P21, especially in the principal cell layers, as shown at higher magnification of the fields framed in the CA1 region (**E-H**), the CA3 region (**I-L**), and the DG (**M-P**) of pictures **A** to **D**. At all ages, the neuropil layers were strongly stained, except stratum lacunosum-moleculare (l-m). Note the mossy fiber terminals (mf) and alveus fiber tract (av) becoming intensely stained at P14 and P21. Other abbreviations: g, granule cell layer; l/r, stratum lucidum/radiatum; m, molecular layer; o, stratum oriens; p, pyramidal cell layer; po, polymorphic layer. Scale bars: 500 μm.

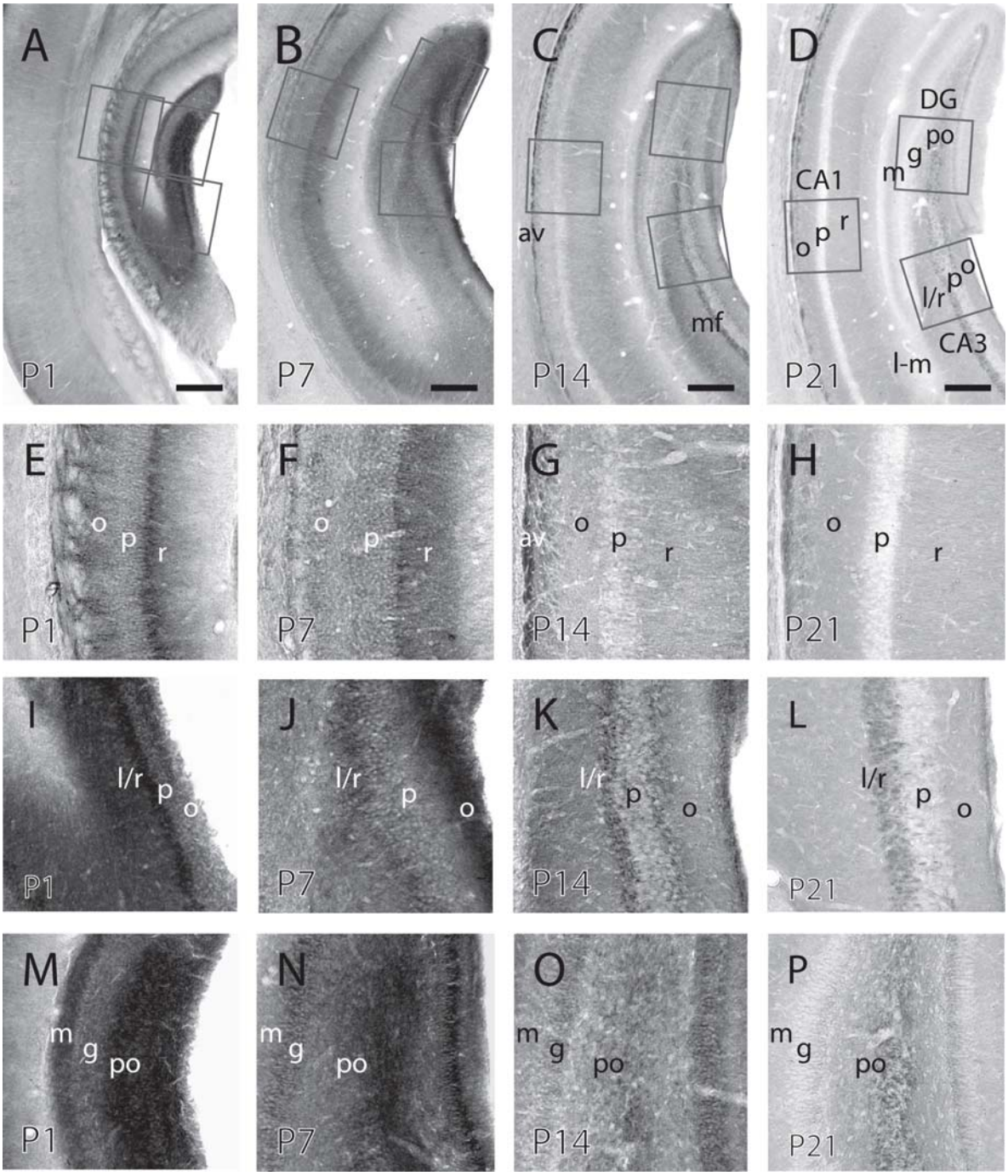

Figure 1. Light photomicrographs of EphA4-immunostained sections of the hippocampus from P1, P7, P14, and P21 rats

Figure 2. Electron micrographs showing EphA4 immunolabeling in neuronal cell bodies (colored in light pink) from the granule cell layer at P1 (**A**), the CA3 pyramidal cell layer at P7 (**B**) and the CA1 pyramidal cell layer at P14 (**C**). In **A** to **C**, the intracellular labeling (open arrows) is associated with the Golgi apparatus (g), endoplasmic reticulum (er) or various types of vesicles. It is also present at the cell surface, as spots on the plasma membrane (filled arrows), often at sites of contact with unlabeled axon terminals (t-; **A**) or adjacent cell bodies (**B**). N, nucleus. Scale bars: 500 nm.

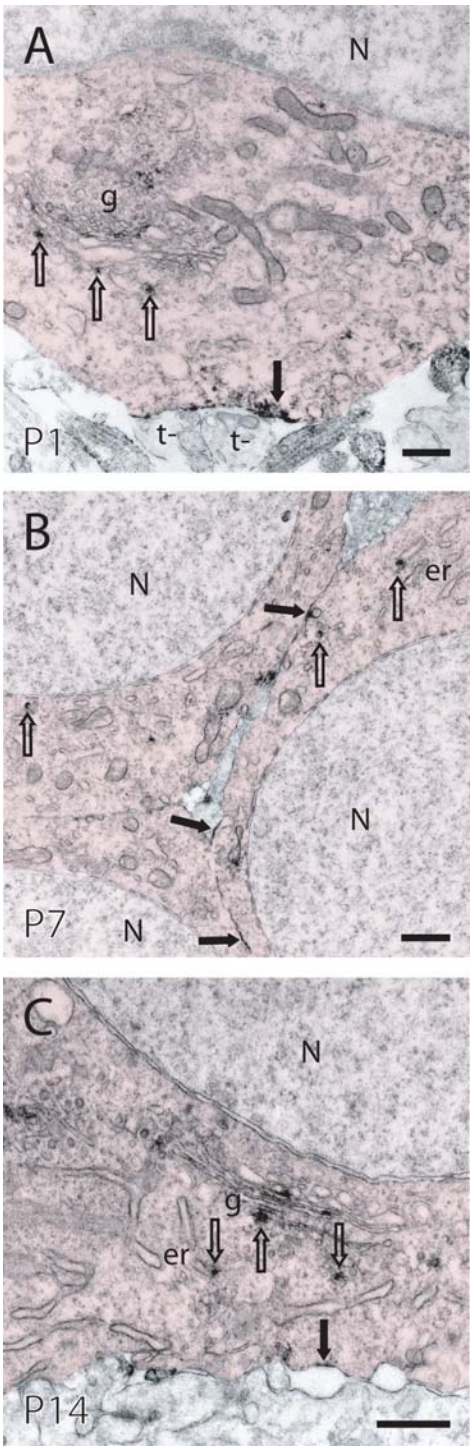

Figure 2. Electron micrographs showing EphA4 immunolabeling in neuronal cell bodies

Figure 3. EphA4-immunoreactive dendritic branches (colored in light yellow) from P1 (**A**), P7 (**B**) and P14 rats (**C**,**D**). In **A**, two dendritic branches from CA1 stratum radiatum display labeled vesicles (open arrows), in addition to spots of labeling on their plasma membrane (filled arrows), notably near a synapse (arrowhead) with an unlabeled axon terminal (t-). **B**: A dendritic branch containing a labeled vesicle (open arrow), and spots of labeling on the plasma membrane at the head of two short extending filopodia (filled arrows) near labeled axonal filopodia (colored in light blue; filled arrows), in CA3 stratum lucidum/radiatum. **C**: Two labeled dendritic branches with one presenting labeling on the plasma membrane (filled arrow) near a labeled axonal filopodia (light blue), and the other with staining on microtubules (open arrow), also in CA3 stratum lucidum/radiatum. **D**: Apical dendritic branch from a CA1 pyramidal cell, in stratum radiatum, with spots of staining on the plasma membrane (top filled arrows), some juxtaposed to an unlabeled axon terminal. Labeled vesicles are also visible (open arrow on the left), as well as labeled microtubules (open arrow at the bottom) near spots of plasma membrane labeling (filled arrow). Scale bars: 500 nm.

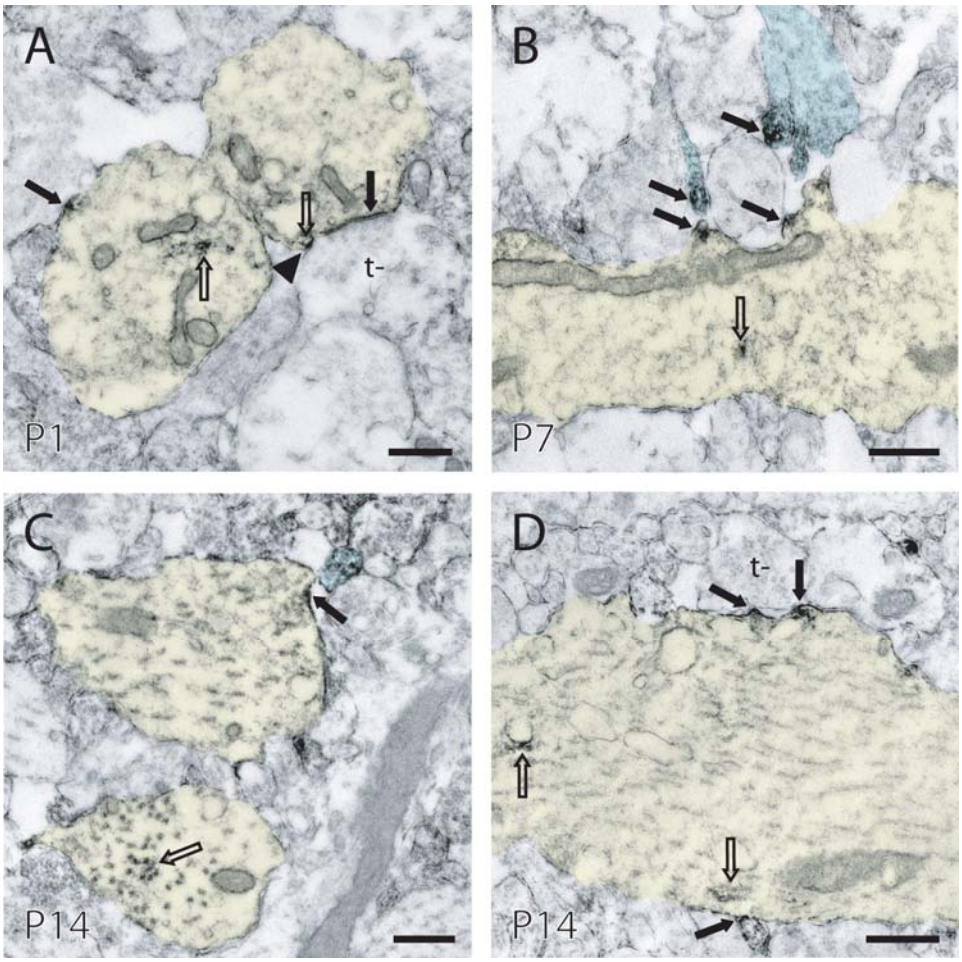

Figure 3. EphA4-immunoreactive dendritic branches

Figure 4. Dendritic spines showing EphA4-immunoreactivity on the plasma membrane, spiny apparatus and post-synaptic density, in the neuropil of P14 (**A**,**B**) and P21 (**C**) rats. **A**: This strongly labeled spine (s+) from the DG polymorphic layer is contacted (arrowhead) by an unlabeled axon terminal (t-). **B**) Labeled dendritic spine (s+) in CA1 stratum radiatum in asymmetric synaptic contact with an unlabeled axon terminal (t-). **C**: Axospinous synapse (arrowhead) between a labeled dendritic spine (s+) and an unlabeled axon terminal (t-) in the DG polymorphic layer. Scale bars: 500 nm.

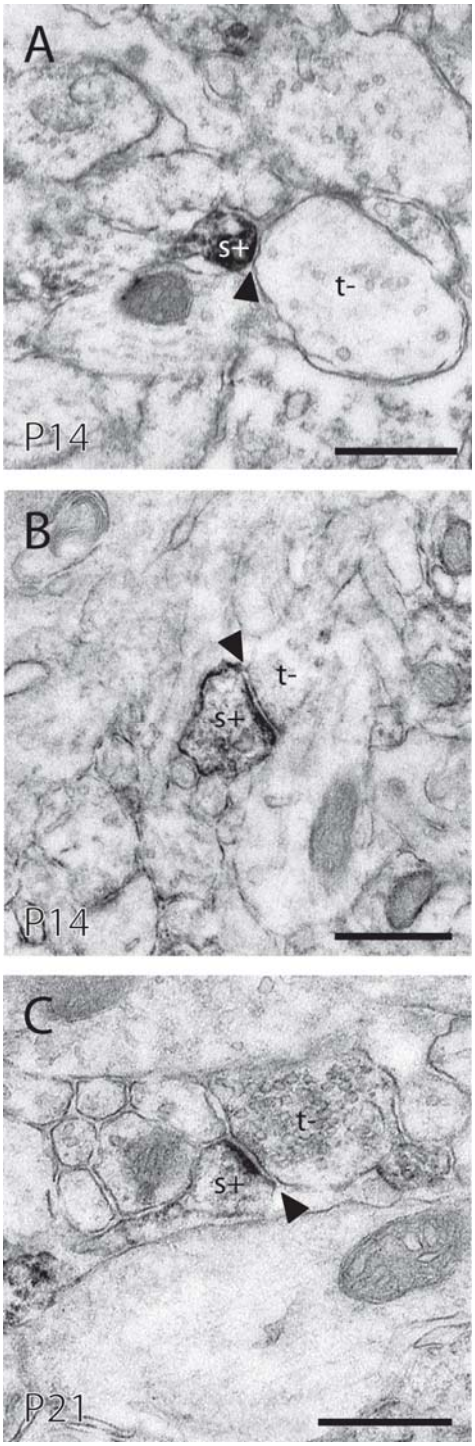

Figure 4. Dendritic spines showing EphA4-immunoreactivity

Figure 5. EphA4-immunolabeled axonal growth cones and filopodia (colored in light blue), at P1 (**A**) and P7 (**B**,**C**). **A**: Axonal growth cones from the DG polymorphic layer displaying intracellular vesicular, as well as spots of surface labeling (filled arrows). **B**: Axonal growth cone near an unlabeled dendritic branch (d-) and displaying vesicle and plasma membrane (filled arrows) labeling in the CA1 stratum radiatum. **C**: Axonal filopodia observed in DG polymorphic layer, growing along an unlabeled dendritic filopodia (colored in light yellow), is labeled at the plasma membrane (arrows) at sites of contact with the dendritic filopodia, as well as near an unlabeled dendritic branch (d-). Scale bars = 500 nm.

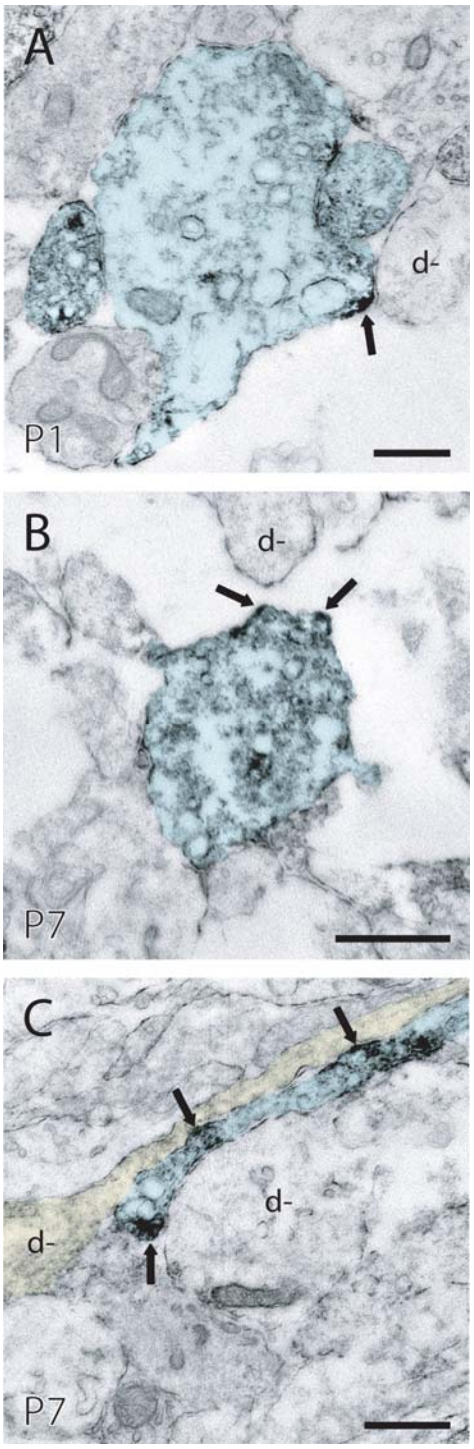

Figure 5. EphA4-immunolabeled axonal growth cones and filopodia

Figure 6. EphA4-immunolabeled axons, axon terminals, and axonal filopodia at P1 (**A**), P7 (**B**,**C**), P14 (**D**,**E**), and P21 (**F**). **A**) A labeled axon terminal (t+) contacts an unlabeled dendritic branch (d-; arrowhead) in CA3 stratum lucidum/radiatum. af+, labeled axonal filopodium. **B**) A labeled axon terminal or filopodium (af+) contacting a dendritic filopodium (df-) protruding from an unlabeled dendritic branch in CA1 stratum radiatum. **C**) Labeled axon terminal (t+) in synaptic contact (arrowhead) with an unlabeled dendritic branch (d-), and presenting a strong extrasynaptic spot of labeling on the plasma membrane (arrow; CA1 stratum radiatum). Note the labeling of the external membrane of the nearby mitochondrion. **D** and **E**) Numerous axon terminals (t+), axons (a+), and an axonal filopodium (af+), display labeled subsets of vesicles (open arrows) or spots of labeling on plasma membrane (filled arrows; d-, unlabeled dendritic branch; s-, unlabeled dendritic spine) in CA1 stratum radiatum. **F**) In DG polymorphic layer, an axon terminal (t+) presents labeled vesicles (open arrow), near a synaptic junction (arrowhead) with an unlabeled dendritic spine (s-), while an axon (a+) linking two unlabeled terminals (t-) shows strong labeling at one spot of its plasma membrane (arrow). Scale bars = 500 nm.

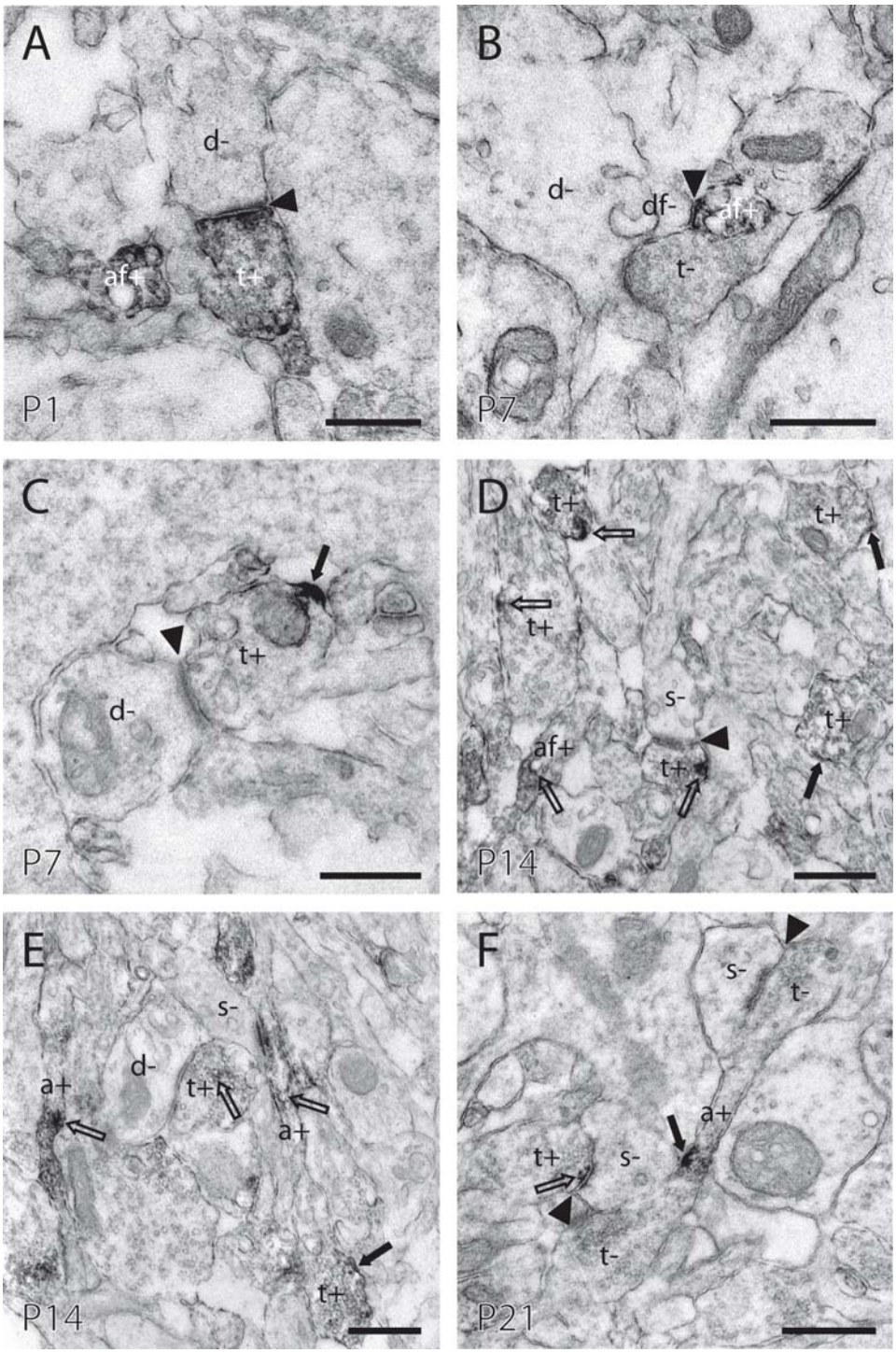

Figure 6. EphA4-immunolabeled axons, axon terminals, and axonal filopodia

Figure 7. EphA4-immunolabeled mossy fiber axons and axon terminals (both colored in light blue), in CA3 stratum lucidum from P14 rats (**A-C**). In **A**, immunonegative axons (a-) are intermingled within a fascicle with immunopositive ones (blue), displaying labeling at their plasma membrane (e.g., filled arrows). **B**: Two large mossy fiber terminals present subsets of immunoreactive vesicles (open arrows), sometimes near the plasma membrane (filled arrow) opposite to a synaptic contact with an unlabeled dendritic branch (d-). **C**: The complex mossy fiber terminal shows subsets of labeled vesicles (open arrows) and makes synaptic contacts with a dendrite (d+) containing a labeled vesicle (open arrow) and two unlabeled thorny excrescences (s-). Scale bars = 500 nm.

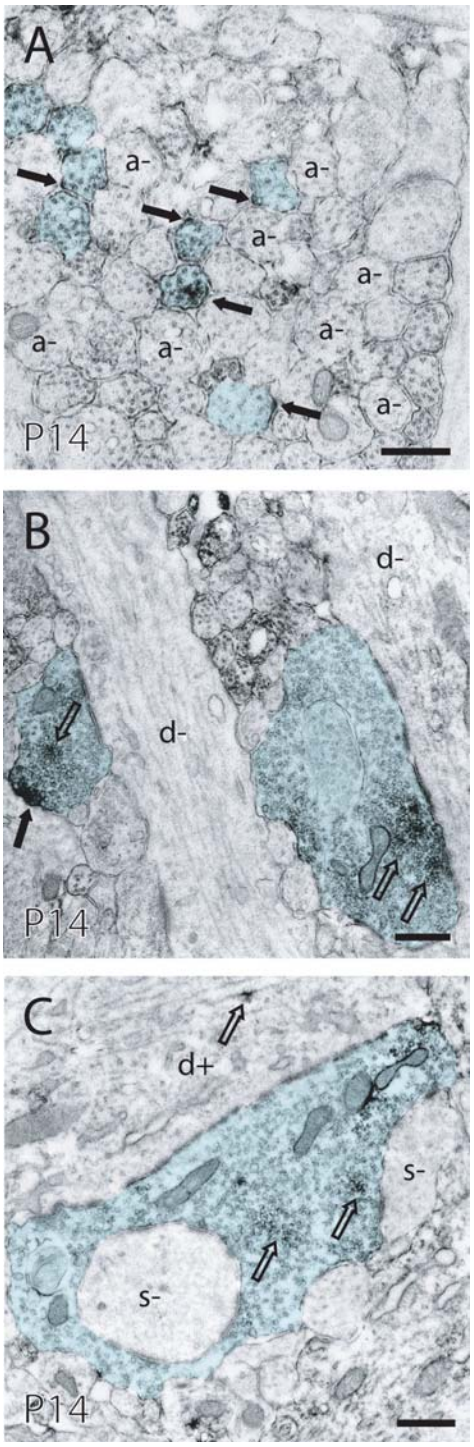

Figure 7. EphA4-immunolabeled mossy fiber axons and axon terminals

Figure 8. EphA4-immunoreactive astrocytic cell bodies and processes (colored in light green), at P1 (**A**,**B**), P7 (**C**,**D**), and P14 (**E**,**F**). **A**) An astrocytic perikaryon from CA1 stratum radiatum displays spots of labeling on the endoplasmic reticulum (er), Golgi apparatus (g), vesicles (open arrow), and plasma membrane (filled arrow) juxtaposing the endothelial cell of a blood vessel (BV; N, nucleus). **B**) A large-caliber process adjacent to an unlabeled axon terminal (t-) shows reactive vesicles (open arrows) and plasma membrane (filled arrows), in CA3 stratum lucidum/radiatum. **C**) A large-caliber process with spots of immunostaining on the plasma membrane, where it contacts the basal membrane of a blood vessel (BV; CA1 stratum radiatum). **D**) Astrocyte, with vesicle (bottom open arrow) and extending processes near synapses (arrowheads) between unlabeled dendritic branches (d-) and axon terminals (t-), in CA3 stratum lucidum/radiatum. **E**: In CA1 stratum radiatum, a small-caliber astrocytic process adjacent to a blood vessel (BV) is stained at the plasma membrane (filled arrow). **F**: An astrocytic process with immunopositive vesicles (open arrow) and plasma membrane (arrows) is found near an axon terminal containing labeled vesicles (t+) and small unmyelinated axons (a+), in DG polymorphic layer. Scale bars = 500 nm.

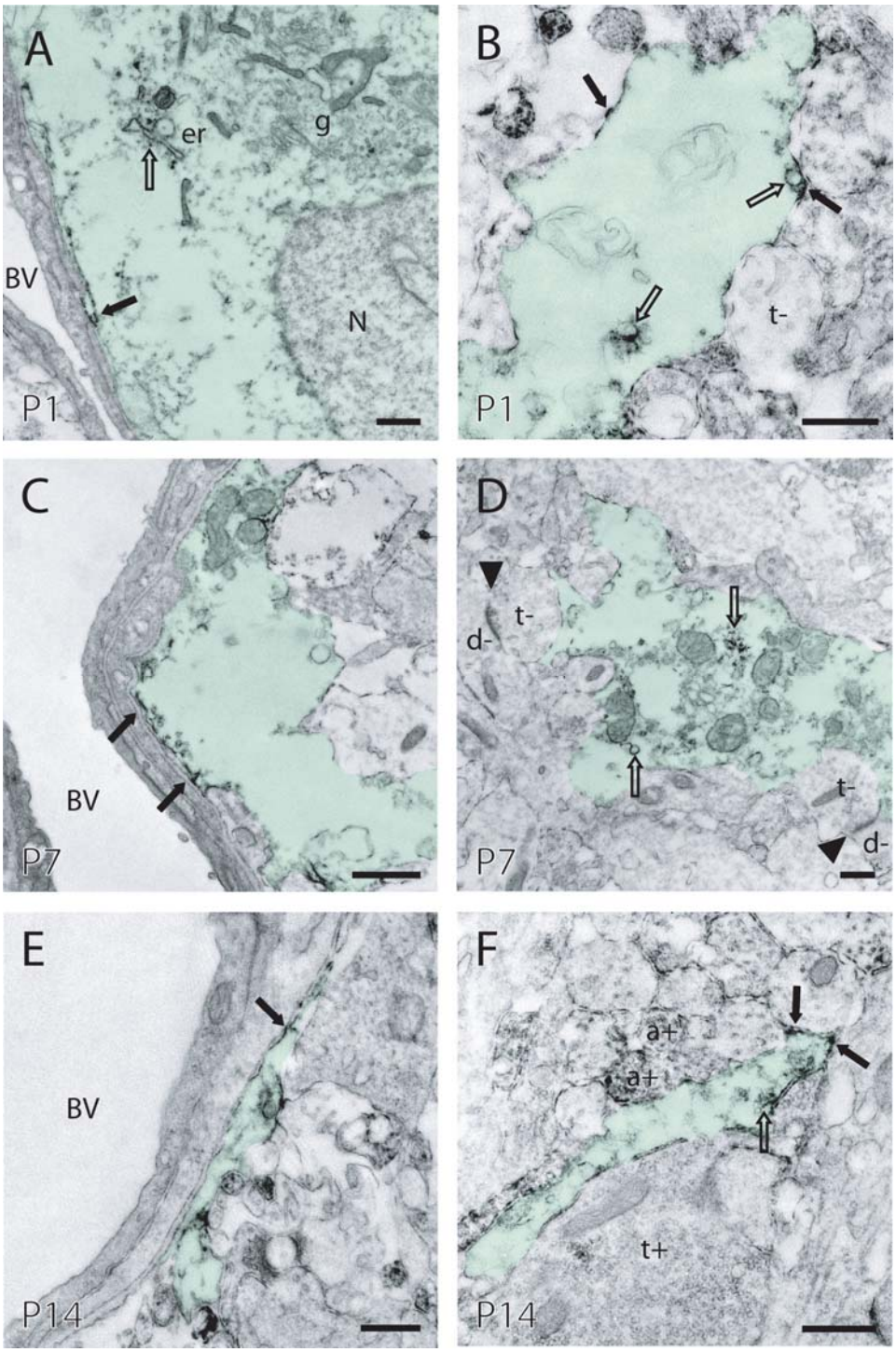

Figure 8. EphA4-immunoreactive astrocytic cell bodies and processes

Figure 9. Myelinating axons displaying intense spots of EphA4 immunostaining (colored in light blue), in CA1 stratum oriens and the alveus fiber tract, at P14 (**A**-**D**) and P21 (**E**,**F**). **A)** A labeled axon (left) is encircled by an oligodendrocytic process (colored in light lavender) and displays a spot of intense reactivity beneath its plasma membrane. Note that the nearby microtubules and mitochondrion are also stained. In **B** (P14), **E** (P21) and **F** (P21) are displayed other examples of axons encircled by olidendrocytic processes and displaying intense spots of immunostaining at the surface between the two cell types. **C**) An axon at more advanced stage of myelination, also showing a similar spot of labeling (arrow), at the site of contact with the cytoplasm-filled oligodendrocytic process (light lavender). **D**) Myelinated axon showing labeled microtubules (bottom right corner). Another axon (a-), encircled by oligodendrocytic process (o-) shows no labeling. Scale bars = 500 nm.

