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par

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Relevance of glial cells in cellular mechanisms underlying the Niemann-Pick type C disease

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n'auraient sûrement pas
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Abbreviations

AGJs	axo-glial junctions
AMPA	amino-3-hydroxy-5-methylisoxazol-4-propionic acid
AP	action potential
ApoE	apolipoprotein E
APP	amyeloid precursor protein
BDNF	brain derived neurotrophic factor
BrdU	bromodeoxy-uridine
BSA	bovine serum albumin
cGMP	cyclic guanosine monophosphate
CNPase	2',3'-cyclic nucleotide 3'-phosphodiesterase
CNS	central nervous system
CNTF	ciliary neurotrophic factor
DIV	day in vitro
DMEM	Dulbecco's modified eagle's medium
DNA	desoxyribonucleic acid
D-PBS	Dulbecco's phosphate buffered saline
EBBS	Earle's balanced salt solution
EDTA	ethylene-diamine-tetraacetic acid
EGL	external granular layer
EPSC	excitatory postsynaptic current
FCS	fetal calf serum
FSM	fully supplement medium
GABA	γ-aminobutyric acid
GAD	glutamate decarboxylase
GalNAcT	N-acetylgalactosaminyltransferase
GAT-3	GABA transporter-3
GC	granule cells
GCM	Glia-conditioned medium
GFAP	glial fibrillary acid protein
GFP	green fluorescent protein
GLAST	Glutamate astrocyte-specific transporter

GLT-1	glial glutamate-transporter
GluR	glutamate receptor
HMG-R	3-hydroxy-3-methylglutaryl coenzyme A reductase
IGF-I	insulin growth factor I
IgG	Immunoglobulin G
IP3R1	inositol 3-phosphate receptor
IPSC	inhibitory postsynaptic current
LDL	Low-density lipoprotein
MAP	microtubule-associated protein
MSM	minimally supplemented medium
MW	molecular weight
NMDA	N-methyl-D-aspartate
NT-3	Neurotrophin-3
NPC	Niemann-Pick type c disease
P	Postnatal day
PC	Purkinje cell
PCR	polymerase chain reaction
PDL	poly-D-lysine
PNS	peripheral nervous system
Ptc	Patched
mRNA	messenger ribonucleic acid
RGC	retinal ganglion cell
SCAP	SREBP cleavage activating protein
Shh	Sonic hedgehog
SNARE	soluble NSF attachment protein receptor
SRE	sterol regulatory element
SREBP	sterol regulatory element binding protein
SSD	sterol-sensing demain
TAE	Tris-acetate-EDTA
TNFα	tumor necrosis factor α
TTX	tetrodotoxin
WT	wild-type

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Résumé en français

Le but de cette thèse était de mettre en place un modèle pratique afin d'analyser le rôle des cellules gliales dans la neurodégénérescence des cellules de Purkinje, chez une souris modèle de la maladie de Niemann-Pick de type C.

La maladie de Niemann-Pick de type C (NPC) est une maladie génétique qui appartient au groupe des pathologies lysosomiales (Patterson MC, 2003). La prévalence estimée est autour de 1:150.000 individus. Elle est caractérisée par une hépatosplénomégalie progressive et par une neurodégénérescence du système nerveux central. Deux gènes ont été découverts: *npc1* et *npc2* et dans lesquels certaines mutations ponctuelles ont pour conséquence la traduction des protéines NPC1 et NPC2 sous forme non fonctionnelle. Les mutations dans *npc1* concernent 95% des cas. Ces deux protéines joueraient un rôle dans la régulation de l'homéostasie du cholestérol cellulaire. Le dysfonctionnement des protéines NPC a pour conséquence une accumulation intracellulaire anormale de cholestérol et de glycosphingolipides dans un certain nombre de tissus. Les individus affectés meurent principalement au cours des deux premières décades. Parmi les modèles biologiques de NPC, la découverte d'une lignée murine naturelle dont le génotype et le phénotype sont similaires aux patients humains (Voikar *et al.*, 2002) a permis un avancement considérable dans la compréhension des mécanismes pathologiques qui sous-tendent cette maladie.

Les récentes découvertes soulignent l'importance première de la pathologie neuronale dans cette maladie, et indiquent que la neurodégénérescence est un événement précoce plutôt qu'un effet secondaire consécutif du défaut viscéral (Paul *et al*., 2004). Au niveau du cervelet, les cellules de Purkinje dégénèrent massivement et présentent une morphologie anormale au niveau de l'arbre dendritique et de l'axone (Sarna *et al*., 2003). Jusqu'à présent, la raison pour laquelle ces neurones sont les seuls à mourir dans le cervelet reste un des points obscurs de la maladie de NPC. Autour des neurones, les cellules gliales (astrocytes, oligodendrocytes et microglie) semblent être aussi affectées par NPC. Entre autres, des études ont montré l'accumulation de cholestérol dans les neurones mais également dans les astrocytes qui expriment aussi la protéine NPC1. La glie, longtemps vue comme un simple support neuronal, est maintenant considérée comme un partenaire actif dans le fonctionnement des circuits neuronaux. L'association fine entre neurones et astrocytes jouerait un rôle important dans la régulation et l'organisation des synapses. Ainsi, le cholestérol sécrété par les astrocytes agirait

comme facteur synaptogénique (Mauch DH et coll. 2001). Dans le cervelet, la cytodifférentiation de la glie de Bergmann est corrélée avec la genèse des synapses et des dendrites ainsi qu'avec la maturation des cellules de Purkinje.

L'ensemble de ces données nous a conduit à nous poser la question suivante: La dégénérescence neuronale est-elle intrinsèque à la cellule déficiente en NPC1 ou est-elle induite par le dysfonctionnement des interactions entre neurones et glie au cours des premiers stades de la maladie?

Afin de répondre à cette question, mon travail de thèse a consisté à établir un modèle de neurones cérébelleux séparés de la glie à partir d'animaux NPC et "wild-type" (WT) à un stade postnatal. Nous avons utilisé une méthode dite d'immunoisolation permettant la séparation des cellules grâce à leurs marqueurs de surface spécifiques. Cette technique nous a permis d'isoler les neurones du cervelet de façon ultra pure (>99% de neurones) où les cellules granulaires (GCs) étaient dominantes. L'addition d'une étape supplémentaire de sélection nous a permis d'enrichir hautement (100 fois) nos cultures en cellules de Purkinje (PCs). Nous avons établi la composition du milieu de culture nécessaire à leur survie pendant 7 jours. Le protocole d'isolation de neurones a été appliqué avec succès à partir de souris NPC à l'exception du rendement de purification qui s'est révélé plus faible. Il serait donc possible que les GCs du cervelet soient aussi sujettes à dégénérescence. En revanche, leur taux de survie était similaire, indiquant que la mort cellulaire liée à NPC ne se manifeste pas dans nos cultures. C'est probablement dû au jeune âge des animaux, alors que la dégénérescence n'a été reportée qu'à 40 jours postnataux (Tanaka *et al*., 1988). Enfin, la dernière étape concernant l'établissement du modèle a consisté à vérifier le contenu en cholestérol de chaque type cellulaire grâce à la filipin, un composé liant les stérols de façon spécifique. Comme décrit chez les patients et souris NPC, nous avons montré une forte accumulation de cholestérol dans une zone spécifique (probablement, le système endosomal/lysosomal) du corps cellulaire des neurones et des cellules gliales dans nos cultures NPC. En revanche, la distribution du cholestérol s'est avérée très homogène dans les cellules WT et leurs extensions. Le traitement par les facteurs solubles provenant de la glie WT a amplifié cette distribution du cholestérol dans les neurones WT et NPC; également, les facteurs sécrétés par la glie NPC ont eu un effet similaire. Ceci indique que les glies WT et NPC libèrent de façon comparable du cholestérol qui est internalisé par les neurones via des

lipoprotéines (Mauch *et al*., 2001). Cet effet est probablement dû à l'homologie en densité et en taille des lipoprotéines sécrétées par les glies WT et NPC (Karten *et al*., 2005).

La seconde partie de notre analyse a consisté à utiliser nos modèles de culture après 7 jours *in vitro* pour étudier le développement des synapses entre les neurones purifiés en absence ou en présence des cellules gliales. Nous nous sommes également demandés si la présence de NPC1 dans les neurones ou dans la glie était impliquée dans cette synaptogenèse. Nous avons donc enregistré l'activité synaptique des cellules granulaires et des cellules de Purkinje. En absence de glie, les GCs purifiées ont montré un niveau d'activité au moins similaire à celui observé dans des cultures mixtes (Virginio *et al.*, 1995; Losi *et al.*, 2002; Fiszman *et al.*, 2005) de cervelet de rats postnataux. De plus, NPC1 est apparue facultative dans cet aspect. En revanche, le niveau d'activité des PCs était très bas, indiquant qu'elles ne pouvaient pas établir des connexions synaptiques en absence de glie. De plus, les PCs présentaient des différences selon la présence de NPC1 sous forme fonctionnelle. Nous avons voulu savoir si les différences d'activité entre les GCs et les PCs étaient dues à un changement au niveau du développement dendritique, mais ce ne fut pas le cas.

Notre étude a révélé que les signaux gliaux induisaient des conséquences différentes dans les PCs et dans les GCs. Nous avons donc analysé séparément les deux populations de neurones.

En présence de glie, l'activité synaptique excitatrice a été fortement augmentée dans les PCs. Cet effet s'est avéré indépendant de la présence de NPC1 dans les neurones ou dans la glie. Nous avons montré qu'il était dû à un renforcement au niveau des synapses individuelles via une augmentation de la réponse à la libération quantique. Ce résultat n'était pas lié à une modification au niveau de l'excitabilité, mais corrélé avec une augmentation du développement des dendrites ainsi que de la densité des synapses somato-dendritiques. En revanche, la glie a décuplé le nombre de PCs (et de GCs) WT montrant une activité inhibitrice mais pas dans les neurones NPC. Ainsi, la présence de NPC1 dans les PCs semble être superflue pour la réponse glutamatergique alors qu'elle paraîtrait indispensable dans la réponse GABAergique aux astrocytes. Cet aspect est intéressant si on considère que la population de GCs, qui sont les seuls neurones libérant du glutamate, ne subit pas de perte au cours de NPC alors que les PCs, partiellement responsables de la transmission GABAergique, sont dramatiquement affectés au cours de la maladie.

La modulation par la glie de l'activité synaptique excitatrice des GCs était différente du cas des PCs, alors que les effets sur l'activité inhibitrice se sont avérés similaires. La

formation des synapses excitatrices n'a pas été modifiée par la glie mais la taille des courants excitateurs a était augmentée, comme observé dans les PCs. De plus, nous avons montré que ce résultat provenait de changements d'activité synaptique potentiel d'action-dépendante. De façon intéressante, notre étude a révélé un rôle critique de la protéine NPC1. Contrairement à la glie WT, la glie NPC a montré globalement des effets négatifs ou un manque d'effets positifs (ceux de la glie WT) sur les activités synaptiques excitatrice et inhibitrice des GCs. Ceci indique que la formation ou le fonctionnement des synapses dans les GCs, ainsi que leur sensibilité aux signaux gliaux sont fortement liés à la fonctionnalité de NPC1 dans les neurones et dans la glie.

L'ensemble de cette étude permet tout d'abord d'introduire un nouveau modèle fonctionnel d'étude in vitro de pathologies cérébelleuses dans lequel le rôle de la glie peut être finement analysé. Ce modèle nous a ainsi permis de mettre en évidence que les interactions entre neurones et cellules gliales étaient cruciales pour la mise en place des synapses. Ensuite, nous avons mis en évidence que l'effet positif des glies sur les synapses cérébelleuses impliquait des mécanismes totalement différents de l'effet négatif lié à la déficience de NPC1 dans la glie sur les neurones. Enfin, nos résultats nous permettent de proposer une hypothèse qui pourrait expliquer le rôle de la glie NPC dans les altérations que subissent les cellules de Purkinje au cours de la maladie de NPC: la glie NPC pourrait perturber les PCs NPC de façon indirecte, via les GCs NPC. En effet, les GCs connectent spécifiquement les PCs par leur axone, les fibres parallèles. Une PC est connectée par un nombre considérable de GCs. Par exemple, l'arbre dendritique d'une PC de rat adulte reçoit 175,000 synapses provenant de fibres parallèles (Napper *et al.*, 1988). On peut alors imaginer l'effet dévastateur d'un si grand nombre de GCs sur la faible proportion de PCs, dans le cas où les GCs seraient porteurs de signaux néfastes.

Il pourrait alors être intéressant de voir s'il existe une corrélation entre les défauts de synaptogenèse et des changements dans les protéines spécifiques des synapses glutamatergique et GABAergique. De plus, nous pourrions mettre en culture des GCs NPC avec des PCs WT afin de voir, par exemple, si la glie NPC peut affecter les PCs WT via les GCs NPC.

Dans tous les cas, nos résultats indiquent qu'un déficit en NPC1 dans les cellules autres que les PCs, que sont les GCs et la glie de Bergmann, pourrait contribuer à leur perte dans NPC. Cette hypothèse nécessiterait d'être testée par d'autres expériences incluant probablement une approche *in vivo* à l'aide d'animaux transgéniques. Dans le cas où elle s'avérerait correcte, cela

servirait à définir des cibles cellulaires potentielles pour une approche thérapeutique visant à combattre cette maladie dévastatrice.

Introduction

A fundamental question is whether in neurodegenerative diseases, neurons die in a cell-autonomous manner or due to a breakdown of interactions with non-neuronal cells, namely glial cells. I have addressed this question in Niemann-Pick type c disease, a rare but particularly devastating human genetic disorder.

1. Niemann-Pick type C disease

1.1 History and clinical course

** History*

The first synopsis concerning Niemann-Pick disease type C (NPC; OMIM number 257220) was published in 1958 by Crocker and Farber (Crocker and Farber, 1958). The authors summarized the clinical phenotype, as described in 1914, of an infant with massive hepatosplenomegaly, *i.e.* an increase in the volume of both liver and spleen, who died at 18 months after rapid neurologic deterioration (Niemann, 1914). Niemann had recognized that this disorder differs from Gaucher disease, but it was Pick who delineated the distinctive pathology (Pick, 1927). In 1961, Crocker classified Niemann-Pick (NP) disease into four groups (Crocker, 1961). Group A denotes the aggressive infantile form firstly described by Niemann, group B includes patients with only visceral manifestations and groups C and D comprise later onset neurologic diseases. Five years later, Brady's description of acid sphingomyelinase (SM) as the defective enzyme in NP type A helped to regroup the different NP diseases (Brady *et al.*., 1966). His observations led to a classification of NPs in two groups: group I (Crocker A and B) caused by primary SM deficiency and group II (C and D) as secondary to SM deficiency.

** Clinical phenotype*

The majority of NPC patients exhibits in early childhood hepatosplenomegaly, ataxia, dystonia, seizures, progressive dementia and reduced cognitive abilities associated with brain atrophy (Pentchev *et al.*, 1995). Children diagnosed with NPC present increasing physical

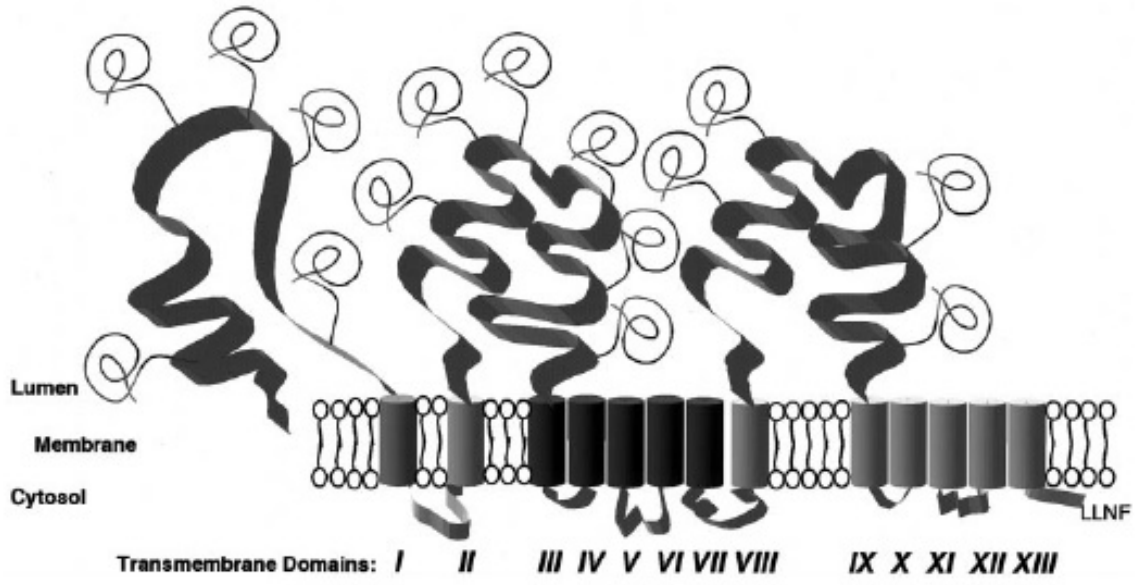

Fig.1: Membrane topology of NPC1
NPC1 contains 13 transmembrane domains (barrels), 3 large hydrophilic loops, facing the lumen of endosomes and lysosomes, and a number of small cytoplasmic loops including a cytoplasmic tail containing the LLNF endosome localization motif. From Ioannou, 2000.

disabilities and live typically into their teen years. Disease onset and progression is variable, including early infantile onset with hypotonia and delayed developmental stages, rapidly fatal neonatal hepatic failure, and adult onset with dementia and psychosis (Schofer *et al.*, 1998). In general, life expectancy of NPC patients is variable, as they succumb to different pathological changes from hepatic dysfunction or pulmonary infiltration to progressive neurodegeneration and associated defects.

1.2 Molecular basis of NPC disease

Although the cellular mechanisms underlying NPC are still not known, the last ten years provided new insights and new tools for research. It is now well established that NPC disease is an inherited autosomal recessive disorder with an estimated frequency of 1/150.000. Genetics studies have identified two NPC complementation groups. The major disease locus *npc1* maps to 18q11 and is responsible for 95% of NPC disease (Millat *et al.*, 1999). In the remaining 5%, the minor disease locus *npc2* maps to 14q24. Deficiency of either NPC1 or NPC2 results in a similar lysosomal accumulation of lipids (Sleat *et al.*, 2004), which is why NPC is classified as a lysosomal storage disease.

** Biological models of NPC*

Two murine models share most of the clinical symptoms observed in human patients: C57BLKS/J spm and BALB/c npc^{nih} (Voikar *et al.*, 2002). Each mouse line has spontaneous mutations independently localized on chromosome 18 in a region syntenic to the human NPC1 locus (Loftus *et al.*, 1997). In addition, a natural feline model is phenotypically and biochemically similar to human NPC1 (Somers *et al.*, 2003). Moreover, the discovery that genetically modifiable organisms have one or two homologs of the human NPC1 gene allowed recently to create new NPC models by gene disruption. These models comprise the nematode *Caenorhabditis elegans* (Sym *et al.*, 2000), Drosophila (Huang *et al.*, 2005) and the yeast *Saccharomyces cerevisae* (Berger *et al.*, 2005).

** NPC1 gene*

The *npc1* gene was identified by positional cloning (Carstea *et al.*, 1997). The gene product is predicted to be a large 1278-amino acid glycoprotein with 13-16 transmembrane domains (**Fig.1**). Transmembrane domains 3-7 show homology to the morphogen receptor Patched. This membrane-bound receptor interacts with Sonic Hedgehog (Stone *et al.*, 1996), a developmental signaling molecule, which in its active state contains a covalently attached

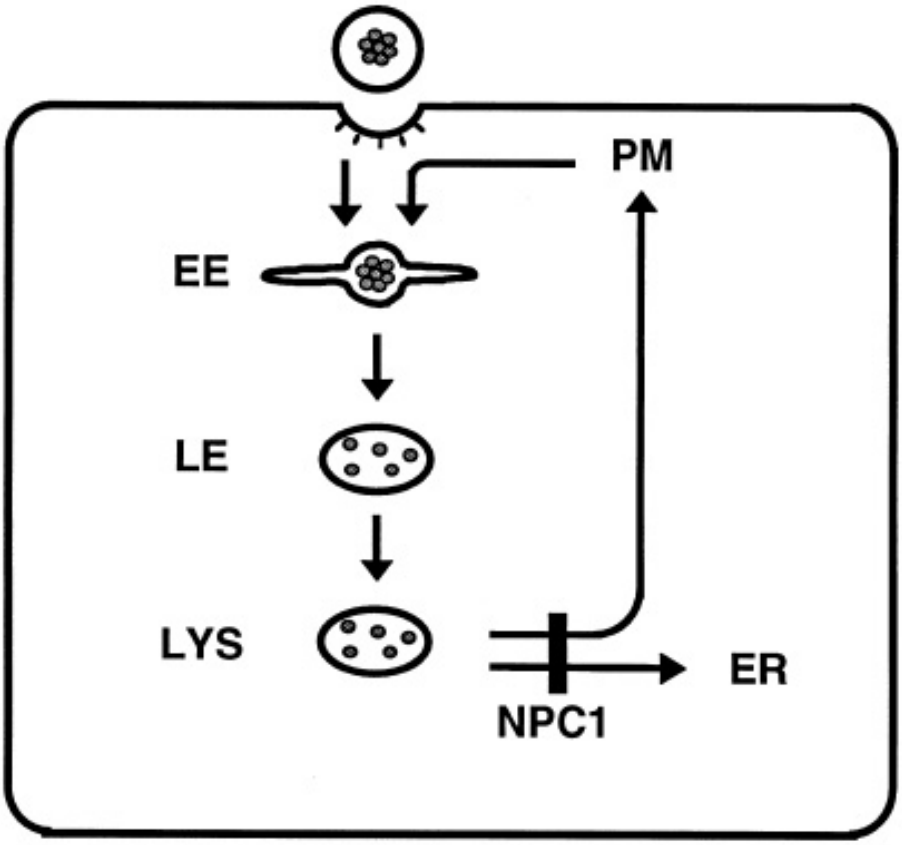

Fig.2: Model for NPC1 function in cellular cholesterol trafficking
Following uptake, LDL-cholesterol enters the endocytic pathway (EE) and is hydrolyzed to free cholesterol in late endosomal (LE) and lysosomal (LYS) compartments. The bulk of free cholesterol is rapidly mobilized to the plasma membrane (PM). PM-cholesterol, which includes both LDL-derived and *de novo* synthesized cholesterol, is endocytosed and delivered to the endoplasmic reticulum (ER) for esterification. It is hypothesized that mutations in NPC1 disrupt trafficking of lysosomal cholesterol to the PM and ER. From Ory, 2000.

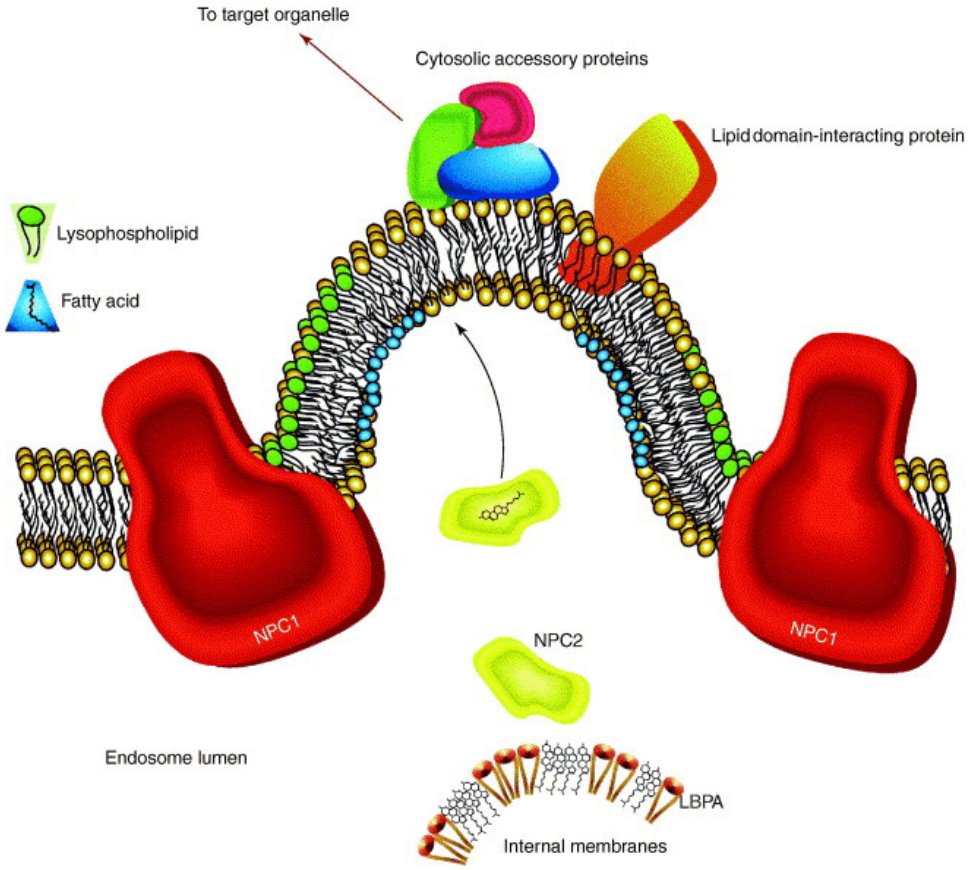

Fig.3: A proposed model for NPC1 function
By flipping specific lipids in the endosomal membrane, NPC1 could create lipid asymmetry that favors increased membrane curvature. Here, NPC2 is shown to extract cholesterol from internal vesicle membranes rich in lyso-bisphosphatidic acid (LBPA). From Ioannou, 2005.

cholesterol moiety (Porter *et al.*, 1996). The sequence similarity with Patched concerns the sterol sensing domains (SSD) that is located in the membrane-spanning region and that is also common to HMG-R and SCAP. Near the NH_2 terminus of NPC1, a leucine zipper motif and surrounding sequences form the "NPC1 domain", which is conserved among all NPC1 orthologs (Carstea *et al.*, 1997). Human patients' mutations in *npc1* are distributed throughout the gene with two hot spots: the cysteine-rich region and the putative SSD (Park *et al.*, 2003). However, there is no correlation between phenotype and specific mutations or location of mutations, and no correlation between severity at the cellular level (*i.e.*, cholesterol accumulation) and at the clinical level (*i.e.*, age at onset and degree of neurological impairment) (Vanier and Suzuki, 1998).

Up to now, the biological function of NPC1 is still unknown. Its structural homology with the resistance-nodulation-division family of prokaryotic permeases and its ability to transport acriflavine from the *E.coli* periplasmic space into the cytosol suggests that NPC1 acts as a permease for lipophilic molecules (Davies *et al.*, 2000). However, several models for NPC1 function were proposed. Ory suggests that a non-functional NPC1 impairs the traffic of unesterified cholesterol from the endosomal/lysosomal system to the endoplasmic reticulum and to the plasma membrane (see below) (Ory, 2000; **Fig.2**). Recently, Ioannou proposed a new hypothesis explaining the pleiotropic effects caused by a defective NPC1 protein (Ioannou, 2005): NPC1 could function as a lipid (free fatty acids and lysophospholipids) flippase that induces membrane curvature and fission for generation of transport vesicles targeted from the late endosome to the *trans*-golgi network (**Fig.3**).

Several studies have examined which organs and cells express NPC proteins. *Npc1* mRNA is present in different tissues in the mouse, as shown by northern-blot (Garver *et al.*, 2005). Its levels vary from very low in kidney to high in liver and lung. In the brain, both wild-type (WT) and npc^{nih} (NPC) mice exhibit the presence of the transcript as shown by *in situ* hybridization (Prasad *et al.*, 2000). There are regional differences in the transcription levels, with the highest being observed in the cerebellum and in the pons. At the cellular level, the NPC1 transcript is detected both in neurons and glial cells in the lateral vestibular nucleus. In the cerebellum, Purkinje cells (PCs) and sub-populations of glial cells also express the Npc1 transcript during early development as well as in the adult (Falk *et al.*, 1999).

The NPC1 protein is predominantly located within the late endosomal membrane but is also transiently associated with lysosomes and the trans-Golgi network (Zhang *et al.*, 2001) as shown by immunocytochemistry. The only immunohistochemical study so far was performed on monkey brain (Hu *et al.*, 2000). The authors report dense NPC1

immunoreactivity in hippocampus, inferior olive and caude-putamen, whilst light staining is observed in the cerebellum and pons as well as in thalamus and hypothalamus. In addition, co-localization of NPC1 and GFAP identified only glial cells as NPC1 positive. Moreover, the NPC1 protein appears to be expressed in perisynaptic astrocytic glial processes of monkey temporal cortex (Patel *et al.*, 1999). Another study reports NPC1 immunoreactivity in recycling endosomes of sympathetic neurons nerve terminals (Karten *et al.*, 2006). It should be noted that, for unknown reasons, it appears difficult to produce good antibodies against NPC1 for immunocyto- and immunohistochemistry. Thus, the distribution of NPC1 at the regional, cellular and subcellular level remains unclear.

A homolog of NPC1, named NPC1L1, has recently been discovered (Davies *et al.*, 2000). NPC1L1 deficient mice exhibit impaired cholesterol absorption by the intestine where NPC1L1 mRNA is mainly expressed (Altmann *et al.*, 2004).

** NPC2*

NPC2 has been identified as the protein HE1 that, along with homologs from numerous mammalian species, represents a major secretory component of epididymal fluid (Fouchecourt *et al.*, 2000). Subcellular fractionation of rat liver demonstrates that HE1 is a soluble lysosomal protein (Naureckiene *et al.*, 2000) and mutations in *he1* are specifically associated with NPC2 phenotype. Translation of *he1/npc2* leads to a mature protein of 132 amino acids, with a predicted molecular mass of 14.5 kD. The crystal structure of bovine apoNPC2 reveals a loosely packed region in the protein interior that appears to be an incipient cholesterol-binding site (Friedland *et al.*, 2003). This hydrophobic binding pocket critically depends on the presence of single amino acids in which any replacement prevents both cholesterol binding and the restoration of normal cholesterol levels in mutant cells (Ko *et al.*, 2003). NPC2 is expressed in human and murine liver (Klein *et al.*, 2006). In the mouse brain, this protein is expressed predominantly in neurons but not in astrocytes, as shown by immunochemistry. In the adult cerebellum, NPC2 has been found specifically in Purkinje cell bodies and dendrites and in the cytosol of dendrites and in post-synaptic densities in neurons from temporal neocortex (Ong *et al.*, 2004).

Sleat *et al.* (2004) generated a double mutant mouse, whose phenotype is similar to that of each single mutant mouse, in terms of disease onset and progression, pathology, neuronal storage and biochemistry of lipid accumulation. This suggests that NPC2 cannot complement the lack of function of NPC1, and *vice-versa*.

