IV. DISCUSSION

4.1 Multiple mechanisms mediate glia-induced synaptogenesis in RGCs

The first part of this work reveals that the GCM- and cholesterol-induced increase in synapse number requires several days of treatment and identifies dendrite differentiation as rate limiting step for glia-induced synaptogenesis. Cholesterol was indispensable for the differentiation of dendrites and glia-derived laminin containing the γ1 chain acted as dendrite-promoting signal for RGCs. Finally, cholesterol was essential for continuous synaptogenesis and for the functional stability of evoked transmitter release.

4.1.1 Dendrite differentiation limits the rate of glia-induced synaptogenesis, requires cholesterol and is promoted by laminin γ1

My observation that GCM and cholesterol induced dendrite differentiation with a remarkably similar time course as the increase in synapse number suggested it as the rate limiting step in glia-induced synaptogenesis. This is in line with reports that dendrite development determines the competence of neurons to form synapses (Fletcher et al., 1994) and that dendritic filopodia play an active role in the establishment of contacts (Ziv & Smith, 1996). My findings corroborate the idea that glial cells are a source of dendrite promoting signals. This has been shown in vitro for RGCs isolated from embryonic chicken (Bauch et al., 1998), for sympathetic neurons from newborn mice (Tropea et al., 1988) and for cortical neurons from embryonic mice (Le Roux & Esquenazi, 2002; Higgins et al., 1997; Keith & Wilson, 2001). In addition, regional differences in the dendrite-promoting activity of glial cells have been reported (Le Roux & Reh, 1994; Dijkstra et al., 1999).

My results suggest that cholesterol and laminin promote the differentiation of dendrites. Under control conditions, most RGCs extended several neurites from their soma, which grew and branched extensively, but lacked MAP2 or GluR2/3. Cholesterol together with laminin induced a redistribution of MAP2 and GluR2/3 receptors to neurites without changing their level of expression. This indicates that they promote the maturation of dendrites. So far, signals and mechanisms that shape the dendritic tree are well established (Gao & Bogert, 2003; Jan & Jan, 2003; Miller & Kaplan, 2003; Scott & Luo, 2001;

Whitford et al., 2002; Wong & Ghosh, 2002), but much less is known about the pathways that regulate their differentiation (Libersat & Duch, 2004).

As mentioned in the introduction, cholesterol could serve as precursor for neurosteroids, as building material or it may act by determining the functional properties of membrane proteins as a component of rafts. It has been shown that progesterone promotes dose-dependent dendritic outgrowth of Purkinje cells, using cerebellar slice cultures from newborn rats (Sakamoto et al., 2001). However, treatment of RGCs with progesterone, pregnenolone and several of their derivatives showed no synaptogenic effects as measured by electrophysiological recordings (D. Mauch, C. Göritz; F.W. Pfrieger, unpublished results). It appears possible that cholesterol serves as building material. The differentiation of dendrites may require large amounts of lipids due to the presence of intracellular organelles like endoplasmatic reticulum and Golgi apparatus (Horton & Ehlers, 2004). A specific dependence of dendrites on the neuronal cholesterol level has been shown previously in primary cortical cultures from embryonic rats, where experimentally induced cholesterol deficiency decreased selectively the number and length of dendrites, but not axonal elongation (Fan et al., 2002). Furthermore, it cannot be excluded at present, that cholesterol promotes dendrite differentiation by clustering of specific signaling components in rafts.

The finding that laminin promotes the differentiation of dendrites is surprising. Laminin is well known to enhance neurite growth in RGCs from different vertebrate species (Manthorpe et al., 1983; Rogers et al., 1983; Smalheiser et al., 1984; Hopkins et al., 1985; Ivins et al., 1998) and in many other neuronal cell types (Luckenbill-Edds, 1997; Powell & Kleinman, 1997; Patton, 2000), but a dendrite-promoting activity of this matrix component has never been reported. This effect may have gone unnoticed, because neurites were not further characterized as axons or dendrites or because the presence of serum in culture medium influenced the neuronal response to laminin (Bates & Meyer, 1994). In glia- and granule cell-deprived organotypic cultures of rat cerebellum, laminin promoted spine proliferation in Purkinje cells, but this effect was not mimicked by laminin fragments (Seil, 1998).

Barres and coworkers proposed recently that around birth, rat RGCs switch from axonal to dendritic growth and that this switch is induced by contact to amacrine cells (Goldberg et al., 2002). Furthermore, it has been shown that during development, axon formation in RGCs becomes independent from laminin (Cohen et al., 1986; Hall et al., 1987; Ivins et al., 1998). Based on my results, I hypothesize that the amacrine-derived

signal changes the way how RGCs respond to laminin: Before birth, laminin induces axons, whereas after birth, it promote dendrite differentiation. But so far, it remains to be studied which signaling mechanisms are involved in laminin promoted dendrite differentiation.

