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par

Céline Clémentine STEINMETZ

Glia influence on synapse development in different brain regions

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Directeur de Thèse : Frank W. PFRIEGER
Rapporteur Interne : Dominique BAGNARD
Rapporteur Externe : Kurt GOTTMANN
Rapporteur Externe : Stephan SIGRIST
Examinateur : Pierrick POISBEAU

INCI – UMR 7168 / LC2 Dept. Neurotransmission et Sécrétion Neuroendocrine

«Un homme qui n'est plus capable
de s'émerveiller a pratiquement
cessé de vivre.»

Albert Einstein

A toutes les petites filles,
jeunes femmes et femmes
privées des libertés
d'Apprendre, d' Aimer,
de Vivre....

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RESUME EN FRANCAIS

Les synapses constituent les connections intercellulaires hautement spécialisées régulant la transmission des signaux entre les neurones. Malgré leur rôle essentiel pour la fonction du système nerveux central, les mécanismes permettant leur formation sont encore très peu connus.

Mon projet de thèse a pour but d'étudier le rôle de la glie dans la formation des synapses du système nerveux central.

Etat des lieux: différents facteurs contenus dans le milieu glial facilitent le développement synaptique

L'hypothèse à la base de ce projet est que des signaux provenant des cellules gliales, en particulier des astrocytes, sont nécessaires au développement des synapses. Cette hypothèse s'appuie sur des travaux antérieurs montrant que des signaux gliaux facilitent la sformation des synapses. Tout d'abord, en utilisant une culture de cellules ganglionnaires de la rétine (RGC) de rat au stade post-natal (Barres *et al.,* 1988), Pfrieger et Barres ont étudié la formation et la fonction synaptique en absence et en présence de cellules gliales (Pfrieger and Barres, 1997). L'addition de cellules gliales dans la préparation neuronale augmente de manière drastique le niveau de l'activité synaptique spontanée ainsi que l'efficacité synaptique, et cela sans modification ni de la survie des neurones ni de leur excitabilité. De plus, le milieu conditionné par les glies (GCM) agit sur la formation synaptique et augmente l'efficacité de la transmission synaptique des RGCs en microculture (Nägler *et al.,* 2001). Par ailleurs, toujours sur le même modèle, Mauch *et al.* (2001) ont identifié le cholestérol comme étant l'un des facteurs contenus dans le milieu glial qui influence le développement synaptique. Récemment dans le laboratoire, Göritz *et al.* (2005) ont montré que le GCM et le cholestérol induisent une augmentation du nombre de synapses avec un délai de quelques jours et que l'étape limitante de la synaptogenèse des RGCs est en fait la différenciation des dendrites. Enfin, toujours sur ce modèle, Christopherson *et al.* (2005) ont identifié les thrombospondines-1 et-2 comme étant des protéines «synaptogéniques». Ces résultats suggèrent donc que, durant le développement des RGCs, une combinaison de différents facteurs dérivés des cellules gliales environnantes est essentielle pour la formation et la fonction des synapses.

Résultats obtenus: rôle de la glie dans la synaptogenèse de neurones du SNC.

Mon projet de thèse a pour but d'étudier la dépendance gliale de différents types neuronaux du système nerveux central lors du développement synaptique et si les effets induits par la glie et observés chez le rat sont spécifiques de l'espèce. Afin de réaliser cette étude il convenait dans un premier temps de mettre au point l'isolement de neurones chez la souris au stade post-natal. Puis dans un second temps, j'ai étudié l'effet de la glie sur la formation et l'activité synaptique de ces neurones par électrophysiologie et immunocytochimie.

1.1. Mise en place d'une procédure permettant la purification de neurones de souris post-natales : immunoisolation des neurones hippocampaux, cérébelleux et RGC.

Afin de répondre à la problématique, un protocole expérimental permettant d'isoler et de cultiver des neurones de trois régions (hippocampe, cervelet et rétine) du cervelet chez la souris au stade post-natal a été établi. Après dissection de l'hippocampe, du cervelet et de la rétine, les trois tissus sont dissociés séparément de manière enzymatique puis mécanique. Les cellules sont ensuite soumises à un protocole d'immuno-isolation où les cellules non neuronales sont soustraites puis les neurones sélectionnés par des combinaisons d'anticorps bien spécifiques. De manière similaire à la purification des RGCs de rats, les RGCs de souris sont isolées grâce à un anticorps dirigé contre la protéine Thy1.2. Les neurones d'hippocampe et de cervelet sont isolés grâce à un anticorps dirigé contre l'épitope extracellulaire de la molécule d'adhésion L1, exprimée exclusivement dans les neurones. Des expériences d'immunocytochimie à l'aide d'anticorps dirigés contre des marqueurs spécifiques des différents types de cellules gliales et des fibroblastes ont montré que cette procédure d'immunoisolation permet d'obtenir une pureté neuronale de plus de 99% avec un rendement qui est fonction du type cellulaire. De plus, comme la procédure d'immuno-isolation est longue, la viabilité des cellules peut en être affectée. Nous avons donc étudié la survie cellulaire initiale. Après 3h *in vitro*, 70 à 80% des neurones sont vivants. Afin d'établir les conditions de culture optimales pour leur survie, différents milieux de culture ont été testés. Après 3 jours en culture dans un milieu de culture basique, le taux de survie est très faible pour les trois types neuronaux. L'addition de facteurs de croissance (brain-dervied neurotrophic factor, ciliary neurotrophic factor) et de forskoline (qui induit une augmentation du niveau intracellulaire d'adénosine monophosphate cyclique) augmente drastiquement la

survie neuronale. Le but du projet étant d'étudier le rôle des cellules gliales sur la formation des synapses, il est nécessaire de les maintenir en culture pendant au moins une semaine. C'est pourquoi la survie neuronale a également été testée après une semaine *in vitro*. Ce taux varie entre 25 et 45 % en fonction du type neuronal. De plus, nous avons montré que moins de 1% des neurones cérébelleux continuaient à proliférer dans la culture. Enfin, des expériences d'immunocytochimie ont permis de montrer que les cultures de neurones d'hippocampe et de cervelet contiennent respectivement 15 % et 5% de neurones gabaergiques. Par ailleurs, des cellules pyramidales et des cellules de Purkinje ont également été identifié dans les cultures d'hippocampepocampe et de cerveletvelet, respectivement. Les cultures de RGCs contiennent excusivement des neurones glutamatergiques. Ainsi, ces résultats montrent que les neurones d'hippocampe, de cervelet et les RGCs de souris peuvent être purifiés et maintenus en vie *in vitro* en l'absence de facteurs gliaux pour une semaine au minimum.

1.2. Effets de la glie sur la formation et l'activité synaptique.

Dans un second temps, j'ai étudié l'effet des signaux gliaux sur la formation et l'activité synaptique de ces neurones par électrophysiologie et immunocytochimie. Afin de mettre en évidence la présence de synapses fonctionnelles dans les préparations neuronales, l'activité synaptique spontanée a été enregistrée par "patch-clamp" en configuration cellule-entière à un potentiel de maintien de -70 mV pour les courants post-synaptiques excitateurs (EPSC) et de -30 mV pour les courants post-synaptiques inhibiteurs (IPSC).

1.2.1. En absence de glie

Les neurones de chaque région sont cultivés pendant une semaine à densité égale et dans un milieu de culture identique. De manière intéressante, en l'absence de facteurs gliaux, la grande majorité des neurones d'hippocampe et de cervelet présentent une activité synaptique spontanée importante, pouvant dépasser 1000 évènements par minute (evts/min) pour les les fréquences des EPSCs, alors que pour les IPSCs les fréquences sont de l'ordre de 250 evts/min. Ainsi, la présence d'une activité synaptique importante indique que les neurones d'hippocampe et de cervelet sont capables de former des synapses excitatrices et inhibitrices en absence de glie. Ceci a été confirmé par des doubles marquages immunocytochimiques contre des protéines pré- et post-synaptiques montrant une importante coïncidence entre ces marqueurs.

A l'inverse, en l'absence de facteurs gliaux, moins de 12% des RGCs présentent une activité synaptique spontanée qui, de plus, est relativement faible (90 evts/min). Ceci indique

que contrairement aux neurones d'hippocampe et de cervelet et indépendemment de l'espèce, les RGCs ne forment que peu de synapses en absence de glie.

Ainsi, l'ensemble de ces résultats montrent que lors de la synaptogenèse, les neurones du SNC diffèrent quant à leur dépendance vis-à-vis de la glie: les neurones d'hippocampe et de cervelet forment des synapses fonctionnelles en absence de glie alors que les RGCs requièrent des signaux gliaux pour former des synapses.

1.2.2. En présence de glie

Afin d'étudier si les signaux gliaux affectent le développement synaptique, les neurones immuno-isolés sont cultivés en absence de glie (situation contrôle), en présence de cellules gliales ou traités avec du GCM (contienant des facteurs sécrétés par la glie). De manière surprenante, ni la présence de glie ni de GCM ne modifie la fréquence des EPSCs et des IPSCs spontanés des neurones d'hippocampe et de cervelet comparativement à la situation contrôle. Cependant, en présence de glie, le pourcentage de neurones d'hippocampe présentant une activité synaptique spontanée excitatrice et de cervelet présentant une activité synaptique spontanée inhibitrice augmente légèrement alors que le pourcentage de RGCs présentant une activité synaptique et la fréquence des EPSCs de RGCs sont drastiquement augmentés. Ceci confirme les observations antérieures obtenues sur des cultures purifiées de RGCs de rat, qui montrent que les cellules gliales induisent la synaptogenèse dans ces neurones (Nägler *et al.,* 2001; Ullian *et al.,* 2001). La taille des EPSCs spontanés est également significativement augmentée pour les trois types neuronaux en présence de glie.
Cependant le GCM ne mime que partiellement les effets induits par la glie. L'ajout de GCM dans le milieu de culture augmente le pourcentage de neurones d'hippocampe présentant des EPSC et l'incidence des EPSC des RGCs.

Les changements induits par la glie peuvent s'expliquer par une augmentation de l'excitabilité synaptique et donc par un niveau d'activité synaptique potentiel d'action indépendant plus important. Afin d'étudier de tester si la glie modifie la libération quantique de neurotransmetteurs (libération potentiel d'action-indépendante) les courants postsynaptiques miniatures excitateurs (mEPSCs) et inhibiteurs (mIPSCs) ont été également enregistrés. La fréquence des mEPSCs et des mIPSCs, ainsi que la taille des mIPSCs restent inchangées en présence de glie, pour les neurones d'hippocampe et de cervelet. De même, la taille des mEPSCs des neurones de cervelet n'est pas modifiée en présence de glie. L'augmentation de la taille des EPSCs spontanés de cervelet est donc due à l'augmentation de la libération évoquée par les potentiels d'action. Par ailleurs, la glie induit une augmentation

drastique de la taille des mEPSCs de neurones d'hippocampe suggérant ainsi que l'augmentation de la taille des EPSCs spontanés passe par des modifications de la libération quantique. Cet effet n'est pas mimé par le GCM mais par le TNF alpha, qui est un facteur sécrété par la glie uniquement lorsqu'elle est en contact des neurones. La fréquence et la taille des mEPSCs de RGCs et le pourcentage de RGCs présentant des mEPCS augmentent également en présence de glie, confirmant ainsi que les cellules gliales favorisent le développement des synapses de RGCs.

Ces résultats pris dans leur ensemble montrent que, lors de la synaptogenèse les neurones du SNC diffèrent quant à leur dépendance vis à vis de la glie et que cette dernière a des effets différents sur le développement et la maturation synaptique en fonction du type neuronal.

1.2. Effets de la glie sur la plasticité synaptique.

Les neurones sont capables d'ajuster leur excitabilité synaptique suite à l'altération du niveau d'activité dans le réseau neuronal dans le but de maintenir leur taux de décharge constant (Turrigiano *et al*. 1994; O'Brien *et al*., 1998; Murthy *et al*., 2001). Afin d'étudier si la glie est nécessaire pour cette forme de plasticité synaptique, l'activité electrique dans les cultures de neurones d'hippocampe, en présence ou non de glie, est bloquée par application tétrodoxine (TTX) ou augmentée par application de BICucculine (BIC) de manière chronique (48 heures). En absence de glie, ni un traitement chronique au TTX ni à la BIC ne modifient pas la fréquence et la taille des mEPSCs et mIPSCs des neurones d'hippocampe. En présence de glie, la fréquence et la taille des mEPSCs et mIPSCs sont modulées par le TTX et la BIC. En réponse à l'application chronique de TTX, la fréquence et la taille de l'activité synaptique excitatrice et inibitrice sont augmentées, de manière à compenser la baisse du niveau d'activité dans le réseau neuronal. En réponse à l'application chronique de BIC, la fréquence et la taille de l'activité synaptique excitatrice présentent le même niveau que dans la situation contrôle, permettant ainsi de compenser l'augmentation du niveau d'activité dans le réseau neuronal, alors qu'elles sont sensiblement augmentées dans le cas de l'activité synaptique inhibitrice.

Ainsi,ces données suggèrent que les neurones requièrent la présence de la glie pour maintenir un taux de décharge constant suite à l'altération du niveau d'activité dans le réseau neuronal.

Conclusions

Lors de ma thèse, j'ai pu montré que certains neurones du système nerveux central nécessitent la présence de glie pour former des synapses (RGCs) alors que d'autres non (hippocampe et cervelet) et que les neurones d'hippocampe ne sont capables de maintenir leur taux de décharge constant suite à l'altération du niveau d'activité dans le réseau neuronal qu'en présence de glie.

ABBREVIATIONS

AMPA	Amino-3-hydroxy-5-methylisoxazol-4-propionic acid
Bicucculline	BIC
ATP	Adenosine tri-phosphate
BDNF	Brain derived neurotrophic factor
BrdU	5-bromo-2-deoxyuridine
CA1	Cornu ammonis 1
BSA	Bovine serum albumin
CER	Cerebellum
CNPase	2',3'-cyclic nucleotide 3'-phosphodiesterase
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
COR	Cortex
DIV	Days in vitro
DMEM	Dulbecco's modified Eagle's medium
DNA	Desoxyribonucleic acid
DNAse	Desoxyribonuclease
D-PBS	Dulbecco's phosphate buffered saline
EAAT	Excitatory amino acid transporter
dEAAT1	*Drosophila* excitatory amino acid transporter 1
dEAAT2	*Drosophila* excitatory amino acid transporter 2
EBSS	Earle's balanced salt solution
EDTA	Ethylene-diamine-tetraacetic acid
EGTA	Ethylene glycol bis(beta-aminoethyl ether)- tetraacetic acid
EphA4	Ephrin A4 receptor tyrosin kinase
EPSC	Excitatory postsynaptic current
mEPSC	Miniature excitatory postsynaptic current
sEPSC	Spontaneous excitatory postsynaptic current
FCS	Fetal calf serum
FITC	Fluorescein isocyanate
FSM	Fully supplemented medium
GABA	Gamma-aminobutyric acid
GAD	Glutamate decarboxylase

GCM	Glia-conditioned medium
GFAP	Glial fibrillary acid protein
GFP	Green fluorescent protein
GS	Glutamine synthetase
gs2	Glutamine synthetase 2
Gs	*Griffonia simplicifolia*
HEPES	4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid
HIP	Hippocampus
IgG	Immunoglobulin G
IP3	Inositol triphosphate
IPSC	Inhibitory postsynaptic current
mIPSC	Miniature inhibitory postsynaptic current
sIPSC	Spontaneous inhibitory postsynaptic current
LDH	Lactate deshydrogenase
LTP	Long Term potentiation
MAP	microtubule-associated protein
MSM	Minimally supplemented medium
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NMJ	Neuromuscular junction
NMR	Nuclear magnetic resonance
NT	Neurotransmitter
NTR	NT receptor
PNS	Peripheral nervous system
PSC	Perisynaptic Schwann cell
PSD	Postsynaptic density
RET	Retina
RGC	Retinal ganglion cell
SIC	Slow inward current
SNARE	Soluble N-ethylmaleimid sensitive factor attachment protein receptor
SON	Supraoptic nucleus

TCA	Tricarboxilic acid cycle
TNFα	Tumor necrosis factor α
Tris	Tris (hydroxymethyl) aminomethane
TSP	Thrombospondin
TTX	Tetrodotoxin

INTRODUCTION

The development of a functional nervous system requires the correct specification of a large number of cells. These cells are divided in two major categories, neurons and neuroglia, the latter of which outnumbers neurons in the vertebrate brain. In humans for example, there are roughly five glial cells for every neuron (Bass *et al.*, 1971).

Neurons interconnect through synapses to form active circuits. Charles Sherrington introduced the term *synapse* (from the Greek, meaning to clasp) in 1897 to describe the specialized zone of contact between neurons (Sherrington, 1906), which was first described histologically by Ramon y Cajal (Ramon y Cajal, 1894). Otto Loewi showed in the 20s that a chemical, acetylcholine, mediates transmission from the vagal nerve to heart (Loewi and Navratil, 1926) and in 1957 Furshpan and Potter provided the first evidence of electrical synaptic transmission at the giant motor synapse of the crayfish (Furshpan and Potter, 1957). Thus, in the nervous system, two types of connection between neurons have been identified: chemical and electrical synapses. Chemical synapses are specialized cell junctions with machinery for the release of neurotransmitter (NT) on the presynaptic side, precisely opposite to the machinery for NT reception on the postsynaptic side. At electrical synapses, the zone of apposition between two neurons is called “gap junction” and is bridged by channels called “gap junction channels”, which conduct the flow of ionic current and thus mediate electrical transmission.

Neuroglia (Greek for “nerve glue”) was first identified as substance filling the space between nervous tissues in the nervous system by Rudolf Virchow in 1856 (Virchow, 1856). Originally, neuroglia was thought to play merely a structural or support role to neurons and synapses. Today however, more than a century after their description there is increasing evidence that synapses and glial cells have an intimate and plastic morphological and functional relationship. Recently a new concept has emerged, named ‘tripartite synapse”, that considers synapse-associated glial cells as active participants in synapse development, function and plasticity (Araque *et al*., 1999).

The aim of my thesis was to study the influence of glial cells on synapse development in different regions of the CNS. First I will give an overview of the morphological arrangement of glial cells and synapses in invertebrates and vertebrates. Then, I will introduce the hypotheses that glial cells play a role in synapse development and function.

1. Morphologic constellation of synapses and glial cells

This part will focus on the structural constellation between glia-like cells and synapses in invertebrates and vertebrates.

1.1. The invertebrates

In this part, I will focus the fly (*Drosophilia melanogaster*) and the worm (*Caenorabditis elegans*). *Drosophilia melanogaster* and *Caenorabditis elegans* (*C. elegans*) are well-established animal models to dissect the molecular mechanism of synaptic development and function because they allow for forward genetic screens to identify novel components of the synapse and to target pre- and postsynaptic elements genetically (Richmond and Broadie, 2002).

