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Dendritic Morphology and Spine Density is not Altered in Motor Cortex and Dentate Granular Cells in Mice Lacking the Ganglioside Biosynthetic Gene *B4galnt1* – A Quantitative Golgi Cox Study

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A B S T R A C T

Gangliosides are characteristic plasma membrane constituents of vertebrate brain used as milestones of neuronal development. As neuronal morphology is a good indicator of neuronal differentiation, we analyzed how lack of the ganglioside biosynthetic gene Galgt1 whose product is critical for production of four major adult mammalian brain complex gangliosides (GM1, GD1a, GD1b and GT1b) affects neuronal maturation in vivo. To define maturation of cortical neurons in mice lacking B4galnt1 we performed a morphological analysis of Golgi-Cox impregnated pyramidal neurons in primary motor cortex and granular cells of dentate gyrus in 3, 21 and 150 days old B4galnt1-null and wild type mice. Quantitative analysis of basal dendritic tree on layer III pyramidal neurons in the motor cortex showed very immature dendritic picture in both mice at postnatal day 3. At postnatal day 21 both mice reached adult values in dendritic length, complexity and spine density. No quantitative differences were found between B4galnt1-null and wild type mice in pyramidal cells of motor cortex or granular cells of dentate gyrus at any examined age. In addition, the general structural and neuronal organization of all brain structures, qualitatively observed on Nissl and Golgi-Cox, were similar. Our results demonstrate that neurons can develop normal dendritic complexity and length without presence of complex gangliosides in vivo. Therefore, behavioral differences observed in B4galnt1-nulll mice may be attributed to functional rather than morphological level of dendrites and spines of cortical pyramidal neurons.

***Key words**: B4galnt1-null mice, glutamatergic neurons, cerebral cortex, hippocampus, Alzheimer disease, epilepsy, developmental plasticity*

Introduction

Gangliosides, sialic acid containing glycosphingolipids, are present in all vertebrate tissues and they are especially abundant in brain. Different studies, especially these on knockout animals, have shown that certain glycosphingolipid structures perform important role in function and long term maintenance of nervous system[1].

However, the exact role of distinct gangliosides and their complexity level in neuronal differentiation is still not clear. Decrease of complex to simple ganglioside ratio by inhibiting the enzyme GM2/GD2 synthase (EC 2.4. 1.92), that direct synthesis from simple to more complex structures in all pathways, blocks neuritogenesis in chicken retinal neuronal culture[2]. In contrast, disruption of GD3 synthase (EC 2.4.99.8) knocks just synthesis of b-series gangliosides in embryonic stem cells and has no effect on neuritogenesis[3]. Finally, development and survival of cerebellar Purkinje cells in vitro demands normal glycosphingolipid synthesis[4], indicating cell type speci-

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ficity in requirements of distinct gangliosides for neuritogenesis.

GM3/GD3 synthase is a Golgi-resident enzyme critical for production of GM1, GD1a, GD1b and GT1b gangliosides which are specifically reduced in the majority of brain regions (particularly temporal cortex and hippocampus) in Alzheimer's disease[5–8]. Therefore, to determine ganglioside role in neuronal differentiation *in vivo*, we carried out the morphological analysis of Golgi impregnated neurons of *B4galnt1-null* mice (KO) lacking enzyme UDP-*N*-acetyl-D-galactosamin: GM3/GD3 *N*-acetyl-D-galactosaminyltransferase (EC 2.4.1.92) (GM2/GD2 synthase) and age-matched wild type animals (WT). Those knock out mice express no complex gangliosides, but do express high concentrations of simpler structures GM3 and GD3[9]. The complex gangliosides GM1, GD1a, GD1b and GT1b appear with the first cortical neurons[10], while simple gangliosides are more inherent for early embryogenesis[11]. Excitability defect of neurons derived from *B4galnt1-null* mice were demonstrated; when exposed to depolarization they die because of inability to regulate intracellular calcium content[12]. The morphological examination of 12 to 16 week old animals revealed decreased central and peripheral myelinization, changes of axonal diameter that progress to axonal degeneration[13], motor deficit with tremor[14] and locomotor hyperactivity[15]. These data further support role of complex gangliosides as functional receptors[16] for myelin associated glycoprotein (MAG) in maintenance of axon-myelin stability[17].