Treatment of RGCs with laminin fragments revealed that the γ1 chain contains the dendrite-promoting activity. However, I should mention that laminin and peptides were used at the same weight per volume, and therefore differ widely in their molar concentrations. Immunohistochemical and in situ hybridization studies showed that this chain is present in the ganglion cell layer in rodents (Dong & Chung, 1991; Libby et al., 2000; Yin et al., 2003), but its exact distribution and cellular source around birth, when RGCs form dendrites (Maslim et al., 1986; Tucker & Matus, 1988; Sernagor et al., 2001) remains to be studied. Other laminins including newly discovered forms (Koch et al., 2000; Yin et al., 2002) may exert dendrite promoting activity, but their function in the retina or other brain regions is unknown.

A fraction of RGCs failed to differentiate dendrites even in the presence of soluble glial factors, and these cells are mainly found on microislands with more than one neuron. This suggests that contact with neighboring RGCs impede dendrite differentiation. Alternatively, dendrite differentiation in these cells may require signals from other neurons or direct neuron-glia contact. A previous study on cultured cerebellar neurons has shown that granule cells induce dendrites in Purkinje cells (Baptista et al., 1994).

4.1.2 Cholesterol is required for ongoing synaptogenesis and the stability of evoked release

My observation that removal of GCM from cultured RGCs stopped the continuous increase in synaptic activity during the culture period and that this effect was rescued, if GCM was replaced by cholesterol indicates that RGCs require cholesterol for continuous synaptogenesis. After removal of GCM, synaptic activity remained at a baseline level indicating that those synapses that had formed, remained active. Interestingly, removal of GCM diminished selectively the size of evoked EPSCs and increased the number of RGCs lacking evoked synaptic responses and these effects were eliminated when GCM was replaced by cholesterol. This indicates that the functional stability of evoked release requires the presence of cholesterol. This is further supported by previous reports that exocytosis is sensitive to the removal of cholesterol, and probably occurs at cholesterol-rich domains in the plasma membrane (Chamberlain et al., 2001; Lang et al., 2001;

Pfrieger, 2003b; Ohara-Imaizumi et al., 2004). Cholesterol may also promote the biogenesis and maturation of synaptic vesicles as suggested previously (Mauch et al., 2001). It appears essential for the biogenesis of secretory vesicles in neurosecretory cell lines (Thiele et al., 2000; Wang et al., 2000) and for the formation of vesicle protein complexes *in vivo* (Mitter et al., 2003; Pfrieger, 2003). The idea of an enhanced vesicle production is further supported by the observation that cholesterol enhanced the number of synapsin-positive puncta, which were dispersed along axons. This is in contrast to GCM-treated cells, where synapsin-puncta were concentrated at synaptic sites along dendrites. The dispersed puncta probably represent clusters of surplus synaptic vesicles that cannot be delivered to synapses due to the lower incidence of dendrites. Alternative, cholesterol may have increased the transmitter concentration in synaptic vesicles, for example by enhancing the efficacy of glutamate transporters (Canolle et al., 2004).

4.2 Influence of soluble glial factors and cholesterol on gene expression of cultured postnatal RGCs

My GeneChip experiments revealed 82 genes whose level of expression changed under the influence of GCM and 38 genes for cholesterol treatment. Interestingly, there was only little overlap between the two groups. The 18 genes which were upregulated by GCM and cholesterol are mainly involved in cholesterol biosynthesis and homeostasis. The supply of external cholesterol, directly or contained in GCM, downregulates genes involved in cholesterol synthesis and uptake, and additional GCM upregulate ABC-G1 involved in cholesterol release. MGP and HO1 are the highest upregulated genes provoked by GCM treatment. The neuronal regulation of cholesterol synthesis and release as well as the upregulation of MGP and HO1 by GCM were confirmed on lipid and protein level and will be discussed in the following.

4.2.1 RGCs synthesize cholesterol and fatty acids, and regulate their homeostasis in reaction to external supply

The gene expression profile of RGCs showed that neurons express the complete enzymatic machinery for cholesterol and fatty acid synthesis. Further metabolic labeling emphasized the ability of RGCs to synthesize cholesterol and phosphatidylcholine. Treatment with GCM downregulated genes involved in both, cholesterol and fatty acid synthesis, whereas cholesterol treatment only affected genes of the cholesterol synthesis pathway. Lipid