Although the role of glial cells during embryonic development has been well studied in *Drosophila* (Lemke, 2001; Parker and Auld, 2006), less is known about structural interaction of glia with synapses. Few studies have shown that glia is closely apposed to synapse. At photoreceptor terminals, specific glial invaginations, named capitate projections, have been reported (Fabian-Fine *et al*., 2003; Prokop and Meinertzhagen, 2006). They are involved in membrane retrieval by clathrin-mediated endocytosis, probably to accommodate the high rates of vesicle recycling required at fly photoreceptor terminals (Fabian-Fine *et al*., 2003). In addition, glia is thought to participate in the reuptake and recycling of NT. dEAAT1 and -2 and gs2, which are involved in glutamate uptake from the synaptic cleft and in glutamate to glutamine conversion respectively, are expressed by glia in embryonic central nervous system (Freeman *et al*., 2003; Soustelle *et al*., 2002). At the NMJ, only dEAAT1 is expressed by glia (Rival *et al*., 2006).

The nervous system of an adult hermaphrodite *C. elegans* consists of only 302 neurons (sensory neurons, interneurons, and motor neurons) that form approximately 7000 synapses. The major synapse-rich regions are the nerve ring, the ventral nerve cord and the dorsal nerve cord and the vast majority of axons, synapses, and neuronal somata are organized into ganglia which surround the synapse-rich nerve ring. Most somata of sensory neurons of *C. elegans* are positioned in the "brain of the animal", the nerve ring and ganglia that surround it. Most motor neuron cell bodies reside in the ventral nerve cord (White *et al.,* 1986). C. elegans yields a list of 50 cells that show striking morphological similarities to glia of other organisms, and that are divided in two classes, sheath glia and socket glia. In *C. elegans*, sheath glial cells are mostly present at sensory synapses. Sheath glia cells have been shown to

play a key role in the development and maintenance of sensory synapses. Indeed, animals failed to develop "dendritic spines" on sensory neurons after ablation of precursors of sheath glial cells, whereas the role of sheath glia cells at neuronal synapses is not known (Shaham, 2006).

These studies suggest that glia is intimately associated at least at some synapses in *Drosophila* and *C. elegans*.

1.2. The vertebrates

In the following, I will give an overview of the morphologic constellation between synapses and glial cells in the vertebrate nervous system with a focus on mammals.

In mammals, glial cells are divided into three different classes (Fields and Stevens-Graham, 2002) (Fig. 1): 1/ Schwann cells and oligodendrocytes which are the myelin forming cells of the PNS and CNS, respectively; 2/ Microglia which respond to injury or disease by engulfing cellular debris and triggering inflammatory responses and 3/ Astroglia-like cells, which are composed of astrocytes in the CNS and PSCs in the PNS, are in close contact with synapses (Fig. 2).

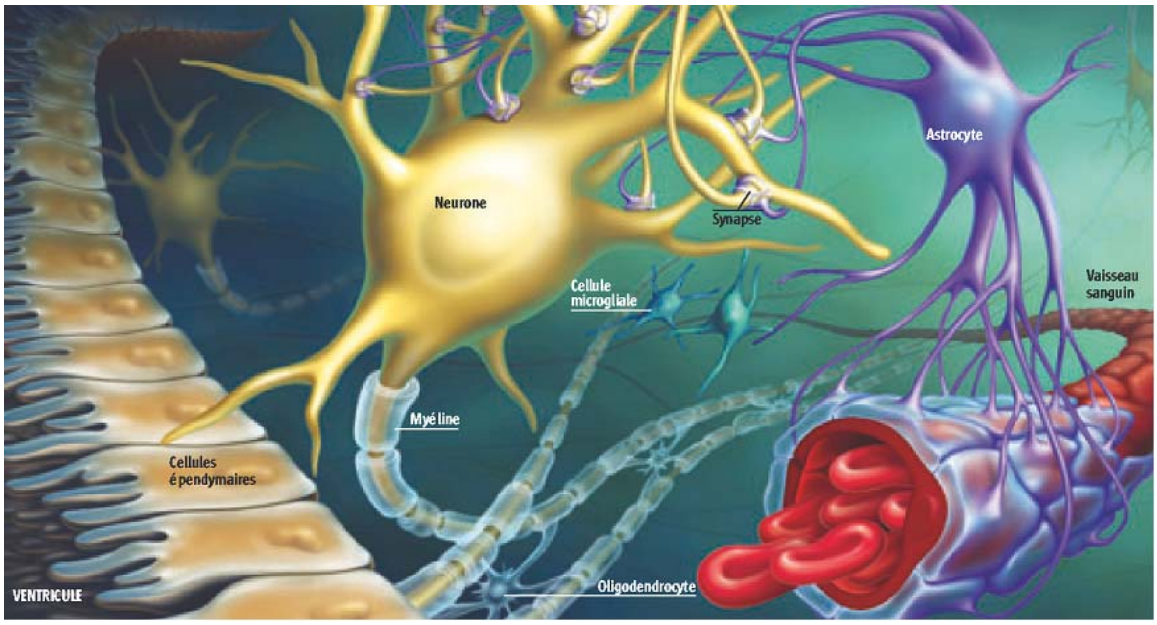

Figure 1 Glia cell type present in the CNS

The degree to which astrocytes cover synapses varies. Some synapses are devoid of perisynaptic glia. For example, thin sensory axons terminating on synapses that lack

perisynaptic glia have been described in the cochlear nucleus and in the spinal cord (Hurd *et al*., 1999). For other synapses, the synaptic perimeter percentage that is covered by astrocyte protrusion varies. In the neocortex, astrocyte apposition to synaptic elements occupies 56% of the synaptic perimeter of astro-dendritic synapses (Wolff, 1970) and astrocyte surface extension often covers the sides of the synaptic clefts to a higher percentage (70-90%) than the pre- and the postsynaptic element (25-55%) (Wolff, 1976). Similar values have been reported for axo-spine synapses in the stratum radiatum of hippocampal area CA1 (43% of the synaptic perimeters; Ventura and Harris, 1999).

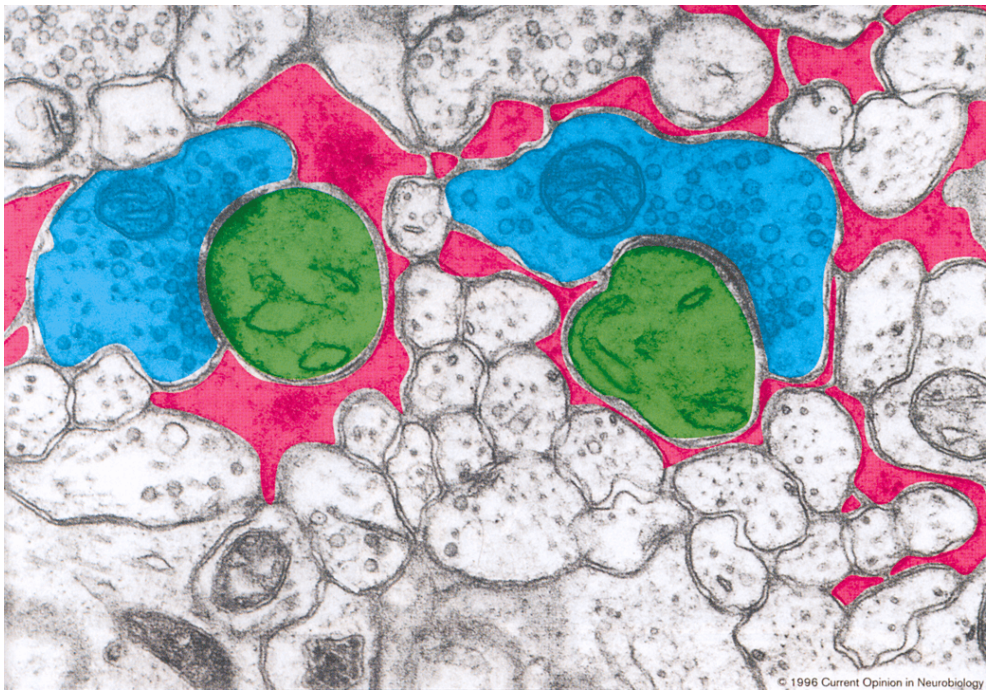

Figure 2: Astrocytic processes ensheath synapses between CNS neurons
Electron microscopic image of the molecular layer of the monkey cerebellum. Blue, presynaptic terminals; green, postsynaptic spines; red, astrocytic processes. Magnification 55 000x (Pfrieger and Barres, 1996).

In addition, it has also been reported that in the HIP, astrocytic processes preferentially contact large synapse (Ventura and Harris, 1999) and target synapses with greater NT release probability (Harris and Sultan, 1995). Haber et al. (2006) recently showed *in situ* on hippocampal slices, that astrocytes contacting postsynaptic spines extended and retracted processes over minutes using the actin-based cytoskeleton. In addition, these investigators also showed that the association between astrocytic processes and the postsynaptic surface

was more stable around larger spines. In the molecular layer of the cerebellar cortex, synaptic junctions between parallel fibers and spines of Purkinje cells are almost completely ensheathed by Bergmann glia cells (Grosche *et al*., 1999), whereas synapse that innervate basket and stellate neurons in the same layer show less glia coverage (Palay and Chan-Palay, 1974). In addition, the arborisation of Bergmann glia is organized into discrete microdomains (Grosche *et al*., 2002). Glia coverage is generally higher at complex synapses than at single synapses (Wolff, 1976). Synaptic glomeruli or complex synapses are composed of multiple synaptic junctions that are enclosed in a common glia sheath. Indeed, it has been showed that synaptic glomeruli of rat thalamic nuclei are covered by multilamellar glia sheaths and that these sheaths do not penetrate the interior of complex synapse which is then a glia free compartment (Spacek and Lieberman, 1971). These studies indicate a high variability of synaptic coverage by astrocytes. Astrocyte endfeet can cover more the presynaptic or the postsynaptic element or can preferentially cover the sides of the synaptic cleft, whereas notably synapses are even devoid of glial processes (Wolff, 1976). Like other synapses, the NMJ closely associated glial cells which are called perisynaptic Schwann cells (PSCs; Sanes and Lichtman, 1999). They have been identified for the first time as cluster of nuclei distinct of muscle nuclei at motor endplate of the vertebrate NMJ (Ranvier, 1878). At the frog NMJ, PSCs form a thin sheath that covers nerve terminals and extended fine processes that cover active zones (Astrow *et al*, 1998).

To conclude, morphological data indicate that many synapses in the CNS and the PNS are covered by astroglia processes, therefore suggesting that astrocyte endfeet in the CNS and PSCs at the NMJ constitute a crucial “third element” of the typical synapse, in addition to the pre- and postsynaptic element (Araque *et al.,* 1999). These cells may therefore not only act as passive bystander but also play a more active role in synapse development and function. In the two following part, I will describe current evidences for this idea.

2. Role of astrocytes in synaptic transmission and plasticity

The intimate association of astrocytes and synapses provides a strategic position to control synaptic activity. As mentioned before, astrocytes were originally thought to play mainly passive roles. During the last decade, however, new findings have indicated a bidirectional communication between astrocytes and synapses.

2.1. Astrocytes integrate synaptic inputs

In the following paragraphs, I will first give an overview on the role of astrocytes in the glutamate/GABA-glutamine cycle and in coupling synaptic activity to glucose utilization and I will then summarize evidence that these cells sense, integrate and modulate synaptic signals (Fig. 3).

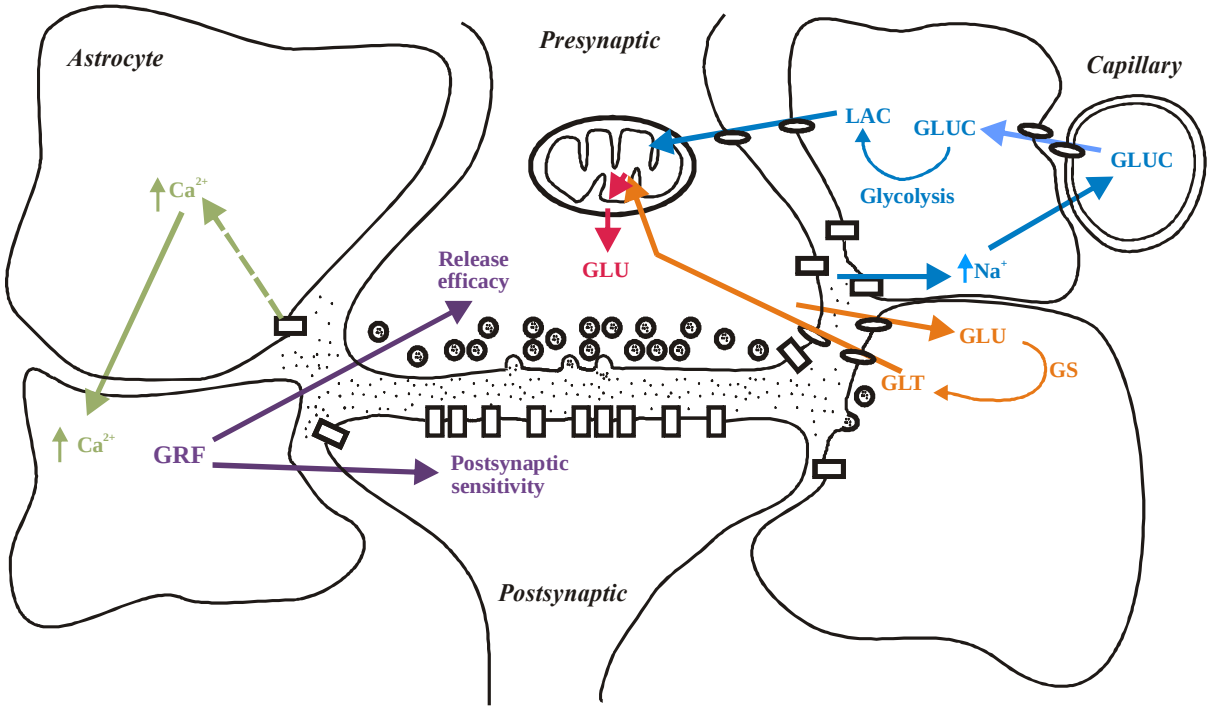

Figure 3: Astrocytic influence on synaptic function

Astrocytes provide metabolic support through the glutamate-glutamine cycle and through glucose uptake and supply of glucose-derived lactate. Astrocytes also integrate and modify synaptic signals by intracellular Ca^{2+} transients and by release of factors that modify pre- and postsynaptic efficacy.

Glutamate/GABA-glutamine cycle

The Glutamate/GABA-glutamine cycle has been studied extensively in vitro on acute slices as well as in slice culture of different brain regions. Neurons are not able to perform *de novo* synthesis of glutamate and GABA from glucose because of the lack of pyruvate carboxylase (Patel, 1974; Hertz *et al.*, 1999). Therefore, glutamatergic and GABAergic terminals are thought to rely on astrocytes to provide transmitter precursors. In the so-called glutamate-glutamine cycle, released glutamate is taken up by surrounding astrocytes by EAAT-1 and -2

transporters which are specific for excitatory amino acid (Danbolt, 2001). These transporters are electrogenic and co-transport three Na^{2+} and one H^{+} and counter-transport one K^{+} (Zerangue and Kavanaugh, 1996). In astrocytes glutamate is transformed into glutamine by GS, which is specific for this cell type (Norenberg and Martinez-Hernandez, 1979). In the GABA-glutamine cycle, Guastella *et al.* (1990) showed that four astrocytic GABA transporters mediate GABA uptake into surrounding astrocytes together with cotransport of two Na^{2+} and one Cl^{-} (Larsson *et al.,* 1980; Gu *et al.,* 1996). In astrocytes, GABA is metabolized in two steps to succinate which is then metabolized to alpha-ketoglutarate in the TCA and finally to glutamate (Peng *et al.,* 1993). In a last step, glutamate is transformed into glutamine by GS. Glutamine efflux from astrocytes and glutamine influx in neuron is mediated by amino acid transporter system, which cotransported Na^{2+} or neutral amino acid and counter-transported H^{+} (Varoqui *et al*., 2000; Chaudhry *et al*., 2002). Finally, in neurons, glutamine is converted to glutamate by phosphate-activated glutaminase and glutamate into GABA by glutamate decarboxylase (Kvamme *et al*., 2001). Recently, studies have confirmed the existence of these cycles *in vivo* in rat and human cortex by C^{13} NMR during intravenous infusion of C^{13}-labelled glucose (Lebon *et al.,* 2002; Oz *et al.,* 2004). Patel *et al.* (2005) used intravenous infusion of C^{13}-labelled glucose and C^{13}-labelled acetate to separately determine rates of glutamate-glutamine and GABA-glutamine cycling and their respective TCA cycles on rat cortex. They showed that both glutamatergic and GABAergic cycling and energy consumption increase with neuronal activity and that cycling of GABA consume less energy than that of glutamate.

Coupling of glutamate uptake and glycolysis in astrocytes.

In addition to synapses, astrocytes also surround capillaries with processes that express specific glucose transporter (Morgello *et al.,* 1995; Yu and Ding, 1998). This provided the hypothesis that astrocytes couple synaptic activity and glucose utilization (Pellerin and Magistretti, 1994). As mentioned above, at glutamatergic synapses, presynaptically released glutamate is taken up by astrocytes through specific transporters (EAAT) and is co-transported with Na^{2+}. Voutsinos-Porche et al. (2003) showed in the developing somatosensory cortex of knock out mice for either EAAT1 or -2 that astrocytic EAATs are critical for glucose utilization and lactate release from astrocytes. Increase of intracellular Na^{2+} concentration in astrocytes leads to the activation of the Na^{2+}/K^{+} ATPase, which stimulates glycolysis (Pellerin and Magistretti, 1996, 1997). In astrocytes, glucose is converted to lactate by the LDH-5, which is specifically expressed in astrocytes (Bittar *et al.,*

1996). The exchange of lactate between astrocytes and neurons is mediated by monocarboxylate transporter isoforms selectively expressed in either astrocyte or neurons (Bröer *et al.,* 1997) and in neurons lactate is converted to pyruvate by LDH-1 (Bittar *et al.,* 1996). Pyruvate can then enter the TCA cycle. *In vivo* studies on rat brain showed that neuronal activation induces the release of astrocytic lactate into the extracellular fluid (Demestre *et al.,* 1997). Recently, Serres *et al*. (2004) analysed glucose and lactate metabolism by *ex vivo* NMR spectroscopy in rats presenting different cerebral activities. They showed that brain lactate production and consumption increases with brain activation, therefore suggesting that brain lactate is involved in the coupling between brain activity and metabolism.

