Differential methylation of the gene encoding *myo*-inositol 3-phosphate synthase (*Isyna1*) in rat tissues

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Abstract

Aims—*Myo*-inositol levels are frequently altered in several brain disorders. *Myo*-inositol 3-phosphate synthase, encoded by the *Isyna1* gene, catalyzes the synthesis of *myo*-inositol in cells. Very little is known about the mechanisms regulating *Isyna1* expression in brain and other tissues. In this study, we have examined the role of DNA methylation in regulating *Isyna1* expression in rat tissues.

Materials & methods—Transfection analysis using *in vitro* methylated promoter constructs, Southern blot analysis of genomic DNA from various tissues digested with a methylation-sensitive enzyme and CpG methylation profiling of genomic DNA from different tissues were used to determine differential methylation of *Isyna1* in tissues. Transfection analysis using plasmids harboring mutated CpG residues in the 5'-upstream region of *Isyna1* was used to identify critical residues mediating promoter activity.

Results—The −700 bp to −500 bp region (region 1) of *Isyna1* exhibited increased methylation in brain cortex compared with other tissues; it also exhibited sex-specific methylation differences between matched male and female brain cortices. Mutation analysis identified one CpG residue in region 1 necessary for promoter activity in neuronal cells. A tissue-specific differentially methylated region (T-DMR) was found to be localized between +450 bp and +650 bp (region 3). This DMR was comparatively highly methylated in spleen, moderately methylated in brain cortex and poorly methylated in testis, consistent with mRNA levels observed in these tissues.

Conclusion—Rat *Isyna1* exhibits tissue-specific DNA methylation. Brain DNA was uniquely methylated in the 5'-upstream region and displayed gender specificity. A T-DMR was identified within the gene body of *Isyna1*. These findings suggest that *Isyna1* is regulated, in part, by DNA methylation and that significant alterations in methylation patterns during development could have a major impact on inositol phosphate synthase expression in later life.

Keywords

Myo-inositol 3-phosphate synthase (E.C. 5.5.1.4; IP synthase) is a rate-limiting enzyme that catalyzes the first step in the biosynthesis of all inositol containing compounds. It converts glucose 6-phosphate to *Myo*-inositol 3-phosphate. Free *Myo*-inositol (MI) is generated when the phosphate moiety is removed by inositol monophosphatase 1 (IMPase1). IP synthase was first purified by Maeda and Eisenberg from rat testis, the richest source of this enzyme [1]. The native mammalian enzyme is considered to be a homotrimer made up of approximately 68-kDa subunits [1]. In rats, these subunits arise by translation of a full-length mRNA, spliced from 11 exons, encoded by the *Isyna1* gene (*ISYNA1* in human). Recently, we and others have identified and characterized a plethora of shorter isoforms [2,3], arising by alternative splicing and intron retention mechanisms. The identification of novel isoforms in different tissues, in addition to the full-length isoform (now termed the α-isoform), suggests that the regulation of IP synthase gene expression is complex.

Myo-inositol is an ubiquitous six-carbon cyclic sugar which is an important component of membrane phospholipids and a key precursor for the phosphoinositide (PI) signaling pathway. MI can be phosphorylated at multiple positions in its ring to yield an array of inositol phosphates (IPs) and PIs that can participate in a multitude of cellular processes. Brain exhibits high levels of MI [4] which may, in part, be attributed to MI's active role in the PI signaling pathway, which also generates the two second messengers – diacylglycerol and inositol triphosphate [5]. MI plays a critical role in the developing axons of sympathetic neurons, as *IMPA1* transcripts encoding IMPase1 constitute the single most abundant transcript in these neurons [6,7]. A plethora of data indicate that alterations in brain MI levels can lead to severe behavioral problems and neurological deficits. Altered MI levels have been observed in the brains of Alzheimer's [8], obsessive compulsive disorder [9,10] and autism spectrum disorder patients [11], as well as in suicide [12] and stroke [13] victims. High fetal MI concentrations in the cerebrospinal fluid have been attributed to the pathogenesis of Down Syndrome [14], while a deficiency is known to cause neural tube defects [15,16]. Oral administration of MI has been reported to be therapeutic for panic disorder [17]. The role of MI in bipolar disorder is an area that has garnered considerable attention and research focus. Bipolar patients in the manic phase of the disorder are often treated with lithium or valproate, drugs that are presumed to decrease free MI levels by inhibiting IMPase1 and IP synthase, respectively [18], while oral administration of MI has been reported to alleviate the depressive phase of bipolar disorder [19]. IP synthase, therefore, is a potential drug target for effecting mood stabilization.

Insights into MI metabolism and its role in brain function can be obtained through a comprehensive understanding of the processes that regulate both *Isyna1* and *IMPA1* expression. We have previously demonstrated that *ISYNA1* is upregulated by E2F1 [20], an observation that has been confirmed in yeast [21]. More recently, we demonstrated that *Isyna1* generates a number of alternatively spliced isoforms, one of which was shown to negatively modulate enzyme activity [2]. Studies presented here clearly implicate epigenetic mechanisms (DNA methylation) in the tissue-specific expression of *Isyna1*. These

observations provide fundamental new insights into the complexity of IP synthase regulation and add a new dimension to the understanding of MI's role in neuropsychiatric and neurological disorders.

