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Abstract

Myoclonus-Dystonia (M-D) is a neurological movement disorder with involuntary jerky and dystonic movements as major symptoms. About 50% of M-D patients have a mutation in epsilon-sarcoglycan (*SGCE*), a maternally imprinted gene that is widely expressed. Since little is known about SGCE function one can only speculate about the pathomechanisms of the exclusively neurological phenotype in M-D. We characterized different *SGCE* isoforms in the human brain using ultra deep sequencing. We show that a major brain-specific isoform is differentially expressed in the human brain with a notably high expression in the cerebellum, namely in the Purkinje cells and neurons of the dentate nucleus. Its expression was low in the globus pallidus and moderate to low in caudate nucleus, putamen and substantia nigra. Our data is compatible with a model in which dysfunction of the cerebellum is involved in the pathogenesis of M-D.

Keywords: Myoclonus-Dystonia, SGCE, deep sequencing, alternative splicing, imprinting, cerebellum

Introduction

Myoclonus-Dystonia (M-D) is a movement disorder characterized by myoclonic jerks and dystonic features, usually affecting the upper part of the body. Little is known about the pathological basis of M-D symptoms. Electrophysiological studies in man suggest that myoclonic symptoms are of subcortical origin.[1-3] In general, dystonia is thought to arise from dysfunction of the basal ganglia.[4] About 50% of M-D patients that were classified as definite M-D carry a mutation in the widely expressed ε-sarcoglycan (*SGCE*).[5-7] The genetic cause in the remaining patients is still unclear. A second locus has been reported in one large M-D family (DYT15, 18p11), but no gene has been identified yet.[8;9] SGCE is part of the sarcoglycan family that consists of N-glycosylated transmembrane proteins. Six different sarcoglycans have been identified so far (α-, β-, γ-, δ-, ε-, ζ-) but little is known about the function of particular sarcoglycan members and their function in different tissues.[10] In muscle, sarcoglycans form a heterotetrameric complex that is constituent of the dystrophin-associated protein complex. This complex mediates the structural stability of the plasma membrane and interactions between the extracellular matrix and the cytoskeleton.[11] Mutations in other sarcoglycans, α-, β-, γ- and δ-sarcoglycan, lead to different forms of limb-girdle muscular dystrophy, characterized by progressive muscle weakness.[10] Little is known about composition and function of the dystrophin-associated protein complex in brain. *SGCE* is highly homologous to α-sarcoglycan,[12] but no muscle or myocardial muscle abnormalities have been identified in M-D patients.[13]

Understanding the exclusively neurological phenotype of the disease is still a major challenge. *SGCE* is an imprinted gene[14;15] meaning that a loss of function mutation in the

expressed allele will be dominant. All reported mutations behave as null alleles, since they result in premature stop codons and are predicted to lead to nonsense-mediated decay. There is also evidence that *SGCE* missense mutations lead to loss of function.[16] SGCE is widely expressed which suggests either redundancy for this protein in non-brain tissue or a brain-specific function for SGCE.

There are four known alternatively spliced exons in the *SGCE* gene (exon 2, 8, 10 and 11b, Fig. 1A) with exon 11b as a brain-specific exon.[17] Recently, a new brain-specific alternatively spliced exon (exon 11c, an elongated exon 11b) has been identified in mice.[18] Transcripts containing either exon 11b or 11c encode proteins with a different C-terminal sequence containing a PDZ-binding motif. This motif is a protein-interaction domain and thus may contribute to a unique SGCE function in brain. The ubiquitous SGCE protein and the brain-specific protein (exon 11b) are localized in different synaptosomal membrane fractions: post- and pre-synaptic membrane, respectively.[17] It has been speculated that both isoforms play different roles at neuronal synapses.

The aim of our study was to characterize the quantitative and qualitative expression pattern of *SGCE* isoforms in human brain in order to identify brain regions associated with M-D.

Methods

Sample collection and preparation

Human tissue was obtained from six control subjects without neurological symptoms (Table 1). In addition, two blood samples were obtained from control subjects. *SGCE* mutations were excluded by direct sequencing of the coding region. Human tissues

were obtained from the Department of neuropathology of the Academic Medical Center (University of Amsterdam, The Netherlands) and informed consent was obtained for research purposes in all control subjects. Mouse (cerebral cortex), rat (cerebral cortex) and zebrafish (100 embryos, 24 hours after fertilization) tissue was obtained from wild type animals (approved by local committee). DNA extractions from frozen brain tissues and whole-blood samples were performed using standard procedures. Twenty to 40 sections (depending on tissue size, 20µm) were cut per sample and dissolved in TRIzol Reagent (Invitrogen, Breda, The Netherlands) for subsequent RNA isolation, or in SE buffer (750mM NaCl pH 8, 250mM EDTA, 1% SDS) containing Proteinase K for DNA isolation. Total RNA was isolated with QIAcube instrument (Qiagen, Venlo, The Netherlands) using RNeasy Mini protocol or using the PAXgene system (Qiagen) for blood samples.

Methylation-sensitive high resolution melting assay

To determine the degree of methylation in the promotor region of *SGCE* we applied a methylation-sensitive high resolution melting (MS-HRM) assay. The region amplified (nucleotide position -1148 to -773 relative to the start ATG) contains 25 CpG sites and has been shown to be differentially methylated.[14] 1µg of genomic DNA was chemically modified with sodium bisulfite using the EZ Methylation kit (Zymo Research, Orange, CA, USA) and amplified in the presence of ResoLight HRM dye (Roche Diagnostics, Almere, The Netherlands). Both, methylated and unmethylated, strands were amplified.[14] MS-HRM analysis was performed with the LightCycler 480 (Roche Diagnostics) and analyzed with LightCycler 480 gene scanning software module. In each run a set of standards of the identical *SGCE* promotor region was included (100%, 50%, 25%, 12.5%, 6.25% and 0%

methylated). Different brain regions of five control subjects (Subject 1, 2, 4, 5, 6), muscle tissue of two control subjects (Subject 2, 3) and blood of 12 definite M-D *SGCE* non-mutation carriers[7] were tested. A subset of PCR amplified samples was cloned (pGEM-T Easy vector, Promega, Leiden, The Netherlands) and sequenced to verify results of the methylation assay (ABI big dye v3.1 chemistry, ABI 3730 sequencer, Applied Biosystems, Foster City, CA, USA).