1.3 Cellular phenotype in NPC

Lysosomal storage disorders which count more than 40 and which comprise NPC, are characterized by an intra-lysosomal accumulation of undegraded metabolites (Futerman and van Meer, 2004).

** Intracellular accumulation of lipids*

In human NPC patients, liver and spleen are accumulating diverse lipids including unesterified cholesterol, sphingomyelin, bis(monoacylglycero)phosphate, neutral and acidic glycosphingolipids, and phospholipids. At the cellular level, accumulation occurs in the late endosome/lysosome. The endosomal/lysosomal accumulation of cholesterol has mainly been revealed by staining with filipin, a fluorescent antibiotic that binds specifically to cholesterol (Pentchev *et al.*, 1995). In human NPC brains, neurons of the cerebral cortex, cerebellum and hippocampus also show endosomal/lysosomal accumulation of cholesterol and gangliosides as shown by immunocytochemistry and filipin histochemistry (Zervas *et al.*, 2001). Cells from NPC mice show a similar phenotype in every tissue studied (Xie *et al.*, 1999).

Recently, a new reagent has been developed that allows to visualize the intracellular distribution of cholesterol at higher sensitivity than filipin (Reid *et al*., 2004). The peptide, named BCϴ, is derived from theta-toxin, a bacterial toxin produced by *C perfringens,* by proteolytic cleavage and biotinylation. Staining of cerebellar sections from NPC mice with BCϴ revealed punctate cholesterol accumulation in PCs as early as postnatal day 9 (P9). Furthermore, the authors observed cholesterol aggregates in dendritic arbors and in cell bodies and processes of astrocytes.

The intracellular accumulation of cholesterol suggests that the principal cellular defect in NPC is an impaired intracellular traffic of cholesterol (and other lipids). This appears to apply to cholesterol that is obtained by intracellular synthesis and by uptake from external source via low density lipoproteins (LDL) (Ory, 2000). Thus, in NPC cells cholesterol cannot be delivered effectively to the plasma membrane or to the endoplasmic reticulum for esterification (**Fig.2**; Ory, 2000). Notably, Karten *et al.*. (2002) report that in NPC1-deficient sympathetic neurons, endogenously produced cholesterol, but not the externally aquired material, cannot be effectively delivered from the cell body to distal axons. The defect in intracellular cholesterol traffic also causes a delayed sterol homeostatic responses in NPC deficient cells. Thus, cultured mutant fibroblasts exhibit impaired LDL-mediated downregulation of LDL receptor activity and cellular cholesterol synthesis (Liscum *et al.*, 1989). Despite the intracellular accumulation of cholesterol in NPC mice, the total level of

cholesterol in the brain is similar as in wildtype mice (Liu *et al.*, 2000). This could be due to a reduced cholesterol synthesis (Quan *et al.*, 2003) but it remains controversial (Frolov *et al.*, 2003). It should be noted that so far, it remains unclear whether NPC deficiency directly affects cholesterol trafficking or whether its endosomal/lysosomal accumulation is secondary to defects in the intracellular transport of other lipids. This possibility is indicated by a study on mice that are doubly deficient in NPC1 and GalNAcT, the enzyme that synthesizes gangliosides GM2/GD2 (Gondre-Lewis *et al*., 2003). These animals exhibit reduced accumulation of cholesterol and GM2 indicating that aberrant cholesterol storage in NPC is ganglioside-dependent.

** Other cellular impairments*

Similarities between Alzheimer's disease and NPC disease have been pointed out in recent studies. They revealed accumulation of c-terminal fragments of APP in PCs (Jin *et al.*, 2004) and in the whole brain of NPC mice (Burns *et al.*, 2003) and tau hyperphosphorylation linked to elevated MAP kinase activity in the NPC mouse brain (Sawamura *et al.*, 2003). Among other kinases that may be involved in NPC disease, an increase of cdk5 kinase activity has been observed in NPC mouse brain (Bu *et al.*, 2002). This is particularly interesting, given the role of this kinase in anterograde and retrograde transport of protein and organellar elements in axons and in the modulation of membrane dynamics and endocytosis at synapses (Vincent *et al.*, 2003). A further aspect of neuronal metabolism that appears to be compromised in NPC is the synthesis of neurosteroids (Griffin *et al.*, 2004). This may of course be a consequence of impaired intracellular transport of cholesterol to mitochondria, where the first step in steroid synthesis takes place (Costa *et al.*, 1994). But it remains controversial (Yu *et al.*, 2005). In any case, lack of neuroactive steroids in PCs can impair dendritic growth, synaptogenesis (Sakamoto *et al.*, 2001) and GABAergic neurotransmission (Poisbeau *et al.*, 1997). Accordingly, Griffin *et al*. (2004) showed that intraperitoneal injections of progesterone in NPC mice slowed the progress of the disease.

1.4 Neuronal and glial degeneration in NPC

Although the characteristic lipid storage and processing defects of NPC occur in both peripheral and neural cells, the clinical hallmark of NPC is a progressive neurodegeneration (Patel *et al.*, 1991; Pentchev *et al.*, 1995). In the cerebellum, the degeneration targets selectively Purkinje cells, as these neurons almost completely disappear in the course of the disease both in human patients (Harzer *et al.*, 1978) as well as in NPC mice (Tanaka *et al.*,

1988). Early pathological changes in PCs have been observed in cerebellar cultures from E18 NPC mice including swelling of their somata and reduced dendritic arborization (Sarna *et al.*, 2003). In 1 month old NPC mice, PCs show swollen dendrites and axon hillocks with irregular contours, focally enlarged branch points and decreased number of spines (Zervas *et al.*, 2001). Moreover, GABAergic neurons from other brain regions including cerebral cortex and hippocampus exhibit characteristic axonal spheroids and ectopic dendritogenesis.

Using silver staining to visualize neuronal degeneration, Ong *et al.* (2001) observed dense signal in the diencephalon and the cerebellum of adult NPC mouse, but less dense staining in the telencephalon. The corpus callosum and cerebellar white matter show degeneration starting at postnatal day 9 and are severely affected in the adult NPC mouse. Interestingly, Ong and colleagues noticed that the earliest signs of degeneration occur in nerve terminals and axons, rather than the soma itself (Ong *et al.* 2001). For instance, degenerating axons are observed in the cerebellum even before PC bodies succumb whereas cell bodies of hippocampal neurons are never affected despite strong degeneration of their nerve terminals. Based on their observations, Ong *et al.*. suggest that neurodegeneration starts at synapses and proceeds in a retrograde manner.

Despite the tremendous progress within the last years, the cause of neurodegeneration in NPC still remains elusive. TUNEL staining in the cerebrum and cerebellum of human patients and the mouse model (Wu *et al.*, 2005) reveal the presence of apoptotic cells. An up-regulation of genes involved in the TNF-α death pathway suggests that neuronal cell loss is caused by apoptosis. In contrary, no significant delay in the onset of neurological disorders is present in a transgenic mouse double knock-out for *npc1* and the pro-apoptotic gene *bcl2* (Erickson *et al.*, 2002) indicating that cell loss does not proceed by a caspase-mediated apoptotic pathway. Interestingly, elimination of intracellular cholesterol accumulation in the brain of mice double deficient for both *npc1* and GalNAcT (GM2/GD2 gangliosides synthase; Gondre-Lewis *et al.*, 2003) or by cholesterol-lowering therapy in a clinical trial (Sturley *et al.*, 2004) did not reduce severity or delay onset of NPC disease. This suggests that accumulation of cholesterol is not the primary cause of neuronal degeneration.

Several recent studies also indicate pathological changes in glial cells. While neurodegeneration commences in NPC during early development (Ong *et al.*, 2001), postnatal inflammation also occurs, with activation of microglia and astroglia (Baudry *et al.*, 2003). Ultrastructural analysis of NPC mouse reveal an age-related increase of inclusion bodies in microglia and astrocytes in the cerebral cortex, similar to those observed in neurons (German

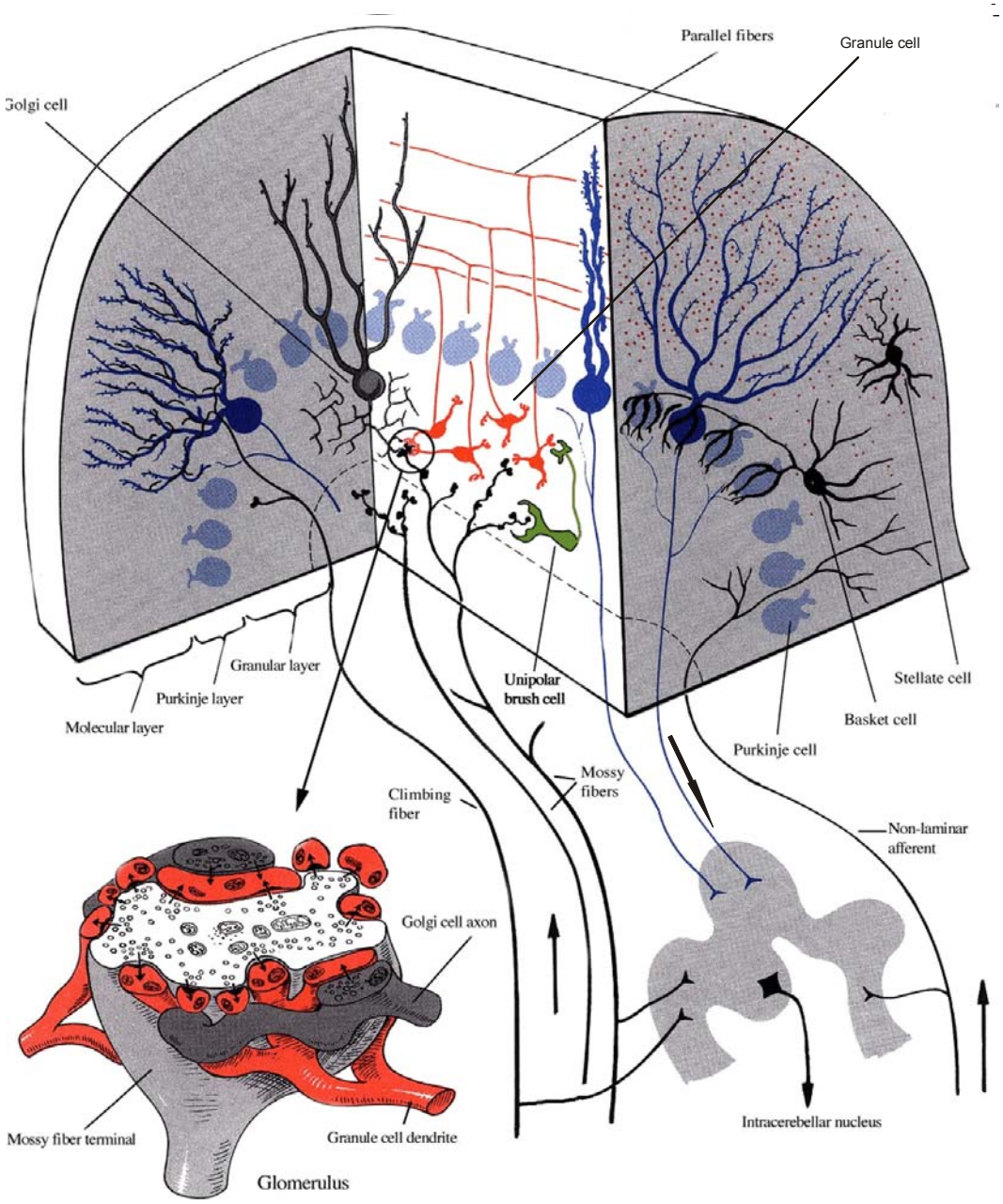

Fig.4: Cells and circuitry of the cerebellar cortex
Modified from Squire *et al.* (2003)

et al., 2002, Suzuki *et al.*, 2003). Glial cells swell consistent with an accumulation of storage material in inclusion bodies. More recently, proteomic analysis shows an alteration of glial transporters in the hippocampus of the mutant mouse (Byun K *et al.*, 2006): expression of glial transporters for GABA (GAT-3) and glutamate increased whereas expression glial excitatory amino acids carrier-1 (EAAC1) decreased in a 8-week-old NPC mouse compared to WT mouse. A marked hypomyelination has also been observed in the CNS and PNS of NPC1 deficient mice (Weintraub *et al.*, 1985; Goodrum *et al.*, 1997; Takikita *et al.*, 2004).

A striking feature of Niemann-Pick type C disease is that it affects only specific cell types, namely in the cerebellum. This is why this brain region constituted the object of our study.

2. The cells and molecules that make a cerebellum

This chapter consists on a brief introduction of cerebellar cells and their interactions during development.

2.1 Overview of mouse cerebellar development

Neuronal populations in the cerebellum (**Fig.4**) arise from at least two different germinal zones (Altman and Bayer, 1997). The initial germinal matrix consists of a neuro-epithelial ventricular zone and a caudal germinal region, also known as the rhombic lip. The first neurons to leave the ventricular zone are the nuclear neurons on embryonic day 10 to 12 (E10-12) that will settle deep in the cerebellar cortex, followed by Purkinje cells (PCs), which are born between E11-13. The formation of a secondary germinal matrix from the rhombic lip –the external granular layer (EGL), which generates granule cells– begins when nuclear and Purkinje neurons have stopped dividing. When EGL cells migrate over the cerebellar surface to a subpial position, Golgi neurons originate from the diminishing ventricular zone. During the first three postnatal weeks, granule cell neuroblasts migrate in an inwardly directed, radial manner through the molecular layer passing developing PCs, to reach the internal granular layer. This period also features the maturation of the two major inputs to the cerebellar cortex: the inferior's olive climbing fibers to PCs and the spinal and reticular mossy fibers to granule cells. The cerebellum undergoes over a 1000-fold increase in volume during this time due to intensive proliferation of granule cell precursors (Goldowitz and Hamre, 1998). The total number of granule cells (GCs), the only glutamatergic neurons within the cerebellum, is

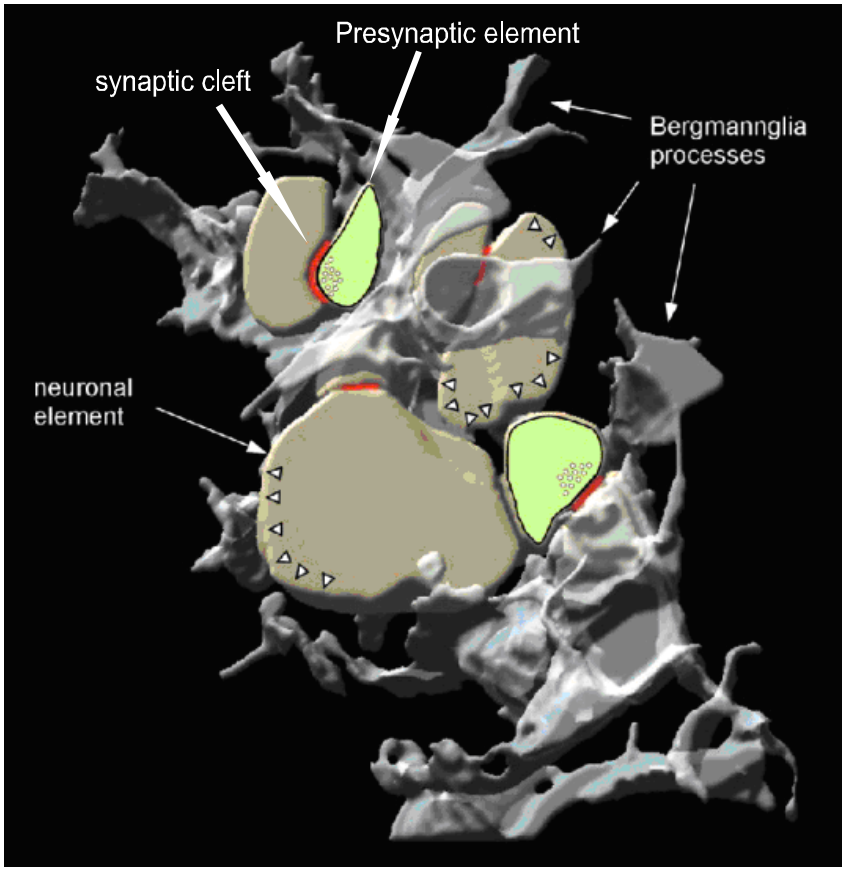

Fig.5: Bergmann glial cell unsheating cerebellar synapses
Reconstruction of a group a neighboring cerebellar synapses with with surrounding Bergmann glial cell. Small arrowheads point to neuronal surfaces not covered by glial sheats from the labeled cell. Modified from Grosche *et al.* (2002).

estimated to about 25 millions in adult mouse (Zanjani *et al.*, 1997). Given that they account for 90% of all cerebellar neurons, there should be a total of 28 millions of neuronal cells in the mature mouse cerebellum. The 10% remaining neurons, which are all GABAergic, include basket and stellate cells but also Golgi and Purkinje neurons. Despite their small number (less than 0.5% of all cerebellar neurons in the mouse), PCs are the principal neurons of the cerebellar cortex because of their central role in the cerebellar circuit: they integrate signals from all other cerebellar neurons and form the only output from the cerebellum to the deep cerebellar nuclei.

Astrocytes in the cerebellar cortex are morphologically divided into three subclasses (Palay and Chang-Palay, 1974). The bushy or velate protoplasmic astrocyte is stellate in shape and commonly seen in the granular layer. The smooth protoplasmic astrocyte is a far less common variety in both molecular and granular layers, and is characterized by long radial processes without lamellar appendages. The third type, named Bergmann glia, is a unipolar radial astrocyte, whose fibers extend through the molecular layer and whose endfeet terminate at the pial surface. Bergmann glia develop during the last embryonic and first postnatal week from radial glia as they acquire their characteristic location, unipolar shape and association with PCs. After P7, Bergmann glia undergo cytoarchitectonic changes, where their radial organization is replaced by a reticular glial meshwork (Yamada and Watanabe, 2002). In the adult brain, the Bergmann glia provide one of the best examples for the intimate relationship between astrocytes and synapses (**Fig.5**).

2.2 Cellular interactions shape cerebellar development

The development of the cerebellum, as any other part of the brain, requires cell-autonomous programs, but depends also critically on interactions between different types of cells. Evidence for some of these interactions will be presented in the following paragraphs.

2.2.1 Neuron-neuron interactions

** Regulation of granule cell number*

The cerebellum is a highly ordered structure, in which the ratio of PCs to granule cells is tightly regulated. Smeyne *et al.* (1995) produced transgenic mice, in which subpopulations of PCs are ablated at various developmental stages. In areas of the EGL that are close to deleted PCs, the mitotic activity of EGL cells is significantly diminished in comparison to regions where PCs are still present. This indicates that PCs control the mitotic activity of

granule cell neuroblasts within the EGL. The Lurcher mouse, which bears a mutation in the delta2 glutamate receptor, exhibits a degeneration of all cerebellar Purkinje cells. Studies on Lurcher chimeric mice are used as a model system since in these animals the number of PCs can be varied genetically and its effect on granule cell number can be examined. It has been shown that 'numeric matching' occurs between the presynaptic granule cell population and its postsynaptic target, the Purkinje cell (Wetts and Herrup, 1983). Recent data suggest that two candidate molecules regulate granule cell proliferation: the ligand morphogen Sonic hedgehog (Shh) and its receptor Patched (Ptc). In the developing mouse cerebellum, PCs express Shh and dividing cells in the EGL express Ptc. Intracranial injection of hybridoma cells secreting anti-Shh antibodies disrupts cerebellar development and reduces proliferation in the EGL as shown by BrdU incorporation (Wallace, 1998). In addition, treatment of dissociated cerebellar cells in culture with recombinant Shh stimulates BrdU incorporation. This suggests that PC-derived Shh normally promotes the proliferation of granule cell precursors in the EGL.

** Survival and differentiation of PCs*

There is also evidence for an inverse relation, namely that development of PCs strongly depends on GCs. Cerebellar granule cells are thought to induce differentiation of PCs. PCs that are purified by a density gradient centrifugation survive and differentiate poorly, whereas coculture with granule neurons enhances their survival and dendritic development (Morrison and Mason, 1998). This effect seems to involve neurotrophins and glutamate signaling (Hirai and Launey, 2000), as NMDA and BDNF both restore impaired PC dendritic differentiation induced by a competitive NMDA receptor antagonist and by a TrkB-specific antibody, respectively. In addition, all of these treatments affect granule cell number, which is correlated with dendritic differentiation of PC, as the survival rate of granule cells decreased significantly in each treatment. Because the BDNF-mediated dendrite differentiation acts via activation of NMDA receptors on granule cells, the authors suggest that granule-PC interaction are essential to PC differentiation (Hirai and Launey, 2000), but this remains controversial (Adcock *et al.*., 2004).

2.2.2 Neuron-glia interactions

How important are glial cells during cerebellar development? Despite being the most abundant cells in the mammalian CNS, they were long believed to be simple support cells for neurons. It is only recently that their role in the formation and function of neural circuits has

been revealed. The following paragraphs aim to illustrate to which extent cerebellar development depends on glia.

** Role of glia as a guide of neuronal migration*

Up until recently, the supposed role of radial glial cells was to guide neuronal migration (Rakic, 1971). After the first ultrastructural evidence (Rakic, 1971), a combination of video time-lapse and ultrastructural observations identified granule cells migrating along astroglial processes and revealed morphological details of cell-cell interactions (Gregory *et al.*, 1988). Later on, this mechanism was refined, as Komuro and Rakic (1998) showed the existence of different migration speeds depending on layer through they go. Recently, inactivation of the *Large* gene (that is required for glycosylation of dystroglycan and whose protein is shown to be expressed in PCs and Bergmann glia throughout cerebellar development) in the Largemyd mouse disrupts the organization of the EGL during postnatal cerebellar development. This impaired location of GCs within the cerebellum is correlated with delayed granule cell migration and a disorganization of Bergmann glial fibers (Qu and Smith, 2005). The authors proposed that the latter effects are causally related.

** Glial cells as precursors of neurons*

The neuronal guidance hypothesis has recently been put into question, at least enhanced. Precisely, cell lineage analysis shows that radial glial cells of the cerebral cortex act as neuronal precursors (Malatesta *et al.*, 2000; Noctor *et al.*, 2001). Using a transgenic mouse line expressing the green fluorescent protein (GFP) under the human GFAP promoter, Malatesta *et al.* studied the expression pattern of astroglial markers in precursor cells. Interestingly, they found that radial glial cells from cortices of embryonic rats represent a heterogeneous population of precursor cells and give rise to neurons and astroglial cells. These authors further specified that the progeny of radial glia differs profoundly between brain regions (Malatesta *et al.*, 2003). Thus, radial glia may not guide but produce neurons.

** Bergmann glia promote neuronal cell survival/death*

A striking demonstration of the glial relevance for neuronal survival in the cerebellum has been revealed by ablation of Bergmann glial cells *in vivo*. This has been accomplished in transgenic mouse lines expressing cytotoxic genes under the control of the GFAP promoter. Oral administration of the cytotoxicity-inducing drug in adult mice induces degeneration of PC dendrites, granule cell death and impaired motor coordination (Cui *et al.*, 2001). This

indicates that Bergmann glia is the essential organizer of the cerebellar cortex. Glia are thought to promote neuronal survival by secreting trophic factors. In the cerebellum, the nonessential amino acids L-Serine and glycine have been shown to be secreted by cerebellar astroglial cells and to promote survival of PCs (Furuya *et al.*, 2000).

If glial cells have the power to favor neuron survival, one can imagine that the contrary is also possible. In fact, some studies have highlighted their putative role in one type of neuronal death: apoptosis. In the developing mouse cerebellum, a large number of PCs express activated caspase-3 (Marin-Teva *et al.*, 2004) and undergo apoptosis (Zanjani *et al.*, 1996; Fan *et al.*, 2001). Around 64% of caspase-3 activated PCs are found partially engulfed by or in close contact to microglial processes (Marin-Teva *et al.*, 2004), suggesting that microglia could promote neuronal death through engulfment. Macroglial cells are also incriminated in apoptosis: astrocytes produce several cytokines and trophic factors, when they are activated in response to injury. One of them, the pro-apoptotic mediator FasL is induced in reactive astrocytes in Alzheimer's disease (Ferrer *et al.*, 2001). In that manner, macroglia may also contribute to neuronal loss in neurodegenerative diseases. It should be noted, however, that there is still no experimental proof that glial cells are actively involved in neuronal apoptosis *in vivo*.

** Glia as scaffold for neurites*

In the molecular layer, PC dendrites are contacted by numerous axons of granule cells and they are also intimately connected with processes of Bergmann glia (Altman, 1972). Cultured dissociated PCs form dendrites but their dendritic arbor loses its characteristic shape and its polarity (Baptista *et al.*, 1994), suggesting that extrinsic factors are involved in shaping or maintaining PC dendrites *in vivo*. Using organotypic slice cultures from mice expressing green fluorescent protein (GFP) controled by the GFAP promoter, Lordkipanidze and Dunaevsky (2005) examined how the dendritic tree of PCs expands. They report that vertical growth of PC dendrites occurs preferentially in contact with glial radial processes. This supports the idea that Bergmann glial processes provide a substrate for the directional growth of PC dendrites, thus influencing the shape of the dendritic tree.

Axonal differentiation also depends on glial signals. Signal exchange between myelinating glial cells and axons appears necessary to form specialized molecular domains like the node of Ranvier, which are the basis for the saltatory mode of action potential propagation. Axo-glial junctions (AGJs) play a critical role in the organization and maintenance of theses domains in myelinated axons. Disruption of AGJs in transgenic mice

deficient for one major protein of AGJs (Neurexin IV/Caspr1/paranodin) induces cytoskeletal disorganization and degeneration of PC axons (Garcia-Fresco *et al.*, 2006). Among the multiple proteins localized at AGJs, the cell adhesion molecule F3/contactin, which is expressed on the axonal surface, mediates axo-glia contacts through its association with extracellular matrix components produced by glia (Revest *et al.*, 1999). The F3/contactin deficient mouse displays severe ataxia correlated with impaired axonal and dendritic projections in the cerebellum (Falk *et al.*, 2002).

** Role of glia in synapse formation and elimination*

Although direct evidence for the cerebellum is lacking, there is increasing evidence from *in vitro* studies on other brain regions that glial cells play a role in synapse development (Slezak and Pfrieger, 2003). A glia-derived factor that may shape synaptogenesis in the cerebellum is the amino acid serine. Interestingly, glial cells, but not neurons, can convert L-Serine to its racemate D-serine, which is an effective coagonist at the "glycine-binding" site of the N-methyl-D-aspartate (NMDA) glutamate receptors (Mothet *et al.*, 2000). In the neonatal rat, D-serine levels are high and NMDA receptors are transiently expressed in PCs. The transient expression of NMDA receptors appears to be essential for the climbing and parallel fibers to make normal connections with PCs, because blocking NMDA receptors at the postnatal stage (P5-P15) prevents the establishment of normal connections (Rabacchi *et al.*, 1992). Following this critical period of synapse formation, D-serine levels drop substantially and glial cells begin to express D-amino acid oxidase, which degrades D-serine (Miller, 2004). Thus, in the developing brain, the transient expression of D-serine in Bergmann glia could influence synapse formation with PCs through NMDA receptors.

There is also evidence that Bergmann glia are involved in the elimination of synapses. Although Bergmann glia are non excitable, they express both *in vitro* and *in situ* receptors for neurotransmitters, including ionotropic receptors for glutamate and γ-aminobutyric acid (GABA) (Verkhratsky and Steinhauser, 2000). Iino *et al.* (2001) took advantage of the fact that Bergmann glia express Ca^{2+}-permeable glutamate receptors (GluRs) containing GluR1 and 4 but not GluR2 subunits. Expression of GluR2 is sufficient to make these receptors Ca^{2+}-impermeant. By injecting a genetically modified adenovirus in 3-week-old rats, the authors made Bergmann glia GluRs Ca^{2+}-impermeable. The conversion leads to a retraction of glial processes that ensheath synapses on PC dendritic spines, and induces multiple innervation of PC by climbing fibers.

** Role of astrocytes in neuronal degeneration processes*

For some time it had been thought that astrogliosis was a response to neuronal death leading to an astrocyte scar to isolate dead neurons. However, recent observations have raised the possibility that astrocytes may induce neuronal degeneration. Levine *et al.* (1999) studied the link between astrogliosis and neurodegeneration in a well-characterized mouse model of amyotrophic lateral sclerosis. Immunohistochemistry followed by optical sectioning and three-dimensional reconstruction of spinal cord sections reveal that astrocytic processes extend toward, wrap around, and sometimes penetrate vacuoles derived from degenerating neuronal mitochondria in dendrites of motor neurons. This indicates that abnormal neuron-glia interactions occur within degeneration.

A link between astrocytes and neuronal death was also incriminated in Huntington's disease. The selective nature of cell death in this pathology indicates that it is unlikely to be caused by a cell-autonomous process. One of current hypotheses is that the characteristic polyQ expansion may result in aberrant neurotransmission and signaling that induce specific toxic products in cells to which certain neuronal populations are selectively vulnerable (cells from the striatum and prefrontal cortex and also in PCs, that were all degenerating in this disease). Lievens *et al.* (2001) investigated the role of astrocytes in a mouse model of Huntington's disease. *In situ* hybridization shows that the mRNA levels of the glial glutamate transporter GLT-1 are decreased as early as 8 weeks of age in the striatum and cerebral cortex of the mutant mouse compared to the WT. This was confirmed by western-blot analysis on the protein expression levels. This result is correlated with a decrease in [3H]D-aspartate binding as a measure of glutamate uptake in frontal brain regions. This indicates that impaired glutamate uptake by glial cells occur within degeneration.

Together, the studies presented to far provide ample evidence how glial cells, namely Bergmann glia, contribute to the construction of the cerebellum and possibly participate in neurodegenerative processes.

3. Aim of this PhD thesis

NPC is a complex disease in which the heterogeneity of neuronal and visceral symptoms is not yet explained at the cellular level. The brain region most affected in this pathology is the cerebellum and more specifically the Purkinje cells, which degenerate progressively. Despite tremendous progress, it is not clear if these neurons die in a cell-autonomous manner or due to a break-down of interactions with glia. Based on previous studies showing that glia-derived cholesterol promoted synaptogenesis (Mauch *et al.*, 2001), it has been proposed that neurons reduce their cholesterol synthesis at post-natal stages and that they rely on cholesterol delivery by astrocytes via lipoproteins (Pfrieger, 2003). Thus, defective NPC1 protein in neurons could impair either the neuronal handling of glia-derived cholesterol. Alternatively, a defect in glial NPC1 could impair production and secretion of cholesterol. To address this question, we developed a new culture model of neurons immunoisolated from glia, that allowed us to study the development of mutant and wild-type cells in the absence and in the presence of glial factors from wild-type and mutant animals.