Astrocytic sense synaptic activity

Astrocytes sense synaptic activity thanks to the expression of a wide variety of functional NT receptors (NTR; Verkhratsky *et al.,* 1998). Some of these receptors are coupled to second messenger pathways that lead to Ca^{2+} release from IP_3-sensitive intracellular stores (Porter and McCarthy, 1996; Bezzi *et al*., 1998; Araque *et al.*, 2001). The elevation of astrocytic intracellular Ca^{2+} concentration was shown in rat hippocampal cultures to be mediated by activation of metabotropic glutamate receptors (Porter and McCarthy, 1996; Pasti *et al*., 1997), of $GABA_B$ receptors (Kang *et al*., 1998) and of muscarinic cholinergic receptors (Araque *et al*., 2002). Importantly it has been demonstrated, *ex vivo*, that synaptic activity induces Ca^{2+} elevation in the retina (Newman, 2004), the cerebellum (Grosche *et al*., 1999) and at NMJs (Colomar and Robitaille, 2004). Astrocytic Ca^{2+} responses to physiological activity *in vivo* have been reported in a very recent study (Wang *et al*., 2006). The authors investigated the relation between sensory stimulation and astrocyte activation by measuring astrocytic Ca^{2+} response to whisker stimulation in layer 2 of the barrel cortex in adult mice. Astrocytes responded to whisker stimulation with an elevation in cytosolic Ca^{2+} and this effect was blocked by metabotropic glutamate receptors antagonists, suggesting that release of synaptic glutamate is implicated in this neuron-astrocyte signaling. These findings demonstrate for the first time that astrocytic Ca^{2+} signaling response to synaptic input *in vivo*. Glutamate-induced increases in cytoplasmic Ca^{2+} concentration of astrocytes frequently propagates as waves within the cytoplasm of individual astrocytes and between adjacent astrocytes (Cornell-Bell *et al*., 1990; Dani *et al.*, 1992; Pasti *et al*., 1997). *In situ,* in hippocampal astrocytes and cerebellar Bergmann glia this synaptic-induced astrocytic Ca^{2+} elevation occurs in spatially restricted areas of astrocytic processes called “microdomains”

(Pasti *et al*., 1997; Grosche *et al*., 1999; Araque *et al*., 2002) In addition, it has been shown that astrocytes respond in a graded manner to synaptic signaling. Pasti *et al*. (1997) showed on rat hippocampal slices and Hirase *et al*. (2004) showed *in vivo* in primary somatosensory cerebral cortical layers of rats, that astrocytic Ca^{2+} signals scale with the intensity of synaptic activity. Moreover, Perea and Araque (2005) showed by stimulating two different axonal pathways in area CA1 of the HIP that astrocytes can discriminate between NT released from different sources and that astrocytic Ca^{2+} signals were potentiated or depressed at relatively low or high level of neuronal activity, respectively.

To conclude, astrocytes sense and integrate synaptic activity through the activation of NT transporters and NTRs, which then induce transient increases in intracellular Na^{2+} and Ca^{2+}.

2.2. Astrocytes modulate synaptic function

Astrocytes not only integrate but also modulate synaptic activity. They release a variety of substances including excitatory and inhibitory amino acids, ATP, nitric oxide, lipid mediators, cytokines, neurosteroids and neurotrophins that can act on adjacent synapses (Volterra and Bezzi, 2002). Different mechanisms have been suggested to underlie the release of these substances: Ca^{2+}- and SNARE-dependent vesicular release similar to that in neurons, osmotic release via volume-activated anion channels, transporter-mediated release, receptor-activated ectodomain-shedding of protein factors and gap junction hemi-channels (Volterra and Bezzi, 2002; Fellin and Carmignoto, 2004).

Astrocytes modulate presynaptic transmitter release.

By releasing factors in response to synaptic activity, astrocytes can act on presynaptic terminal to increase or decrease the efficacy of NT release.

Araque *et al.* (1998a) investigated the effects of astrocytic glutamate release on action potential-evoked synaptic transmission in cultured hippocampal cells. They showed that Ca^{2+} elevation evoked by electrical or mechanical stimulations in astrocytes induced a transient reduction of evoked synaptic transmission by activation of metabotropic glutamate receptors, probably located on presynaptic terminals. On the other hand, they showed in the same experimental model that glutamate released by astrocytes also transiently increases the frequency of mEPSCs and mIPSCs, by acting on presynaptic NMDA receptors (Araque *et al.,* 1998b). On hippocampal slices, Kang *et al.* (1998) showed that direct stimulation of astrocytes by the patch-clamp pipette in close proximity to interneurons increased the

frequency of mIPSCs through glutamate release. Liu *et al.* (2004) later showed that this regulatory loop was mediated by kainate receptors on GABAergic terminals.

In the rat retina, Newman (2003) demonstrated that activation of astrocytes and Müller cells by mechanical stimulation or by focal injection of different substances induce ATP release from these cells, which then inhibited neuronal transmission by A1 receptor activation on RGCs. Indeed, the time course of the neuronal current was reduced and slowed by an A1 adenosine receptor antagonist, an ecto-ATPase inhibitor and an ectonucleotidase inhibitor. Zhang *et al*. (2003) demonstrated in cultured rat hippocampal neurons that ATP decreased the frequency but not the amplitude of mEPSC and increased the paired-pulse ratio. In addition, by using several purinergic antagonists, they also showed that ATP mediated these effects via P2Y receptors and A1 receptors activation. They therefore suggested that synaptic release of glutamate induced the release of ATP from adjacent astrocytes, which caused homo- and heterosynaptic depression by acting presynaptically.

Astrocytes modulate postsynaptic receptor sensitivity.

Astrocyte-derived factors can also regulate synaptic activity by modulating postsynaptic NTRs sensitivity.

In the study mentioned above on rat hippocampal cultures, Araque *et al*. (1998a) showed that elevation of the intracellular Ca^{2+} concentration by different stimuli in astrocytes induced slow inward currents in adjacent neurons and that these currents were induced by glutamate release from astrocytes. Subsequent studies on hippocampal and thalamic slices showed that these slow inward currents were mediated by postsynaptic ionotropic glutamate receptors (Fellin *et al.,* 2004; Parri *et al*., 2001). In the paraventricular nucleus of the hypothalamus, glia-released ATP potentiates the amplitude of glutamate-mediated mEPSCs (Gordon *et al.,* 2005) and this effect is due to the insertion of AMPA receptors at the postsynaptic site. Activation of AMPA/kainate receptors of cultured astrocytes induces a robust release of another prominent neuromodulator substance, namely, D-serine (Schell *et al.,* 1995), which binds to the glycine-binding site of postsynaptic NMDAR (Mothet *et al*., 2000). The serine racemase, which converts L-serine to D-serine, can only be found in glial cells enwrapping NMDAR-bearing synapses (Wolosker *et al.,* 1999). Taken together these studies prompted the hypothesis that D-serine is released from astrocytes after their activation by glutamate and that D-serine then activates postsynaptic NMDAR (Martineau *et al*., 2006). A very recent study from the Oliet lab on the rat hypothalamic SON confirmed this hypothesis (Panatier *et al*., 2006). In the SON, astrocytic coverage of glutamatergic synapse is

drastically reduced as females begin lactation. The authors demonstrated that when glial coverage of the synapse is reduced, the NMDA component of the synaptic connection is smaller and that this was due to reduced release of D-serine. A final example for astrocytic modulation of postsynaptic NTRs comes from the groups of Volterra and Malenka. Bezzi *et al.* (2001) showed on hippocampal slices that astrocytes release TNFα when activated and that TNFα is involved in astrocytic glutamate release. In hippocampal mixed neuronal-glia culture and in hippocampal slices from postnatal rats, Beattie *et al.* (2002) showed that astrocyte-released TNFα increased mEPSCs frequency and AMPA receptor insertion on the postsynaptic site.

Taken together these studies showed that astrocyte-released factors regulate synaptic activity through the modulation of postsynaptic receptors sensitivity.

Changes in astrocyte structure influence synaptic environment

The examples discussed above demonstrate the ability of glia to modulate synaptic activity. Recent studies suggested that astrocytes change their morphology in response to synaptic activity and thereby influence synaptic transmission.

Murai *et al.* (2003) showed on hippocampal slice from adult mice that dendritic spines of pyramidal neurons are enriched with EphA4 whereas its ligand, ephrin-A3 is localized on astrocytic processes that envelop spines. They found that pharmacologic or genetic modulation of EphA4 induced spine retraction and spine irregularities. Thus, their study suggested that transient interactions between spine-localised EphA4 and ephrin-A3 localised on astrocytic processes regulate spine morphology. The Kirchhof lab (Hirrlinger *et al*., 2004) investigated structural changes of astrocytes at synaptic terminals in mice that express the GFP under the control of astrocyte specific human GFAP-promoter. Using *in situ* time-lapse imaging in acutly isolated brainstem slices, they observed that astrocytes exhibit two types of motility at synapses, lamellipodial gliding and filopodia extension. The majority of filopodia stayed elongated for a few minutes and retracted again. This paper presented the first demonstration that astrocytic processes are dynamic. Haber *et al*. (2006) recently characterized astrocyte-synapse interaction *in situ* using organotypic hippocampal slices and viral infection with either the genes for farnesylated enhanced green fluorescent protein or farnesylated red fluorescent protein, therefore allowing visualization of dendritic spines and ramified astrocytic processes. They showed that astrocyte displayed extension and retraction of their processes near dendritic spines over minutes and that their motility was driven by actin cytoskeleton.

These examples mentioned above provide the first evidence that astrocytes are structurally dynamic and suggest that morphological changes can modulate synaptic transmission.

2.3. Astrocytes modulate synaptic plasticity.

In the following, I will describe evidence that astrocytes modulate synaptic plasticity.

A study by Yang *et al.* (2003) demonstrated on hippocampal slices that D-serine can be released from astrocytes in response to neuronal activity and that this release is necessary for LTP. Hippocampal neurons from embryonic rats, cultured in contact with astrocytes showed LTP induced by repetitive correlated pre- and postsynaptic activation. Neurons that grew in media conditioned by astrocytes (but without contact) didn't showed LTP but LTP was restored by the addition of D-serine to the medium. This study suggested that astrocytes are actively involved in the induction of LTP by regulating the availability of D-serine. In addition, in the hypothalamic SON, Panatier *et al.* (2006) also asked whether glia-derived D-serine regulates plasticity. They showed that when the astrocytic processes retract (in lactating rats) and the level of synaptic D-serine therefore decreases, long term depression is induced by membrane depolarization paired with afferents stimulation. In virgin rats that have a high degree of synaptic coverage, the same stimulus induces LTP. Thus, their study suggested that astrocyte-released D-serine, determine the activity-induced direction of synaptic changes.

Pascual *et al.* (2005) investigated the role of gliotransmitters in synaptic network by using acute isolated hippocampal slices from transgenic mice that allow for reversible expression of a dominant negative-SNARE domain in astrocyte. The expression of this dominant negative-SNARE domain in astrocyte specifically blocks the release of gliotransmitters. They showed that astrocytic ATP, after being hydrolyzed into adenosine, suppresses excitatory synaptic transmission. This suppression was mediated by A1 receptors. They also showed in mice expressing dominant negative-SNARE that the magnitude of LTP induced by theta-burst stimulation was less than in control mice. They therefore suggested that astrocytic ATP enhance the capability of synapses to express synaptic plasticity.

To conclude, those three very recent studies indicate that in the CNS, astrocytes are actively involved in synaptic transmission and plasticity.

3. Role of glia in synapse development and maturation

The evidence that astrocytes control synaptic activity raises the question whether they influence synapse development. A temporal correlation between synaptogenesis and the differentiation of astrocytes supported this idea. In the rodent retinocollicular pathway, for example, axons of RGCs innervate the superior colliculus around embryonic day 16. The main phase of synaptogenesis starts only at the end of the first postnatal week and continues for two to three weeks (Pfrieger and Barres, 1996). There is therefore a delay of at least a week between these two processes. This delay period together with the fact that astrocytes are generated around birth suggests the possibility that signals from astrocytes are necessary to trigger synapse formation.

One experimental approach to investigate astrocyte involvement in synaptogenesis consists of glia-free cultures of neurons. Neurons can be prepared from embryonic rats or mice, when astrocytes are not born yet. Alternatively, neurons from postnatal animals can be separated from glia by immunoisolation. Currently only a few i*n vivo* approaches are possible in vertebrates because ablation of astrocytes in living animals causes neurodegeneration (Cui *et al.,* 2001). Therefore, the influence of astrocytes on neuronal differentiation can not be studied *in vivo*.

3.1. Glia control synaptic birth and maturation in rat RGCs.

Pfrieger and Barres (1997) studied whether purified CNS neurons form synapses when cultured in the presence or absence of astrocytes. They used RGCs from one week old postnatal rats which can be immunoisolated to greater than 99.5% purity (Barres *et al*, 1988) and cultured in defined serum-free medium in the absence of other cell types (Meyer-Franke *et al*., 1995). Using electron microscopy and electrophysiology, they found that cultured RGCs were able to form ultrastructurally defined synapses in the absence of glia but that they showed only little spontaneous activity. Coculture of RGCs with glia cells from their target region (superior colliculus), specifically macroglia cells, or culturing RGCs on top of macroglial feeding layers, strongly increased the frequency and the size of sEPSCs and decrease the failure rate of evoked transmission, compared to the glia-free cultures.

These experiments showed that macroglia-derived soluble factors enhanced the efficacy of excitatory synapses *in vitro*, without affecting neuronal survival, neurite outgrowth, or electrical excitability.

Subsequent studies on the same model revealed that under glia-free conditions only a few neurons (10%) showed synaptic activity, with low frequencies and small amplitude of spontaneous events, high failure rates of evoked transmission (more than 90%) and no asynchronous release (Nägler *et al*, 2001) (Fig. 4). Immunostaining with pre- and postsynaptic markers showed that without glia only few synapses are formed (Nägler *et al*, 2001; Ullian *et al.,* 2001) and electron microscopy revealed that these synapses were ultrastructurally normal (Ullian *et al.,* 2001). When RGCs were cultured in direct contact with glia the level of synaptic activity was enhanced in about 30% of the neurons with a strong increase in the size and frequency of spontaneous events, an increase in the frequency of asynchronous release and a decrease in the failure rates of evoked transmission. Glial cells increased the number of synapses as shown immunocytochemically with pre- and postsynaptic markers that correspond to the site of a single functional synapse (Nägler *et al*., 2001). Similar results were reported by Ullian *et al* (2001). This was a first clear demonstration that glial cells promote synaptogenesis. Glia conditioned medium (GCM) mimicked the effects of glial cells on synapse development and affected synaptic activity not immediately but with a delay of 24h, suggesting that existing synapses are strengthened first and that the synapse number is then increased (Nägler *et al*., 2001). Taken together these results showed that soluble glia-derived signals induce RGCs synapse formation.

By a combination of biochemical and electrophysiological techniques, Mauch *et al.* (2001) identified cholesterol as a glia-derived factor that mimicked effects of GCM on synapse number and efficacy. They showed, by double-labeling RGCs in microculture with pre- and postsynaptic markers, that cholesterol raised the number of synapses in a similar manner than GCM. Cholesterol lowered the failure rate of evoked synaptic currents and increased the frequency of spontaneous currents and the quantal content, thus indicating that cholesterol increases the efficacy of transmitter release. To determine how cholesterol influences synapse development in RGCs, Göritz *et al*. (2005) treated microcultures of highly purified rat RGCs with GCM or cholesterol. By double-labeling synapses with pre- and postsynaptic markers and by recording synaptic activity, they showed that GCM and cholesterol increased synapse numbers with a delay of at least 48 hours. Immunostaining with an antibody against MAP2 identified dendrite differentiation as rate limiting for synaptogenesis in RGCs. In conclusion, glia-secreted cholesterol promotes RGC synaptogenesis *in vitro* by promoting differentiation of dendrites and of presynaptic terminals. In a parallel study, Christopherson *et al.* (2005) identified TSPs as glia-derived factors that regulate synapse development of highly purified rat RGCs. They found that TSP-1 enhanced

synapse formation to a similar degree as astrocyte-conditioned medium. These synapses were ultrastructurally normal and presynaptically active as indicated by electron microscopy and by presynaptic uptake of an anti-synaptotagmin luminal domain antibody, respectively, but postsynaptic silent as indicated by recording mEPSCs. Double mutant mice for TSP-1 and -2 exhibited a reduction in the number of synapses *in vivo*. The authors concluded that glia secretes TSPs to promote formation of silent synapse and that other factor(s) convert these silent synapses into functional ones.

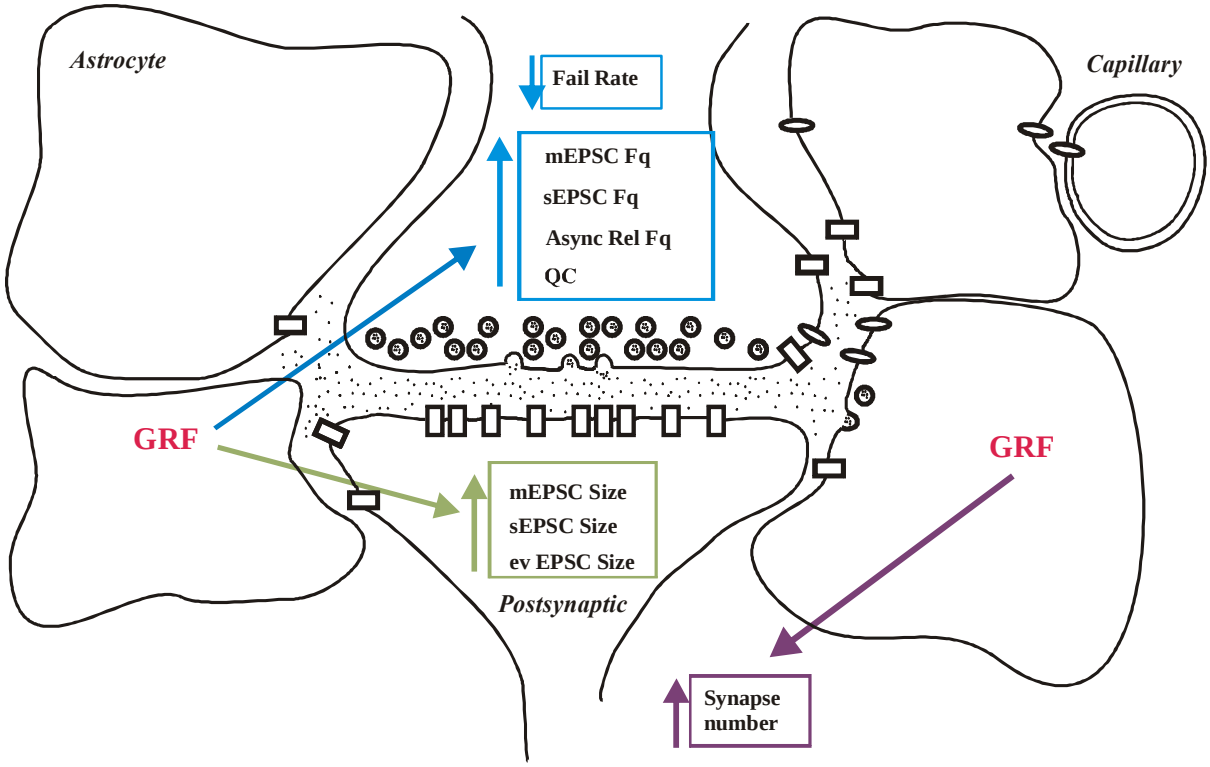

Figure 4: Astrocytic influence on synapse development

Astrocytic signals enhance presynaptic transmitter release, postsynaptic sensitivity and the number of synapses, as indicated by previous studies (Nägler *et al.*, 2001; Ullian *et al.*, 2001).