Ultra deep amplicon sequencing

cDNA was synthesized from 1µg of total RNA with oligodT_{12}-VN primers and 1µl cDNA was subjected to a 10µl PCR reaction. PCR reactions were performed using fusion primers consisting of a 19 bp fixed sequence (Roche/454 GS FLX, A or B sequence at the 5'end) and a target-specific sequence (3'end). We added a 5 nucleotide multiplex identifier-tag to allow for multiplexing of samples (primer sequences and PCR conditions are available on request). Amplicons were processed and ultra deep sequenced using the 454 GS FLX system (Roche Diagnostics) according to manufacturer's instructions.

Data analysis

In order to identify all alternatively spliced events, sequences were first grouped by a multiplex identifier-tag. Secondly, we used the BLAT algorithm (BLAST-like alignment tool) to compare each sequence with all other sequences to identify similar exon combinations per sample. Sequences were grouped when they encompassed at least 210 nt, had a percent identity of at least 98%, a score above 105 and a query coverage above 98%. The resulting groups were mapped to the respective chromosome (BLAT algorithm, hg18

build) and analyzed in the UCSC genome browser and using CodonCode Aligner software 3.0.1 (Dedham, MA, USA).

Real-time quantitative PCR

1µl cDNA was amplified in triplicates using the LightCycler480 (Roche Diagnostics) in a final volume of 10µl containing SYBR Green I Master mix (Roche Diagnostics). Intron spanning primers were designed to target either all *SGCE* isoforms or the brain-specific isoform containing exon 11b. All quantitative PCR (qPCR) results were normalized against the housekeeping genes *GAPDH* and *eEF-1 alpha*. Data was analyzed using LinRegPCR analysis program.[19]

***In situ* hybridization**

In situ hybridization **(**ISH) was performed as previously described.[20] Two 5'-fluorescein-labeled 19mer antisense oligonucleotides containing locked nucleic acid and 2'-O-methyl (2OME) RNA moieties (Ribotask ApS, Odense, Denmark) were designed: one targeting the brain-specific alternatively spliced exon 11b (5'-AacGaaAauCucCugTagT-3', locked nucleic acid residues are given in capital letters, 2'-O-methyl-RNA in lower case) and one detecting all isoforms targeting the constitutive *SGCE* exon 3 (5'-TagGacTccAucAcuAuaT-3'). Brain tissue was treated with Proteinase K (200µg/ml, Roche Diagnostics) at 50°C for 10 minutes. Probe annealing was performed at 53°C for 1.5 hours. Peroxidase activity was visualized using NovaRED (Vectorlabs, Burlingame, CA) and counterstained with hematoxylin. In an adult control subject we tested different human brain regions (cerebellum, mesencephalon, caudate nucleus, putamen, globus pallidus,

cerebral cortex and hippocampus). Skeletal muscle, heart, kidney and liver sections were used to confirm specificity of the brain-specific probe. As a control for specific binding, brain material of a *SGCE* mutation carrier (c.835_839del, p.Lys280SerfsX16, 50 h post mortem) was analyzed. This tissue was obtained from the NICHD Brain and Tissue Bank (Baltimore, MD, USA).

Results

SGCE imprinting and M-D

SGCE is widely expressed and there is thus far no explanation for the brain-specific phenotype of *SGCE* mutation carriers. One explanation could be that brain regions which are not affected are rescued by expression of the maternally imprinted allele. Therefore, we analyzed the *SGCE* imprinting throughout the human brain with a MS-HRM assay. We observed that the imprinting pattern was maintained in all brain regions and tissues tested (Supplementary Fig. 2). Results of the MS-HRM assay were confirmed by cloning and direct sequencing of a subset of samples. Only minor variations in the number of methylated/unmethylated CpGs were detected by cloning but not by the MS-HRM assay.

SGCE mRNA expression by ultra deep amplicon sequencing

Different *SGCE* isoforms have been reported so far, but little is known about their distribution and expression levels throughout the brain. To qualitatively and quantitatively characterize alternatively spliced *SGCE* exons, we performed a systematic analysis of *SGCE* cDNA by ultra deep amplicon sequencing. This technique provides a powerful and accurate tool to investigate relative mRNA expression levels and detects rare and unknown

splice events. To cover all exon-exon boundaries, four overlapping *SGCE* amplicons were made (exon 1 to 5, exon 4 to 6, exon 5 to 9, exon 7 to 12) and sequenced with a 454 GS FLX sequencer. Ultra deep sequencing was achieved by aiming for 10 000 sequence reads per amplicon (average (± SD): 11 107 (±4 273) reads, Supplementary Table 1).

SGCE mRNA isoforms

To get an overview of all alternative splicing events and their tissue specificity, we analyzed alternative splicing events of the entire *SGCE* gene in one control subject (Subject 1) in the primary somatosensory cortex (SM1), heart and blood. Twenty-three different splicing events of the *SGCE* gene were detected, but only four of them occurred at frequencies above 1%: exon 1c and the known alternatively spliced exons 2, 8 and 11b (Fig. 1B, Table 2). In-frame exon 1c was expressed in brain (5.7%), but also in non-brain tissue (2% - 2.3%). The in-frame exon 2 showed an overall high inclusion level in all tissues tested, whereas exon 8 was highly represented in muscle and blood and low in brain. Exon 11b was mainly expressed in brain (34%); it was not expressed in blood and at very low levels in muscle (0.05%). These four major variants result in a partially altered protein coding region and are extended into the last exon. Therefore, we expect these to be translated and stable. Of the low frequency variants only one, exon 4a, retains a long open reading frame. All other mRNA isoforms, except all 11b variants, are expected to result in nonsense mediated decay.[21] Splice site predictions and positions of known and novel exons are listed in Table 2. Most of the new low frequent variants lead to a frameshift (17/19) resulting in a premature stop codon and were conserved among primate lineage only (Table 2, Supplementary Table 2). All known constitutive *SGCE* exons were present to 100%

except for exon 11: Skipping of this exon was observed in all tissues and species tested, leading to a premature stop after amino acid 409, albeit at low frequency (brain: 0.8%, muscle: 1.4%, blood: 0.3%). The recently identified brain-specific exon 11c was expressed at very low levels in brain but also non-brain tissue (≤0.7%, Table 2). Also, in mouse and rat brain it was expressed at very low levels (< 2.1%), it was not expressed in zebrafish.