labeling experiments showed that both, GCM and cholesterol drastically reduced the level of de novo synthesized neuronal cholesterol. In general, regulation of cholesterol bio-synthesis takes place at the level of gene transcription, mRNA stability, translation, enzyme phosphorylation and enzyme degradation. Cellular cholesterol levels are also modulated by conversion of cholesterol to cholesteryl esters, bile acids and oxysterols (Liscum, 2002). The transcriptional regulation of lipid homeostasis in vertebrate cells is regulated by a family of membrane-bound transcription factors designated SREBPs (Horton et al., 2002). SREBPs are activated by proteolytical cleavage of membrane bound precursors regulated by a lipid sensing mechanism (Brown & Goldstein, 1997). They directly activate the expression of more than 30 genes dedicated to the synthesis and uptake of cholesterol, fatty acids, triglycerides, and phospholipids, as well as the NADPH cofactor required to synthesize these molecules (Brown & Goldstein, 1997; Horton & Shimomura, 1999; Sakakura et al., 2001). The mammalian genome encodes three SREBP isoforms, designated SREBP-1a, SREBP-1c, and SREBP-2 (Yokoyama et al., 1993; Hua et al., 1993). SREBP-1a is a potent activator of all SREBP-responsive genes, including those that mediate the synthesis of cholesterol, fatty acids, and triglycerides. The Steroidogenic acute regulatory protein, downregulated by GCM, was shown to be regulated by SREBP-1a mediated by sterol regulatory element (SRE) binding sites in its promoter region (Christenson et al., 2001; Shea-Eaton et al., 2001). SREBP-1c preferentially enhances transcription of genes required for fatty acid synthesis (Shimano et al., 1997). This regulation includes the genes for ATP citrate lyase, and fatty acid synthase, necessary to produce palmitate, fatty acid elongase, which converts palmitate to stearate, stearoyl-CoA desaturase, which converts stearate to oleate, and glycerol-3-phosphate acyltransferase, the first committed enzyme in triglyceride and phospholipid synthesis. All these genes were downregulated by GCM, indicating that SREBP-1c activity may be reduced by GCM. Both SREBP-1 splice variants have been shown to mediate the GCM and cholesterol caused downregulation of the LDL receptor by binding to an SRE-1 binding side in its promoter region (Yokoyama et al., 1993). SREBP-2, itself downregulated by GCM, preferentially controls cholesterol synthesis as shown for the entire pathway (Sakakura et al., 2001). My observation that GCM and cholesterol downregulated 15 genes involved in this pathway in a similar manner, suggested that glia derived cholesterol contained in GCM stopped SREBP-2 activation mediated by a cholesterol sensing mechanism (Brown & Goldstein, 1997). Finally, SREBP-1c and SREBP-2 activate three genes required to generate NADPH, which is consumed at multiple stages in these lipid biosynthetic pathways

(Horton et al., 2002). One of them is isocitrate dehydrogenase, which was downregulated by GCM.

The difference regarding gene expression of fatty acids synthesizing enzymes between GCM and cholesterol treatment is possibly caused by the fact that GCM also contains fatty acids, which may act on the SREBP-1c pathway and which are not supplied to cells treated with cholesterol alone. The difference between GCM and cholesterol treatment for neuronal cholesterol release, observed by pulse chase labeling, may be related to the same fact. Since cholesterol is water insoluble, released cholesterol needs to be taken up by, or released with a carrier. Physiologically, active cellular cholesterol release appears together with phosphatidylcholine release and is mediated by specific transporters like ABC-A1 and ABC-G1 (Zheng et al., 2001; Klucken et al., 2000; Schmitz et al., 2001). The latter was upregulated by GCM. The release of cholesterol from untreated RGCs seems to be unphysiological since only cholesterol is released, probably to BSA in the culture medium. Treatment of RGCs with GCM or cholesterol caused additional release of phosphatidylcholine. The higher cholesterol release by GCM compared to cholesterol treatment is probably related to lipoproteins contained in GCM, which mediate transporter dependent cholesterol uptake by apolipoproteins like A-1 (Wang et al., 2000 & 2001; Chambenoit et al., 2001; Ito et al., 2002). Another possibility was suggested by Sun et al. (2003) who showed that co-transfection of ABC-A1 with either stearoyl CoA desaturases1 or 2, both downregulated by GCM but not by Cholesterol, inhibited ABC-A1-mediated cholesterol efflux but not phospholipid efflux in HEK 293 cells.