In summary, studies on highly purified RGCs showed that signals secreted by glial cells, namely from astrocytes, are involved in synaptogenesis. A main question that is at the origin of my thesis project was whether the glia requirement for synaptogenesis is a general principle or depends on the neuronal cell type. Before I started my thesis, a few other groups working on neurons from embryonic or newborn rats also reported that glia-derived signals control synaptogenesis. In the following part, I will briefly summarize these studies.

3.2. Glia and synapse development in other models.

A series of recent papers suggested that PSCs control NMJ formation, maturation and stability. The pioneering work of Trachtenberg and Thompson (1997) showed that, in young rats, application of a soluble form of neuroligin, which plays an important role in cell-cell interactions at the developing NMJ, alters terminal PSCs and the stability of synapses. They also showed by using Schwann cell transplants that these synaptic alterations were due to the action of neuroligin on PSCs. They therefore concluded that PSCs are necessary for synaptic maintenance. This idea has been confirmed by different groups (Woldeyesus *et al*., 1999; Lin *et al*., 2000) who showed that in the absence of PSCs due to knock out of neuregulin receptor ErbB2 and-3, NMJs formed transiently but were not maintained. At developing NMJ from embryonic and adult frogs, it was shown that PSCs were not required for initial nerve-muscle contacts, but that PSC sprouts were necessary for synaptic formation (Herrera *et al*., 2000; Koirala et *al.*, 2000). To directly test whether PSCs are require for synaptic growth, PSCs were selectively ablated from developing NMJs *in vivo* using complement-mediated cell lysis (Reddy *et al.*, 2003). The authors showed that PSCs are essential for the maintenance of adult NMJs as well as for the growth and stability of developing NMJ, *in vivo*. Ullian *et al.* (2004) showed *in vitro* on highly purified motor neurons isolated from rat spinal cords, that so far unidentified Schwann cell-derived factor(s) promoted synapse formation and efficacy. Schwann cell-derived factor(s) also enhanced spontaneous synaptic transmission on developing NMJs in embryonic *Xenopus* nerve-muscle culture (Peng *et al*., 2003) and transforming growth factor-β1 was identified as a glia factor that promoted NMJs formation *in vitro* (Feng *et al.,* 2005). Yang *et al*. (2001) showed in adult *Xenopus* nerve-muscle culture that PSCs also expressed active agrin, which is essential for acetylcholine receptor aggregation on NMJs. Lesuisse *et al.* (2000) and Peng *et al.* (2003) showed that PSCs-derived factor(s) regulates neuronal agrin expression and the synaptogenic potential of cultured motoneurons by enhancing agrin deposition.

Studies on hippocampal neurons from embryonic or newborn rats and on retinal neurons from embryonic chicken provided further evidences that astrocytes enhance synapse development and stability. Blondel *et al*. (2000) showed in hippocampal or cortical cultures from embryonic rats that neurons stimulated astrocytes by intestinal vasopeptide, which then induced secretion of activity dependent neurotrophic factors by astrocytes. Activity dependent neurotrophic in term increased synapse number and postsynaptic NMDAR density. In the chicken retina, Belmonte *et al.* (2000) described a glia-derived signal, termed muscarinic-

acethylcholine-receptor-inducing activity that controls the expression of the M2 subtype of the muscarinic acethylcholine receptors in retinal neurons *in vitro* and *in ovo.*

Taken together, these studies suggest that astrocytes play a role in synapse formation, maintenance and elimination by releasing synaptogenic factors.

4. Goal of the thesis

The finding that cholesterol-released by glia promotes synaptogenesis in immunoisolated rat RGCs (Mauch *et al*., 2001) raises the question whether glia requirement for synaptogenesis is a general principle or varies with the neuronal cell type. However, so far, no methods were available to isolate neurons from postnatal brain to a sufficiently high degree and to cultivate them under defined glia-free conditions.

To address this question, my PhD thesis work focused on two steps. First, we developed new methods to immunoisolate neurons from different brain regions of postnatal mice and to culture them in a chemically defined medium. Second, I studied the effect of glia on synaptogenesis of these immunoisolated neurons by electrophysiology and immunocytochemical staining.

MATERIAL AND METHODS

1. Immunoisolation and culture of CNS neurons from postnatal mice

Balb/c mice (7 days old; animal facility, Faculty of Medicine, Universite Louis Pasteur, Strasbourg) were killed by decapitation according to institutional guidelines. Hippocampi and cerebella were dissected, cut in small pieces and incubated (5% CO_2, 37°C) for 60 min in EBSS (Gibco/Invitrogen, Cergy-Pontoise, France) containing 200 U ml^{-1} papain (Worthington Biochemical Corporation, Lakewood, NJ, USA), 200 U ml^{-1} DNAse (Sigma, St-Quentin Fallavier, France), 1.5 mM $CaCl_2$, 1 mM $MgSO_4$, 0.5 mM EDTA (Sigma). Retinae from the same animals were incubated for 45 min at 37°C in D-PBS (Gibco/Invitrogen) containing 160 U ml^{-1} papain, 200 U ml^{-1} DNAse. The tissues were then sequentially triturated in D-PBS containing 0.15% trypsin inhibitor and 0,15% BSA (Roche Diagnostics, Meylan, France), 650 U ml^{-1} DNAse and 1:75 rabbit anti-rat macrophage antibody (Sigma) to remove microglial cells using Eppendorf tips with defined diameters (20X trituration at 3mm; 15X trituration at 2 mm; 20X trituration at 1 mm). Cells were spun down (800 × g for 13 min), resuspended in 1% trypsin inhibitor and 1% BSA in D-PBS; spun down again and then resuspended in D-PBS containing 0.02% BSA (fraction V; Sigma). For immunopanning, one (HIP, CER) or two (RET) subtractionplates (Ø 150 mm petri-dish; Falcon; BD Biosciences/VWR, Fontenay sous Bois, France) and one selectionplate (Ø 100 mm petri-dish) were incubated for > 12 h at 4°C with 10 µg ml^{-1} secondary antibody in 50 mM Tris-HCl (pH 9.5) [subtraction: goat anti-rabbit-IgG; selection: HIP-CER, goat anti-rat-IgG; RGCs, goat anti-mouse-IgG (Jackson Immunoresearch Laboratories/Beckman Coulter, Marseille, France)]. After washing three times with PBS, selection plates were covered with 0.2% BSA (fraction V; in D-PBS) and incubated for >4 hrs (CER, RET) or >12 hrs (HIP) at room temperature with 2 µg/ml primary antibody (HIP, CER: rat IgG anti-L1 clone 324, Chemicon/Euromedex, Mundolsheim, France; RET: mouse IgM anti-Thy1.2, clone MCA01, Serotec, Cergy Saint-Christophe, France) and then washed with D-PBS. The cell-suspensions were filtered through a nylon-mesh (Nitex 20 µm, Tetko/Sefar Filtration, Rüschlikon, Switzerland) and sequentially incubated on each subtraction-plate for 30 min. The supernatant was filtered and incubated on the selection-plate (HIP, CER: 60 min, RET: 45 min). Non-adherent cells were thoroughly washed off and bound cells were released by washing with 0.2% BSA (HIP, CER) or by trypsination (RET: 12,000 U ml^{-1} in EBSS for 10 min in 5% CO_2 at 37°C). Following washing (HIP and CER) or inactivation (RET) of trypsin with heat-inactivated 20% FCS (Gibco/Invitrogen), the cells were spun down and resuspended in

culture medium. The number of viable cells was determined in a hemocytometer using tryptan blue staining (Sigma).

Table 1: Neurobasal medium medium for cultivation of RGCs, hippocampal and cerebellar neurons (modified from Meyer-Franke *et al.,* 1995)

MSM components	**Concentration**
Neurobasal medium (Invitrogen)	
Glutamine (Invitrogen)	2 mM
Insulin (Sigma)	5 µg/ml
N-acetylcysteine (Sigma)	60 µg/ml
Penicillin (Invitrogen)	100 units/ml
Progesterone (Sigma)	62 ng/ml
Putrescine (Sigma)	16 µg/ml
Sodium selenite (Sigma)	40 ng/ml
Bovine serum albumin (rystalline grade #A4161, Sigma)	100 µg/ml
Sodium pyruvate (Invitrogen)	1 mM
Streptomycin (Invitrogen)	100 µg/ml
Triiodothyronine (Sigma)	40 ng/ml
Holo-transferrin (Sigma)	100 µg/ml
Additional FSM components	
B27 (Gibco/Invitrogen)	1:50,
Brain-derived neurotrophic factor (BDNF; PeproTech, London),	25 ng ml^{-1}
Ciliary neurotrophic factor (CNTF; PeproTech)	10 ng ml^{-1}
Forskolin (Sigma)	10 µM

For electrophysiological recordings and immunocytochemical staining, neurons were plated at 600 cells mm^{-2} in a small circle (Ø 10 mm) centered on tissue culture plates (Ø 35 mm, Falcon, BD Biosciences) coated with 5 µg ml^{-1} poly-D-lysine (MW ~ 40 kDa; Sigma) with (HIP) or without (CER, RET) 10 µg ml^{-1} laminin (Sigma) and culture medium was added 4 hours after the plating. For survival assays, cells were plated on tissue culture plates in indicated culture media: initial viability 3 hrs after plating and survival at 3 DIV (170 cell mm^{-2} on 96-well plate); survival at 7 DIV (600 cells/mm^2 in a small circle, Ø 10 mm, centered on tissue culture plates, Ø 35 mm, Falcon, BD Biosciences). Cells were cultured in

Neurobasal medium (Gibco/Invitrogen) as indicated in Table 1. This medium is referred to as minimally supplemented medium (MSM). For long-term culture, this medium was further supplemented as indicated in table 1 and referred to as fully fully supplemented medium (FSM). For some experiments, FSM was further supplemented with D-serine (5 μM) or TTX (1 μM; Sigma) after 5 DIV and for 48 hours, with cholesterol (5μg/ml from an 1000X ethanol stock solution; Sigma) after 1 DIV for 6 DIV, with TNFα, (100 ng/ml; R&D Systems, Lille, France) either after 5 DIV and for 48h or after 7 DIV for 20 minutes.

2. Preparation of glia-neuron coculture and glia-conditioned medium

Mouse-GCM was obtained from primary cultures of hippocampal, cerebellar and cortical glial cells according to a standard protocol. P7 mice were killed by decapitation and hippocampi, cerebella and cortices were dissected out. The preparation of hippocampal, cerebellar and cortical cell suspensions followed the same procedures as described before respectively for HIP, CER and RGCs. After resuspension in BSA, cells were spun down and resuspended in DMEM/FCS culture medium (see Tab.2). Cells were plated in 5 ml tissue flask (Falcon, BD Bioscience, France) coated with 5 μg ml^{-1} poly-D-lysine. After one week, culture flasks were washed with PBS and glial cells were then cultured in DMEM supplemented (DMEM+) by the same components as for FSM except that BDNF, CNTF and B27 were omitted. Three times a week, half of the GCM was harvested and replaced by freshly concentrated DMEM+. GCM was centrifuged for 5 min at 300*g* to remove cellular debris and added to 1-day-old neuronal cultures (1.7 ml GCM to 2 ml culture medium).

Table 2: Glia culture medium DMEM/FCS

Components (all Invitrogen)	**Concentration**
DMEM without glutamine and phenol red, with 1000 mg/ml glucose	
FCS, heat inactivated, sterile filtered	10%
Glutamine	2 mM
Penicillin	100 units/ml
Sodium pyruvat	1 mM
Streptomycin	100 μg/ml

For co-culture experiments, glial cells grown in flasks were released by trypsination (12.000 U ml^{-1} in EBSS for 10 min in 5% CO_2 at 37°C). Following inactivation of trypsin

with 20% heat inactivated FCS (Gibco/Invitrogen), glial cells were counted, spun down, resuspended in culture medium and added to 1 day old purified neuronal cultures at a ratio of 2/1.

3. Survival assay

For survival assays, cells were plated at 170 cell/mm^2 per well in 96-well culture plates (Falcon BD bioscience, France) in indicated culture media. The neuronal survival rate was determined after indicated times by the LIVE/DEAD assay (Molecular Probes/Invitrogen) according to the manufacturer's instructions.

This two-color fluorescence assay simultaneously determines the number of living cells and dead cells. Living cells are labeled by fluorescent calcein that is converted from nonfluorescent cell-permeable calcein acetoxymethyl to a fluorescent form by intracellular esterases. Dead cells are labeled by ethidium homodimer-1 that produces a bright red fluorescence when bound to nucleic acids in damaged or dead cells. The DNA marker bis-benzimide (20 µg ml^{-1}; Sigma) was simultaneously added to the Live-dead solution. The proportion of living cells was calculated as the number of calcein-positiv cells divided by the number of all cells (live cells + cells ethidium-positive + bis-benzimide-positive that were calcein- and ethidium-negative). Calcein, ethidium and bis-benzimide fluorescence were viewed on an inverted microscope (Axiovert 135TV, Zeiss, Göttingen, Germany) using suitable filter sets (Omega Optical/PhotoMed GmbH, Seefeld, Germany) and a objective (20x, Zeiss). Images were digitized by an air-cooled monochrome charge-coupled device camera (Sensicam, 1280 x 1024 pixels, 8-bit digitization width, PCO Computer Optics, Kelheim, Germany). The percentage of living cells was analysed in fluorescence micrographs by a custom-routine written with Labview (National Instruments, Le Blanc Mesnil, France).

4. Immunocytochemistry

Immunostaining was carried out using standard procedures (Nägler *et al*., 2001). Cells growing on tissue culture plates were washed (PBS), fixed (5 min in 4% formaldehyde at room temperature), blocked and permeabilized [30 min at room temperature; 50% goat serum in antibody buffer containing 150 mM NaCl, 50 mM Tris, 1% BSA (Sigma A2153), 100 mM l-lysine, 0.1% Triton X-100, pH 7.4] and incubated overnight with primary antibodies (diluted in antibody buffer).

To determine the purity of neuronal preparations, cells were stained 24 hrs after plating with cell-type specific antibodies. Oligodendrocytes were immunostained by two antibodies, mouse anti-CNPase (HIP, 1:500; CER, 1/1000; RET, 1/300; Sigma), and mouse anti-O4 (1:200; Roche Diagnostics). Fibroblasts were stained with a rabbit anti-fibronectin antibody (HIP, 1:500; CER, 1/2000; RET, 1/800; Sigma). Astrocytes were detected by two antibodies, rabbit anti-GFAP (HIP and RET, 1:2000; CER, 1/8000; Dako Cytomation, Trappes, France) and rabbit anti-S100β (HIP and RET, 1:1000; CER, 1/4000; Swant, Bellinzona, Switzerland). A marker for microglial cells *Griffonia (Bandeiraea) Simplicifolia* lectin I isolectin B4-FITC (Streit and Kreutzberg, 1987) (1:100; Vector Laboratories/Alexis, Grunberg, Germany) was applied together with secondary antibodies.

Inhibitory neurons were identified by staining for the GABA producing enzyme GAD (rabbit anti-GAD65, 1:1000; Chemicon/Euromedex). Control staining on neuron-glia cocultures ensured that the antibodies detect the respected cell type. To determine the total number of cells in a field of view, the DNA-marker H33342/bis-benzimide (20 μg ml^{-1}; Sigma) was added together with the secondary antibody. The percentage of specific cell-types was determined by counting the total number of fluorescent nuclei and then the number of antibody-labelled cells.

For synapse staining, neurons were labeled by mouse anti-synapsin I (clone 46.1; 1:200; Synaptic Systems, Göttingen, Germany) and rabbit anti-PSD95 (1:100, Synaptic Systems) or rabbit anti-GABAA receptor (1:400; Upstate Biotechnology/Euromedex).
As secondary antibodies, Alexa 488-, Alexa 555- and Alexa 594- (1h, room temperature, 1/400, Molecular Probes/Invitrogen), Cy2- and Cy3-conjugated (1h, room temperature; 1/400, Jackson Immunoresearch Laboratories) goat anti-mouse or goat anti-rabbit IgG antibodies were used. Fluorescence was viewed through an appropriate emission filter and a 40x objective (water-immersion, n.a. 0.8, Zeiss) on a upright microscope (Axioscop 2 FS mot). Images were acquired by an air-cooled monochrome CCD camera (Sensicam; PCO Computer Optics, Kelheim, Germany). Control experiments showed absence of background staining by secondary antibodies. The percentage of each cell type was determined by counting first the total number of fluorescent nuclei and then the number of labeled cells.

5. Electrophysiological recordings

Recordings of spontaneous postsynaptic currents were performed as described (Nägler *et al.*, 2001; Göritz *et al.*, 2005). Whole-cell currents were recorded at room temperature (20 - 24°C) on an inverted microscope (Axiovert 135TV, Zeiss) with patch pipettes made of borosilicate glass (5-10 MOhm; World Precision Instruments) by a pipette puller (P97, Sutter Instruments)] using an Axopatch 200B amplifier (Axon Instruments), a data acquisition board (PCI-MIO-16E1, National Instruments) and custom-written Labview programs (National Instruments). The intracellular recording solution contained (all Sigma) 100 mM potassium gluconate, 10 mM KCl, 10 mM EGTA, 10 mM HEPES adjusted to pH 7.4 with KOH. The extracellular solution contained (all Sigma) 120 mM NaCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM KCl, 10 mM HEPES adjusted to pH 7.4 with NaOH. Currents were low-pass filtered at 5 kHz and digitized at 20 kHz. For each cell, spontaneously occurring postsynaptic currents were recorded during 2 x 30 sec. Analysis of EPSCs and IPSCs was performed automatically by a custom-written Labview routine. The frequency of EPSCs was determined from inward currents recorded at −70 mV holding potential. EPSCs could be distinguished from IPSCs, which were also inwardly directed under our recording conditions, due to their faster time course: halfwidths of spontaneous EPSCs ranged from 0.8 to 6 ms whereas those from IPSCs ranged between 6 to 40 ms. The frequency of IPSCs was determined from outward currents recorded at −40 mV, as the chloride ions reversal potential is -64 mV in our conditions.

mEPSCs and mIPSCs, which are due to action potential-independent transmitter release, were recorded in the presence of TTX (1 µM; Sigma). All neurons tested were electrically excitable as indicated by the presence of large voltage-activated sodium currents in response to depolarizing voltage steps to 0 mV holding potential.

For some experiments miniature currents were also recorded in the presence of glutamine (2 mM).