In order, to exclude that these low frequency variants were experimental artifacts, we sequenced a synthetic *SGCE* RNA using the same procedure. Analysis of 12 000 reads gave only sequence reads identical to the input RNA (data not shown).

Brain region-specific SGCE expression

SGCE exon 11b was the most abundant and a highly conserved brain-specific splice variant. Therefore, we analyzed exon 11b expression in more detail and tested nine different human brain regions of two control subjects and three different organisms (mouse and rat brain, zebrafish) by ultra deep amplicon sequencing.

Exon 11b showed differential expression among the different brain regions in both control subjects. Levels were highest in SM1 and motor cortex (M1), low in the globus pallidus, intermediate in caudate nucleus and substantia nigra, and interindividual variation was found in the cerebellum and putamen (Table 3). In mouse and rat brain, we observed a higher rate of exon 11b inclusion compared to human brain (SM1: 50% in mouse, 73% in rat vs. 35% in human). In whole zebrafish RNA, exon 11b was present in 20% of transcripts.

SGCE mRNA expression by qPCR

Ultra deep amplicon sequencing revealed that exon 11b is the major brain-specific alternatively spliced *SGCE* exon and that it is differentially expressed in the human brain. Similar trends in expression levels of exon 11b among the different brain regions were identified, but we also observed interindividual variability for some regions. To further characterize the expression pattern of *SGCE* transcripts we tested the same brain regions of more control subjects by qPCR (Fig. 2). We found similar trends of exon 11b expression in five control subjects: Expression in the cerebellum was significantly higher compared to globus pallidus (Fig.2, $p<0.001$, Friedman test, Dunn's multiple comparison test) and substantia nigra ($p<0.05$). Also SM1 and pallidum were significantly different ($p<0.05$). The total *SGCE* expression level (all isoforms together) was determined in all five control subjects for the various brain regions. Their means did not differ significantly in the different brain regions ($p=0.5$), suggesting that high exon 11b expression is indeed due to differential isoform expression and not only due to differences in total *SGCE* expression levels. Overall, *SGCE* and *SGCE* exon 11b expression levels varied among individuals. Normalization with reference genes *GAPDH* or *eEF-1* showed comparable results. Furthermore, the 454 sequencing and qPCR results are in support of each other (Supplementary Fig. 1).

Localization of *SGCE* mRNA isoforms in human brain

In order, to study *SGCE* mRNA localization of the major brain-specific and the ubiquitously expressed transcripts we performed isoform-specific ISH in human brain (Fig. 3). *SGCE* exon 11b and total *SGCE* expression was most prominent in neurons in all regions tested: high signal in the cerebral cortex, cerebellum (Purkinje cells, dentate

nucleus), hippocampus (pyramidal neurons of all CA regions) and moderate signal in the mesencephalon (substantia nigra) and basal ganglia. There was only a faint signal in glial cells and no staining in the white matter. The exon 11b probe did not detect a signal in skeletal muscle, heart, kidney and liver, in contrast to the *SGCE* probe targeting all isoforms. Probe specificity was confirmed with brain tissue of a *SGCE* null mutation carrier (c.835_839del, p.Lys280SerfsX16) showing no staining for both probes, whereas it stained for control genes (*CD68, TSEN54*).

In view of the localized expression of *SGCE* in the cerebellum, we tested whether the interindividual variation of *SGCE* exon 11b expression in the cerebellum is due to variation in Purkinje cell number in the tissue examined. qPCR analysis of Purkinje cell marker Calbindin-D28k showed no correlation with *SGCE* expression (data not shown).

Mutation analysis of novel *SGCE* exons

We performed mutation analysis in our cohort of definite M-D patients where no mutation was identified[7] and sequenced novel alternatively spliced *SGCE* exons. This included all new in-frame exons (1c, 4a) and frameshift exon 11b with all extended versions (exon 11d, 11c, 11f). Also, frameshift exons 3b and 3d were sequenced since their combination was observed in a transcript, and maintains the reading frame. No mutations were identified in the 12 patients analyzed.

Discussion

Mutations in *SGCE* lead to M-D, but how mutations in this ubiquitously expressed gene result only in neurological impairments is unknown. In this study, we propose a link between the cerebellum and the M-D phenotype.

M-D is an autosomal-dominant disease with a reduced penetrance due to maternal imprinting. Monoallelic paternal *SGCE* expression has been shown in human blood leukocytes and in human and mice brain.[15;18] Several genes are known to show tissue-specific imprinting[22] and brain cell-type or region-specific imprinting.[23] The latter has not yet been investigated for *SGCE*, but is important to address, since only regions in which the imprint is maintained can be involved in the M-D pathogenesis. In this study, we show that the *SGCE* imprinting pattern is retained in different human brain regions and different human tissues (muscle, blood) similar to what has been shown in mice.[18] Thus, the brain-specific M-D phenotype cannot be explained by a brain region-specific or tissue-specific imprinting. Since not all M-D patients have mutations in *SGCE*, silencing of *SGCE* expression due to imprinting defects could also be a possible disease mechanism. We tested our cohort of patients in which no mutation was identified and found a normal imprint in blood. Imprinting defects in the brain cannot be excluded, however.

More likely explanations for the brain-specific symptoms in M-D are the presence of a brain-specific SGCE isoform with a brain-specific function or a brain-specific function for ubiquitous SGCE. Our analysis of *SGCE* expression in human brain using ultra deep amplicon sequencing shows the presence of 23 alternatively spliced exons, of which 19 at very low frequencies (Table 2). Five of the 23 events retained an open reading frame and are expected to be translated (exon 1c, 2, 4a, 8, and 10). Exon 1c and 4a have not been identified thus far. The majority of the new splice variants have splice sites implying

involvement of the splicing machinery. However, most of the new isoforms lead to a frameshift and a premature stop codon which makes them likely a target for nonsense mediated decay. Despite the overall low frequency of all new exons, we cannot exclude that they contribute to the physiological function of SGCE.