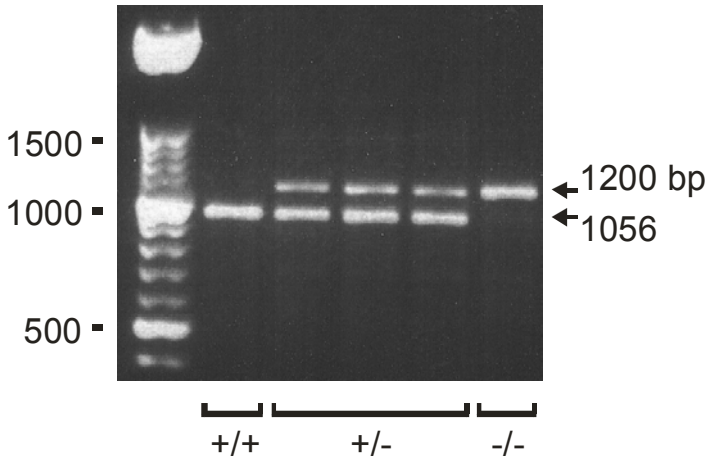

Fig.6: Genotyping of littermates
PCR products for WT mouse pup (+/+; only one band of 1056 base pairs-bp-); Heterozygous pups (+/-; one band of 1056 bp and one band of 1200 bp); NPC mouse pup (-/-; only one band of 1200 bp).

Material and Methods

1. Genotyping of NPC/WT mice

Two breeding pairs of heterozygous mice were purchased from Jackson Laboratories (BALB/cNctr-Npc1N/+, Jax Mice Bar Harbor, MA, USA) and a stable colony was established. For genotyping, a piece of tail of each offspring animal, referred to the remaining-fingers-number, was removed from 3-4 day old pups. Tails were incubated overnight at 55°C in 270 µl of tail buffer (50 mM Tris buffer pH 8, 100 mM EDTA –ethylenediaminetetraacetic– , 100 mM NaCl and 1% SDS –sodium dodecyl sulfate–) and 30 µl of proteinase K (10 mg ml^{-1} in bidistilled water, SIGMA). Proteinase K was destroyed by boiling for 5 min. Tissue extracts were then vortexed and spun down for 2 min at 16000g at room temperature. 1 µl of DNA extract was mixed with 12.5 µl of PCR premix (FailSafe™ PCR System, Epicentre Biotechnologies, Madison, USA), 8.75 µl of bidistilled water and 1.25 µl of each primer solution (20 µM). The following primers were used to identify mutant and wildtype NPC1 mice (Loftus *et al.*, 1997):

Sense primer (TM=60°C): GGT GCT GGA CAG CCA AGT A

Antisense primer (TM=58°C): GAT GGT CTG TTC TCC CAT G

All compounds were mixed, 0.25 µl of TAQ polymerase (FailSafe™ PCR System, Epicentre) were added and the sample was mixed a last time before the amplification reaction under the following conditions:

94°C 3 min, 35 cycles of {94°C 30 sec + 54°C 45 sec + 72°C 1 min} and 72°C 2 min.

PCR products were separated on a 1% agarose gel in TAE (Tris-acetate-EDTA buffer) at 120V for 2 hours. **Fig.6** shows an example of PCR products obtained from 3 types of mice. WT possess the 1056 base pair (bp) allele, NPC have only the 1200 bp allele and heterozygous animals possess both alleles.

2. Immunoisolation and culture of CNS neurons from postnatal mice

Seven day old (P7) postnatal mice (balb/c; Animal facility, Louis Pasteur University, Strasbourg or from our NPC colony) were killed by decapitation according to institutional guidelines. Cerebella were dissected out, cut in small pieces and incubated (5% CO_2, 37°C)

for 60 min in Earle's buffered salt solution (EBSS) (Gibco/Invitrogen, Cergy-Pontoise, France) containing 200 U ml^{-1} papain (Worthington Biochemical Corporation/Cell Systems, France), 200 U ml^{-1} DNAse (D4527; Sigma, St Quentin Fallavier, France), 1.5 mM $CaCl_2$, 1 mM $MgSO_4$, 0.5 mM EDTA (Sigma). Cerebella were then sequentially triturated in D-PBS (Gibco/Invitrogen) containing 0.15% trypsin inhibitor (Boehringer-Mannheim/Roche Diagnostics, France), 650 U ml^{-1} DNAse and 1:75 rabbit anti-rat macrophage antibody (Sigma) to remove microglial cells. Cells were spun down (800g for 13 min), resuspended in 1% trypsin inhibitor in D-PBS, spun down again and then resuspended D-PBS containing 0.02% bovine serum-albumin (fraction V; Sigma). For immunopanning, one subtraction plate (Ø 150 mm petri-dishes; Falcon; BD Biosciences, France) and two selection plates (one to isolate GCs and one for PCs; 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: goat anti-rat-IgG (1^{st} plate); anti-mouse-IgG (2^{nd}) (Jackson Immunoresearch Laboratories/Beckman Coulter, Marseille, France)]. After washing for three times with PBS, selection plates were covered with 0.2% bovine serum albumin (fraction V; in D-PBS) and incubated for > 4 h at room temperature with 2 µg/ml primary antibody (1^{st} selection plate: rat IgG anti-L1 clone 324, Chemicon-Euromedex, Mundolsheim, France; 2^{nd} selection plate: mouse IgM anti-Thy1.2, 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 the subtraction plate for 30 min. The supernatant was filtered and incubated for 30 min at 37°C with triiodothyronine (20 ng ml^{-1}; Sigma), insulin (2.5 µg ml^{-1}; Sigma), putrescine (8 µg ml^{-1}; Sigma), sodium-selenite (20 ng ml^{-1}; Sigma), bovine serum albumin (BSA, 50 µg ml^{-1}; fraction V, crystalline grade, Sigma), progesterone (31 ng ml^{-1}; Sigma) and holotransferrin (50 µg ml^{-1}; Sigma) and then added for 45 min on the 1^{st} selection plate. Non-adherent cells were thoroughly washed off and bound cells were released mechanically with 0.2% BSA. The cells were spun down and resuspended in medium. This population contained mainly granule cells (GCs) (see Results for explanation). The number of viable cells was determined in a hematocytometer using tryptan blue staining (Sigma). To immunoisolate Purkinje cells (PCs), the cells from the L1 selection were then transferred to a Thy1 selection plate for 45 min and the Thy1.2+ cells were released mechanically.

For electrophysiological recordings and immunocytochemical staining, neurons were plated at 600 cells per mm2 in a small circle (Ø 10 mm) centered on tissue culture plates (Ø 35 mm, Falcon, BD Bioscience, France) coated with 5 µg ml^{-1} poly-D-lysine (MW ~ 40 kDa;

Sigma). For survival assays, cells were plated on tissue culture plates (initial viability 3 hrs after plating and survival at 3 days in vitro: 5000 cells per well on 96-well plate; survival at 7 DIV: 600 cells mm^2 on Ø 35 mm plate, Falcon, BD Bioscience) in indicated culture media. Cells were cultured in Neurobasal medium (Gibco/Invitrogen) supplemented with pyruvate (1 mM; Sigma), glutamine (2 mM; Gibco/Invitrogen), *N*-acetyl-L-cysteine (60 µg ml^{-1}; Sigma), putrescine (16 µg ml^{-1}; Sigma), sodium-selenite (40 ng ml^{-1}; Sigma), bovine serum albumin (BSA, 100 µg ml^{-1}; fraction V, crystalline grade, Sigma), progesterone (62 ng ml^{-1}; Sigma), holotransferrin (100 µg ml^{-1}; Sigma), streptomycin (100 µg ml^{-1}; Sigma), triiodothyronine (40 ng ml^{-1}; Sigma), insulin (5 µg ml^{-1}; Sigma) and penicillin (100 U ml^{-1}; Sigma). This basal medium is referred to as minimally supplemented medium (MSM). For long-term culture, MSM was further supplemented with B27 (1:50, Gibco/Invitrogen), brain-derived neurotrophic factor (BDNF; 25 ng ml^{-1}; Regeneron Pharmaceuticals, USA), ciliary neurotrophic factor (CNTF; 10 ng ml^{-1}; PeproTech/Tebu) or forskolin (10 µM; Sigma). This medium is referred to as fully supplemented medium (FSM). For PCs, FSM was further supplemented with insulin-growth factor I (IGF-I; 25 ng ml^{-1}; PeproTech/Tebu). In some cultures, the influence of neurotrophin-3 (NT-3; 25 ng ml^{-1}; PeproTech/Tebu) on PC survival was tested.

3. Culture of glial cells

Glia-conditioned medium (GCM) was obtained from primary cultures of mouse cerebellar glial cells from WT or NPC mice, according to a standard protocol (McCarthy and de Vellis, 1980). Briefly, papain-digested and triturated cerebella of P7 mice were cultured in PDL-coated tissue culture flasks (25 cm2, TPP, Trasadingen, Switzerland) in a medium that does not support survival of neurons containing DMEM, heat-inactivated fetal calf serum (10%), penicillin (100 U ml-1), streptomycin (100 µg ml-1), glutamine (2 mM) and sodium pyruvate (1 mM, all Invitrogen). After one week, culture flasks were washed with PBS and glial cells were cultured in MSM. Three times a week, half of the GCM was harvested and replaced by fresh MSM. GCM was spun down (4 min at 3000 g) to remove cellular debris and added to 1 day old cerebellar neuronal cultures (1.7 ml to 2 ml culture medium).
For co-culture experiments, glial cells grown in theses 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% fetal calf serum (Gibco/Invitrogen), glial cells were counted, spun down,

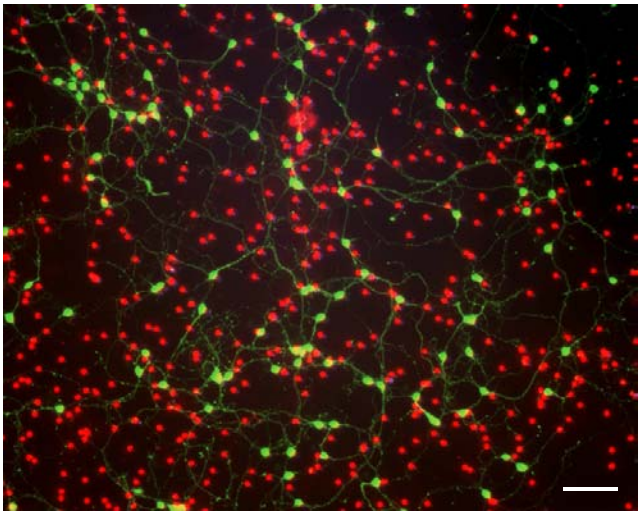

Fig.7: Three components of the Live-dead assay
Micrograph showing live cells (in green), nuclei of all cells (in blue) and dead cells (in red). Cultured cells were incubated in the Live-dead assay solution for 30 min. Scale bar= 80µm.

resuspended in culture medium and added to 1 day old cerebellar purified neurons at a ratio of 2:1.

4. Survival and proliferation assay

For survival assays, cells were plated at 5000 cells/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. It is a two-color fluorescence assay (**Fig.7**) that simultaneously determines:

· *Live cell number* - Live cells have intracellular esterases that convert nonfluorescent, cell-permeable calcein acetoxymethyl to the intensely fluorescent calcein. Cleaved calcein is retained within cells.

· *Dead cell number* - Dead cells have damaged membranes; the ethidium homodimer-1 (EthD-1) enters damaged cells and is fluorescent when bound to nucleic acids. EthD-1 produces a bright red fluorescence 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 live cells divided by the number of total 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 two objectives (10x and 40 x, Zeiss). Images were digitized by an air-cooled monochrome CCD 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).

To detect dividing cells, cells were plated at 1000 mm^{-2} in 24-well culture plates (Falcon, BD Bioscience) and incubated 3 (n = 2) and 21 hrs (n = 2) after plating for six hours in FSM supplemented by 5-bromo-2-deoxyuridine (BrdU; 10µM; Sigma). In this manner, cells in S phase of mitosis incorporate BrdU instead of thymidin in neosynthesized DNA. We incubated cells with BrdU for 6 hours, which were sufficient for each dividing cell to complete an entire mitosis cycle (Alberts *et al.*, 2002). Cells were then fixed with ethanol for 90 min at -20°C, incubated with HCl 2N for 30 min and immunostained (mouse anti-BrdU; clone BU33; 1:1000; Sigma) and viewed on the same setup as described above.

5. Immunocytochemistry

Immunostaining was carried out using standard procedures (Nagler *et al*., 2001). Cells growing on tissue culture plates were washed (PBS), fixed (10 min in Formaldehyde 4%, or 7 min in ethanol at 4°C), permeabilized and blocked (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, 0.04% sodium azide, pH 7.4). For some stainings, cells were simultaneously fixed and permeabilized in methanol 100% for 7 min at -20°C. Cells were then incubated overnight with the following primary antibodies (diluted in antibody buffer):

Purity of neuronal culture: Oligodendrocytes were immunostained against established markers 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase; mouse monoclonal clone C5922; 1/1000; Sigma), and O4 (oligodendrocyte lineage; Schachner, 1982; 1:200; Boehringer-Mannheim/Roche Diagnostics, France). Fibroblasts were stained with a rabbit anti-fibronectin antibody (1/2000; Sigma). Astrocytes were detected by two antibodies, rabbit anti-GFAP (Eng, 1985; 1/8000; Dako Diagnostika, France) and rabbit anti-S100β (Donato, 1999; 1/4000; Swant, Bellinzona, Switzerland). The 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.

Characterization of cerebellar cultured cells: cerebellar PCs were immunostained with specific markers including calbindin 28kD (mouse monoclonal clone CB-955; 1/1000; Sigma) and IP3R1 (Inositol 3 phosphate receptor type 1; rabbit polyclonal; 1/3000; Sigma). Inhibitory cerebellar neurons were identified by staining for the γ-aminobutyric acid (GABA) producing enzyme glutamate decarboxylase (GAD: rabbit anti-GAD65; 1/1000; Chemicon International, Hofheim, Germany). Bergmann glial cells were detected by a guinea pig polyclonal antibody against glutamate/aspartate transporter (GLAST; 1:1000; Chemicon International). Dendrite differentiation and synaptogenesis were monitored by a mouse or rabbit anti-MAP2 (1/500; Sigma) and mouse anti-synaptophysin (1:1000; Sigma).

As secondary antibodies Cy2- or Cy3-conjugated goat anti-mouse- or goat anti-rabbit- or goat anti guinea pig-IgG antibodies were used (1/500; Jackson Immunoresearch Laboratories/Dianova). The non-selective DNA-marker bis-benzimide (20 μg ml^{-1}; Sigma) was applied together with the secondary antibody. Fluorescence was viewed through an appropriate emission filter (Filter Set 2, Zeiss) and a 40x objective (water-immersion, n.a. 0.8, Zeiss). 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 Cy2- or Cy3-labeled cells.

Dendrite differentiation and synapse formation: To allow for a first quantification of dendrite differentiation in cultured neurons, we used an arbitrary classification of differentiation levels (**Fig.34**). When Map2 was expressed only in the cell body, it corresponded to endogenous synthesis, that is why we referred this level to: no dendrite (**Fig.34A**). When Map2 was expressed in neurites whose lengh was maximal to the soma diameter, we referred this level to: "first dendrites" (**Fig.34B**). When Map2 was expressed in neurites whose lengh was longer than the soma diameter, we referred this level to "prolonged dendrites" (**Fig.34C**). Finally, when Map2 expression showed well-developed dendrites with branches, we referred this level to "fully differentiated dendrites" (**Fig.34D**). In a same manner and in order to simplify the quantification, cells were classified in three categories where "0" indicates no colocalization of synaptophysin with MAP2-positive dendrites, "<5" indicates fewer than five colocalized puncta and ">5" indicates more than 5 co-localizing puncta (**Fig.35**). Note that all colored fluorescence micrgraphs are false-color.

6. Filipin staining

To observe the cholesterol distribution in neurons, cultured cells were fixed (4 % paraformaldehyde for 30 min) and incubated for 2 hours with filipin (10 µg ml-1 from a 100x 95% ethanolic stock solution, Sigma), a polyene antibiotic that selectively binds to sterols with a hydroxy group at the 3rd C-atom (Norman *et al.*, 1972). Filipin fluorescence was then excited by monochromatic light (356 nm, provided by a xenon-lamp and a monochromator, Polychrome Junior, TILL Photonics), fed into the epi-illumination port of an upright microscope (Axioskop II FS, Zeiss) and digitized by the air-cooled CCD camera (Sensicam). The measured fluorescence intensity is correlated with the concentration of cholesterol in the cell (Muller *et al.*, 1984). Extensive tests showed that it was impossible to compare intensities of filipin fluorescence between independent staining experiments probably due to the instability of the compound. Thus, comparisons between different cell types or treatments were performed on parallel experiments.

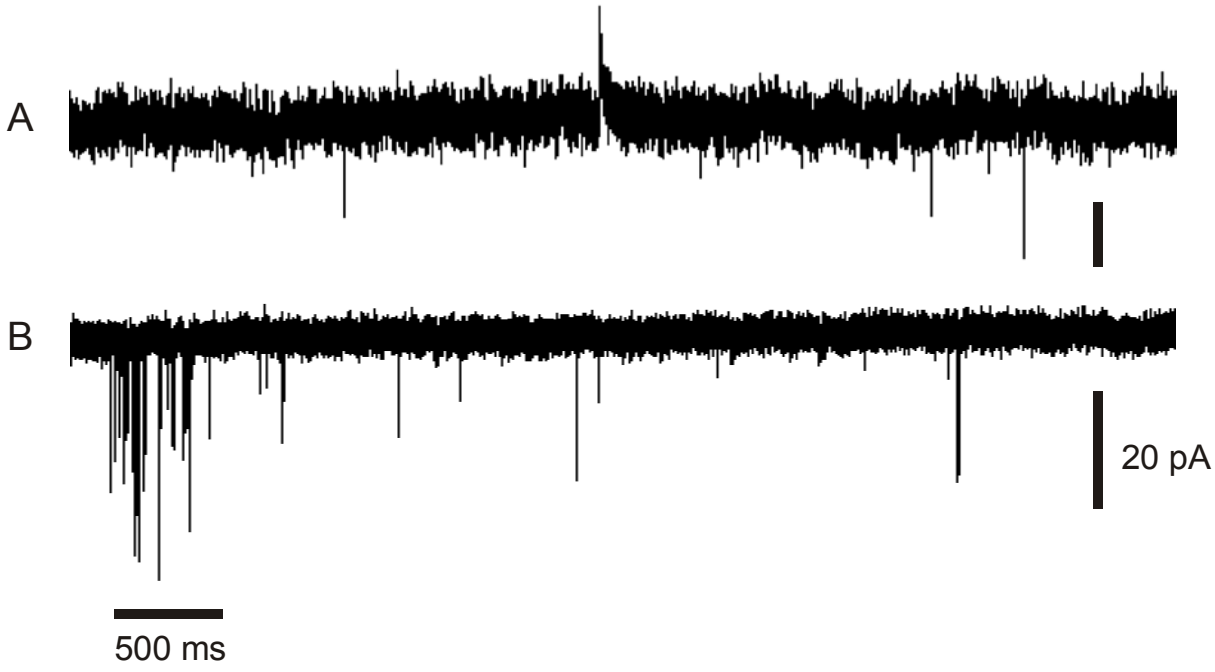

Fig.8: Traces of post-synaptic currents (PSCs)
Recordings from a GC cultured for 7DIV at -40 (A; inhibitory, outward) and -70mV (B; excitatory, inward) holding potential.

7. Electrophysiological recordings

** Voltage Clamp recordings: synaptic activity*

Recordings of spontaneous postsynaptic currents were performed as described (Nagler *et al.*, 2001; Goritz *et al.*, 2005) from GCs and PCs, which could both be recognized by their characteristic size in the respective culture preparations. 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 [2 – 5 MOhm, (World Precision Instruments) prepared using 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 (**Fig.8**) were recorded during 1 min. Analysis of EPSCs and IPSCs was performed automatically by a custom-written Labview routine. The frequency of excitatory postsynaptic currents (EPSCs) was determined from inward currents recorded at −70 mV holding potential. EPSCs could be distinguished from inhibitory postsynaptic currents (IPSCs), which were also inwardly directed under our recording conditions, due to their faster time course. The frequency of IPSCs was determined from outward currents recorded at −40 mV. Miniature EPSCs (mEPSCs) and IPSCs (mIPSCs), which are due to action potential-independent transmitter release, were recorded in the presence of tetrodotoxin (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.

** Current Clamp recordings: Spike activity in Purkinje cells*

Spontaneous and evoked spike activity was recorded from PCs after 7 DIV. Depolarizing currents pulses were applied at 5 (50) pA increments to the cells in current clamp mode and the responses were recorded with a custom-made Labview program (National Instruments). Two parameters characterizing action potential (AP) firing were noted: the minimum effective current to generate an AP – referred to as threshold current (nA) – and the maximal number of APs induced by depolarization – referred to as AP max

frequency (Hz) –. In addition, the resting membrane potential and the capacitance of each cell were noted.

8. 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) and statistically significant changes were detected by appropriate tests; multiple independent samples: Krustall-Wallis ANOVA, two independent samples: Mann-Whitney U test and two dependent samples: Wilcoxon signed rank test. Levels of significance are indicated by asterisks (*$p<0.05$; ** $p<0.01$; *** $p<0.001$).

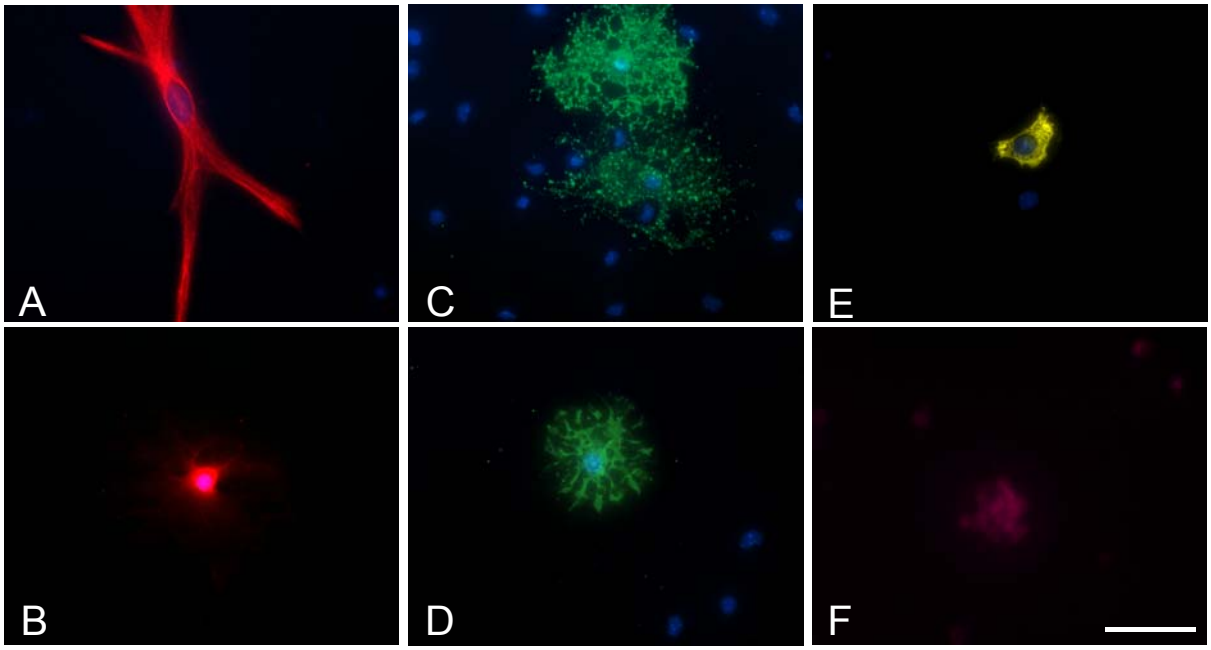

Fig.9: Immunocytochemical staining of cerebellar glial cells
Fluorescence micrographs showing cultured cerebellar cells immunostained after 1 DIV for marker proteins specific to astrocytes (**A**: GFAP; **B**: S100ß), oligodendrocytes (**C**: CNPase; **D**: O4), fibroblasts (**E**: fibronectin) and microglia (**F**: *Griffonia Simplicifolia* lectin I isolectin B4). Cell nuclei were stained with a DNA marker (bisbenzimide; blue). Scale bar= 50µm.

Results

1. Purification and culture of immunoisolated cerebellar neurons

To study the role of glial cells in the development of cerebellar neurons and their degeneration in NPC disease, we developed protocols to immunoisolate distinct types of cerebellar neurons, namely granule cells (GCs) and Purkinje cells (PCs) from postnatal mice. To separate cerebellar neurons from glia, we used an antibody against the cell adhesion molecule L1. This surface marker is expressed by GCs, on PC axons and in the molecular layer of the cerebellum (Rathjen and Schachner, 1984). The L1 isolation yielded 1.6 ± 1.0 million cells (mean ± SD; n = 8 preparations) per animal. The initial viability of the L1+ positive neurons after 3 hours averaged at 77 ± 12% (n=3). PCs are the only cerebellar cell type that degenerates in NPC disease (Harzer *et al.*, 1978; Tanaka *et al.*, 1988), but they represent less than 0.5% of all cerebellar neurons. Therefore, it was important to enrich our cell preparation with PCs. To accomplish this, we performed an additional immunopanning step with the L1+ positive fraction using an antibody against the cell surface glycoprotein Thy1.2, which is expressed by PCs (Bolin *et al.*, 1986) and we obtained approximatively 10,000 Thy1.2 cells per animal.

** Immunoisolated cells are glia-free*

To investigate whether our L1-based immunoisolation protocol provided a pure neuronal preparation, we performed immunocytochemical stainings with specific markers for glial cells, including astrocytes, oligodendrocytes and microglia. As positive controls, we stained mixed cerebellar cells from 6-7 day old mice. Two astrocyte-specific markers GFAP (**Fig.9A**) and S100β (**Fig.9B**) were used to detect cerebellar astrocytes (Levitt and Rakic, 1980**)**. Oligodendrocytes were characterized with the markers O4 (**Fig.9C**) and CNPase (**Fig.9D**). Additionally, a lectin (*Griffonia Simplicifolia* lectin I isolectin B4-FITC) was used to stain microglial cells (**Fig.9E**). Staining the cell population from the L1 selection plate showed that only 0.8 ± 0.7% (n = 2100 cells; 3 preparations) of the cells stained positive for glial markers indicating that the immunoisolation yielded an essentially glia-free neuronal cell population. Since the Thy1+ cells, which we isolated in a second selection step, were derived from this population, they were considered glia-free as well.

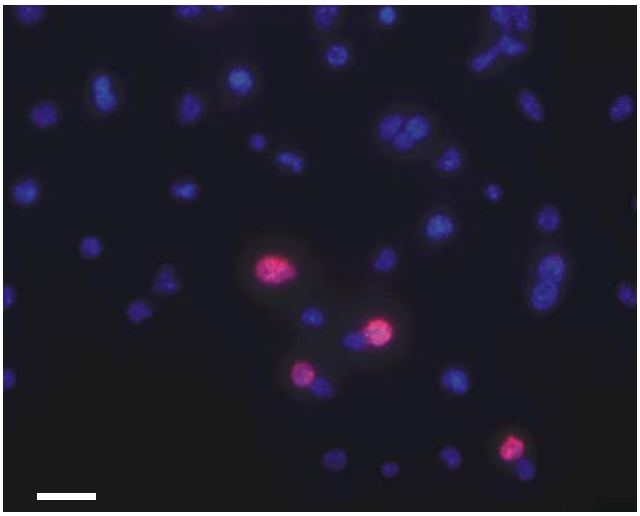

Fig.10: Detection of dividing cells in L1+ cells
Fluorescence micrograph of immunoisolated GCs that were incubated one day after plating with BrdU for 3 hours and then stained with an anti-BrdU antibody (red). Cell nuclei were stained with the DNA marker bisbenzimide (blue). Scale bar= 20µm.

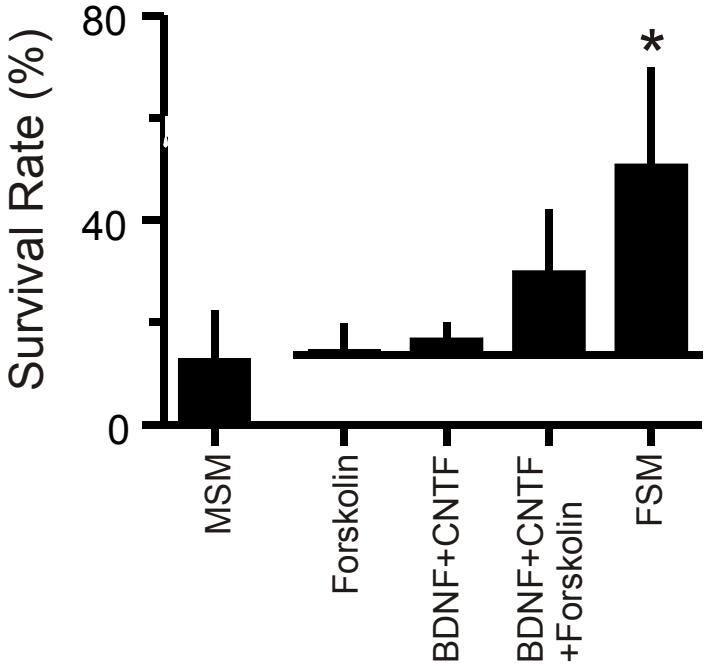

Fig.11: Survival of L1+ cells in chemically defined serum-free medium
Column plot indicates survival rates determined by a microfluorometric assay of L1+ cells that were cultured for three days in MSM (n = 19 preparations) or in MSM supplemented by forskolin (n = 5), by BDNF + CNTF (n = 5), by forskolin + BDNF + CNTF (n = 3) or in FSM (n = 8). Columns and whiskers indicate mean and standard deviation, respectively. For conditions other than MSM, columns indicate survival rates added to basal survival in MSM-treated control cultures by each respective treatment. Asterisks indicate significant changes compared to paralelle MSM-treated cultures (paired samples; Wilcoxon signed rank test).