Materials & methods

Materials

Sodium bisulfite and hydroquinone were obtained from Sigma-Aldrich Corp. (MO, USA); cell culture media were obtained from ATCC (Manassas, VA, USA) or Invitrogen (CA, USA); P^{32}-dCTP (3000 Ci/mmol) and Microspin G-25 columns were from Amersham Biosciences (NJ, USA); PCR primers were synthesized by Integrated DNA Technologies, Inc. (IA, USA). The plasmid pFRL2 was a kind gift from Dr Richard Hodin, Massachusetts General Hospital, MA, USA [22]. Unless otherwise specified, all chemicals and reagents were of analytical grade procured from established manufacturers.

Cell culture

Cell lines SK-N-AS (CRL-2137), HEK (CRL-1573) and NTERA-2cl.D1 (CRL-1973) were obtained from ATCC and cultured according to their protocols. All cell lines were grown in the presence of 10 U/ml penicillin and 10 µg/ml streptomycin.

Animals

Adult Sprague-Dawley rats, obtained from Harlan Bioproducts for Science Inc. (IN, USA) were fed standard rat chow (Purina rat chow 5001) and water *ad libitum*. Animal use was approved by the Institutional Review Board of the Veterans Affairs Medical Center, KY, USA, and were conducted as per NIH guidelines. Rats were housed according to Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) recommendations.

In vitro promoter methylation analysis

The minimal promoter of *ISYNA1* in pGL3 luciferase reporter vector (Promega, WI, USA) has been extensively characterized in our laboratory [20]. A plasmid harboring the minimal promoter region (*Pst*I/pGL-3) was methylated *in vitro* using *Hha*I, *Hae*III and *Sss*I methylases according to the supplier's (New England BioLabs, Inc., MA, USA) protocol [20]. Methylation was ascertained by digestion with *Hae*III (for plasmids methylated by *Hae*III and *Sss*I methylases) and *Hha*I (for the plasmid methylated by *Hha*I methylase) and resolving them on gels. Absence of digested products seen with the methylated plasmids and their presence with the unmethylated plasmid indicated completion of methylation. Equal amounts (2 µg/ml) of methylated and unmethylated promoter plasmids were transfected into SK-N-AS human neuroblastoma cells along with 50 ng/ml of a control vector (pRL-TK; Promega) expressing *Renilla* luciferase. Transfection was performed in 2 ml media containing 3×10^5 cells using FuGENE6® Reagent (Roche, IN, USA) in 6-well plates. Cells were harvested after 48 h, and firefly and *Renilla* luciferase activities were assayed using the dual luciferase® reporter assay system (Promega) in a Turner 20/20 luminometer. Normalized luciferase activities were then determined. The experiment was repeated thrice with duplicates and expressed as mean ± standard error of the mean. The reporter activity of the unmethylated parent plasmid was taken as 100%.

Southern blot analysis

A total of 20 µg of genomic DNA from various rat tissues (testis, brain cortex, spleen, heart and pancreas) was digested with *Eco*RI and *Hind*III, followed by digestion with *Sma*I and resolved on 1% agarose gels. Gels were Southern blotted onto Zeta-Probe GT membranes

(BioRad, CA, USA) and probed with a full-length *Isyna1* cDNA labeled with α-P[32]dCTP using the Prime-A-Gene (Promega) system and purified through Microspin™ G-25 columns (GE healthcare, WI, USA). Membranes were hybridized overnight and washed first in 1XSSC/0.1% sodium dodecyl sulfate for 10 min at room temperature, followed by another wash at 68°C, and finally with two washes in 0.1X SSC/0.1% sodium dodecyl sulfate for 10 min at 68°C. Membranes were placed in Saran Wrap and exposed to Fuji Medical X-Ray film (Super HR-G30) overnight in −80°C.

Bisulfite conversion, sequencing & determination of CpG methylation profiles

Approximately 10–15 µg of genomic DNA was digested with *EcoR*I (an enzyme that does not digest the amplicons) and subjected to the bisulfite reaction in the presence of 3.1 M sodium bisulfite, pH 5.0, and 0.5 mM hydroquinone at 55°C for 16 h under mineral oil. DNA was purified using the QIAquick PCR purification kit (Qiagen, CA, USA). DNA, after denaturation in 0.3M NaOH, was ethanol precipitated and dissolved in Tris-ethylene diamine tetra acetate buffer and used for PCR amplification using primers specific for the bisulfite-modified DNA. Primers (Table 1) were designed using the BiSearch program [23]. Each amplicon was ligated into pGEM-T Easy vector (Promega) and transformed into *Escherichia coli* DH5α competent cells. Approximately 10 random colonies were selected for mini-prep plasmid DNA preparation (Qiagen), sequenced at the University of Louisville Sequencing Core facility (KY, USA), and methylated CpG residues were identified and their methylation levels determined.

Mutation analysis of CpG sites in region 1 of the 5' flanking region

To determine the effect of differential methylation in region 1 (−700 bp–−500 bp) on promoter activity, each CpG residue (CpG 1–9; Figure 1) in region 1 was subjected to mutation analysis. A 758-bp amplicon from the 5´-flanking region of rat *Isyna1*, terminating 72 bp upstream of the ATG start site, and containing CpG 1–9, was cloned into pGEM®-T Easy vector (Promega). CpG residues were mutated by conversion of the CG residue to AG using the QuikChange® site-directed mutagenesis kit (Stratagene, CA, USA). The mutated insert, after confirmation by sequencing, was recovered and recloned into the pFRL2 vector [22]. This vector is unique in that it carries both the firefly luciferase gene for determining the cloned promoter activity and a *Renilla* luciferase gene that serves as a control to normalize transfection efficiency in the same vector, obviating the need for cotransfections. Only one CpG residue was mutated per plasmid thus permitting the assessment of the effect of each specific residue on total promoter activity. The mutated and unmutated parent plasmids were transfected (as described earlier) into neuroblastoma (SK-N-AS), human embryonic kidney (HEK) and human testicular embryonic carcinoma (NTerra-2) cell lines and normalized promoter activity was determined. Cell lines were grown as described previously [20], or following ATCC protocols. Transfection was carried out at least three-times, in duplicate wells, and relative promoter activity was expressed as mean ± standard error of the mean. The promoter activity of each mutant construct was expressed as a ratio of the unmutated construct (taken as 100%). Statistical significance was computed using the unpaired two-tailed Student's *t*-test using software from GraphPad Software, Inc. (CA, USA).

