

## Table of contents

<b>List of figures.....</b>	<b>VII</b>
<b>List of tables .....</b>	<b>X</b>
<b>Abbreviations .....</b>	<b>XII</b>
<b>1    Introduction .....</b>	<b>1</b>
1.1    Glycosphingolipids .....	1
1.2    Glucosylceramide: key lipid in glycosphingolipid metabolism .....	2
1.2.1    Degradation of glycosphingolipids .....	5
1.2.1.1    The lysosomal glucosylceramidase GBA1.....	6
1.2.1.2    The non-lysosomal glucosylceramidase GBA2.....	6
1.3    Glycosphingolipid-associated disorders.....	8
1.3.1    Lysosomal storage disorders.....	8
1.3.2    GBA2-associated disorders.....	9
1.3.2.1    Male subfertility in GBA2-knockout mice .....	9
1.3.2.2    Mutations in <i>GBA2</i> in neurological disorders.....	10
1.4    Aim of this thesis .....	13
<b>2    Material and methods.....</b>	<b>14</b>
2.1    Chemicals .....	14
2.2    Cell culture material.....	14
2.3    Antibodies .....	14
2.3.1    Primary antibodies .....	14
2.3.2    Secondary antibodies.....	16
2.3.3    Dyes.....	17
2.4    Molecular biology .....	17
2.4.1    Cloning of wild-type and mutant mGBA2 proteins .....	17
2.4.1.1    Vectors.....	17
2.4.1.2    Primers .....	17
2.4.1.3    Polymerase Chain Reaction (PCR) .....	21
2.4.1.4    Agarose gel electrophoresis for detection of nucleic acids .....	22
2.4.1.5    DNA purification using Sure Clean .....	23

## Table of contents

---

2.4.1.6	Restriction digest of plasmid DNA .....	23
2.4.1.7	Extraction of DNA from agarose gels .....	24
2.4.1.8	Ligation of DNA fragments with vector .....	24
2.4.1.9	Determining nucleic acid concentrations by spectrophotometry .....	24
2.5	<i>Escherichia coli</i> culture.....	24
2.5.1	Bacterial strains.....	24
2.5.2	Culture medium.....	25
2.5.3	Generation of competent <i>E.coli</i> .....	25
2.5.4	DNA amplification in <i>E.coli</i> .....	25
2.5.4.1	Transformation of competent bacteria .....	25
2.5.4.2	Small-scale (Mini) plasmid preparation via alkaline lysis .....	26
2.5.4.3	Sequencing of amplified plasmid DNA .....	26
2.5.4.4	Large-scale (Midi/Maxi) plasmid preparation .....	26
2.6	Mice .....	27
2.6.1	Isolation of genomic DNA from mouse tails .....	27
2.6.2	Genotyping of mice by PCR .....	28
2.7	Mammalian cell culture.....	29
2.7.1	Buffers and media used for cell culture.....	29
2.7.1	Cell line .....	29
2.7.1	Preparing back-ups of cultured cells.....	29
2.7.1	Re-culturing of cells frozen as back-ups.....	30
2.7.1	Poly-L-lysine (PLL) coating of glass coverslips.....	30
2.7.2	Transient transfection using PEI.....	30
2.7.3	Stable cell line expressing mGBA2.....	30
2.7.4	Isolation of murine dermal fibroblasts .....	31
2.7.4.1	Transient transfection of murine fibroblasts via electroporation .....	31
2.7.5	Isolation of murine cerebellar neurons.....	31
2.7.5.1	Treatment of cultured cerebellar neurons with NB-DNJ or AMP-DNM.....	32
2.8	Immunocytochemistry .....	32
2.8.1	Fixation of cells .....	32
2.8.2	Immunocytochemical (ICC) staining .....	33

2.9	Isolation of murine tissue.....	33
2.9.1	Dissection of mice .....	33
2.9.2	Fixation of tissue in glutaraldehyde .....	34
2.9.3	Perfusion of mice with paraformaldehyde (PFA).....	34
2.9.4	Cryopreservation in sucrose gradient .....	34
2.9.5	Cryosectioning of murine brain and spinal cord.....	34
2.10	Histochemical stainings.....	35
2.10.1	Detection of $\beta$ -galactosidase expression using X-gal .....	35
2.10.2	Nissl body staining .....	35
2.11	Protein biochemistry.....	36
2.11.1	Preparation of proteins .....	36
2.11.1.1	Protein lysates of cultured cells .....	36
2.11.1.2	Protein lysates of murine tissue.....	36
2.11.2	Protein concentration determination .....	37
2.11.2.1	Bradford assay .....	37
2.11.2.2	Bicinchoninic (BCA) test.....	37
2.11.3	Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).....	38
2.11.3.1	Self-casted SDS gels .....	38
2.11.3.2	Protein marker.....	39
2.11.3.3	Coomassie staining to detect proteins on polyacrylamide gels .....	39
2.11.4	Western blot analysis .....	40
2.11.4.1	Immunostaining of immobilized proteins.....	40
2.11.5	Protein expression in <i>E.coli</i> .....	41
2.11.5.1	Test expression of recombinant protein.....	41
2.11.5.2	Large-scale expression of recombinant mGBA2 protein .....	42
2.11.5.3	Activity measurements of recombinant mGBA2.....	42
2.11.6	Purification of recombinant protein .....	42
2.11.6.1	Affinity chromatography to purify recombinant mGBA2 .....	43
2.11.6.2	Tobacco Etch Virus (tev) protease cleavage .....	44
2.11.6.3	Buffer exchange using NaP <sup>TM</sup> -5 columns .....	44
2.11.6.4	Calibration of the Superdex <sup>TM</sup> 200 Increase 10/300 column .....	44