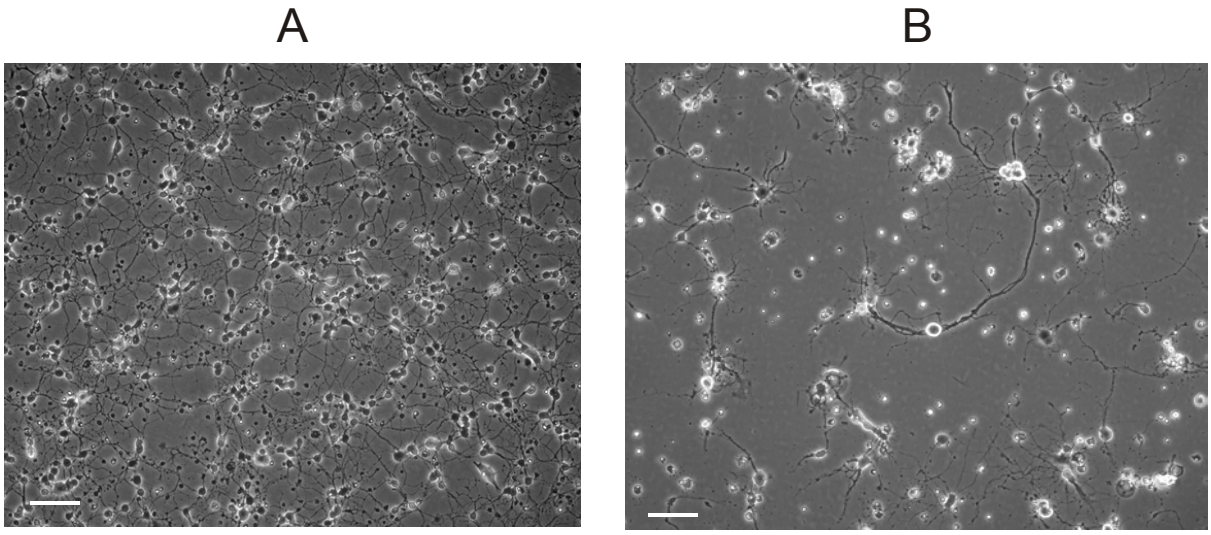

Fig.12: Immunoisolated cerebellar neurons survive and grow in the absence of glia
Phase-contrast micrographs of immunoisolated L1+ (**A**) and Thy1+ (**B**) cells cultured for 7 days under defined, glia-free conditions. Scale bar= 80µm.

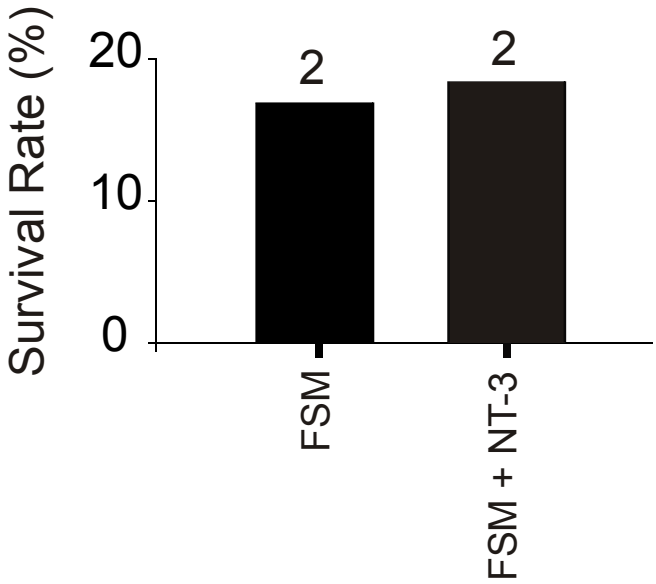

Fig.13: Survival of immunoisolated Thy1+ cells in chemically defined, serum-free medium
Column plot indicating survival rates of Thy1+ cells that were cultured for 7 DIV in FSM, and in FSM supplemented by NT-3. Columns and whiskers indicate mean and standard deviation of survival rates (n = 2 preparations).

** Immunoisolated cells are post-mitotic*

We next determined whether the L1+ neurons contained dividing granule cell precursors, as it is known that these cells are still present in the cerebellum of 7 day old mice (Swisher and Wilson, 1977). Cells were incubated for 6 hrs with BrdU 3 hrs or 24 hrs after plating. Incorporated BrdU was then visualized by immunocytochemistry (**Fig.10**). We found that the cerebellar preparation contained only very few dividing cells (1.1 ± 0.8%; n = 4) indicating the absence of precursors.

** A chemically defined medium supports survival of cultured neurons*

The culture conditions supporting survival and differentiation of isolated cells were established using a microfluorometric viability assay. As a basis, we used a chemically defined medium, referred to as minimally supplemented medium (MSM), whose components (see Material and Methods) are known to support survival of CNS neurons in conventional, glia-containing cultures (Brewer *et al.*, 1995). In this condition, only a weak number of L1+ cells survived at 3 DIV (**Fig.11**), indicating that neurons require external signals to survive. Interestingly, a previous study reported that a combination of peptide growth factors together with an increase in the level of cyclic adenosine monophosphate enhanced survival rates of RGCs (Meyer-Franke *et al..*, 1995) and motoneurons (Hanson *et al..*, 1998). Thus, MSM was supplemented with a combination of forskolin, BDNF, CNTF and B27. This medium, which is further referred to as fully supplemented medium (FSM), stabilized survival of L1+ cells (**Fig.11**). When cultured for one week in FSM, L1+ cells showed vigorous neurite outgrowth (**Fig.12A**) with survival rates averaging at 40 ± 15% (n = 3). For survival of Thy1+ cells, which should comprise also PCs, the FSM was further supplemented with insulin-like growth factor 1 (IGF-I) that favors *in vitro* development of PCs (Fukudome *et al.*, 2003). Under this condition, Thy1+ cells survived and formed an extensive network (**Fig.12B**). It has also been shown that neurotrophin-3 (NT-3) increases the survival of dissociated PCs in culture (Mount *et al.*, 1994; Larkfors *et al.*, 1996). However, this component did not enhance significantly the survival of Thy1+ cells (**Fig. 13**).

** Characterization of L1- and Thy1-positive neurons*

Next, we determined the type of cerebellar neurons that were present in our immunoisolated cell populations. Since the cerebellum contains both excitatory (GCs) and inhibitory (basket, stellate, Golgi and Purkinje) neurons, L1+ cells were submitted after 7 DIV to immunocytochemical staining against GAD 65 in order to determine the proportion of

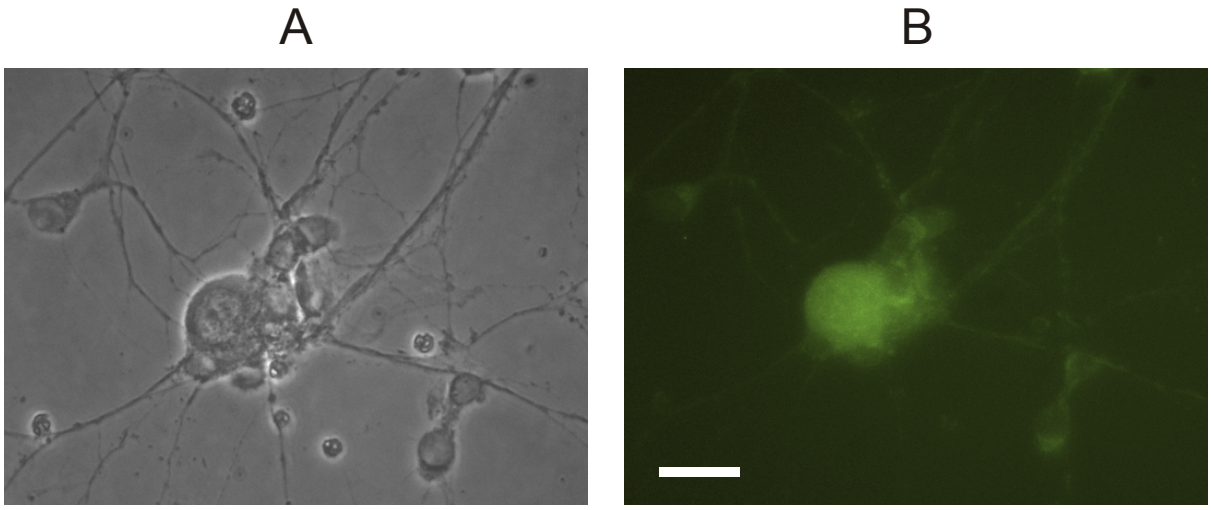

Fig.14: Characterization of L1+ cells. Phase-contrast (**A**) and fluorescence (**B**) micrograph of L1+ cells after 7 DIV showing a GAD-positive PC surrounded by GCs. Scale bar = 20 µm.

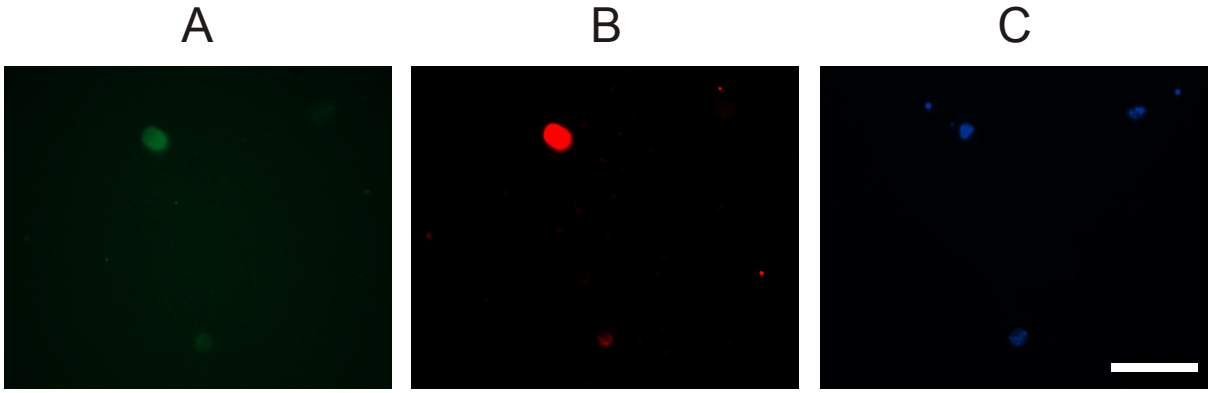

Fig.15: Immunocytochemical characterization of immunoisolated Thy1+ cells
Fluorescence micrographs of immunoisolated cells stained on the Thy1.2-selection plate with antibodies against calbindin (**A**) and IP3R1 (**B**) and with the nuclear marker bisbenzimide (**C**). Scale bar= 80µm.

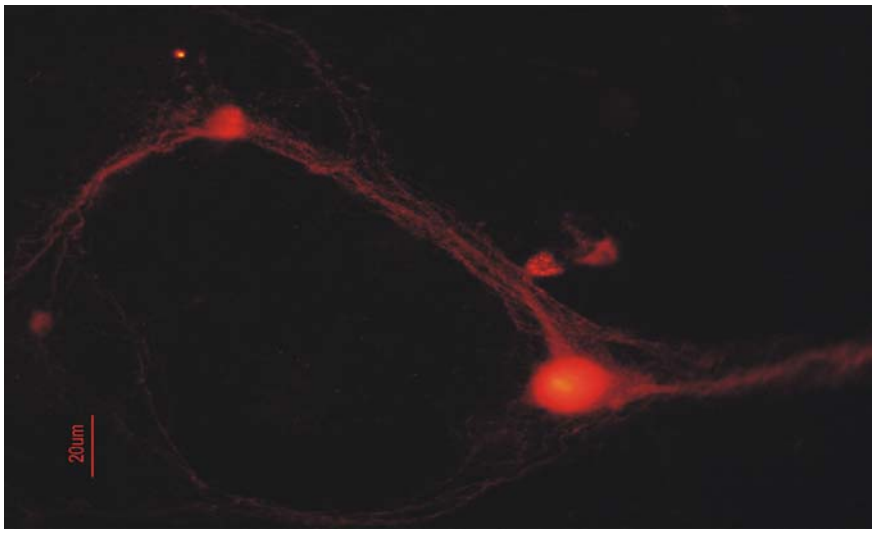

Fig.16: Immmunocytochemical characterization of a PC after 7 DIV
Fluorescence micrograph showing an immunoisolated PC cultured for 7 DIV in FSM supplemented by IGF-I and then stained with an IP3R1-specific antibody. Scale bar= 20µm.

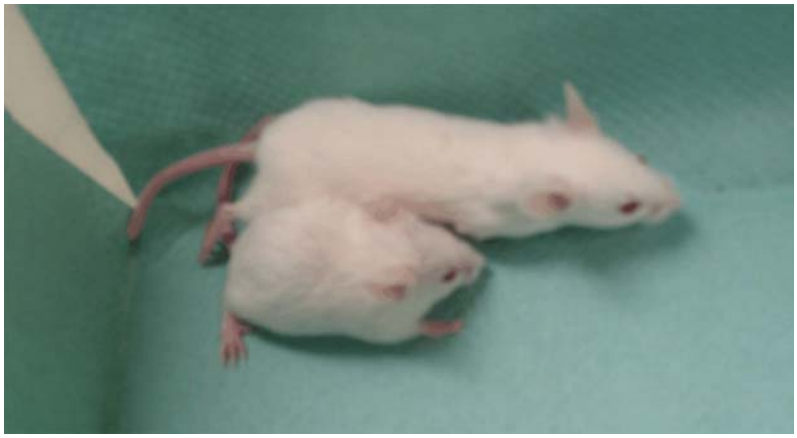

Fig.17: Phenotype of WT and NPC mice
Photographs of 2 month old littermates with WT (top) and NPC (bottom) mice exhibiting a remarkable difference in size.

GABAergic neurons. The results showed that only 7 ± 2% (n = 6678 cells; 3 preparations) of L1+ cells were GAD-positive (**Fig.14**). This finding indicated that this population contained mostly (~90%) GCs, which reflects their abundance *in vivo*. Therefore, we further referred to the L1+ cells as GCs (Fig. 12A). GCs could be easily identified by the small size of their somata. In this preparation, PCs, which could be identified by their large somata, were rarely detected (less than 1 out of 20,000 cells per culture plate) (**Fig.14A**).

We next examined the proportion of PCs in the Thy1+ culture by immunocytochemical staining against two well-established PC markers. On the selection plate, 59 ± 1% and 58 ± 3% of the cells tested were positive for calbindin and IP3R1 (n = 2 preparations, 568 cells), respectively (**Fig.15**). This fraction remained stable, as after 7 DIV still 50 ± 12% (n = 2) of Thy1+ cells were IP3R1-positive (**Fig.16**). This indicates that PCs are the predominant cell type in the Thy1-positive neuronal preparation and that the culture conditions supported their survival. We therefore further referred to Thy1+ cells as PCs.

2. Purification and culture of GCs and PCs from NPC mice

** Obtention of NPC mice*

From two pairs of heterozygous NPC founder animals, we established within six months a colony that delivered animals for our project. Homozygous NPC mice showed clearly the previously described symptoms. A few days after birth, 75% of mutant pups were smaller than wildtype pups. One month later, mutant animals continued to lose weight, showed tremor and ataxic gait that worsened until death at about 12 to 14 weeks of age (**Fig.17**) as observed previously (Morris *et al.*, 1982). The limiting aspect of this mouse model of NPC is the sterility of the homozygous mutants (Erickson *et al.*, 2002). Therefore, we were obliged to breed heterozygous mice and to genotype 4 day old pups before their experimental use. Given the high yield of GCs per animal, only one homozygous mutant mouse (P6-7) was sufficient for a single GC preparation. On the other hand, the PC preparation was only possible with a minimum of four homozygous mice. Therefore, the number of PC preparations that could be obtained was limited. All experiments were performed on wildtype (WT) mice or on mice that were homozygous for the mutation (NPC).

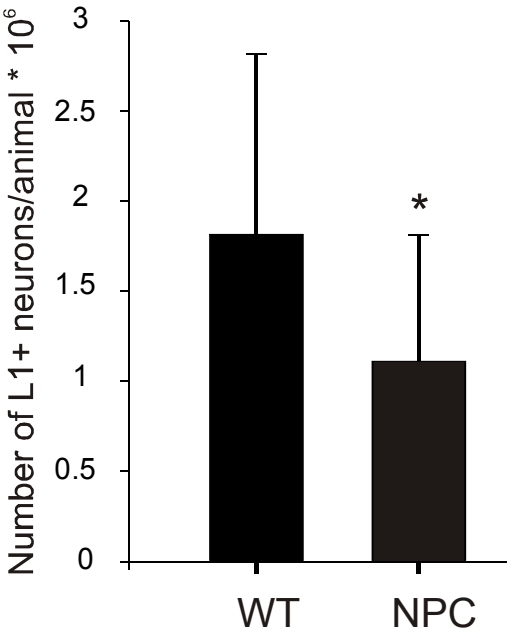

Fig.18: Yield of immunoisolated GCs from WT and NPC mice
Column plot indicating mean numbers of neurons immunoisolated from 6-7 days old WT and NPC mice (paired samples, Wilcoxon paired rank test; n=8 preparations).

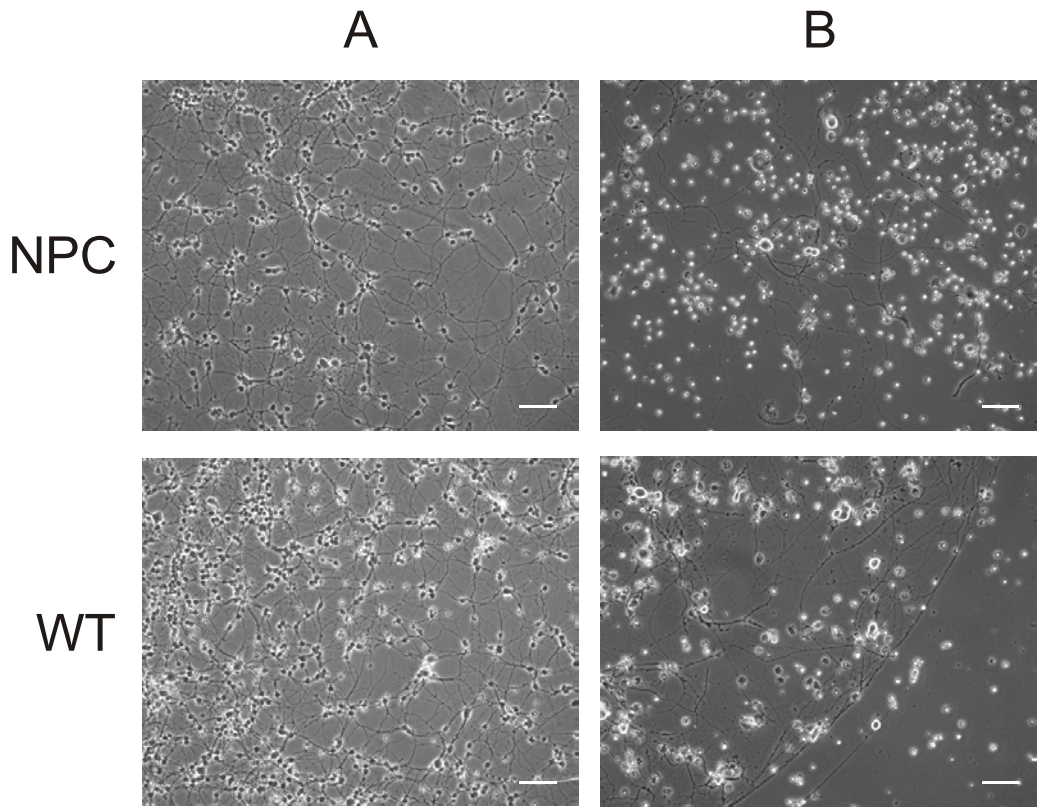

Fig.19: Cultured WT and NPC neurons exhibit similar morphology
Phase-contrast micrographs of GCs (**A**) and PCs (**B**) immunoisolated from NPC (top) and WT (bottom) mice that were cultured for 7 DIV in FSM supplemented by WT GCM.
Scale bar= 80µm.

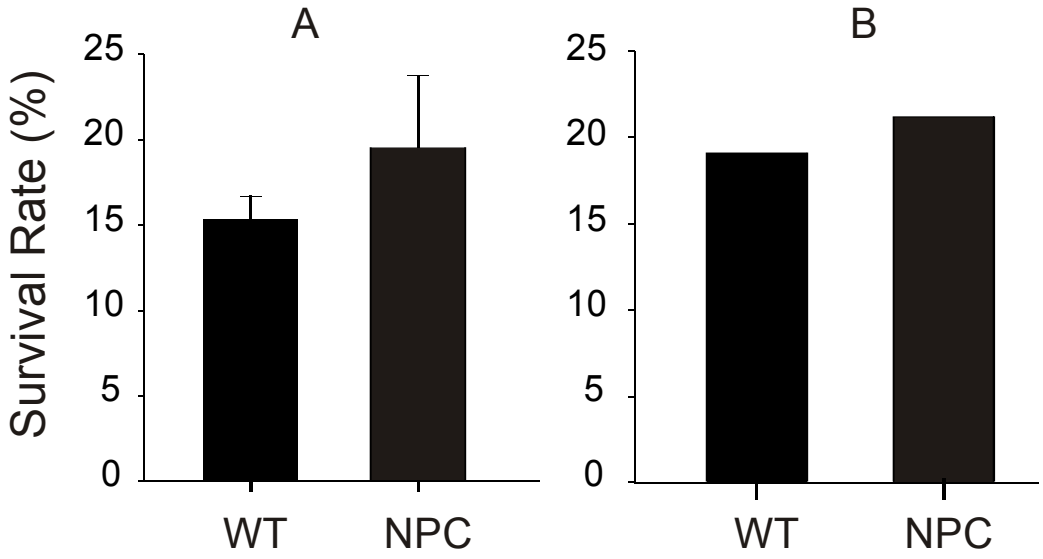

Fig.20: Survival rates of immunoisolated neurons from WT and NPC mice
Column plots of of GCs (**A**; n= 4 preparations) and PCs (**B**; n = 1) immunoisolated from WT or NPC animals and cultured for 7 days in FSM.

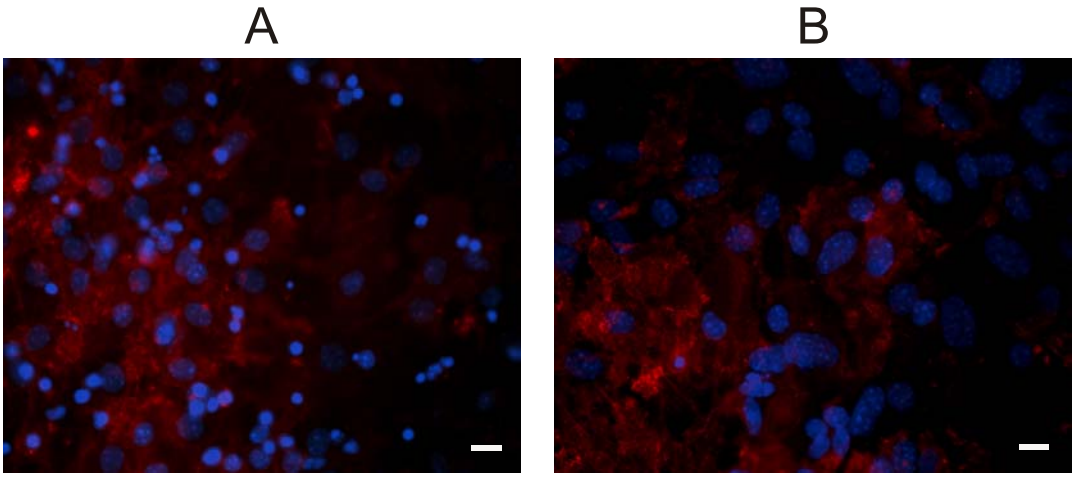

Fig.21: Cultured glial cells from WT and NPC mice contain Bergmann glia
Fluorescence micrographs of glial cells from P7 WT (**A**) and NPC (**B**) mice immunostained after 14 DIV with a GLAST-specific antibody and with nuclear staining with bisbenzimide. Scale bar = 20 µm.

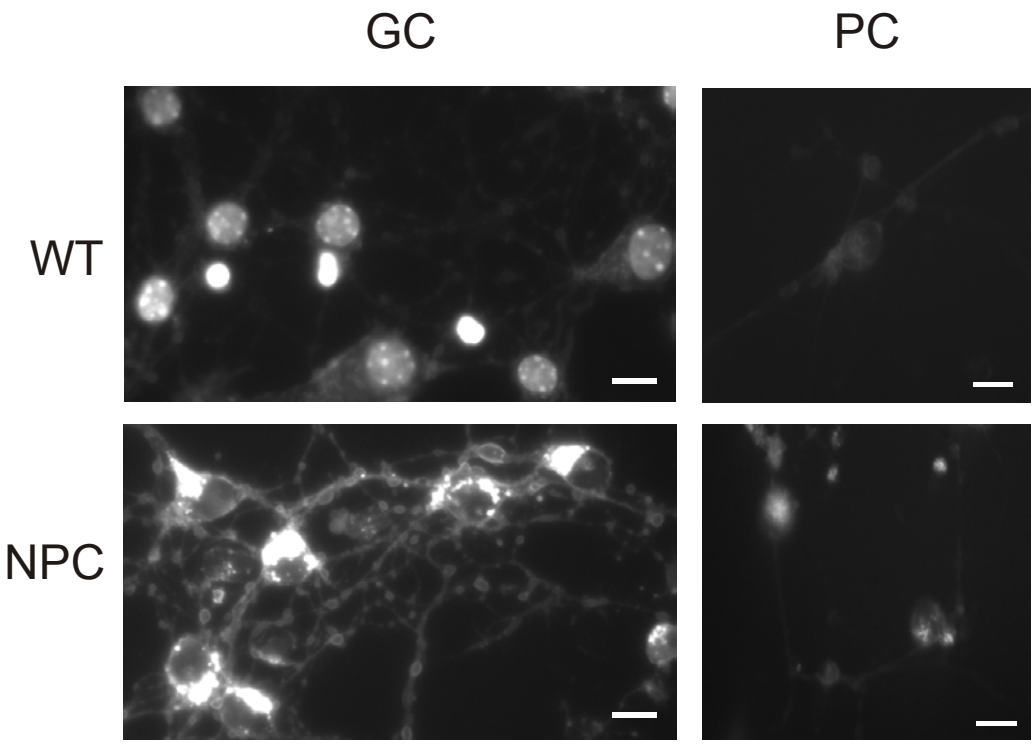

Fig.22: Intracellular cholesterol distribution in GCs and PCs from WT and NPC animals growing under defined, glia-free conditions
Fluorescence micrographs of GCs (left) and PCs (right) that were immunoisolated from WT (top) and NPC (bottom) mice and cultured for 7 DIV in FSM. Cells were fixed and stained with filipin. Scale bar= 5µm (GC); 25µm (PC).

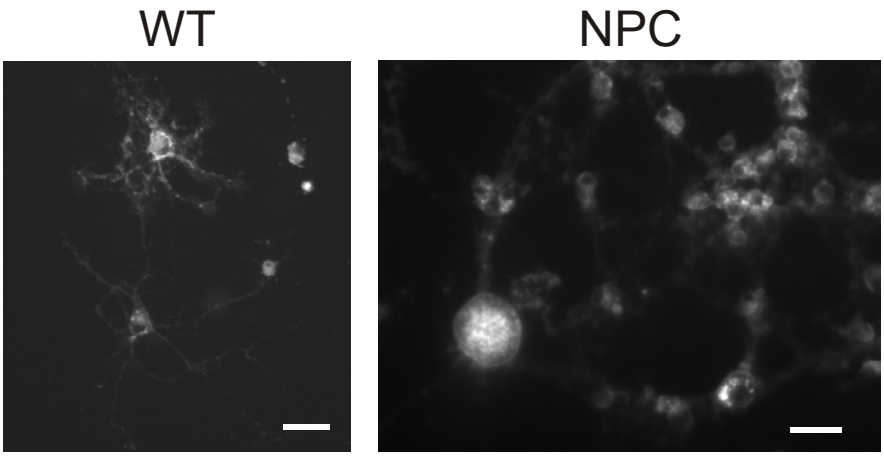

Fig.23: Cholesterol distribution in GCs from WT and NPC animals after treatment with GCM
Fluorescence micrographs of GCs from WT (left) and NPC (right) mice cultured in FSM that was supplemented with GCM or cholesterol. After 7DIV, cells were fixed and stained with filipin. Scale bar= 10µm (WT); 25µm (NPC).

** Isolation and culture of GCs and PCs from NPC mice*

We next tested if the immunoisolation protocols were applicable to NPC postnatal mice. The yield of GCs was slightly but significantly ($p <= 0.05$; Wilcoxon matched pair test) lower in NPC than in WT mice (**Fig.18**). Due to the very low yield of PCs, it was impossible to obtain reliable numbers for NPC animals. After one week in culture, GCs and PCs from NPC and WT animals exhibited similar morphologies (**Fig.19**). Furthermore, there was no difference in survival rates of GCs ($p=0.0678$; $n=4$; Wilcoxon matched pair test; **Fig.20A**) and PCs between NPC and WT animals after 7DIV in FSM (**Fig.20B**).

** Culture of glial cells from both NPC mutant and wildtype mice*

Our goal was to define the role of glial cells in cerebellar development and in NPC-related neurodegeneration. Since Bergmann glia are prominent astroglial cells in the cerebellum, we wanted to know if these cells were present in the glial cultures that we used for co-culture experiments and for production of GCM and whether their presence was different in cultures from WT and NPC animals. We performed immunocytochemical staining of cerebellar glial cells that were grown under the same conditions as GCs using an antibody against GLAST that is specific to Bergmann glia at postnatal stages (Yamada and Watanabe, 2002). We observed that glial cultures derived from WT and NPC animals contained Bergmann glia at 21% (**Fig.21A**) and 18% (**Fig.21B**), respectively ($n=1$). This suggested that cultures derived from NPC animals contained a similar proportion of Bergmann glia as those from wildtype animals.

** Cholesterol accumulation in neurons and glial cells from NPC mice*

A hallmark of cells from NPC animals or patients is accumulation of cholesterol in the endosomal/lysosomal system (Zervas *et al.*, 2001). We next determined, if neurons and glia in our culture preparations also showed this phenotype using staining with filipin, a fluorescent sterol-binding antibiotic that is commonly used to determine the intracellular cholesterol distribution. As expected, the filipin signal was more intense in NPC-GCs and -PCs compared to WT cells (**Fig.22**) and concentrated in an intracellular compartment that resembled the endosomal/lysosomal system. Treatment of GCs or PCs with GCM from WT animals (**Fig.23**) enhanced the level of cholesterol within cell bodies and dendrites, as previously shown in rat RGCs (Goritz *et al.*, 2005), indicating cholesterol uptake via lipoproteins.