Results

In vitro methylation of human *ISYNA1* promoter decreases reporter activity

Because the rat *Isyna1* gene promoter was uncharacterized, a fully characterized human *ISYNA1* minimal promoter construct, available in our laboratory, was used [20]. The rat and human genes are highly conserved, including in the 5´-flanking region. Transfection analysis with the methylated and unmethylated promoter constructs indicated that promoter activity

was significantly decreased upon methylation (Figure 2). The greatest decrease (~78%) was observed with *Sss*I methylase, followed by *Hha*I (~70%) and *Hae*III (~36%) methylases. *Sss*I methylase methylates C residues at all CG dinucleotides whereas the *Hha*I and *Hae*III methylases are sequence-specific and methylate the internal Cs at GCGC and GGCC motifs, respectively. The decrease in reporter activity observed with the *Sss*I methylase is consistent with more CpG sites being available for methylation. These results not only indicate that the promoter activity of *ISYNA1* can be suppressed by methylation but also that a number of CpG sites participate in the regulation of gene expression.

Differential methylation of rat *Isyna1* in various tissues

To examine if the rat *Isyna1* gene is differentially methylated in various tissues, genomic DNA from four (adult male) tissues – brain cortex, heart, pancreas and testis – was isolated, digested with *Sma*I (which does not cleave DNA when the 3´C in its recognition sequence – CCCGGG – is methylated) and analyzed by Southern blot using a rat *Isyna1* cDNA probe (Figure 3). Three major *Sma*I fragments were identified – a 3.0 kb partially undigested fragment and the 1.23-kb and 1.84-kb fragments derived from the complete digestion of the 3.0-kb fragment. The 3.0-kb fragment can be detected if either or both internal *Sma*I sites (marked by asterisks in Figure 3) are methylated and the flanking *Sma*I sites are unmethylated. Thus, the extent of methylation can be assessed by visual comparison of the intensity of the 3.0-kb band with that of its digested products. It is evident from the Southern blot that the internal *Sma*I sites are methylated to varying degrees in the four tissues analyzed. This is exemplified in the brain cortex where the internal *Sma*I sites appeared to be relatively highly methylated because the intensity of the two completely digested bands were barely evident compared with the 3.0-kb band. Conversely, in testis and pancreas, the digested bands were comparatively more intense than the 3.0-kb band suggesting that the internal *Sma*I sites are poorly methylated. The observation with the testis is consistent with this tissue expressing high levels of IP synthase [2]. In the heart, all three bands appeared equally pronounced with the 3.0-kb band slightly more intense than the two digested bands. Comparison of the intensities of the 3.0-kb fragment with that of its digested products suggests the following order of decreasing *Isyna1* methylation: first, brain cortex; then the heart; followed by the pancreas/testis. These results imply that methylation of rat *Isyna1* may contribute to differential gene expression in various tissues.

Mean methylation index

We next sought to characterize the CpG methylation profiles of *Isyna1* in different tissues. To compare methylation of identical CpG regions amongst different tissues, we determined the mean methylation index (MMI), a value that describes the average methylation level of CpG residues within a defined region. It is computed by dividing the sum of the methylation levels of all CpG sites present within a region by the number of CpG sites present in that region. The MMI can have a maximum value of 100 (complete methylation) or a minimum of 0 (no methylation). The methylation level of a CpG residue is the percentage of analyzed clones (~10) that harbor a methylated residue at that site. Methylation levels more than or equal to 30% were considered significant. Significant methylation levels were further characterized as being highly methylated (>60%) or moderately methylated (30–60%). Methylation levels less than 30% were considered poorly methylated and insignificant.

CpG methylation profile of rat *Isyna1* in various tissues

We examined the CpG methylation status of a 1.35-kb region extending from nucleotide −700 bp in the 5´-flanking region (relative to the ATG start site) to approximately +650 bp in intron 4 in genomic DNA from five (adult male) tissues – brain cortex, heart, pancreas, spleen and testis (Figure 1). Over 250 clones were analyzed to decipher the overall CpG methylation profiles of *Isyna1* in DNA from these tissues. Specifically, methylation of all

but 25 (CpG10 and CpG71–94) of the 106 CpG residues spanning this region were examined. The 25 CpG residues probably reside in a region of poor sequence context generated after bisulfite conversion making them refractory to PCR amplification, a typical problem encountered in bisulfite sequencing [24]. Upon bisulfite conversion, there are a large number of C to T conversions, which end up creating long stretches of Ts. Hence, primer design programs do not always identify appropriate primer pairs for amplifying the genomic region of interest. Second, amplicons larger than approximately 300 bp are typically difficult to obtain as the harsh bisulfite treatment results in large-scale degradation [25]. Unique tissue-specific methylation signatures were apparent in the 5′-flanking region between −700 bp and −500 bp (hereafter, region 1) and in the intron 3–intron 4 region between +450 bp and +650 bp (region 3); no significant changes were observed between −350 bp and +200 bp (region 2) which, interestingly, is located within a CpG island. Methylation differences in region 1 of *Isyna1* were most prominent in the brain cortex while the most pronounced differences in region 3 were apparent in spleen (see the 'Discussion' section for more details).