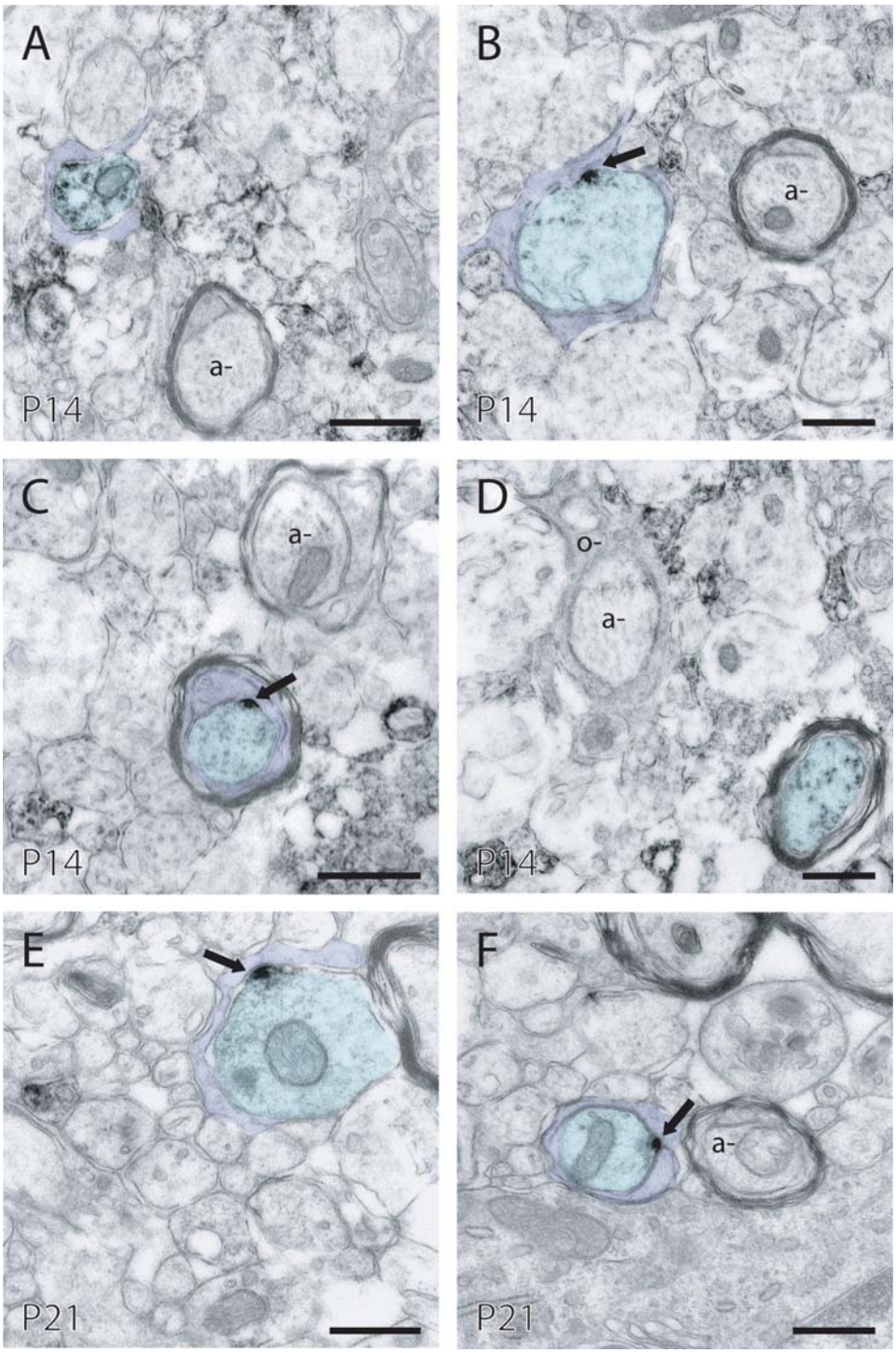

Figure 9. Myelinating axons displaying intense spots of EphA4 immunostaining

Participation d'EphA4 à la migration des cellules granulaires générées dans l'hippocampe adulte

Marie-Ève Tremblay, Mustapha Riad, Karl J. Fernandes et Guy Doucet

Département de pathologie et biologie cellulaire et Groupe de recherche sur le système nerveux central, Université de Montréal.

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1. Introduction

Chez les mammifères adultes, la neurogenèse se poursuit dans deux niches neurogéniques, la zone sous-ventriculaire (ZSV) du ventricule latéral et la zone sous-granulaire (ZSG) du gyrus dentelé de l'hippocampe. Les neuroblastes générés dans la ZSV, à partir de cellules souches astrocytaires, migrent vers le bulbe olfactif, où ils se différencient en interneurones inhibiteurs, nommément les cellules granulaires et périglomérulaires. Dans la ZSG, les cellules souches astrocytaires génèrent des cellules progénitrices, qui se différencient en cellules granulaires excitatrices, lesquelles sont intégrées dans la circuiterie hippocampique locale. La neurogenèse dans la ZSV semble participer à la rétention de la mémoire olfactive (Enwere et al., 2004; Mak et al., 2007; Rochefort et al., 2002), alors que celle dans la ZSG serait requise pour la rétention de la mémoire spatiale et l'apprentissage contextuel de la peur, tel que récemment démontré par la performance comportementale de souris transgéniques adultes chez lesquelles les cellules progénitrices multipotentes sont sélectivement éliminées par apoptose, au moment où elles débutent leur différenciation neuronale (Imayoshi et al., 2008). En outre, la prolifération, la migration, la maturation et la survie des nouveaux neurones sont étroitement régulées en fonction du stade développemental, la région du SNC, l'expérience et l'état de santé de l'animal. Plusieurs études ont identifié des neurotransmetteurs, hormones, facteurs de croissance et stimuli environnementaux qui participent à leur régulation. Des molécules de guidage axonal sont aussi impliquées, notamment les Eph et les éphrines (Efn) (voir Kempermann, 2006; Lledo et al., 2006).

Quelques études ont révélé la participation d'Eph et d'Efn à la genèse, la migration, la maturation ou la survie de neurones chez l'adulte (Chumley et al., 2007; Conover et al., 2000; Furne et al., 2008; Holmberg et al., 2005; Jiao et al., 2008; Ricard et al., 2006). Dans l'hippocampe, EphB1 et EphB2 sont impliqués dans la prolifération, la migration et le développement dendritique des cellules progénitrices ou granulaires immatures (Chumley et al., 2007). En effet, des souris knockouts pour EphB1 ou doublement knockouts pour EphB1 et EphB2 ont un nombre significativement réduit de cellules progénitrices (nestine-positives) dans le gyrus dentelé, comparativement à des souris de type sauvage. De plus, ces souris ont des cellules progénitrices, prolifératives (5-bromo-2-désoxyuridine (BrdU) -positives) et granulaires immatures (doublecortine (DCX) -positives) anormalement

positionnées en dehors de la ZSG, ainsi que des cellules granulaires immatures ayant des embranchements dendritiques précoces, dans la couche granulaire, et une arborisation dendritique basale anormale, dans la couche polymorphe (Chumley et al., 2007). Des souris knockouts pour l'EfnB3 ont aussi des cellules progénitrices et granulaires immatures en position ectopique ou ayant une arborisation basale anormale, ce qui suggère que la migration cellulaire et le développement dendritique soient régulés par des interactions entre EphB1 ou EphB1 et l'EfnB3. Le marquage X-gal chez des souris knockins exprimant la β-galactosidase sous le contrôle du promoteur d'EphB1 ou de l'EfnB3 a aussi révélé la distribution d'EphB1 dans la ZSG et celle de l'EfnB3 dans les couches polymorphe et moléculaire interne du gyrus dentelé (Chumley et al., 2007).

Nous avons récemment étudié chez le rat la distribution laminaire et la localisation subcellulaire d'EphA4, par immunocytochimie en microscopie photonique et électronique (Chapitres II et III). Dans le gyrus dentelé, EphA4 est localisé, entre autres, dans les couches de cellules granulaires (incluant la ZSG), polymorphe et moléculaire interne, de la naissance jusqu'à l'âge adulte, avec un niveau d'immunoréactivité qui diminue graduellement pendant le développement postnatal, surtout dans la couche de cellules granulaires. Dans les cellules granulaires, EphA4 décore la surface des corps cellulaires et de leurs prolongements dendritiques et axonaux, entre les jours 1 et 14 suivant la naissance (P1 et P14). Il est ensuite principalement localisé dans les épines dendritiques et les terminaisons axonales associées aux synapses excitatrices, à P21 et chez l'adulte. En lien avec les rôles connus d'EphA4 dans la migration et le positionnement des corps cellulaires (Barrios et al., 2003; Smith et al., 1997), la croissance et le guidage axonaux (Coonan et al., 2001; Eberhart et al., 2002; Egea et al., 2005; Helmbacher et al., 2000; Huffman and Cramer, 2007; Marin et al., 2001; Vanderhaeghen et al., 2000), puis la maturation et la plasticité des synapses excitatrices (Bourgin et al., 2007; Grunwald et al., 2004; Murai et al., 2003a; Richter et al., 2007; Zhou et al., 2007), nos observations appuient l'hypothèse de rôles similaires lors de la maturation des cellules granulaires générées chez l'adulte. Par ailleurs, la ressemblance entre la distribution laminaire d'EphA4 et celle de l'EfnB3 suggère leurs interactions potentielles, lesquelles participeraient à la migration cellulaire et à la maturation dendritique des cellules granulaires générées chez l'adulte (Chumley et al., 2007). En outre, la présence d'EphA4 dans la ZSG suggère qu'il pourrait aussi exercer un rôle dans la prolifération ou la migration des cellules progénitrices.

Pour vérifier l'hypothèse selon laquelle EphA4 serait impliqué dans la prolifération, la migration ou la maturation cellulaires, lors de la neurogenèse chez l'adulte, nous avons d'une part examiné sa présence dans les cellules progénitrices, prolifératives et granulaires immatures dans la ZSG de l'hippocampe ventral et dorsal du rat adulte et, d'autre part, le positionnement cellulaire, la morphologie et la polarité de l'arborisation dendritique des cellules granulaires immatures, chez des souris adultes knockouts pour EphA4 et leurs congénères de type sauvage. Pour ce faire, nous avons utilisé l'immunocytochimie en microscopie confocale à fluorescence, envers des indicateurs de cellules progénitrices (nestine), prolifératives (Ki-67) et granulaires immatures (DCX) (voir Ming and Song, 2005; Figure 6 du Chapitre I).

2. Matériel et méthode

2.1. Animaux

Nous avons utilisé trois rats adultes Sprague-Dawley (Charles River, St-Constant, Québec, Canada), deux souris adultes knockouts pour EphA4 et deux souris de type sauvage des mêmes portées, fournies par Dre Mirella Dottori (Center for Reproduction and Development, Monash Institute of Medical Research, Clayton, Australia) (Dottori et al., 1998). Les animaux étaient hébergés à température (21°C) et humidité (60%) constantes, sous un cycle de 12 h de clarté/noirceur, avec accès libre à la nourriture et à l'eau. Les protocoles expérimentaux ont été approuvés par le Comité de déontologie de l'expérimentation sur les animaux de l'Université de Montréal. Toutes les procédures ont été réalisées en respectant les directives du Manuel sur le soin et l'utilisation des animaux d'expérimentation (Ed2) du Conseil canadien de protection des animaux. Les animaux ont été profondément anesthésiés avec une solution de pentobarbital sodique (80 mg/kg, i.p.) et perfusés par l'arc aortique avec une solution d'acroléine 0,5% suivie d'une solution de paraformaldéhyde (PFA) 4,0%, dans du tampon phosphate sodique 0,1M (TP), à pH 7,4. Le cerveau a été prélevé, immergé dans la PFA 4,0% pendant 1 heure à 4^{o}C, puis rincé dans du TP salin (TPS; 0,9% NaCl dans TP 50 mM, pH 7.4).

2.2. Anticorps

La production et la caractérisation de l'anticorps anti-EphA4 (Ab11) généré chez le lapin contre les 11 derniers acides aminés C-terminaux d'EphA4, lesquels sont identiques chez le rat, la souris, le poulet et l'humain, ont précédemment été décrites en détails (Soans et al., 1994). Nous avons aussi démontré la spécificité du marquage immunocytochimique obtenu avec cet anticorps, telle que révélée par l'absence de marquage sur des coupes du cerveau de souris néonatales ou adultes knockouts pour EphA4 traitées en parallèle avec des coupes de souris congénères de type sauvage (Chapitres II et III). Des anticorps dirigés contre les marqueurs Ki-67 (BD Pharmingen), nestine (BD Pharmingen) et DCX (Santa Cruz Biotechnology) ont aussi été utilisés.

2.3. Immunocytochimie

Des coupes transversales du cerveau (50 μm d'épaisseur) ont été obtenues avec un vibratome dans du TPS froid, à des niveaux rostrocaudaux incluant les parties ventrale et dorsale de l'hippocampe. Elles ont été traitées au borohydrure de sodium à 0,1% dans du TPS, pendant 30 min à la température de la pièce, rincées dans du TPS, puis incubées flottantes à la température de la pièce pendant 2 h dans une solution de blocage contenant du sérum normal d'âne (EphA4 avec DCX) ou de chèvre (EphA4 avec nestine ou Ki-67) à 5% et de la gélatine à 0,5%, dans du TPS, puis pendant 48 h en présence des anticorps primaires de lapin anti-EphA4 (1:500), de souris anti-nestine (1:200), de souris anti-Ki-67 (1:100), ou de chèvre anti-DCX (1:500) dans les mêmes solutions de blocage.

Pour les simples marquages d'EphA4, de la nestine et du Ki-67, ainsi que les doubles marquages d'EphA4 avec la nestine ou le Ki-67, les coupes ont été incubées pendant 3 h à la température de la pièce, en présence d'un anticorps secondaire de chèvre anti-lapin conjugué à l'Alexa Fluor 488 (Molecular Probes, 1:500 dans la solution de blocage correspondante) pour révéler EphA4, d'un anticorps secondaire de chèvre anti-souris conjugué à l'Alexa Fluor 568 pour révéler la nestine ou le Ki-67, ou en présence des deux anticorps secondaires pour révéler simultanément EphA4 avec la nestine ou le Ki-67. Pour les simples marquages d'EphA4 et de DCX, ainsi que les doubles marquages d'EphA4 avec la DCX, un anticorps secondaire d'âne anti-chèvre conjugué à l'Alexa Fluor 568 a aussi été utilisé (Molecular Probes, 1:500, 3 h à la température de la pièce) pour

révéler DCX. Les coupes ont ensuite été rincées dans du TPS puis incubées à la température de la pièce avec une solution de sérum normal de chèvre à 5% dans du TPS pendant 1 h, puis avec un anticorps secondaire de chèvre anti-lapin conjugué à l'Alexa Fluor 488 pendant 3 h pour révéler EphA4.

Afin de confirmer l'absence de réactivité croisée non spécifique entre les anticorps primaires et secondaires utilisés, nous avons utilisé un anticorps secondaire d'âne anti-chèvre conjugué à l'Alexa Fluor 568 pour révéler EphA4, ainsi qu'un anticorps secondaire de chèvre anti-lapin conjugué à l'Alexa Fluor 488 pour révéler DCX, ce qui a résulté en une absence de marquage. Nous avons aussi comparé la distribution laminaire et la localisation cellulaire du marquage d'EphA4, nestine, Ki-67 et DCX obtenues lors des simples et doubles marquages. Par ailleurs, afin de visualiser les cellules de la couche granulaire, nous avons incubé des coupes pendant 15 min à la température de la pièce avec une solution de Hoescht (Sigma; 1 mM), un marqueur fluorescent de l'ADN nucléaire.

Les coupes ont enfin été rincées dans du TP, montées entre lame et lamelle avec le milieu VectaShield (Vector Laboratories; Burlingame, CA), puis examinées au microscope confocal Leica TCS ou au microscope à fluorescence Zeiss Axiophot.

2.4. Échantillonnage et analyse

La colocalisation d'EphA4 avec la nestine, le Ki-67 ou la DCX dans les cellules prolifératives ou granulaires immatures a été examinée dans des coupes d'hippocampe ventral et dorsal provenant de trois rats adultes (>10 coupes examinées par animal). La colocalisation de Ki-67 et de DCX a aussi été examinée chez une souris knockout pour EphA4 ou sa congénère de type sauvage. Enfin, la position des corps cellulaires dans la couche granulaire, ainsi que la polarité (apicale, basale) et la morphologie (longueur, embranchement) de l'arborisation dendritique des cellules granulaires immatures exprimant le marqueur DCX ont été examinées dans des coupes d'hippocampe ventral et dorsal provenant de deux paires de souris knockouts pour EphA4 et de leurs congénères de type sauvage (>20 coupes d'hippocampe examinées par animal).

3. Résultats

3.1. Localisation d'EphA4 dans les cellules granulaires immatures

Afin de déterminer les types cellulaires de la ZSG exprimant EphA4, nous avons d'abord examiné sa colocalisation avec la nestine, le Ki-67 ou la DCX. Ces doubles marquages immunocytochimiques ont d'abord révélé qu'EphA4 n'est pas colocalisé avec la nestine ni avec le Ki-67; suggérant qu'il n'est pas exprimé par les cellules progénitrices ou prolifératives de la ZSG (non montré). Toutefois, EphA4, qui présente un marquage associé avec certains corps cellulaires de la couche granulaire et de la ZSG, ainsi qu'avec des prolongements cellulaires dans le neuropile (voir Chapitre II), était clairement colocalisé avec la DCX dans des corps cellulaires et des dendrites apicales (Fig. 1C,F; montré par des flèches) de cellules granulaires immatures (DCX-positives) positionnées dans la ZSG. Cette observation démontre la présence d'EphA4 dans les cellules granulaires générées chez l'adulte, comme au cours du développement postnatal précoce (voir Chapitre III).

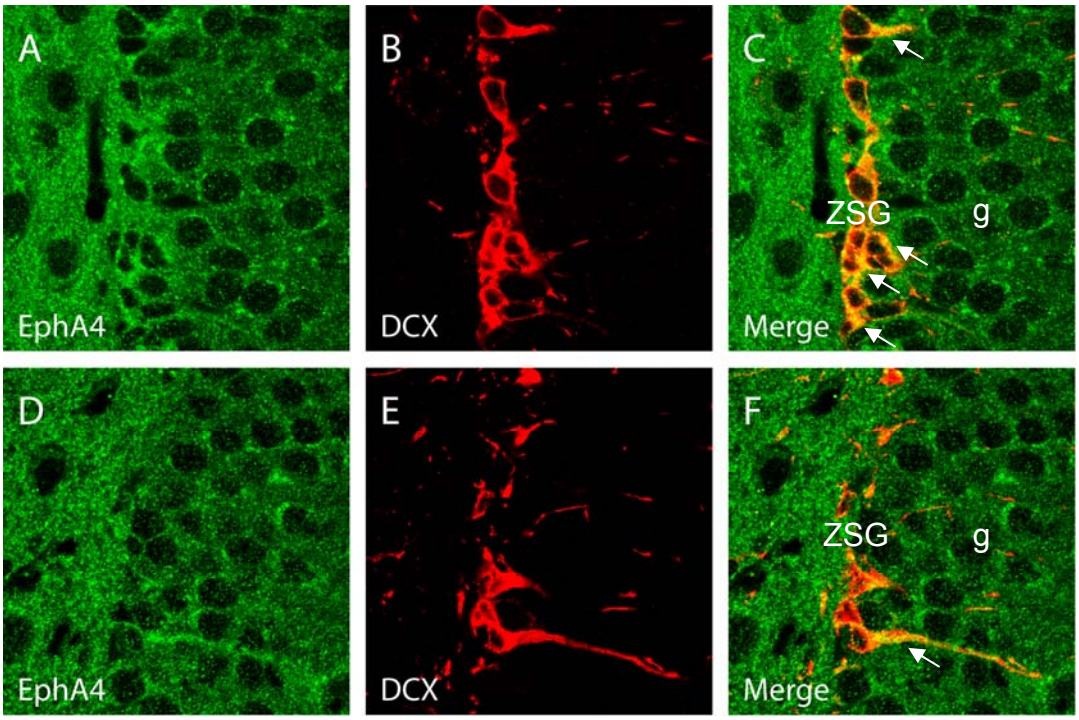

Figure 1. Localisation d'EphA4 dans les cellules granulaires immatures (DCX-positives)

3.2. Cellules granulaires immatures ectopiques chez des souris knockouts pour EphA4

Pour explorer les rôles potentiels d'EphA4 dans la maturation des cellules granulaires générées dans l'hippocampe adulte, nous avons comparé le positionnement du corps cellulaire de ces cellules ainsi que la polarité apicale ou basale et la morphologie de leur arborisation dendritique, dans des coupes d'hippocampe ventral et dorsal chez des souris knockouts pour EphA4 et leurs types sauvages. Nous avons ainsi observé des cellules granulaires immatures DCX-positives ectopiques, leur corps cellulaire étant positionné en dehors de la ZSG (Figure 2B, C, E, F; montrées par des flèches) et parfois même à l'extérieur de la couche de cellules granulaires (Fig. 2F), uniquement chez les souris knockouts. Nous avons compté entre 1 et 5 cellules ectopiques par coupe d'hippocampe (50 µm d'épaisseur). Les cellules granulaires DCX-positives présentaient toutefois une arborisation dendritique uniquement apicale comme chez leurs congénères de type sauvage. Enfin, les cellules granulaires immatures DCX-positives ectopiques observées chez les souris knockouts pour EphA4 n'exprimaient pas le Ki-67, un marqueur des cellules prolifératives (non montré).

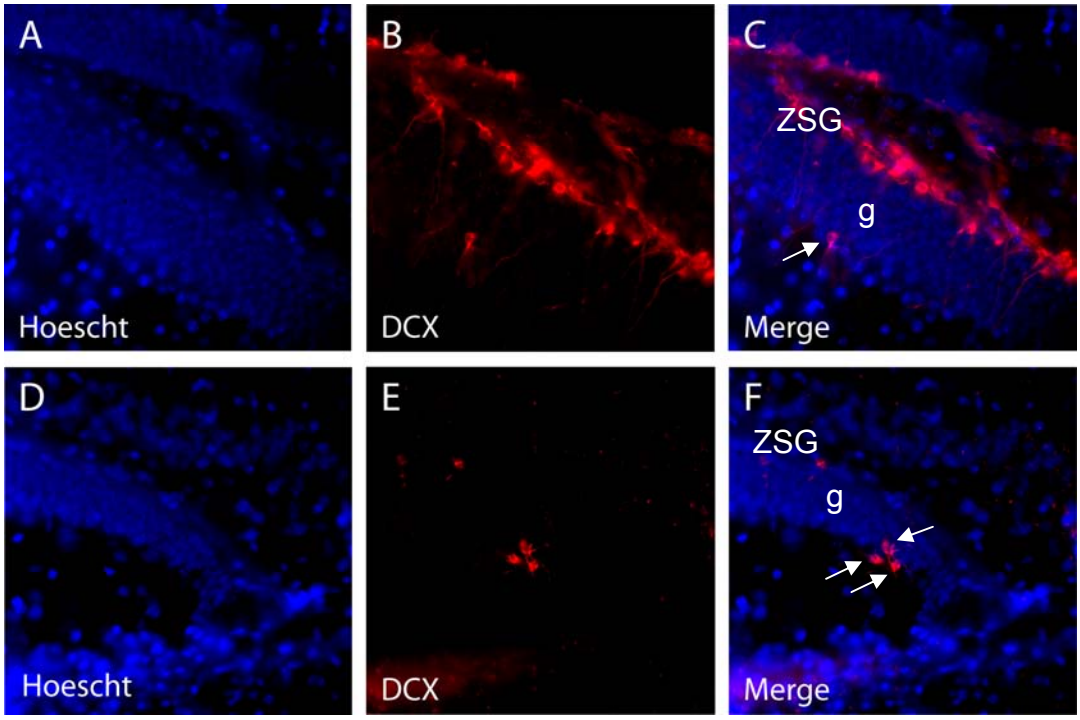

Figure 2. Cellules DCX-positives ectopiques chez des souris knockouts pour EphA4

4. Discussion

Nous avons vérifié la présence d'EphA4 dans les cellules progénitrices (nestine-positives), prolifératives (Ki-67-positives) ou granulaires immatures (DCX-positives) dans la ZSG de l'hippocampe du rat adulte, puis le positionnement cellulaire, la polarité et la morphologie dendritiques des cellules granulaires immatures (DCX-positives), chez des souris adultes knockouts pour EphA4 et leurs congénères de type sauvage. Il s'agit de la première étude du rôle d'EphA4 dans la neurogenèse hippocampique chez l'adulte.

Nos observations montrent qu'EphA4 est colocalisé avec la DCX, mais pas avec la nestine ou le Ki-67; ce qui suggère son expression dans les cellules granulaires immatures, mais pas dans les cellules progénitrices ou prolifératives. De plus, des cellules DCX-positives ectopiques, dont le corps cellulaire était positionné en-dehors de la ZSG et parfois même en dehors de la couche granulaire, ont été observées chez des souris knockouts pour EphA4, mais non chez des souris de type sauvage des mêmes portées. Ces cellules ectopiques étaient non prolifératives, tel que révélé par l'absence de colocalisation de DCX avec Ki-67. Enfin, la polarité et la morphologie de l'arborisation dendritique de ces cellules ne semblaient pas affectées chez les souris knockouts pour EphA4.

4.1. EphA4 et la migration ou la prolifération des cellules progénitrices

L'absence apparente d'EphA4 dans les cellules progénitrices et prolifératives de la ZSG indique que ce récepteur n'est pas impliqué dans la prolifération ou la migration des cellules progénitrices. Au contraire, Chumley et al. (2007) ont récemment montré que les ARNm d'EphB1 et d'EphB2 sont localisés dans les cellules progénitrices de la ZSG, lesquelles expriment la nestine ou la forme polysialylée de la molécule d'adhérence cellulaire neurale (PSA-NCAM), tel que montré par RT-PCR sur des cellules isolées par FACS, à partir de souris exprimant la GFP sous le contrôle du promoteur de la nestine. De plus, EphB1 et EphB2 jouent un rôle dans la prolifération et la migration des cellules progénitrices, tel que révélé notamment par la diminution du nombre de cellules progénitrices dans le gyrus dentelé et leur position ectopique en dehors de la ZSG, chez des souris knockouts pour EphB1 ou doublement knockouts pour EphB1 et EphB2 (voir Introduction et Chumley et al., 2007).

Toutefois, il n'est pas exclu qu'EphA4 puisse participer à la prolifération dans la ZSV. En effet, Conover et al. (2000) ont montré qu'une infusion dans les ventricules latéraux d'EphB2-Fc ou d'EfnB2-Fc pré-agglomérés, mais pas d'EfnB1-Fc pré-agglomérée, perturbe la migration des neuroblastes, en plus d'accroître le nombre de cellules prolifératives dans la ZSV, telles que reconnues par leur incorporation de BrdU et leur morphologie astrocytaire en microscopie électronique (Conover et al., 2000). Mentionnons que la protéine de fusion EphB2-Fc peut lier les EfnB endogènes et bloquer les récepteurs EphB et EphA4, alors que l'EfnB2-Fc et l'EfnB1-Fc peuvent bloquer les interactions entre EphB et EphA4 endogènes, et leurs interactions avec toutes les Efn auxquelles ils se lient normalement. Ainsi, ces observations sont compatibles avec une implication possible d'EphA4 dans la prolifération cellulaire dans la ZSV ou la migration des neuroblastes dans le courant de migration rostral (RMS). En effet, les différences observées à la suite de l'infusion d'EfnB2-Fc ou d'EfnB1-Fc pourraient s'expliquer par l'affinité de liaison plus faible entre EphA4 et l'EfnB1, qu'entre EphA4 et les autres EfnA ou EfnB (Gale et al., 1996b; Mellitzer et al., 1999; North et al., 2007).