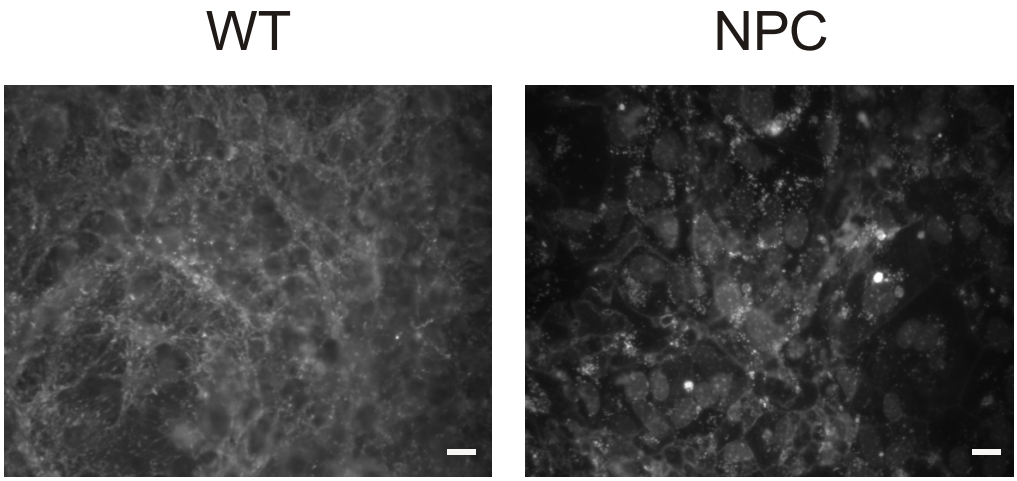

Fig.24: Cholesterol accumulation in NPC glia
Fluorescence micrographs of cerebellar glial cells from WT and NPC animals that were fixed after 14 DIV and stained with filipin. Scale bar = 50µm.

Treatment of NPC-GCs or -PCs with GCM (**Fig.23**) enhanced their cholesterol content mainly in the cell bodies, whereas no increase in neurites was seen. We could also see thistype-specific cholesterol distribution in PCs. Treatment with exogenous cholesterol also induced similar effects as GCM in both GCs and PCs. The enhanced signal appeared to be localized in the endosomal/lysosomal system, but this would need to be verified by counterstaining with compartment-specific markers. We also stained cultured glial cells from WT and NPC mice with filipin. Again, NPC cells exhibited cholesterol aggregates within cell bodies and processes as indicated by a punctate filipin signal, whereas cholesterol was distributed much more homogenously in WT glial cells (**Fig.24)**. These results showed that the well-established differences in sterol distribution between WT and NPC cells occurred in our cultures.

3. Electrophysiological properties of neurons from GC and PC cultures from NPC and WT mice

3.1. Synaptic activity in GCs and PCs from WT and NPC animals in the absence of glia

Next, we tested, whether GCs and PCs established functional excitatory and inhibitory synaptic connections in the absence of glial cells and whether the lack of functional NPC1 in neurons influenced development. Isolated neurons were cultured at similar densities in FSM and, after one week in culture, the presence of functional synapses was analyzed by electrophysiological recordings.

Interestingly, the large majority of GCs from WT and NPC mice (**Fig.25A**) showed vigorous excitatory synaptic activity under control conditions with frequencies of sEPSCs reaching up to 800 events per min (**Fig.25B**). The size distribution of sEPSCs in WT- and NPC-GCs was similar (**Fig.25C**). The incidence of spontaneous inhibitory activity was considerably lower as only a small fraction of both WT and NPC GCs showed sIPSCs (**Fig.27A**) occurring at frequencies of up to 40 events per minute (**Fig. 27B**). The low level of inhibitory activity was probably due to the small proportion of GABAergic cells in the GC culture. Nevertheless, there was no difference in the incidence (**Fig. 27A**), frequency (**Fig. 27B**) or size (**Fig. 27C**) of sIPSCs between WT and NPC-GCs.

In marked contrast to GCs, only half of the PCs exhibited spontaneous excitatory synaptic activity regardless of the presence of functional NPC1 (**Fig.28A**). In WT-PCs, frequencies of sEPSCs reached maximally 120 events per min and were therefore

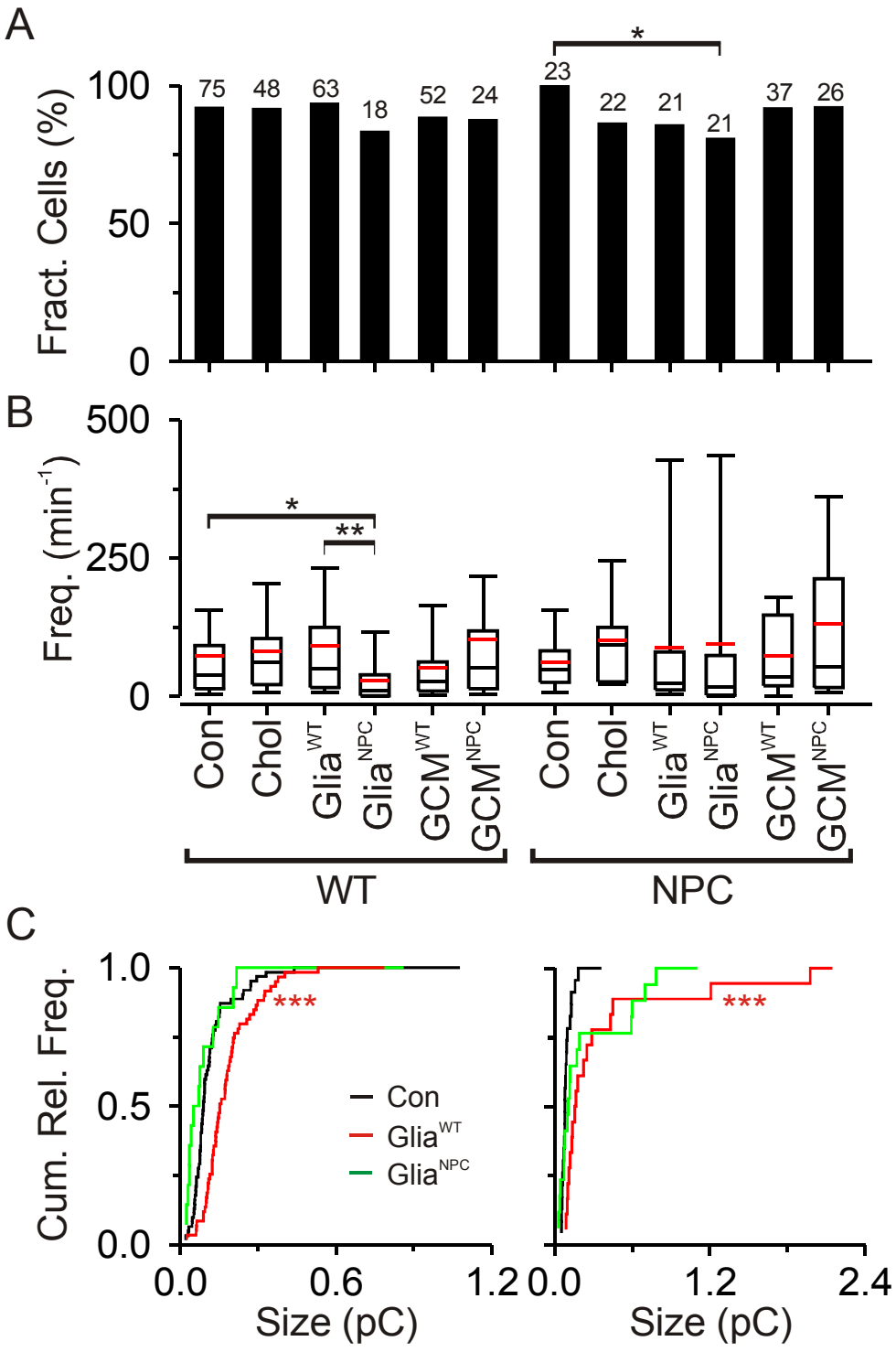

Fig.25: Level of spontaneous excitatory synaptic activity in GCs cultures from WT and NPC mice
A, Column plot showing the fraction of WT (left) or NPC (right) GCs with spontaneous EPSCs. Numbers above columns indicate cell numbers. **B**, Box-plots representing frequencies of spontaneous EPSCs in WT- (left) and NPC-GCs (right) with excitatory activity (red lines, mean value). **C**, Cumulative relative frequency distribution of charge transfer of EPSCs representing the 90th percentile in each cell in glia-free control cultures (black), in coculture with $Glia^{WT}$ (red) or with $Glia^{NPC}$ (green). GCs from WT and NPC animals were cultured for 7 DIV in the absence of glia (Con), together with glial cells from WT ($Glia^{WT}$) or NPC ($Glia^{NPC}$) animals, treated with cholesterol (Chol) or with GCM from WT (GCM^{WT}) or NPC (GCM^{NPC}) animals. Asterisks indicate significant changes compared to control cultures (A: Pearson's Chi square test; B, C: Kruskal-Wallis ANOVA by ranks test with post-hoc comparison of mean ranks).

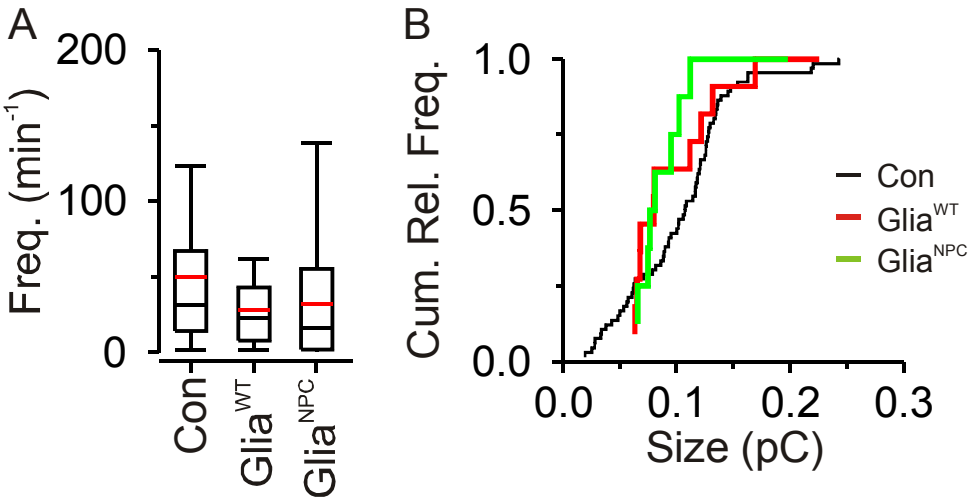

Fig.26: Effects of cocultures on mEPSCs in WT GCs
A, Box-plots representing frequencies of miniature EPSCs in WT GCs with excitatory activity (red lines, mean value). **B**, Cumulative relative frequency distribution of charge transfer of EPSCs representing the 90th percentile in each cell in glia-free control cultures (black), in coculture with Glia^{WT} (red) or with Glia^{NPC} (green). GCs from WT and NPC animals were cultured for 7 DIV in the absence of glia (Con) or together with glial cells from WT (Glia^{WT}) or NPC (Glia^{NPC}) animals. Asterisks indicate significant changes compared to control cultures (A: Pearson's Chi square test; B, C: Kruskal-Wallis ANOVA by ranks test with post-hoc comparison of mean ranks).

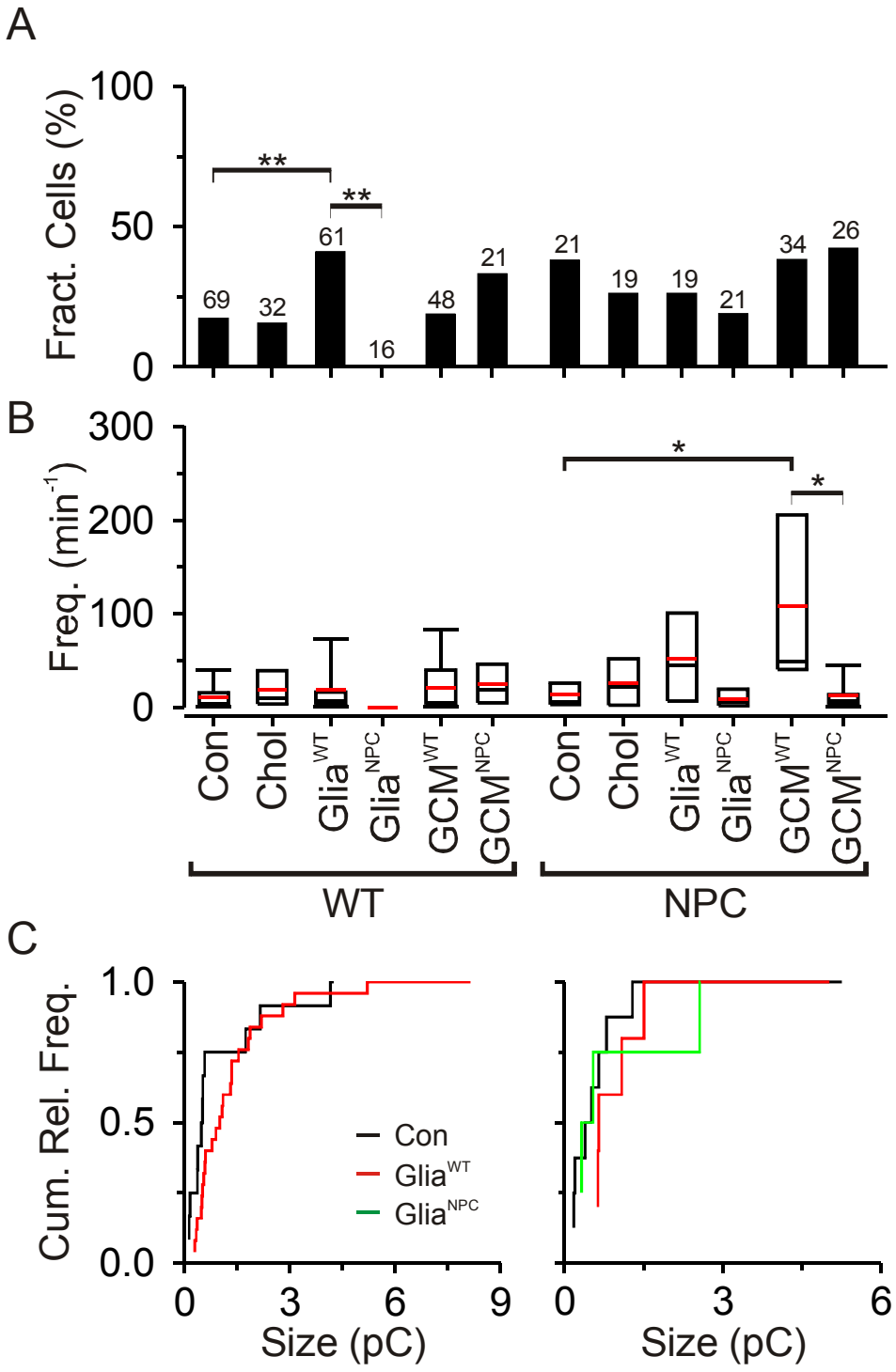

Fig.27: Level of spontaneous inhibitory synaptic activity in GCs cultures from WT and NPC mice
A, Column plot showing the fraction of WT (left) or NPC (right) GCs with spontaneous IPSCs. Numbers above columns indicate cell numbers. **B**, Box-plots representing frequencies of spontaneous IPSCs in WT- (left) and NPC-GCs (right) with inhibitory activity (red lines, mean value). **C**, Cumulative relative frequency distribution of charge transfer of IPSCs representing the 90th percentile in each cell in glia-free control cultures (black), in coculture with $Glia^{WT}$ (red) or with $Glia^{NPC}$ (green). GCs from WT and NPC animals were cultured for 7 DIV in the absence of glia (Con), together with glial cells from WT ($Glia^{WT}$) or NPC ($Glia^{NPC}$) animals, treated with cholesterol (Chol) or with GCM from WT (GCM^{WT}) or NPC (GCM^{NPC}) animals. Asterisks indicate significant changes compared to control cultures (A: Pearson's Chi square test; B, C: Kruskal-Wallis ANOVA by ranks test with post-hoc comparison of mean ranks).

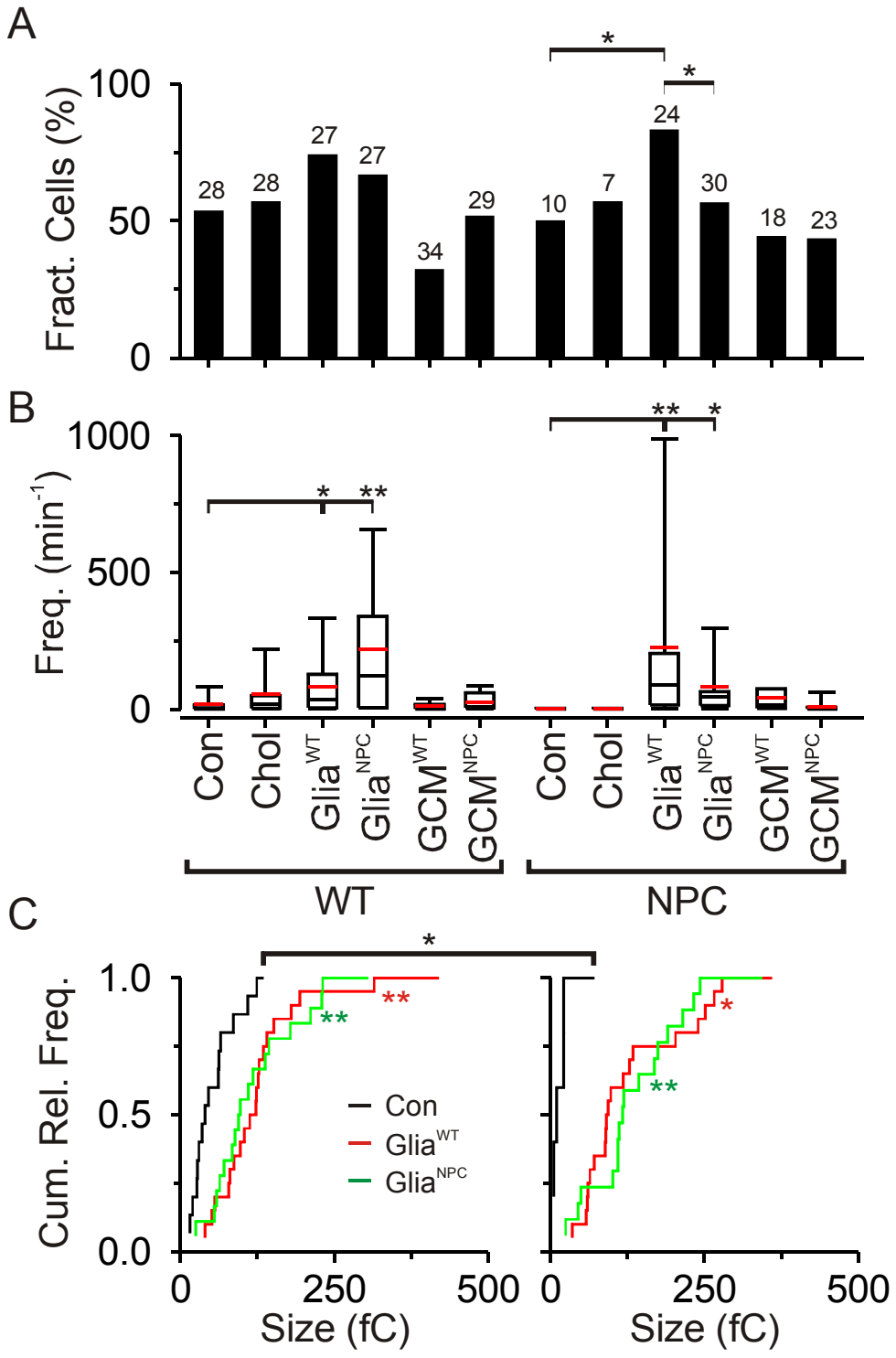

Fig.28: Level of spontaneous excitatory synaptic activity in PCs cultures from WT and NPC mice
A, Column plot showing the fraction of WT (left) or NPC (right) PCs with spontaneous EPSCs. Numbers above columns indicate cell numbers. **B**, Box-plots representing frequencies of spontaneous EPSCs in WT- (left) and NPC-PCs (right) with excitatory activity (red lines, mean value). **C**, Cumulative relative frequency distribution of charge transfer of EPSCs representing the 90th percentile in each cell in glia-free control cultures (black), in coculture with $Glia^{WT}$ (red) or with $Glia^{NPC}$ (green). PCs from WT and NPC animals were cultured for 7 DIV in the absence of glia (Con), together with glial cells from WT ($Glia^{WT}$) or NPC ($Glia^{NPC}$) animals, treated with cholesterol (Chol) or with GCM from WT (GCM^{WT}) or NPC (GCM^{NPC}) animals. Asterisks indicate significant changes compared to control cultures (A: Pearson's Chi square test; B, C: Kruskal-Wallis ANOVA by ranks test with post-hoc comparison of mean ranks).

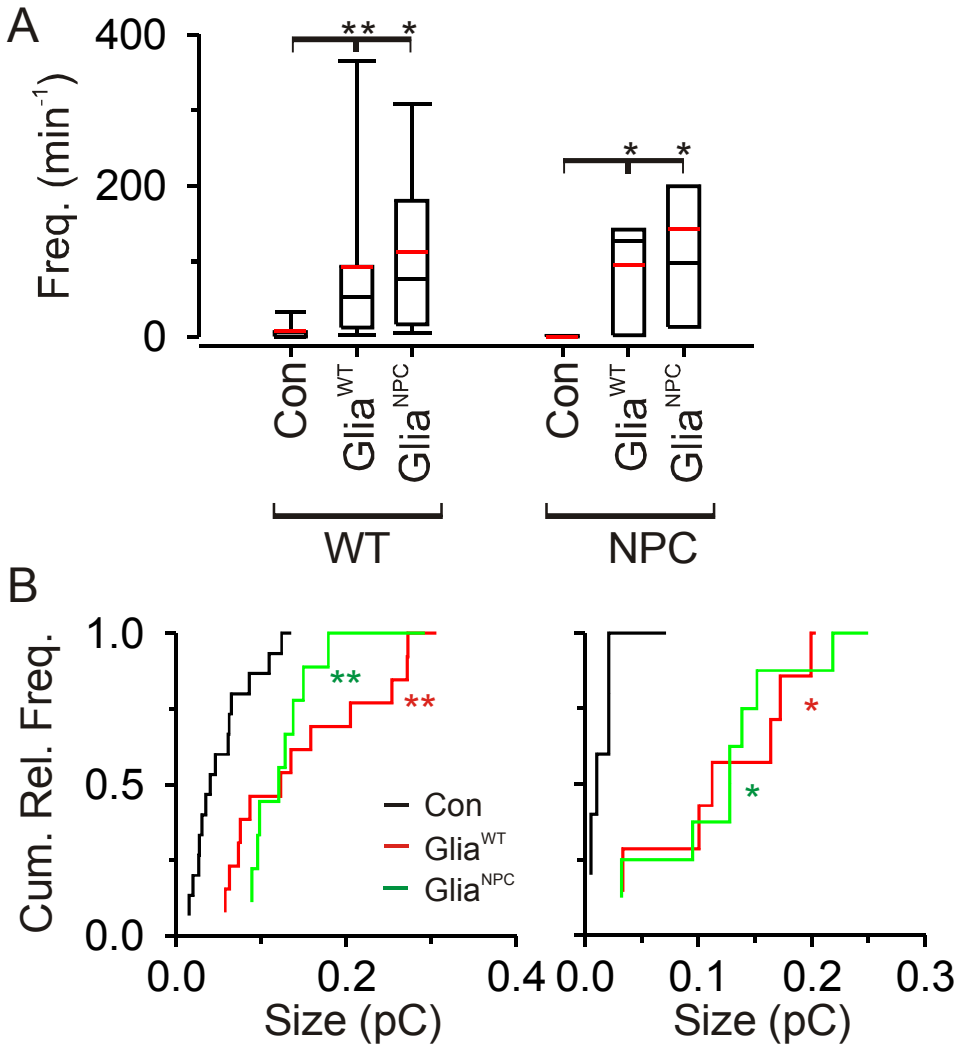

Fig.29: Level of miniature excitatory synaptic activity in PCs cultures from WT and NPC mice
A, Box-plots representing frequencies of miniature EPSCs in WT- (left) and NPC-PCs (right) with excitatory activity (red lines, mean value). **B**, Cumulative relative frequency distribution of charge transfer of EPSCs representing the 90th percentile in each cell in glia-free control cultures (black), in coculture with Glia^{WT} (red) or with Glia^{NPC} (green). GCs from WT and NPC animals were cultured for 7 DIV in the absence of glia (Con) or together with glial cells from WT (Glia^{WT}) or NPC (Glia^{NPC}) animals. Asterisks indicate significant changes compared to control cultures (A: Pearson's Chi square test; B, C: Kruskal-Wallis ANOVA by ranks test with post-hoc comparison of mean ranks). Note that datas for control conditions come from spontaneous recordings.

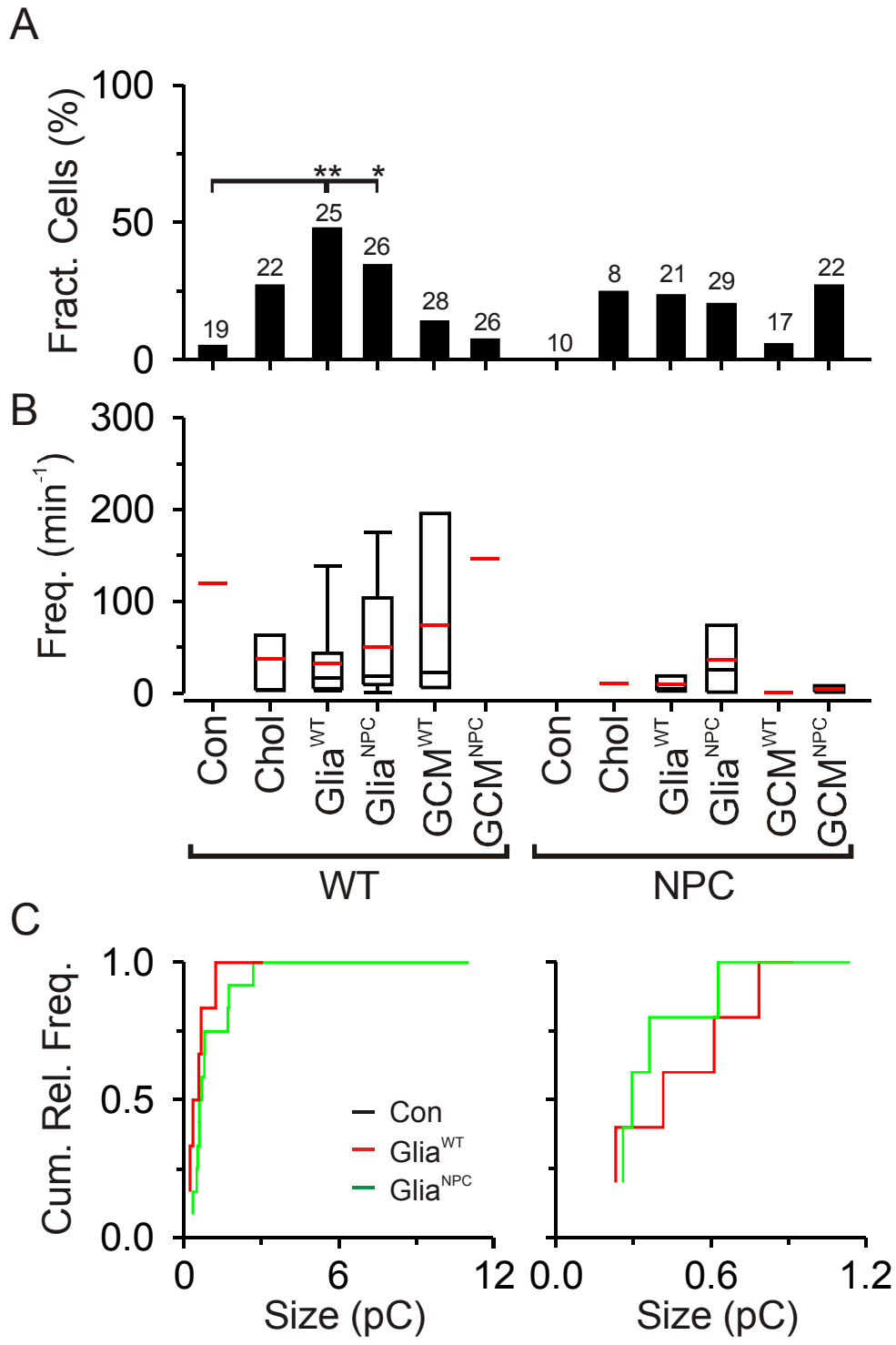

Fig.30: Level of spontaneous inhibitory synaptic activity in PCs cultures from WT and NPC mice
A, Column plot showing the fraction of WT (left) or NPC (right) PCs with spontaneous IPSCs. Numbers above columns indicate cell numbers. **B**, Box-plots representing frequencies of spontaneous IPSCs in WT- (left) and NPC-PCs (right) with inhibitory activity (red lines, mean value). **C**, Cumulative relative frequency distribution of charge transfer of IPSCs representing the 90th percentile in each cell in glia-free control cultures (black), in coculture with $Glia^{WT}$ (red) or with $Glia^{NPC}$ (green). PCs from WT and NPC animals were cultured for 7 DIV in the absence of glia (Con), together with glial cells from WT ($Glia^{WT}$) or NPC ($Glia^{NPC}$) animals, treated with cholesterol (Chol) or with GCM from WT (GCM^{WT}) or NPC (GCM^{NPC}) animals. Asterisks indicate significant changes compared to control cultures (A: Pearson's Chi square test; B, C: Kruskal-Wallis ANOVA by ranks test with post-hoc comparison of mean ranks).

considerably lower than in GCs. NPC-PCs showed even lower frequencies of sEPSCs compared to WT-PCs, but the difference did not reach statistical significance (Mann-Whitney U test). In addition, sEPSC in PCs from NPC animals were significantly smaller than those from WT animals (**Fig.28C**). PCs showing sIPSCs were very rare in the WT preparation (5%) and completely absent in the NPC preparation (**Fig. 30A**), which precluded their further analysis.

Taken together, these results indicated that GCs and PCs differ in their ability to form synapses. In the absence of glia, GCs showed excitatory and inhibitory activity indicating the formation of synapses, whereas PCs showed a very low level of synaptic activity. In GCs, a functional NPC1 protein appeared dispensible for excitatory and inhibitory synaptic activity. On the other hand, PCs showed differences in the frequency and size of sEPSCs between WT and NPC animals indicating that absence of NPC1 in PCs affects excitatory activity in these neurons.

** Effects of cholesterol on synaptic activity*

To investigate, whether glia-derived cholesterol promotes synaptogenesis in GCs and PCs, as shown for RGCs (Mauch *et al.*, 2001), we treated cells with cholesterol at a similar concentration (5 μg ml^{-1}) as in GCM (Mauch *et al.*, 2001). In GCs and PCs, addition of cholesterol for one week did not change the incidence or level of excitatory and inhibitory activity in cells derived from WT or NPC animals (**Figs.25-30**), although filipin staining revealed accumulation of cholesterol within their cell bodies (**Fig.23**).