Besides having a role on axonal membrane, the complex gangliosides are expressed on neuronal bodies and synapses[18]. Each one of ganglioside structures was expected to have specific function in chaperoning neurotransmitter receptors and formation of lipid rafts[19]. To examine if lack of major complex gangliosides would also reflect on dendritic morphology and spine density, we found the best candidate to be principal neurons of the motor cortex and dentate gyrus granule cells of the hippocampal formation.

Materials and Methods

Animals and tissue staining

B4galnt1-null mice were kindly provided by Dr. Ronald L. Schnaar, Johns Hopkins School of Medicine, Baltimore, USA. The mice generation and rearing has been previously described by Sheikh et al.[13] The knockouts and age-matched wild type controls were 3 (P3), 21 (P21) and 150 days (i.e. 5 months – 5MO) old. The experimental procedure was approved by Ethical Committee of School of Medicine, »Josip Juraj Strossmayer« University (No. 0219021). The mice were deeply anaesthetized in isofluran vapors and decapitated. The brains were removed and divided in the medial sagittal direction. Both hemispheres were alternatively cut in a coronal plane into 3 blocks of tissue that were placed in Golgi-Cox solution for 3 weeks, with one change of solution after 3 days[20]. After impregnation, the tissue was dehydrated, embedded in celloidin and sectioned coronaly at 180 μm. For developing of staining, the sections were immersed in 20% ammonium hydroxide for 5 min and then transferred in 15% solution for 25 min. After rinsing they were further processed through 1% thiosulfate for 7 min, dehydrated in alcohol, cleared in Histoclear (National Diagnostic, Atlanta, Georgia) and covered with Histomount mounting media[21] (National Diagnostic, Atlanta, Georgia). In 5MO mice quantitative dendritic tree analysis was performed on layer III and layer V pyramidal neurons in the primary motor cortex and granular cells of in the middle part of dentate gyrus upper blade[22]. In P3 and P21 only layer III pyramidal neurons were quantitatively analyzed. The quantitative analysis was performed on 3 KO and 3WT animals *per* age group, and at least 5 neurons *per* animal were reconstructed for purpose of quantitative analysis. Reconstructed neurons have their cell body positioned in the middle third of sections thickens, because neurons that lie in the middle third of section thickness have highest level of impregnation[20] and this criterion could reduce the number of cut segments at the section surface. Since all brain tissue was processed under same laboratory conditions, obtained values were not corrected for the shrinkage factor.

The quantitative morphometric analysis was performed using Neurolucida 3,18 software (Microbrightfield Inc., Colchester, VT) and automatic dendrite measuring system that provides three-dimensional data of the dendritic tree[23–25]. The measurements were made using a 60x air objective with actual enlargement on screen equal to 4200x. The neurons were drawn over the live picture on the PC screen, bringing the signed point in the sharp focus when drawing. Changes in depth (z-dimension) were identified for each drawn point, and automatically corrected according to Snell's law for diffraction air correction factor (1.515). The X-Y coordinates were also given to the each point in relation to the reference point. The subjects were coded, so that the investigators were not aware of the genetic background. On reconstructed neurons we did not found signs of incomplete impregnation. The following dendritic parameters were analyzed[26,27]: a) number of primary dendrites, b) total number of segments, c) total dendritic length *per* neuron, d) spine density. For pyramidal neurons only the basal dendritic tree was quantitatively analyzed. Obtained data are presented as median value +/– standard deviation. Parameters were compared using Student's t-test, with significance level set at $p<0.05$.

Results and Discussion

In neither one stage qualitative differences between *B4galnt1-null* and wild type mice were not observed on Nissl (data not shown) either Golgi Cox slices (Figure 1). The motor cortex pyramidal neurons (Figure 2A) and fascia dentata granular cells (Figure 2B) quantitative analysis did not reveal statistically significant differences in dendritic trunk parameters either (Table 1, 2).

