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AN ELEVATED LEVEL OF CIRCULATING GALANIN PROMOTES DEVELOPMENTAL EXPRESSION OF MYELIN BASIC PROTEIN IN THE MOUSE BRAIN

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Abstract—Myelinogenesis is a scheduled process that is regulated by the intrinsic properties of the cell and extracellular signals. Galanin (GAL) is a bioactive neuropeptide that is widely distributed throughout the nervous system. Chronic increase in circulating GAL levels protects the demyelination processes. Furthermore, GAL is synthesized in myelin-producing glial cells, such as oligodendrocytes and its expression level is at its highest between postnatal days 10 and 40. In the present study, we use our GAL transgenic mouse model to examine the effects of GAL on postnatal myelinogenesis in the CNS. Although we observed no difference in the proliferation of oligodendrocyte precursor cells, we found that GAL has a strong pro-myelinating effect. The transgenic mice at postnatal day 10 appeared to undergo myelinogenesis at an accelerated rate, as demonstrated by the increase in myelin basic protein (MBP) synthesis. The immunohistochemical results are consistent with our preliminary findings that suggest that GAL is a regulator of myelination and may be one of the myelination promoters. This finding is especially important for studies focusing on endogenous molecules for treating myelin-related diseases, such as multiple sclerosis and other leukodystrophies.

Key words: galanin, myelin basic protein, immunohistochemistry, oligodendrocyte development.

INTRODUCTION

The process of myelination in the central nervous system begins with the presence of oligodendrocyte precursor cells (OPCs), which originate from areas around the ventricles of the developing brain, specifically from the neuroectoderm (Kamholz, 1996). When the OPCs are no longer influenced by inhibitory signals (Aggarwal et al., 2011), they differentiate and promote the synthesis of the major components that make myelin. In rodents, most OPCs begin to differentiate between birth and postnatal day 5 (Huang et al., 2013). These cells then migrate to myelination sites, mature and begin myelin production. The OPC maturation timeline proposed by Ishibashi et al. (2009) suggests that in rodents migration occurs at approximately postnatal day 7 and maturation starts at approximately postnatal day 10 (Ishibashi et al., 2009). In the absence of inhibitory signals, a variety of transcription factors and other regulatory factors are involved in regulating OPCs, their differentiation, their maturation and the synthesis of myelin. Timing appears to be especially important for oligodendrocyte maturation and the onset of myelination. The literature suggests that there is only a brief time frame during which oligodendrocytes are able to myelinate (Glenn and Talbot, 2013).

Galanin (GAL) is a bioactive neuropeptide that is 29 amino acids long and widely distributed throughout the rat, mouse and human nervous systems. GAL has diverse neuromodulatory effects and acts as a ''classical neurotransmitter'' (Vrontakis, 2002; Lang et al., 2007). Furthermore, GAL has neurotrophic effects in the developing and adult brain (Shen et al., 2005). It has also been suggested that GAL plays a role in neurogenesis, specifically in the proliferation and differentiation of neural stem cells (Agasse et al., 2013) and that GAL acts as a growth and survival factor (Holmes et al., 2000; O'Meara et al., 2000; Elliott-Hunt et al., 2004; Butzkueven and Gundlach, 2010) for various neurons (Shen et al., 2003) and oligodendrocytes (Habert-Ortoli et al., 1994; Ubink et al., 2003; Zhang et al., 2012). GAL acts through the three receptor subtypes GalR1, GalR2 and GalR3 (Habert-Ortoli et al., 1994; Fathi et al., 1997; Howard et al., 1997; Wang et al., 1997; Lang et al., 2007). Although all three of these receptors are members of the G-protein-coupled receptor family, they demonstrate differences in their functional coupling, which might explain the variety of physiological effects exhibited by GAL (Lundstrom et al., 2005a,b; Lang et al., 2007). GAL and its receptors have been found in the

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Abbreviations: ANOVA, analysis of variance; BBB, blood–brain barrier; CNTF, ciliary neurotrophic factor; GAL, galanin; GH, growth hormone; IHC, immunohistochemistry; MBP, myelin basic protein; OPCs, oligodendrocyte precursor cells; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; RT, reverse transcription; RT-PCR, reverse transcription polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gels; TG, transgenic; WT, wild-type.

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corpus callosum, where GAL may play a role in regulating OPCs and, thus, myelination (Shen et al., 2005; Lang et al., 2007). Additionally, *in situ* hybridization has shown that GAL expression can be detected in the corpus callosum region as early as postnatal day 2–5 and is strongly expressed through postnatal day 40 (Shen et al., 2005). Furthermore, a study on the rat brain showed that some non-neuronal cells express high levels of GAL and that there is a temporally restricted GAL mRNA expression pattern in the corpus callosum (Butzkueven and Gundlach, 2010). Recently, we demonstrated that GAL has pronounced neuroprotective effects on demyelination and remyelination in a multiple sclerosis animal model (Zhang et al., 2012). In the present study, GAL is assessed as a potential regulator of oligodendrocyte maturation and as a potential factor that promotes myelinogenesis.