3.2. Effects of glial signals on synapse development of GCs and the relevance of functional NPC1 in neurons and glial cells

To study whether glial signals influence synapse development in GCs and whether a possible glial influence requires functional NPC1 in glial cells or neurons, GCs from WT and NPC animals were cultured in the presence or absence of glial cells or of GCM from WT or NPC animals. The fraction of GCs showing sEPSCs was not affected by glial signals except for a small but significant decrease in cocultures of NPC-PCs with NPC-glia compared to glia-free cultures of NPC-GCs (**Fig. 25A**). However, this effect was not mimicked by GCM. The frequency of sEPSCs in GCs from WT or NPC animals was not changed by WT glial cells. Notably, when cocultured with glial cells from NPC animals, GCs from WT animals showed a significantly lower frequency of sEPSCs compared to glia-free controls and to glia from WT animals. This effect did not occur in GCs from NPCs. Again, GCM did not mimick

the effects (**Fig.25B**). Interestingly, the size of sEPSCs was increased, when GCs from WT and NPC animals were cocultured with glial cells from WT animals, but this effect was not mimicked by glia from NPC animals (**Fig.25C**).

To test whether the decrease and increase in the frequency and size of sEPSCs in WT GCs was due to changes in action potential-dependent and -independent synaptic transmission, we recorded mEPSCs in the presence of TTX. Coculturing WT-GCs with WT- and NPC-glia did not change the frequency or size of mEPSCs (**Fig.26**) indicating that the observed changes in sEPSCs were due to changes in action potential-dependent synaptic responses.

Glial signals affected spontaneous inhibitory activity in GCs in a different manner than excitatory events. First, the fraction of WT GC cells showing sIPSCs was enhanced significantly by glial cells from WT animals, but not from NPC animals (**Fig.27A**). This indicated that glial cells lacking NPC1 could not induce inhibitory synaptic activity in GCs. On the other hand, this change was not observed in GCs from NPC animals indicating that the lack of functional NPC1 in neurons abolished their sensitivity to this effect. Despite the increase in the fraction of WT-GCs showing sIPSCs, their frequencies were not enhanced by glia (**Fig.27B**). Interestingly, in NPC-GCs, GCM from WT, but not from NPC animals enhanced the frequency of IPSCs indicating again that absence of NPC1 from glia impairs its ability to enhance inhibitory synaptic activity. Surprisingly, this effect was not mimicked by cocultures (**Fig.27B**). Finally, glial signals in cocultures or GCM did not affect the size of sIPSCs. Unfortunately, a very low incidence of mIPSCs in GCs did not allow to determine whether the observed effects on sIPSCs were due to changes in action potential-dependent or -independent synaptic activity.

Taken together, the results suggested first, that glial signals influenced differentially excitatory and inhibitory synaptic activity in GCs and second, that the lack of functional NPC1 in neurons and glia determined how glia changes synaptic activity. Coculturing GCs with glia enhanced the size of sEPSCs probably by increasing action potential-dependent transmitter release. This effect required functional NPC1 in glia, but not in neurons. On the other hand, glia lowered the frequency of sEPSCs in GCs, again in an action potential-dependent manner, but this effect required functional NPC1 in neurons and its absence in glia. On the other hand, glia enhanced the frequency of sIPSCs but this effect required absence of NPC1 in neurons and its presence in glia.

3.3. Effects of glial signals on synapse development of PCs and the relevance of functional NPC1 and glia

To study whether glial signals influence synapse development in PCs and whether a possible glial influence requires functional NPC1 in glial cells or neurons, we cultured WT and NPC PCs under similar culture conditions as described for GCs. The fraction of NPC-PCs showing sEPSCs was enhanced significantly by glial cells from WT animals, but not from those of NPC animals (**Fig.28A**) indicating that glial cells without functional NPC1 could not induce excitatory synaptic activity in PCs. On the other hand, this change was not observed in PCs from WT animals indicating that glial cells could induce this effect only in neurons lacking functional NPC1. Notably, this is somehow a reverse effect as the one observed for the presence of sIPSCs in GCs. Coculture with glial cells from WT or NPC animals enhanced significantly the frequency of sEPSCs in WT and NPC PCs (**Fig.28B**). Thus, mouse PCs showed a remarkably similar behaviour as rat RGCs (Pfrieger and Barres, 1997). Although overall, the glia-induced increase in synaptic activity did not require functional NPC1 in PCs or glia, there was a remarkable difference: Whereas NPC-Glia induced a stronger effect than WT-Glia in WT neurons, the reverse was true for NPC neurons, where WT-Glia was more efficient than NPC-Glia. It appears as if the lack of either neuronal or glial NPC1 led to a stronger synaptic activity than its presence or absence in both. Coculture with glial cells significantly increased the size of sEPSCs in PCs compared to glia-free cultures regardless of the presence of functional NPC1 in neurons or glia (**Fig.28C**). None of the effects on excitatory synaptic activity was mimicked by GCM.

To further test whether the increase in the frequency and size of sEPSCs in PCs was due to changes in action potential-dependent and -independent synaptic transmission, we recorded mEPSCs in the presence of TTX. As for spontaneous excitatory activity, contact with glial cells significantly enhanced the frequency (**Fig.29B**) and size (**Fig.29C**) of mEPSCs regardless of the presence of functional NPC1 in PCs or glia. This indicated that glial cells enhanced the level of spontaneous excitatory synaptic activity by increasing the number or efficacy of individual synapses independently from action potential-dependent transmitter release. Clearly, these effects did not require functional NPC1 in neurons or glial cells.

As for GCs, glial signals affected spontaneous inhibitory activity in PCs differently than spontaneous excitatory events. The fraction of WT-PCs showing sIPSCs was enhanced significantly by glial cells from WT and NPC animals (**Fig.30A**), but GCM did not mimick .

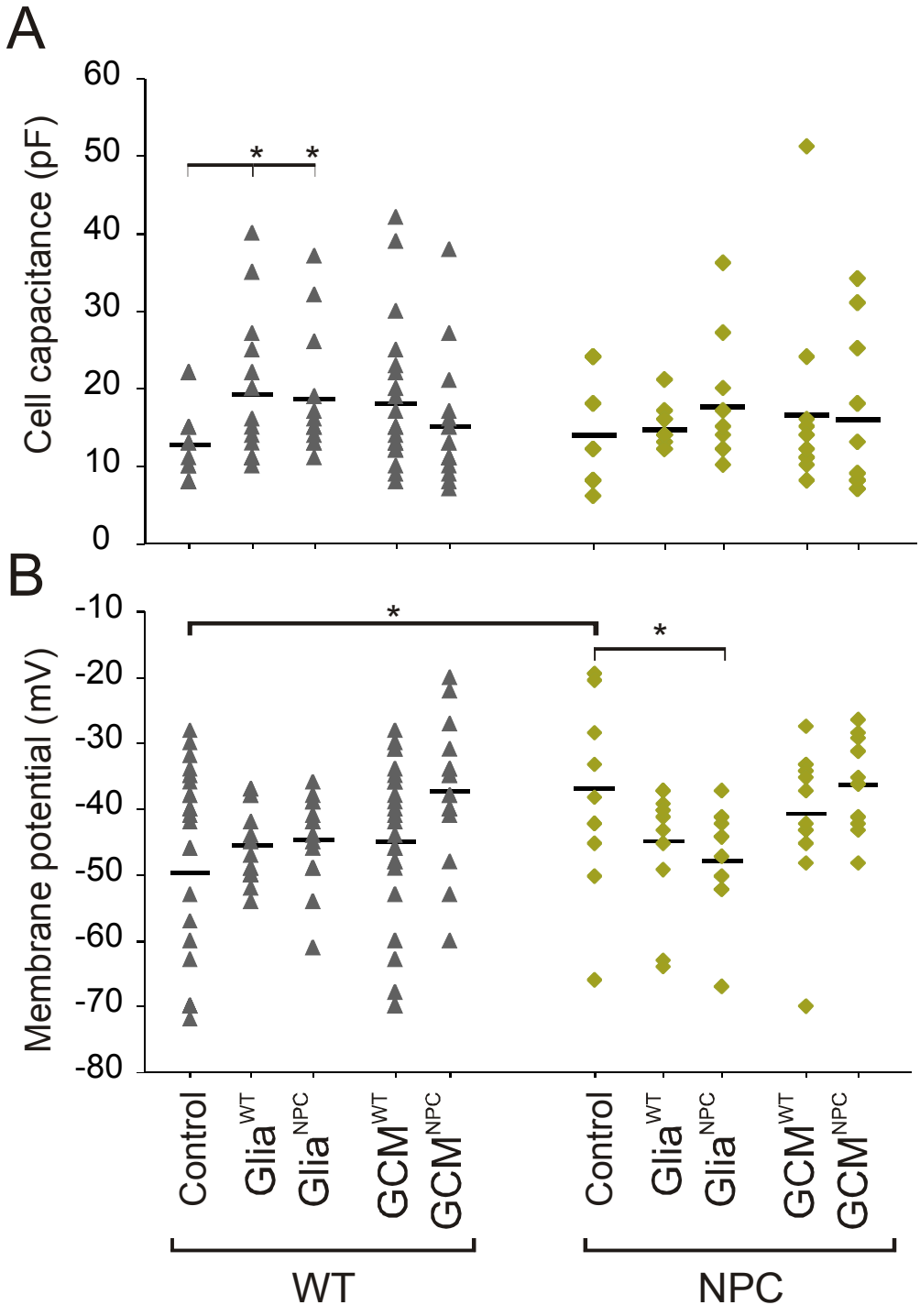

Fig.31: Cell capacitance and resting membrane potential in WT and NPC PCs
Scatterplots of cell capacitance (**A**) and resting membrane potentials (**B**) of individual PCs that were immunoisolated from WT (left) and NPC (right) animals and cultured for seven days in the absence of glia (control; n= 29 WT-, n = 11 NPC-PCs), together with WT or NPC glia (WT-PC: n = 15 $Glia^{WT}$; 14 $Glia^{NPC}$; NPC-PC: n = 12 $Glia^{WT}$; 10 $Glia^{NPC}$), or after treatment with or WT or NPC GCM (WT-PC: n = 32 GCM^{WT}; 25 GCM^{NPC}; NPC-PC: n = 8 GCM^{WT}; 13 GCM^{NPC}). Horizontal bars indicate mean values and asterisks indicate statistically significant changes (Kruskal-Wallis ANOVA followed by post-hoc comparison of mean ranks for A and B and Mann-Whitney U test in B)

this effect. In PCs from NPC animals, glial cells from WT and NPC animals also enhanced the fraction of PCs with sIPSCS, but the effect did not reach statistical significance (**Fig.30A**). Frequencies of sIPSCs are shown in **Fig.30B**, but we note that due to the low incidence, in particular under glia-free conditions (for WT-PC: n = 1 out of 19; for NPC-PC: 0 out of 10), no statistical comparison could be performed. Taking into account the effects on the fraction of PCs showing inhibitory activity (**Fig.30A**) and on the frequencies of sIPSCs (**Fig.30B**), we can conclude that glial cells from WT and NPC animals enhanced inhibitory activity in PCs from WT animals, but not or to a much lesser extent in PCs from mutant animals. The low incidence of inhibitory activity in PCs precluded an analysis of mIPSCs.

Taken together, glial cells affected synaptic activity in PCs more strongly than in GCs. Interestingly, glial cells induced excitatory synaptic activity in PCs, but this effect required functional NPC1 in glia, but its absence from neurons. Glial signals strongly enhanced the frequency and size of sEPSCs and of mEPSCs indicating that they affected the number or efficacy of individual synapses. Overall, these effects did not require presence of functional NPC1 in neurons or glia, but interestingly, the increase in the frequency of sEPSCs was stronger, if glia or neurons contained functional NPC1, than if it was absent or present in both. Glial cells also enhanced the level of inhibitory synaptic activity in PCs, but this effect was mainly seen in WT PCs.

3.4. Influence of glial signals on the excitability of PCs and the relevance of functional NPC1 in PCs and glial

The prominent glia-induced increase in the frequency and size of sEPSCs in PCs could have been due to an enhanced excitability. To address this issue, current-clamp recordings were performed on PCs from WT and NPC animals in the absence or presence of glial cells or GCM from WT and NPC animals. We first examined whether the cell capacitance and the membrane potential of PCs were affected by glial cells from WT and NPC animals. The capacitance of PCs from WT and NPC animals was very similar (**Fig.31A**). Coculture with WT or NPC glia enhanced significantly the capacitance of WT PCs (**Fig.31A**).These effects were not mimicked by WT- or NPC-GCM. On the other hand, the capacitance of NPC-PCs was unaffected by glial signals (**Fig.31A**). This indicated that glial signals enhanced the size of PCs with a functional NPC1, but failed to do so in neurons with a mutated protein. This increased capacitance could explain the increased EPSC size in WT-PCs, but not for NPC-PCs, which precludes this hypothesis.

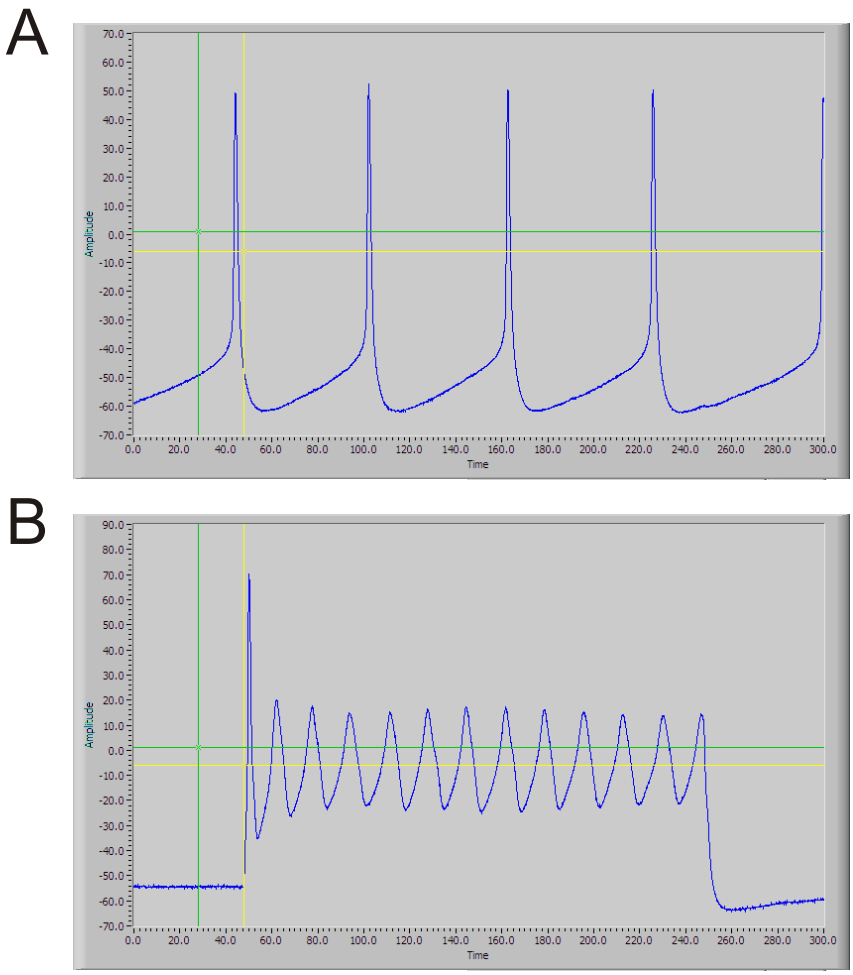

Fig.32: Action potentials in PCs
Traces of APs that occurred spontaneously (**A**) or after current injection (**B**; 0.5 nA) recorded in current-clamped mode from PCs that were cultured for 7 DIV.

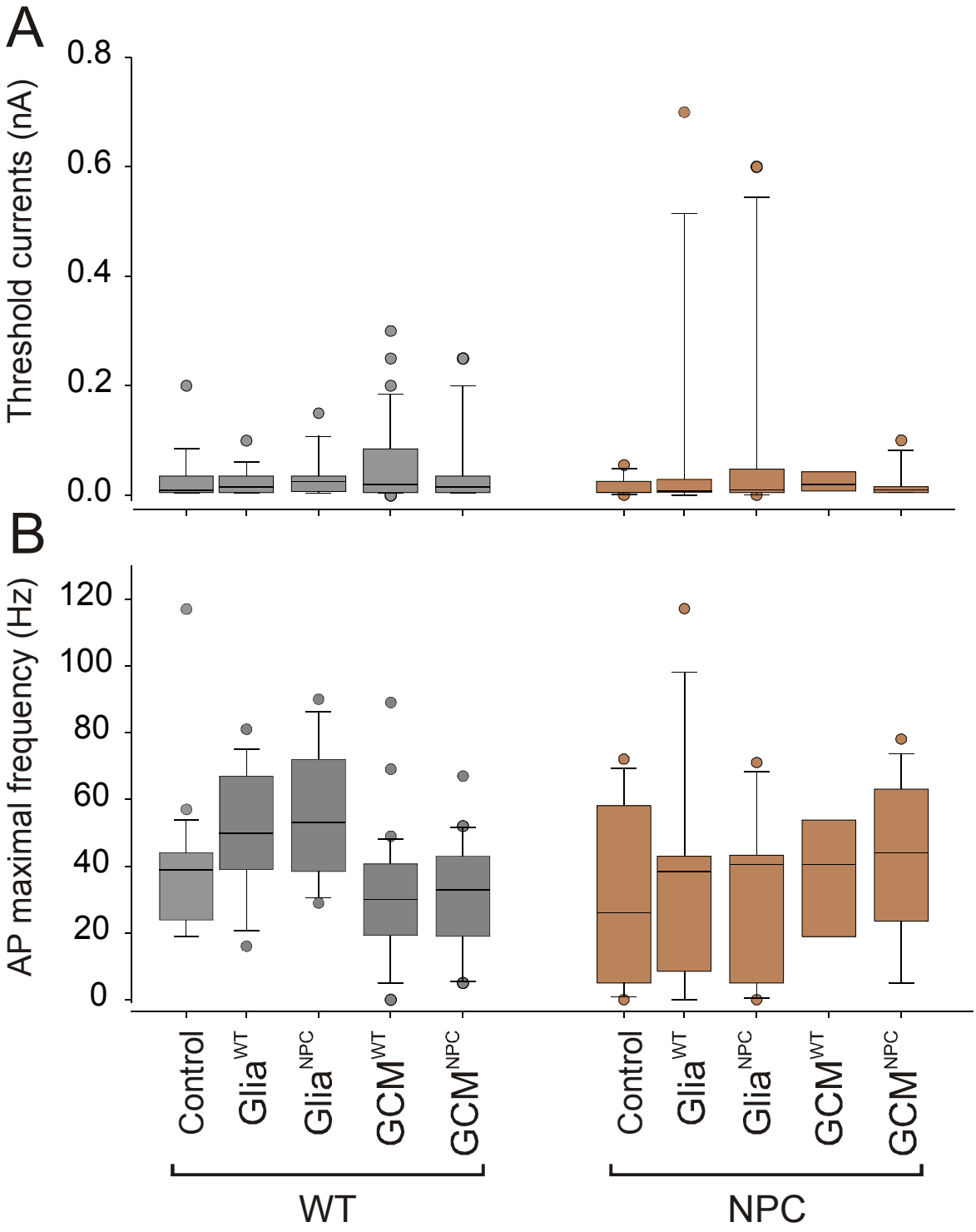

Fig.33: Action potential firing of Purkinje cells from WT and NPC mice
Boxplots of injected threshold currents necessary to induce action potentials (**A**) and of the maximal frequency of action potentials elicited by 0.5 nA depolarizing currents (**B**). PCs were cultured for 7 DIV in the absence of glia (control), together with WT/NPC Glia or after treatment with WT/NPC GCM.

The resting membrane potential of NPC-PCs was significantly more depolarized than that of WT-PCs (**Fig.31B**) indicating that functional NPC1 helped to maintain a normal hyperpolarized resting potential in these neurons. Glial signals did not affect the membrane potential of PCs from WT or NPC animals (**Fig.31B**) except for a small, but significant shift to more hyperpolarized values in coculture of NPC-PCs with NPC-glia (**Fig.31B**). We next investigated whether the glia-induced increase in synaptic activity in PCs was due to an enhanced action potential firing. Similar fractions of WT (12%; n=100 cells) and NPC (17%; n=73 cells) PCs exhibited spontaneous AP firing at frequencies of up to 20 Hz. Almost all cells from WT (96%) or NPC (88%) mice fired APs in response to current injection and the discharge of elicited APs was always tonic (**Fig.32**).

To explore possible glial effects on the excitability of PCs, we determined threshold currents necessary to trigger an AP (**Fig.33A**) and maximal frequencies of APs in response to depolarizing current steps of fixed size (0.5 nA) (**Fig.33B**). Boxplots in **Fig.33** indicate that the threshold current and the maximal frequency were non-normally distributed. Overall, threshold currents were very similar in PCs from WT and NPC animals, and no glia-induced changes were observed (**Fig.33A**). The maximal firing frequency of PCs was variable ranging from 5 to 120 Hz with no significant difference between WT and NPC animals under control conditions (**Fig.33B**). Coculture with WT- and NPC-glia appeared to enhance the AP frequency in WT-PCs, but this effect did not reach statistical significance. PCs from NPC animals did not show this effect and GCM did not change this parameter under any condition (**Fig.33B**).

Taken together, these results suggest that the effects of glial cells on synaptic activity of WT and NPC PCs were not due to changes in their excitability.

4. Dendrite differentiation and synapse formation in GCs and PCs and relevance of glial cells and functional NPC1 in neurons and glia

The glia-induced increase in the frequency and size of mEPSCs in PCs of WT and NPC animals could have been due to changes in neuronal differentiation, particularly in changes in the number of synapses. To test this, we labeled PCs under different culture conditions using antibodies against MAP2 (**Fig.34**), a marker for mature dendrites, and synaptophysin, a marker for presynaptic terminals (**Fig.35**).

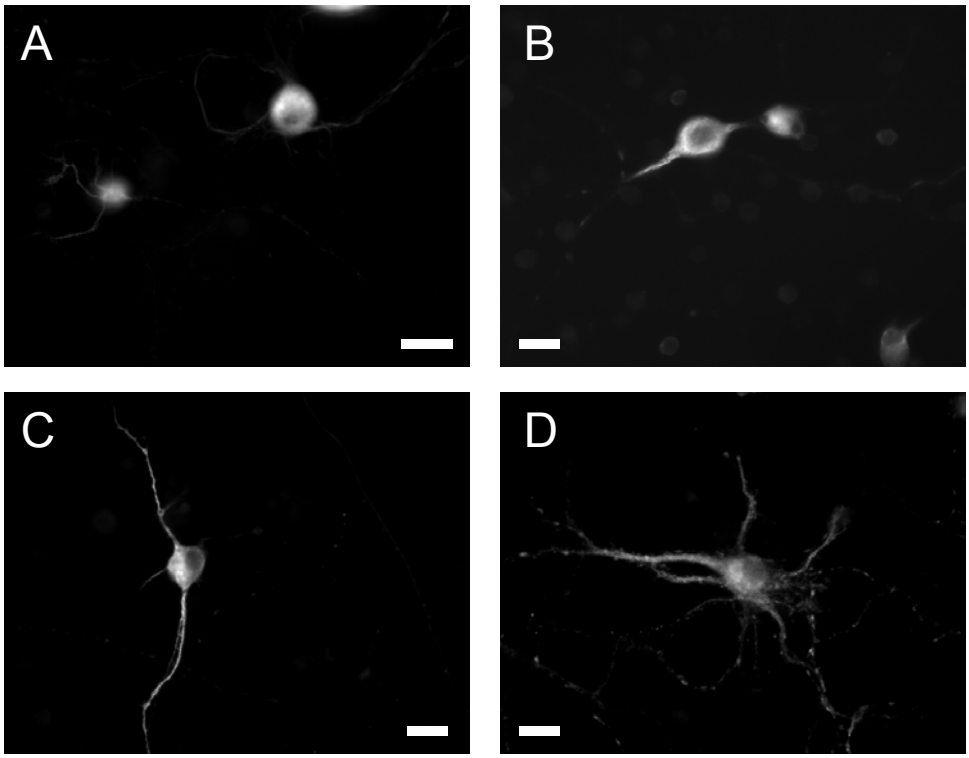

Fig.34: Stages of dendrite differentiation in immunoisolated PCs
Representative fluorescence micrographs of immunoisolated PCs that were cultured for 7DIV in the presence of WT glia and then immunostained with a MAP2-specific antibody. **A**: MAP2 only in cell bodies (= no dendrite). **B**: Map2 localized in single neurites close to the soma (first dendrites). **C**: Map2 localized in entire neurites (prolonged dendrites). **D**: neurons with Map2 staining in dendrites and branches (mature dendrites). Scale bar = 20µm.

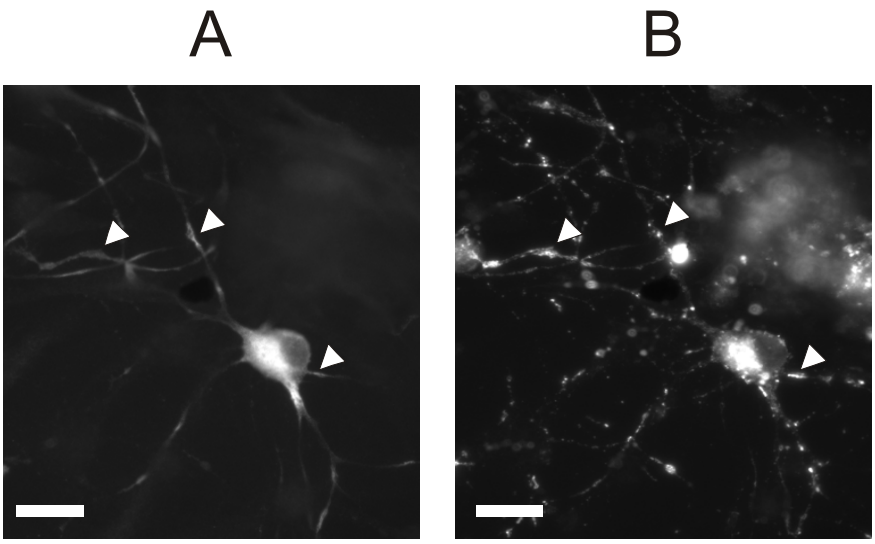

Fig.35: Visualization of axodendritic synapses
Fluorescence micrographs of PCs cultured for 7 DIV in the presence of WT glia and then double-stained with antibodies against MAP2 (**A**) and synaptophysin (**B**). Scale bar = 20µm.

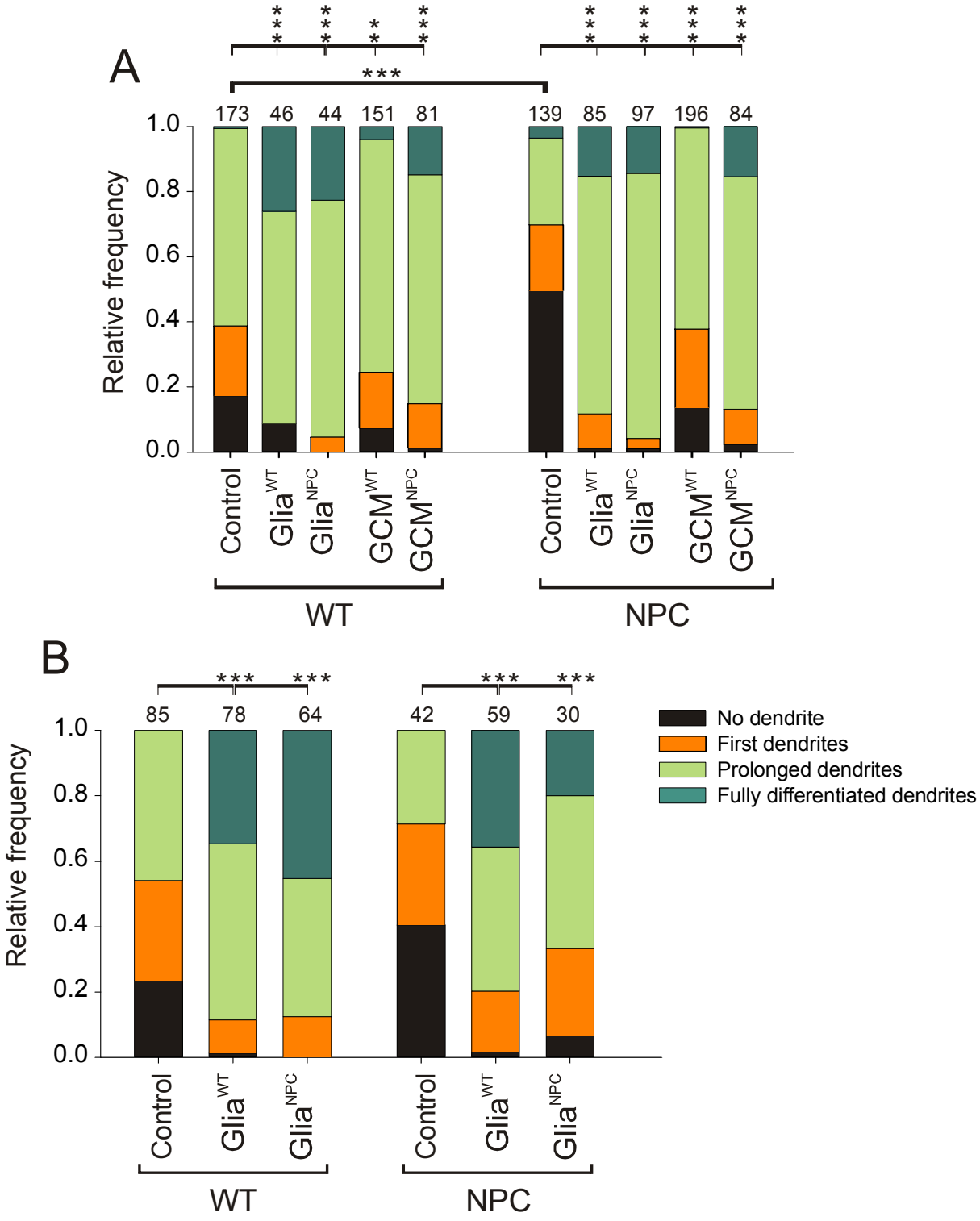

Fig.36: Dendrite differentiation in GC and PC neurons from WT and NPC mice
Stacked bars representing different stages of MAP2-based dendrite differentiation in GCs (**A**) and PCs (**B**) when cultured for 7 DIV in the absence of glia (control), together with WT/NPC Glia or after treatment with WT/NPC GCM.