Of the four major alternatively spliced exons (exon 1c, 2, 8, 11b), exon 11b was of most interest with respect to the disease: It has a brain-specific expression pattern, was highly conserved and expressed at high levels. Exon 8 expression was low in brain (7.2%) compared to heart and muscle (96%), but its expression in brain appears to be variable. We further analyzed expression of *SGCE* exon 11b throughout the human brain. We identified differential expression of exon 11b in human brain areas, whereas total *SGCE* expression levels did not differ. Quantitative PCR analysis confirmed this observation and revealed trends: A consistently high expression in the cerebellum, moderate to high expression in SM1, moderate expression in substantia nigra and putamen and consistently low levels in globus pallidus. We observed a high interindividual variability of exon 11b expression in the cerebellum. Interindividual differences in expression of isoforms could account for this observation[24;25].

We show that exon 11b is the major brain-specific alternatively spliced *SGCE* exon and that it is differentially expressed in five control subjects. Its expression pattern is compatible with a role for the brain-specific SGCE isoform in the cerebellum and a link to M-D. This hypothesis is supported by recent literature: (1) there is a shift from dysfunction of the basal ganglia towards involvement of the cerebellum in dystonia pathogenesis;[26] (2) several animal models linked dystonia to abnormal cerebellar signaling and cerebellar defects, and cerebellar lesions leading to dystonia have been observed in patients;[26;27] (3)

fMRI analysis of genetically confirmed M-D patients suggests involvement of thalamus and cerebellum (dentate nucleus)[28] and involvement of different cortical areas and cerebellum.[29]

A role of the cerebellum in M-D is in contrast with striatal changes on dopamine levels in SGCE deficient mice and a reduced striatal D2 receptor binding in M-D patients.[30;31] Moderate expression levels of the major brain-specific *SGCE* isoform in the striatum and lowest expression levels in the globus pallidus were found in all control subjects. We propose that observed striatal changes may be secondary due to abnormal cerebellar signaling. This is supported by an anatomical link between the cerebellum and the basal ganglia, namely projections from the dentate nucleus to the striatum,[32] and by cerebellar lesions or stimulations that have been shown to alter striatal dopamine signaling.[33-35] Our isoform-specific ISH showed that the major brain-specific *SGCE* transcript is highly expressed in Purkinje cells and neurons of the dentate nucleus in the cerebellum. No signals were detected in the cerebellar granule cell layer in the cerebellum as it has been shown in mouse brain.[17] Interestingly, alcohol consumption alleviates the symptoms in M-D and the cerebellum reacts very sensitively to alcohol.[36] Also, symptoms of patients with essential tremor, a common neurological movement disorder with suggested involvement of the cerebellum,[37-39] are known to respond to alcohol as seen in M-D.

We propose that the loss of function of the brain-specific SGCE isoform underlies the exclusively neurological M-D phenotype. We hypothesize that the general function of the abundant SGCE isoform is redundant or not essential since no non-neurological

symptoms have been reported. The brain-specific protein may have a unique function and cannot be replaced.

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Conflict of interest. The authors declare no conflict of interest.

Supplementary information is available at EJHG's website.