Pour infirmer un rôle d'EphA4 dans la prolifération des cellules progénitrices de la ZSG, il sera nécessaire d'examiner le nombre de cellules progénitrices ou prolifératives dans la ZSG chez des souris knockouts pour EphA4 et de les comparer avec leurs congénères de type sauvage.

4.2. EphA4 et la migration des cellules granulaires immatures

Dans le contexte de la neurogenèse chez l'adulte, l'EfnB3 a récemment été impliquée dans la régulation de la migration des cellules progénitrices et granulaires immatures, puisque ces cellules étaient observées en position ectopique, dans les couches granulaire ou polymorphe, chez des souris knockouts pour cette molécule (Chumley et al., 2007). Puisque EphA4 peut lier l'EfnB3 avec une grande affinité, il pourrait aussi être responsable de la traduction de ces signaux.

Par ailleurs, l'EfnB3 n'est pas localisée dans les cellules progénitrices de la ZSG, tout comme EphA4, mais elle jouerait cependant un rôle dans la régulation de leur migration ou positionnement. Pour vérifier l'hypothèse selon laquelle EphA4 soit aussi impliqué dans régulation de la migration des cellules progénitrices, il sera nécessaire de

comparer, chez des souris knockouts pour EphA4 et leurs congénères de type sauvage, la position, dans la ZSG et la couche de cellules granulaires du gyrus dentelé, des cellules progénitrices qui expriment la nestine ou des cellules prolifératives marquées au Ki-67. Nos observations suggèrent toutefois que les cellules ectopiques (DCX-positives) observées chez des souris knockouts pour EphA4 ne sont pas prolifératives, tel que révélé par l'absence de colocalisation entre la DCX et le Ki-67.

4.3. EphA4 et le développement dendritique des cellules granulaires

Nous avons aussi examiné l'arborisation dendritique des cellules granulaires immatures qui expriment la DCX chez des souris adultes knockouts pour EphA4 et leurs congénères de type sauvage. Nos observations préliminaires ne montrent pas de différence apparente dans la polarité ou la morphologie dendritique. Cependant, la présence de dendrites basales anormales dans la couche polymorphe des souris knockouts pour l'EfnB3 (Chumley et al., 2007) suggère qu'une telle anomalie pourrait être observée chez des souris knockouts pour EphA4, ce qui devra être examiné plus en détails.

4.4. EphA4 et la survie cellulaire

Enfin, Furne et al. (2008) ont récemment démontré qu'EphA4 est impliqué dans la régulation de la survie cellulaire. En effet, la surexpression d'isoformes d'EphA4, pourvues ou pas de domaine catalytique, dans des cellules HEK 293T, induit leur mort cellulaire par apoptose, tel que notamment révélé par un marquage de la portion terminale des acides nucléiques de l'ADN en dégradation (*Terminal deoxynucleotidyl transferase dUTP nick end labeling* ou TUNEL), comparativement à leur transfection avec un vecteur témoin. De plus, l'ajout au milieu de culture d'EfnB3 pré-agrégée diminuait la proportion de cellules apoptotiques, de façon dose-dépendante. En outre, des souris knockouts pour EphA4 ont davantage de neuroblastes immunopositifs pour la PSA-NCAM dans la ZSV ou le RMS, que des souris de type sauvage. Le nombre de cellules prolifératives n'est pas augmenté chez des souris knockouts pour EphA4, à la différence des souris de type sauvage, selon un dénombrement des cellules exprimant le Ki-67 ou marquées au BrdU, mais au contraire, leur proportion des cellules marquées par TUNEL est diminuée. Ainsi, l'augmentation du nombre de neuroblastes chez des souris knockouts pour EphA4 résulterait d'une diminution de l'apoptose. Au contraire, des souris knockouts pour l'EfnB3 ont davantage de cellules

marquées par TUNEL, que des souris de type sauvage. Toutes ces données suggèrent ainsi qu'EphA4 puisse induire la mort cellulaire en l'absence de l'EfnB3 (*dependence receptor*).

Afin de vérifier l'hypothèse selon laquelle EphA4 jouerait un rôle similaire dans l'hippocampe, il sera nécessaire de comparer la densité ou la proportion de cellules marquées par TUNEL dans la ZSG, entre souris adultes knockouts pour EphA4 ou l'EfnB3, et leurs congénères de type sauvage. Par ailleurs, afin de vérifier l'hypothèse selon laquelle le niveau d'expression ou la localisation subcellulaire d'EphA4 ou de l'EfnB3 affecteraient leur rôle dans l'induction de l'apoptose, il serait intéressant de comparer l'expression et la localisation subcellulaire d'EphA4 chez des souris knockouts pour l'EfnB3, et inversement, celles de l'EfnB3 chez des souris knockouts pour EphA4.

4.5. Conclusion

Somme toute, EphA4 est localisé dans les corps cellulaires et les dendrites apicales des cellules granulaires immatures (DCX-positives), dans la ZSG du rat adulte, alors que des cellules DCX-positives sont en position ectopique chez des souris adultes knockouts pour EphA4. Ces observations soutiennent ainsi un rôle d'EphA4 dans la régulation de la migration des cellules granulaires immatures, lors de la neurogenèse hippocampique adulte.

En outre, l'absence apparente d'EphA4 dans les cellules progénitrices ou prolifératives de la ZSG chez le rat adulte suggère qu'il n'est pas impliqué dans la régulation de la prolifération ou de la migration des cellules progénitrices. Pour infirmer un tel rôle d'EphA4, il sera toutefois nécessaire de comparer, entre souris knockouts pour EphA4 et congénères de type sauvage, le nombre et la position des cellules progénitrices et prolifératives dans la ZSG et la couche de cellules granulaires du gyrus dentelé.

Discussion générale

1. Résumé des observations

Nous avons étudié la localisation cellulaire et subcellulaire d'EphA4 dans différentes couches de l'hippocampe du rat, de la naissance jusqu'à l'âge adulte (P1, P7, P14, P21 et adulte), ainsi que ses rôles potentiels dans la genèse, la migration et la maturation des cellules granulaires dans l'hippocampe du rat et de la souris adultes.

1.1. Distribution laminaire d'EphA4

En microscopie photonique, une forte immunoréactivité (peroxydase/DAB) pour EphA4 est observée à P1 et P7 dans les couches de corps cellulaires du gyrus dentelé et des secteurs CA3 et CA1, avec un marquage notamment associé aux corps cellulaires des cellules granulaires et pyramidales. L'intensité du marquage diminue progressivement entre P7 et P14, pour devenir plutôt faible à P21 et chez l'adulte. Par ailleurs, les cellules granulaires du gyrus dentelé sont fortement marquées chez l'adulte, dans la couche de cellules granulaires (incluant la ZSG), pendant leur stade de maturation où elles expriment la DCX. Enfin, les interneurones ne sont pas marqués pour EphA4, dans toutes les couches de corps cellulaires et de neuropile de l'hippocampe.

Par ailleurs, toutes les couches de neuropile des secteurs CA3, CA1 et du gyrus dentelé, excepté la couche lacunosum-moleculare de CA3 et CA1, ainsi que la portion externe de la couche moléculaire du gyrus dentelé, restent fortement marquées de P1 jusque chez l'adulte. À tous les âges examinés, les couches EphA4-positives sont celles qui reçoivent les projections commissurales et associatives, les fibres moussues et les collatérales de Schaffer, alors que les couches EphA4-négatives sont innervées par la voie perforante en provenance du cortex entorhinal (voir Andersen, 2007; Paxinos, 2004).

1.2. Localisation cellulaire et subcellulaire d'EphA4

En microscopie électronique, l'immunoréactivité (peroxydase/DAB) pour EphA4 décore toute la membrane plasmique des cellules pyramidales et granulaires, du corps cellulaire jusqu'aux extrémités distales, entre P1 et P14, pour devenir confiné aux

extrémités synaptiques à P21 et chez l'adulte. En particulier, leurs extrémités distales EphA4-positives se développent progressivement, pendant la maturation cellulaire, à partir de petites branches ou filopodes dendritiques et de cônes de croissance ou filopodes axonaux, pour devenir des épines dendritiques et des terminaisons axonales associées aux synapses excitatrices. Mentionnons aussi que les axones en cours de myélinisation (P14 et P21) présentent une forte immunoréactivité punctiforme à leur membrane plasmique. À la membrane plasmique des astrocytes protoplasmiques, EphA4 est similairement redistribué, décorant les corps cellulaires et les prolongements proximaux à P1 et P7, pour devenir progressivement restreint aux petits prolongements distaux, incluant les structures périsynaptiques, à partir de P14. Mentionnons que ces observations sont aussi appuyées par le marquage préférentiel d'EphA4 à la membrane plasmique, après immunocytochimie à l'or colloïdal, chez le rat ou la souris, à P1, P7, P14 (résultats non publiés) et à l'âge adulte (Chapitre I et Bouvier et al. (2008) en Annexe I).

Enfin, les organelles impliquées dans la synthèse, la modification post-traductionnelle et le transport des protéines glycosylées comme EphA4, soit le réticulum endoplasmique, l'appareil de Golgi et les vésicules de transport, sont aussi marquées dans les neurones et les astrocytes, et plus abondamment chez les jeunes animaux, reflétant une synthèse plus élevée de la protéine EphA4 pendant le développement neural.

2. Rôles d'EphA4 suggérés par ses localisations subcellulaires

Ces localisations subcellulaires d'EphA4 suivent les sites cellulaires où un remodelage structurel se produit pendant la migration des corps cellulaires, la croissance des prolongements, puis la maturation et la plasticité synaptiques. Elles soutiennent ainsi différents rôles, qui sont similairement exercés par la régulation locale et dynamique du cytosquelette d'actine et des microtubules (voir la section 2.5. du Chapitre I, pour les voies signalétiques activées en aval des Eph et des Efn).

2.1. Localisation d'EphA4 dans les corps cellulaires neuronaux et leurs prolongements axonaux

Entre P1 et P14, EphA4 est localisé à la surface des corps cellulaires granulaires et pyramidaux, souvent aux sites de contact avec des prolongements astrocytaires ou des

corps cellulaires neuronaux voisins. Pendant le développement postnatal, ces neurones migrent et se positionnent les uns par rapport aux autres dans leurs couches cellulaires respectives (voir Andersen, 2007). EphA4 pourrait ainsi participer à la régulation de leur comportement motile, en lien avec des interactions entre EphA4 et l'EfnB2 qui sont impliquées dans la régulation de la migration des cellules des crêtes neurales (Smith et al., 1997) et du remodelage de la morphologie cellulaire et des relations intercellulaires pendant la formation des somites (Barrios et al., 2003), ainsi que des rhombomères (Cooke et al., 2005). En outre, EphA4 est localisé dans les corps cellulaires et les prolongements dendritiques des cellules granulaires immatures (DCX-positives) dans la ZSG du rat adulte alors que des souris knockouts pour EphA4 ont des cellules granulaires immatures (DCX-positives) en position ectopique, c'est-à-dire en dehors de la ZSG et parfois même de la couche granulaire. Chumley et al. (2007) ont aussi rapporté la position ectopique de cellules progénitrices (nestine-positives), prolifératives (Ki-67-positives) et granulaires immatures (DCX-positives), dans la couche de cellules granulaires chez des souris postnatales ou adultes, knockouts pour EphB1, doublement knockouts pour EphB1 et EphB2, ou knockouts pour l'EfnB3 (voir la section 4.2.4. du Chapitre I). Puisqu'EphA4 peut lier l'EfnB3 avec une forte affinité, il pourrait ainsi être responsable de la transduction des signaux impliqués dans la migration cellulaire. Prises ensemble, ces données suggèrent ainsi qu'EphA4 puisse participer de façon similaire dans la maturation des cellules granulaires, pendant le développement postnatal et lors de la neurogenèse chez l'adulte.

Peu après la naissance, particulièrement à P1 et P7, EphA4 est aussi localisé à la surface des axones, cônes de croissance et filopodes axonaux. Ces observations soutiennent un rôle d'EphA4 dans la croissance et le guidage axonaux. En effet, la fonction d'EphA4 dans le guidage axonal a déjà été démontrée dans l'établissement de plusieurs projections du SNC (Coonan et al., 2001; Dufour et al., 2006; Eberhart et al., 2002; Egea et al., 2005; Helmbacher et al., 2000; Huffman and Cramer, 2007; Kullander et al., 2003; Marin et al., 2001; Vanderhaeghen et al., 2000). Particulièrement dans l'hippocampe, Otal et al. (2006) ont révélé des défauts de guidage des projections commissurales et associatives, tel que montré par la distribution anormale de leurs terminaisons axonales, en plus d'une diminution de la densité des terminaisons axonales de la projection commissurale et des fibres moussues dans leurs couches respectives, chez des souris knockouts pour l'EfnA5. De plus, des études *in vitro* ont démontré que plusieurs EfnA peuvent réguler la croissance,

le branchement et l'élagage des axones (Brownlee et al., 2000; Gao et al., 1999), ainsi que l'affaissement des cônes de croissance (Dail et al., 2006; Richter et al., 2007) de cellules pyramidales de l'hippocampe. Puisque toutes les EfnA peuvent se lier à EphA4, il pourrait donc être responsable de la transduction de ces signaux (voir section 4.2.1. du Chapitre I). Par ailleurs, en raison de leur distribution laminaire et nature glutamatergique, révélée par les jonctions de type asymétrique et la colocalisation d'EphA4 avec le transporteur vésiculaire du glutamate de type 1 (Vglut1) chez l'adulte (voir Bouvier et al. (2008) en Annexe I), les structures axonales EphA4-positives paraissent appartenir aux projections commissurales et associatives, aux fibres moussues et aux collatérales de Schaffer. Ainsi, nos observations vont dans le sens d'un rôle d'EphA4 dans la croissance et le guidage axonaux, pendant le développement postnatal de l'hippocampe, particulièrement des projections commissurales et associatives, des fibres moussues et des collatérales de Schaffer.

EphA4 est également localisé à la membrane plasmique de petits axones non myélinisés, tel qu'observé dans les faisceaux de fibres moussues, de P14 jusque chez l'adulte, ce qui suggère un rôle dans la fasciculation axonale. Un tel rôle a d'ailleurs déjà été proposé lors des interactions attractives entre EphA4 et les EfnA2 et EfnA5 dans des axones moteurs périphériques (Eberhart et al., 2000). Par contre, des interactions répulsives entre EphA4 et les EfnA, localisés à la surface d'axones adjacents, ont été impliquées dans la ségrégation des projections sensorielles et motrices des nerfs périphériques, pendant l'établissement de l'innervation neuromusculaire (Gallarda et al., 2008). Ces données suggèrent ainsi que des interactions attractives entre EphA4 et les EfnA localisés à la surface des axones d'une même projection, par exemple les fibres moussues, servent à leur fasciculation, alors que des interactions répulsives entre axones de projections différentes, notamment sensorielles et motrices, induisent plutôt leur ségrégation. Mentionnons enfin que les faisceaux des fibres moussues pourraient avoir des besoins particuliers en fasciculation axonale, en raison de l'intégration fonctionnelle de nouvelles cellules granulaires qui se poursuit chez l'adulte (voir Lledo et al., 2006; Ming and Song, 2005).

En outre, EphA4 décore la surface des axones en cours de myélinisation, tel qu'observé dans l'alveus et la couche oriens du CA1, à P14 et P21. Cette localisation suggère un nouveau type d'interaction neurone-glie entre les Eph et les Efn, c'est-à-dire

entre les axones et les oligodendrocytes dans le processus de myélinisation. L'EfnB3 pourrait être impliquée, puisqu'elle est exprimée par les oligodendrocytes myélinisants, du moins dans la moelle épinière chez la souris, de P5 jusque chez l'adulte (Benson et al., 2005). Afin de vérifier l'hypothèse selon laquelle EphA4 participerait dans la myélinisation axonale au niveau d'autres faisceaux de fibres en développement, tant dans le SNC que dans le système nerveux périphérique, il sera important d'y examiner sa localisation subcellulaire ainsi que celle d'Efn spécifiques. Par exemple, des interactions entre EphA4 et l'EfnB3 pourraient être impliquées dans la myélinisation de la voie corticospinale.

Somme toute, la localisation d'EphA4 à la surface des cellules neuronales en maturation supporte ses rôles dans la migration et le positionnement des corps cellulaires, pendant le développement postnatal ou lors de la neurogenèse chez l'adulte, puis dans la croissance, le guidage, la fasciculation et la myélinisation de leurs axones.

2.2. Localisation d'EphA4 dans les épines dendritiques et les terminaisons axonales

La localisation d'EphA4 dans les prolongements dendritiques, petites branches, filopodes et épines, est conséquente avec son rôle dans la morphogenèse des épines dendritiques pendant le développement postnatal, puis dans leur plasticité et maintien structurels chez l'adulte (Bourgin et al., 2007; Fu et al., 2007b; Murai et al., 2003a; Nishida and Okabe, 2007; Richter et al., 2007; Zhou et al., 2007). Sa localisation dans les terminaisons axonales, de la naissance jusque chez l'adulte, suggère aussi sa participation au remodelage morphologique des terminaisons axonales, en lien avec son rôle dans le remodelage morphologique des épines dendritiques. En effet, les deux partenaires synaptiques subissent tous deux des changements morphologiques semblables, quoique les épines dendritiques sont généralement plus motiles que les terminaisons axonales (Deng and Dunaevsky, 2005; Holtmaat et al., 2008; Konur and Yuste, 2004; Majewska et al., 2006). La localisation d'EphA4 aux synapses excitatrices, aussi bien dans les éléments pré- que postsynaptiques, réconcilie ainsi les observations de Martone et al. (1997), Murai et al. (2003a) et Grunwald et al. (2004) qui suggéraient des localisations dans les épines dendritiques ou les terminaisons axonales. En outre, la distribution laminaire des éléments synaptiques EphA4-positifs suggère l'implication d'EphA4 dans la plasticité synaptique

structurelle ou fonctionnelle de plusieurs synapses hippocampiques, soient celles des fibres moussues, des projections commissurales et associatives, en plus des collatérales de Schaffer où ces rôles ont précédemment été examinés (Grunwald et al., 2004; Murai et al., 2003a). Pour ce faire, des expériences similaires à celles de Murai et al. (2003a) ou Grunwald et al. (2004) pourraient être réalisées sur des tranches d'hippocampe, mais spécifiquement dans le gyrus dentelé ou le secteur CA3.

La participation d'EphA4 au remodelage morphologique des structures synaptiques est aussi conséquente avec sa fonction apparente dans la plasticité synaptique fonctionnelle de types LTP et LTD à la synapse des collatérales de Schaffer (sous-section 4.2.3. du Chapitre I). En effet, plusieurs études *in vitro* ont démontré une relation directe entre les plasticités structurelle et fonctionnelle, dans des cellules pyramidales de l'hippocampe (voir Alvarez and Sabatini, 2007; Hayashi and Majewska, 2005; Kasai et al., 2003; Matsuzaki, 2007). Par exemple, la stimulation à haute fréquence (Matsuzaki et al., 2004; Okamoto et al., 2004; Zhou et al., 2004) et les traitements chimiques induisant la LTP (Kopec et al., 2006; Otmakhov et al., 2004) causent un élargissement significatif des têtes épineuses. Au contraire, une stimulation à faible fréquence induisant la LTD entraîne une diminution du volume des têtes épineuses et même leur élimination dans certains cas (Okamoto et al., 2004; Zhou et al., 2004). Bien que ces observations n'écartent pas la possibilité que la plasticité structurelle et la plasticité fonctionnelle soient des phénomènes indépendants, on doit admettre que la géométrie des épines influence la concentration calcique intracellulaire. Noguchi et al. (2005) ont en effet montré une augmentation du volume des têtes épineuses à la suite d'une potentialisation, ainsi que des changements de la forme du cou des épines dendritiques favorisant une diffusion plus rapide du calcium entre l'épine et la dendrite et limitant ainsi l'accumulation du calcium intracellulaire suivant la stimulation des récepteurs NMDA. Ces changements structurels, ainsi que les changements calciques qui en résultent, auraient donc des conséquences importantes sur la fonction synaptique (Holthoff et al., 2002). Ainsi, les problèmes d'induction de la LTP et de la LTD observés chez les souris knockouts pour EphA4 (voir la section 4.2.3 du Chapitre I) pourraient être des conséquences d'une perturbation du remodelage structurel des épines dendritiques.

En ce sens, d'autres études ont révélé des effets comportementaux de la perturbation des interactions entre les Eph et les Efn. Les études de Gerlai et al. (1999) et Gerlai et

McNamara (2000) ont ainsi démontré *in vivo* leur participation à la mémoire et à l'apprentissage. En effet, une diminution de performance est observée dans deux tâches d'apprentissage qui impliquent l'hippocampe, après l'infusion chronique de protéines de fusion EphA5-Fc chez la souris (Gerlai et al., 1999). Quant à l'infusion chronique de l'EfnA5-Fc, elle réduit significativement l'amnésie rétrograde qui succède à l'anesthésie générale (Gerlai and McNamara, 2000). Dans ce cas-ci, l'infusion d'EphA5-Fc ou d'EfnA5-Fc peut, respectivement, perturber les interactions endogènes entre les EfnA et les EphA ou EphB2 (l'EfnA5 pouvant lier EphB2) ou stimuler celles entre les EphA et les EfnA ou EfnB (EphA4 pouvant lier toutes les EfnB) (voir la section 2.5. du Chapitre I).

Par ailleurs, une dérégulation de l'expression ou de la localisation subcellulaire d'EphA4 pourrait être impliquée dans la pathogenèse de l'autisme, du retard mental, de la maladie bipolaire ou de la schizophrénie, qui sont associés à des anomalies de morphogenèse ou de stabilisation des épines dendritiques (Fiala et al., 2002; Kaufmann and Moser, 2000). Cette hypothèse a été proposée sur la base de la localisation de plusieurs gènes codant pour des Eph ou des Efn dans des régions chromosomiques précédemment associées, par analyse de linkage chez l'humain, avec la susceptibilité pour ces maladies mentales (voir Cowan et al., 2002; Murai and Pasquale, 2004). Par exemple, parmi les régions du chromosome 11 potentiellement liées à l'autisme, six correspondent à la localisation de gènes codant pour des Eph ou des Efn, incluant deux locus ayant une liaison forte en 2q (EphA4) et en 7q (EphB4, EphB6 et EphA1).

En bref, la localisation d'EphA4 à la surface des petites branches, filopodes et épines dendritiques est conséquente avec ses rôles dans la morphogenèse des épines dendritiques pendant le développement postnatal, puis dans leur plasticité structurelle et fonctionnelle, ainsi que dans la mémoire et l'apprentissage chez l'adulte.

2.3. Localisation d'EphA4 dans les petits prolongements astrocytaires périsynaptiques

Les petits prolongements astrocytaires sont hautement motiles, davantage même que les épines dendritiques (Benediktsson et al., 2005; Haber et al., 2006; Hirrlinger et al., 2004; Lippman et al., 2008; Nestor et al., 2007; Nishida and Okabe, 2007), tel que montré par microscopie confocale ou à multiphotons, dans des tranches d'hippocampe en culture

organotypique, des tranches de cervelet ou de tronc cérébral de souris juvéniles et adultes. De plus, les petits prolongements astrocytaires forment rapidement des contacts, transitoires ou persistants, avec les épines dendritiques et les terminaisons axonales. Ces contacts sont particulièrement fréquents dans le cas d'épines dendritiques stables et volumineuses ou de terminaisons axonales actives (Haber et al., 2006; Hirrlinger et al., 2004; Nishida and Okabe, 2007). Par la visualisation simultanée des prolongements astrocytaires et dendritiques dans des tranches d'hippocampe de souris juvénile (P4-P5) en culture organotypique, Nishida et Okabe (2007) ont notamment montré que l'infusion d'EphA4-Fc, qui peut perturber les interactions endogènes entre les EfnA ou EfnB et les EphA ou EphB auxquels elles se lient habituellement (incluant EphA4) (voir section 2.5. du Chapitre I), diminue aussi bien la durée des contacts entre prolongements astrocytaires et prolongements dendritiques, la durée de vie des prolongements dendritiques, que leur probabilité de se développer en épines dendritiques (voir section 4.2.3. du Chapitre I).

Nestor et al. (2007) ont aussi démontré *in vitro* un rôle des interactions entre les Eph et les Efn dans le remodelage structurel des petits prolongements astrocytaires, dans des tranches d'hippocampe de rats ou souris juvéniles (P6) en culture organotypique. En effet, l'application d'EfnA3-Fc induit une croissance rapide des filopodes astrocytaires chez des astrocytes témoins, contrairement aux astrocytes exprimant des isoformes d'EphA4 dépourvues de domaines catalytiques. Rappelons que ces isoformes pourraient perturber les interactions endogènes entre les Efn (A et B) et tous les Eph auxquels celles-ci se lient normalement (incluant EphA4), mais aussi agir comme des formes dominantes-négatives et perturber la signalisation des EphA4 endogènes en s'introduisant dans leurs regroupements multimériques (Murai et al., 2003a) (voir la section 4.2.3. du Chapitre I). De plus, Haber et al. (2006) ont mis en lumière un remodelage coordonné de prolongements astrocytaires et d'épines dendritiques, dans les mêmes conditions. Combinées avec les données de Murai et al. (2003a) sur le rôle d'EphA4 dans la régulation de la morphologie des épines dendritiques, les données que nous venons de présenter pointent vers l'hypothèse selon laquelle les interactions entre prolongements astrocytaires et prolongements dendritiques, qui impliquent notamment les Eph et les Efn, auraient un effet instructif sur la morphogenèse des épines dendritiques, mais aussi sur le maintien et le remodelage de leur morphologie.

Le positionnement des prolongements astrocytaires autour des synapses excitatrices et inhibitrices affecte aussi l'homéostasie des ions, la neurotransmission, ainsi que la gliotransmission, notamment par des changements des propriétés diffusionnelles et de la géométrie de l'espace extracellulaire (voir Oberheim et al., 2006; Theodosis et al., 2008). En effet, Panatier et al. (2006) ont démontré que le changement du niveau d'association entre les prolongements astrocytaires et les structures neuronales, particulièrement les épines dendritiques et les terminaisons axonales associées aux synapses excitatrices, modifie le seuil d'induction de la plasticité synaptique. Pendant la lactation chez la ratte, la rétraction des prolongements astrocytaires dans le noyau supraoptique (SON) de l'hypothalamus diminue leur couverture des synapses glutamatergiques, en plus de causer une augmentation du seuil d'induction de la LTP, en raison d'une diminution du taux d'occupation des sites glycinergiques des récepteurs NMDA par le gliotransmetteur D-sérine. Quoique EphA4 ne soit pas exprimé dans le SON de l'hypothalamus (observations non publiées), des changements du niveau d'association structurelle entre les petits prolongements astrocytaires et les synapses ont été observés dans plusieurs autres régions du SNC, incluant l'hippocampe (voir Theodosis et al., 2008).

Plusieurs études ultrastructurales ont révélé que le degré de couverture astrocytaire des synapses excitatrices est significativement augmenté lors de la plasticité synaptique liée à l'activité, notamment la LTP dans le gyrus dentelé de l'hippocampe *in vitro* (Wenzel et al., 1991), ou liée à l'expérience sensorielle et à l'apprentissage moteur *in vivo*, comme l'enrichissement de l'environnement (Jones and Greenough, 1996; couches II/III du cortex visuel primaire), l'apprentissage moteur acrobatique, par exemple la navigation dans un parcours avec des cordes, des échelles, des chaînes et des barres parallèles en guise d'obstacles (Kleim et al., 2007; cervelet) et la stimulation des vibrisses (Genoud et al., 2006; cortex somatosensoriel). Ainsi, EphA4 pourrait réguler le niveau de couverture astrocytaire des synapses excitatrices, par le remodelage structurel des petits prolongements astrocytaires périsynaptiques, en fonction de l'activité ou de l'expérience comportementale. En ce sens, il devient intéressant d'analyser chez des souris knockouts pour EphA4 ou des Efn spécifiques le niveau d'association des prolongements astrocytaires avec les synapses excitatrices, notamment par microscopie électronique combinée avec des reconstructions tridimensionnelles. Toutefois, les différences potentielles pourraient être fort subtiles, Smith (1992) ayant calculé qu'une augmentation de la distance entre les prolongements

astrocytaires et les synapses d'environ 10 nm suffirait pour augmenter la probabilité de libération de neurotransmetteur d'approximativement 200%.

En somme, ces données soutiennent des rôles pour EphA4 astrocytaire dans le remodelage structurel des prolongements astrocytaires et par conséquent, la morphogenèse et le remodelage structurel des épines dendritiques, en plus de la plasticité synaptique liée à l'activité ou à l'expérience comportementale. Les rôles astrocytaires d'EphA4 rendraient ainsi compte de plusieurs phénotypes connus d'une perturbation des interactions entre EphA4 et les Efn chez la souris knockout pour EphA4 ou suivant l'infusion d'EphA-Fc.

3. Fonction d'EphA4 dans la régulation des capacités plastiques du SNC

L'expression régionale, laminaire, cellulaire et subcellulaire d'EphA4, observée dans le cadre de mes travaux de doctorat ou précédemment décrite dans la littérature, soutient l'hypothèse selon laquelle sa fonction principale concernerait la régulation des capacités plastiques du SNC, selon le stade développemental, la région, l'état de santé et l'expérience sensorielle de l'animal (voir sous-section 1.1. du Chapitre I).