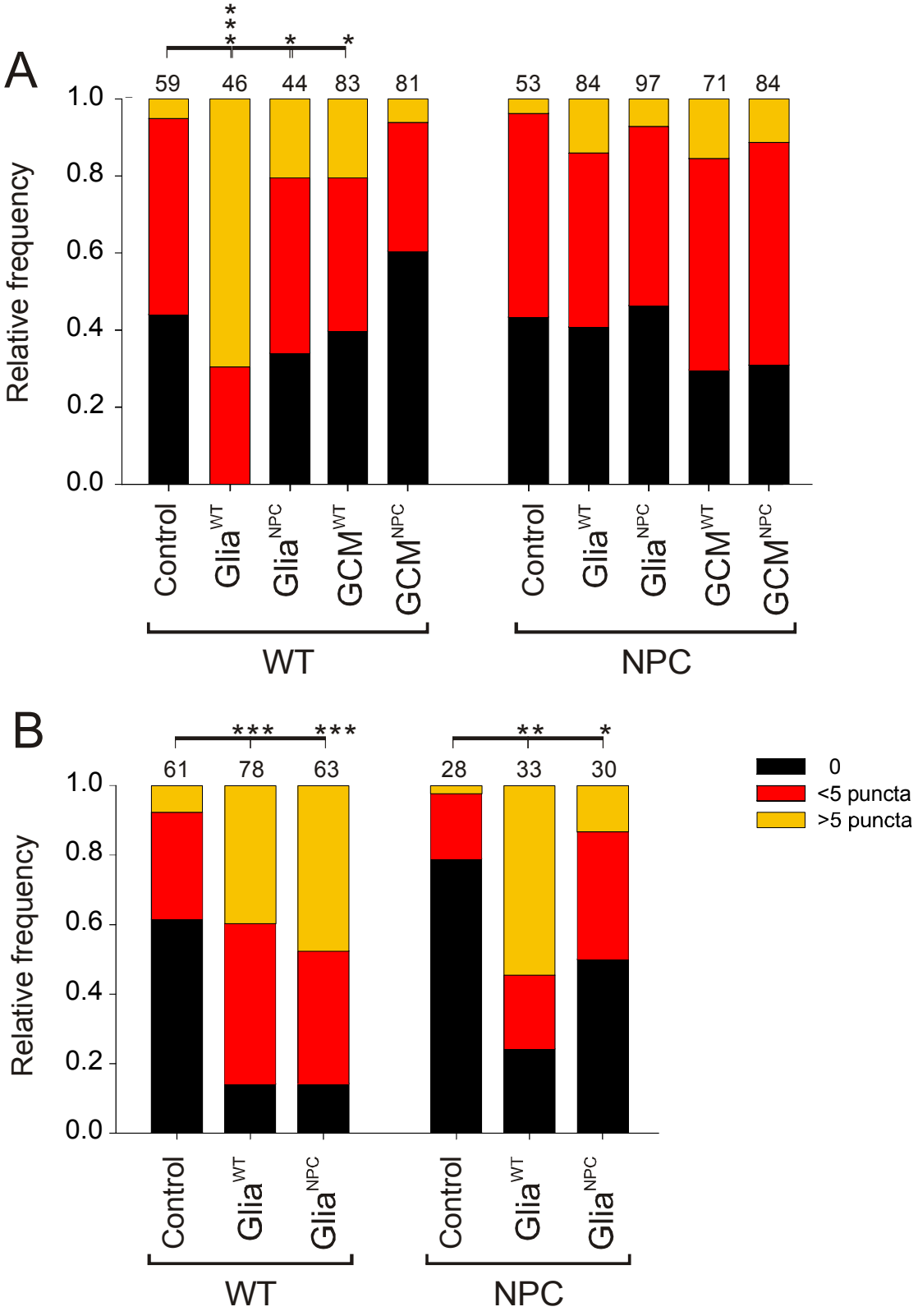

Fig.37: Formation of axo-dendritic synapses in immunisolated GCs and PCs from WT and NPC mice
Stacked bars representing the number of synaptophysin-positive puncta that impinged on MAP2-positive dendrites in GCs (**A**) or PCs (**B**) that were cultured for 7 DIV in the absence of glia (control), together with WT/NPC Glia or after treatment with WT/NPC GCM.

Interestingly, GCs from WT animals exhibited a significantly higher level of Map2-based dendrite differentiation (**Fig.36A**) compared to GCs from NPC animals, where nearly have of the neurons lacked MAP2-positive dendrites . This indicated that absence of NPC1 impairs dendritogenesis in these neurons. Coculture with glia, but also treatment with GCM from WT and NPC animals strongly enhanced the level of dendrite differentiation in WT-GCs and in NPC-GCs (**Fig.36A**). This indicated that glial signals enhanced dendritogenesis regardless whether they contain functional glial NPC1 and that they could overcome the impaired dendritogenesis in NPC-GCs.

In contrast, PCs from WT and NPC animals showed the same, moderate degree of dendrite differentiation under control conditions: none of the cells examined showed mature, branching dendrites (**Fig.36B**). Coculture with WT and NPC glia significantly enhanced the number of WT- and NPC-PCs exhibiting dendrites with a prominent induction of branches (**Fig.36B**). These results indicated that in the absence of glia, PCs show a moderate level of dendritogenesis and synaptogenesis regardless of the presence of functional NPC1. Glial signals strongly promoted dendrite formation in PCs again irrespective of the presence of functional NPC1.

To study the level of synapse formation in GCs and in PCs in the presence or absence of glia, we determined the number of synaptophysin-positive puncta that impinged on MAP2-positive dendrites (**Fig.35**).

Under glia-free conditions, the large majority of WT- and NPC-GCs showed no or only few synaptophysin/MAP2-positive puncta (**Fig.37A**). Notably, coculture with WT glia strongly increased the number of double-stained puncta so that the large majority showed more than >5 synapses. (**Fig.37A**). Contact with NPC glia also enriched the number of synapses in WT-GCs but to a lesser degree than WT glia, and GCM from WT animals mimicked this effect partially, whereas WT-PCs treated with NPC-GCM showed essentially no change compared to untreated controls (**Fig.37A**). Notably, in NPC-GCs neither contact with glia nor GCM changed the number of synaptophysin/MAP2-positive puncta (**Fig.37A**).

Under control conditions, less than half of PCs from WT or NPC animals showed immunocytochemically defined synapses (**Fig.37B**). Contact with WT glia greatly increased the number of synapses in PCs from WT and NPC animals with half of PCs showing >5 synaptophysin/MAP2 positive puncta (**Fig.37B**). Coculture with NPC glia enhanced the number of synapses similarly to WT glia in WT-PCs, but showed less of an effect in NPC-PCs (**Fig.37B**).

Taken together, these results indicated glial signals promote dendrite differentiation and synapse formation in WT cells. In contrast, their effect on NPC seemed to be cell-specific.

Discussion

1. Immunoisolated cerebellar neurons as model to study the relevance of neuron-glia interactions in WT and NPC postnatal mice

We have established a new primary culture model that allows us to purify cerebellar granule and Purkinje neurons from postnatal mice and to culture them under chemically defined, glia-free conditions. To our knowledge, this allows for the first time to study the relevance of glia for postnatal development of dissociated GCs and PCs. Up to now, methods for separating different cerebellar cell types from postnatal mice included density gradient centrifugation for GCs (Barkley *et al.*, 1973) and FACs sorting of fluorescently labeled PCs in transgenic mice, where expression of GFP is under control of the L7 promoter (Tomomura *et al*., 2001). As GCs are the smallest cells of the cerebellum, centrifugation on a density gradient allows their separation from macroglial cells and other neurons. The choice to immunoisolate cerebellar neurons including GCs and PCs originated from a parallel study in our group, where we immunoisolated neurons from different brain regions of postnatal mice including hippocampus and retina to study the relevance of glial cells for synapse development (Steinmetz, Buard, Claudepierre, Nagler, Pfrieger; manuscript in preparation). The isolation procedure yielded about 2 million cells per animal, whereas the total number of granule cells averages around 25 millions per animal. This indicates that we only isolated a fraction of ~8%. This is probably due to the fact that L1 is expressed, in early postnatal stages, only by a subset of GCs that are mainly located in the internal part of the EGL (Rathjen and Schachner, 1984). Our experiments showed that the cultures did not contain dividing cells, thus, even if our procedure selected GC precursors, they stopped mitosis under the defined culture conditions. In contrast to GCs, PCs cannot be isolated by density gradients from postnatal animals, as they accumulate with macroglial cells in high density fractions (Barkley *et al*., 1973). That is why we first separated L1+ cerebellar neurons from glia, and then enriched this pure neuronal population for PCs by an additional immunoisolation step. A previous study reported that pre-plating of cerebellar cells on Thy1 antibody coated culture dish (Bossu *et al*., 1989) allowed the selection of PCs, but they were always "contaminated" by glial cells. Moreover, cultivation of PCs on a culture plate covered with Thy1 antibodies enhanced the survival of PCs (Messer *et al*, 1984) indicating that this surface marker selects for PCs. Our two-step immunoisolation increased the proportion of PCs from around 0.5% *in vivo* to 50% and thus around 100-fold. Our isolation procedure yielded around 5,000 PCs per

animal which corresponds to 3% of the total number of PCs averaging 150,000 per animal (Zanjani *et al*, 2004).

We showed that isolated neurons required a chemically defined medium supplemented with BDNF, CNTF and forskolin to survive and grow indicating that they clearly need external signals. Isolated rat RGCs also showed this requirement (Mauch *et al.*, 2001). Previous studies had also reported that survival of GCs requires constant depolarization by high concentrations of potassium chloride (Armanino *et al*., 2005). This effect was possibly mimicked by forskolin, which is thought to mimick electrical activity (Meyer-Franke *et al*., 1995). In addition, our GC culture contained around 7% GABAergic neurons. This reflects their proportion *in vivo* indicating that these neurons survive in our conditions.

The immunoisolation procedures were successfully applied to NPC postnatal mice which allowed us to study the relevance of neuron-glia interactions in this disease, and specifically, whether possible NPC-induced changes in neurons occur cell-autonomously or due to glial defects. The yield of L1+ NPC cells was lower compared to WT. This probably reflected a loss of GCs rather than PCs, as the latter contributed only a small fraction to the L1+ cells. It appears possible that loss of GCs occurs in NPC animals. However, because of their huge number in the cerebellum, it is probably hard to detect. Thus, most studies did not describe any GC loss in the NPC mouse, except for Griffin *et al.* (2004) who reported a decrease of NeuN-immunolabeled granule cells in adult NPC mice. As the yield of PCs was very low even in WT animals, it was not possible to compare the yield in WT and NPC mice.

Our chemically defined FSM supported similar survival rates of GCs from WT and NPC animals indicating that their survival requirements at least at the early postnatal stage are not different. Furthermore, we have no evidence that immunoisolated PCs from NPC mice degenerated specifically in our culture model. This is probably due to the fact that we isolated PCs from one week old animals and thus well before PCs degenerate in NPC mice (Tanaka *et al*, 1988).

Filipin staining revealed that the sterol accumulation that is characteristic for cells from NPC mice or patients occurred also in GCs and PCs immunoisolated from P7 NPC mice as well as in glial cells. An early postnatal onset of sterol accumulation has been already been reported in nine day old NPC PCs and in thalamic astrocytes from three week old animals (Reid *et al.*, 2004). The sterol accumulation was observed even in control conditions, *i.e.* in the absence of glia, indicating that our culture conditions allowed for the expression of this characteristic feature of NPC-affected cells. Given that the accumulation occurred in the absence of an external source of cholesterol, it is possible that cholesterol accumulation is an

intrinsic process. Addition of glia-secreted factors to NPC neurons increased the intracellular cholesterol accumulation, regardless of the presence of NPC1 in glial cells. This observation confirms the idea that glial cells produce and secrete cholesterol that is internalized by neurons via lipoproteins (Mauch *et al.*, 2001). Moreover, the fact that both WT and NPC glial cells induced cholesterol accumulation is probably due to similar density and size of lipoproteins secreted by WT and NPC glial cells (Karten *et al.*, 2005). Treatment with GCM or cholesterol enhanced the cholesterol content in WT cell bodies and dendrites, as previously shown in rat RGCs (Mauch *et al.*, 2001; Goritz *et al.*, 2005), whereas in NPC neurons, cholesterol accumulated mainly in the cell body but not in dendrites. This result is consistent with the study of Karten *et al.* (2002) who reported that cholesterol accumulates in cell bodies of sympathetic neurons from NPC animals, but is decreased in distal axons, due to an impaired cholesterol transport. However, their observation concerned only endogenously-synthesized cholesterol while LDL-cholesterol transport was normal. In contrast, our study suggests that exogenously supplied cholesterol becomes entrapped in the soma of GCs and PCs of NPC animals.

We studied the electrophysiological properties of isolated granule and Purkinje neurons with and without functional NPC1. To our knowledge, our study together with the abstract of the Maue group (Boegle *et al*., 2005) is the first on this subject. The majority of previous studies on NPC concerned intracellular trafficking defects, and there has been only one study, which examined electrophysiological properties of cortical neurons in culture and in slices from NPC animals (Deisz *et al*, 2005). This study did not observe differences in cortical neurons from embryonic mutant and WT animals. In contrast to this, our recordings revealed, however, a prominent difference as PCs from NPC animals showed clearly more depolarized resting membrane potentials than those from WT animals. Since the maintenance of the resting membrane potential requires energy, this NPC-effect could be due to an involvement of NPC in mitochondrial function as proposed previously (Yu *et al.*, 2005). Thus, NPC deficient PCs may be less capable to generate the energy required for the maintenance of ionic gradients. This is further supported by the fact that the resting potential is restored in the presence of glia from WT or NPC animals, which provide neurons with energy metabolites (Tsacopoulos and Magistretti, 1996).

2. Synaptic development of isolated neurons in the absence of glia

Our results showed that GCs and PCs differed in their ability to form synapses in the absence of glial cells. GCs showed robust excitatory synaptic activity and thus appeared to make readily synaptic connections among themselves. Evidently, one could argue that such synapses are non-natural, as GCs normally make contact exclusively to PCs. However, the same argument applies for other preparations of immunoisolated neurons like motoneurons (Ullian *et al*., 2004) and RGCs (Pfrieger and Barres, 1997) in which synaptogenesis has been studied and which normally do not form synapses among each other. In contrast to these cells, which required glial signals for synaptogenesis, GCs did not and showed robust synaptic activity in the absence of glia. Frequencies of EPSCs and IPSCs in glia-free GC cultures were in fact similar or even higher as those reported previously in conventional cerebellar cultures (Virginio *et al.*, 1995; Losi *et al.*, 2002; Fiszman *et al.*, 2005) derived from postnatal rats or mice. This indicated that under our culture conditions, purified cerebellar neurons reach the same level of connectivity as those growing in glia-containing primary cultures. The low incidence of inhibitory synaptic activity probably reflected the fact that these cultures contain only few inhibitory neurons. Comparison of synaptic activity in GCs derived from WT and NPC animals revealed no difference suggesting that in GCs, functional NPC1 protein is dispensible for the establishment of excitatory and inhibitory synapses.

On the other hand, PCs showed a very low level of excitatory synaptic activity indicating that they cannot receive synaptic input from GCs in the absence of glia. The low incidence of inhibitory activity in WT and NPC animals was probably due to the low incidence of other GABAergic neurons or due to the fact that PCs do not form synapses among each other, at least in the absence of glia. Functional NPC1 protein appeared relevant for synaptic activity in PCs, as the frequency and size of spontaneous excitatory events were remarkably smaller in PCs from NPC compared to WT animals. The low level of synaptic activity in PCs under glia-free conditions precluded an analysis of mEPSCs. However, given that mEPSCs in the presence of glia were significantly larger than sEPSCs under control condition the spontaneous events were probably mainly due to quantal release. This implies that NPC1-dependent changes in size and frequency of spontaneous activity are due to changes at individual synapses. The reduced EPSC size in NPC-PCs could also be a consequence of defective cholesterol transport in NPC cells. Recent studies have shown that changes in cholesterol content affect action potential-dependent transmitter release and exocytosis by modifying protein complexes that are involved in exocytosis (Mitter *et al*,

2003; Salaun *et al*, 2004; Pfrieger, 2003). Thus, one can imagine that a variation in the level of membrane cholesterol in vesicles can impair the efficiency of exocytosis, membrane fusion or neurotransmitter release. This could results also result in a reduction of synaptic vesicles that are available for action potential-evoked release, as observed in another neurodegenerative lysosmal storage disease, the neuronal ceroid lipofuscinosis-1 (Virmani *et al*, 2005). Moreover, the association of NPC1 with recycling endosomes in presynaptic nerve terminals of sympathetic neurons (Karten *et al*, 2006) may explain why a deficiency in NPC1 could affect exocytosis.

The differences in synaptic levels between GCs and PCs in glia-free conditions could have been partially due to impaired dendrite differentiation in GCs compared to PCs. However, both exhibited the same level of differentiation which precludes this hypothesis. When isolated from glia, only few immunoisolated GCs or PCs differentiated mature dendrites. This is particularly the case for PCs, whose highly branched dendritic arbor enables the generation of complex spikes (Ito, 1987). Immunoisolated PCs developed only few dendrites with no branches and no complex spike under control conditions. Evidently, the short culture period may not have allowed for dendrite development, which in many neurons requires several weeks (Kapfhammer, 2004). However, in occasional three week old cultures, PCs did not show more developed dendrites. Until now, the factors controlling dendritic development of PCs remain unclear.

Interestingly, the lack of functional NPC1 appeared to impair dendrite development: our immunocytochemical staining with a dendrite marker MAP2 revealed a lower degree of dendritic differentiation in GCs lacking functional NPC1 compared to those from wildtype animals. Although this difference was most prominent in GCs, there was also a tendency in PCs. Consistent with this observation, Sarna *et al.* (2003) described a reduced dendritic arborization and dendritic and axonal alterations of NPC PCs compared to WT (Zervas *et al.*, 2001). So far, there has been no report that also dendrite differentiation in GCs is impaired in NPC animals. NPC1 may be required for dendrite differentiation due to its role in cholesterol transport, as a previous study showed that cholesterol availability limits the degree of dendritic development in immunoisolated RGCs (Goritz *et al*., 2005). An impaired intracellular transport of cholesterol due to NPC1 deficiency may have the same effect. Despite the lower degree of dendrite differentiation in NPC1-deficient GCs, the density of axo-dendritic synapses appeared unchanged in these cells indicating that this did not impair synaptogenesis.

3. Glial cells promote development of synapses between GCs and PCs independently from the presence of functional NPC1

Our study revealed that glial signals induced a very strong increase in the frequency and size of excitatory synaptic events in PCs indicating that they promote the formation of natural synapses from GCs onto PCs. Notably, this effect resembled the glia-induced increase in synaptic activity in RGCs (Pfrieger and Barres, 1997) and motoneurons (Ullian *et al*., 2004). We note that all three types of neurons are long-projecting and thus it appears possible that cells that form synapses in regions distant from their soma require glia to accomplish this. In contrast to RGCs and motoneurons, secreted factors from glia including cholesterol did not mimick this effect suggesting that it was mediated by contact-dependent signaling or that glial cells require the presence of neurons in order to release the synapse promoting activity. This possibility is supported by a previous study in which long-term potentiation is promoted in hippocampal neurons when they are grown in a layer of astrocytes but not when cultured with GCM (Hama *et al*., 2004).

Our immunocytochemical staining revealed that the enhancement of synaptic activity was at least in part due to an increase in dendrite maturation and in the number of synapses that impinged on PC dendrites. This effect is similar as observed in RGCs (Goritz *et al*., 2005) and adds further evidence that signals from glia promote dendritogenesis in CNS neurons (Lein *et al*., 2002). So far, there has been no study about the influence of glial cells on dendritic development of PCs. This is mostly due to the fact that the current experimental models to study PCs contain glial cells and thus do not allow to evaluate their contribution to dendritogenesis. It should be noted that in previous studies, pharmacological ablation of glial cells *in vivo* and *in vitro* affected the dendrites of PCs (Cui *et al.*, 2001; Drake-Baumann and Seil, 1999) but the underlying mechanisms remain unclear.

Coculture with glia enhanced the size of miniature EPSCs in PCs indicating that glial signals strengthened individual synapses by enhancing their response to quantal release or the concentration of glutamate in synaptic vesicles of GCs. The fact that similar changes in the size of mEPSCs were not observed in GC cultures argues against a presynaptic effect and indicates that glial cells enhance the postsynaptic sensitivity of PCs to glutamate. This may be linked to the presence of more mature dendrites in cocultures, which may facilitate production of glutamate receptors and their postsynaptic insertion.

Notably, the effects of glial cells on excitatory synaptic activity, dendrite differentiation and synaptogenesis in PCs were independent from the presence of functional

NPC1 in PCs or in glial cells. Thus, loss of NPC1 from PCs and GCs did not impair their ability to establish synaptic connections in the presence of glial signals. On the other hand, the synapse-promoting activity of glial cells was similarly independent from functional NPC1. Our study further revealed that coculture with glia increased the number of cells showing spontaneous IPSCs in PCs from WT animals, and to a lesser extent in NPC animals. A remarkably similar effect occurred in GCs. This could have been due to the fact that glia enhanced the number of inhibitory neurons in both cultures, for example by supporting their survival or glial cells may have increased the ability of PCs to receive GABAergic input. At present, it is not possible to distinguish between these two possibilities.
Taken together, our study provides evidence that glial cells strongly promote the formation of excitatory connections between GCs and PCs and this does not require functional NPC1 in neurons or glial cells.

4. The absence of functional NPC1 in GCs and/or in glial cells impairs development of excitatory and inhibitory synapses

In contrast to their effects on PCs, glial cells did not induce major changes in the level of excitatory synaptic activity in GCs. On the other hand, they enhanced the incidence of inhibitory activity similarly as in PCs. This further supports the idea that CNS neurons have different requirements for the establishment of excitatory and inhibitory synapses.

First, coculture with glial cells did not enhance the frequency of excitatory synaptic activity in GCs as observed for PCs. Our immunocytochemical staining revealed on the other hand that coculture with glial cells promoted dendritogenesis and synaptogenesis in GCs, but this did not result in a higher level of activity. It is possible that the additional synapses were functionally silent or immature. As observed for PCs, coculture with glia also enhanced the size of sEPSCs in GCs. But in contrast to PCs, this effect did not occur in mEPSCs indicating that it was due to changes in action potential-dependent synaptic activity, for example a change in the excitability of GCs or in the efficacy of calcium-dependent transmitter release. Coculture with glia doubled the fraction of GCs with sIPSCs similarly as in PCs indicating that in both cultures, glial signals enhance the chance that GCs receive inhibitory synaptic input. As outlined above, this may be due to an increase in the number of GABAergic neurons that survive, their ability to form synapses or the ability of GCs to receive inhibitory input. On the other hand, no changes in the frequency and size of sIPSCs occurred.

Importantly, our study revealed that the presence of functional NPC1 in glia and GCs determined how glial cells influence synaptic activity in GCs. This was in stark contrast to

PCs, where the absence of NPC1 in neurons or glia did not influence glia-induced changes in the level of synaptic activity. Glial cells lacking NPC1 diminished the frequency of sEPSCs in GCs compared to WT glia. This effect did not occur in mEPSCs and involved therefore changes in action potential-dependent activity. Furthermore, glial cells lacking NPC1 failed to mimick the glia-induced increase in the size of sEPSCs, which also was due to changes in action potential-dependent synaptic transmission. Finally, absence of functional NPC1 in glial cells abolished the increase in the fraction of GCs with sIPSCs observed in cocultures with WT glia. Globally, absence of NPC1 from neurons reversed or abolished all glial effects on excitatory or inhibitory activity observed in WT GCs. Thus, GCs from NPC mice did not exhibit the glia-induced decrease in the frequency of sEPSCs or the increase in the fraction of neurons with as observed in WT neurons. On the other hand, glial signals strongly enhanced the frequency of sIPSCs only in NPC1-deficient neurons. Notably, whereas glial signals enhanced dendritogenesis in GCs regardless of the presence or absence of functional NPC1 in neurons or glia, only glial cells with functional NPC1 enhanced the number of synapses.

Taken together, the formation and/or function of excitatory and inhibitory synapses in GCs and their sensitivity to glial signals were profoundly affected by functional NPC1 in neurons and glia. So far, it is unclear how this influence comes about. The accumulation of cholesterol (and possibly other lipids) in the endosomal-lysosomal system, which, as shown by filipin stainings, occurs both in neurons and glia, may impair the function of this organelle. This then may impair the generation or release of glial signals that modify the formation or function of synapses or change the sensitivity to glial signals or otherwise disable their effects in neurons. It has been shown that astrocytes undergo pathological changes during NPC progression (Suzuki *et al.*, 2003). This includes alteration of glial transporters in the hippocampus of the mutant mouse (Byun *et al.*, 2006). Since glial transporters are involved in the neurotransmitter uptake, a change in the ambient concentration of glutamate or GABA may desensitize neuronal transmitter receptors on GCs and explain for example the lower levels induced by NPC glia.

5. Conclusion and outlook

We have established cell culture preparations of immunoisolated GCs and PCs from postnatal mice and we used them in combination with a natural mouse model of NPC disease to determine whether the absence of functional NPC1 protein causes changes in synaptic development and function, and whether such changes occur in a cell-autonomous manner, or

are due to deficit of NPC1 in glia. Our main findings are first, that PCs require glial signals to receive excitatory synaptic input from GCs with glia-induced dendritogenesis playing a major role in this process. Based on previous studies showing a similar dependency on glia in RGCs and motoneurons, we hypothesize that long-projecting neurons may require glia to receive and establish synaptic connections with their pre- and postsynaptic partners. Second, glial signals enhanced the incidence of inhibitory activity both in PCs and GCs indicating that the ability of GABAergic interneurons (or PCs) to form connections or the ability of PCs and GCs to receive inhibitory input was enhanced by glia. Third, the absence of functional NPC1 in neurons and glial cells had profound, but distinct effects on synapse development and function in PCs and GCs. In PCs, but not in GCs, lack of NPC1 reduced the frequency and size of synaptic events originating from individual synapses, whereas glia cells promoted dendritogenesis and synapse formation in PCs independently from functional NPC1 in neurons or glia. In GCs, on the other hand, effects of glial cells on synaptic activity were abolished or reversed, when neurons or glia lacked NPC1. Based on our results, we propose a new hypothesis concerning the cellular culprit of PC loss in NPC disease. Since a deficit in NPC1 in neurons and glia affects more strongly synapses in GCs than in PCs, we hypothesize that the impairment and ultimately degeneration of PCs in NPC is due to a deficient synapse development or function in GCs and a breakdown of their interactions with glial cells. Given the massive input of PCs from GCs (175,000 parallel fibers; Napper *et al.*, 1988), PCs appear particularly vulnerable to changes in GCs In the future, it will be important to corroborate our findings and to test our hypotheses. Thus, for example it would be interesting to compare synapse formation and function in cocultures between PCs and GCs from WT and NPC1 mice.

In any case, our results indicate that a deficit of NPC1 in cells other than PCs, namely GCs and Bergmann glia, may contribute to their loss in NPC disease. This hypothesis needs to be tested with further experiments, including probably in vivo approach involving transgenic animals. If proven to be correct, this may also provide more defined cellular targets for a therapeutical approach to this devastating disease.

References

Adcock KH, Metzger F, Kapfhammer JP (2004) Purkinje cell dendritic tree development in the absence of excitatory neurotransmission and of brain-derived neurotrophic factor in organotypic slice cultures. Neuroscience 127: 137-145.

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (2002) Molecular Biology of the Cell

Altman J (1972a) Postnatal development of the cerebellar cortex in the rat. 3. Maturation of the components of the granular layer. J Comp Neurol 145: 465-513.

Altman J (1972b) Postnatal development of the cerebellar cortex in the rat. I. The external germinal layer and the transitional molecular layer. J Comp Neurol 145: 353-397.

Altman J (1972c) Postnatal development of the cerebellar cortex in the rat. II. Phases in the maturation of Purkinje cells and of the molecular layer. J Comp Neurol 145: 399-463.

Altman J and Bayer SA (1997). The Development of the Cerebellar System: In Relation to its Evolution, Structure, and Functions, CRC Press

Altmann SW, Davis HR, Jr., Zhu LJ, Yao X, Hoos LM, Tetzloff G, Iyer SP, Maguire M, Golovko A, Zeng M, Wang L, Murgolo N, Graziano MP (2004) Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. Science 303: 1201-1204.

Armanino M, Gravielle MC, Natalia MM, Fiszman ML (2005) NMDA receptors contribute to the survival promoting effect of high potassium in cultured cerebellar granule cells. Int J Dev Neurosci 23: 545-548.

Baptista CA, Hatten ME, Blazeski R, Mason CA (1994) Cell-cell interactions influence survival and differentiation of purified Purkinje cells in vitro. Neuron 12: 243-260.

Barkley DS, Rakic LL, Chaffee JK, Wong DL (1973) Cell separation by velocity sedimentation of postnatal mouse cerebellum. J Cell Physiol 81: 271-279.

Baudry M, Yao Y, Simmons D, Liu J, Bi X (2003) Postnatal development of inflammation in a murine model of Niemann-Pick type C disease: immunohistochemical observations of microglia and astroglia. Exp Neurol 184: 887-903.

Berger AC, Hanson PK, Wylie NJ, Corbett AH (2005) A yeast model system for functional analysis of the Niemann-Pick type C protein 1 homolog, Ncr1p. Traffic 6: 907-917.

Boegle AK; Paul CA; Fry M; Penatti CAA; Jarvinen MK; Yang P; Jones BL; Henderson LP; Maue RA (2005) Impaired electrical activity of Purkinje neurons in Niemann Pick type C disease is associated with altered sodium currents and synaptic function. Abstract No. 965.16. Washington, DC: Society for Neuroscience, 2005.

Bolin LM, Rouse RV (1986) Localization of Thy-1 expression during postnatal development of the mouse cerebellar cortex. J Neurocytol 15: 29-36.

Bossu JL, Dupont JL, Feltz A (1989) Calcium currents in rat cerebellar Purkinje cells maintained in culture. Neuroscience 30: 605-617.

Brady RO, Kanfer JN, Mock MB, Fredrickson DS (1966) The metabolism of sphingomyelin. II. Evidence of an enzymatic deficiency in Niemann-Pick diseae. Proc Natl Acad Sci U S A 55: 366-369.

Brewer GJ (1995) Serum-free B27/neurobasal medium supports differentiated growth of neurons from the striatum, substantia nigra, septum, cerebral cortex, cerebellum, and dentate gyrus. J Neurosci Res 42: 674-683.

Bu B, Li J, Davies P, Vincent I (2002) Deregulation of cdk5, hyperphosphorylation, and cytoskeletal pathology in the Niemann-Pick type C murine model. J Neurosci 22: 6515-6525.