CpG methylation profile of rat *Isyna1* in male & female brain cortex

As a preliminary step to identify possible gender bias (sex differences) in brain genomic DNA methylation patterns, the CpG methylation profiles of *Isyna1* in age-matched male and female rat brain cortex were compared. The CpG profile of the 1.35-kb *Isyna1* region from male brain cortex DNA (as shown in Figure 1) was compared with that from an age-matched adult female (Figure 4). The most pronounced difference in methylation was in region 1 of the gene. In DNA from female brain cortex, region 1 of the gene was relatively unmethylated (MMI: 3.0) compared with DNA from the male cortex (MMI: 12.2) – indeed, all the methylated residues in region 1 of the gene from female cortex were found in only one out of 11 clones analyzed. Methylation levels were essentially similar in regions 2 (MMI: 4.9 for males vs 4.5 for females) and 3 (MMI: 5.5 for both sexes) of the *Isyna1* gene. CpG103 in region 3 of the gene was the lone exception being moderately methylated in DNA from male cortex (30%) but essentially unmethylated (10%) in the DNA from female cortex. Interestingly, CpG103 was highly methylated (70%) in DNA from spleen (Figure 1), the only other tissue showing significant overall methylation. These preliminary data, though speculative, indicate that the *Isyna1* gene in male brain cortex is comparatively more methylated than the gene in female brain cortex in region 1 and, therefore, might harbor putative sex-specific regulatory element(s) required for IP synthase expression in this tissue.

Mutation analysis of CpG residues in the 5' flanking region of rat *Isyna1*

To ascertain if the increased methylation observed in region 1 of *Isyna1* in male brain cortex had any functional consequences, all CpG residues in region 1 (i.e., CpG1–9; Figure 1) were individually altered by site-directed mutagenesis (CG>AG) and the effect of each mutation (Mut1–9, respectively) was examined in three different cell lines: SK-N-AS, HEK and NTerra-2 cells (Figure 5). Two CpG sites exhibited significant downregulation of promoter activity when mutated: Mut9 decreased promoter activity in the neuroblastoma cell line by 17% (p = 0.002) and Mut2 decreased activity by 23% (p = 0.01) in the kidney cell line. These results indicate that CpG9 and CpG2 may be components of distinct regulatory elements required for gene expression in neuronal and kidney cells, respectively. None of the nine mutations had any significant effect on *Isyna1* promoter activity in the testis cell line.

Discussion

In this study, we provide evidence that the differential expression of rat *Isyna1* in tissues is regulated, in part, by DNA methylation. Evidence for the role of DNA methylation comes

from three different experimental approaches. First, *in vitro* methylation of the cognate human (*ISYNA1*) promoter, cloned into a luciferase reporter vector and transfected into a neuroblastoma cell line, significantly decreased reporter activity. Of the three methylases used for *in vitro* methylation – *Sss*I, *Hae*III and *Hha*I methylases – the greatest decrease (78%) was observed with *Sss*I methylase, an enzyme that methylates all CpG residues. The remaining two enzymes which act in a sequence-specific context caused decreases of 70 and 36%, respectively (Figure 2). Because all three enzymes significantly decreased *Isyna1* reporter activity to different extents, a number of CpG residues in the promoter are probably required for gene expression. Given that the rat and human genes are highly conserved, including in the 5´-flanking region (data not shown), it is likely that CpG methylation of the upstream region may also play an important role in rat *Isyna1* gene expression.

Second, Southern blot analysis of *Sma*I-digested rat genomic DNA from four different tissues, probed with P³²-labeled *Isyna1* cDNA, revealed differing band intensities. As the CG dinucleotide in the *Sma*I recognition sequence – CCCGGG – is sensitive to methylation, the amount of digested/undigested DNA depends on the extent of methylation of the *Sma*I sites that are located within the gene segment detectable by the probe (Figure 3). Comparison of the intensities of the undigested and digested fragments clearly suggests that the *Sma*I sites located within *Isyna1* are differentially methylated in the four tissues. When tissues are ranked by decreasing methylation levels, based on the degree of digestion of the 3.0-kb fragment (see 'Results' section), the order is: first brain cortex, second the heart, and third the pancreas/testis.

Third, CpG methylation profiling of rat *Isyna1* provided direct evidence for differential methylation in tissues. CpG methylation profiling was undertaken on a 1.35-kb region, extending from −700 bp to +650 bp of the rat *Isyna1* gene in genomic DNA from five different tissues isolated from a single male rat. Because CpG methylation patterns can be influenced by extraneous factors such as diet, maternal health during gestation and growth environment, we did not pool tissues from different animals; rather, we chose to compare CpG methylation patterns among different tissues from a single animal. The strength of this study, therefore, is that it allows direct comparison of tissue-specific *Isyna1* methylation patterns in the same genetic background with little or no contribution from confounding factors. It also allows the identification of subtle methylation changes in the gene that may not be evident in DNA from pooled samples, especially for a housekeeping gene such as *Isyna1*, which is anticipated to exhibit low methylation levels overall. This is also a drawback, as the data, though derived from an apparently healthy rat, may not be representative of the population. Nevertheless, the present study provides novel insights into the epigenetic regulation of *Isyna1*. The analyzed region encompasses 106 CpG residues, of which the methylation levels of 81 CpG residues were successfully determined through the use of overlapping amplicons. For CpG methylation profiling, the *Isyna1* gene was arbitrarily divided into three regions, based on amplicon contiguity: region 1 (−700 bp– −500 bp in the 5´-flanking region), region 2 (−350 bp–+200 bp flanking the ATG start site) and region 3 (+450 bp–+650 bp in the gene body). As anticipated, overall methylation levels for *Isyna1* were expectedly low, consistent with the housekeeping nature of this gene. Tissue-specific differentially methylated regions (T-DMRs) were, however, apparent in regions 1 and 3.