3.1. Fonction d'EphA4 dans la régulation des capacités plastiques du SNC selon le stade développemental

D'abord, l'évolution développementale de la localisation cellulaire et subcellulaire d'EphA4 corrèle avec une diminution des capacités plastiques entre la naissance et l'âge adulte (voir section 1.2.), la plasticité concernant surtout la formation de nouvelles synapses et le renforcement des connexions synaptiques existantes chez l'adulte.

3.2. Fonction d'EphA4 dans la régulation des capacités plastiques du SNC selon la région, le secteur ou la couche

Chez l'adulte, EphA4 est abondamment exprimé dans plusieurs régions du SNC où la plasticité est accrue, notamment le cervelet et le cortex cérébral, mais aussi l'hippocampe et le bulbe olfactif, qui intègrent à leur circuiterie de nouveaux neurones tout au long de la

vie (voir Andersen, 2007; Boyden et al., 2004; Buonomano and Merzenich, 1998; Kempermann, 2006; Liebl et al., 2003; Martone et al., 1997; Ming and Song, 2005).

Dans le cortex cérébral, la plasticité structurelle des épines dendritiques varie entre régions ou couches corticales. Deux études ont démontré des différences significatives de motilité des épines dendritiques chez la souris juvénile à P28, soit à l'apogée de la période critique qui s'étend de P19 à P35. En effet, Majewska et al. (2006) ont révélé par microscopie à multiphotons *in vivo* que les épines dendritiques du cortex auditif sont plus motiles et celles du cortex visuel moins motiles, que celles du cortex somatosensoriel. Oray et al. (2004) ont aussi rapporté *in vitro* que la motilité des épines dendritiques est accrue dans les couches II/III en comparaison de la couche IV du cortex visuel. Or, la modalité des afférences au cortex auditif n'était pas la cause de cette différence de motilité des épines dendritiques entre régions corticales (Majewska et al., 2006). En effet, une lésion bilatérale des collicules inférieurs réalisée à la naissance, laquelle entraîne l'innervation du corps genouillé médian par les axones des cellules ganglionnaires de la rétine et donc l'innervation du cortex auditif par des projections visuelles, ne réduit pas le dynamisme des épines dendritiques du cortex auditif. En conséquence, des indices moléculaires intrinsèques aux régions corticales réguleraient la dynamique structurelle des épines dendritiques, tel que suggéré par Majewska et al. (2006).

Des populations d'astrocytes particulières pourraient affecter, positivement ou négativement selon la région ou la couche, la propension à la plasticité. Par exemple, Jiao et al. (2008) ont récemment démontré que les astrocytes diffèrent entre les zones non neurogéniques et neurogéniques du SNC adulte, par leur expression des EfnA2 et EfnA3 qui inhibent la prolifération des cellules progénitrices multipotentes *in vitro* (voir la section 4.2.4. du Chapitre I), en lien avec la précédente démonstration de Song et al. (2002) que les astrocytes issus de l'hippocampe adulte, et non ceux issus de la moelle épinière adulte, ont la capacité d'induire la différenciation neuronale de cellules progénitrices multipotentes. Comme nous l'avons déjà vu dans la section 2.3., des associations structurelles entre prolongements astrocytaires et prolongements dendritiques, notamment par des interactions entre Eph et Efn, pourraient réguler la plasticité ou le maintien de la morphologie dendritique. Plus précisément, nous proposons l'hypothèse selon laquelle des populations d'astrocytes qui diffèrent entre régions ou couches corticales, notamment par leur niveau

d'expression ou leur localisation subcellulaire d'EphA4, rendraient compte des différences de motilité des épines dendritiques observées. Liebl et al. (2003) ont rapporté l'expression de l'ARNm d'EphA4 dans les couches II à IV du cortex cérébral du rat adulte, sans toutefois préciser dans quelles régions. Nos données sur la localisation subcellulaire d'EphA4 dans les couches profondes du cortex moteur primaire de la souris adulte montrent une grande proportion d'épines dendritiques immunopositives (voir Bouvier et al. (2008) en Annexe I), alors que mes observations préliminaires dans les couches I et II/III du cortex visuel primaire du rat adulte montrent qu'EphA4 est surtout localisé dans les prolongements astrocytaires périsynaptiques, en plus de quelques épines dendritiques. Afin de vérifier l'hypothèse d'un effet instructif des astrocytes sur le remodelage morphologique des prolongements dendritiques, par des interactions entre EphA4 et des Efn particulières, il serait utile de comparer la motilité des prolongements astrocytaires et dendritiques ainsi que leurs associations structurelles, par microscopie à multiphotons, tout comme l'expression et la localisation subcellulaire d'EphA4 et des Efn, par immunocytochimie en microscopie électronique, dans différentes régions ou couches corticales.

Dans l'hippocampe, quelques études ont rapporté des différences entre les épines dendritiques des couches de neuropile EphA4-positives et EphA4-négatives. Matsuda et al. (2004) et Megias et al. (2001) ont d'abord montré une densité d'épines dendritiques et de synapses excitatrices moins élevée dans la couche lacunosum-moleculare, EphA4-négative, comparativement aux couches radiatum, oriens et de cellules pyramidales, EphA4-positives, des secteurs CA3 et CA1 du rat adulte. De plus, Nicholson et al. (2006) ont révélé une proportion plus grande d'épines dendritiques avec une densité postsynaptique perforée dans la couche lacunosum-moleculare, dans le secteur CA1 du rat adulte. Puisque les épines dendritiques perforées sont généralement plus volumineuses que les épines dendritiques non perforées, les épines dendritiques de la couche lacunosum-moleculare seraient donc plus stables que celles de la couche radiatum (voir Alvarez and Sabatini, 2007). Ainsi, la distribution laminaire d'EphA4 corrèle avec celle des synapses excitatrices ayant une propension élevée à la plasticité. Il sera intéressant d'examiner la motilité des éléments synaptiques astrocytaires et dendritiques par microscopie à multiphotons sur des tranches d'hippocampe en culture organotypique (Haber et al., 2006), dans différents secteurs et couches, puis de comparer ces observations avec nos données sur la distribution laminaire et la localisation subcellulaire d'EphA4 dans l'hippocampe du rat adulte.

Enfin, les astrocytes protoplasmiques du SNC occupent chacun des territoires ou îlots synaptiques distincts (Bushong et al., 2004; Bushong et al., 2002; Halassa et al., 2007; Ogata and Kosaka, 2002). Dans l'hippocampe, un seul astrocyte influencerait la fonction d'environ 140 000 synapses excitatrices (Bushong et al., 2002). Des différences significatives de densité numérique des astrocytes et du volume occupé par leurs prolongements ont été rapportées entre les couches EphA4-positives et EphA4-négatives. Ogata et Kosaga (2002) ont montré que les astrocytes de la couche lacunosum-moleculare sont distinctement caractérisés par une densité numérique plus élevée et des territoires plus petits par rapport aux astrocytes des autres couches de l'hippocampe, chez la souris adulte. Bushong et al. (2003) ont d'autre part examiné la morphologie et les territoires astrocytaires dans la couche moléculaire du gyrus dentelé. Dans cette étude, l'immunocytochimie pour EphA4 a été utilisée pour distinguer les couches moléculaires interne, EphA4-positive, et externe, EphA4-négative, chez le rat adulte. Deux populations astrocytaires ont été observées : une première confinée à la couche moléculaire externe et dépourvue de prolongements qui franchissent la ligne de séparation avec la couches interne, et une deuxième située dans la couche moléculaire interne et ayant des prolongements qui se ramifient dans les deux couches moléculaires. Ces données appuient donc l'hypothèse selon laquelle EphA4 serait associé à des populations astrocytaires différentes, ayant des distributions laminaires distinctes et régulant différemment la propension à la plasticité des synapses excitatrices localisées à l'intérieur de leur territoire.

3.3. Fonction d'EphA4 dans la régulation des capacités plastiques selon l'état d'intégrité du SNC

Dans l'hippocampe postnatal, surtout entre P1 et P14, EphA4 est localisé à la membrane plasmique des prolongements astrocytaires en contact avec des cellules endothéliales ou des péricytes associés aux microvaisseaux sanguins. Or, des interactions entre les cellules endothéliales, qui constituent la paroi des microvaisseaux cérébraux, les neurones, les péricytes et les astrocytes contribuent au maintien de la barrière hématoencéphalique (voir Banerjee and Bhat, 2007). Par suite d'une lésion de la moelle épinière chez des souris adultes de type sauvage, l'expression d'EphA4 est augmentée dans les astrocytes, qui sont alors étroitement associés avec les vaisseaux sanguins, tandis que chez des souris knockouts pour EphA4, les prolongements astrocytaires sont plus courts et

ne parviennent pas à former des réseaux périvasculaires denses (Goldshmit et al., 2006a). Cette observation, conjuguée à nos propres observations, va dans le sens d'un rôle d'EphA4 astrocytaire dans l'induction de la barrière hématoencéphalique pendant le développement postnatal précoce, aussi bien que pendant la réparation du SNC suite à une lésion.

Par ailleurs, Fabes et al. (2007; 2006) et Goldshmit et al. (2004) ont montré, après une hémisection de la moelle épinière, que l'expression d'EphA4 est aussi régulée à la hausse dans les astrocytes réactifs ainsi que dans la portion proximale des axones corticospinaux lésés. Son activité est alors inhibitrice à la régénération axonale (voir la section 2.7.3. du Chapitre I). Il serait intéressant d'examiner les conséquences d'une lésion de la voie perforante ou des projections commissurales, par exemple, sur le niveau d'expression et la localisation subcellulaire d'EphA4 dans l'hippocampe du rat adulte, ainsi que de comparer la propension à la croissance et au bourgeonnement axonaux entre souris knockouts pour EphA4 et leurs types sauvages, afin de vérifier si EphA4 exercerait un rôle inhibiteur similaire dans l'hippocampe comparativement à la moelle épinière.

3.4. Fonction d'EphA4 dans la régulation des capacités plastiques du SNC selon l'expérience comportementale

Les rôles d'EphA4 dans le remodelage structurel des prolongements dendritiques et astrocytaires associés aux synapses excitatrices pointent vers sa participation dans la régulation de la formation et de la stabilisation des synapses, en fonction de l'expérience sensorielle (voir sections 2.2. et 2.3). Afin de vérifier cette hypothèse, il sera d'abord nécessaire de comparer le niveau d'expression et la localisation subcellulaire d'EphA4 et d'Efn particulières, entre différents paradigmes comportementaux. Il sera aussi utile de caractériser le dynamisme structurel des prolongements astrocytaires et dendritiques (mais aussi des terminaisons axonales), les modalités de leurs interactions structurelles réciproques, ainsi que les conséquences de ces interactions sur la formation et la stabilisation des synapses, *in vivo*, pendant le développement et l'apprentissage, par des observations chroniques en microscopie à multiphotons. La plasticité des colonnes de dominance oculaire, induite par l'occlusion monoculaire, est un modèle qui pourrait être employé pour étudier les mécanismes moléculaires de la plasticité de la zone binoculaire du cortex visuel primaire, pendant la période critique ou bien chez l'adulte (voir Majewska and

Sur, 2006). Une telle étude permettrait aussi d'examiner *in vivo* les conséquences d'une infusion chronique des protéines de fusion EphA-Fc ou EfnA-Fc spécifiques, qui pourraient être utilisées pour perturber les interactions entre Eph et Efn. Enfin, des souris knockouts conditionnelles et inductibles pour EphA4 pourraient aussi être employées, afin de bloquer sélectivement son expression dans les neurones ou les astrocytes chez l'adulte, ce qui permettrait de différencier ses rôles exercés dans ses divers sites d'action astrocytaires et neuronaux.

4. Conclusions

Somme toute, nos observations en microscopie photonique montrent que l'intensité de l'immunoréactivité pour EphA4 diminue progressivement, entre la naissance et l'âge adulte, dans les couches de corps cellulaires des secteurs CA3, CA1 et du gyrus dentelé, alors qu'elle persiste dans les couches de neuropile. Les couches de neuropile EphA4-positives reçoivent les projections commissurales et associatives, tandis que celles EphA4-négatives sont innervées par la voie perforante en provenance du cortex entorhinal. Nos observations en microscopie électronique ont révélé des changements au niveau de la localisation subcellulaire d'EphA4 pendant le développement postnatal, ce qui confirme notre hypothèse de départ (voir section 5. du Chapitre I). En effet, EphA4 décore d'abord toute la surface des cellules pyramidales et granulaires, du corps cellulaire jusqu'aux extrémités distales, entre P1 et P14, puis devient confiné aux extrémités synaptiques, à P21 et chez l'adulte. À la surface des astrocytes, EphA4 marque d'abord le corps cellulaire et ses prolongements proximaux à distaux, à P1 et P7, puis devient restreint aux prolongements périsynaptiques distaux, à partir de P14. En outre, des axones en cours de myélinisation, surtout observés à P14 et P21, sont aussi fortement immunoréactifs à leur membrane plasmique. Enfin, EphA4 est localisé dans le corps cellulaire et les dendrites des cellules granulaires générées dans l'hippocampe adulte, au stade de maturation où elles expriment la DCX. De plus, des souris adultes knockouts pour EphA4 présentent des cellules granulaires (DCX-positives) positionnées en dehors de la ZSG, ce qui suggère un rôle d'EphA4 dans la régulation de leur migration.

Nos observations soutiennent ainsi des rôles d'EphA4 dans la migration cellulaire, la croissance et le guidage des axones, la formation des épines dendritiques, puis la

plasticité et le maintien des synapses excitatrices chez l'adulte. En outre, elles suggèrent des rôles potentiels d'EphA4 dans la myélinisation axonale, le remodelage morphologique des terminaisons axonales, ainsi que la migration des cellules granulaires immatures (DCX-positives) générées lors de la neurogenèse hippocampique chez l'adulte. Les rôles d'EphA4 dans le remodelage morphologique des structures synaptiques, neuronales ou astrocytaires, pourraient aussi rendre compte de son implication dans la plasticité synaptique de types LTP ou LTD, la mémoire et l'apprentissage, en plus de suggérer que des dérégulations de son expression ou de sa localisation subcellulaire contribuent à la pathogenèse de l'autisme, du retard mental, de la schizophrénie ou de la maladie bipolaire.

Prises ensemble, ces données pointent vers une fonction importante d'EphA4 dans la régulation des capacités plastiques des neurones et des astrocytes, selon le stade développemental, la région du SNC, l'état de santé de l'animal, ainsi que son expérience comportementale. Ce présent travail démontre ainsi l'importance primordiale que revêt l'analyse de la localisation ultrastructurale des Eph et des Efn pour accéder à une meilleure compréhension de leurs rôles fonctionnels. En lien avec cette fonction d'EphA4 dans la régulation des capacités plastiques du SNC, il serait aussi très intéressant d'étendre l'étude de sa localisation ultrastructurale vers d'autres systèmes physiologiques (voir section 2.7. du Chapitre I pour les fonctions des Eph et des Efn dans la physiologie des organes matures), chez les vertébrés mais aussi chez des organismes pluricellulaires de niveaux de complexité divers, dans le but de mettre en lumière la fonction globale des Eph et des Efn dans le développement et le maintien de l'organisation cellulaire.

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Annexe I. Presynaptic and postsynaptic localization of EphA4 and EphB2 in adult mouse forebrain

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Comme troisième auteure, j'ai contribué à cette étude par la réalisation des expériences d'immunocytochimie en microscopie photonique et électronique, puis par la collaboration à l'analyse des résultats, la préparation des figures et la rédaction du manuscrit.

Presynaptic and postsynaptic localization of EphA4 and EphB2 in adult mouse forebrain

David Bouvier[1], Amadou T. Corera[2], Marie-Ève Tremblay[1], Mustapha Riad[1], Miguel Chagnon[5], Keith K. Murai[3], Elena B. Pasquale[4], Edward A. Fon[2†], Guy Doucet[1†*]

[1] Département de pathologie et biologie cellulaire and Groupe de recherche sur le système nerveux central (GRSNC), Université de Montréal, Montréal, QC

[2] Department of Neurology and Neurosurgery, McGill University, and Montreal Neurological Institute, Montréal, QC

[3] Department of Neurology and Neurosurgery, McGill University, and Centre for Research in Neuroscience, Montreal General Hospital, Montréal, QC

[4] The Burnham Institute for Medical Research, La Jolla, CA

[5] Département de mathématiques et de statistiques, Université de Montréal, Montréal, QC

**Corresponding author*, Département de pathologie et biologie cellulaire, Université de Montréal, C.P. 6128, succ. Centre-ville, Montréal, QC, Canada H3C 3J7. E-mail: [redacted], Telephone: 514-343-6255, Fax: 514-343-5755.

[†]EAF and GD contributed equally to the supervision of this study.

Abbreviations

AMPA, alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate; CA1/CA3, Cornus ammonis sectors of hippocampus; CNS, central nervous system; CtxD, deep layers, v-vi, of primary motor cortex; EEA1, early endosome antigen-1; GluR2/3, subunits of AMPA-type glutamate receptors; GM130, Golgi membrane-130; GST, glutathione-s-transferase; NR1, NMDA receptor subunit; PMSF, phenylmethanesulphonylfluoride; PSD, postsynaptic density; SIGP, silver-intensified immunogold particle; VP, virtual particles.

ABSTRACT

The ephrin receptors EphA4 and EphB2 have been implicated in synaptogenesis and long-term potentiation in the cerebral cortex and hippocampus, where they are generally viewed as postsynaptic receptors. To determine the precise distribution of EphA4 and EphB2 in mature brain synapses, we used subcellular fractionation and electron microscopy to examine the adult mouse forebrain/midbrain. EphA4 and EphB2 were both enriched in microsomes and synaptosomes. In synaptosomes, they were present in the membrane and the synaptic vesicle fractions. While EphA4 was tightly associated with PSD-95-enriched postsynaptic density fractions, EphB2 was easily extracted with detergents. In contrast, both receptors were found in the presynaptic active zone fraction. By electron microscopy, EphA4 was mainly detected in axon terminals, whereas EphB2 was more frequently detected in large dendritic shafts, in the hippocampus and cerebral cortex. However, in the ventrobasal thalamus EphB2 was detected most frequently in axon terminals and thin dendritic shafts. The localization of EphA4 and EphB2 in multiple compartments of neurons and synaptic junctions suggests that they interact with several distinct scaffolding proteins and play diverse roles at synapses.

Keywords: Cerebral cortex, hippocampus, thalamus, synaptogenesis, ephrin receptors, cell fractionation, electron microscopy, immunohistochemistry

Running title: EphA4 and EphB2 at mature CNS synapses

INTRODUCTION

The Eph receptor family has 14 members in mammals and is divided into two classes, EphA receptors (A1-8, and A10) and EphB receptors (B1-4, and B6), based on their sequence homologies and their binding affinities for ephrinA (A1-A5) or ephrinB (B1-B3) ligands (Himanen & Nikolov 2003). EphA receptors interact with ephrinA ligands, and EphB receptors with ephrinB ligands, with two known exceptions: EphA4 is also activated by ephrinB2 and ephrinB3 (Gale *et al.* 1996), and EphB2 by ephrinA5 (Himanen *et al.* 2004). Reciprocal trans-activation of Eph receptors and ephrins on adjacent cells leads to their clustering and induces bidirectional signaling, notably through cytoskeletal proteins involved in cell motility. Eph-ephrin interactions can activate both repulsive and attractive forces between cells (Klein 2001) and have been implicated in cell migration, axon guidance, and topographic mapping in the developing nervous system (Klein 2004). Several Eph receptors and ephrins are also widely expressed in the adult central nervous system (CNS), where they appear to regulate synaptic function and plasticity (Martinez & Soriano 2005, Yamaguchi & Pasquale 2004).

EphA4 and EphB2 are the Eph receptors most intensively studied in the adult CNS. By *in situ* hybridization and immunohistochemistry, they have been detected in many regions and cell types, notably in the neocortex and hippocampus (Liebl *et al.* 2003, Moreno-Flores & Wandosell 1999, Grunwald *et al.* 2001, Goldshmit *et al.* 2006, Henderson *et al.* 2001, Tremblay *et al.* 2007). At the subcellular level, these receptors are believed to be mainly associated with dendritic spines and postsynaptic densities (PSDs) (Martone *et al.* 1997, Buchert *et al.* 1999, Murai *et al.* 2003, Torres *et al.* 1998, Grunwald *et al.* 2004) and have been implicated in dendritic spine morphogenesis and LTP (Murai et al. 2003, Armstrong *et al.* 2006, Contractor *et al.* 2002, Grunwald et al. 2004, Grunwald et al. 2001, Henderson et al. 2001). However, a qualitative and quantitative overall assessment of the distributions of EphA and EphB receptors in discrete neuronal compartments has not been performed. Accurate knowledge of their precise localization will help clarify their functions in the mature CNS.

In the present study, we have examined the subsynaptic distribution of EphA4 and EphB2 by cell and synaptosome fractionation, and by pre-embedding immunohistochemistry (immunoperoxidase and immunogold with silver intensification) in

conjunction with electron microscopy. Our results shed new light on the precise synaptic localization of the EphA4 and EphB2 receptors in the adult mouse forebrain and will help to define their respective roles in synaptogenesis and synaptic plasticity, particularly in the hippocampus where they have been most extensively characterized.

MATERIALS AND METHODS

Animals

All procedures were conducted in strict accordance with the *Guide to the Care and Use of Experimental Animals* (Ed2) of the Canadian Council on Animal Care. The experimental protocols were approved by the Animal Care Committee of the Université de Montréal. The animals were housed at constant temperature (21°C) and humidity (60%), under a fixed 12 h light/dark cycle and with free access to food and water.

Cell fractionation experiments were conducted on 36 adult male C57BL/6 mice (4 for each fractionation). These animals were decapitated under deep halothane anesthesia.

The electron microscopic analysis was carried out on brain tissue from one EphA4 KO (EphA4 $^{-/-}$), one wild-type littermate (EphA4 $^{+/+}$) (Dottori *et al.* 1998) and 4 C57BL/6 adult male mice. These animals were deeply anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and perfused through the aortic arch with a solution of 3.5 % acrolein, followed by 4 % paraformaldehyde (PFA), both in 0.1 M sodium phosphate buffer (PB), pH 7.4. Brains were further fixed by immersion in 4% PFA for 1 h at 4°C, washed in sodium phosphate-buffered saline (PBS; 0.9% NaCl in 50 mM PB, pH 7.4). Transverse sections, 50 µm thick, were cut in cooled PBS with a vibratome.

Antibodies

The anti-EphA4 antibody (Ab11), raised in rabbit against the last 11 carboxyterminal amino acids of EphA4, has been shown to recognize EphA4 in rat, mouse and human (Soans *et al.* 1994, Martone et al. 1997, Murai et al. 2003). The specificity of this antibody has been previously demonstrated by the absence of labeling in EphA4-/- mice (Tremblay et al. 2007).

The anti-EphB2 antibody was made using a glutathione-s-transferase- (GST) fusion protein containing the 99 carboxy-terminal amino acids of chicken EphB2 as the antigen, as described (Holash & Pasquale 1995). The antibody was affinity purified using the antigen and adsorbed on a GST fusion protein of the equivalent region of EphB4 to eliminate possible cross-reacting antibodies and anti-GST antibodies. This antibody was shown to recognize mouse and human EphB2 (91 of the 99 carboxyterminal amino acids are identical between chicken and mouse): it has also been used to immunoprecipitate EphB2 from mouse hippocampal neurons (Henkemeyer *et al.* 2003), and produces no staining in Western blots of human cancer cells known to lack EphB2, but expressing EphB3 and EphB4 (N. Noren and E. B. Pasquale, unpublished results). It also recognizes EphB2, and not the closely related EphB4, in Western blots of transfected HEK293 cells (Supplementary Fig. S1). Labelling of the mouse hippocampus with the anti-EphB2 antibody (Fig. S2) is similar to that previously published using a different anti-EphB2 antibody (Grunwald et al. 2001, Fig. 1I). The staining with the anti-EphB2 antibody is also consistent with *in situ* hybridization results for EphB2 (Grunwald et al. 2001, Fig. 1C), but not for EphB1 or EphB3 (Grunwald et al. 2001, Fig. 1A,E), and with β-galactosidase staining in knock-in mice expressing EphB2 fused to β-galactosidase in place of wild-type EphB2 (Grunwald et al. 2001, Fig. 1H).

The rabbit polyclonal anti-synaptosome-associated protein-25 (SNAP-25) antibody was a gift from Dr. Peter McPherson (Montreal Neurological Institute, Montréal, QC, Canada). The monoclonal anti-synaptophysin antibody was purchased from Sigma, the monoclonal anti-GluR2/3 from Chemicon, and the monoclonal anti-postsynaptic density protein-95 (PSD95), anti-NR1, anti-early endosome antigen-1 (EEA1) and anti-Golgi membrane-130 (GM130) antibodies, from BD Transduction Laboratories (BD Biosciences, Mississauga, ON, Canada).

Subcellular fractionation, purification of PSD fractions, and separation of pre- and postsynaptic fractions

All the following fractionation procedures were reproduced at least 3 times in separate experiments. Subcellular fractions were prepared from mouse forebrain/midbrain, as described by Huttner et al (1983, see supplementary material in Zhou *et al.* 2007, for details). Briefly, the forebrain/midbrain was dissected on ice, following a transverse cut

between the occipital cortex and cerebellum. The whole forebrain/midbrain was used for fractionation experiments in order to obtain sufficient amount of proteins in the end fractions. All subsequent procedures were done at 0-4°C. Brain tissues were homogenized in ice-cold sucrose/HEPES buffer (0.32 M sucrose, 10 mM HEPES, pH 7.4) containing protease inhibitors. The nuclear material (P1) was discarded following centrifugation at 1 000 x g, and the supernatant (S1) was centrifuged again at 17 500 x g. The resulting supernatant (S2) was used to prepare the cytosolic (S3) and microsomal (P3) fractions. The pellet, containing the crude synaptosomal fraction (P2), was re-suspended in sucrose/HEPES buffer, layered on top of a discontinuous sucrose gradient and ultra-centrifuged**.** Six milliliters of the fraction collected at the 0.8 / 1.2 M interface were washed with sucrose/HEPES buffer and centrifuged at 20 000 x g. The resulting pellet, constituting the purified synaptosomal fraction (P2'), was re-suspended in approximately 2 ml of sucrose/HEPES buffer. One to 1.5 ml of this P2' fraction was lysed osmotically in 10 mM HEPES buffer containing protease inhibitors, homogenized, and centrifuged at 33,000 x g. The pellet, representing the total synaptosomal membrane fraction (LP1), was re-suspended in sucrose/HEPES buffer. The supernatant (LS1) and the S2 fraction were ultra-centrifuged at 260 000 x g, to obtain, respectively, the crude vesicle fraction (LP2) and the cytosolic (S3) and microsomal (P3) fractions.

Postsynaptic density (PSD) fractions were obtained as described (Cho *et al.* 1992 , see supplementary material, in Zhou et al. 2007). The P2' fraction was incubated in 50 mM Tris buffer containing 0.5% Triton X-100, followed by centrifugation (32 000 x g, 20 min). The resulting pellet (PSD-I) was re-suspended in Tris buffer. The PSD-II and PSD-III fractions were obtained by treating aliquots of PSD-I fraction with 0.5% Triton X-100 in 50 mM Tris buffer (pH 7.4), or with 3% Sarcosyl in 50 mM Tris buffer containing 1 mM EDTA, respectively. The suspensions were centrifuged at 200 000 x g. The resulting PSD-II and PSD-III fractions were solubilized in 5% SDS.

Presynaptic, postsynaptic and extra-junctional synaptic proteins were separated according to a method previously described (Phillips *et al.* 2001, Pinheiro *et al.* 2005, Rebola *et al.* 2005, Rebola *et al.* 2003), with some modifications (see Zhou et al. 2007, supplementary material). Briefly, purified synaptosomes (P2') were suspended in ice-cold $CaCl_2$, before adding a solubilization buffer (2% Triton X-100 in 40 mM Tris, pH 6.0).

They were incubated on ice with gentle agitation, and the insoluble synaptic junctions were pelleted (40 000 x g). At this pH, the junctional synaptic complexes are isolated at high yield and purity. The supernatant (extra-junctional membranes fraction, including plasma and vesicle membranes) was decanted, and its proteins were precipitated with acetone, at -20°C, and recovered by centrifugation at 18 000 x g. The pellet of the 40 000 x g centrifugation, above (synaptic junctions), was washed in pH 6.0 solubilization buffer, re-suspended in pH 8.0 solubilization buffer (1% Triton X-100, 20 mM Tris, pH 8.0), incubated on ice, and centrifuged at 40 000 x g. This pH increase (6 to 8) released presynaptic active zones (supernatant) from the PSDs, which remained in the pellet. The latter was subjected to a further extraction with pH 8.0 solubilization buffer and centrifuged as above for maximal recovery of the presynaptic active zone proteins. The two supernatants were combined, precipitated with acetone and spun down at 18 000 x g. These sub-synaptic fractions (extra-junctional membranes, presynaptic active zones and PSDs) were solubilized in 5% SDS.

Protein concentration was assessed in every fraction with the BCA protein assay kit (Pierce; Rockford, IL), using bovine serum albumins as a standard. Aliquots of all brain fractions were used for Western blotting. Equal amounts (10 µg) of protein from every fraction were separated on SDS-PAGE, blotted following standard procedures, and revealed with enhanced luminol-based chemiluminescence (ECL Western Blotting Substrate; Pierce).