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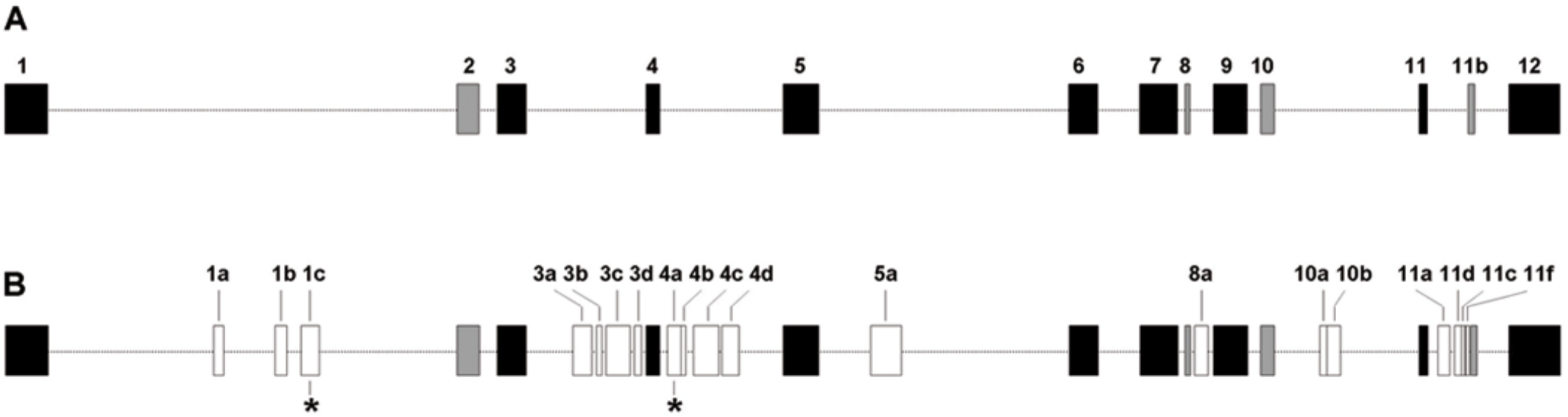
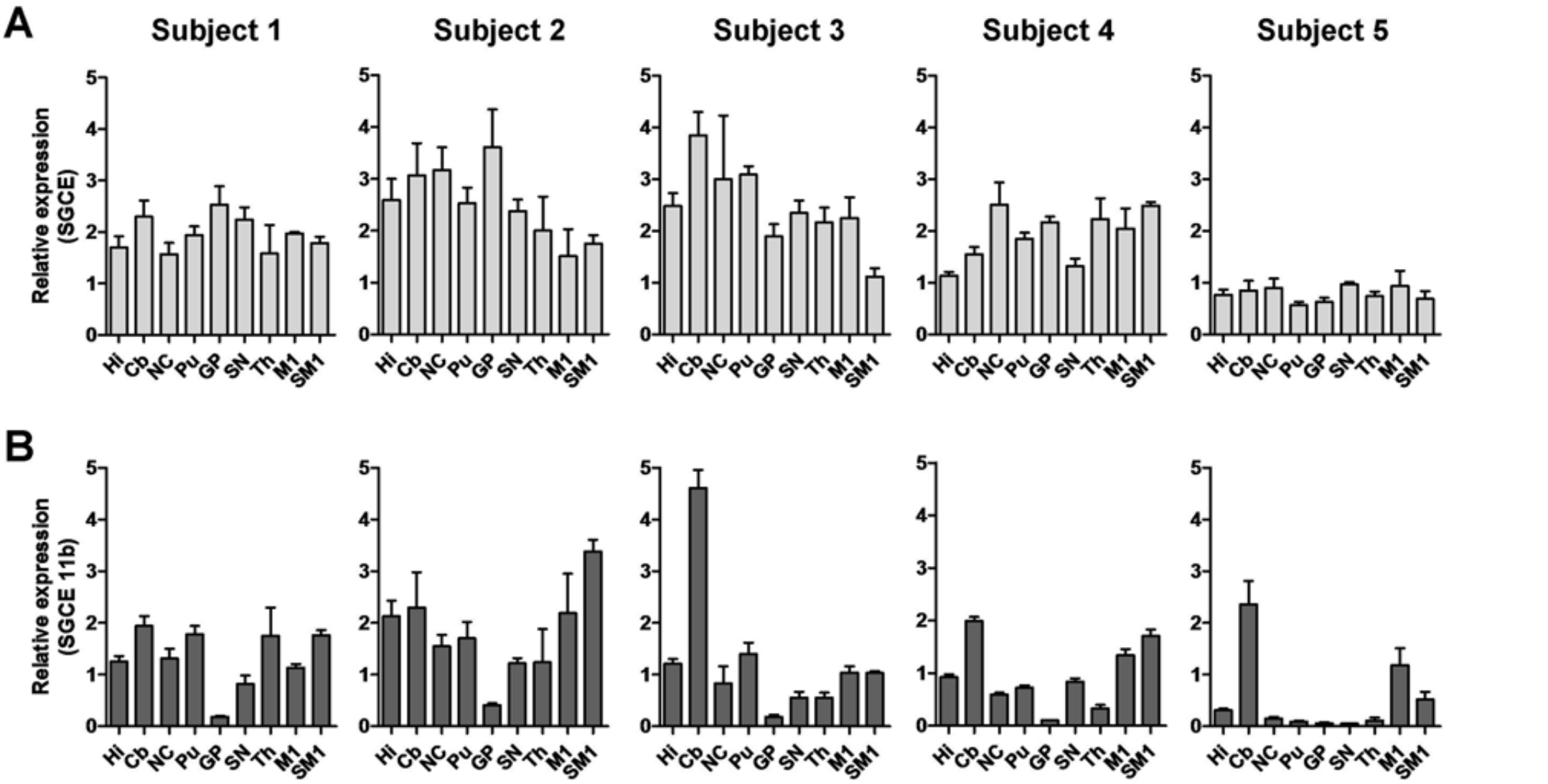
Titles and legends to figures

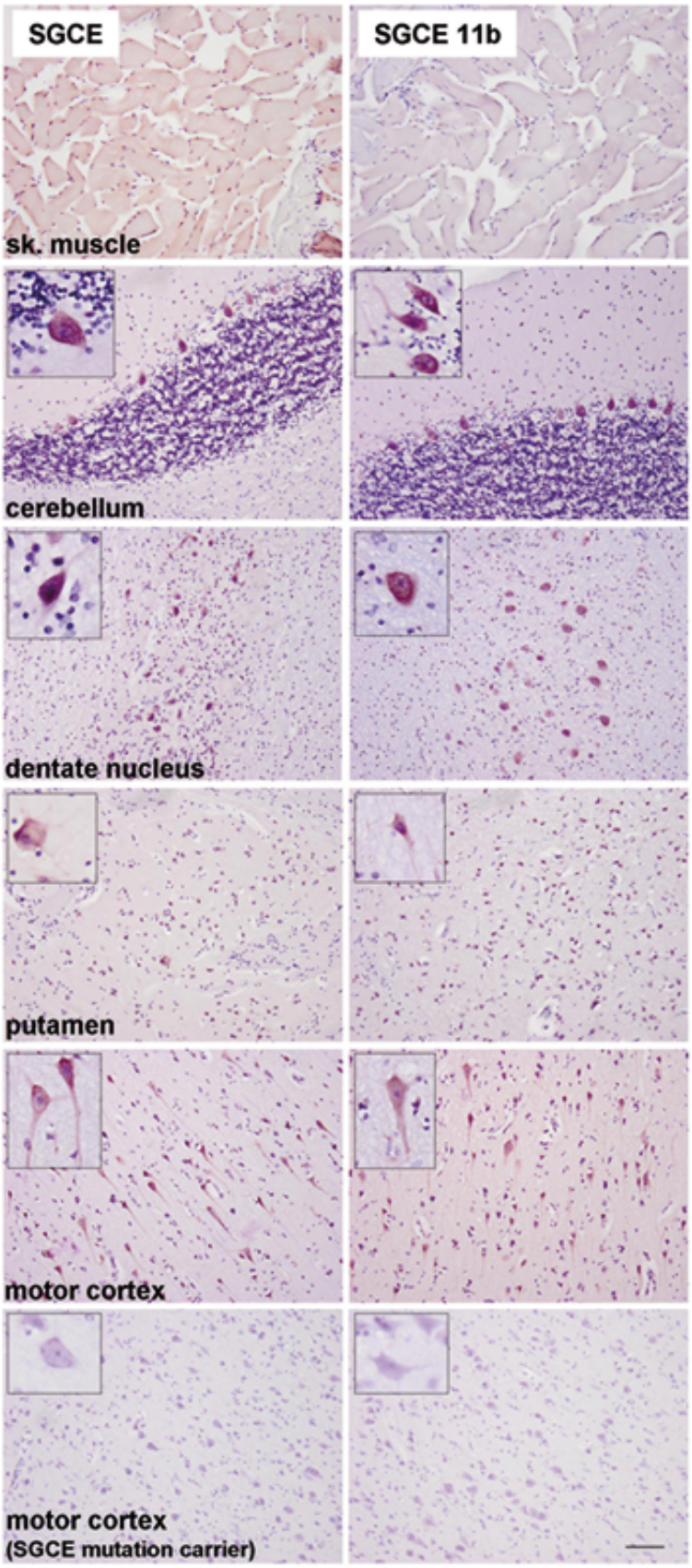
Figure 1: Overview of novel alternatively spliced exons identified by 454 ultra deep sequencing. A) *SGCE* gene including all known exons. Constitutive exons are depicted in black, alternatively spliced exons in grey. B) *SGCE* gene including novel alternatively spliced exons (in white). Asterisks represent novel in-frame exons. In-frame exons were observed with a higher frequency (range: 0.3-5.7%) than frameshift exons (range: 0.01-3%). Relative position of the exons and sizes of exons are correct, but exon sizes are not represented in the same scale as the introns.