6. Data representation and statistical analysis

Statistical analysis was performed using STATISTICA 7.1 (StatSoft Inc., Maison-Alfort, France). Graphs were created by SigmaPlot 9.01 (Systat Software GmbH, Erkrath, Germany). Non-normally distributed values were represented by box-plots (horizontal line indicates median; lower and upper box limits show 1^{st} and 3^{rd} quartiles, respectively, and whiskers indicate the 10^{th} and 90^{th} percentile, thin red lines indicate mean values), statistically significant changes were detected by appropriate tests and in some cases statistical tests were

performed on subsamples. Levels of significance are indicated by asterisks (*p‹0.05; ** p‹0.01; *** p‹0.001).

RESULTS

1. Purification and culture of neurons from different regions of the postnatal mouse brain

To test, whether neurons of the mouse CNS depend on glia to form synapses, we developed protocols to immunoisolate hippocampal and cerebellar neurons from postnatal mice using an antibody against the cell adhesion molecule L1 (Rathjen and Schachner, 1984). For comparison we also purified RGCs from postnatal mice using a modified version of the protocol for rat RGCs (Barres *et al.*, 1988).

The yield of cells per animal that could be harvested from the selection plates varied with the brain region. As indicated in Table 3, the yield of cerebellar cells per animal that could be obtained is much higher than that obtained for RGCs and hippocampal neurons. Immunopanning of mouse RGCs yielded about a third of cells (Table 3) compared to their isolation from postnatal rats (71,000 ± 14,000; n = 75).

Table 3 Characterization of immunoisolated neurons

Region	Cells per Animal (/1000)	Fraction of Non-Neuronal Cells (%)		Viability 3 hrs (%)
		Astroc./Oligodend.	Fibroblast/Microglia	
HIP	23 ± 8 (36)	0.09 ± 0.18 (4; 2451)	0 (3; 1363)	74 ± 11 (n= 3)
CER	1,715 ± 763 (13)	0.85 ± 0.67 (3; 1726)	0.05 ± 0.08 (1332)	77 ± 12 (n = 3)
RGCs	27 ± 9 (27)	1.18 ± 0.8 (8; 1691)		75 ± 12 (n = 3)

Yield, fraction of non-neuronal cells and viability were determined by hemocytometer counts of tryptan-blue excluding cells, by immunocytochemical staining with cell-type specific markers and by a microfluorometric survival assay, respectively. Values indicate mean ± standard deviation with number of preparations or cells in parantheses.

The immunoisolation procedure takes several hours and the viability of the isolated cells could therefore be diminished. To address this, we determined the survival rate of the cells three hours after plating by a microfluorometric assay. We could show that it averaged at 75% regardless of the brain region (Table 3).

We next determined the purity of the neuronal preparations by performing immunocytochemical staining with combinations of cell-type specific markers. Fractions of GFAP/S100β-positive astrocytes, of O4/CNPase-positive oligodendrocytes, of fibronectin-

positive fibroblasts and of Gs lectin I-positive microglia were very low (Table 3) indicating that the procedure delivered very pure neuronal populations from the three brain regions. Indeed, less then 1% of non-neuronal cells have been detected in both preparation of mouse hippocampal and cerebellar neurons immunoisolated by the L1 specific antibody. In addition, as expected from previous studies on rat (Barres *et al.*, 1988) and chicken (Butowt *et al.*, 2000; Annies & Kroger, 2002), our preparation of mouse RGCs immunoisolated by a Thy-1 specific antibody also contained very few non-neuronal cells (Table 3).

The HIP and the CER consist of various neuronal cell populations including pyramidal cells, Purkinje cells, respectively, as well as interneurons. In both regions, granule cells outnumber pyramidal cells, Purkinje cells and interneurons. To explore, whether our hippocampal and cerebellar cultures contained inhibitory interneurons, GABAergic neurons were stained with an antibody against the enzyme GAD65, 24 hours after plating. This enzyme converts glutamate to GABA and is therefore expressed by GABAergic inhibitory neurons. In the hippocampal preparation, immunostaining revealed that on average 18 ± 2% of neurons (n = 3 preparations; 12576 cells examined) were GABAergic. After 7 DIV, about 10% of neurons (n = 154 cells total) resembled pyramidal cells (Fig. 5*A*, inset). Taken together, these observations indicated that hippocampal culture contained different neuronal cell populations, thus mimicking the *in vivo* situation. In the cerebellar preparation, immunostaining revealed that on average 7 ± 2% of cerebellar neurons (n = 3 preps; 6678 cells) were GABAergic. Purkinje cells, which can be recognized by their large somata, were very rare in these cultures (about 1 out of 4000). This incidence was even lower than *in vivo* where Purkinje cells represent 0.5% of all cerebellar neurons. The low incidence of GABAergic interneurons and Purkinje cells in cerebellar preparations indicated that these cultures consisted mainly of glutamatergic granule cells, as expected from the situation *in vivo*. This was further supported by the small somata of most cerebellar neurons, which is characteristic for granule cells.

The cerebellum of 7 day old mice contains granule cell precursors (Swisher and Wilson, 1977). It has been shown that these cultures contained only a very small fraction of dividing cells (1.1 ± 0.8%; n = 4; Isabelle Buard personnal communication).

To test whether neurons require glia to form synapses, they have to be maintained in culture for at least one week. We therefore first established chemically defined culture conditions that supported neuronal survival in the absence of glia after 3 DIV by a microfluorometric assay. When immunoisolated neurons were cultured for 3 days in MSM, survival rates were very low regardless of the brain region (Fig. 5).

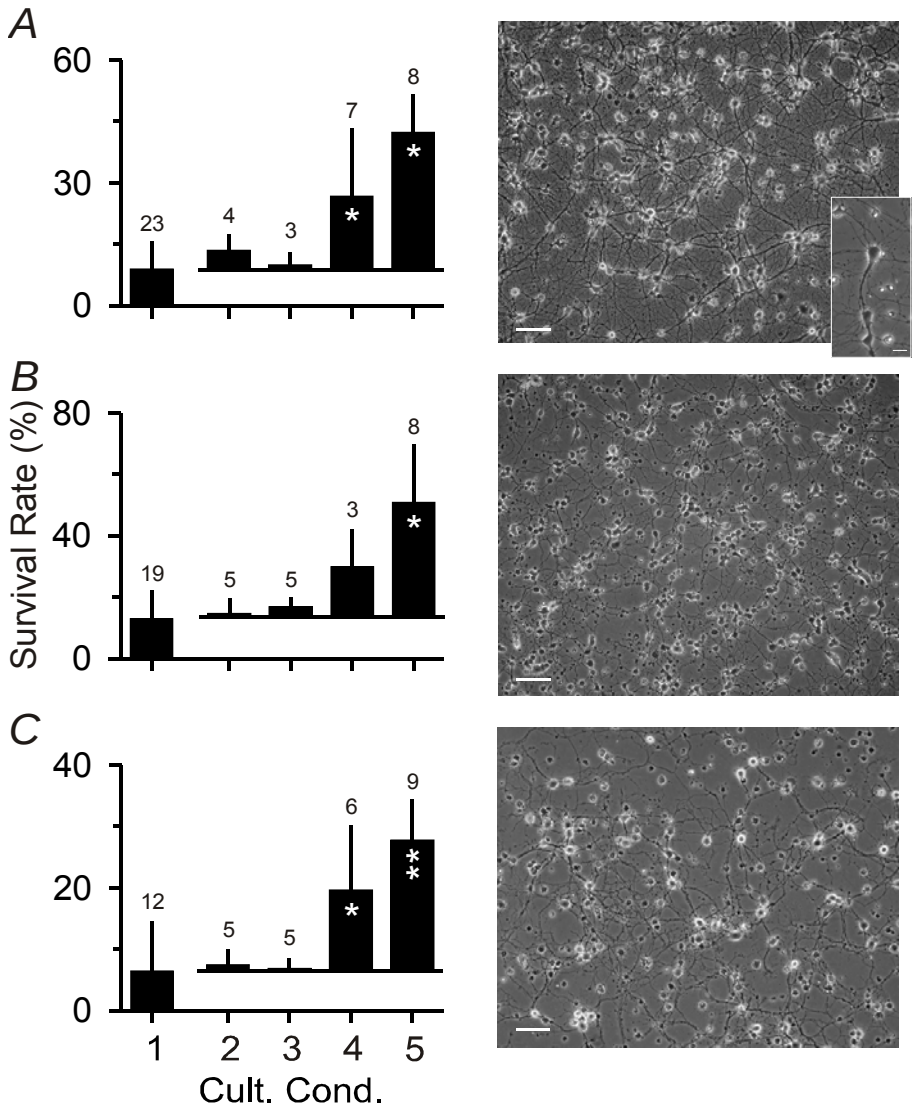

Figure 5: Serum-free culture of immunoisolated CNS neurons

Survival rates (left; mean ± SD; 3 DIV) and phase-contrast micrographs (right; 7 DIV in FSM; scale bars, 80 µm) of neurons from hippocampus (*A*), cerebellum (*B*) and retina (RGCs) (*C*) that were immunoisolated from postnatal mice. Inset in *A*, phase-contrast micrograph of a hippocampal pyramidal cell. Scale bar, 20 µm. For survival assays, neurons were cultured for three days in MSM (1) and in MSM supplemented by forskolin (2), by BDNF / CNTF (3), by forskolin / BDNF / CNTF (4) or by forskolin / BDNF / CNTF / B27 (5). Numbers above columns indicate sample size (number of preparations). For conditions 2-5, columns indicate mean of additional rates compared to basal survival in MSM-treated control cultures induced by each respective treatment. Asterisks indicate significant changes compared to MSM (1) (paired samples; Wilcoxon signed rank test).

This indicated that neuronal survival required additional signals. Previous studies on immunoisolated RGCs (Meyer-Franke *et al.*, 1995) and motoneurons (Hanson *et al.*, 1998), revealed that peptide growth factors (CNTF and BDNF) combined with an increase in the level of cyclic adenosine monophosphate, induced by forskolin, enhanced survival rates. We could show that the same applies to RGCs and to cerebellar and hippocampal neurons from mice (Fig. 5). Addition of a commercially available medium supplement (B27) further stabilized neuronal survival (Fig. 5). We next determined the survival rate after one week in culture with FSM. It averaged at 43 ± 11% (n = 4) for hippocampal neurons, at 40 ± 15% for cerebellar neurons and at 25 ± 6% for RGCs. The lower survival rate of RGCs was due to occasional preparations with very low (<20%) viability, which were excluded from further analysis. Phase-contrast micrographs at 7 DIV showed that in each preparation, neurons grew long branching neurites (Fig. 5).

Taken together these experiments showed that immunoisolation delivered pure hippocampal and cerebellar neurons as well as RGCs and that these neurons could be cultured in defined medium. This allowed to study synapse development in the presence and absence of glial cells.

2. Synapse development in CNS neurons in the absence of glia

Next, I used our new culture preparations to test whether hippocampal and cerebellar neurons establish functional synaptic connections in the absence of glial cells. Neurons from each region were cultured for one week at similar densities under defined, glia-free conditions. The presence of functional synapses was determined by whole-cell patch-clamp recordings of excitatory and inhibitory synaptic activity from randomly selected cells.

After one week in culture, the large majority of hippocampal (Fig. 6, 11*A*) and cerebellar (Fig. 7 and 11*B*) neurons showed excitatory synaptic activity without glia. Spontaneous excitatory synaptic activity was observed in more than 50% of neurons (Fig. 11*A* and *B*) with frequencies reaching up to 1000 events per min and higher (Fig. 6*B* and 7*B*). Spontaneous inhibitory synaptic activity was observed in more than half of hippocampal neurons (Fig. 6, 13*A*) with frequencies reaching up to 250 events per minute (Fig. 6*B* and 13*A*). The fraction of cerebellar neurons showing sIPSCs and their frequencies (Fig. 7*B* and 13*B*) were lower than in hippocampal neurons (Fig. 6*B* and 13*A*). This probably reflected the smaller percentage of GABAergic neurons in the cerebellar culture preparation.

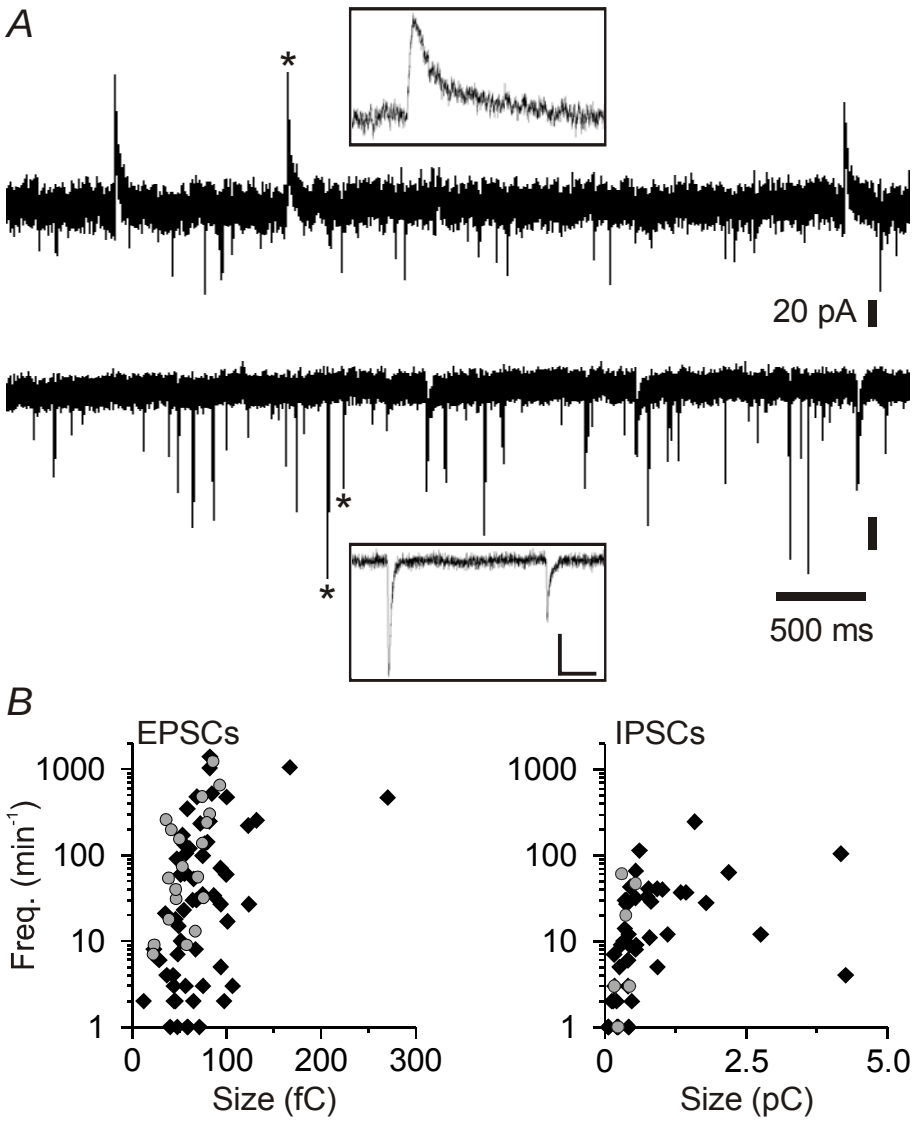

Figure 6: Presence of functional excitatory and inhibitory synapses in glia-free cultures of hippocampal neurons immunoisolated from postnatal mice

A, recordings of spontaneous synaptic activity from hippocampal neurons at –30 mV (top trace) and at –70 mV (bottom trace). Prior to recordings, neurons were immunoisolated and cultured for seven days in FSM. Asterisks label individual synaptic currents shown in boxes with different time and amplitude scales (scale bars, 40 pA; 20 ms). *B*, scatter plots of frequencies and sizes (charge transfer) of sEPSCs (left) and sIPSCs (right) in individual hippocampal neurons (black diamonds, randomly selected cells; grey circles, pyramidal neurons).

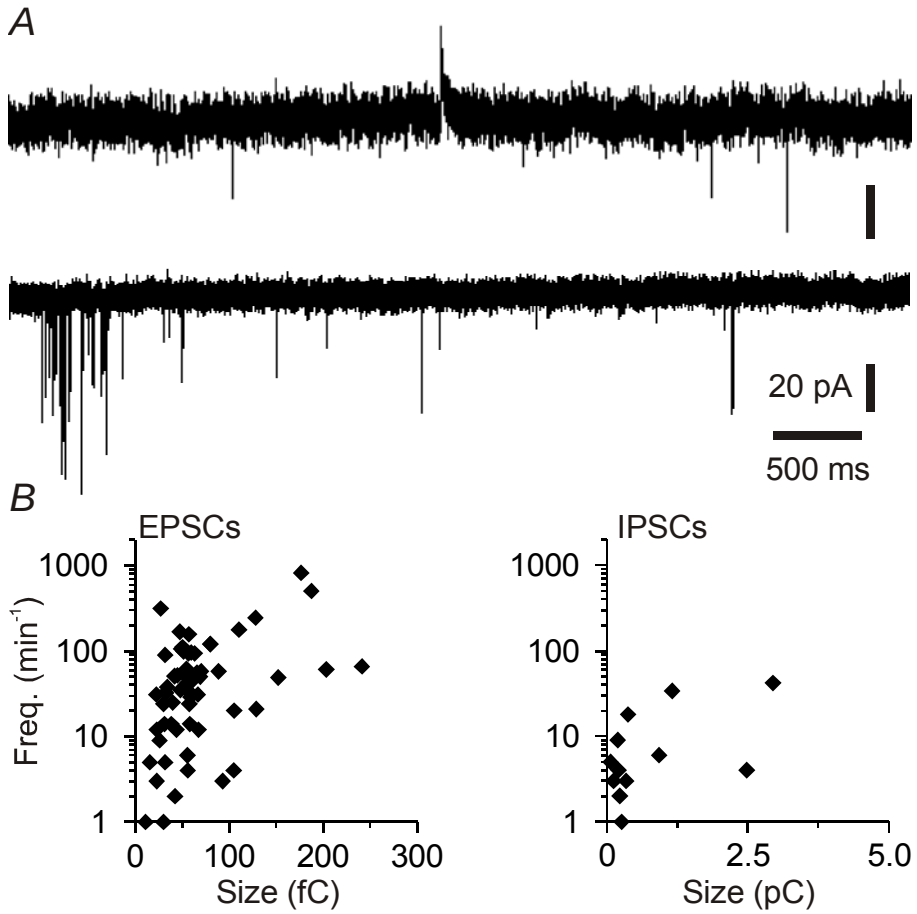

Figure 7: Presence of functional excitatory and inhibitory synapses in glia-free cultures of cerebellar neurons immunoisolated from postnatal mice

A, recordings of spontaneous synaptic activity from cerebellar neurons at –30 mV (top trace) and at –70 mV (bottom trace). Prior to recordings, neurons were immunoisolated and cultured for seven days in FSM. *B*, scatter plots of frequencies and sizes (charge transfer) of sEPSCs (left) and sIPSCs (right) in individual cerebellar neurons.