For region 1, the most intriguing difference pertained to the (male) brain cortex where a relatively higher level of methylation (MMI: 12.2) of *Isyna1* was observed, compared with that in all other tissues (MMI: 0–4.4; Figure 1) examined. A total of six CpG sites in region 1 were methylated in genomic DNA from brain cortex compared with three in DNA from spleen, one each in DNA from testis/heart and none in DNA from pancreas (see also Table 2). In addition to having more methylated sites, genomic DNA from brain cortex also had

the only significantly methylated site (CpG8; 30%) in region 1 of the gene. The unique gene methylation pattern in DNA from the brain cortex could imply the probable presence of regulatory element(s) required for fine-tuning *Isyna1* gene expression in the brain. To test this hypothesis, substitution mutations (CG>AG) were introduced into each of the 9 CpG residues present in region 1 (CpG 1–9; Figure 1) of *Isyna1*. The effect of each of these altered CpG sites on *Isyna1* promoter activity was examined in SK-N-AS, HEK and NTerra2 cell lines (Figure 5). A significant decrease in promoter activity was observed with Mut9 in the neuronal cell line and Mut2 in the HEK cell line. None of the *Isyna1* mutations had any significant effect in the testis cell line. This result is consistent with the data on the recently characterized human methylome where cell lines were observed to have different methylation and expression levels [26]. The data suggest that CpG9 may be important for brain/neural gene expression and likely defines a putative brain-specific regulatory element. CpG9, however, is poorly methylated in DNA from the brain cortex (10%; Figure 1), but is located one nucleotide away from CpG8, which is significantly methylated (30%). Methylation of CpG8 of *Isyna1*, therefore, could sterically hinder the binding of a transcription factor/complex to CpG9, or confer a more compact and less accessible DNA conformation [27], thereby accounting for a decrease in *Isyna1* expression in this tissue. In line with this argument, elimination of CpG8 of *Isyna1* by substitution mutation (Mut8) did not affect promoter activity (Figure 5), presumably because the putative transcription factor/ complex can now access its binding site at CpG9 to drive promoter activity. CpGs 8 and 9 are located within a putative Sp1 binding site, CCTGCCCGCC, that matches the consensus Sp1 sequence, MSNNCCCSSC (inferred by AliBaba2.1 [101]). The methylation of region 1 of *Isyna1*, and CpG8 in particular, observed in normal brain cortex likely contributes to decreased MI levels. However, unlike the other tissues analyzed, regulation in the brain may be more complex because region 3 is also methylated (see below). How these two regions contribute to *Isyna1* expression in brain cortex remains to be determined. On a cautionary note, we cannot rule out that the observed decrease in gene expression may be owing to the mutations disrupting a transcription factor binding site.

The brain is a sexually dimorphic organ as the male and female brain follow different developmental trajectories [28]. The human male brain is masculinized and defeminized by estrogens during development, a process that is countered by α-fetoprotein in the developing female brain [28]. Male/female brain patterning could involve selective methylation of target genes to fine-tune gene expression. As an exploratory step, we sought to determine if the CpG profile of the *Isyna1* gene in male brain cortex and that from an age-matched female exhibited differences in methylation signatures. As seen in Figure 4, the most salient difference was observed only in region 1, which was essentially unmethylated in the female brain cortex (MMI: 3 for female vs 12.2 for male); regions 2 and 3 were identical. Indeed, the CpG profile of region 1 in DNA from a female brain resembled that in DNA from other tissues more than it did in DNA from the male brain cortex. This indicates that the regulatory element(s) putatively identified in region 1 (discussed above) is likely associated with male-specific gene expression in the brain cortex. This intriguing observation, though highly speculative, warrants more detailed follow-up analyses.

None of the five tissues analyzed displayed notable differences in *Isyna1* CpG profiles for region 2 (MMI: 4.8–5.5 for all tissues within the methylated segment used for comparison; Figure 1). Region 2 also had two of the most methylated CpG residues in *Isyna1* – CpG31, bordering the exon 1/intron 1 junction, was methylated 55–72% and CpG70, in intron 2, was methylated 90–100% in all tissues. The only exception appeared to be CpG45, which was moderately methylated in all tissues (30–40%), but poorly methylated in both male and female brain cortex (20%) (Figures 1, 4 & Table 2). It is also noteworthy that region 2 of the gene, located within a CpG island, did not demonstrate tissue-specific DNA methylation differences. Interestingly, the CpG islands in rat and human genes, as determined by

CpGPlot [102], are located in identical regions, extending from exon 1 to intron 3 (data not shown). Although sequence divergence is expected in the upstream regions of both genes, it would be interesting to see if similar features – namely, the lack of tissue-specific methylation differences in the CpG island and the presence of a brain-specific methylated region upstream of the CpG island – are also present in human tissues.