Figure 2: *SGCE* expression levels (qPCR). Displayed are expression levels for total *SGCE* (panel A) and *SGCE* exon 11b (panel B) transcripts of the five tested control subjects normalized to GAPDH. Y-axis shows *SGCE* or *SGCE11b*/*GAPDH* *100. Abbreviations: Hi: Hippocampus, Cb: Cerebellum, NC: Caudate Nucleus, Pu: Putamen, GP: Globus Pallidus, SN: Substantia Nigra, Th: Thalamus, M1: Primary Motor Cortex, SM1: Primary Somatosensory Cortex.

Figure 3: Localization of total *SGCE* transcripts and those containing exon 11b. Depicted are ISH results for total *SGCE* and brain-specific *SGCE* in different human brain regions and human skeletal muscle. *SGCE* showed neuronal staining in all brain regions tested as well as staining in muscle. Brain-specific *SGCE* transcripts containing exon 11b showed the same expression pattern in brain but were not expressed in skeletal muscle. No *SGCE* mRNA was detected in the motor cortex of an M-D patient carrying a *SGCE* nonsense mutation. Inserts in each panel show high magnification of neurons. Scale bar represents 100µm.


A
1
2
3
4
5
6
7
8
9
10
11
11b
12
B
1a
1b
1c
3a
3b
3c
3d
4a
4b
4c
4d
5a
8a
10a
10b
11a
11d
11c
11f
*
*


A
Subject 1
Subject 2
Subject 3
Subject 4
Subject 5
Relative expression (SGCE)
B
Relative expression (SGCE 11b)
0
1
2
3
4
5
Hi
Cb
NC
Pu
GP
SN
Th
M1
SM1


SGCE
SGCE 11b
sk. muscle
cerebellum
dentate nucleus
putamen
motor cortex
motor cortex
(SGCE mutation carrier)

Tables 1-3

Table 1: Sample material

Subject	Gender	Age (years)	Post-mortem (h)	Brain tissue	Other	Assay
1	male	77	7	Cb GP Hi M1 NC Pu SM1 SN Th	He MP	454 qPCR
2	male	76	8	Cb GP Hi M1 NC Pu SM1 SN Th	na	454 qPCR
3	female	76	5	na	He MP	454
4	male	74	5	Cb GP Hi M1 NC Pu SM1 SN Th	na	qPCR
5	female	88	12	Cb GP Hi M1 NC Pu SM1 SN Th	na	qPCR
6	female	45	24	Cb GP Hi M1 NC Pu SM1 SN Th	na	qPCR

Abbreviations: Cb: Cerebellum, GP: Globus Pallidus, Hi: Hippocampus, M1: Primary Motor Cortex, NC: Caudate Nucleus, Pu: Putamen, SM1: Primary Somatosensory Cortex, SN: Substantia Nigra, Th: Thalamus, He: Heart, MP: M.Psoas, na: not available

Table 2: Known and new alternatively spliced *SGCE* exons revealed by screening the entire *SGCE* gene in one control subject with ultra deep sequencing. Additional information of alternatively spliced *SGCE* exons include: occurrence of respective exons in brain (SM1), heart and blood of control subject 1 in percent, splice sites according to www.fruitfly.org, NNSPLICE 0.9 version (minimum score for 3'/ 5'splice site 0.5): splice donor (D)/acceptor (A), frameshift (FS) or in-frame (IF) exons, isoform subject to nonsense mediated decay (NMD), exon size in bp, nucleotide position according to NG_008893.1. Constitutive exons (100% expression) are not shown. *: Exons that were sequenced in definite M-D patients where no mutation could be identified.