We investigated the effects of GAL by comparing the myelin development in GAL transgenic (TG) mice with a chronic increase of circulating GAL levels and wild-type mice (WT) with the same genetic background. Tests for detecting the expression of biomarkers for myelin synthesis (such as myelin basic protein (MBP) protein) were conducted at postnatal days 10, 15 and 30 (P10, P15 and P30), and the results were used to monitor the rate of myelination.

EXPERIMENTAL PROCEDURES

Experimental animals

All mice, including the wild-type (WT; C57BL/6) and homozygous TG mice (TG) maintained on a C57BL/6 background, were housed in the University of Manitoba animal facility in a temperature-controlled environment (at 20 °C under a 12-h light/dark cycle). Food and drinking water were available *ad libitum*. WT mice were obtained from the University of Manitoba Genetic Modeling of Disease Centre; the TG mice were generated as previously reported (Perumal and Vrontakis, 2003), using a 320-bp fragment of the rat growth hormone (GH) promoter to the full-length rat preprogalanin cDNA clone. In these TG animals GAL was over expressed in the pituitary and over secreted in the circulation. Thus, the creation of these TG mice, with chronic elevation of circulating GAL allows for the study of the effect of GAL in a variety of regions. Before the experiment began, homozygous TG mice were backcrossed to WT; C57BL/6 from Charles River and bred again to homozygosity. The WT and TG mice were collected at P10, P15, and P30 and sorted into three groups per genotype. Each group consisted of 4–6 mice, and experiments were repeated twice. To confirm the results of day 10, six more male mice were added to the control group and four more TG male mice were added to the experimental group. All of the procedures were conducted in accordance with the Animal Protocol Review Board of the University of Manitoba, which approved this study under protocol #10-013/1/2.

Tissue collection and preparation

Mice were anesthetized and intracardially perfused with 0.1 M phosphate-buffered saline (PBS) containing 50 U/ml heparin, followed by 4% paraformaldehyde prepared in 0.1 M PBS Whole brains were carefully harvested after decapitation. To exclude the regional differences in the myelination rate (Smith, 1973), the cerebellums were not included in this study. The dissected brains were cut along the longitudinal fissure to generate two hemispheres of equal size. Half of the hemispheres were collected and stored at −80 °C until protein extraction for western blot analysis, and the remaining half of the hemispheres were incubated in RNAlater solution (Cat. # AM7020, Life Technologies™, Burlington, Ontario, Canada) at 4 °C overnight and then stored at −80 °C until RNA extraction for real-time polymerase chain reaction (PCR). Animals intended for the histological studies were perfused and fixed with 4% paraformaldehyde. The fixed brains were cryoprotected in PBS containing 30% sucrose. The brains were then snap frozen on dry ice and stored at −80 °C until the samples were sectioned.

Western blot analysis

The protocol used in this study is a modified version of a protocol provided by Abcam, Toronto, Ontario, Canada. The frozen tissues were homogenized in radioimmunoprecipitation assay (RIPA) lysis buffer containing a protease inhibitor cocktail (CKT, Cat. # P8340-1ML, Sigma–Aldrich, Oakville, Ontario, Canada). The protein concentration was quantified using a BCA Protein Assay Kit (Product # 23225, Pierce, Thermo Scientific, Fisher Scientific, Nepean, Ontario, Canada). Protein samples were denatured and reduced following the manufacturer's protocol and were then stored in aliquots at −80 °C.

Samples of 25 μg protein and a protein marker (EZ-Run Prestained REC Protein Ladder, Cat. # BP3603-500, Fisher BioReagents, Fisher Scientific, Nepean, Ontario, Canada) were separated on 10% sodium dodecyl sulfate polyacrylamide gels (SDS–PAGE) using a Mini Protean 3 cell system at room temperature (Cat. # 165-3301, BIO-RAD, Mississauga, Ontario, Canada). Membranes were blocked using a solution of 5% milk in Tris-buffered saline, 0.1% Tween-20 (TBST) buffer for 1 h and then incubated in the primary antibodies (Table 1) overnight at 4 °C. The next day, the membranes were incubated in the horseradish peroxidase (HRP)-conjugated secondary antibodies (Table 1) for 1 h at room temperature on a rocking shaker. Finally, the bands were visualized using the Western Lightning® Plus-ECL kit (Cat. # NEL104001EA, PerkinElmer, Cedarlane, Burlington, Ontario, Canada) and autoradiography films.

Histology staining

Serial 25-μm coronal sections were cut from the frozen brains. The sections used for staining were in the 185–195 level according to the High Resolution Mouse Brain Atlas by Sidman et al. (http://www.hms.harvard.edu/research/brain/atlas.html).

MBP staining was performed using the avidin–biotin–peroxidase complex technique. The full description of the immunohistochemistry (IHC) technique in our lab was previously reported by Zhang et al., 2012).