4.2.2 Dendritic localization of MGP and HO1

GCM and cholesterol enhanced the expression of several genes, but no overlap between these two groups was observed. Choosing the two most upregulated genes by GCM treatment, I confirmed the expression results on the protein level. Using immunostaining with specific antibodies, I detected a somatodendritic localization of MGP and HO1 in GCM treated microculture RGCs, whereas control cells showed only labeled somata. These findings suggested the specific upregulation of dendrite associated proteins and is a further hint for the observed glia-induced dendrite differentiation.
MGP is a small matrix protein containing carboxyglutamic acid (GLA), initially isolated from bone and characterized by Price et al., (1985). It affects differentiation in developing cartilage and bone and has an effect on the mineralization in chondrozytes (Luo et al.,

1997; Yagami et al., 1999). Urist et al., (1984) found that MGP is tightly associated with bone morphogenetic protein (BMP) *in vitro* during protein purification and that strong denaturants are required to break the association. Using the multipotent mouse embryonic cell line C3H10T1/2 Boström et al., (2001) showed that MGP inhibits BMP-induced cell differentiation, by application of BMP2 on MGP transfected cells. Deletion of MGP by antisense transfection or cell preparation from MGP-deficient mice enhanced BMP2 induced differentiation. Together, these results suggest that MGP modulates BMP activity. Highly interesting, BMP-7, a glia-derived factor, promotes dendrite formation in sympathetic (Lein et al., 1995 & 2002) and different types of CNS neurons (Le Roux et al., 1999; Withers et al., 2000). This has also been shown for BMP-2, -5, and -6 at sympathetic neurons *in vitro* (Guo et al., 1998; Beck et al., 2001). My findings that MGP localized at RGC dendrites and was upregulated by GCM, which promotes dendrite differentiation, could be a hint for its involvement in BMP-regulated neuronal differentiation.

The second highly upregulated gene in response to GCM treatment was HO1. This enzyme cleaves the heme ring at the alpha methene bridge to form biliverdin, ferric iron and CO (Tenhunen et al., 1968). HO1 activity can be induced in almost all cell types by cellular stressors, including ultraviolet radiation, hydrogen peroxide, heavy metals, and arsenite, and has also been referred to as heat-shock protein 32 (HSP32) (Keyse & Tyrrell, 1989; Kothary & Candido, 1982) and p32 (Kageyama et al., 1988). Molecular cloning revealed three heme oxygenase genes, the highly inducible isoform HO1 (Shibahara et al., 1985) and two constitutive expressed isoforms termed HO2 (Rotenberg & Maines, 1990) and HO3 (McCoubrey et al., 1997). But the catalyzed reaction of all three enzymes is the same. Unlike HO1, HO2 is selectively concentrated in the brain and testes. Therefore the majority of studies concerning the brain had focused on HO2. I observed that HO1 and GluR2/3 were co-localized at dendrites, which opens the possibility for an involvement in neurotransmission. Recently, CO as one of the products formed by HO reaction has been shown to be involved in neurotransmission by activating the soluble guanylyl cyclase (sGC) (Verma et al., 1993; Zakhary et al., 1997). Interestingly, the GABA-B receptor 2, which is upregulated by GCM but not by cholesterol, could be involved in the regulation of HO activity. Several studies have suggested that metabotropic glutamate receptor (mGluR) activity regulates HO2 activity. Nathanson et al., (1995) found that exogenous CO and mGluR agonists both increase cGMP and Na-, K-ATPase activity in cerebellar slices, and mGluR agonists also enhance endogenous CO production. These effects are completely blocked by inhibitors of HO2 and protein kinase C (PKC) (which is activated by mGluR1).

Similarly, Dore et al., (1999), found activation of HO2 in response to phorbol ester treatment of hippocampal and cortical cultures, effects that are blocked by PKC inhibitors. HO2 is activated by glutamate in cerebral vascular endothelium (Parfenova et al. 2001). Glutamate, NMDA, and AMPA injections into mouse spinal cord stimulate cGMP production that is blocked by HO inhibitors and is absent in HO2 knockout animals (Li & Clark 2002). It has to be noted, however, that the HO inhibitors used in these studies (protoporphyrins substituted with metals other than iron) exhibit poor specificity and can directly inhibit sGC (Grundemar & Ny, 1997).

HO1 has also a neuroprotective role by producing an antioxidant precursor. Biliverdin formed by HO is rapidly reduced to bilirubin because of the high levels of biliverdin reductase in most tissues. Bilirubin is neuroprotective (Dore et al., 1999[a]) and exerts this effect by redox cycling. Each molecule of bilirubin that acts as an antioxidant is thereby itself oxidized to biliverdin. The high tissue levels of biliverdin reductase immediately reduce the biliverdin back to bilirubin (Baranano & Snyder, 2001). In brain cultures of HO2-/- mice neurotoxicity is markedly detectable (Dore et al., 1999[a]). Augmented neurotoxicity is associated with a selective increase in apoptotic death and is reduced by HO2 transfection (Dore et al., 2000). HO2-/- animals also display increased neuronal damage after middle cerebral artery occlusion (Dore et al., 1999[b]). Moreover, HO1-/- mice, which are notably debilitated and die when 3-4 months old, do not display augmented stroke damage.