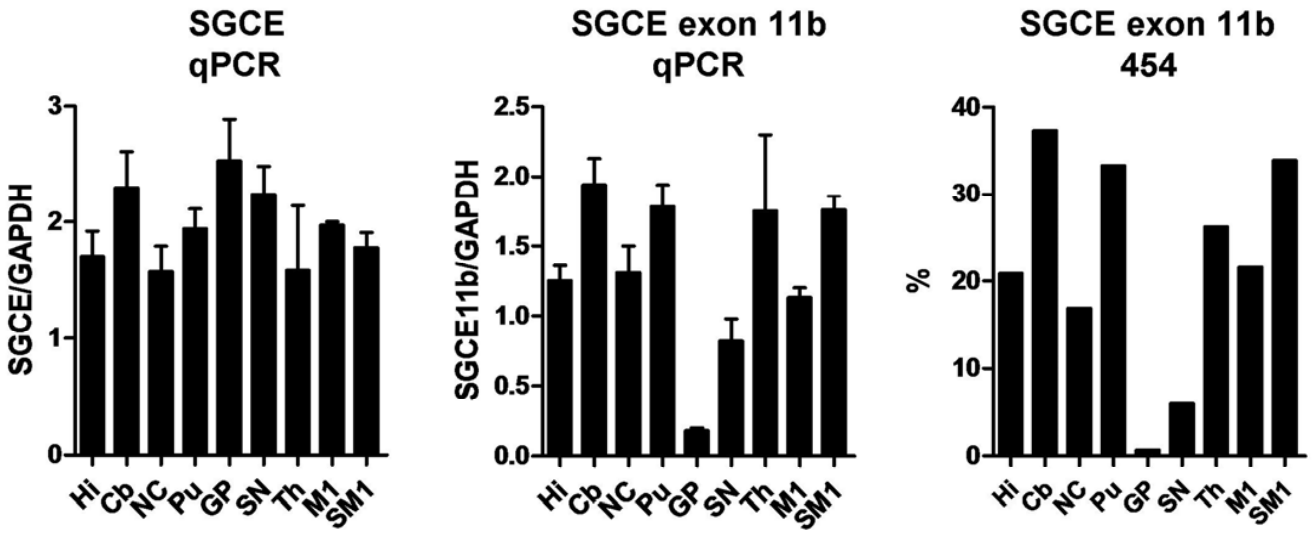
Alternatively spliced exons	Exon	Brain (%)	Heart (%)	Blood (%)	Splice site		FS/IF	NMD	Exon size (Bp)	NG_008893.1	
					D	A				Start	End
known	**2***	98.20	94.80	78.50	+	+	IF	-	123	31369	31491
	8*	7.20	96.00	96.00	+	+	IF	-	27	61487	61513
	10*	0.03	0.03	1.25	-	+	IF	-	75	63206	63280
	11b*	33.92	0.05	-	-	-	FS	-	35	73398	73432
new	**1a**	0.66	0.31	1.08	+	+	FS	+	62	16543	16604
	1b	0.03	0.11	-	+	+	FS	+	68	20246	20313
	1c*	5.70	1.96	2.25	+	-	IF	-	108	21762	21869
	3a	-	0.03	-	+	-	FS	+	102	36163	36264
	3b*	0.43	0.02	-	+	+	FS	+	31	36961	36991
	3c	0.05	-	-	+	+	FS	+	130	37052	37181
	3d*	-	0.17	-	+	-	FS	+	35	37655	37689
	4a*	0.80	0.94	1.66	+	+	IF	-	81	38584	38664
	4b	0.14	0.37	-	-	+	FS	+	110	38584	38693
	4c	0.07	0.12	-	+	+	FS	+	147	39215	39361
	4d	-	0.01	-	+	+	FS	+	94	39504	39597
	5a	-	-	0.06	-	+	FS	+	176	45589	45764
	8a	0.08	0.11	0.51	+	-	FS	+	74	61810	61883
	10a	0.01	-	-	-	+	FS	+	76	66703	66778
	10b	0.02	-	-	+	+	FS	+	115	66664	66778
	11a	0.04	-	-	+	-	FS	-	68	73177	73244
	11d*	0.03	-	0.12	-	-	FS	-	107	73326	73432
	11c*	0.70	0.28	0.41	-	-	FS	-	72	73361	73432
	11f*	0.33	-	-	-	-	FS	-	51	73382	73432

Table 3: Expression levels of alternatively spliced *SGCE* exon 11b in human brain regions of two control subjects (454 sequencing).

Expression levels of exon 11b are given as percentage of all analyzed sequence reads obtained by 454 sequencing. Abbreviations: HS: Homo sapiens, RN: Rattus norvegicus, MM: Mus musculus, Hi: Hippocampus, Cb: Cerebellum, NC: Caudate Nucleus, Pu: Putamen, GP: Globus Pallidus, SN: Substantia Nigra, Th: Thalamus, M1: Primary Motor Cortex, SM1: Primary Somatosensory Cortex.

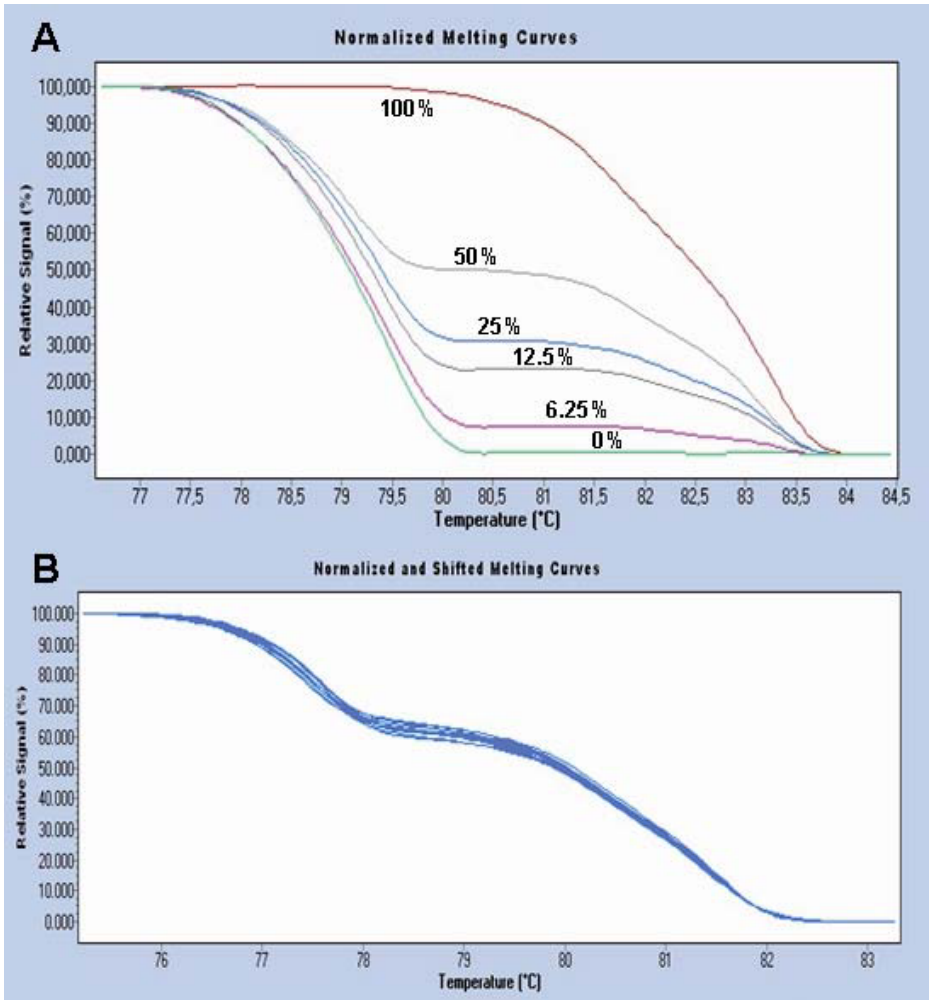
		Exon 11b	
	Region	**Subject 1 (%)**	**Subject 2 (%)**
HS	**Hi**	20.84	16.59
	Cb	37.31	20.99
	NC	16.89	12.48
	Pu	33.29	11.63
	GP	0.68	4.09
	SN	5.98	12.60
	Th	26.22	16.71
	M1	21.58	25.51
	SM1	33.92	36.79
RN	**Brain**	73.25	
MM	**Brain**	49.80	
ZF	**Embryo**	20.38	