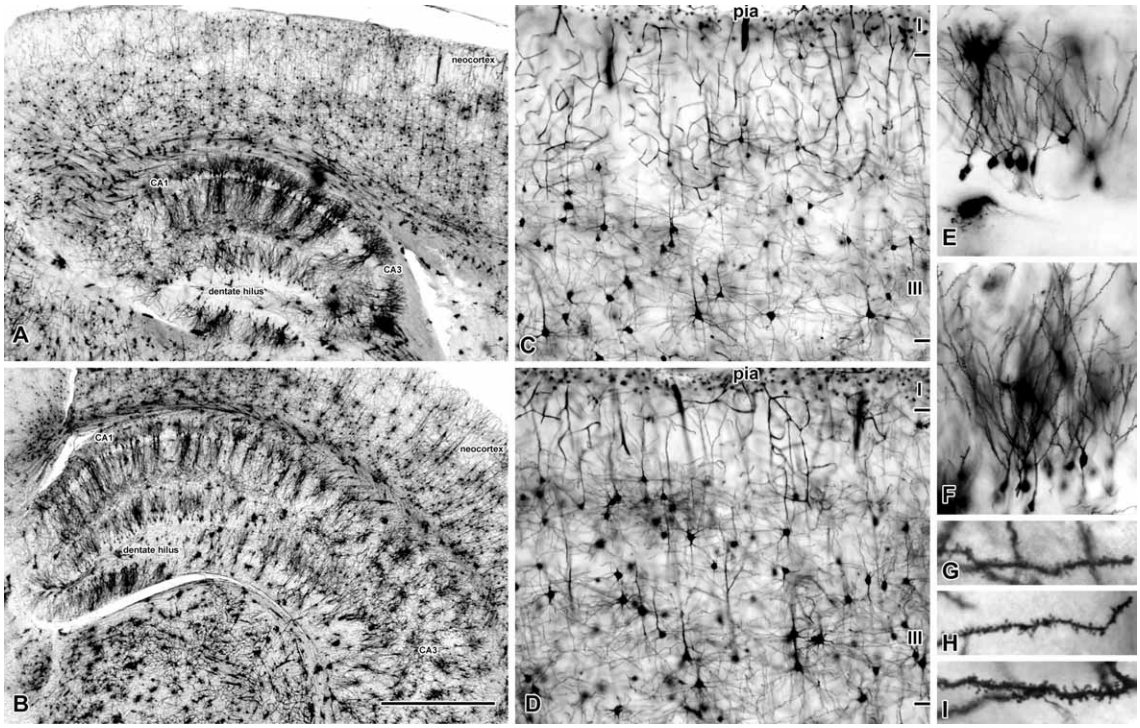

Fig. 1. Microphotography of Golgi-Cox impregnated brain slices of wild type (A,C,E,G) and knock out mouse (B,D,F,H,I) at age of 5 months. Qualitatively no differences were observed in general neuronal organization (A,B), dendritic morphology and spine density in the motor cortex (C,D) and the dentate gyrus (E,F). High magnification of oblique pyramidal neuron dendrites in motor cortex showed no differences in spine morphology (G,H,I). Bar scale is 500 μm (A,B), 250 μm (C,D) 120 μm (E,F) and 20 μm (G,H,I).