CpG methylation of region 3 of *Isyna1* showed pronounced differences among tissues. When ranked according to decreasing levels of DNA methylation (based on region 3 MMI values), the order is: first spleen, second the brain cortex, third the pancreas, fourth the heart and last the testis. The *Isyna1* gene from spleen was comparatively the most methylated (MMI: 18.2) based not only on the number of sites methylated, but also on the number of sites significantly methylated – a total of nine CpG residues (seven within the region of comparison) were methylated with four residues showing significant methylation (Figure 1 & Table 2). Indeed, the most highly methylated residue in region 3 of the gene was CpG103 (70%) in DNA from spleen, which implicates this residue in regulating expression in this tissue. By contrast, region 3 of the *Isyna1* gene from testis, the tissue expressing highest levels of *Isyna1* was hardly methylated (MMI: 1.0) with methylation observed at only one site, CpG103 (11%). Region 3 of *Isyna1* from male brain cortex (MMI: 5.5) was ranked second in levels of DNA methylation and showed an intermediate methylation pattern with four methylated CpGs; it was also the only other tissue exhibiting significant DNA methylation (30%) at CpG103. While the total number of significantly methylated (≥30%) sites in region 3 of the *Isyna1* gene was highest in DNA from spleen (four CpG sites), followed by brain cortex (one CpG site), no significant methylated residue in region 3 of the gene was observed in DNA from the remaining tissues. The CpG methylation profiles in region 3 of the gene in DNA from spleen, testis and brain cortex appear to be inversely correlated to *Isyna1* mRNA expression levels in both rat and human tissues [2,29], which suggests that methylation in this region of the gene may dictate tissue-specific expression. Northern blot analysis of *Isyna1* mRNA indicates that expression is highest in testis [2], moderate in brain, and poor in spleen, a pattern also seen in humans [29], which correlates extremely well with increasing MMI values of 1.0, 5.5 and 18.2, respectively, for the gene in these tissues. Interestingly, male and female brain cortex exhibited the same methylation levels in region 3 of *Isyna1* (MMI: 5.5 for both sexes; Figure 4), in keeping with their brain-specific expression, as any significant deviation in methylation levels will negate the argument that these differentially methylated regions (DMRs) are associated with tissue-specificity. This observation presupposes that the region 3 T-DMR likely harbors positive regulatory element(s) necessary for increased gene expression. Methylation of these putative element(s), as seen in DNA from spleen, and to a lesser extent in that from brain cortex, may prevent a positively acting factor (activator) from binding to these elements, thereby, downregulating *Isyna1* gene expression. On the other hand, in testis, in which region 3 of the *Isyna1* gene is relatively unmethylated, the putative activator can presumably bind facilitating increased gene expression. However, as noted before, regulation of *Isyna1* in the brain may be more complex as region 1 also appears to contribute to the moderate levels seen in this tissue. Many of these promoter-distal DMRs are also typically associated with enhancer elements [30].

Alternatively, the methylation of the T-DMR in region 3 of *Isyna1* may be associated with alternative splicing. The full-length transcript of *Isyna1* encodes a polypeptide (the α-isoform) that is 557 amino acids long. Recently, we identified a number of *Isyna1* splice variants, one of which – a short γ_c-isoform – was shown to modulate IP synthase enzyme activity negatively [2]. This mRNA initiates at the normal ATG codon, in exon 2, but terminates in intron 4, encoding a polypeptide of only 148 amino acids. Interestingly, the termination site for this isoform coincides with the T-DMR in region 3 of the gene. Increased methylation of region 3, therefore, could affect splicing at the intron 4 splice

donor site, facilitating the expression of the γ_c-isoform which would result in the negative modulation of *Isyna1* expression in spleen. This observation is concordant with recent observations on the human methylome where sites of significant methylation differences were found to occur at splice junctions, suggesting an association between transcription and splicing [31]. Mutational analysis of individual CpG residues in region 3 of the *Isyna1* gene cloned in the correct positional context (i.e., cloned downstream of a promoter) should permit a better understanding of the role of the T-DMR in region 3. Of note, our observations on the methylation of the region 3 DMR is in contrast with the prevailing view that increased gene body methylation correlates with increased gene expression [32]. Testis, a tissue that expresses high levels of *Isyna1*, shows negligible methylation in the gene body.

CpG methylation profiling of the entire analyzed region (regions 1–3) of the *Isyna1* gene indicates that the gene in spleen and male brain cortex exhibits comparatively higher overall methylation as evidenced by the presence of 16 methylated sites each (Table 2), followed by the gene in female brain cortex (eleven CpG sites), heart (eight CpG sites), pancreas (seven CpG sites) and testis (six CpG sites). The *Isyna1* gene in heart, pancreas and testis show identical, and insignificant, methylation levels overall. Methylation is most pronounced in region 3 of the gene in spleen, and to a lesser extent in this region in the male brain cortex. Increased methylation of region 1 of the gene is unique to male brain cortex. The comparatively higher level of methylation of region 1 of the gene in brain cortex is consistent with Southern blot data (Figure 3). While MMIs have been used to identify regions of differential methylation, it should be noted that different CpG methylation profiles could also yield identical MMI values. Therefore, the role of a specific CpG residue playing a key functional role in *Isyna1* expression cannot be over-looked. Specific CpG residues in *Isyna1* exhibiting significant methylation levels (>30%) in the various tissues analyzed are also identified and listed in Table 2.