Immunohistochemistry

Sections were processed free-floating by pre-embedding immunoperoxidase and immunogold protocols (see Tremblay et al. 2007) using 1 µg/ml of the primary rabbit anti-EphA4 or anti-EphB2 antibodies, without Triton X-100. For electron microscopy, sections were post-fixed flat in 1% osmium tetroxide, embedded in resin (Durcupan ACM; Sigma), and mounted onto ACLAR embedding film (EMS, Hatfield, PA). Small pieces of the latero-ventral hippocampus (CA1 radiatum and CA3 lucidum-radiatum), the deep layers (V-VI) of the primary motor cortex, and the ventrobasal thalamus were excised from the embedding film, re-embedded at the tip of resin blocks, sectioned (70-80 nm-thick) with an ultramicrotome (Ultracut S, Leica), collected on bare square-mesh copper grids, stained with lead citrate, and examined at 60 kV with a Philips CM100 electron microscope.

Data analysis

Electron microscopic examination was carried out on material from 4 C57BL/6 mice, as well as from the EphA4 $^{-/-}$ and EphA4 $^{+/+}$ adult male mice (the latter two processed together, in the same vials). The immunoperoxidase labeling was first examined to identify the types of cellular profiles (dendrites, axons, dendritic spines, axon terminals, glial processes, etc.) that were immunoreactive with the EphA4 and EphB2 antibodies. Six pictures per region randomly taken at 8 900 X, covering ~300 μm^2, were used to assess the relative proportions of each type of profiles labeled.

To refine the subcellular localization (plasma membranes, synaptic vesicles, PSDs, etc.), we used the pre-embedding silver-intensified immunogold procedure (attempts with a post-embedding approach were unsuccessful). To analyze the distribution of the silver-intensified immunogold particles (SIGP), 10 pictures per region (CA1 radiatum, CA3 lucidum-radiatum, deep layers of primary motor cortex, ventrobasal thalamus), covering ~500 μm^2, were randomly taken at 8 900 X, in one animal (except for CA3 lucidum-radiatum, which was analyzed in 3 mice), for each of EphA4 and EphB2. Film negatives were scanned (Epson, Perfection 3200) and converted into positive pictures at a final magnification of 28 500. Images were adjusted for brightness and contrast with Adobe Photoshop (CS) software.

The identification of neuronal or glial cell bodies or processes (axons, dendrites, dendritic spines, axon terminals, glial processes) was made using criteria defined in Peters *et al.* (1991), as in Tremblay *et al.* (2007). Compared to abundant proteins, such as neurotransmitter receptors (e.g., see Riad *et al.* 2001), the relatively low labeling for EphA4 or EphB2 yielded few SIGP per small cellular profile in the electron microscope images. A statistical analysis of the distribution of SIGP was thus required to determine the labeled structures. SIGP were scored in separate compartments: perikarya; astrocytes (the latter two were eventually pooled with "other", with unidentified processes); pre-synaptic active zones, vesicles, plasma membrane and "others" (unidentifiable organelles) of axon terminals; plasma membrane and core of dendrites; and PSD, plasma membrane and "others" of dendritic spines.

In order to estimate the relative area covered by each type of cellular element, as well as the relative probability of each cellular element of being labeled by randomly attached particles, the distribution of a comparable number of virtual particles (VP) of the same size was also scored. To this end, patterns of uniformly distributed VP, made with Photoshop, were overlaid onto the same EM pictures. Indeed, a regular array of particles becomes random in relation to the irregular distribution of the various types of cellular profiles such as dendrites, axon terminals axons dendritic spines, glial processes and so forth, as they appear in EM pictures (see Descarries *et al.* 1982 and Figure S3 in Supplementary Material).

The distributions of SIGPs vs VPs were compared using the Pearson's chi-square test (FREQ procedure, SAS Program, version 8.0, The SAS Institute, www.sas.com). In addition, comparing the numbers of EphA4-SIGPs in area CA3 of the hippocampus in EphA4 $^{-/-}$ and EphA4 ^{wt} mice and the distributions of SIGPs and VPs in EphA4 $^{-/-}$ mice, served to assess the ratios and distribution of background particles.

Pearson's chi-square analysis was first used to compare the distribution of the particles among neuropil cellular profiles in the thin sections (axons, axon terminals, dendrites, and dendritic spines, see Table 1: for each type of profile, we used the summation of the particles scored on their various organelles). In a second step, we analyzed the distribution of the particles among the different organelles or compartments of each type of neuronal profiles (membrane vs core of dendrites; pre-synaptic active zone, synaptic vesicles, plasma membrane or "others" of axon terminals; and PSD, plasma membrane or "others" of dendritic spines). We used the calculation of the exact chi-square distribution, for organelles, to take into account the small size of some samples. To account for the multiple comparisons made on the same data set, differences were considered significant only when $p < 0.01$. Results where p was between 0.01 and 0.05 are described as trends.

RESULTS

EphA4 and EphB2 are detected in microsomes and various synaptosomal compartments

Only limited data exist that address the subcellular distribution of Eph receptors in neurons and glia of the adult rodent brain (Torres et al. 1998, Grunwald et al. 2004). We used differential centrifugation to compare the distribution of Eph receptors from each subclass, EphA4 and EphB2, in the adult mouse forebrain/midbrain. A fractionation protocol (Huttner et al. 1983) was used to separate non-synaptic fractions (cytosol and microsomes) from synaptosomes (P2') (Fig. 1). Synaptosomes were further separated into synaptosomal membranes (LP1, enriched in plasma membrane, large internal membranes and mitochondria) and synaptosomal vesicles (LP2). Several markers were use to characterize the various fractions: NR1 and GluR2/3 for synaptosomal and PSD fractions, PSD95 for PSDs, synaptophysin for synaptic vesicles and presynaptic active zones, SNAP25 for presynaptic active zones, and EEA1 and GM130, respectively, for early endosomes and Golgi membranes.

The two Eph receptors had a similar distribution in these fractions, which represent different cellular compartments. They were abundant in the microsomal fraction, which was enriched in the Golgi membrane marker, GM130, and the endosomal marker, EEA1. The detection of synaptophysin in this fraction is likely due to its presence in transport vesicles. Furthermore, both EphA4 and EphB2 were present at high levels in the purified synaptosomal fraction, which was also enriched in the AMPA and NMDA glutamate receptor subunits, GluR2/3 and NR1 respectively, as well as the synaptic vesicle protein, synaptophysin. The absence of the transmembrane glutamate receptors in the cytosolic fraction further confirmed the purity of the fractions. Separation of membranes (LP1) and synaptic vesicles (LP2) from the synaptosomal fraction showed that EphA4 and EphB2 were enriched in the synaptosomal membranes marked by GluR2/3 and NR1, which are associated with postsynaptic membranes. The two Eph receptors were also detectable in the synaptic vesicle fraction, which was enriched in synaptophysin and devoid of the glutamate receptor markers.

These results indicate that EphA4 and EphB2 are associated with intracellular organelles, such as microsomes and synaptic vesicles, as well as with the synaptosomal membranes, which contain both pre- and postsynaptic membranes.

Strong association of EphA4, but not EphB2, with the postsynaptic density

Since Eph receptors had previously been reported to be localized in postsynaptic structures, we used a second protocol to purify PSDs in order to examine the association of Eph receptors with postsynaptic elements. Different PSD protein fractions were prepared from purified synaptosomes using sequential Triton X-100 and Sarcosyl extractions (Cho et al. 1992) (Fig. 2). Treatment of purified synaptosomes with Triton X-100 resulted in a relative enrichment of EphA4 in the PSD-I pellet, although not as strongly as that of NR1, GluR2/3 or PSD-95, which are known to be concentrated in PSDs. In contrast, the Triton X-100 treatment almost totally extracted EphB2 from the PSDs.

To further purify postsynaptic density components, the PSD-I fraction was extracted again with Triton X-100, which slightly enriched GluR2/3, NR1 and PSD-95 in the PSD-II fraction. EphA4 was also slightly further enriched following this second extraction with Triton X-100, but EphB2 was not. Extraction of the PSD-I fraction with Sarcosyl reduced

the concentration of EphA4 in the resulting PSD-III fraction, in a fashion similar to NR1 and GluR2/3, whereas PSD-95 was further enriched. EphB2 was not detected in PSD-III. Thus, EphA4 is associated with PSD fractions and behaves similarly to glutamate receptor subunits in this extraction protocol. A substantial amount of EphA4 was resistant to extraction with Triton X100 and Sarcosyl, suggesting a strong association with the core (or junctional lattice) proteins of the PSDs. Surprisingly, EphB2 was only weakly associated with the PSD fraction, as it was extracted with Triton X-100 and Sarcosyl.

EphA4 and EphB2 are present in the presynaptic active zone

The subcellular fractionation experiments described above show that EphA4 and EphB2 are associated with synaptic membranes, but do not provide information as to the possible presynaptic localization of the two receptors. Therefore, we used a different synaptic fractionation protocol to separate the pre- and postsynaptic junctional densities from the "*extra-junctional*" membranes (pre- and postsynaptic plasma and vesicle membranes of synaptosomes) and the pre- and postsynaptic *junctional* elements (presynaptic active zones and PSDs) from each other (Phillips et al. 2001, Pinheiro et al. 2005, Rebola et al. 2005, Rebola et al. 2003) (Fig. 3).

Purified synaptosomes were first extracted with 1% Triton X-100 at pH 6, which solubilizes the plasma membrane and vesicle membranes, yielding a soluble extra-junctional fraction and an insoluble pellet consisting of pre- and postsynaptic junctional lattices. Treatment of these synaptic junctions with 1% Triton X-100 at pH 8 detached the presynaptic web from the insoluble PSDs, yielding the presynaptic active zone and PSD fractions. This protocol yielded the expected distributions of markers for the presynaptic active zone (SNAP-25, a protein of the SNARE complex responsible for the fusion of vesicles with the presynaptic active zone) and of markers for synaptic vesicles (synaptophysin) and for the PSDs (PSD-95, NR1 and GluR2/3). A portion of the synaptophysin remained in the presynaptic active zone fraction and likely represents synaptophysin associated with docked or fused vesicles, as recently shown by immunogold labeling (Tao-Cheng 2006). On the other hand, some SNAP-25 was detected in the PSD fraction, suggesting that some presynaptic active zone proteins remained associated with the PSDs (Phillips et al. 2001).

EphA4 and EphB2 were both detected in the extra-junctional membrane fraction and in the junctional lattices. EphB2 was again only weakly detectable in PSDs, whereas EphA4 was abundant in this compartment. Both receptors were abundant in the presynaptic active zone fraction. These results demonstrate that EphA4 and EphB2 can be both found presynaptically, associated with synaptic vesicles and the presynaptic active zone, whereas perhaps only EphA4 is present in the PSDs.

EphA4 and EphB2 have distinct distributions in dendrites versus axon terminals in the adult mouse hippocampus and frontal cortex

To complement the cell fractionation results, we examined the ultrastructural localization of EphA4 and EphB2 in major regions of the forebrain, i.e., the hippocampus and cerebral cortex.

The subcellular distribution of EphA4 in the adult rat hippocampus, as detected by electron microscopy, was recently reported (Tremblay et al. 2007). We show here that EphA4 immunoperoxidase labeling has a similar distribution in the adult mouse hippocampus, with axon terminals, dendritic spines, astrocytic processes (not shown), and axon shafts (not shown) being the most frequently labeled elements (Fig. 4A). Furthermore, some dendritic shafts were occasionally labeled in the mouse hippocampus (Fig. 4A). We also examined the deep layers of the mouse and rat cerebral cortex, where the relative proportion of labeled profiles was similar (not shown). No immunoperoxidase staining was found in EphA4-/- mice.

EphB2 immunoperoxidase labeling was detected mainly associated with relatively large dendritic shafts and dendritic spines in the hippocampus and deep layers of the primary motor cortex, by electron microscopy, while relatively few nerve terminals were labeled (Fig. 4B-D). Dendritic shafts, spines and nerve terminals represented respectively 44, 49 and 3 % of the labeled neuronal profiles in area CA1; 56, 29 and 7 % in area CA3; and 70, 22 and 0 % in the deep layers of the primary motor cortex. Occasionally, some fine, usually perisynaptic astrocytic processes were labeled for EphB2, but less frequently than for EphA4. Thus, EphB2 appears to have a mostly dendritic rather than axonal localization in the regions of hippocampus and cerebral cortex examined.

EphA4 and EphB2 are also distributed differently at the organelle level

The immunoperoxidase reaction product can diffuse locally inside the labelled processes and therefore does not allow precise determination of subcellular localization. Hence, we used a pre-embedding immunogold protocol combined with silver intensification to examine the association of the EphA4 and EphB2 receptors with the plasma membrane, synaptic vesicles, presynaptic active zones and the PSDs. The silver-intensified immunogold particles (SIGPs) labeled the same neuronal processes that were also positive by immunoperoxidase labeling (Figs. 5,6). However, the number of SIGPs in small individual processes such as axons, dendritic spines, and axon terminals was small. We therefore performed a statistical analysis of the distribution of the SIGPs and comparable numbers of randomly distributed virtual particles (VPs; Pearson's chi-square analysis; see Materials and Methods and supplementary Fig. S3). The results are shown in Table 1.

As a control, we also analyzed the distribution of the SIGP labeling for EphA4 in the hippocampus of an EphA4-/- mouse. This analysis did not reveal significant differences between the distributions of SIGPs and VPs among the profiles of neuronal process or at the organelle level, in the knockout mouse. Background labeling thus appeared uniform and the various neuronal compartments were labeled according to their surface area in the ultrathin sections. The number of SIGPs in the CA3 region of hippocampus of the EphA4-/- mouse were 27% of those found in the same region of the hippocampus of a EphA4+/+ littermate that was processed in parallel (Table 1). This feature therefore represents an estimate of the background labeling. Subtracting this background to obtain the specific labeling for EphA4 had only a small impact on the calculation of the relative EphA4 labeling in different subcellular structures and did not change the conclusions of the analyses (not shown). Therefore, we expressed the results and conducted the chi-square statistical analyses for both EphA4 and EphB2 without background subtraction (Fig. 6).

In all regions examined, and for both EphA4 and EphB2, there was a significant difference between the distributions of the SIGPs and VPs among the various types of neuronal profiles ($p < 0.0005$, except for EphA4 in CA3, where $p<0.02$, 0.005 and 0.0001, respectively in the 3 animals; Table 1, last column; glial processes were excluded from this analysis). These differences between SIGPs and VPs were attributable to EphA4-associated

SIGPs, which were more concentrated than the VPs in axon terminals, and to EphB2-associated SIGPs, which were strongly concentrated in dendritic shafts in all hippocampal and cortical areas examined. The distribution of SIGPs (Fig. 6) was consistent with the immunoperoxidase labeling for both EphA4 and EphB2, except for a somewhat lower number of EphB2 SIGPs in dendritic spines. There was no significant difference in the distributions of EphA4 or EphB2 SIGPs, respectively, between the 3 animals examined in CA3, confirming the reproducibility of these results.

Since the fractionation experiments indicated higher EphB2 levels in axon terminals of the forebrain/midbrain than detected in electron microscopy in terminals of the hippocampus and parietal cortex, we also examined the distribution of EphB2 in the ventrobasal thalamus. In this region, EphB2-associated SIGPs were more frequently found in axon terminals and also in small dendritic shafts (Fig. 6).

These results demonstrate that EphA4 and EphB2 are localized in overlapping yet distinct patterns in the hippocampus and cerebral cortex. EphA4 is more frequently localized in axon terminals, while EphB2 predominates in dendrites. Moreover, the subcellular localization of EphB2 appears to vary regionally, since this receptor is frequently found in axon terminals in the thalamus but not in the hippocampus and cortex.

EphB2 is more associated with the plasma membrane than EphA4

The distribution of SIGPs was then analyzed at the organelle level (i.e. in the plasma membrane, synaptic vesicles, pre-synaptic active zone, and PSDs; Figs. 7,8). In this analysis, EphA4-associated SIGPs were found to be distributed rather uniformly and similarly to the VPs, with one exception (Table 1). In the CA1 region, they were more frequently associated with the plasma membrane and vesicles of the axon terminals ($p < 0.003$). This pattern of distribution was not observed in the CA3 region.

EphB2-associated SIGPs were more heterogeneously distributed, at the sub-profile level, in the hippocampus and motor cortex. They were more concentrated on the plasma membrane of dendritic shafts than the VPs ($p < 0.0001$). This was confirmed in 3 animals, in the CA3 region of the hippocampus ($p < 0.0001$ in every case). The EphB2-associated SIGPs also appeared concentrated on the plasma membrane of axon terminals in CA1 ($p < 0.0001$).

In the ventrobasal thalamus, EphB2-associated SIGPs were uniformly distributed at the plasma membrane and the core of nerve terminals and small dendrites. Thus, their distribution in the ventrobasal thalamus was more similar to that of EphA4-associated SIGPs in the hippocampus and cortex, with a major location in axon terminals and no clear preference for the plasma membrane versus the core of the profiles.

Chi-square analyses of the distributions of SIGPs associated with EphA4 and EphB2 showed that they were significantly different from each other in all brain regions where both receptors were examined (CA1, CA3, and deep layers of motor cortex - or CtxD; $p < 0.0001$). This was also true in the 3 animals where the analysis was performed in CA3.

Overall, the biochemical and immunohistochemical observations are in agreement. Both EphA4 and EphB2 were detected in pre-synaptic elements (nerve terminals) as well as postsynaptic elements (dendritic shafts and spines). EphA4 was detected on vesicles and plasma membranes of synaptic terminals, in both the hippocampus and the cerebral cortex. EphB2 was also detected on vesicles in axon terminals, with higher frequency in the ventrobasal thalamus. In electron microscopy, both receptors were also detected in small astrocytic processes, as previously reported for EphA4 by Tremblay et al. (2007). Several Eph receptors, including EphA4 and EphB2, have also been detected by RT-PCR in freshly isolated astrocytes (Nestor *et al.* 2007). However, EphA4 labelling on PSDs, in electron microscopy, did not appear as abundant as expected from fractionation results, presumably because of a limited access to the epitopes in these dense structures.

DISCUSSION

The functions of the Eph receptors in the adult brain are beginning to be elucidated, and information on the precise localization of these receptors at the subcellular level is needed to fully interpret the functional data. To date, most reports have emphasized a postsynaptic localization and function for the Eph receptors. This is largely based on limited electron microscopy analyses in the adult brain (Buchert et al. 1999, Martone et al. 1997) and biochemical demonstrations of interactions between some Eph receptors and postsynaptic proteins (see below).

We have investigated the subcellular and sub-synaptic distributions of EphA4 and EphB2 by cell fractionation and electron microscopy. Cell fractionation showed many similarities, but also some major differences in the distribution of the two receptors in different subcellular compartments. Both receptors were abundant in the microsomal fraction; findings that will require further investigation to identify the precise organelles with which they are associated. They might be present in transport vesicles, as synaptophysin which was also detected in this fraction (Morin *et al.* 1991). Interestingly, EphB2 has been previously localized in the Golgi apparatus and associated with peri-Golgi vesicles (Hoogenraad *et al.* 2005). In the synaptosomal fractions, EphA4 and EphB2 were associated with membranes, synaptic vesicles and presynaptic active zones. Previous cell fractionation studies of adult rat brain also detected EphB2 in the synaptosomal fraction (Torres et al. 1998), and detected EphB2 and EphA4 in PSD as well as non PSD fractions (Grunwald et al. 2004). We found that the two receptors differ markedly in their association with PSDs. Surprisingly, EphB2 was easily extracted from PSDs with Triton X-100, whereas a subpopulation of EphA4 remained tightly associated with PSDs, even following further treatment with Sarcosyl. Our finding of an association of both Eph receptors with the presynaptic active zone and synaptic vesicles in the adult brain raises new questions about their synaptic roles and may warrant reconsideration of previous functional models based on an exclusively postsynaptic localization.

Electron microscopic examination also showed that EphA4 and EphB2 have rather different locations in the hippocampus and cerebral cortex, with EphA4 being associated more with axon terminals, and EphB2 with dendritic shafts, while both appear similarly associated with dendritic spines. At the organelle level, the statistical analyses of the distributions of SIGPs versus VPs demonstrated that EphB2 is more concentrated at the plasma membrane of relatively large dendritic shafts in the hippocampus and cerebral cortex. The fractionation results indicate that EphA4 and EphB2 are also more concentrated in other types of organelles, such as synaptic vesicles. However, the limited resolution of the electron microscopy approach used, which combines pre-embedding immunogold labeling with statistical analysis of the distribution of the resulting SIGPs, did not allow conclusive demonstration of a more precise localization of the two receptors in the smaller processes.

EphA4 is more tightly associated with the PSDs than EphB2

EphA4 was previously detected by confocal microscopy in dendritic spines of pyramidal neurons of area CA1 in the adult mouse hippocampus, where it appears to regulate spine morphology and density through interactions with astroglial ephrinA3 (Murai et al. 2003). The present demonstration of its association with the extra-junctional plasma membrane of spines fits nicely with such interactions with astrocytic processes, whereas the EphA4 located in the PSDs most likely plays a distinct role at the synaptic junction. Therefore, the localization of EphA4 suggests that this receptor plays multiple roles in dendritic spines. To date, no direct interaction with other receptors or scaffolding proteins has been demonstrated for EphA4, but its cytoplasmic domain contains a sterile alpha motif (SAM) and a PDZ- (postsynaptic density-95, disc large, zonula occludens-) binding motif that could mediate an interaction with such proteins, as already demonstrated for EphA7 (Torres et al. 1998).

It was surprising to find that EphB2 is weakly associated with the PSDs. Moreover, the detection of EphB2 in the PSD fraction might even be due to contamination by presynaptic proteins, as indicated by the presence of SNAP-25 in this fraction. EphB2 has been implicated in functions thought to depend on its postsynaptic location in excitatory synapses (Buchert et al. 1999, Grunwald et al. 2001, Dalva *et al.* 2000, Henderson et al. 2001). The present fractionation and electron microscopic results suggest that the EphB2 localized in the extrajunctional plasma membranes of dendritic shafts or spines (or in astrocytes), rather than the PSDs, might be fulfilling these functions.

The expression of EphA4 overlaps with that of EphB receptors in the hippocampus (Liebl et al. 2003, Grunwald et al. 2001), and EphA4 has a high affinity for ephrinB2 and ephrinB3 (Gale et al. 1996). Therefore, EphA4 might also contribute to ephrinB-regulated spine maturation in concert with EphB receptors (Henkemeyer et al. 2003). Considering that EphA4 is tightly associated with the PSDs, and that it does not seem to interact with the same proteins as EphB2 (Dalva et al. 2000, Ethell *et al.* 2001), its role may be complementary rather than redundant with that of EphB2.

EphA4 and EphB2 are also presynaptic

The EphA4 and EphB2 receptors were both detected in the synaptic vesicles and the presynaptic active zone fraction. Since synaptophysin was also detected in the presynaptic active zone fraction, it is possible that some vesicle membrane remained in this fraction. Indeed, synaptophysin has recently been detected in the active zone by immunogold electron microscopy, following vesicle fusion with the plasma membrane during transmitter release (Tao-Cheng 2006). Therefore, these results demonstrate that both receptors are present in axon terminals. Their presence in these presynaptic fractions suggests that they follow vesicles to the active zone. Moreover, immunoperoxidase staining showed that EphA4 and EphB2 antibodies often, but not always, labeled only subsets of the vesicles in axon terminals (see also Tremblay et al. 2007). This suggests that they are then localized in special types of vesicles, perhaps transport, or recycled vesicles with internalized receptors. In such cases, it is possible that EphB2 localized in presynaptic terminals represents the internalized receptor that was trans-endocytozed on its ephrinB ligand side following activation in its postsynaptic localization (Zimmer *et al.* 2003). Nevertheless, in view of its possible absence from PSDs, suggested by its weak association with the PSD fractions, this possibility appears improbable.

EphB2 has been reported to use GRIP-1 as an adaptor for kinesin-dependent transport into dendrites, using the motor protein KIF5 (Hoogenraad et al. 2005). KIF5 is also involved in the transport of newly synthesized membrane proteins into axons (Munakata *et al.* 2003). In addition, synaptojanin-1, a nerve terminal protein involved in the recycling of synaptic vesicles, is a phosphorylation substrate for EphB2 (Harris *et al.* 2000, McPherson *et al.* 1996), which could thus influence clathrin-mediated endocytosis (Irie *et al.* 2005, Schuske *et al.* 2003). Synaptojanin-null drosophila mutants also display increased amounts of presynaptic active zone per unit of surface area, at the neuromuscular junction (Dickman *et al.* 2006). Eph receptors could thus participate in the presynaptic cycling of synaptic vesicles and regulation of the active zone.

Defining the roles and molecular associations of EphA4 and EphB2 on the presynaptic side of mature synapses will be important to fully understand their functions in the adult CNS.

Roles of EphA4 and EphB2 in LTP

The mechanisms of LTP differ between the CA3 and CA1 regions of hippocampus, and EphA4 and EphB2 are thought to play distinct roles in these mechanisms (Contractor et al. 2002, Henderson et al. 2001, Grunwald et al. 2001, Grunwald et al. 2004). Electrophysiological data suggest that EphB2 is involved postsynaptically in mossy fiber-CA3 LTP (Contractor et al. 2002). Our observations indicate that this receptor is indeed localized preferentially in dendritic shafts and spines, but not strongly associated with the PSDs. Its role in LTP must therefore involve the "*extrajunctional"* pool of this receptor (or the small pool present in astrocytes). On the other hand, EphA4 is present both in mossy fibers and in the spines of the CA3 pyramidal neurons (Tremblay et al. 2007). It would thus be well situated to play a dual role in mossy fiber-CA3 LTP. Currently, there is only functional evidence for a role of EphA4 in regulating LTP at the Shaffer collateral-CA1 synapses, where it could serve as a presynaptic ligand for postsynaptic ephrin-B2 and ephrin-B3 localized in dendritic spines of CA1 pyramidal neurons (Grunwald et al. 2004, Rodenas-Ruano *et al.* 2006, Liebl et al. 2003). The data presented here and our previous results (Tremblay et al. 2007), which localized EphA4 in the Schaffer collateral terminals, are consistent with a presynaptic role of EphA4 in synapses of area CA1 of the hippocampus. However, further work is necessary to clarify the respective roles of pre- and postsynaptic (or glial) EphA4 and EphB2 in LTP mechanisms in the CA3 versus CA1 sectors of hippocampus.

CONCLUSIONS

The present results show that EphA4 and EphB2 are both localized in several pre- and postsynaptic compartments of synapses in the adult forebrain/midbrain: synaptic vesicles, presynaptic active zones, PSDS, and extrajunctional membranes. However, their relative distributions among these compartments differ in cortical structures (hippocampus and cerebral cortex), where EphA4 is more abundant in axon terminals and EphB2 in dendritic shafts (and particularly in the plasma membrane of the latter). At the synaptic level, the major difference between the two receptors concerns the PSDs, where EphA4 appears tightly linked to the junctional lattice, whereas EphB2 was easily extracted with detergents. It will be interesting to associate each of these locations with specific functions, such as regulation of dendritic spine maturation or of different types of LTP.

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Table 1. **Neuropil distribution of the silver-intensified immunogold particles for EphA4 and EphB2**.

Silver intensified immunogold particles (SIGPs) were scored in axons, axon terminals, dendritic spines, and dendritic shafts (those in neuronal perikarya, astrocytic processes and unidenfiable profiles were pooled as "Others", after the statistical analyses). A similar number of virtual particles (VPs) of the same size was then overlaid onto the same micrographs and scored in the same manner (see Fig. S3, in supplementary material). The distributions among neuronal processes were then compared, using the Pearson's chi-square test (last column). In a second step, chi-square test analyses compared the distribution of SIGPs and VPs among the organelles (plasma membrane, vesicles, PSDs, active zones, etc) of each type of process. The results of the latter comparisons are indicated by an asterisk and bold type (*, when $p<0.003$). Since the number of VPs is proportional to the area of the various elements in the pictures, a significantly higher proportion of SIGPs than VPs scored over a given type of structure signifies that this structure is more strongly labelled than others. An absence of specific labelling can be concluded when the number of SIGPs is inferior to the background, i.e., less than 27% of the VPs counted in the same type of process or organelle. **[a]** In area CA3, the analysis was done in 3 animals, and means ± SEM are reported, to verify the reproducibility of the analyses. No significant difference was found in the distribution of SIGPs between the 3 animals. **[b]** For EphA4 in CA3, the resulting "p" differed among animals. It was smaller than 0.02 (considered as a tendency), 0.005 and 0.0001 (significant), respectively in each animal. It was thus considered as significant overall. AZ, presynaptic active zone; CtxD, deep layers of primary motor cortex; KO, EphA4 $^{-/-}$ mouse; mb, plasma membrane; PSD, postsynaptic density; TOT, total; Vs, vesicles.