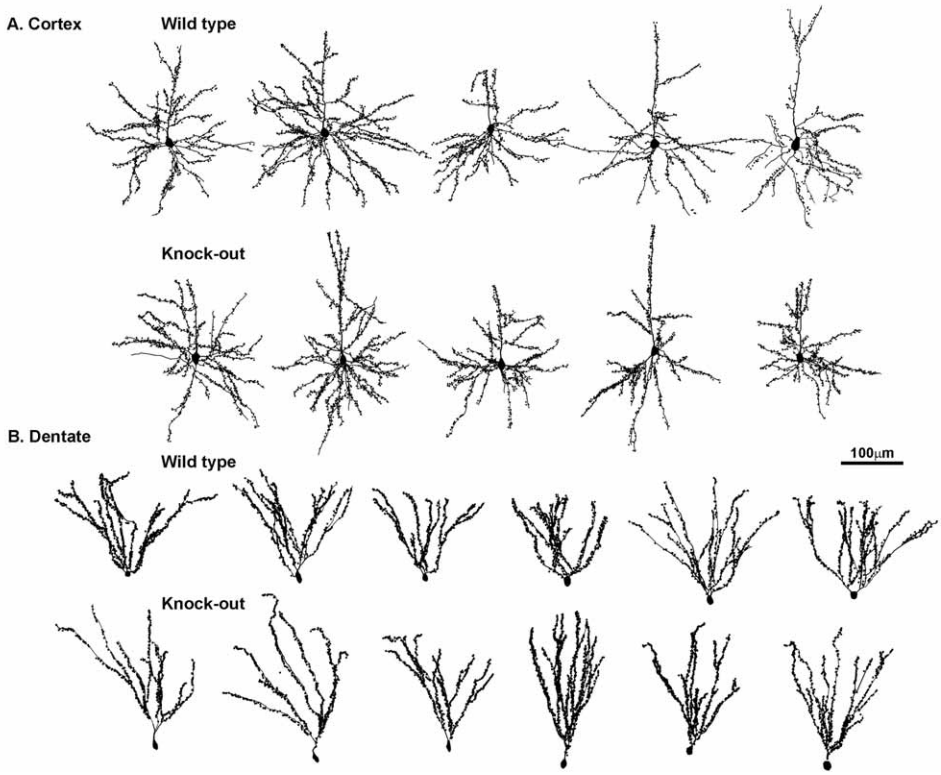

Fig. 2. Representative neurons three-dimensionally reconstructed by Neurolucida system. No differences could be quantitatively seen between wild type and knock-out mouse, both on layer III pyramidal neurons in the motor cortex (A) and dentate gyrus granular cells (B). Bar scale 100μm.

TABLE 1
MORPHOMETRIC VALUES OF ANALYZED NEURONS ON LAYER III IN PRIMARY MOTOR CORTEX OF 21 DAYS AND 3 DAYS OLD MICE (X±SD)

3 days old mice	Wild type	Knockout
Dendrite		
Total length (μm)	131±36	118±39
Number of primary dendrites	4.9±0.8	4.8±1.5
Number of segments	10±3	10±3
Spine density (1/μm)	0.045±0.028	0.044±0.021
21 days old mice		
Dendrite		
Total length (μm)	1988±674	1900±645
Number of primary dendrites	7.2±1.3	6.6±1.4
Number of segments	48±10	45±12
Spine density (n/μm)	0.356±0.040	0.340±0.046

TABLE 2
MORPHOMETRIC VALUES OF ANALYZED NEURONS IN PRIMARY MOTOR CORTEX AND DENTATE GYRUS OF 5 MONTHS OLD MICE (X±SD)

Layer III pyramidal neurons	Wild type	Knockout
Dendrite		
Total length (μm)	1972±458	2034±459
Number of primary dendrites	6.9±1.2	6.2±1.3
Number of segments	41±10	46±11
Spine density (n/μm)	0.298±0.094	0.297±0.090
Layer V pyramidal neurons		
Dendrite		
Total length (μm)	1971±640	2211±489
Number of primary dendrites	6.9±1.1	7.1±1.7
Number of segments	49±10	51±12
Spine density (n/μm)	0.311±0.047	0.334±0.073
Dentate granular cells		
Dendrite		
Total length (μm)	1556±348	1551±321
Number of segments	25±8	22±6
Spine density (n/μm)	0.539±0.113	0.529±0.110

In P3 mice, Golgi staining showed undeveloped cortex (Table 1). The pyramidal neurons at different stages of differentiation could be observed. In the most superficial cortical layers neurons have just started to grow one or two basal dendrites. The neurons in deeper layers have few basal and oblique dendrites and some of them are already branching. Relatively few stubby spines and spine-like outgrowth on neuron bodies can be observed, both indicating immaturity. The layer V pyramidal neurons are more mature; having larger cell bodies and more branched dendritic trunk. At this age, the hippocampal formation was poorly impregnated and unsuitable for further analysis.

In motor cortex of P21 a Golgi impregnation shows neurons of developed morphology (Table 1), reaching the dendritic complexity and length of 5MO animal (Table 2). Compared to P3 mice, the neuronal cells and dendritic trunks matured. The dendrites were covered with developed spines, without somatic spines. There was no visible difference in development of pyramidal neuron basal dendritic trunks between layers III and V, but neuronal bodies in layer V were much larger than those in layers II or III. Data of this research showed absence of large morphological abnormalities in cortical circuitry of *B4galnt1-null* adult mice implying undisturbed development. However, we cannot exclude the possibility that some abnormalities are present at fine ultra structural and molecular level. Also, some selective neuronal population[28], like different types of interneurons[29], not included in this study, could have abnormalities in arborization. However, the quantitative methodology and obtained data of this study are in line with present literature data[22,24,26–28,30–43]. Also, the brains from *B4galnt1-null* animals and wild type mice were preceded and analyzed at the same conditions; we do not found that peculiarities of Golgi method could affect the final conclusion.