The frequencies of spontaneous postsynaptic currents, particularly of sEPSC, in hippocampal and cerebellar neurons were broadly dispersed. To test whether this variability was due to the presence of neuronal cell types with distinct levels of synaptic activity, I recorded spontaneous synaptic activity from hippocampal pyramidal cells. These cells could be identified based on their characteristic morphology (Fig. 5*A*, inset). In pyramidal cells cultured for seven days, the cell fraction that showed sEPSCs and their frequency range were similar as in randomly selected hippocampal neurons (Fig. 6*B* and 11*A*). This indicates that the variability did not originate from cell type-specific differences.

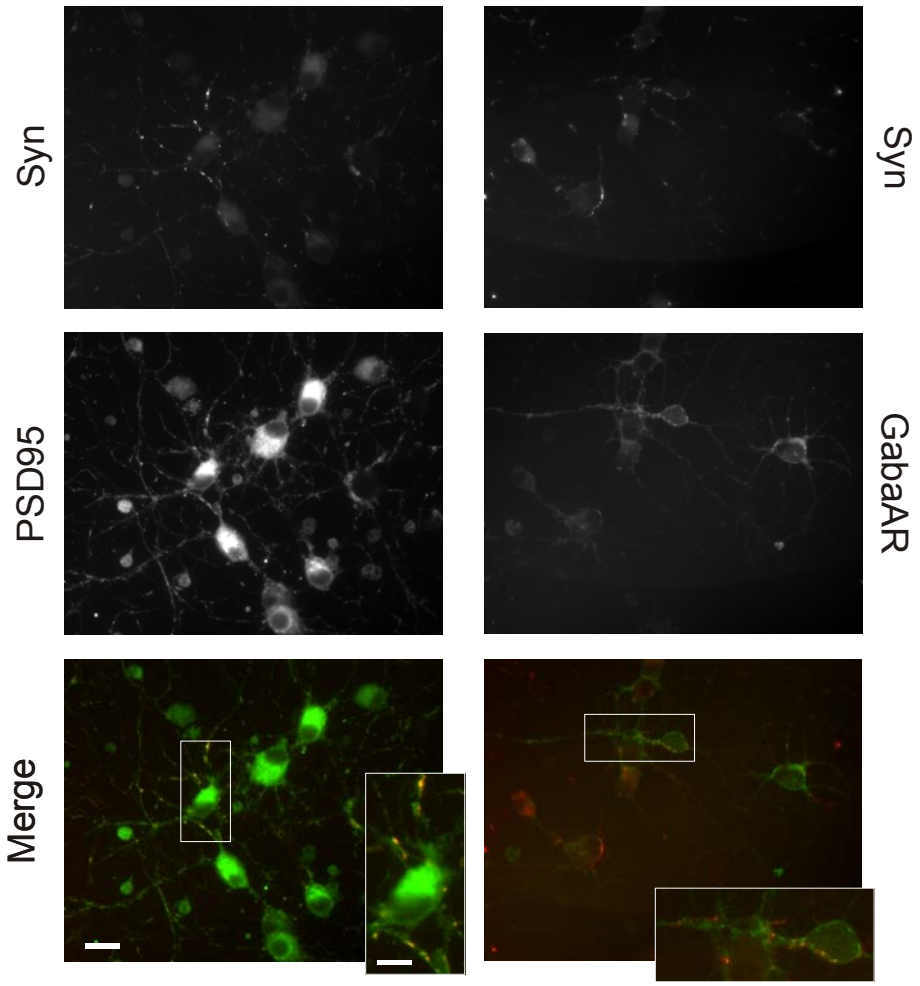

Figure 8: Formation of excitatory and inhibitory synapses by immunoisolated hippocampal neurons in the absence of glia

Representative fluorescence micrographs of hippocampal neurons that were cultured for seven days in FSM and then processed for immunostaining of excitatory (left) and inhibitory (right) synapses with antibodies against the presynaptic marker synapsin (top) and against postsynaptic proteins (middle left, PSD95; middle right: GABAA receptor; green bottom). Bottom, merged false color micrographs with red and green indicating pre- and postsynaptic markers, respectively. Scale bar, 20 µm. White rectangles indicate magnified areas shown in inset. Scale bar, 10µm.

The presence of robust synaptic activity indicated that the majority of hippocampal and cerebellar neurons formed functional excitatory and inhibitory synapses in the absence of glia. To confirm this, I performed immunocytochemical double-staining of seven day old glia-free hippocampal and cerebellar cultures with antibodies against pre- and postsynaptic

proteins. As illustrated in Figs. 8 and 9, neurons in both cultures showed frequent colocalization of pre- and postsynaptic markers. This confirmed the formation of excitatory and inhibitory synapses.

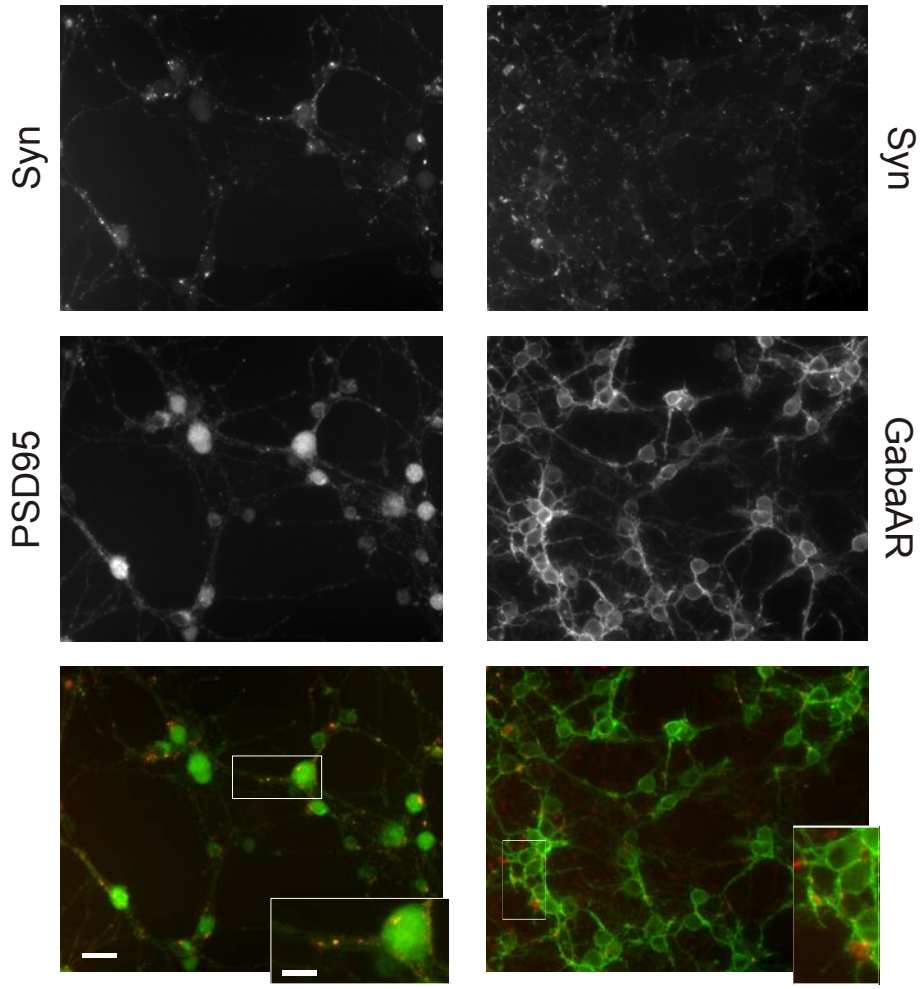

Figure 9: Formation of excitatory and inhibitory synapses by immunoisolated cerebellar neurons in the absence of glia

Representative fluorescence micrographs of cerebellar neurons that were cultured for seven days in FSM and then processed for immunostaining of excitatory (left) and inhibitory (right) synapses with antibodies against the presynaptic marker synapsin (top) and against postsynaptic proteins (middle left, PSD95; middle right: GABAA receptor; green bottom). Bottom, merged false color micrographs with red and green indicating pre- and postsynaptic markers, respectively. Scale bar, 20 µm.

For comparison, I next analysed excitatory synaptic activity in purified mouse RGCs. Previous studies on RGCs immunoisolated from rats reported a low level of sEPSCs and a

low incidence of synapses under glia-free conditions (Pfrieger and Barres, 1997; Nägler *et al.*, 2001; Ullian *et al.*, 2001). In our preparation, only 12% of all RGCs tested (n = 83) showed sEPSCs when grown for seven days in the absence of glia and their frequencies reached maximally 90 events per minute (Fig. 10*B* and 11*C*). Double immunostaining after 7 DIV showed a low incidence of synapses (Fig. 10*C*). This confirmed previous observations on immunoisolated RGCs from rats.

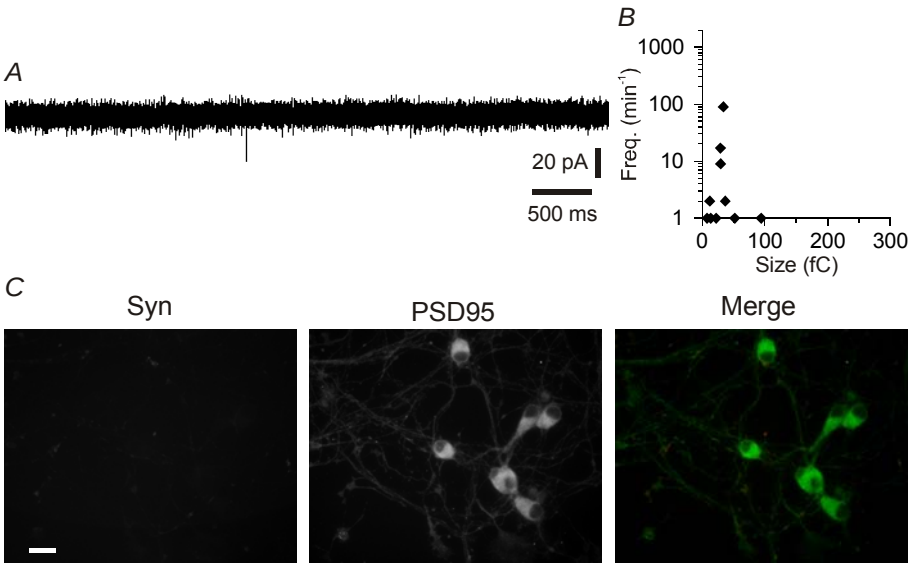

Figure 10: Low incidence of excitatory synapses in glia-free cultures of RGCs immunoisolated from postnatal mice

A, recordings of spontaneous synaptic activity from RGCs at –70 mV. Prior to recordings, neurons were immunoisolated and cultured for seven days in FSM. *B*, scatter plot of frequencies and sizes (charge transfer) of sEPSCs in individual RGCs. *C*, Representative fluorescence micrographs of RGCs that were cultured for seven days in FSM and then processed for immunostaining of excitatory synapses with antibodies against the presynaptic marker synapsin (left) and against the postsynaptic protein PSD95 (middle). Right, merged false color micrographs with red and green indicating pre- and postsynaptic markers, respectively. Scale bar, 20 µm.

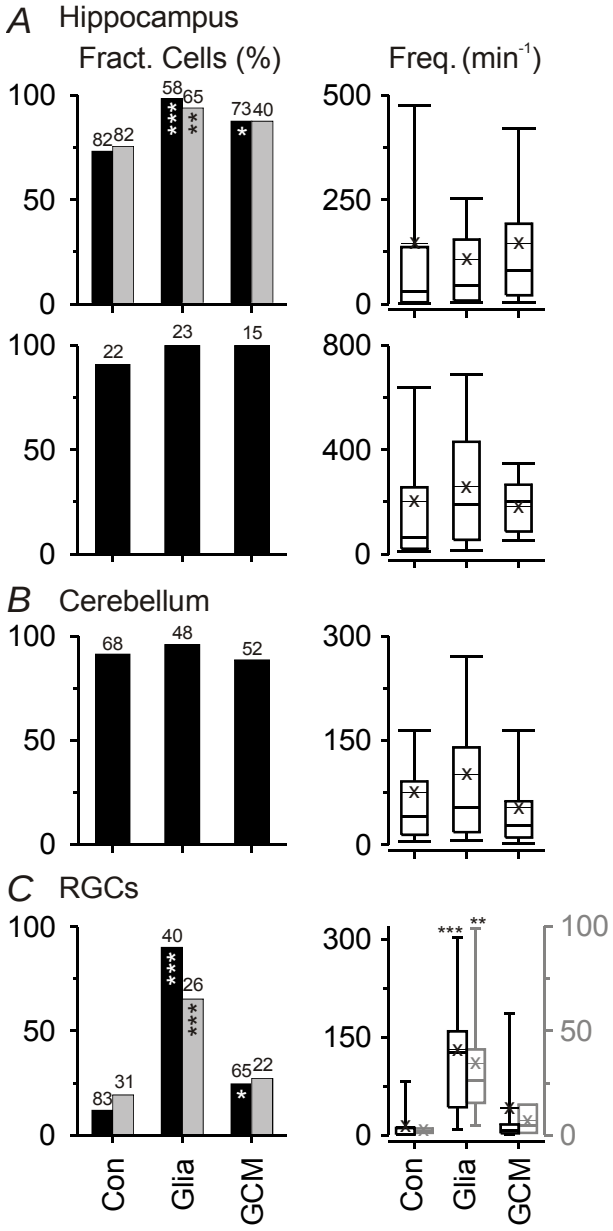

Figure 11: Effects of glial signals on incidence and frequency of spontaneous EPSCs in cultures of immunoisolated CNS neurons

Level of spontaneous excitatory synaptic activity in hippocampal neurons (*A*; upper row: randomly selected cells; lower row: pyramidal cells), in cerebellar neurons (*B*) and in RGCs (*C*) cultured for 7 days in the absence of glia (Con), cocultured with glial cells (Glia) or treated with GCM (GCM). Left, column plots showing fraction of neurons with sEPSCs (numbers indicate sample size). Right, box plots representing frequencies of sEPSCs in neurons with excitatory activity (thin lines with crosses, mean values). For parameters showing statistically significant glia-induced changes in spontaneous activity compared to

control cultures, the incidence (left, grey columns) and frequency (right, grey box plots) of action potential-independent mEPSCs are indicated. Asterisks indicate significant changes compared to control cultures (left, Pearson's Chi square test; right, Kruskal-Wallis ANOVA by ranks test with post-hoc comparison of mean ranks).

Taken together, RGCs formed few and inefficient synapses in the absence of glia regardless of the species of origin, whereas hippocampal and cerebellar neurons were able to form functional synapse without glia.

3. Glia requirement for synapse development

3.1. Effects of glial cells on spontaneous synaptic activity

To study, whether glial signals affected synapse development, immunoisolated neurons were cocultured with glial cells or treated with GCM, to test effects of neuron-glia contact and of factors released by glial cells, respectively.

In randomly selected hippocampal neurons, coculture with glial cells induced a small but significant enhancement in the fraction of cells showing sEPSCs, without changing the frequency of sEPSCs (Fig. 11*A*). In addition, coculture with glia enhanced the size of sEPSCs in a large fraction of neurons (Fig. 12*A*). Inhibitory activity was not affected by the presence of glia (Fig. 13*A*). In pyramidal cells, glial cells also enhanced strongly the size of sEPSCs (Fig. 12*A*), but had no effect on their incidence and their frequency (Fig. 11*A*). Glial cells also significantly increased the incidence of sIPSCs and their size (Fig. 13*A*). In cerebellar neurons, coculture with glia increased the size of sEPSCs (Fig. 12*B*) and raised the incidence of inhibitory activity (Fig. 13*B*), but left all other parameters unaffected.

The changes observed in neuron-glia cocultures were only mimicked in part by GCM which only increased the fraction of cells showing sEPSCs in randomly selected hippocampal neurons.

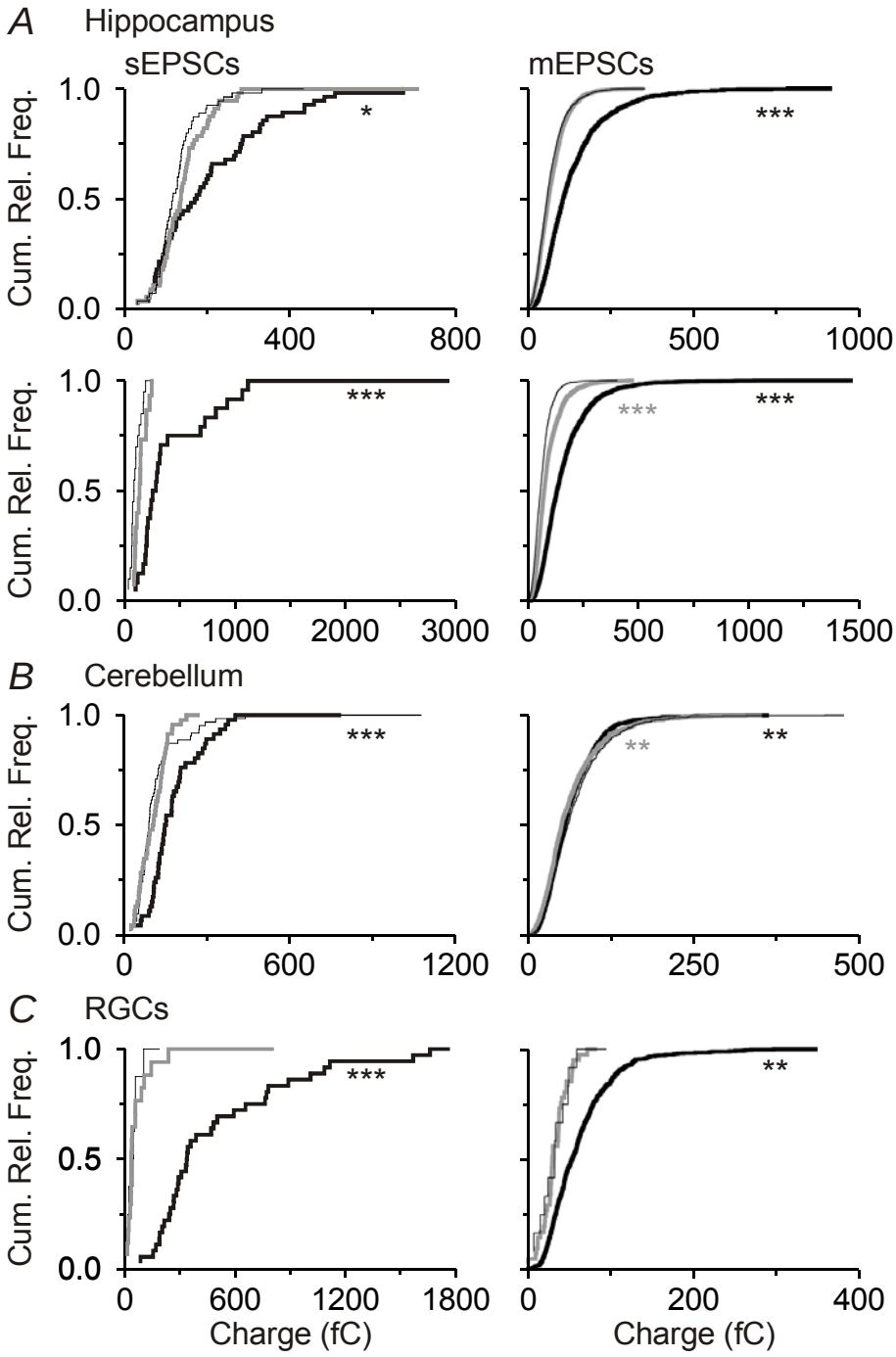

Figure 12: Glia-induced changes in the size of spontaneous and miniature EPSCs in cultures of immunoisolated neurons

Cumulative relative frequency distributions of charge transfer of sEPSCs representing the 90th percentile in each cell (left column) and of charge transfers of individual mEPSCs pooled from all cells analysed (right column) that were recorded from hippocampal (*A*; upper row: randomly selected cells; lower row: pyramidal cells) and cerebellar neurons (*B*) and from

RGCs (*C*). Prior to recordings, neurons were cultured for 7 days in the absence of glia (thin black lines), cocultured with glial cells (thick black lines) or treated with GCM (grey lines). For hippocampal (n ~ 2000) and cerebellar neurons (n ~ 800), random subsamples of mEPSCs were analysed. Asterisks indicate significant changes compared to control cultures (left, Kruskal-Wallis ANOVA by ranks test with post-hoc comparison of mean ranks; right, ANOVA with Dunnett's posthoc test). For RGCs, the number of individual mEPSCs (n = 12) recorded under glia-free conditions was very low compared to cocultures (n = 582) or GCM-treated cultures (n = 41) and therefore a subsample could not be analysed. The asterisk therefore indicates a statistically significant change in random subsamples (~40 events) from cocultures and from GCM-treated cultures (Mann-Whitney U test).