A) EphA4 immunolabeling

Region	Labeling	axons			axon terminals				dendritic spines			dendrites		Other	TOT	χ2
		mb	core	myelin	AZ	Vs	mb	other	PSD	mb	other	mb	other			
CA1	SIGP	16	2	0	3	*60**	*48**	*38**	8	24	44	32	69	66	410	p<0.0002
CA1	VP	19	17	1	2	40	22	55	6	16	38	15	76	92	400	
CA3 [a]	SIGP	32 ± 6	20 ± 6	0	3 ± 1	46 ± 3	25 ± 1	33 ± 2	2 ± 1	9 ± 1	24 ± 3	23 ± 1	67 ± 10	41 ± 2	326 ± 20	p<0.02 [b]
CA3 [a]	VP	26 ± 5	29 ± 9	5 ± 2	1 ± 1	31 ± 2	15 ± 2	29 ± 3	2 ± 1	9 ± 2	15 ± 2	13 ± 1	76 ± 12	72 ± 3	323 ± 23	
CA3 KO	SIGP	0	3	2	0	10	2	5	2	1	5	8	34	15	87	NS
CA3 KO	VP	7	7	2	0	11	4	5	1	2	6	7	20	15	87	
CtxD	SIGP	10	12	2	2	35	15	17	7	14	25	17	32	32	220	p<0.0001
CtxD	VP	3	19	8	0	27	13	15	3	3	9	18	38	44	200	

B) EphB2 immunolabeling

Region	Labeling	axons			axon terminals				dendritic spines			dendrites		Other	TOT	χ2
		mb	core	myelin	AZ	Vs	mb	other	PSD	mb	other	mb	other			
CA1	SIGP	4	3	1	1	*11**	*34**	*20**	13	26	28	*135**	*121**	87	484	p<0.0001
CA1	VP	9	1	2	2	25	14	40	7	6	12	81	212	89	500	
CA3 [a]	SIGP	9 ± 2	4 ± 3	0	2 ± 1	25 ± 3	22 ± 6	23 ± 3	6 ± 2	20 ± 2	27 ± 4	*167 ± 13**	140 ± 10	65 ± 4	510 ± 20	p<0.0001
CA3 [a]	VP	21 ± 3	17 ± 4	5 ± 2	1 ± 1	27 ± 1	25 ± 5	56 ± 4	4 ± 1	12 ± 2	21 ± 3	42 ± 3	140 ± 10	95 ± 7	467 ± 20	
CtxD	SIGP	8	6	2	0	7	19	8	3	3	2	*109**	39	31	237	p<0.0001
CtxD	VP	10	16	12	2	21	19	15	1	6	7	12	32	46	200	
Thal	SIGP	*22**	*16**	*1**	4	35	27	40				37	77	42	301	p<0.0001
Thal	VP	11	23	28	0	19	15	28				22	65	89	300	

Figure legends

Figure 1: Representative examples of Western blots following cell fractionation of the adult mouse forebrain/midbrain. In this and the next 2 figures, the reported fractionation experiments were repeated entirely at least 3 times. The same amount of proteins (10 μg) was loaded in every lane. EphA4 and EphB2 show similar distributions in microsome and synaptosome fractions. Both are detected in non-synaptic (microsomes) and in synaptic membrane (LP1) and vesicle (LP2) fractions in the adult mouse brain. GluR2/3 and NR1 are subunits of NMDA and AMPA glutamate receptors; EEA1 and GM130 are, respectively, the "early endosome antigen-1" and the "golgi membrane-130" markers.

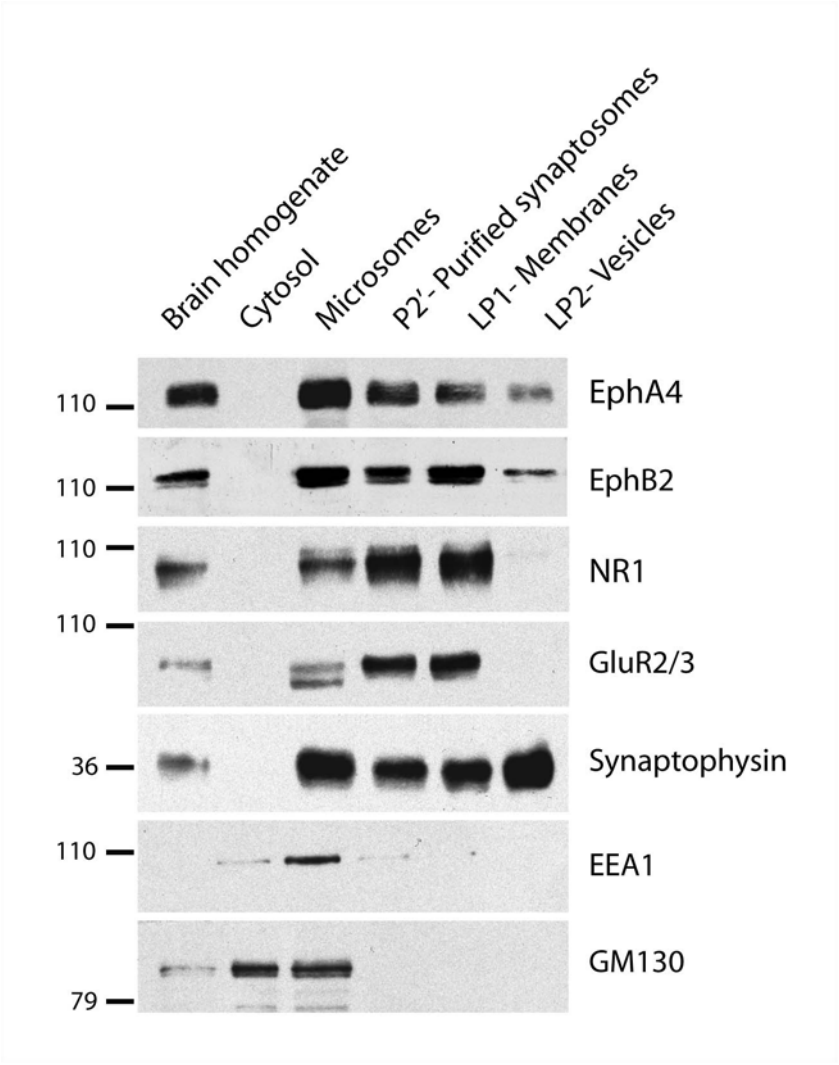

Brain homogenate
Cytosol
Microsomes
P2'- Purified synaptosomes
LP1- Membranes
LP2- Vesicles
110
EphA4
110
EphB2
110
NR1
110
GluR2/3
36
Synaptophysin
110
EEA1
79
GM130

Figure 2: Extraction of postsynaptic densities (PSDs) from purified synaptosomes. EphA4 is strongly associated with PSD-I and PSD-II, after TritonX-100 extraction, and partly resists extraction with Sarcosyl (PSD-III), as do the NR1 and GluR2/3 subunits of glutamate receptors, known as PSD-associated proteins. In contrast, EphB2 was easily extracted with Triton X- 100 and detected at very low levels only in PSD-I and PSD-II. PSD-95 is a core protein of PSDs, and synaptophysin is a marker for synaptic vesicles. Although not apparent in this figure, PSD-95 and NR1 were detectable in the P2' fraction, as in Fig. 1, but only with longer exposure of the autoradiographic film. Since the film rapidly reached saturation for these proteins in the PSD fractions, where they are highly concentrated, we had to shorten the exposure so as to be able to detect the differences in density between the three PSD fractions.

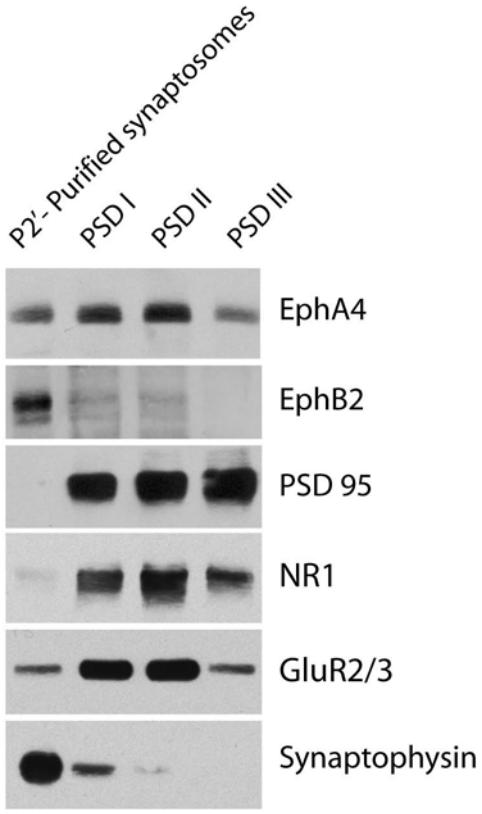

P2- Purified synaptosomes
PSD I
PSD II
PSD III
EphA4
EphB2
PSD 95
NR1
GluR2/3
Synaptophysin

Figure 3: Separation of pre- and post-synaptic densities from purified synaptosomes shows that EphA4, but maybe not EphB2, is associated with both sides of synapses. EphA4 and EphB2 are found in the presynaptic active zones and *extra-junctional* membrane fractions (pre- and postsynaptic plasma and vesicle membranes), but only EphA4 is abundant in PSDs. SNAP-25 is a marker for presynaptic active zones. It was still detected in the PSD fraction, indicating that some presynaptic proteins remained in this fraction. GluR2/3 was detected rapidly in the PSD fraction, before it became apparent in the purified synaptosomal fraction. With longer exposure (not shown), it was also detected in the latter fraction, as in Figs. 1 and 2, where the same amount of proteins were loaded (10 µg per lane).

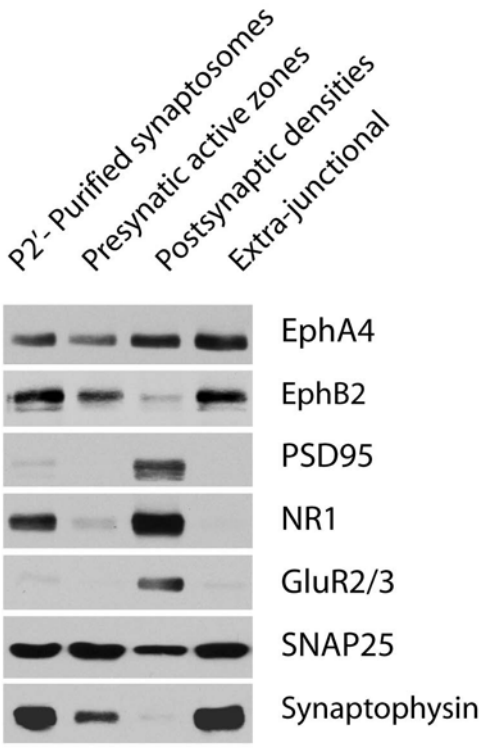

P2′- Purified synaptosomes
Presynatic active zones
Postsynaptic densities
Extra-junctional
EphA4
EphB2
PSD95
NR1
GluR2/3
SNAP25
Synaptophysin

Figure 4. A) EphA4 is preferentially expressed in axon terminals and dendritic spines in area CA3 of the hippocampus. EphA4 immunoperoxidase in electron microscopy shows labelled axon terminals (t+) and dendritic spines (s+). In addition, a dendritic shaft (d+) and an axon terminal display spots of labelling (arrows) while the rest of these profiles appear unlabelled. B) EphB2 immunoperoxidase labeling in area CA3 of the hippocampus is found primarily in dendritic shafts (d+), but dendritic spines (s+) and rare axon terminals (t+) are also labelled. t-, unlabeled terminals. C and D show examples of such labelled dendritic spines (s+). Scale bars: 0.5 μm.

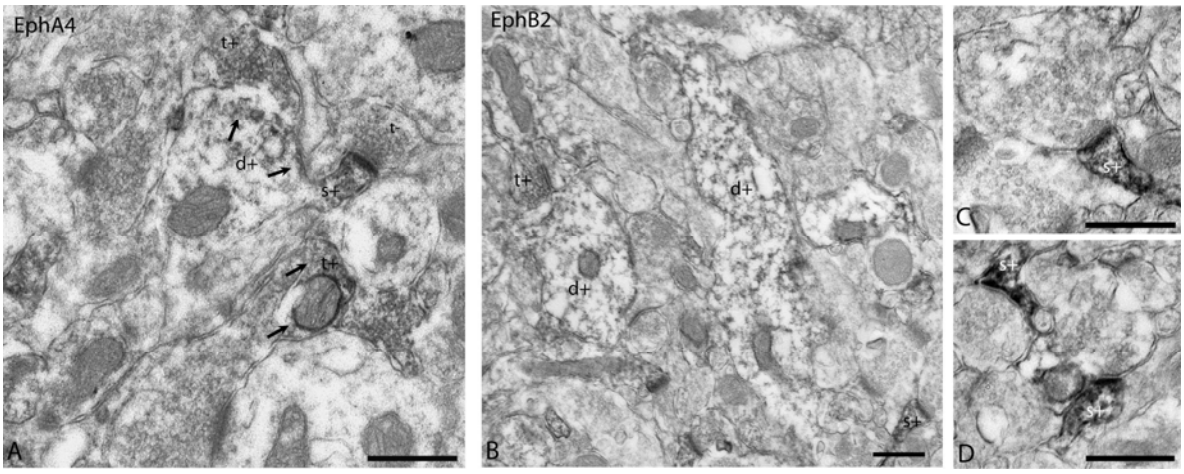
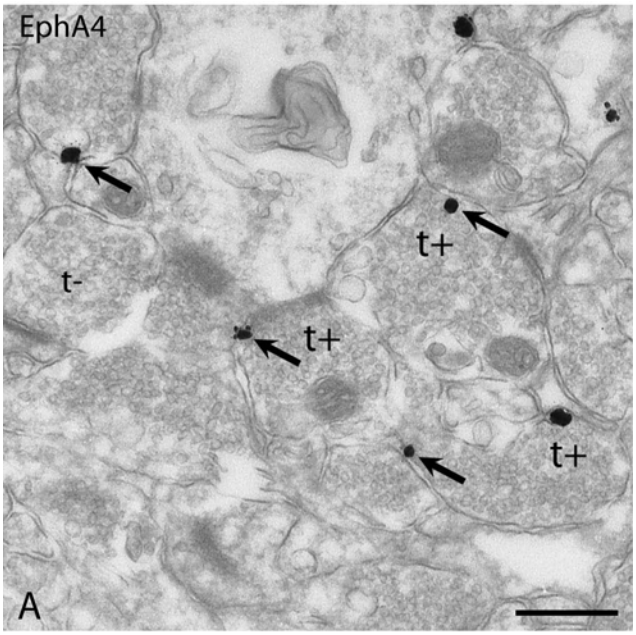

EphA4
t+
t-
d+
s+
t+
A
EphB2
t+
d+
d+
s+
B
s+
C
s+
s+
D

Figure 5. Pre-embedding immunogold labeling of the hippocampus showing the distributions of EphA4 and EphB2. A) In area CA1, EphA4-associated silver-enhanced immunogold particles (SIGPs, arrows) are frequent in axon terminals. B) In area CA3, EphB2-associated SIGPs predominate in dendritic shafts. Arrows show examples of SIGPs on the plasma membrane of dendritic shafts. Scale bars: 0.5 μm.


EphA4
t+
t-
t+
t+
A

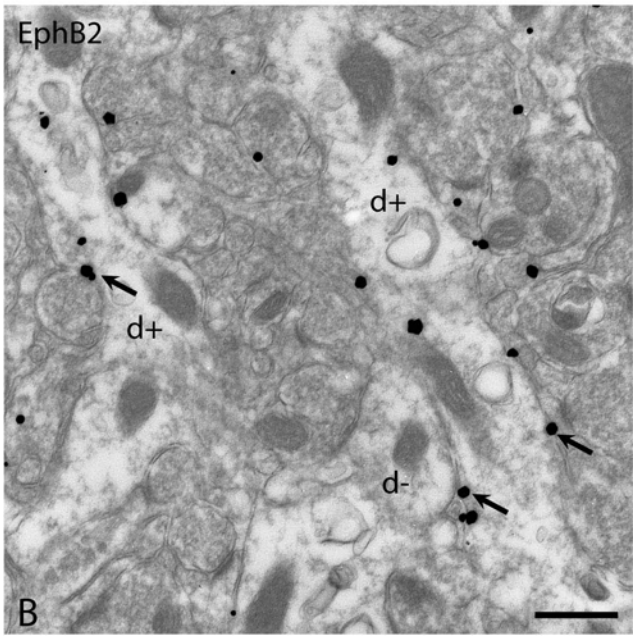

EphB2
d+
d+
d-
B

Figure 6. Distribution of A) EphA4-associated SIGPs in areas CA1 and CA3 of the hippocampus and deep layers of the primary motor cortex (CtxD), and B) EphB2-associated SIGPs in the same regions and in the ventrobasal thalamus (Thal). The small numbers of SIGPs scored in perikarya, glia, or unidentified profiles are not represented. Pearson's chi-square analyses showed that the distributions of both EphA4 and EphB2 SIGPs differed significantly from those of random VPs and that the distributions of EphA4 SIGPs differed from those of EphB2 in every analyzed hippocampal and cortical region. This was also true in the 3 animals analysed in the CA3 region (see text and Table 1).

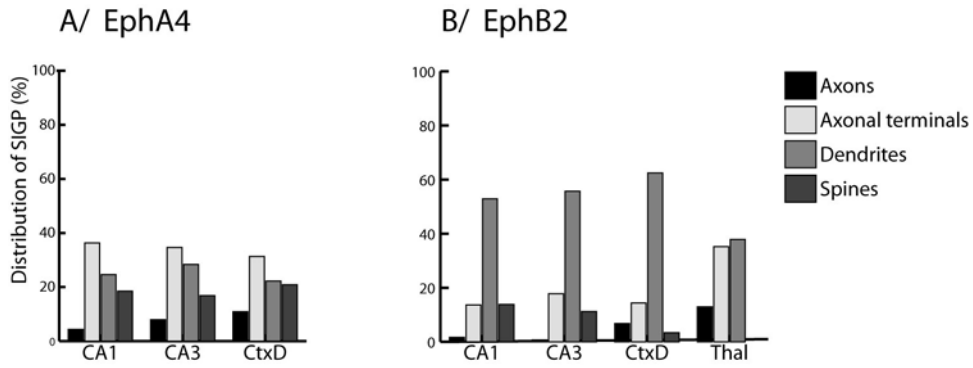

A/ EphA4
B/ EphB2
Distribution of SIGP (%)
100
80
60
40
20
0
CA1
CA3
CtxD
Thal
Axons
Axonal terminals
Dendrites
Spines

Figure 7. Localisation of SIGPs at the organelle level. A-C: EphA4 SIGPs touching A) the plasma membrane of axon terminals in area CA3 of the hippocampus (arrows), B) the core of a dendritic spine in CA1 (arrow, spine apparatus), C) the PSD of a dendritic spine in the parietal cortex. D-F: EphB2 SIGPs touching D) synaptic vesicles of axon terminals in the ventrobasal thalamus (arrows), E) the presynaptic active zone of a nerve terminal in the ventrobasal thalamus (arrow), or F) the plasma membrane of a dendritic spine in CA1 (arrow). Scale bars: 0.25 μm.

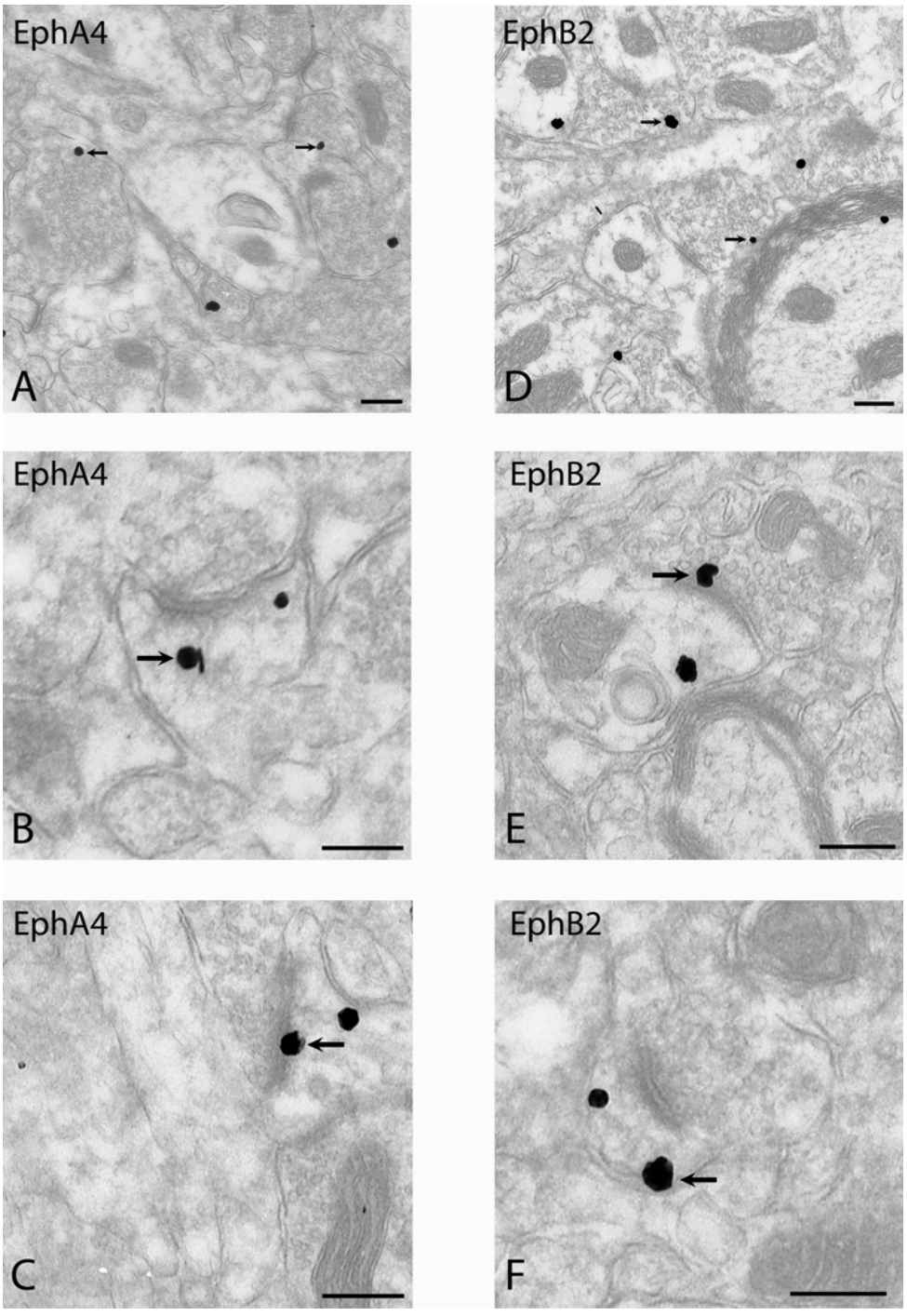
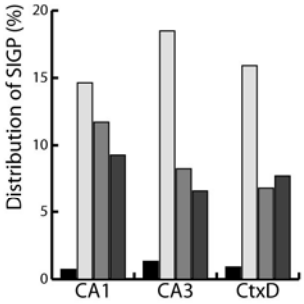

EphA4
A
EphB2
D
EphA4
B
EphB2
E
EphA4
C
EphB2
F

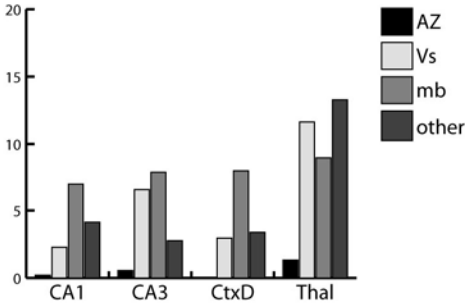
Figure 8. Distribution of SIGPs in axon terminals (A,B) or dendritic spines (C,D), for EphA4 (A,C) and EphB2 (B,D). AZ, presynaptic active zones; mb, plasma membrane; PSDS, postsynaptic densities ; Vs, synaptic vesicles; “other” indicate SIGPs present in the core of the profiles, but not clearly attributable to specific organelles.

Axon terminals

A/ EphA4

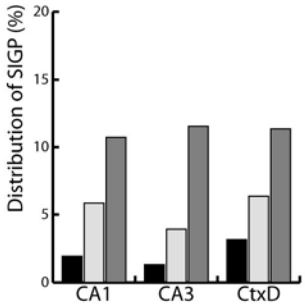


B/ EphB2

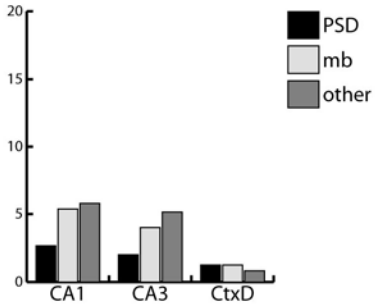


Dendritic spines

C/ EphA4



D/ EphB2



Supplementary Material

Figure S1. Western blots from lysates of HEK293 human embryonal epithelial cells transfected with EphB2 or EphB4 and probed with anti-EphB2 or anti-EphB4 antibodies demonstrate the specificity of the anti-EphB2 antibody used in the present study.

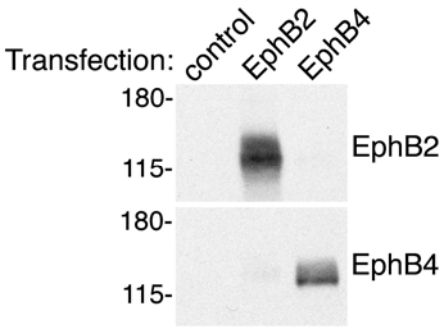

Transfection:
control
EphB2
EphB4
180-
115-
EphB2
180-
115-
EphB4

Figure S2. EphB2 immunoperoxidase staining of the hippocampus and dentate gyrus (DG) in light miscroscopy. l-m, stratum lacunosum moleculare; l/r, strata lucidum radiatum; o, stratum oriens; p, stratum pyramidale; po, polymorph layer; r, stratum radiatum. Scale bar: 500 μm.

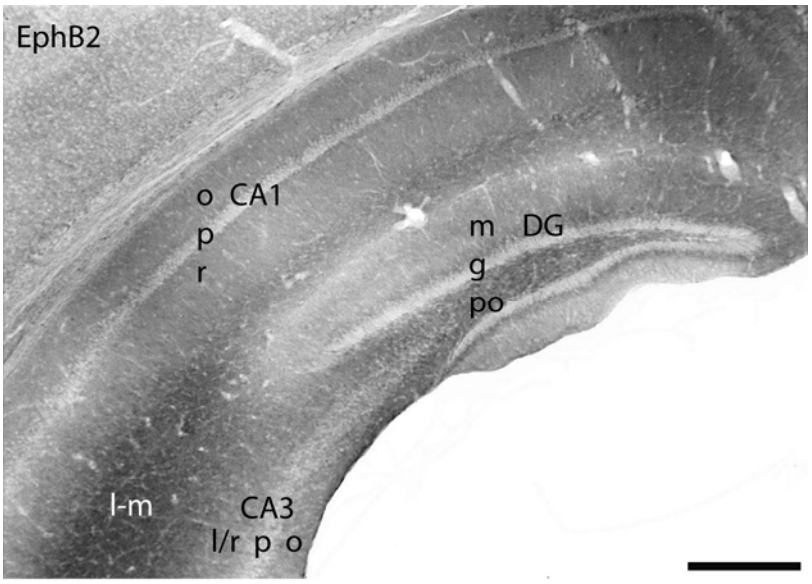
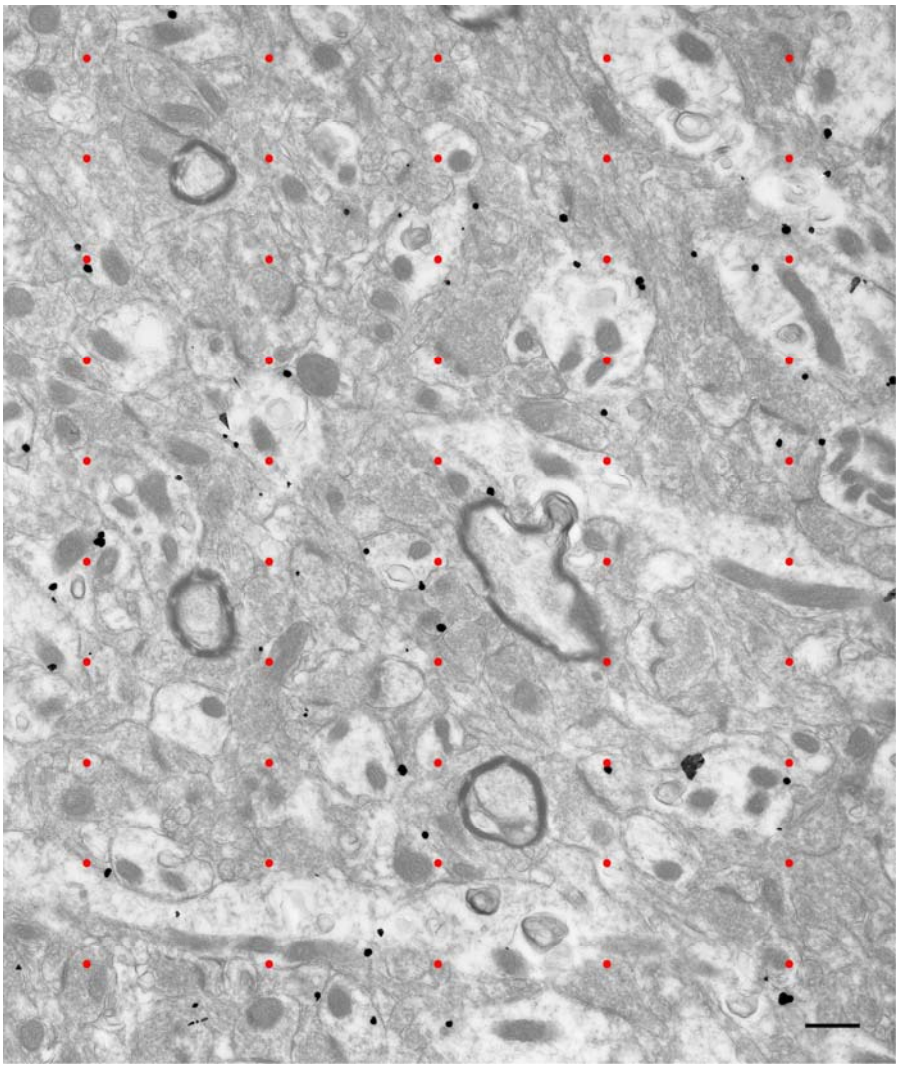

EphB2
o CA1
p
r
m DG
g
po
l-m
CA3
l/r p o

Figure S3. Distribution of EphB2-associated SIGPs (black particles) compared to a similar number of virtual particles, or VPs (red) of a comparable size, overlaid on the same electron micrograph. The VP were drawn in Photoshop, as a regular grid of dots, and were thus distributed at random relative to the profiles in the pictures. Scale bar: 0.5 μm. This method is based on a stereological approach used by Descarries et al. (1982). Other basic stereological references discussing this approach are cited in this paper.