Numerous *in vitro* experiments assumed that gangliosides could participate in neuritogenesis, without providing clear image on their role in differentiation. While some authors showed that gangliosides are necessary for normal axonogenesis in cultured hippocampal neurons[44,45], others find them important for process of dendritogenesis. Moreover, it was suggested that ganglioside GM2 itself is responsible for new dendrite formation in normal pyramidal neurons during dendritic growth and that excess of GM2 in some sphingolipid storage diseases leads to dendrite outgrowth on axonal hillock[46]. It was also demonstrated that nanomolar concentrations of GT1b, or solely its oligosaccharide part, induce fillopodium and dendrite formation by activation of Ca^{2+}/calmodulin-dependant protein kinase II (cdc42 GTP-ase intervened) in cultured hippocampal and Purkinje neurons. These data indicate existence of membrane receptor specifically recognizing certain gangliosides[47].

There were few hypothesis how gangliosides could affect dendritogenesis. For instance, it was considered that transport of glycosilphosphatidilinositol anchored proteins (GPI-anchored proteins) to membrane depends on glycosphingolipids and for some of GPI-anchored proteins, like CPG15[48], is known that they can stimulate dendrite growth. Moreover, lipid rafts (with gangliosides as integral part) are associated with signaling molecules like tyrosine kinase c-Src or GTP-ases Rho-A and Ras, which could regulate dendritogenesis. The gangliosides also could regulate growth factor receptor activity, for example, nerve growth factor (NGF) receptor Trk A[49].

Results of our study demonstrate that cortical and hippocampal neurons '*in vivo*' can develop normal size of dendritic tree and spine density without presence of complex gangliosides. Although it is possible that ganglio-

sides participate in processes important for dendritogenesis, it appears that *B4galnt1-null* mouse brain has regulatory mechanisms to compensate role of complex gangliosides in neuronal morphogenesis. The gangliosides probably can mutually compensate the deficit, in this case, simple GM3 and GD3 for the complex ones. It is also possible that other regulatory pathways substitute complex ganglioside dependant pathways.

This study does not support specific role of certain complex gangliosides in neuron differentiation *in vivo*. Nevertheless, their role in some physiological processes, due to possible adaptation of other regulatory pathways, cannot be excluded. It also cannot be excluded that complex gangliosides regulate neuronal maturation in cerebellum, parts of hippocampus, cortex or subcortical regions, which were not analyzed.

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MORFOLOGIJA DENDRITA I GUSTOĆA SPINA NIJE PROMIJENJENA U MIŠEVA S ISKLJUČENIM *B4GALNT1* GENOM ZA BIOSINTEZU GANGLIOZIDA: KVANTITATIVNA GOLGI-COX STUDIJA

S A Ž E T A K

Gangliozidi su karakteristične sastavnice stanične membrane stanica mozga kralješnjaka koje se rabe kao pokazatelji razvoja neurona. Kako je neuronska morfologija dobar pokazatelj diferencijacije neurona, mi smo analizirali kako nedostatak gena *B4galnt1* za biosintezu gangliozida čiji je produkt od kritične važnosti za produkciju četiri glavna kompleksna gangliozida odraslog mozga sisavaca (GM1, GD1a, GD1b i GT1b) utječe na sazrijevanje neurona *in vivo*. Kako bi utvrdili zrelost kortikalnih neurona miševa s inaktiviranim *B4galnt1* genom proveli smo morfološku analizu Golgi-Cox impregniranih piramidnih neurona primarne motoričke kore i granularnih stanica girusa dentatusa u miševa s isključenim *B4galnt1* genom i u miševa divljeg soja, starih 3, 21 i 150 dana. Kvantitativna analiza bazalnog dendritičkog stabla sloja IIIc piramidnih neurona motoričke kore pokazala je vrlo nezrelu sliku dendrita u obje skupine miševa starih 3 dana. Miševi stari 21 dana su obje skupine postigli vrijednosti dužine dendrita, složenosti i gustoće trnova (spina) poput one u odraslih životinja. U niti jednoj dobnoj skupini nisu nađene kvantitativne razlike između *B4galnt1-null* miševa i miševa divljeg tipa među piramidnim neuronima motoričke kore, niti među granularnim stanicama girusa dentatusa bilo koje promatrane dobi. Osim toga, opća strukturna i neuronska organizacija svih moždanih regija, promatrana kvalitativno (Nissl i Golgi-Cox impregnacija neurona), također je bila slična. Naši rezultati pokazuju da neuroni mogu razviti normalnu dendritičku složenost i duljinu i bez prisustva složenih gangliozida *in vivo*. Radi toga, razlike u ponašanju primijećene kod miševa s isključenim *B4galnt1-null* genom prije bi se mogle pripisati funkcijskoj, a ne morfološkoj razlici na razini dendrita i spina kortikalnih piramidnih neurona.