In RGCs, coculture with glial cells strongly enhanced synaptic activity. Glial cells increased the incidence of sEPSCs by seven-fold and the mean frequency (Fig. 11*C*) and size (Fig. 12*C*) by at least six-fold compared to glia free cultures. These effects confirmed previous observations on RGCs from rats (Pfrieger and Barres, 1997; Nägler *et al.*, 2001; Ullian *et al.*, 2001). GCM mimicked the glia-induced enhancement of synaptic activity only partially (Fig. 11*C* and 12*C*). GCM only increased by 3-fold the incidence of sEPSCs and had no effect on sEPSC frequency and size. This could be due to a low concentration of cholesterol in mouse GCM. This component has been identified as synaptogenesis-promoting factor in immunoisolated RGCs from postnatal rats (Mauch *et al.*, 2001; Göritz *et al.*, 2005). Addition of cholesterol to the culture medium for five days increased the fraction of RGCs with synaptic activity nearly three-fold and increased the frequency of sEPSCs significantly compared to control cultures (Fig. 14*A* and *B*). However, cholesterol did not mimick the glia-induced increase in the size of sEPSCs compared to glia-free cultures (Fig. 14*C*).

3.2. Effects of glial cells on miniature synaptic activity

Spontaneous synaptic activity is composed of action potential-independent activity, which corresponds to spontaneous release of transmitter quanta and of action potential-driven activity, which corresponds to simultaneously evoked release of synaptic vesicles from all terminals of an axon. Therefore, glia-induced changes in spontaneous synaptic activity in the different neuronal cell types could have been due to enhanced neuronal excitability and thus a higher level of action potential-driven synaptic transmission. To address this, I recorded miniature postsynaptic currents in the presence of TTX, which are due to action potential-independent quantal transmitter release.

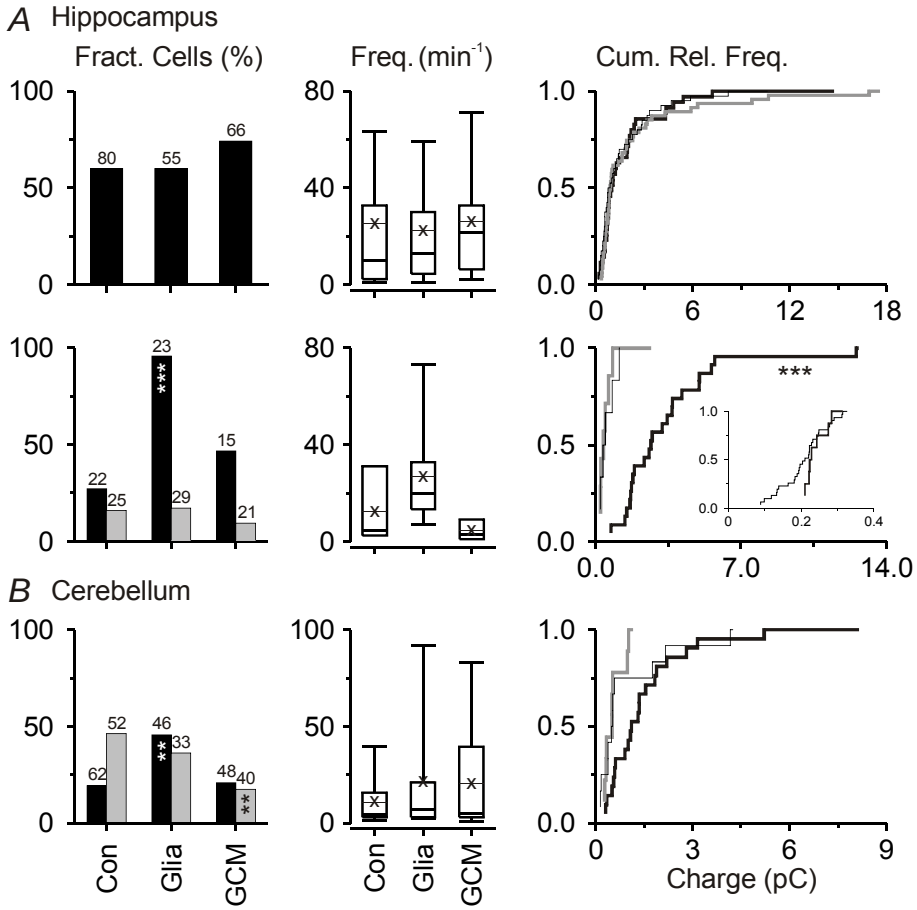

Figure 13: Effects of glial signals on inhibitory synaptic activity in cultures of immunoisolated CNS neurons

Level of spontaneous inhibitory synaptic activity in hippocampal neurons (*A*; upper row: randomly selected cells; lower row: pyramidal cells) and in cerebellar neurons (*B*) cultured for 7 days in the absence of glia (Con), cocultured with glial cells (Glia) or treated with GCM (GCM). Left, column plots showing the fraction of neurons with sIPSCs (numbers indicate sample sizes). Middle, box plots representing frequencies of sIPSCs in neurons with excitatory activity (thin lines with crosses, mean values). Right, cumulative relative frequency distribution of charge transfer of sIPSCs representing the 90th percentile in each cell in glia-free control cultures (thin black lines), in cocultures (thick black) and in GCM-treated cultures (grey). Asterisks indicate significant changes compared to control cultures (left, Pearson's Chi square test; middle and right, Kruskal-Wallis ANOVA by ranks test with post-hoc comparison of mean ranks). For parameters showing statistically significant glia-induced changes in spontaneous activity compared to control cultures, the incidence (left, grey

columns) and size (*A*, right, inset in lower panel, pooled from all neurons tested) of action potential-independent mIPSCs are indicated.

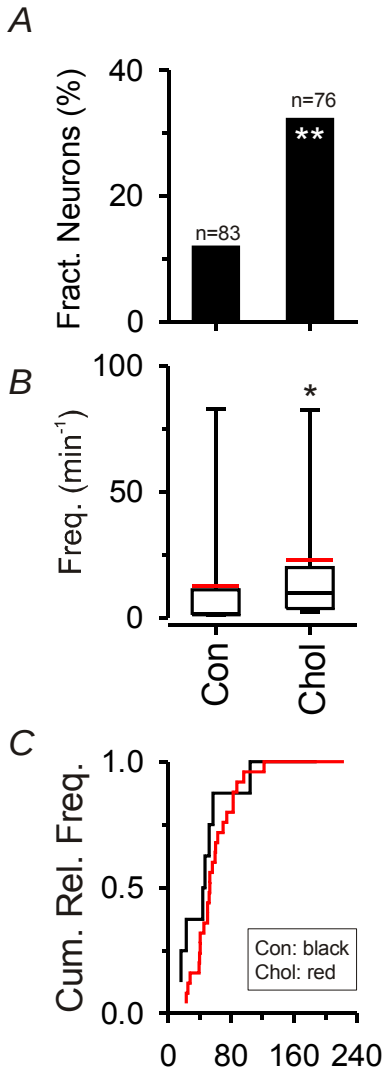

Figure 14: Effects of cholesterol on spontaneous synaptic activity in RGCs.
Fraction of neurons with sEPSCs (*A*, column plots; Pearson's Chi square test), frequencies (*B*, box plots; Mann-Whitney U test) and charge transfer of sEPSCs (*C*, cumulative relative frequency plot of charge transfer representing the 90th percentile in each cell; Mann-Whitney U test) in RGCs cultured in absence (Con; n = 10 with activity) or presence of cholesterol (Chol; n = 25 with activity)

In hippocampal neurons including pyramidal cells, glial cells affected mEPSCs in a similar manner as spontaneous excitatory events: compared to glia-free cultures, coculture

with glia increased the fraction of neurons showing mEPSCs (Fig. 11*A*) and their size (Fig. 12*A*). The glia-induced changes in the incidence and size of sIPSCs of pyramidal cells were not observed in mIPSCs (Fig. 13*A*). This indicated that glial cells affected the development or function of excitatory, but not of inhibitory synaptic connections in hippocampal neurons. The higher incidence and size of inhibitory activity was probably caused indirectly by enhanced excitatory synaptic activity. Recent studies showed that several glia-derived factors enhanced the size of mEPSCs in hippocampal cultures. TNFα (Beattie *et al.*, 2002; Stellwagen and Malenka, 2006) and D-serine (Yang *et al*., 2001) have been shown to increase the size of mEPSCs in hippocampal cultures. In addition, the glutamate precursor, glutamine, is exclusively released by astrocytes and is converted to glutamate in neurons (Hertz *et al.,* 1999; Kvamme *et al*., 2001). Glutamine could therefore also increase NT concentration in vesicles and the size of mEPSCs. I found that 24 hours treatment with different concentrations of D-serine and recordings in the presence of glutamine enhanced the size of mEPSCs, slightly but significantly, but did not affect their frequency (Fig. 15). In contrast, treatment of hippocampal neurons with TNFα for 15 min after 7 DIV increased significantly the frequency and the size of mEPSCs, whereas treatment for 48 hrs had no effect (Fig. 15).

In cerebellar cultures, glial cells decreased the size of mEPSCs (Glia: n = 38; GCM: n = 39) compared to glia-free cultures (n = 66; Fig. 12*B*) indicating that the enhanced size of sEPSCs in these neurons was due to changes in action potential-dependent synaptic activity. In RGCs, the glia-induced increases in the incidence, frequency and size of sEPSCs were also observed for mEPSCs (Fig. 11*C*, 12*C*) thus confirming our previous observations that glial cells promote the development of synapses in these neurons.

Our study on new primary cultures of immunoisolated CNS neurons from postnatal mice revealed that most hippocampal and cerebellar neurons formed functional synapses in the absence of glia, whereas RGCs did not. Coculture with glia strongly promoted synaptic activity only in RGCs, but not in hippocampal and cerebellar neurons and this effect was mimicked in part by cholesterol thus confirming previous results (Mauch *et al.*, 2001; Göritz *et al.,* 2005). On the other hand, glial signals enhanced the size of spontaneous excitatory synaptic events in all three regions, and in hippocampal neurons and RGCs this effect was due to an increase in the size of action potential-independent miniature synaptic events. These effects could have been due to combined activity of the glia-derived factors, D-serine, glutamine and TNFα. Finally, no effect of glia on inhibitory synapses was detected.

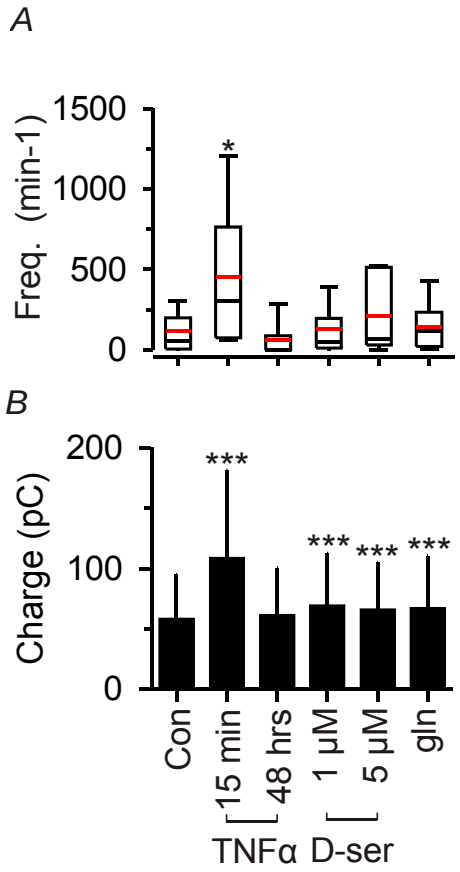

Figure 15: Effects of glia-derived factors on frequency and charge transfer of miniature EPSCs in cultures of immunoisolated hippocampal neurons.

Distribution of frequencies (*A*, box plots) and charge transfer (*B*, column plots: mean values +/- standard deviation) of mEPSCs recorded from hippocampal neurons cultured in the absence of glia (Con; n = 60), after treatment with tumor necrosis factor α (TNF α; n = 12) for 15 minutes and 48 hours (n = 26), with D-serine (D-ser) at 1µM (n = 18) and 5 µM (n = 9), and with glutamine (gln) at 2 mM (n = 9). Asterisks indicate significant changes compared to control culture in random subsamples of mEPSCs pooled from all cells from each condition (n = 1000) (*A*: TNFα and D-serine, Kruskal-Wallis ANOVA by ranks test with post-hoc comparison of mean ranks; glutamine, Mann-Whitney U test; *B*: TNFα and D-serine, ANOVA with Dunnett's posthoc test; glutamine, Kolmogorov-Smirnov test).

4. Glia requirement for activity-dependent changes

Neurons are able to adjust their synaptic connectivity in response to changes in network activity *in vitro* in cortical (Turrigiano *et al*., 1998), spinal (O'Brien *et al*., 1998) and

hippocampal cultures (Murthy *et al*., 2001) and *ex vivo* in cortical slices (Desai *et al*., 2002; Maffei *et al*., 2004; Maffei *et al*., 2006).

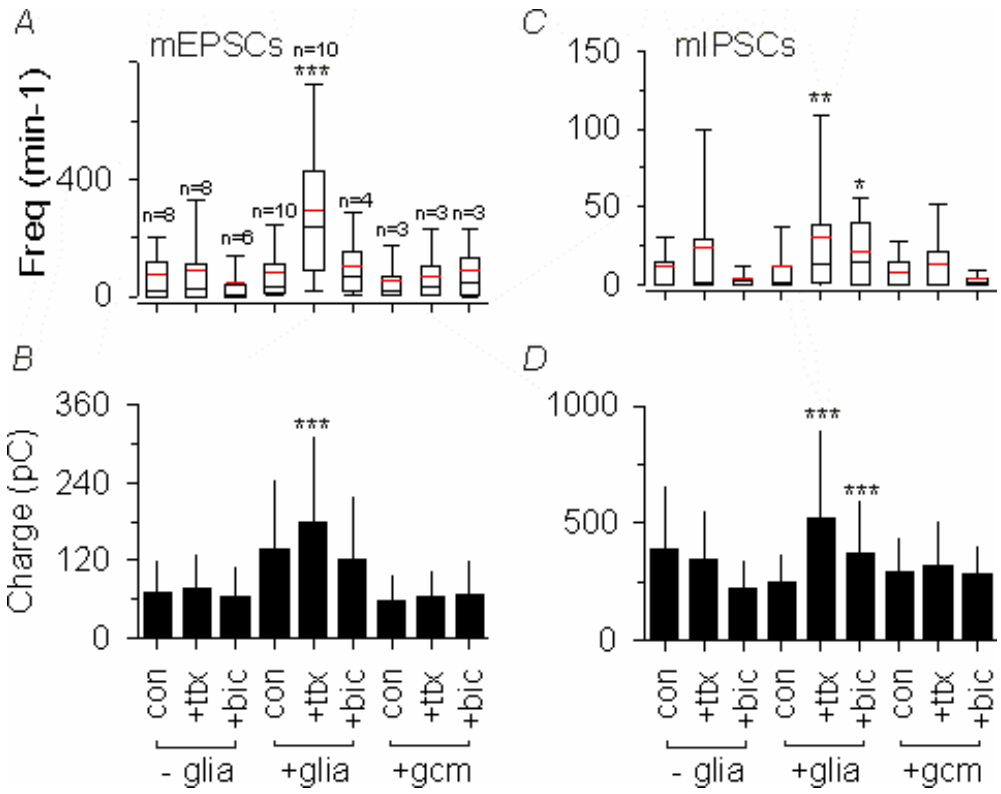

Figure 16: Effects of chronic blockade of electrical activity on synaptic miniature currents in cultures of immunoisolated hippocampal neurons depend on glia presence

Level of miniature activity in hippocampal neurons cultured for 7 days in the absence of glia (-glia), cocultured with glial cells (+glia) or treated with GCM (GCM), without (Con) or with 2 days of treatment with tetrodoxin (+ttx) or bicuculline (+bic).

Frequency of mEPSCs and mIPSCs (*A* and *C*, box plots; red thin lines, mean values) and charge transfer of mEPSCs and mIPSCs (*B* and *D*, column plots). Asterisks indicate significant TTX- and BIC induced changes compared to control condition (Kruskal-Wallis ANOVA by ranks test with post-hoc comparison of mean ranks).

To study whether glial cells were required for this adjustment in immunoisolated hippocampal neurons, electrical activity was blocked or increased by application of TTX or BIC, respectively, after 5 DIV for 2 days. In the absence of glia, nor TTX treatment nor BIC treatment had a effect on the frequency and the size of mEPSCs (Fig. 16). Increasing network activity in pure hippocampal neuronal culture left the frequency of mIPSCs unaffected

(Fig.16). Interestingly, when hippocampal neurons were cultured with glia, TTX treatment increased the frequency and size of mEPSCs, whereas BIC treatment had no effect (Fig. 16). Both TTX and BIC treatment also increased the frequencies and size of mIPSCs when hippocampal neurons were cocultured with glia (Fig. 16). GCM didn't mimicked glia effects. Taken together, these results suggested that glial are required to adjust the strength of synapses in response to changes in the level of electrical activity. However I observed considerable variability in the described effects across different culture preparations.