Indeed, stereological studies had previously been performed to assess the distribution of autoradiographic silver grains in electron micrographs; a situation very similar to the one in this manuscript. A recognized approach to assess the area covered by a given type of profile in electron microscopy (or its chance of being hit by random particles), is to use virtual grains randomly distributed in relation to the structure of the tissue. In the cerebral cortex or hippocampus, EM pictures from the neuropil of brain tissue contain profiles of various types and sizes (dendrites, axons, terminals, spines, glial processes, mitochondria, cell membrane and so forth) that are irregularly distributed. Using an overlay of a regular array of grains of similar size and numbers constitutes an easy means to produce a random distribution of grains relative to these EM pictures, since the tissue itself does not follow such a regular organization.



Annexe II. EphA4 is associated with synaptic and clathrin-coated vesicles in synapses of adult mouse brain

Manuscrit en préparation

Comme deuxième auteure, j'ai contribué à cette étude par la réalisation des expériences, l'analyse des résultats et la préparation des figures d'immunocytochimie en microscopie électronique, ainsi qu'en collaborant à la rédaction du manuscrit.

EphA4 is associated with synaptic and clathrin-coated vesicles in synapses of adult mouse brain

David Bouvier[1], Marie-Ève Tremblay[1], Mustapha Riad[1], Amadou T. Corera[2], Katie Horn[2], Keith K. Murai[3], Timothy Kennedy[2], Peter McPherson[2], Edward A. Fon[2], Guy Doucet[1]*

[1] Département de pathologie et biologie cellulaire, Groupe de recherche sur le système nerveux central, Université de Montréal, Montréal, QC

[2] Department of Neurology and Neurosurgery, McGill University, and Montreal Neurological Institute, Montréal, QC

[3] Department of Neurology and Neurosurgery, McGill University, and Centre for Research in Neuroscience, Montreal General Hospital, Montréal, QC

*_Corresponding author_, Guy Doucet, Département de pathologie et biologie cellulaire, Faculté de médecine, Université de Montréal, C.P. 6128, succ. Centre-ville, Montréal, QC, Canada H3C 3J7. E-mail: [redacted] , Telephone: 514-343-6255, Fax: 514-343-5755.

Running title: EphA4 in synaptic and clathrin-coated vesicles

Key Words: EphA4, synaptic vesicles, clathrin-coated vesicles, trafficking, immunoisolation, electronic microscopy, hippocampus

ABSTRACT

We recently showed that EphA4 is frequently associated with axonal terminals, where it is distributed in different functional compartments, such as subpopulations of vesicles and active zones. The present study aimed at determining the association of EphA4 with the various types of transport, synaptic and clathrin-coated vesicles, using cell fractionation, immunoisolation of vesicles in the forebrain-midbrain and immunocytochemistry in electron microscopy in region CA1 of the hippocampus. EphA4 was detected in vesicles isolated with antibodies anti-synaptophysin, anti-VGLUT1 and anti-VGAT; demonstrating its presence in synaptic vesicles. However, it was not detected in Piccolo-Bassoon transport vesicles, immunoisolated with an anti-piccolo antibody. EphA4 was also found in a fraction enriched in clathrin-coated vesicles, suggesting its recycling together with synaptic vesicles. In CA1 of hippocampus, EphA4 was localized on VGLUT1-positive, but not on VGAT positive terminals. Nevertheless, it was colocalized with both VGLUT1 and VGAT vesicular markers in hippocampal neurons in culture. When such cultures were stimulated with KCl, there was an increase in cell surface EphA4 expression, as detected by immunocytochemistry with an antibody directed against its extracellular domain, in the absence of detergent. These observations constitute strong evidence that EphA4 is present in synaptic vesicles, a novel observation for a tyrosine kinase receptor raising new questions about its functional role in this compartment.

INTRODUCTION

Eph receptors, the largest family of tyrosine kinase receptors, count 14 members in mammals, divided in two subclasses EphA (A1-8, and A10) and EphB (B1-4, and B6) (Himanen and Nikolov, 2003). Their functions have been mostly studied in the development of the central nervous system (CNS), where they participate in various processes as such cell migration, segmentation patterning, and axonal growth cone guidance (Klein, 2004; Pasquale, 2005).

Most Eph receptors are still expressed in the mature CNS (Goldshmit et al., 2006), where they have been implicated in synaptogenesis and synaptic plasticity (Murai and Pasquale, 2004; Yamaguchi and Pasquale, 2004). Eph receptors have been generally viewed as postsynaptic receptors of the mature synapse (Buchert et al., 1999; Martone et al., 1997; Murai et al., 2003; Torres et al., 1998), implicated in the modulation of the morphology of dendritic spines (Ethell et al., 2001; Henkemeyer et al., 2003; Murai and Pasquale, 2004; Penzes et al., 2003).

Defects have also been described in synaptic plasticity, such as in LTP or LTD, in EphA4 or EphB2 null mice, but their precise roles in these processes are still not well understood (Armstrong et al., 2006; Contractor et al., 2002; Grunwald et al., 2004; Henkemeyer et al., 2003; Murai et al., 2003). Again, the role of these Eph receptors in synapse activity and plasticity has generally been interpreted with the view of a postsynaptic localization of the receptor, although EphA4 was suggested to act as a presynaptic partner for postsynaptic ephrin-B2 and -B3 in the modulation of CA3-CA1 LTP (Grunwald et al., 2004).

We recently demonstrated that EphA4 and EphB2 are localized in axon terminals in the mature CNS (Bouvier et al., 2008; Tremblay et al., 2007). The two molecules were found to have different subcellular distributions in the hippocampus and cerebral cortex, where EphA4 was mainly detected in axon terminals, while EphB2 was found predominantly in dendritic shafts and rarely in axon terminals. However, EphB2 was also frequently detected in axon terminals in the ventrobasal thalamus. Both receptors were localized in various compartments of the presynaptic terminals, by cell fractionation and electron microscopy, such as the plasma membrane, the presynaptic active zone and subpopulations of vesicles. The association of these Eph receptors with vesicles and

presynaptic active zones suggests a role in the activity of the mature synapse and needs to be fully characterized.

The presence of Eph receptors in vesicles of axonal terminals could reflect multiple sorting steps in their cellular trafficking, but could also represent a specific function in the vesicle cycle. To address these questions, we examined the association of EphA4 with presynaptic vesicles, including piccolo-bassoon transport (PTVs), synaptic, and clathrin-coated vesicles (CCVs), as a first step to understand how EphA4 could reach the presynaptic active zone and be retrieved.

Analysis of vesicle-enriched fractions on a linear sucrose gradient showed that the receptor is associated with various types of vesicles. Since the receptor might transit through various types of vesicles to reach the presynaptic active zone, such as synaptic vesicles, or Piccolo-Bassoon transport vesicles (Dresbach et al., 2006; Shapira et al., 2003; Tao-Cheng, 2007), we have proceeded to the immunoisolation of synaptic vesicles and PTVs, using anti-synaptophysin and anti-piccolo antibodies, respectively. EphA4 was associated with synaptic vesicles but not with PTVs. We further characterized the association of EphA4 with subpopulations of glutamatergic and gabaergic synaptic vesicles, respectively using anti-VGLUT1 and anti-VGAT antibodies.

Electronic microscope observations with double immunolabeling for EphA4 with VGLUT1 or VGAT in the CA1 sector of the hippocampus revealed that EphA4 was frequently associated with glutamatergic but not with gabaergic axon terminals. However, double immunochemistry, on cultured hippocampal neurons showed frequent colocalizations of EphA4 with VGLUT 1 and VGAT in the neurites, at 15 days in vitro (DIV15). Since EphA4 might reach the presynaptic active zone through synaptic vesicles, we detected the presence of the receptor on CCVs, through which it might be endocytosed with synaptic vesicles components (Bonanomi et al., 2006). Finally, we found an increase in the cell surface expression of EphA4 following intense electrical activity in hippocampal cultures.

These observations unveil new possibilities of trafficking and role of Eph receptors in association with synaptic vesicles and CCVs at excitatory and inhibitory synapses and lead to new questions on their actions on axon terminals in the mature synapse.

RESULTS

EphA4 in multiple subpopulations of vesicles

We recently reported that EphA4 is present in 25 to 40% of the axon terminals in adult murine hippocampus (Tremblay et al., 2007). We also reported that it is distributed in plasma membrane, presynaptic active zone and in vesicles of axon terminals (Bouvier et al., 2008). The EphA4 immunolabeling sometimes decorated all the vesicles of a terminal profile, but sometimes also it was restricted to only a small group of vesicles, usually near the plasma membrane (Fig. 1). These observations suggest that EphA4 is present in different types of vesicles in the axon terminals.

To further characterize the types of EphA4 positive vesicles, we used a cell fraction enriched in vesicles, LP2, from adult mouse forebrain/midbrain, on a linear sucrose density gradient (10-35% sucrose, Fig. 2). The low density organelles, present in fractions 1 to 3, were nearly devoid of synaptic vesicle markers, except for traces of VMAT2 in fraction 2, but contained Rab7, a late endosome marker, starting in fraction 2. The subpopulation of vesicles in fractions 4 to 9 was enriched in the synaptic vesicle markers synaptophysin, VGLUT1 and synaptotagmin. The higher density fractions (11-16) contained a variety of synaptic and endosomal vesicle proteins in lower quantity. All fractions were negative for the early endosome marker, EEA1, consistent with our previous result that LP2 was devoid of this marker (see also Bouvier et al., 2008, Fig.1). EphA4 was absent from fractions 1 to 4, but progressively appeared in fractions 5 to 15. Its distribution appeared bimodal, with peaks in fractions 7-8 and 10-11, suggesting two different pools of vesicles. Thus EphA4 did not follow exactly the distribution of synaptic vesicle markers, but their distributions overlapped to some extent.

EphA4 is present in various subpopulations of immunoisolated vesicles

The above EM and cell fractionation results support the presence of EphA4 in synaptic, as well as other types of vesicles. Since EphA4 has previously been detected in fractions of presynaptic active zones (PAZ) (Bouvier et al., 2008), the question arise as to whether this receptor is transported to this specialized region by synaptic vesicles or by Piccolo-Bassoon transport vesicles (PTVs) (Dresbach et al., 2006; Shapira et al., 2003; Tao-Cheng, 2007), which are known to transport the PAZ material. To examine these

possibilities, we isolated subpopulations of synaptic vesicles and PTVs from the LS1 supernatant (see Bouvier et al., 2008), which is enriched in vesicles and deprived of synaptosomal membranes.

Antibodies directed against the cytoplasmic part of synaptophysin or piccolo, were used to immunoisolate synaptic vesicles and PTVS, respectively (Fig.3.A, B). Labelling for EphA4 was observed in synaptophysin vesicles, but absent from piccolo vesicles.

To further characterize the association of EphA4 with synaptic vesicles, we immunoisolated distinct subpopulations of specific neurotransmitter vesicles, using anti-VGLUT1 and anti-VGAT antibodies (Fig.3.C, D). Both immunoisolates were enriched in synaptophysin, confirming the integrity of the immunoisolated vesicles. Control isolations done with non specific IgGs showed faint spots of reactivity for synaptophysin, and larger ones for VGLUT1 or VGAT, but were negative for EphA4. The presence of IgG around 50 kDa may explain in part the spots of VGLUT1 or VGAT in the controls. Nevertheless, there was a clear enrichment in synaptophysin, VLUT1 and VGAT in the specific immunoisolates, which confirmed the efficiency of the immunoisolations. EphA4 was detected in both populations of VGLUT 1 and VGAT vesicles in comparable amounts.

Importantly, EphA4 did not co-immunoprecipate with VGLUT1 or VGAT after treatment of the LS1 fraction with 0.5 % Triton X-100. The use of detergent disrupted lipid membranes in the vesicle-enriched preparations and, thus, only the proteins recognized by the antibodies and their direct interacting partners were precipitated. This suggests that EphA4 is present in synaptic vesicles, but not in a direct physical interaction with the vesicular transporters.

EphA4 is also associated with clathrin-coated vesicles

The presence of EphA4 in synaptic vesicles as well as in other types of vesicles moved us to examine its presence in a preparation of purified CCVs (Allaire et al., 2006; Girard et al., 2005a). Indeed, clathrin-mediated endocytosis is critical for the reuptake of synaptic material and the reformation of synaptic vesicles (Jung and Haucke, 2007). As shown in Figure 4, EphA4 showed a similar enrichment as the clathrin heavy chain protein in this fractionation protocol (in Pellet 2 (P2), in the Sucrose Gradient pellet or SGp, and in

the Sucrose Gradient supernatant, or SGs). Compared to the clathrin heavy chain, it was less enriched, but still quite abundant in the CCVs fraction.

This pattern of EphA4 distribution is similar to that of synaptotagmin and synaptophysin, described previously (Allaire et al., 2006).

EphA4 is present in axon terminals expressing VGLUT1, but not VGAT in hippocampal CA1 adult

Double EM immunocytochemistry, in region CA1 of the hippocampus showed a frequent co-localization of EphA4 with VGLUT1 (Fig. 5 A,B) but never with VGAT-positive terminals (Fig. 5 C, D). Thus, these results suggest that EphA4 is present in glutamatergic terminal, but not in gabaergic ones, at least in CA1 of the hippocampus.

EphA4 is co-localized with VGLUT1 and VGAT in cultured hippocampal neurons

A dual immunocytochemical labeling was performed for EphA4 with either VGLUT1 or VGAT, on hippocampal neurons at DIV15. Immunostaining for EphA4 with the ab11 antibody, directed against its cytoplasmic part, gave a granular staining along neurites, with a more diffuse staining over cell bodies (Fig.6 A, D, G, J). This staining is consistent with that reported from other studies at DIV15 (Fu et al., 2007). Immunostaining for VGLUT1 or VGAT also gave granules of different sizes all along neurites and around cell bodies, consistent with the known synaptic localization of these vesicular proteins (Fig. 6 B, E, H, K). Double immuno-labeling for either VGAT or VGLUT1, together with EphA4, showed frequent spots of co-localization (Fig. 6 C, F, I, L). EphA4 co-localized with both VGLUT1 and VGAT, suggesting an association with both subpopulations of synaptic vesicle, *in vitro*.

The surface expression of EphA4 is regulated by neuronal activity in hippocampal culture

The above observations constitute strong evidence that EphA4 is present in synaptic vesicles, as well as in endocytotic CCVs, in nerve terminals. This transit through these types of vesicle suggests a role for this tyrosine kinase receptor in association with neuronal activity in the presynaptic terminals. In this context, we tested if cell surface expression of EphA4 changed during neuronal activity. Immunostaining was done with an antibody

raised against the extracellular domain of the receptor, on cultured hippocampal neurons, in a non stimulated condition and after 2 min of KCl depolarization (Fig. 7). The extracellular immunostaining appeared as small intense spots, or granules, on the cell bodies and neurites. We measured only the granules on neurites. Following the KCl treatment, there was a significant increase in the mean optical density of EphA4 staining, the mean surface area covered by the granules, and the numbers of EphA4 granules detected on the neurites in each of three experiments (Fig.7B-D).

DISCUSSION

We previously demonstrated that EphA4 is associated with presynaptic compartments including the active zone and vesicles, in addition to being in postsynaptic elements such as spines and PSD (Bouvier et al., 2008). In the present study, we have further investigated the association of EphA4 with synaptic and endocytotic vesicles by cell fractionation, immunoisolation, confocal and EM dual immunocytochemistry. The results provide strong evidence for the presence of EphA4 in synaptic vesicles, a novel observation for a tyrosine kinase receptor, as well as with CCVs.

EphA4 may be transiting to the active zone through neurotransmitter synaptic vesicles

The EM observations and the linear sucrose density gradient indicated that EphA4 was attached to various subpopulations of vesicles. In EM, the EphA4 immunoreaction product often decorated only small subpopulations of vesicles in axon terminal profiles, while, in fractionation, EphA4 only partly overlapped the distribution of synaptic vesicle markers (VGLUT1, synaptophysin, synaptotagmin) indicating that it is also present in other types of vesicles. Two points have to be considered to read this fractionation. EphA4 is not expressed in all nerve terminals, while it is also located in several other types of organelles. Therefore, its distribution in the fractions is expected to differ from that of synaptic vesicle markers.

According to the immunoisolation and immunocytochemistry on cultured hippocampal neurons, at least two distinct populations of synaptic vesicles, VGLUT1- and VGAT-positive, were carrying EphA4. Indeed, the glutamate and GABA transporters have been demonstrated to reside on separate vesicles which are functionally distinct

subpopulation of synaptic vesicles (Takamori et al., 2000). In contrast, the PTVs immunoisolated with an anti-piccolo antibody, were EphA4-negative, indicating that the receptor is not transported to the presynaptic active zone in these transport vesicles. Indeed, PTVs are clearly distinct from the synaptic vesicle and have been described as the transport vesicles which carry the cytomatrix material composed of piccolo, bassoon, Rim, Munc13, Munc18, and SNAP25 to the active zone (Shapira et al., 2003). Thus, one hypothesis that should be tested is that EphA4 is transported to the axon terminals and presynaptic active zones by synaptic vesicles.

It may seem contradictory that EphA4 was detected only in VGLUT1-positive axon terminals in CA1 of the hippocampus. This result is consistent with our previous EM analysis of EphA4 in the mature rat hippocampus (Tremblay et al., 2007), where it was concluded that most, if not all, of the labelled nerve terminals were excitatory (mostly axo-spinous and asymmetric). However, the LS1 supernatant, the starting material for immunoisolation, was extracted from total forebrain/midbrain of adult mouse. Examination of other forebrain regions where EphA4 is expressed, such as the thalamus, might therefore reveal EphA4-containing GABA terminals. On the other hand, the co-localization of EphA4 and VGAT *in vitro* in hippocampal neurons at DIV15 could be due to the embryonic state of the neurons in culture.

A role for EphA4 in endocytosis?

The localization of EphA4 in CCVs is also a novel observation and raises some questions about the endocytosis of the receptor and its recycling. In the CCVs fractionation, EphA4 showed a distribution similar to that of the synaptic vesicle markers, synaptophysin and synaptotagmin (Allaire et al., 2006). It has been estimated that approximately 90 % of all CCVs isolated from rat brain lysate are involved in SV retrieval (Girard et al., 2005b). Thus this tyrosine kinase receptor could be recycled together with the synaptic vesicle material and its association with CCVs might considered as further evidence of an association of EphA4 with synaptic vesicles.

Other receptor tyrosine kinases, like RET (Richardson et al., 2006), the insulin growth factor receptor (Monami et al., 2008), the epidermal growth factor receptor (Carter and Sorkin, 1998; Jiang et al., 2003; Sorkina et al., 2002), p75 and Trk receptors (Bronfman

et al., 2003; Zheng et al., 2008) have already been described using the clathrin-mediated endocytosis. Internalized TrkA and TrkB have also been shown to be still competent, functional and signaling in the endocytotic vesicles (Grimes et al., 1997; Grimes et al., 1996) while transported from synapses to cell body in a still phosphorylated state (McPherson et al., 2001; Watson et al., 1999). In analogy, Eph receptors might also remain functional in the endocytotic vesicles. Indeed, the ephrin ligand and the Eph receptor have been shown to be endocytosed as complexes. Two different mechanisms have been proposed, cleavage of ephrin-As by a metalloprotease, for EphA-ephrin-A complexes (Hattori et al., 2000; Janes et al., 2005) and trans-endocytosis for EphB-Ephrin-B complexes (Marston et al., 2003; Zimmer et al., 2003), but neither type of endocytosis had been characterised or associated with clathrin-mediated endocytosis.

Although EphA4 had never been directly associated with CCVs, another Eph receptor, EphB2, has previously been implicated in the molecular modulation of the clathrin-dependant endocytosis by influencing the tyrosine phosphorylation of synaptojanin-1 (Hopper and O'Connor, 2005; Irie et al., 2005). Interestingly, the activation of EphA4 has also been shown to result in the recruitment of cyclin-dependent kinase-5 (Cdk5) leading to its phosphorylation (Fu et al., 2007). This interaction has been described in spines, however Cdk5 is also a key component of presynaptic vesicle endocytosis in the clathrin machinery (Nguyen and Bibb, 2003), where it seems to modulate the phosphorylation of the endocytic proteins, dynamin, amphyphisin, and also synaptojanin-1 (Graham et al., 2007; Lee et al., 2004; Tan et al., 2003; Tomizawa et al., 2003).

Furthermore activated EphA4 also recruits and activates PLCγ1 (Zhou et al., 2007), which then generates the ubiquitous second messengers inositol 1,4,5-trisphosphate and diacylglycerol (Rebecchi and Pentyala, 2000). There phosphoinositides are known to play a critical role in clathrin-mediated endocytosis (Gaidarov and Keen, 1999). Likewise, PLCγ1 has the same cellular distribution as EphA4 in cell fractionation experiments and is also associated with synaptic vesicles (Zhou et al., 2007). Thus EphA4 could directly participate in the synaptic vesicle cycle.

A role for EphA4 in synaptic vesicles?

Eph receptors could transit by synaptic vesicles to be delivered to the PAZ where they could play a role in the structural cohesion of synapses. Indeed EphA4 and EphB2 have been also localized in PAZ (Bouvier et al., 2008). Many adhesion molecules, including cadherins, neuroligins, neurexins, and SynCAM (synaptic cell adhesion molecule) are concentrated in PAZ and are essential for synapse formation and stabilization (Dalva, 2007; Rosenmund et al., 2003). Interestingly, EphA4 cell surface expression appeared directly modulated by neuronal activity. Thus, the increase in EphA4 surface expression of hippocampal neurons, after stimulation of neurotransmitters release with KCl, is likely associated with synaptic vesicle fusion with the PAZ.

Furthermore, EphB2 has been demonstrated to interact with GRIP1 and to be an essential partner for the kinesin-dependant transport of AMPA receptors to the postsynaptic part of the synapse (Hoogenraad et al., 2005). EphA4 might therefore play a similar role in the trafficking of synaptic vesicles to terminals.

Conclusion

The present observations unveil potentially novel aspects in the trafficking and function of Eph receptors in axon terminals. The association of EphA4 with synaptic vesicles and CCVs suggest new possibilities for roles in the synaptic vesicle cycle and in endocytosis. Its cell surface expression seemed to be modulated by neuronal activity and is consistent with an implication of Eph receptors in synapse plasticity.

MATERIALS AND METHODS

Animals

All procedures were conducted in strict accordance with the *Guide to the Care and Use of Experimental Animals* (Ed2) of the Canadian Council on Animal Care. The experimental protocols were approved by the Animal Care Committee of the Université de Montreal.

Cell fractionation and immunoisolation experiments were conducted on 14 adult male C57BL/6 mice. These animals were decapitated under deep CO2 anaesthesia.

The electron microscopic analysis was carried out on brain tissue from four adult male C57BL/6 mice. These animals were deeply anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and perfused through the aortic arch with a solution of 0.5 % acrolein diluted in 4 % paraformaldehyde (PFA), in 0.1 M sodium phosphate buffer (PB) pH 7.4, followed by 4 % PFA. Brains were further fixed by immersion in 4% PFA for 1 h at 4°C, and washed in sodium phosphate-buffered saline (PBS; 0.9% NaCl in 50 mM PB, pH 7.4). Transverse sections, 50 µm thick, were cut in ice-cooled PBS with a vibratome.

For hippocampal cell culture, 12 litters of E18-19 rat fetuses were taken from pregnant dams, deeply anaesthetized with CO_2.

Antibodies

The polyclonal anti-EphA4 antibody (Ab11), a generous gift from Dr E.A. Pasquale (The Burnham Institute, La Jolla, CA), has been shown to recognize EphA4 in rat, mouse and human (Martone et al., 1997; Murai et al., 2003; Soans et al., 1994). We also tested its specificity for EM immunocytochemistry with EphA4-/- mice (Tremblay et al., 2007). The monoclonal EphA4 antibody, raised against peptides 279-472 of the extracellular domain of EphA4, was purchased from BD Transduction Laboratories (BD Biosciences, Mississauga, ON, Canada) and its specificity tested by Western blotting from brain homogenate of EphA4-/- mice (Helmbacher et al., 2000).

The anti-VGLUT1 and anti-VGAT monoclonal antibodies, respectively raised against a synthetic peptides corresponding to residues 456-560 of rat VGLUT1, or residues

75-87 of rat VGAT, were purchased from Synaptic systems (Goettingen, Germany). The polyclonal antibody anti-Piccolo, raised against residues 4439-4776 of the rat protein, and the monoclonal anti-synaptophysin antibody were purchased from Synaptic system. The anti-clathrin heavy chain (CHC) antibody was purchased from BD Biosciences.

LS1 and LP2 subcellular fractionation of mouse forebrain/midbrain

Subcellular fractions were prepared from adult mouse forebrain/midbrain (2 for each of 3 experiment), as described (see supplementary material in Zhou et al., 2007). Briefly, the forebrain/midbrain was dissected out on ice, following a transverse cut between the occipital cortex and cerebellum. All subsequent steps were done at 0-4°C. Brain tissues were homogenized by 9 up-and-down strokes at 900 rpm in 20 ml of ice-cold sucrose/HEPES buffer (0.32 M sucrose, 10 mM HEPES, pH 7.4) containing protease inhibitors (Roche complete). The nuclear material (P1) was discarded following centrifugation at 1 000 x g during 10 min, and the supernatant (S1) was centrifuged again at 12 000 x g for 15 min. The new supernatant was discarded and the pellet was homogenized again in 20 ml of ice-cold sucrose/HEPES buffer and centrifuged again at 13 000 x g for 15 min. The resulting pellet, constituting the synaptosomal fraction (P2) was re-suspended in approximately 2 ml of sucrose/HEPES buffer.

Two milliliters of this P2 fraction were lysed osmotically in 20 ml of 10 mM HEPES buffer containing the protease inhibitors, and then homogenized by 3 up-and-down strokes at 2 000 rpm and centrifuged at 33,000 x g for 20 min. The pellet, representing the synaptosomal membrane fraction (LP1) was discarded, while the supernatant (LS1) was used for immunoisolation of vesicles, or centrifuged again at 260 000 x g during 2 h, to obtain the crude vesicle fraction (LP2).

Linear sucrose density gradient (SDG) of crude vesicle fraction:

The crude vesicle fraction, LP2, was re-suspended in 3 ml of sucrose/HEPES buffer (0,04 M sucrose, 10 mM HEPES, pH 7.4). The fraction was homogenized (3 strokes at 900 rpm) followed by repeated extrusion through 22 ½ and 27 ½ gauge needles and layered on a top of a 37 ml linear sucrose density gradient ranging from 10 to 35% of sucrose solution. The gradient was centrifuged at 120 000 x g during 4h using a SW 28 rotor (Beckmann). Sixteen 2,5 ml aliquot fractions were collected from the top (fraction 1) to the bottom of the

tube (fraction 16). Equal volumes of each fraction (80 µl) were added to 2X loading buffer and loaded on a SDS gel for Western blotting.

Immunoisolation of vesicles

Magnetic beads (~10^7) with pre-coupled immunoglobulins (Dynabeads M-280 sheep anti-rabbit or anti-mouse IgG; Dynal, Invitrogen, Oslo, Norway), were washed 3 times with PBS and incubated overnight with the primary polyclonal or monoclonal antibodies (3 µg of each), or rabbit or mouse control immunoglobulins (Chromopur rabbit or mouse IgG, Jackson Immunoresearch Laboratories, West Grove, PA, USA), in PBS containing 1 % bovine serum albumin (BSA). The LS1 supernatant (0.4 µg of protein per µl) was used as an immunoisolation buffer (Ii buffer) consisting of LS1 fraction, 50 mM Tris HCl at pH 7,4, 50 mM KCl, 20 mM $MgCl_2$, 0,2 M sucrose, and protease inhibitors (Complete EDTA-free, Roche, Manheim, Germany). The antibody-coupled beads were washed 3 times with PBS/BSA 1%. One milliliter of the Ii buffer/ BSA 1% was added to the pre-coupled beads for each immunoisolation and incubated for 1h45. The beads were then washed 4 times with PBS and resuspended in 25 µl of loading buffer (Biorad, Hercules, CA, USA), heated at 95 °C for 5 min, for standard SDS PAGE and Western blotting.

Isolation of clathrin-coated vesicles

The isolation of clathrin-coated vesicles (CCVs) from rat adult brain was performed as described (Girard et al., 2005a).

Hippocampal cell culture

Primary neuronal cultures were prepared from the hippocampi of 18-19 days old foetal mice, as described by Banker (Banker and Cowan, 1977). For each experiment (n=3), two litters of C57BL/6 mice were used. Briefly, the hippocampi were dissociated by treatment with trypsin (0.25% for 15 min at 37°C, Sigma), followed by trituration with fire-polished Pasteur pipettes. Dissociated cells were plated at densities of 100 000 to 200 000 on poly-L-lysine-treated coverslips (Fisher, Nepean, Ontario, Canada) in minimum essential medium (MEM, Invitrogen) containing 10 mM pyruvic acid, 0,6% glucose and 10 % heat-inactivated horse serum. After 3 h of incubation at 37ºC, cultures were fed with

Neurobasal medium (Invitrogen) supplemented with 0,5 mM L-glutamine and 2 % B27 (Gibco, Invitrogen). Cultures were fixed after 15 days *in vitro* (DIV15) with 4% paraformaldehyde.