Myo-inositol is found in high levels in the brain probably due to its active role in the PI signaling pathway [4]. As described elsewhere, altered MI levels are found in a range of behavioral and neurological disorders, thereby underlining its importance in brain function and maintenance. Some of these disorders manifest a gender bias that is not fully accounted for by changes in the sex chromosomes [33]. Autism [34,35], alcohol dependence [36], antisocial personality disorder [37] and attention deficit disorder [38], for instance, predominantly affect males, whereas, females are more prone to disorders such as anorexia nervosa [39], major depression [40], multiple sclerosis [41], panic disorder [42] and post-traumatic stress disorder [43]. Preliminary data from the present study indicate the possible existence of sex-specific DNA methylation patterns in regulatory regions of the *Isyna1* gene, which may help elucidate some of the sex-specific differences observed in the etiology of these disorders. Significant alterations in the DNA methylation patterns of the developing pre- and post-natal brain in either sex could have occurred when exposed to environmental toxins that could conceivably explain some of these etiologies in later life. Such sex differences in brain DNA methylation patterns in genes have recently been reported [44]. This area of investigation, therefore, deserves greater in depth analyses.

The prevailing view regarding T-DMRs is that a significant majority are not likely to be located in CpG islands but at the outer edges of these islands in regions called 'CpG island shores' [45]. Consistent with this view, we find no significant change in DNA methylation levels in the CpG island of *Isyna1* (CpG13–70; Figure 1). Regions of differential DNA methylation occur outside the island in region 1 of the gene in male brain cortex and in region 3 in all other tissues analyzed. We cannot discount the possibility that additional, and perhaps, more pronounced DMRs may exist downstream of exon 4 and/or outside the gene body in the 3′ regions. Other factors may also contribute to tissue specificity. For example, although genomic DNA from rat heart is poorly methylated in region 3 of the *Isyna1* gene

(Figure 1), this tissue does not manifest high mRNA levels [2,29]. It is possible that regulation in the heart may be dependent on nonepigenetic mechanisms. Tissues get inositol through three basic mechanisms: by *de novo* synthesis *via* Isyna1, by inositol transport, or by recycling mechanisms which generate free MI from the breakdown of inositol polyphosphates. It is not clear which mechanism predominates in the heart. Perhaps, DNA methylation plays a key role only in tissues that are solely dependent on the synthetic mechanism, as opposed to those that rely more on the other (recycling or transport) mechanisms. Future studies should resolve this issue.

Future perspective

This study opens up several interesting avenues for further investigation and emphasizes the use of animal models to unravel subtle changes in methylation profiles which are especially relevant for housekeeping genes that manifest low methylation levels. Future studies should examine:

- Whether sex-specific factors regulate gene expression in the brain;

- The significance of the T-DMR in region 3 of the gene in spleen;

- Whether methylation of the region 3 T-DMR promotes the synthesis of alternatively-spliced regulatory isoforms;

- Whether similar features of CpG methylation exist in the human *ISYNA1* gene.

This study should provide a useful blueprint to address those questions.

Executive summary

- Understanding *Myo*-inositol 3-phosphate synthase regulation can contribute to our understanding of why *Myo*-inositol levels are frequently altered in several brain disorders.

- This study examines whether the expression of rat *Isyna1*, the gene encoding *Myo*-inositol 3-phosphate synthase, is regulated by DNA methylation in different tissues.

Materials & methods

- Transfection analysis, Southern blot analysis, CpG methylation profiling and mutation analysis were used to determine if *Isyna1* is differentially methylated in rat tissues.

***In vitro* methylation of human *ISYNA1* promoter decreases reporter activity**

- Transfection analysis utilizing plasmids that harbor *in vitro* methylated CpG residues in the human promoter indicate that CpG methylation decreases *ISYNA1* promoter activity.

Differential methylation of rat *Isyna1* in various tissues

- Southern blot analysis of genomic DNA, digested with a methylation-sensitive enzyme, indicates that the gene is differentially methylated in the brain cortex, heart, pancreas and testis in the rat. Ranked by decreasing methylation levels, the order is first the brain cortex, second heart and third pancreas/testis.

CpG methylation profile of rat *Isyna1* in various tissues

- CpG methylation profiling was undertaken on a 1.35-kb region extending from −700 bp to +650 bp of the *Isyna1* gene in rat tissues. Analysis was

divided into three regions: region 1 (− 700 bp to −500 bp, in the 5' upstream region), region 2 (−350 bp to +200 bp spanning the transcriptional start site) and region 3 (+450 bp to +650 bp, in the gene body).

- Region 1 of *Isyna1* was comparatively highly methylated in the male brain cortex when compared with that from other tissues.

- Region 2 of *Isyna1*, localized within a CpG island, showed no distinctive DNA methylation changes among the various tissues.

- Region 3 of *Isyna1*, located within the gene body, was comparatively highly methylated in the spleen, moderately methylated in brain cortex and poorly methylated in testis, a tissue that expresses high levels of the enzyme. The reciprocal relationship observed between the methylation pattern found in this region and mRNA expression in these tissues suggests that region 3 could be a tissue-specific differentially methylated region.

CpG methylation profile of rat *Isyna1* in male & female brain cortex

- A preliminary analysis of matched male and female brain cortex suggests the putative presence of a sex-specific regulatory element within region 1.

Mutation analysis of CpG residues in the 5' flanking region of rat *Isyna1*

- Mutation analysis of each of the nine CpG residues present in region 1 of *Isyna1* identified one CpG residue as significantly contributing to promoter activity in neuronal cells.

Discussion

- Rat *Isyna1* exhibits tissue-specific DNA methylation patterns. Brain DNA was uniquely methylated in the 5' upstream region and harbors a putative sex-specific element.