## Table of contents

---

2.11.6.5	Size-exclusion chromatography .....	45
2.11.7	Co-immunoprecipitation using magnetic beads .....	45
2.11.8	Chemical protein cross-linking using disuccinimidyl suberate (DSS) .....	47
2.11.9	Fluorescence-based $\beta$ -glucosidase activity assay .....	47
2.11.9.1	Setup of the $\beta$ -glucosidase activity assay .....	47
2.11.9.2	Fluorometric measurement using Fluostar plate reader.....	48
2.11.9.3	Dose-response analysis of AMP-DNM .....	49
2.11.10	Rho GTPase pull-down activation assay.....	49
2.12	Lipid analyses .....	50
2.12.1	Isolation of lipids from murine cerebellum.....	50
2.12.2	Lipid extraction for mass spectrometry .....	50
2.12.3	Lipid extraction for thin layer chromatography (TLC) .....	51
2.12.4	Isolation of detergent-resistant (DRM) membranes .....	51
2.13	Behavioral tests.....	52
2.13.1	Weight Test.....	52
2.13.2	Catwalk .....	53
2.14	Validation of mouse genotypes.....	54
2.15	Software applications .....	54
2.16	Statistics.....	54
<b>3</b>	<b>Results .....</b>	<b>55</b>
3.1	Do mutations in GBA2 affect enzyme function?.....	55
3.1.1	Cloning of mGBA2 mutants .....	55
3.1.2	Heterologous expression of mGBA2 mutants .....	55
3.1.3	$\beta$ -Glucosidase activity assay .....	56
3.1.4	Mutations in GBA2 cause a loss of function of the enzyme .....	57
3.2	Structure-function analysis of GBA2.....	58
3.3	Structural modelling of GBA2 .....	61
3.4	Purification of mGBA2 .....	62
3.4.1	Expression of mGBA2 in <i>E.coli</i> .....	63
3.4.2	Affinity chromatography of MBP-tagged mGBA2 via MBPTrap <sup>TM</sup> .....	63

3.4.3	Separation of mGBA2-126/882 from MBP-tev-mGBA2-126/882 via MBPTrap™	64
3.4.4	Calibration of the size-exclusion chromatography column .....	65
3.4.5	Size-exclusion chromatography of mGBA2-126/882 .....	68
3.4.6	MBP-tev-mGBA2-126/882 is not active .....	70
3.5	GBA2 interaction studies .....	72
3.5.1	Co-immunoprecipitation .....	72
3.5.1.1	Does mGBA2-Flag bind to anti-Flag magnetic beads? .....	73
3.5.1.2	Test for specificity .....	74
3.5.1.3	Co-immunoprecipitation of mGBA2-Flag and mGBA2-HA .....	75
3.5.1.4	Independent HA-tagged control protein .....	75
3.5.1.5	Co-immunoprecipitation of mutant and wild-type mGBA2 .....	76
3.5.2	Chemical cross-linking .....	77
3.5.2.1	Optimizing cross-linking conditions .....	78
3.5.2.2	Cross-linking of mutant mGBA2 .....	79
3.5.3	GBA2 activity interaction studies .....	82
3.5.3.1	Cloning of the GBA2-2A and -linker constructs .....	82
3.5.3.2	Activity of mutant and wild-type mGBA2-2A and -linker chimera .....	83
3.6	Genetic ablation of GBA2 expression in mice .....	86
3.7	Expression and activity of GBA2 in the central nervous system .....	90
3.7.1.1	Brain .....	90
3.7.1.2	Spinal Cord .....	94
3.7.1.3	Cerebellum .....	96
3.7.1.3.1	Cerebellar morphology in GBA2-KO mice .....	97
3.8	Expression and activity of GBA2 in skeletal muscle .....	99
3.9	GlcCer accumulates in the cerebellum in GBA2-KO mice .....	101
3.10	Analysis of small Rho GTPases .....	103
3.11	Pharmacological inhibition of GBA2 by iminosugars .....	105
3.11.1	Dose-response relationship of AMP-DNM and GBA2 .....	106
3.12	Loss of GBA2 affects cytoskeletal dynamics .....	110
3.13	Neurite outgrowth of cerebellar neurons .....	111

## Table of contents

---

3.13.1	Pharmacological blocking of GBA2 .....	111
3.13.2	Genetic ablation of GBA2 .....	112
3.14	Behavior studies.....	113
3.14.1	Behavior abnormalities in GBA2-KO mice .....	113
3.14.2	Muscle strength.....	114
3.14.3	Gait and locomotion .....	115
<b>4</b>	<b>Discussion.....</b>	<b>124</b>
4.1	Structural and functional analysis of GBA2.....	124
4.2	Role of GBA2 in the CNS .....	126
4.2.1	Neurons: major site of GBA2 expression and activity in the CNS .....	127
4.2.2	Species-specific functions of GBA2 in the brain .....	129
4.2.3	AMP-DNM – a potent GBA2 inhibitor applicable <i>in vivo</i> .....	132
<b>5</b>	<b>References.....</b>	<b>133</b>
<b>6</b>	<b>Appendix.....</b>	<b>150</b>
	<b>Danksagung.....</b>	<b>154</b>