Immunofluorescence and confocal microscopy

Coverslips were treated for 5 min with 3% Triton X-100 in PBS, rinsed 3 times with PBS, and blocked during 1h with a solution of 5% BSA in PBS, at room temperature. They were incubated overnight with the primary antibodies, at room temperature, in a humid chamber, in 5% BSA in PBS. After 3 rinses in PBS, coverslips were incubated for 1h with FITC-labeled anti rabbit and TRITC-labeled anti-mouse antibodies in PBS/BSA. The coverslips were rinsed 4 times with PBS and mounted with Mowiol. Immunofluorescence was viewed and captured by a Leica DM IRBE confocal microscope. High-resolution pictures were acquired as composites of eight confocal optical sections.

KCl depolarization of hippocampal neurons

Hippocampal neurons at DIV15 were incubated for 2 min with 90 mM KCl in Neurobasal medium supplemented with glutamine, and B27. They were then washed twice with ice-cold PBS, before fixation in 4% PFA. Triton X-100 was omitted to restrict the immunodetection of EphA4 to the cell surface by using an antibody directed against the extracellular domain of the receptor. Immunofluorescence was viewed and captured by a fluorescence microscope (Karl Zeiss, Axiophot, Germany) equipped with a numeric camera (qimaging, Canada). The measurements of cell surface EphA4 was carried out with the Northern Eclipse Imaging V.6 software (Empix Imaging, Missisauga, ON, Canada), on 25 frames covering each 21 000 μm^2 for each condition (control vs KCl treatment). Unstimulated and KCl-treated cultures were analyzed under the same image exposure, with the same threshold values. The analysis was performed in 3 cultures for each condition. All granular spots of labeling were treated as individual objects for the following measurements: object count, selected area, total object area, percent object area, and total gray. Measurements were automatically transferred in an Excel document. Optical density was the result of the gray value of the spot multiplied by its area and divided by the total area of the frame.

Electron microscope immunocytochemistry

Sections were processed free-floating by pre-embedding immunoperoxidase and immunogold protocols. The protocol of double-EphA4 and –VGLUT1 or –VGAT immunolabeling for electron microscopy was slightly modified from the single labeling protocol described previously (Tremblay et al., 2007). After simultaneous incubation with both primary antibodies for 48h, VGLUT1 (monoclonal, 1:200 dilution; Synaptic Systems, Goettingen, Germany) or VGAT (monoclonal, 1:200 dilution; Synaptic Systems) was labeled with immunogold and then EphA4 (Ab11; 1:500 dilution) with immunoperoxidase. For immunogold labeling, sections were incubated overnight at room temperature (RT) in goat anti-mouse IgGs conjugated to 1-nm colloidal gold particles (AuroProbe One; Amersham Biosciences, Oakville, Ontario, Canada) in a blocking solution of PBS containing 5% normal goat serum and 0.5% gelatin. The diameter of the immunogold particles was increased with a silver enhancement kit (IntenSE, Amersham Biosciences) for 12-15 minutes at RT. For immunoperoxidase labeling, the sections were incubated for 2 hours at RT in goat anti-rabbit IgGs conjugated to biotin (Jackson Immunoresearch, West Grove, PA) and with streptavidin-horseradish peroxidase (Jackson Immunoresearch) for 1 hour in blocking solution. Afterwards, labeling was revealed with 0.05% diaminobenzidine (DAB) and 0.01% hydrogen peroxide in Tris/HCl-buffered saline (50 mM, pH 7.4).

Sections were post-fixed flat in 1% osmium tetroxide, embedded in resin (Durcupan ACM; Sigma), and mounted onto ACLAR embedding film (EMS, Hatfield, PA). Small pieces of the latero-ventral hippocampus (CA1 pyramidal cell layer and stratum radiatum) were excised from the embedding film, re-embedded at the tip of resin blocks, sectioned (70-80 nm-thick) with an ultramicrotome (Ultracut S, Leica Canada, St-Laurent, Québec, Canada), collected on bare square-mesh copper grids, stained with lead citrate, and examined at 60 kV with a Philips CM100 electron microscope.

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Abbreviations

CA1, Cornus ammonis sectors of hippocampus; CCVs, clathrin-coated vesicles; Cdk5, cyclin-dependent kinase-5; CNS, central nervous system; EEA1, early endosome antigen-1; LTD, long term depression; LTP, long term potentiation; PAZ, presynaptic active zone; PLCγ, Phospholipase-C gamma; PSD, postsynaptic density; PTVs, Piccolo-bassoon transport vesicles; SynCAMs, synaptic cell adhesion molecule; VGAT, vesicular GABA transporter ; VGLUT, vesicular Glutamate transporter; VMAT2, vesicular monoamine transporter.

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Figure Legends

Figure 1: Electron microscope immunocytochemistry for EphA4 in the CA1 region of adult mouse hippocampus showing that the immunostaining sometimes decorates all synaptic vesicles in an axon terminal profile (A) and sometimes is restricted to only a subgroup of such vesicles (B, C). Scale bars: 250 nm.

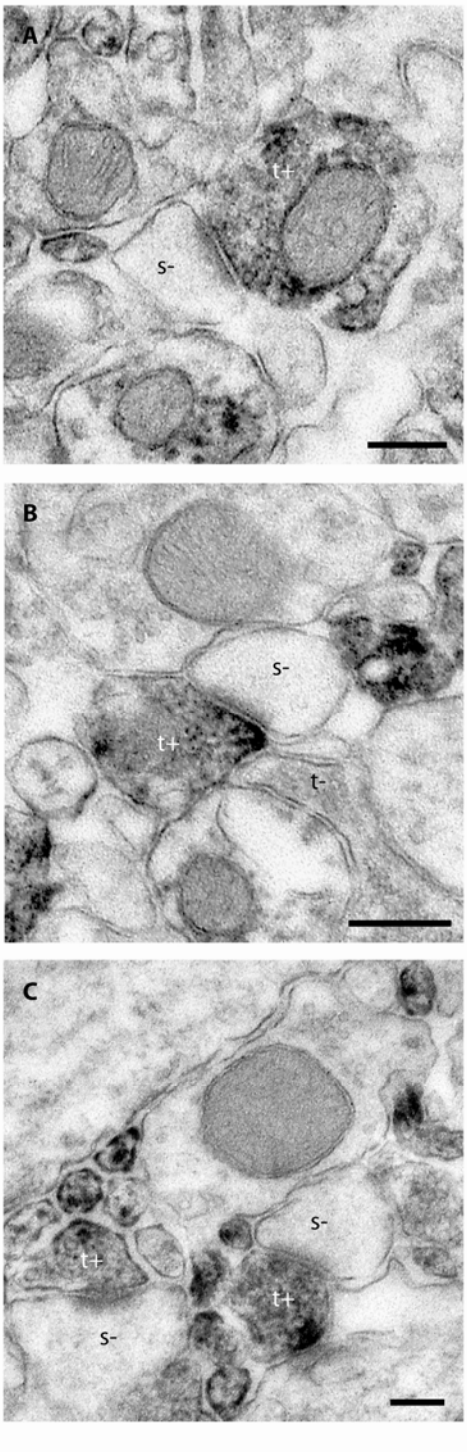

A
t+
s-
B
s-
t+
t-
C
s-
t+
t+
s-

Figure 2: Distribution of EphA4 in an LP2 fraction layered on a linear 10-35% sucrose density gradient. EphA4 distribution overlapped partially that of synaptic vesicle markers (synaptophysin, VGLUT1, synaptotagmin, but not the vesicular monoamine transporter, VMAT2). However, EphA4 did not follow the distribution of the late endosome marker, Rab7.

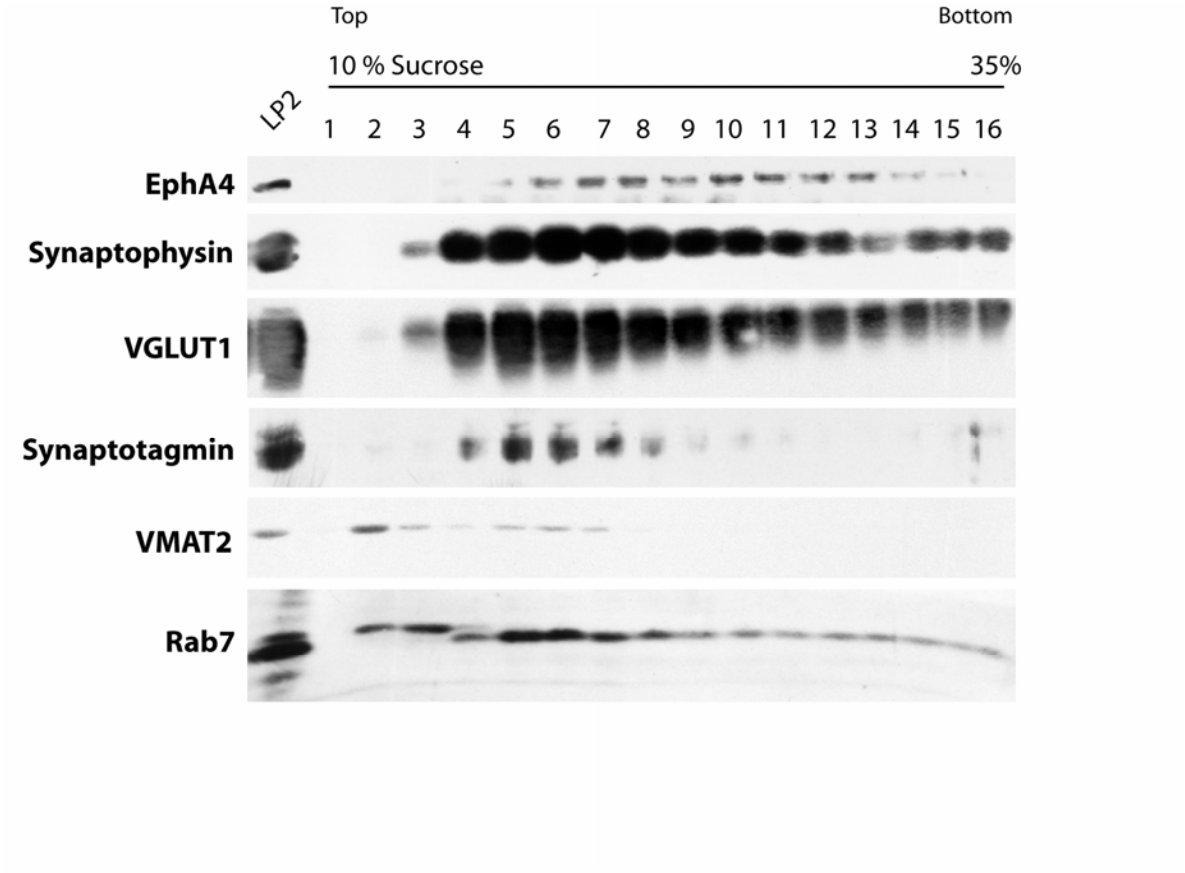

Top
Bottom
10 % Sucrose
35%
LP2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
EphA4
Synaptophysin
VGLUT1
Synaptotagmin
VMAT2
Rab7

Figure 3: Immunoisolation of synaptic and piccolo-bassoon transport vesicles from the LS1 cell fraction. A) Synaptic vesicles were immunoisolated with an anti-synaptophysin antibody (Syn). Immunoisolation with control immunoglobulins (Ctl IgG) was negative for EphA4 and showed only traces of synaptophysin staining, whereas the immunoisolation with synaptophysin was positive for both EphA4 and synaptophysin. B) Piccolo-bassoon transport vesicles (PTVs) were immunoisolated with an anti-piccolo antibody (Pic). The immunoisolation of PTVs was negative for EphA4. C) Immunoisolation of glutamatergic synaptic vesicles (VGLUT1-Ii) and immunoprecipitation of VGLUT1 (VGLUT1-IP) using an anti-VGLUT1 antibody. The immunoisolation with the control IgGs was negative for EphA4 and synaptophysin, but showed a spot at the level of VGLUT1 (~50 KDa) that is probably attributable to the IgGs. The immunoisolation with the VGLUT antibody was positive for EphA4 and synaptophysin (confirming the success of the immunoisolation of synaptic vesicles), while the spot corresponding to VGLUT1 was strongly increased, also confirming the success of the immunoisolation. The immunoprecipitation of VGLUT1 was negative for EphA4, indicating that the co-localisation in the same vesicles is not through direct interactions between the two molecules. D) Immunoisolation of gabaergic synaptic vesicles (VGAT-Ii) and immunoprecipitation of VGAT (VGAT-IP): results similar to VGLUT1, showing that EphA4 is present in these vesicles but does not interact directly with VGAT.

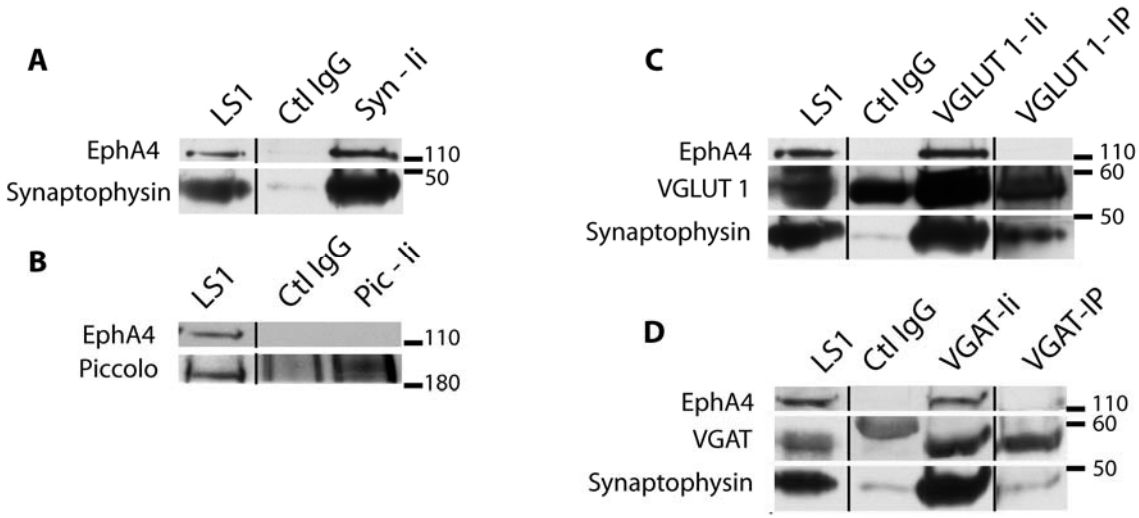

A
LS1
Ctl IgG
Syn - Ii
EphA4
Synaptophysin
110
50
B
LS1
Ctl IgG
Pic - Ii
EphA4
Piccolo
110
180
C
LS1
Ctl IgG
VGLUT 1- Ii
VGLUT 1- IP
EphA4
VGLUT 1
Synaptophysin
110
60
50
D
LS1
Ctl IgG
VGAT-Ii
VGAT-IP
EphA4
VGAT
Synaptophysin
110
60
50

Figure 4: EphA4 in clathrin-coated vesicles isolated by cell fractionation. EphA4 was present in all fractionation steps where the clathrin light (LC) and heavy chains (HC) were detected, including the CCV fraction. H, homogenate; P1, pellet-1; P2, pellet-2 S2; supernatant-2; SGp, pellet of the sucrose gradient; SGs, supernatant of the sucrose gradient, as described (Girard et al., 2005a).

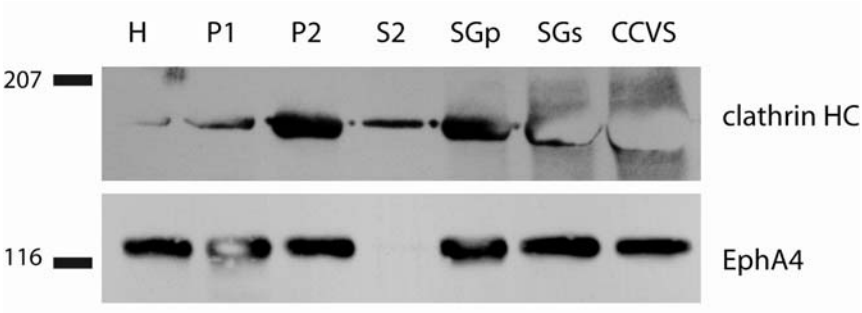

H
P1
P2
S2
SGp
SGs
CCVS
207
clathrin HC
116
EphA4

Figure 5: Double immunocytochemistry for EphA4 (immunoperoxidase) with VGLUT or VGAT (immunogold) in the CA1 region of adult mouse hippocampus show that EphA4 is co-localized with VGLUT1 (A, B) in glutamatergic axon terminals, but appears absent from gabaergic terminals labeled for VGAT (C, D). Scale bars: 250 nm.

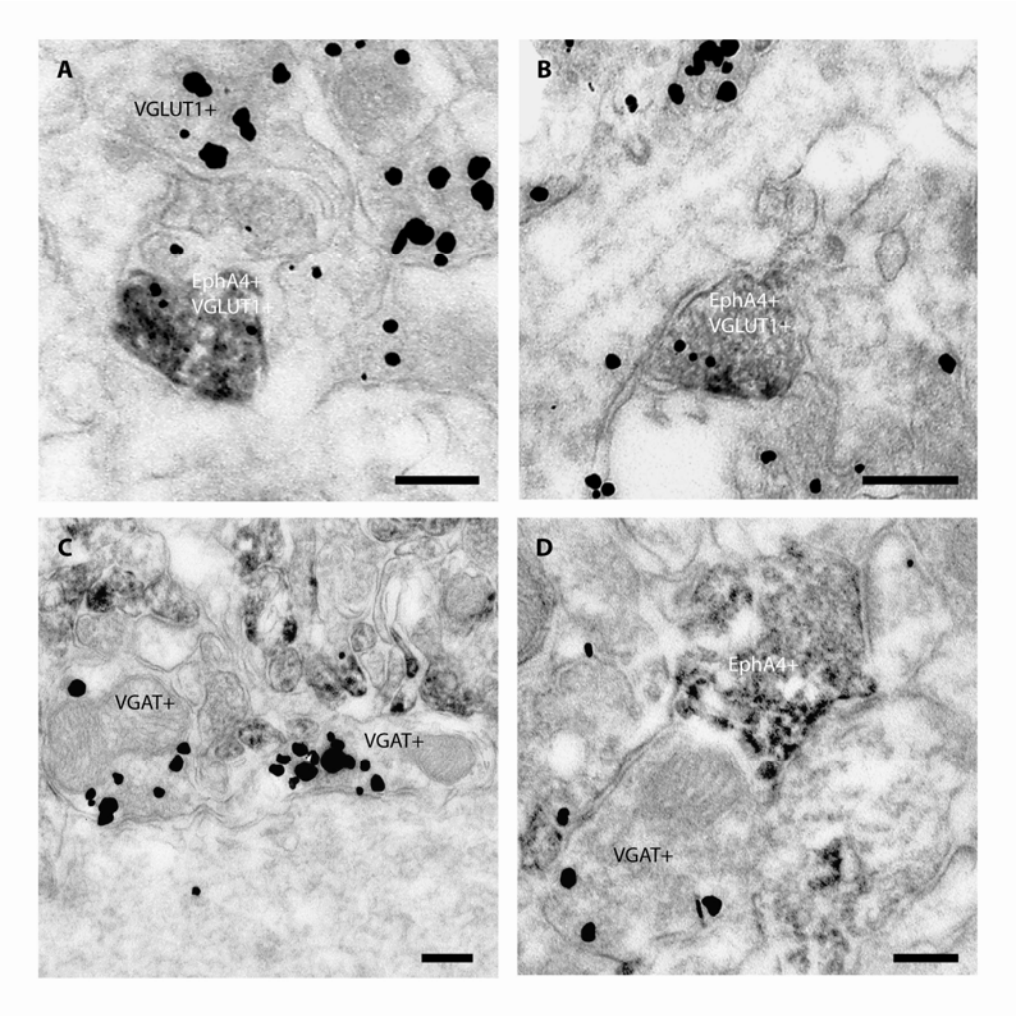

A
VGLUT1+
EphA4+
VGLUT1+
B
EphA4+
VGLUT1+
C
VGAT+
VGAT+
D
EphA4+
VGAT+

Figure 6: Double immunocytochemistry for EphA4 (green in A, D, G, J) with VGLUT1 or VGAT on hippocampal neurons at DIV15 in culture. anti-VGLUT1 (B, E, red) and anti-VGAT (H, K, Red). Co-localization of EphA4 with VGLUT1 (C, F) or VGAT (I, L) appear in yellow on the overlay. Scale bars: 5 μm.

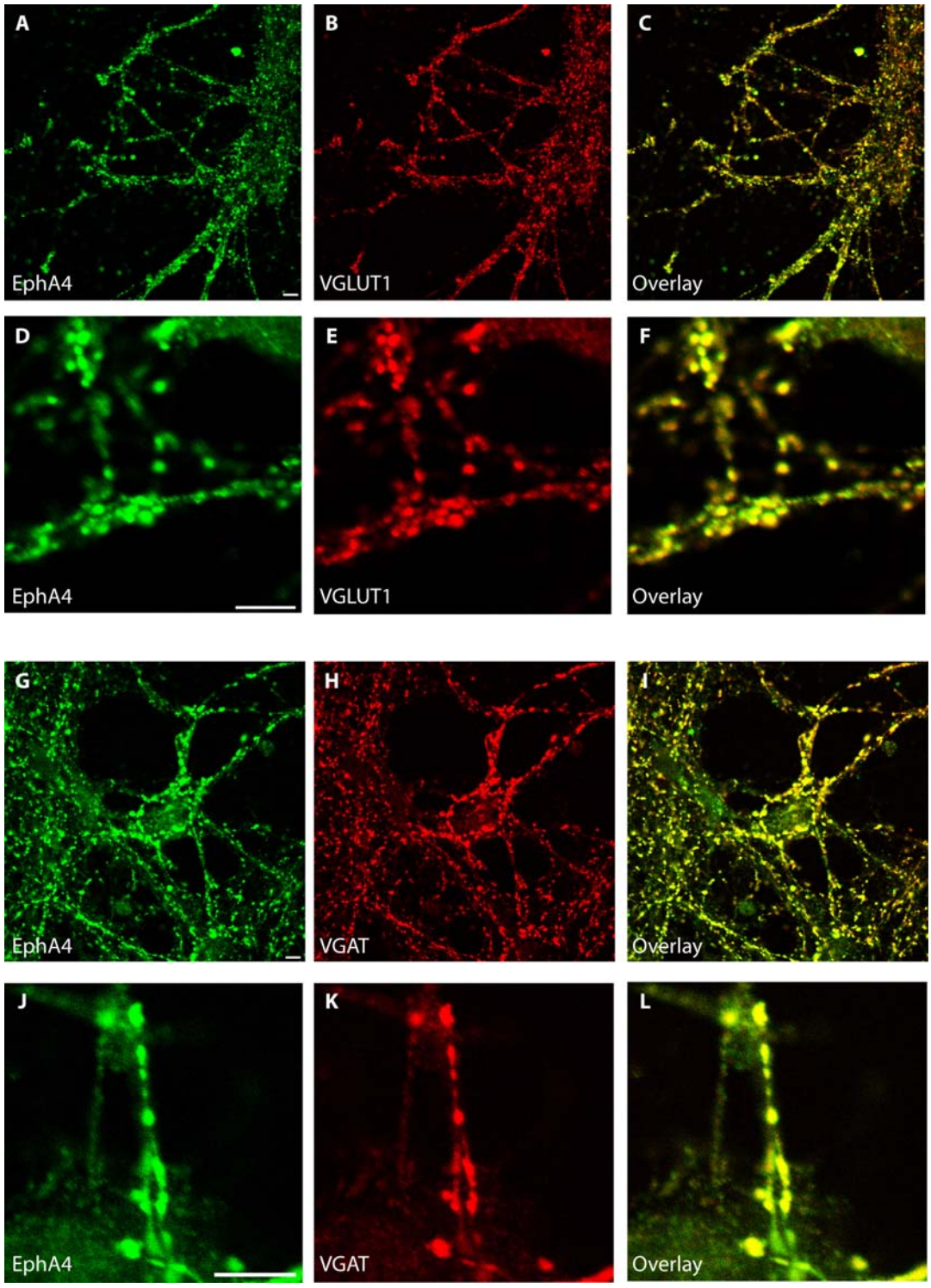
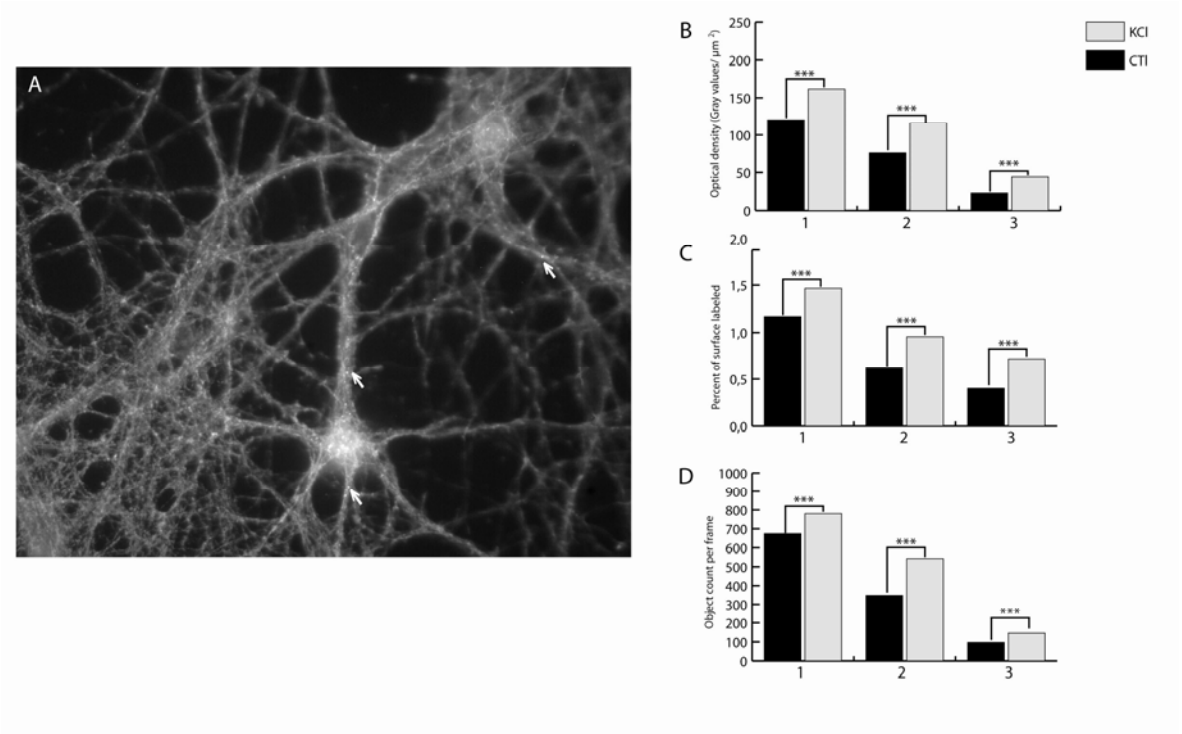

A
B
C
EphA4
VGLUT1
Overlay
D
E
F
EphA4
VGLUT1
Overlay
G
H
I
EphA4
VGAT
Overlay
J
K
L
EphA4
VGAT
Overlay

Figure 7: Cell surface expression EphA4 is modulated by neuronal activity in DIV 15 hippocampal cultures. A) Representative picture of cell surface EphA4 immunolabeling of non stimulated neurons. The labeling is distributed in granules along neurites and on the cell bodies (arrows). B-D) Quantitative analysis of the labeling was performed on 25 frames (each 21 000 μm^2) in each condition (control or CTl; 2 min of 90 mM KCl treatment or KCl) and in each of 3 different experiments (1, 2, 3). Labeling on cell bodies was excluded from the analysis. KCl depolarization increased the mean optical density of the EphA4 labeling (B), the mean percent surface (C), and the mean number of granules (D). Two-ways ANOVA (condition) was performed, and differences between control and KCl treatment were significant for B, C and D and for the 3 experiments (condition, ***= $p<0,001$). Comparisons of the mean difference between control and KCl showed no significant difference between the 3 experiments for any type of measurement (Two-ways ANOVA (experiment*condition), B, p=0,576; C, p=0,978; D, p=0,368).


A
B
KCl
CTl
Optical density (Gray values/ µm ²)
250
200
150
100
50
0
***
***
***
1
2
3
C
Percent of surface labeled
2,0
1,5
1,0
0,5
0,0
***
***
***
1
2
3
D
Object count per frame
1000
900
800
700
600
500
400
300
200
100
0
***
***
***
1
2
3

Annexe III. Désignation des acides aminés

Nom	3 lettres	1 lettre
acide aspartique	Asp	D
acide glutamique	Glu	E
alanine	Ala	A
arginine	Arg	R
asparagine	Asn	N
cystéine	Cys	C
glutamine	Gln	Q
glycine	Gly	G
histidine	His	H
isoleucine	Iso	I
leucine	Leu	L
lysine	Lys	K
méthionine	Met	M
phénylalanine	Phe	F
proline	Pro	P
sérine	Ser	S
thréonine	Thr	T
tryptophane	Trp	W
tyrosine	Tyr	Y
valine	Val	V