Burns M, Gaynor K, Olm V, Mercken M, LaFrancois J, Wang L, Mathews PM, Noble W, Matsuoka Y, Duff K (2003) Presenilin redistribution associated with aberrant cholesterol transport enhances beta-amyloid production in vivo. J Neurosci 23: 5645-5649.

Byun K, Kim J, Cho SY, Hutchinson B, Yang SR, Kang KS, Cho M, Hwang K, Michikawa M, Jeon YW, Paik YK, Lee B (2006) Alteration of the glutamate and GABA transporters in the hippocampus of the Niemann-Pick disease, type C mouse using proteomic analysis. Proteomics 6: 1230-1236.

Carstea ED, Morris JA, Coleman KG, Loftus SK, Zhang D, Cummings C, Gu J, Rosenfeld MA, Pavan WJ, Krizman DB, Nagle J, Polymeropoulos MH, Sturley SL, Ioannou YA, Higgins ME, Comly M, Cooney A, Brown A, Kaneski CR, Blanchette-Mackie EJ, Dwyer NK, Neufeld EB, Chang TY, Liscum L, Strauss III JF, Ohno K, Zeigler M, Carmi R, Sokol J, Markie D, O'Neill RR, van Diggelen OP, Elleder M, Patterson MC, Brady RO, Vanier MT, Pentchev PG, Tagle DA (1997) Niemann-Pick C1 Disease Gene: Homology to Mediators of Cholesterol Homeostasis. Science 277: 228-231.

Costa E, Auta J, Guidotti A, Korneyev A, Romeo E (1994) The pharmacology of neurosteroidogenesis. J Steroid Biochem Mol Biol 49: 385-389.

CROCKER AC (1961) The cerebral defect in Tay-Sachs disease and Niemann-Pick disease. J Neurochem 7: 69-80.

CROCKER AC, FARBER S (1958) Niemann-Pick disease: a review of eighteen patients. Medicine (Baltimore) 37: 1-95.

Cui W, Allen ND, Skynner M, Gusterson B, Clark AJ (2001) Inducible ablation of astrocytes shows that these cells are required for neuronal survival in the adult brain. GLIA 34: 272-282.

Davies JP, Chen FW, Ioannou YA (2000a) Transmembrane molecular pump activity of Niemann-Pick C1 protein. Science 290: 2295-2298.

Davies JP, Levy B, Ioannou YA (2000b) Evidence for a Niemann-pick C (NPC) gene family: identification and characterization of NPC1L1. Genomics 65: 137-145.

Deisz RA, Meske V, Treiber-Held S, Albert F, Ohm TG (2005) Pathological cholesterol metabolism fails to modify electrophysiological properties of afflicted neurones in Niemann-Pick disease type C. Neuroscience 130: 867-873.

Donato R (1999) Functional roles of S100 proteins, calcium-binding proteins of the EF-hand type. Biochim Biophys Acta 1450: 191-231.

Drake-Baumann R, Seil FJ (1999) Influence of functional glia on the electrophysiology of Purkinje cells in organotypic cerebellar cultures. Neuroscience 88: 507-519.

Eng LF (1985) Glial fibrillary acidic protein (GFAP): the major protein of glial intermediate filaments in differentiated astrocytes. J Neuroimmunol 8: 203-214.

Erickson RP, Bernard O (2002) Studies on neuronal death in the mouse model of Niemann-Pick C disease. J Neurosci Res 68: 738-744.

Erickson RP, Kiela M, Devine PJ, Hoyer PB, Heidenreich RA (2002) mdr1a deficiency corrects sterility in Niemann-Pick C1 protein deficient female mice. Mol Reprod Dev 62: 167-173.

Falk J, Bonnon C, Girault JA, Faivre-Sarrailh C (2002) F3/contactin, a neuronal cell adhesion molecule implicated in axogenesis and myelination. Biol Cell 94: 327-334.

Falk T, Garver WS, Erickson RP, Wilson JM, Yool AJ (1999) Expression of Niemann-Pick type C transcript in rodent cerebellum in vivo and in vitro. Brain Res 839: 49-57.

Fan H, Favero M, Vogel MW (2001) Elimination of Bax expression in mice increases cerebellar purkinje cell numbers but not the number of granule cells. J Comp Neurol 436: 82-91.

Ferrer I, Puig B, Krupinsk J, Carmona M, Blanco R (2001) Fas and Fas ligand expression in Alzheimer's disease. Acta Neuropathol (Berl) 102: 121-131.

Fouchecourt S, Metayer S, Locatelli A, Dacheux F, Dacheux JL (2000) Stallion epididymal fluid proteome: qualitative and quantitative characterization; secretion and dynamic changes of major proteins. Biol Reprod 62: 1790-1803.

Friedland N, Liou HL, Lobel P, Stock AM (2003) Structure of a cholesterol-binding protein deficient in Niemann-Pick type C2 disease. Proc Natl Acad Sci U S A 100: 2512-2517.

Fukudome Y, Tabata T, Miyoshi T, Haruki S, Araishi K, Sawada S, Kano M (2003) Insulin-like growth factor-I as a promoting factor for cerebellar Purkinje cell development. Eur J Neurosci 17: 2006-2016.

Furuya S, Tabata T, Mitoma J, Yamada K, Yamasaki M, Makino A, Yamamoto T, Watanabe M, Kano M, Hirabayashi Y (2000) L-serine and glycine serve as major astroglia-derived trophic factors for cerebellar Purkinje neurons. Proc Natl Acad Sci U S A 97: 11528-11533.

Futerman AH, van Meer G (2004) The cell biology of lysosomal storage disorders. Nat Rev Mol Cell Biol 5: 554-565.

Garcia-Fresco GP, Sousa AD, Pillai AM, Moy SS, Crawley JN, Tessarollo L, Dupree JL, Bhat MA (2006) Disruption of axo-glial junctions causes cytoskeletal disorganization and degeneration of Purkinje neuron axons. Proc Natl Acad Sci U S A 103: 5137-5142.

Garver WS, Xie C, Repa JJ, Turley SD, Dietschy JM (2005) Niemann-Pick C1 expression is not regulated by the amount of cholesterol flowing through cells in the mouse. J Lipid Res 46: 1745-1754.

German DC, Liang CL, Song T, Yazdani U, Xie C, Dietschy JM (2002) Neurodegeneration in the Niemann-Pick C mouse: glial involvement. Neuroscience 109: 437-450.

Goldowitz D, Hamre K (1998) The cells and molecules that make a cerebellum. Trends Neurosci 21: 375-382.

Gondre-Lewis MC, McGlynn R, Walkley SU (2003) Cholesterol accumulation in NPC1-deficient neurons is ganglioside dependent. Curr Biol 13: 1324-1329.

Goodrum JF, Pentchev PG (1997) Cholesterol reutilization during myelination of regenerating PNS axons is impaired in Niemann-Pick disease type C mice. J Neurosci Res 49: 389-392.

Goritz C, Mauch DH, Pfrieger FW (2005) Multiple mechanisms mediate cholesterol-induced synaptogenesis in a CNS neuron. Mol Cell Neurosci 29: 190-201.

Gregory WA, Edmondson JC, Hatten ME, Mason CA (1988) Cytology and neuron-glial apposition of migrating cerebellar granule cells in vitro. J Neurosci 8: 1728-1738.

Griffin LD, Gong W, Verot L, Mellon SH (2004) Niemann-Pick type C disease involves disrupted neurosteroidogenesis and responds to allopregnanolone. Nat Med 10: 704-711.

Hama H, Hara C, Yamaguchi K, Miyawaki A (2004) PKC signaling mediates global enhancement of excitatory synaptogenesis in neurons triggered by local contact with astrocytes. Neuron 41: 405-415.

Hanson MG, Jr., Shen S, Wiemelt AP, McMorris FA, Barres BA (1998) Cyclic AMP elevation is sufficient to promote the survival of spinal motor neurons in vitro. J Neurosci 18: 7361-7371.

Harzer K, Schlote W, Peiffer J, Benz HU, Anzil AP (1978) Neurovisceral lipidosis compatible with Niemann-Pick disease type C: morphological and biochemical studies of a late infantile case and enzyme and lipid assays in a prenatal case of the same family. Acta Neuropathol (Berl) 43: 97-104.

Hirai H, Launey T (2000) The regulatory connection between the activity of granule cell NMDA receptors and dendritic differentiation of cerebellar Purkinje cells. J Neurosci 20: 5217-5224.

Hu CY, Ong WY, Patel SC (2000) Regional distribution of NPC1 protein in monkey brain. J Neurocytol 29: 765-773.

Huang X, Suyama K, Buchanan J, Zhu AJ, Scott MP (2005) A Drosophila model of the Niemann-Pick type C lysosome storage disease: dnpc1a is required for molting and sterol homeostasis. Development 132: 5115-5124.

Iino M, Goto K, Kakegawa W, Okado H, Sudo M, Ishiuchi S, Miwa A, Takayasu Y, Saito I, Tsuzuki K, Ozawa S (2001) Glia-synapse interaction through Ca2+-permeable AMPA receptors in Bergmann glia. Science 292: 926-929.

Ioannou YA (2005) Guilty until proven innocent: the case of NPC1 and cholesterol. Trends Biochem Sci 30: 498-505.

Isaacson LG, Tanaka DJ (1988) Cholinergic innervation of canine thalamostriatal projection neurons: an ultrastructural study combining choline acetyltransferase immunocytochemistry and WGA-HRP retrograde labeling. J Comp Neurol 277: 529-540.

Ito M (1987) Signal processing in cerebellar Purkinje cells. Physiol Bohemoslov 36(3):203-16.

Jin LW, Maezawa I, Vincent I, Bird T (2004) Intracellular accumulation of amyloidogenic fragments of amyloid-beta precursor protein in neurons with Niemann-Pick type C defects is associated with endosomal abnormalities. Am J Pathol 164: 975-985.

Kapfhammer JP (2004) Cellular and molecular control of dendritic growth and development of cerebellar Purkinje cells. Prog Histochem Cytochem 39: 131-182.

Karten B, Campenot RB, Vance DE, Vance JE (2006) The Niemann-Pick C1 protein in recycling endosomes of presynaptic nerve terminals. J Lipid Res 47: 504-514.

Karten B, Hayashi H, Francis GA, Campenot RB, Vance DE, Vance JE (2005) Generation and function of astroglial lipoproteins from Niemann-Pick type C1-deficient mice. Biochem J 387: 779-788.

Karten B, Vance DE, Campenot RB, Vance JE (2002) Cholesterol accumulates in cell bodies, but is decreased in distal axons, of Niemann-Pick C1-deficient neurons. J Neurochem 83: 1154-1163.

Klein A, Amigo L, Retamal MJ, Morales MG, Miquel JF, Rigotti A, Zanlungo S (2006) NPC2 is expressed in human and murine liver and secreted into bile: potential implications for body cholesterol homeostasis. Hepatology 43: 126-133.

Ko DC, Binkley J, Sidow A, Scott MP (2003) The integrity of a cholesterol-binding pocket in Niemann-Pick C2 protein is necessary to control lysosome cholesterol levels. Proc Natl Acad Sci U S A 100: 2518-2525.

Komuro H, Rakic P (1998) Distinct modes of neuronal migration in different domains of developing cerebellar cortex. J Neurosci 18: 1478-1490.

Larkfors L, Lindsay RM, Alderson RF (1996) Characterization of the responses of Purkinje cells to neurotrophin treatment. J Neurochem 66: 1362-1373.

Lein PJ, Beck HN, Chandrasekaran V, Gallagher PJ, Chen HL, Lin Y, Guo X, Kaplan PL, Tiedge H, Higgins D (2002) Glia induce dendritic growth in cultured sympathetic neurons by modulating the balance between bone morphogenetic proteins (BMPs) and BMP antagonists. J Neurosci 22: 10377-10387.

Levine JB, Kong J, Nadler M, Xu Z (1999) Astrocytes interact intimately with degenerating motor neurons in mouse amyotrophic lateral sclerosis (ALS). GLIA 28: 215-224.

Levitt P, Rakic P (1980) Immunoperoxidase localization of glial fibrillary acidic protein in radial glial cells and astrocytes of the developing rhesus monkey brain. J Comp Neurol 193: 815-840.

Lievens JC, Woodman B, Mahal A, Spasic-Boscovic O, Samuel D, Kerkerian-Le Goff L, Bates GP (2001) Impaired glutamate uptake in the R6 Huntington's disease transgenic mice. Neurobiol Dis 8: 807-821.

Liscum L, Ruggiero RM, Faust JR (1989) The intracellular transport of low density lipoprotein-derived cholesterol is defective in Niemann-Pick type C fibroblasts. J Cell Biol 108: 1625-1636.

Liu Y, Wu YP, Wada R, Neufeld EB, Mullin KA, Howard AC, Pentchev PG, Vanier MT, Suzuki K, Proia RL (2000) Alleviation of neuronal ganglioside storage does not improve the clinical course of the Niemann-Pick C disease mouse. Hum Mol Genet 9: 1087-1092.

Loftus SK, Morris JA, Carstea ED, Gu JZ, Cummings C, Brown A, Ellison J, Ohno K, Rosenfeld MA, Tagle DA, Pentchev PG, Pavan WJ (1997) Murine model of Niemann-Pick C disease: mutation in a cholesterol homeostasis gene. Science 277: 232-235.

Lordkipanidze T, Dunaevsky A (2005) Purkinje cell dendrites grow in alignment with Bergmann glia. GLIA 51: 229-234.

Losi G, Prybylowski K, Fu Z, Luo JH, Vicini S (2002) Silent synapses in developing cerebellar granule neurons. J Neurophysiol 87: 1263-1270.

Malatesta P, Hack MA, Hartfuss E, Kettenmann H, Klinkert W, Kirchhoff F, Gotz M (2003) Neuronal or glial progeny: regional differences in radial glia fate. Neuron 37: 751-764.

Malatesta P, Hartfuss E, Gotz M (2000) Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. Development 127: 5253-5263.

Mangano TJ, Patel J, Salama AI, Keith RA (1991) Inhibition of K(+)-evoked [3H]D-aspartate release and neuronal calcium influx by verapamil, diltiazem and dextromethorphan: evidence for non-L/non-N voltage-sensitive calcium channels. Eur J Pharmacol 192: 9-17.

Marin-Teva JL, Dusart I, Colin C, Gervais A, van Rooijen N, Mallat M (2004) Microglia promote the death of developing Purkinje cells. Neuron 41: 535-547.

Messer A, Snodgrass GL, Maskin P (1984) Enhanced survival of cultured cerebellar Purkinje cells by plating on antibody to Thy-1. Cell Mol Neurobiol 4: 285-290.

Meyer-Franke A, Kaplan MR, Pfrieger FW, Barres BA (1995) Characterization of the signaling interactions that promote the survival and growth of developing retinal ganglion cells in culture. Neuron 15: 805-819.

Millat G, Marcais C, Rafi MA, Yamamoto T, Morris JA, Pentchev PG, Ohno K, Wenger DA, Vanier MT (1999) Niemann-Pick C1 disease: the I1061T substitution is a frequent mutant allele in patients of Western European descent and correlates with a classic juvenile phenotype. Am J Hum Genet 65: 1321-1329.

Miller RF (2004) D-Serine as a glial modulator of nerve cells. GLIA 47: 275-283.

Mitter D, Reisinger C, Hinz B, Hollmann S, Yelamanchili SV, Treiber-Held S, Ohm TG, Herrmann A, Ahnert-Hilger G (2003) The synaptophysin/synaptobrevin interaction critically depends on the cholesterol content. J Neurochem 84: 35-42.

Morris MD, Bhuvaneswaran C, Shio H, Fowler S (1982) Lysosome lipid storage disorder in NCTR-BALB/c mice. I. Description of the disease and genetics. Am J Pathol 108: 140-149.

Morrison ME, Mason CA (1998) Granule neuron regulation of Purkinje cell development: striking a balance between neurotrophin and glutamate signaling. J Neurosci 18: 3563-3573.

Mothet JP, Parent AT, Wolosker H, Brady RO, Jr., Linden DJ, Ferris CD, Rogawski MA, Snyder SH (2000) D-serine is an endogenous ligand for the glycine site of the N-methyl-D-aspartate receptor. Proc Natl Acad Sci U S A 97: 4926-4931.

Mount HT, Dreyfus CF, Black IB (1994) Neurotrophin-3 selectively increases cultured Purkinje cell survival. Neuroreport 5: 2497-2500.

Muller CP, Stephany DA, Winkler DF, Hoeg JM, Demosky SJ, Jr., Wunderlich JR (1984) Filipin as a flow microfluorometry probe for cellular cholesterol. Cytometry 5: 42-54.

Nagler K, Mauch DH, Pfrieger FW (2001) Glia-derived signals induce synapse formation in neurones of the rat central nervous system. J Physiol 533: 665-679.

Napper RM, Harvey RJ (1988) Number of parallel fiber synapses on an individual Purkinje cell in the cerebellum of the rat. J Comp Neurol 274: 168-177.

Naureckiene S, Sleat DE, Lackland H, Fensom A, Vanier MT, Wattiaux R, Jadot M, Lobel P (2000) Identification of HE1 as the second gene of Niemann-Pick C disease. Science 290: 2298-2301.

Niemann A (1914) Ein unbekanntes Krankheitsbild. Jahrb Kinderheilkd 79:1

Noctor SC, Flint AC, Weissman TA, Dammerman RS, Kriegstein AR (2001) Neurons derived from radial glial cells establish radial units in neocortex. Nature 409: 714-720.

Norman AW, Demel RA, de Kruyff B, van Deenen LL (1972) Studies on the biological properties of polyene antibiotics. Evidence for the direct interaction of filipin with cholesterol. J Biol Chem 247: 1918-1929.

Ong WY, Kumar U, Switzer RC, Sidhu A, Suresh G, Hu CY, Patel SC (2001) Neurodegeneration in Niemann-Pick type C disease mice. Exp Brain Res 141: 218-231.

Ong WY, Sundaram RK, Huang E, Ghoshal S, Kumar U, Pentchev PG, Patel SC (2004) Neuronal localization and association of Niemann Pick C2 protein (HE1/NPC2) with the postsynaptic density. Neuroscience 128: 561-570.

Ory DS (2000) Niemann-Pick type C: a disorder of cellular cholesterol trafficking. Biochim Biophys Acta 1529: 331-339.

Palay S and Chang-Palay V 1974 Cerebellar Cortex Springer-Verlag, New York

Park WD, O'Brien JF, Lundquist PA, Kraft DL, Vockley CW, Karnes PS, Patterson MC, Snow K (2003) Identification of 58 novel mutations in Niemann-Pick disease type C: correlation with biochemical phenotype and importance of PTC1-like domains in NPC1. Hum Mutat 22: 313-325.

Patel SC, Barton NW and Argoff C (1991). Niemann-Pick type A, C and D, Gaucher's disease type I, II and III and Wolman's disease. In: de Jong JMBV (eds). Handbook of clinical neurology, vol 16, Elsevier, Amsterdam, pp 147-164

Patel SC, Suresh S, Kumar U, Hu CY, Cooney A, Blanchette-Mackie EJ, Neufeld EB, Patel RC, Brady RO, Patel YC, Pentchev PG, Ong WY (1999) Localization of Niemann-Pick C1 protein in astrocytes: implications for neuronal degeneration in Niemann- Pick type C disease. Proc Natl Acad Sci U S A 96: 1657-1662.

Pentchev PG, Vanier MT, Suzuki K, Patterson MC (1995) Niemann-Pick disease, type C: a cellular cholesterol lipidosis. In: The metabolic and molecular bases of inherited disease (Scriver CR, Beaudet AL, Sly WS, Valle D, eds), pp 2625-2640. New York: McGraw-Hill.

Pentchev PG, Vanier MT, Suzuki K, Patterson MC (1995). Niemann-Pick type disease, type C: a cellular cholesterol lipidosis. In: Scriver CR, Beaudet AL, Sly WS and Valle D (eds). The metabolic and molecular basis of inherited disease. McGraw-Hill, New-York, pp 2625-2640

Pfrieger FW (2003) Outsourcing in the brain: do neurons depend on cholesterol delivery by astrocytes? Bioessays 25: 72-78.

Pfrieger FW, Barres BA (1997) Synaptic efficacy enhanced by glial cells in vitro. Science 277: 1684-1687.

Pfrieger FW (2003) Role of cholesterol in synapse formation and function. Biochim Biophys Acta 1610: 271-280.

Pick L (1927) Uber die lipoidzellige splenohepatosplenomegalie typus Niemann-Pick als stoffwechselerkrankung. *Med Klein* 23:1483

Poisbeau P, Feltz P, Schlichter R (1997) Modulation of GABAA receptor-mediated IPSCs by neuroactive steroids in a rat hypothalamo-hypophyseal coculture model. J Physiol 500 (Pt 2): 475-485.

Porter JA, Young KE, Beachy PA (1996) Cholesterol modification of hedgehog signaling proteins in animal development. Science 274: 255-259.

Prasad A, Fischer WA, Maue RA, Henderson LP (2000) Regional and developmental expression of the Npc1 mRNA in the mouse brain. J Neurochem 75: 1250-1257.

Qu Q, Smith FI (2005) Neuronal migration defects in cerebellum of the Largemyd mouse are associated with disruptions in Bergmann glia organization and delayed migration of granule neurons. Cerebellum 4: 261-270.

Quan G, Xie C, Dietschy JM, Turley SD (2003) Ontogenesis and regulation of cholesterol metabolism in the central nervous system of the mouse. Brain Res Dev Brain Res 146: 87-98.

Rabacchi S, Bailly Y, Delhaye-Bouchaud N, Mariani J (1992) Involvement of the N-methyl D-aspartate (NMDA) receptor in synapse elimination during cerebellar development. Science 256: 1823-1825.

Rakic P (1971) Guidance of neurons migrating to the fetal monkey neocortex. Brain Res 33: 471-476.

Rathjen FG, Schachner M (1984) Immunocytological and biochemical characterization of a new neuronal cell surface component (L1 antigen) which is involved in cell adhesion. EMBO J 3: 1-10.

Reid PC, Sakashita N, Sugii S, Ohno-Iwashita Y, Shimada Y, Hickey WF, Chang TY (2004) A novel cholesterol stain reveals early neuronal cholesterol accumulation in the Niemann-Pick type C1 mouse brain. J Lipid Res 45: 582-591.

Revest JM, Faivre-Sarrailh C, Schachner M, Rougon G (1999) Bidirectional signaling between neurons and glial cells via the F3 neuronal adhesion molecule. Adv Exp Med Biol 468: 309-318.

Sakamoto H, Ukena K, Tsutsui K (2001) Effects of progesterone synthesized de novo in the developing Purkinje cell on its dendritic growth and synaptogenesis. J Neurosci 21: 6221-6232.

Salaun C, James DJ, Chamberlain LH (2004) Lipid rafts and the regulation of exocytosis. Traffic 5: 255-264.

Sarna JR, Larouche M, Marzban H, Sillitoe RV, Rancourt DE, Hawkes R (2003) Patterned Purkinje cell degeneration in mouse models of Niemann-Pick type C disease. J Comp Neurol 456: 279-291.

Sawamura N, Gong JS, Chang TY, Yanagisawa K, Michikawa M (2003) Promotion of tau phosphorylation by MAP kinase Erk1/2 is accompanied by reduced cholesterol level in detergent-insoluble membrane fraction in Niemann-Pick C1-deficient cells. J Neurochem 84: 1086-1096.

Schachner M (1982) Cell type-specific surface antigens in the mammalian nervous system. J Neurochem 39: 1-8.

Schofer O, Mischo B, Puschel W, Harzer K, Vanier MT (1998) Early-lethal pulmonary form of Niemann-Pick type C disease belonging to a second, rare genetic complementation group. Eur J Pediatr 157: 45-49.

Sleat DE, Wiseman JA, El Banna M, Price SM, Verot L, Shen MM, Tint GS, Vanier MT, Walkley SU, Lobel P (2004) Genetic evidence for nonredundant functional cooperativity between NPC1 and NPC2 in lipid transport. Proc Natl Acad Sci U S A 101: 5886-5891.

Slezak M, Pfrieger FW (2003) New roles for astrocytes: regulation of CNS synaptogenesis. Trends Neurosci 26: 531-535.

Smeyne RJ, Goldowitz D (1989) Development and death of external granular layer cells in the weaver mouse cerebellum: a quantitative study. J Neurosci 9: 1608-1620.

Somers KL, Royals MA, Carstea ED, Rafi MA, Wenger DA, Thrall MA (2003) Mutation analysis of feline Niemann-Pick C1 disease. Mol Genet Metab 79: 99-103.

Squire LR, Bloom FE, McConnell SK, Roberts JL, Spitzer NC, Zigmond MJ. Fundamental Neuroscience 2003

Steinmetz CC, Buard I, Claudepierre T, Nagler K, Pfrieger FW. Regional variations in the glial influence on synapse development in the central nervous system. Manuscript in preparation

Stone DM, Hynes M, Armanini M, Swanson TA, Gu Q, Johnson RL, Scott MP, Pennica D, Goddard A, Phillips H, Noll M, Hooper JE, de Sauvage F, Rosenthal A (1996) The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog. Nature 384: 129-134.

Streit WJ, Kreutzberg GW (1987) Lectin binding by resting and reactive microglia. J Neurocytol 16: 249-260.

Sturley SL, Patterson MC, Balch W, Liscum L (2004) The pathophysiology and mechanisms of NP-C disease. Biochim Biophys Acta 1685: 83-87.

Suzuki H, Sakiyama T, Harada N, Abe M, Tadokoro M (2003) Pathologic changes of glial cells in murine model of Niemann-Pick disease type C: immunohistochemical, lectin-histochemical and ultrastructural observations. Pediatr Int 45: 1-4.

Swisher DA, Wilson DB (1977) Cerebellar histogenesis in the lurcher (Lc) mutant mouse. J Comp Neurol 173: 205-218.

Sym M, Basson M, Johnson C (2000) A model for niemann-pick type C disease in the nematode Caenorhabditis elegans. Curr Biol 10: 527-530.

Takikita S, Fukuda T, Mohri I, Yagi T, Suzuki K (2004) Perturbed myelination process of premyelinating oligodendrocyte in Niemann-Pick type C mouse. J Neuropathol Exp Neurol 63: 660-673.

Tomomura M, Rice DS, Morgan JI, Yuzaki M (2001) Purification of Purkinje cells by fluorescence-activated cell sorting from transgenic mice that express green fluorescent protein. Eur J Neurosci 14: 57-63.

Tsacopoulos M, Magistretti PJ (1996) Metabolic coupling between glia and neurons. J Neurosci 16: 877-885.

Ullian EM, Harris BT, Wu A, Chan JR, Barres BA (2004) Schwann cells and astrocytes induce synapse formation by spinal motor neurons in culture. Mol Cell Neurosci 25: 241-251.

Vanier MT, Suzuki K (1998) Recent advances in elucidating Niemann-Pick C disease. Brain Pathol 8: 163-174.

Verkhratsky A, Steinhauser C (2000) Ion channels in glial cells. Brain Res Brain Res Rev 32: 380-412.

Vincent I, Bu B, Erickson RP (2003) Understanding Niemann-Pick type C disease: a fat problem. Curr Opin Neurol 16: 155-161.

Virginio C, Martina M, Cherubini E (1995) Spontaneous GABA-mediated synaptic currents in cerebellar granule cells in culture. Neuroreport 6: 1285-1289.

Virmani T, Gupta P, Liu X, Kavalali ET, Hofmann SL (2005) Progressively reduced synaptic vesicle pool size in cultured neurons derived from neuronal ceroid lipofuscinosis-1 knockout mice. Neurobiol Dis 20: 314-323.

Voikar V, Rauvala H, Ikonen E (2002) Cognitive deficit and development of motor impairment in a mouse model of Niemann-Pick type C disease. Behav Brain Res 132: 1-10.

Wallace VA (1999) Purkinje-cell-derived Sonic hedgehog regulates granule neuron precursor cell proliferation in the developing mouse cerebellum. Curr Biol 9: 445-448.

Weintraub H, Abramovici A, Sandbank U, Pentchev PG, Brady RO, Sekine M, Suzuki A, Sela B (1985) Neurological mutation characterized by dysmyelination in NCTR-Balb/C mouse with lysosomal lipid storage disease. J Neurochem 45: 665-672.

Wetts R, Herrup K (1983) Direct correlation between Purkinje and granule cell number in the cerebella of lurcher chimeras and wild-type mice. Brain Res 312: 41-47.

Wu YP, Mizukami H, Matsuda J, Saito Y, Proia RL, Suzuki K (2005) Apoptosis accompanied by up-regulation of TNF-alpha death pathway genes in the brain of Niemann-Pick type C disease. Mol Genet Metab 84: 9-17.

Xie C, Turley SD, Pentchev PG, Dietschy JM (1999) Cholesterol balance and metabolism in mice with loss of function of Niemann-Pick C protein. Am J Physiol 276: E336-E344.

Yamada K, Watanabe M (2002) Cytodifferentiation of Bergmann glia and its relationship with Purkinje cells. Anat Sci Int 77: 94-108.

Yu W, Gong JS, Ko M, Garver WS, Yanagisawa K, Michikawa M (2005) Altered cholesterol metabolism in Niemann-Pick type C1 mouse brains affects mitochondrial function. J Biol Chem 280: 11731-11739.

Zanjani H, Lemaigre-Dubreuil Y, Tillakaratne NJ, Blokhin A, McMahon RP, Tobin AJ, Vogel MW, Mariani J (2004) Cerebellar Purkinje cell loss in aging Hu-Bcl-2 transgenic mice. J Comp Neurol 475: 481-492.

Zanjani HS, Vogel MW, Delhaye-Bouchaud N, Martinou JC, Mariani J (1996) Increased cerebellar Purkinje cell numbers in mice overexpressing a human bcl-2 transgene. J Comp Neurol 374: 332-341.

Zanjani HS, Vogel MW, Delhaye-Bouchaud N, Martinou JC, Mariani J (1997) Increased inferior olivary neuron and cerebellar granule cell numbers in transgenic mice overexpressing the human Bcl-2 gene. J Neurobiol 32: 502-516.

Zervas M, Dobrenis K, Walkley SU (2001) Neurons in Niemann-Pick disease type C accumulate gangliosides as well as unesterified cholesterol and undergo dendritic and axonal alterations. J Neuropathol Exp Neurol 60: 49-64.

Zhang M, Dwyer NK, Neufeld EB, Love DC, Cooney A, Comly M, Patel S, Watari H, Strauss JF, III, Pentchev PG, Hanover JA, Blanchette-Mackie EJ (2001) Sterol-modulated glycolipid sorting occurs in niemann-pick C1 late endosomes. J Biol Chem 276: 3417-3425.