Supplementary Fig. 1



Comparison of qPCR and 454 sequencing expression data. Displayed are expression levels of overall *SGCE* and *SGCE* exon 11b transcripts obtained by qPCR and 454 sequencing of a control subject. qPCR results are normalized to *GAPDH* (mean and standard deviation of triplicates). 454 sequencing results show expression levels of transcripts containing exon 11b given as percentage of all sequence reads analyzed. (Hi Hippocampus, Cb Cerebellum, NC Caudate Nucleus, Pu Putamen, GP Globus Pallidus, SN Substantia Nigra, Th Thalamus, M1 Primary Motor Cortex, SM1 Primary Somatosensory Cortex)

Supplementary Fig. 2



***SGCE* imprinting pattern by MS-HRM assay.**
A) Samples with different degrees of methylation of the *SGCE* promotor region were included as standards in each experiment : 100% methylated and unmethylated controls and 50%, 25%, 12.5% and 6.25% methylated samples. B) Example of melting curves of different brain regions and blood samples all showing a normal imprinting pattern with 50% methylation.

Supplementary Tables 1-2

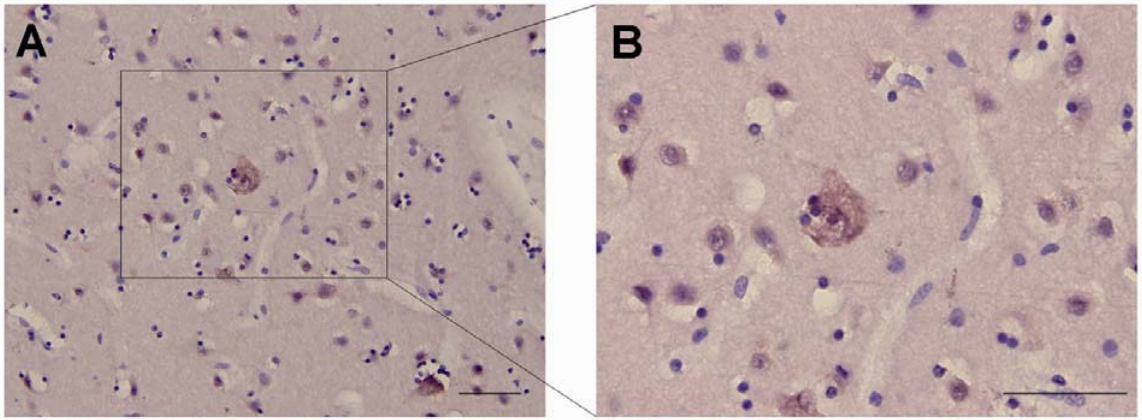
Supplementary Table 1: Number of total sequence reads analyzed with 454 sequencing. The numbers of sequence reads analyzed are given as average (range) of all sequenced samples (brain regions/tissues) or as absolute numbers in case there were one or two regions sequenced only.

	# Samples	Number of reads analyzed
H.sapiens Subject 1 (Brain, Muscle)	10	15 106 (12 643-17 916)
H.sapiens Subject 2 (Brain)	9	7 084 (4 535-11 677)
H.sapiens Subject 3 (Muscle)	1	6 409
H.sapiens Blood	2	12 423, 13 786
R.norvegicus Brain	1	10 435
M.musculus Brain	1	8 213
D.rerio Embryos	1	14 382

Supplementary Table 2: Conservation of alternatively spliced *SGCE* exons.
Conservation was investigated using the "blast assembled genomes" feature and the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Numbers indicate the percentage of identical nucleotides relative to the size of the exon in humans.

Exon	1a	1b	1c	2	3a	3b	3c	3d	4a	4b	4c	4d	5a	8	8a	10	10a	10b	11	11a	11d	11c	11f	11b
P.troglodytes	100	100	100	100	100	100	99	100	100	100	99	98	99	100	100	94	97	98	100	98	98	100	100	100
P.pygmaeus	94	97	100	100	98	100	99	100	97	97	97	95	99	100	98	-	97	98	100	97	97	98	100	100
M.mulatta	97	93	100	99	97	100	96	94	95	95	95	96	97	100	100	-	96	96	100	98	98	100	100	100
B.taurus	80	71	96	93	72	-	86	-	89	89	49	-	-	100	93	-	70	54	100	93	92	97	98	100
R.norvegicus	-	-	-	86	-	-	-	-	-	-	-	-	-	96	77	-	42	-	95	89	93	94	98	100
M.musculus	-	-	91	88	75	-	-	-	-	-	-	-	16	92	68	-	47	-	90	91	93	92	96	100
G.gallus	-	-	82	85	-	-	-	-	-	-	25	-	-	100	60	-	-	-	88	-	67	81	88	91
D.rerio	-	-	-	63	-	-	-	-	-	-	-	-	-	96	-	-	-	-	68	-	-	-	-	63

Optional figure 1:



Localization of brain-specific SGCE in small and large striatal neurons.
Higher magnifications of ISHs for detection of SGCE exon 11b transcripts as an example to illustrate neuronal staining in the putamen. Scale bar represents 50µm.