DISCUSSION

Our study on new primary cultures of immunoisolated CNS neurons from postnatal mice revealed that hippocampal and cerebellar neurons formed functional synapses in the absence of glia, whereas RGCs did not. Coculture with glia strongly promoted synaptic activity only in RGCs, but not in hippocampal and cerebellar neurons and this effect was mimicked in part by cholesterol thus confirming previous results (Mauch *et al.*, 2001; Göritz et al., 2005). On the other hand, glial signals enhanced the size of spontaneous excitatory synaptic events in all three regions, and in hippocampal neurons and RGCs this effect was due to an increase in the size of action potential-independent miniature synaptic events. Finally, no effect of glia on inhibitory synapses was detected. Taken together, our results indicate that in the mammalian CNS the influence of glial signals on synapse development is not a general principle, but varies with the neuronal cell type. This underlines the importance of comparative studies on different neuronal cell types. In addition, a first series of experiments on activity-dependent changes in synaptic efficacy revealed that in hippocampal neurons blockade of chronic activity raised the frequency and size of synaptic currents only if glial cells were present.

1. Validity of the experimental model

Currently, neuronal cultures are often prepared from embryonic rats or mice (Blondel *et al*., 2000; Mazzanti and Haydon, 2003; Yang *et al.,* 2003; Hama *et al*., 2004; Elmariah *et al.*, 2005). These cultures are already "neuron-enriched" because glial cells are not born at the stage of the preparation and so their content is relatively low (<5%). However, because at the embryonic stage astrocytes are not born yet, studying glia requirement for synaptogenesis with these cultures is not optimal. A more stringent approach to study these questions is to test whether neurons form synapse in glia-free cultures derived from postnatal animals. This, however, requires active separation of neurons from glial cells. Our protocols for immunoisolation of CNS neurons obtained around 99% of purity and supported neuronal survival and growth in a defined medium without serum. The absence of serum is important because lipoproteins contained in serum would not allow to study the influence of glia-derived lipids. In addition, the development of the immunoisolation procedures allowed for a direct comparison with previous results obtained on immunoisolated RGCs and motoneurons from postnatal rats (Pfrieger and Barres, 1997; Nägler *et al.,* 2001; Ullian *et al.,* 2001; Mauch *et al.,* 2001; Ullian *et al.,* 2004).

2. Hippocampal and cerebellar neurons, but not RGCs show robust synapse formation in the absence of glia

A main finding of our study is that CNS neurons differ in their ability to form synapses without glia. Within one week in culture, most hippocampal and cerebellar neurons formed functional excitatory and inhibitory synaptic contacts in the absence of glia as indicated by robust spontaneous synaptic activity and by the presence of immunocytochemically stained synapses. The frequencies of EPSCs and IPSCs in glia-free cultures were similar or even higher than those reported previously in conventional hippocampal (Mennerick *et al.*, 1995; Bouron and Reuter, 1997; Boehm, 1999) and cerebellar (Virginio *et al.*, 1995; Losi *et al.*, 2002; Fiszman *et al.*, 2005) cultures derived from postnatal rats or mice. This indicated that under the present culture conditions, purified neurons reach the same level of connectivity as those growing in glia-containing primary cultures. In contrast, cultured RGCs formed only very few connections and showed a very low level of activity in the absence of glia thus confirming previous studies (Pfrieger and Barres, 1997; Nägler *et al.*, 2001; Ullian *et al.*, 2001).

Our isolation procedure and the culture conditions could have selected only neurons that form synapses autonomously. However, hippocampal and cerebellar cultures contained inhibitory neurons and principal neurons like pyramidal at ratios similar to those observed *in vivo*. Thus, our preparation preserved the diversity of neuronal cell types and did not select for neurons that form synapses autonomously. In addition, it could not be excluded that some synaptic connections in our cultures were 'non-natural' and that their formation therefore proceeds independently from glia. In hippocampal cultures, pyramidal cells, which represent principal synaptic targets, received synaptic input from excitatory and inhibitory neurons. So, their synapse formation occurred between natural partners. In cerebellar cultures, only granule cells can form excitatory glutamate connections. In this culture, excitatory synaptic activity originates therefore from synapses between granule cells, which are non-natural. However, the same argument applies for cultures of immunoisolated RGCs (Nägler *et al.*, 2001; Ullian *et al.*, 2001) and motoneurons (Ullian *et al.*, 2004), which lack natural partner neurons. In contrast to cerebellar granule cells, however, these neurons required glia to form non-natural synaptic connections. So, even if the connections were non-natural, neurons differ in their ability to form synapses autonomously. Due to the rare occurrence (<0.5%) of Purkinje cells in our cultures it was not possible to determine whether they receive natural input from

granule cells. Preliminary data from a parallel study indicate however that formation of these connections require glial signal (Buard, Pfrieger; in preparation)

3. Glial signals promote formation of synapses by RGCs, but not by hippocampal or cerebellar neurons

Glial signals did not enhance the frequency of excitatory or inhibitory synaptic events in hippocampal and cerebellar cultures indicating that they did not promote the formation of new connections by these neurons. However it is possible that glial signals promoted the formation of synapses that were not functional (Christopherson *et al.,* 2005). Our observations are in contrast to previous reports that glial signals promote synaptic activity and synapse formation in cultures of hippocampal neurons. Blondel *et al*. (2000) showed in hippocampal cultures from embryonic rats by immunostaining against the presynaptic marker synaptophysin that activity-dependent neurotrophic factor released by astrocytes increased the number of excitatory synapse. They also showed by Western blots that activity-dependent neurotrophic factor increased the density of NMDAR subunits. In addition, three other studies on hippocampal neurons showed that glial cells are required for synapse development. Yang *et al.* (2003) showed by electrophysiological recordings that in hippocampal cultures from embryonic rats, coculture with glial cells enhanced the frequency and size of mEPSCs and that GCM only mimicked glia effects on mEPSCs size. In hippocampal cultures from newborn rats, Mazzanti and Haydon (2003) demonstrated that in astrocyte-enriched cultures, neuronal calcium currents were increased after 1 to 3 DIV. Finally, Hama *et al*. (2004) showed that in hippocampal neurons from embryonic rats cultured on microislands, integrin–mediated contact between astrocytes and neurons and neuronal activation of protein kinase C are required to promote excitatory synapse formation and activity. These effects were not mimicked by GCM, which was required for neuronal survival on microislands. Taken together, these studies on hippocampal cultures from embryonic or newborn rats suggested that glia-contact and glia-secreted factors are required for the formation of excitatory synapses. In addition, it has also been shown in this culture model that astrocytes increase the number of synapses, the frequency of sIPSCs and of GABAA receptor clustering at inhibitory synapses by increasing the neuronal expression of BDNF and TrkB (Elmariah *et al*., 2005). The divergence between our conclusions and those mentioned above may be explained by differences in culture conditions and in the age of animals from which neurons were obtained. All these cited studies prepared neurons from embryonic animals, where only few glial cells

have been generated and where culture preparations can be regarded as glia-free. At the embryonic stage, neurons may require glial signals for synaptogenesis or for neurite growth, whereas postnatally they become autonomous. In our model, glia-induced increase in a small fraction of hippocampal neurons (but not of pyramidal cells) with excitatory synaptic events indicated an induction of synapse formation in a subset of neurons.

In contrast to hippocampal and cerebellar neurons, glial signals strongly promoted synapse formation in RGCs as indicated by the large increase in the incidence of synaptic activity and in the frequency of spontaneous and miniature synaptic events. This confirmed previous studies reports that the glia-induced increase of synaptic activity in these neurons is due to enhanced synaptogenesis (Nägler *et al.*, 2001; Ullian *et al.*, 2001). The lack of effect by mouse GCM, differs from the situation in the rat (Pfrieger and Barres, 1997; Nägler *et al.*, 2001). It was probably due to a lower cholesterol concentration in mouse GCM, since addition of cholesterol mimicked the effects of coculture on the frequency of sEPSCs at least in part. At present, we can not exclude whether a combination of cholesterol (Mauch *et al.,* 2001) with the synaptogenic TSPs (Christopherson *et al.,* 2005) enhances synapse development in these neurons.

4. Glial cells enhance the size of miniature synaptic currents in hippocampal neurons and RGCs

Glial cells enhanced the size of spontaneous excitatory postsynaptic currents in all three culture preparations. In hippocampal neurons and RGCs, this was due to an increase in the size of action potential-independent miniature synaptic currents. In cerebellar cultures, which consist mainly of granule cells, the effect was action potential-dependent and may have been caused by an increased excitability or by an increased efficacy of evoked transmitter release.

The increase in spontaneous inhibitory synaptic currents in pyramidal cells did not occur in miniature inhibitory synaptic currents and was therefore probably action potential-dependent. An indirect activation of synaptic activity via elevated excitatory synaptic activity, could explain this. Indeed, enhanced excitatory synaptic activity could excite GABAergic interneurons providing synaptic input to pyramidal cells. Alternatively, glial cells, namely astrocytes, may also have directly activated interneurons as reported recently (Liu *et al.*, 2004). In this study, the authors showed in hippocampal slices from 11- to 15-day-old rats that Ca^{2+} elevation in astrocytes, induced by calcium uncaging, in turn increased the frequency of sIPSCs on interneurons and had no effects on their size. They also demonstrated

that this effect was mediated by the activation of postsynaptic kainate receptors by astrocyte-derived glutamate. Such a selective effect on glutamatergic rather than GABAergic synapses fits to the fact that inhibitory synapses develop before glutamatergic synapses (Ben-Ari, 2002) and thus may not require glial signals.

To increase the size of mEPSCs in hippocampal neurons and RGCs, glial signals may have enhanced postsynaptically the efficacy of glutamate receptors or presynaptically the glutamate concentration in synaptic vesicles. We could show that TNFα increased the size of mEPSCs at hippocampal synapses, therefore indicating that this component mediate glial effect. Short-term application of astrocyte-derived TNFα has been shown to increase the frequency and size of mEPSCs in hippocampal culture from newborn rats, by enhancing the clustering of AMPA receptors (Beattie *et al.*, 2002). Our data also indicate that acute application of glutamine slightly mimicked glia effects on hippocampal mEPSC, therefore suggesting that glia support of the glutamate glutamine cycle (Hertz & Zielke, 2004) may also induce the increase of mEPSCs. Culture medium contained glutamine but it is possible presynaptic glutamate pools diminished when we removed the culture medium and replaced it by the recording solution. The glia-induced increase in miniature size was only seen in neuron-glia cocultures and was not mimicked by soluble glial factors contained in GCM. GCM may not contain D-serine as neuronal glutamate-release together with spontaneous neuronal activities is required to induce release of D-serine from glial cells in hippocampal cultures (Yang *et al.,* 2003). TNFα has also been shown to be constitutively secreted by glial cells (Beattie *et al.,* 2002) and Stellwagen and Malenka (2006) suggested that glial cells adjust TNFα production to the level of neuronal activity, with a high level of neuronal activity inducing small production of TNFα. In addition, acute application of TNFα or GCM (15 min) increases the size of mEPSCs (Stellwagen and Malenka, 2006). Our data indicate that acute application of TNFα increase the size of mEPSCs whereas its application for longer time had no effect. We only tested the effects of GCM on mEPSCs after 5 days of application and not after acute application. Finally, it is possible that glia-neuron contact was also required to enhance the size of mEPSCs. This is in line with our previous observation on immunoisolated rat RGCs that coculture with glia enhanced the size of mEPSCs more strongly than GCM (Nägler *et al.*, 2001).

5. Further investigation of glia effects on synapse development *in vivo.*

Previous reports (Pfrieger and Barres, 1997; Nägler *et al.*, 2001; Ullian *et al*., 2001) together with our results indicate that in the mammalian CNS the influence of glial cells on synapse development varies with the neuronal cell type. These studies were based on culture preparations of immunoisolated neurons from postnatal rats and mice. This prompts the question whether glia influence synapse development *in vivo*.

So far, it has been shown that ablation of PSCs from live frog muscle *in vivo* by complement-mediated cell lysis decreased presynaptic function and increased nerve terminal retraction one week after ablation at adult NMJs (Reddy *et al.*, 2003). In addition, this ablation reduced growth and addition of nerve terminal. These observations suggest that synapse-associated glial cells are essential for the maintenance of synaptic structure and function of adult NMJs as well as for the growth and stability of the developing NMJ, *in vivo*. In mammals, selective ablation of astrocytes in transgenic mice *in vivo* by the expression of *Escherichia coli* nitroreductase under the control of the GFAP promoter causes neurodegeneration, thus making it impossible to study the astrocytic role in synaptogenesis (Cui *et al.*, 2001). An alternative approach was used by Iino *et al*. (2001). They induced the retraction of Bergmann glia processes in three-week-old rats *in vivo*, by adenoviral-mediated delivery of the glutamate receptor 2 gene. This converted Ca^{2+} - permeable AMPA receptors to Ca^{2+} - impermeable AMPA receptors in Bergmann glia. Retraction of Bergmann glia processes caused multiple innervation of Purkinje cells by climbing fibers *in vivo*, therefore suggesting that Bergmann glia regulates synapse elimination between climbing fibers and Purkinje cells. An approach to bypass deleterious effects of astrocyte ablation on neuronal survival would be to develop mouse models for temporally controled elimination of gene expression exclusively in astrocytes by using for example the Cre/LoxP system (Morozov *et al.*, 2003)

6. Glia is required for activity-dependent changes in synaptic strength in hippocampal neurons

We could show that chronic activity blockade greatly increased the frequency and size of mEPSCs when glial cells were present, whereas enhancing electrical activity had the opposite effects. This suggested that glial cells are required for the regulation of excitatory synaptic strength in response to chronic changes in network activity. Even though GCM did not mimick glial effects, some factors are released by glial cells only when neurons are present

and may be implicated in this regulation. A recent study (Stellwagen and Malenka 2006) indicated that TNFα mediates activity-dependent changes in synaptic activity. They showed that in response to 48 hours of activity blockade, the increase in mEPSCs size is mediated by TNFα. Using combinaitions of wild-type and TNFα-deficient neurons and glia, they also showed that glial cells are the source of TNFα and suggested that by modulating TNFα levels, glia actively participate in the activity-dependent regulation of synaptic connectivity. However, TNFα did not mediate the reduction in synaptic strength produced by increased neuronal firing, suggesting that others factors, released by glia or by neurons themselves are implicated in the process. Our results suggest that TNFα mediated the glial enhancement of mEPSCs size but it is unclear whether it also induced activity-dependant changes that we observed. There are a number of other candidate molecules that could mediate the glial effects. Glia-released ATP has been showed to decrease the frequency of mEPSC (Zhang *et al.,* 2003) in hippocampic cultures through the presynaptic activation of P2Y and A1 receptors. ATP has also been shown to increase the size of mEPSCs in the hypothalamus culture by postsynaptically enhancing the insertion of AMPA receptors (Gordon *et al*., 2005). In addition, it has been shown in hippocampal and cortical cultures that activation of the calcium/calmodulin family of kinases increased the strength of excitatory synaptic transmission (Pratt *et al*., 2003) by enhancing the insertion of AMPA receptors at the postsynaptic side (Hayashi *et al*., 2000). Activity-induced changes in mEPSC size and frequency may have been due to changes in the clustering of postsynaptic glutamate receptors and to changes in presynaptic transmission. Indeed, previous studies on cortical and spinal neurons cocultured with glia have found that the size of glutamatergic currents was enhanced or decreased by neuronal activity blockade or enhancement, respectively (Turrigiano *et al*., 1998; O'Brien *et al*., 1998) and that these changes were accompanied by synaptic and nonsynaptic expression of surface AMPA receptor (Lissin *et al*., 1998; O'Brien *et al.,* 1998; Wierenga *et al.,* 2005). In addition, Murthy *et al.* (2001) showed in hippocampal cultures that increased mEPSC frequency after chronic activity blockade arose from an enhancement of the release probability.

Our experiments also revealed that chronic activity blockade raised the frequency and size of mIPSCs only when glial cells were present. This is in opposition with previous reports which showed in cortical and hippocampal cultures that chronic suppression of activity decreased inhibitory synaptic strength by decreasing the number of synaptic GABAA receptors clusters, the number of functional inhibitory synapses and the amount of neurotransmitter in synaptic vesicles (Kilman *et al.*, 2002; Hartman *et al.*, 2006). The

different results may be due to different culture conditions. We note that we observed such an effect in the absence of glia, where enhancing synaptic activity decreased the size of mIPSCs. This suggests that glial signals regulate activity-dependent changes in inhibition activities. Glial cells may sense network activity alteration and in turn regulate synaptic strength by modulation of GABAA receptor insertion, of presynaptic transmission and by transform inhibitory silent synapses to functional ones. Glutamine released by astrocytes has been shown to increase the frequency of mIPSCs in hippocampal slices by activating kainate receptors on GABAergic terminals (Liu *et al*., 2004) and may therefore be implicated in activity-dependant changes on inhibitory synapses.

Notably, we observed great variability in synaptic activity after blockade or increase of activities in individual cultures. This may be due to variability in the culture density. Indeed, it has been shown that the size of mEPSCs in hippocampal neurons decreases as the number of synaptic inputs increase (Liu and Tsien, 1995). A second parameter is the duration of chronic treatment. Thus it is possible that a longer treatment induces more robust changes.

Taken together, our results indicate a role of glial cells in activity-dependent synaptic changes. However the conditions for a reliable detection of such changes and the molecular mechanisms remain to be defined.

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ABSTRACT

There is increasing evidence that synapse function depends on interactions with glial cells, namely astrocytes. Studies on specific neurons of the central nervous system (CNS) indicated that glial signals also control synapse development, but it remained unclear, whether this is a general principle that applies to other neuronal cell types. To address this question, we developed new methods to immunoisolate neurons from different brain regions of postnatal mice and to culture them in a chemically defined medium. In the absence of glial cells, electrophysiological recordings and immunocytochemical staining, revealed vigorous synaptogenesis in hippocampal and cerebellar neurons, but not in retinal ganglion cells (RGCs). Coculture with glia promoted synapse development in RGCs as indicated by a strong increase in the incidence and frequency of action potential-independent miniature synaptic currents, but showed no such effects in hippocampal or cerebellar neurons. On the other hand, glial signals promoted the maturation or function of excitatory synapses as indicated by an increase in the size of spontaneous synaptic events in all neuronal cell type and of miniature synaptic currents in hippocampal neurons and RGCs. Inhibitory synaptic currents remained largely unaffected by glia. In a first series of experiments on hippocampal cultures, we studied whether glia is required for activity-dependent changes in synaptic efficacy. We observed that chronic blockade of electrical activity in hippocampal neurons raised the frequency and size of synaptic currents only if glia cells were present. Our results indicate that in the mammalian CNS glial signals influence on the development and plasticity of excitatory synapses.