- A tissue-specific differentially methylated region was identified within the gene body of *Isyna1*. In contrast to the prevailing view, increased gene body methylation did not correlate with increased gene expression.

- The study lays the groundwork for future studies to examine the potential role of aberrant *Isyna1* methylation in contributing to altered *Myo*-inositol levels observed in various brain disorders.

Acknowledgments

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▪ of interest

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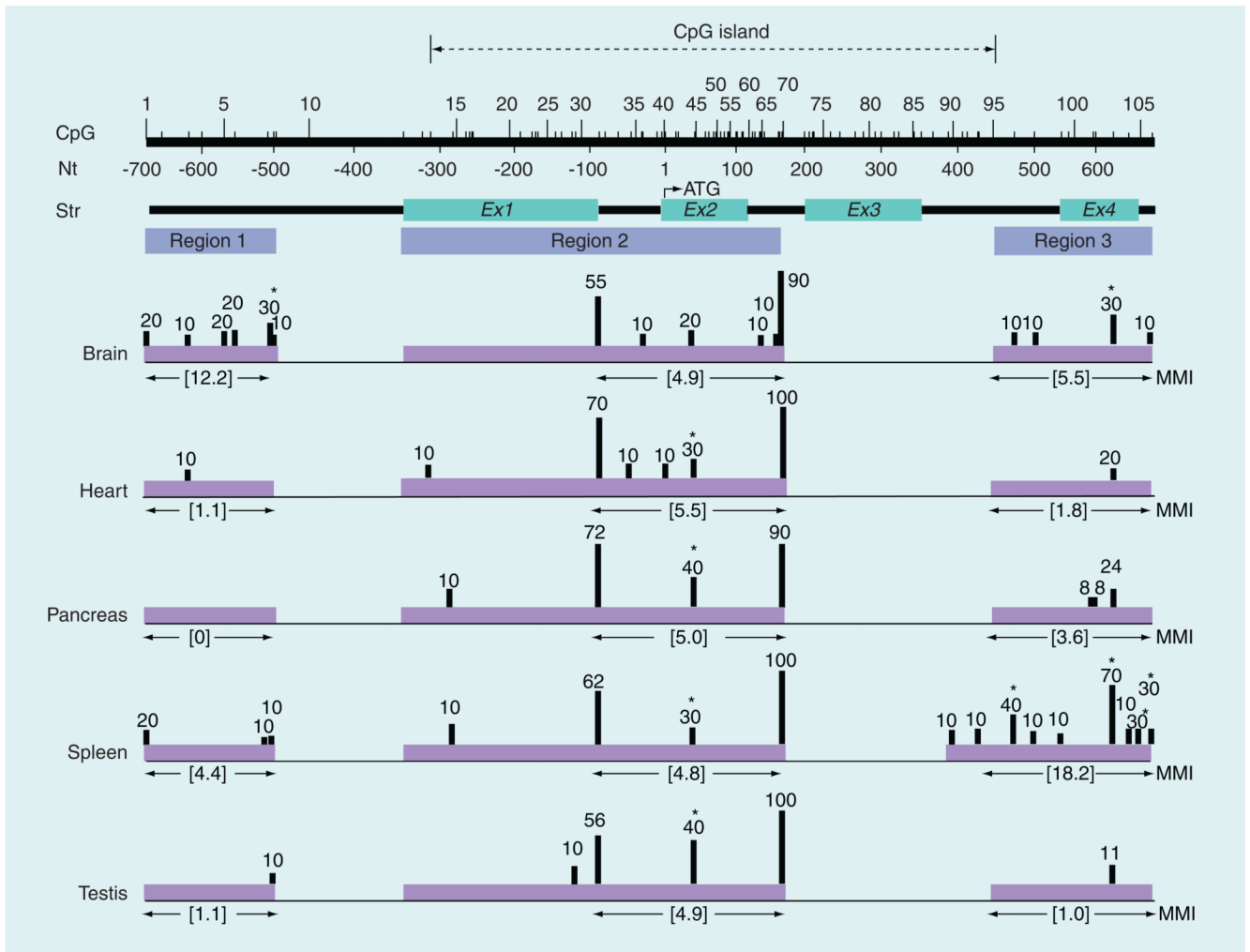102. EMBOSS CpGPlot. www.ebi.ac.uk/Tools/emboss/cpgplot/index.html

Figure 1. CpG methylation profile of rat *Isyna1* in various tissues
CpG methylation profile was determined in five (male) rat tissues – brain cortex, heart, pancreas, spleen and testis. The CpG island (dotted lines with arrowheads), number and position of each CpG residue, nucleotide position and gene structure are indicated on the top of the figure. The CpG island, determined using European Molecular Biology Open Software Suite (EMBOSS) CpGPlot [102], extends from CpG13–CpG95. Regions 1, 2 and 3 represent amplicons used for CpG analysis; region 2 is composed of three overlapping amplicons. Analyzed regions are shown in purple shaded horizontal blocks with the MMI values directly below each block. Methylation levels (%) are denoted by the height of the vertical bars and are based on the percentage of clones analyzed. For CpG31, the methylation level was averaged from two overlapping amplicons. CpG sites that are significantly differentially methylated in tissues are marked by an asterisk. Note, in particular, the pronounced differences in methylation patterns in the brain cortex in region 1 and in spleen in region 3. Except for CpG10 and CpG71–94, the status of all CpG residues spanning a 1.35-kb region from nucleotides −700 bp of the promoter to +650 bp in intron 4 was examined.
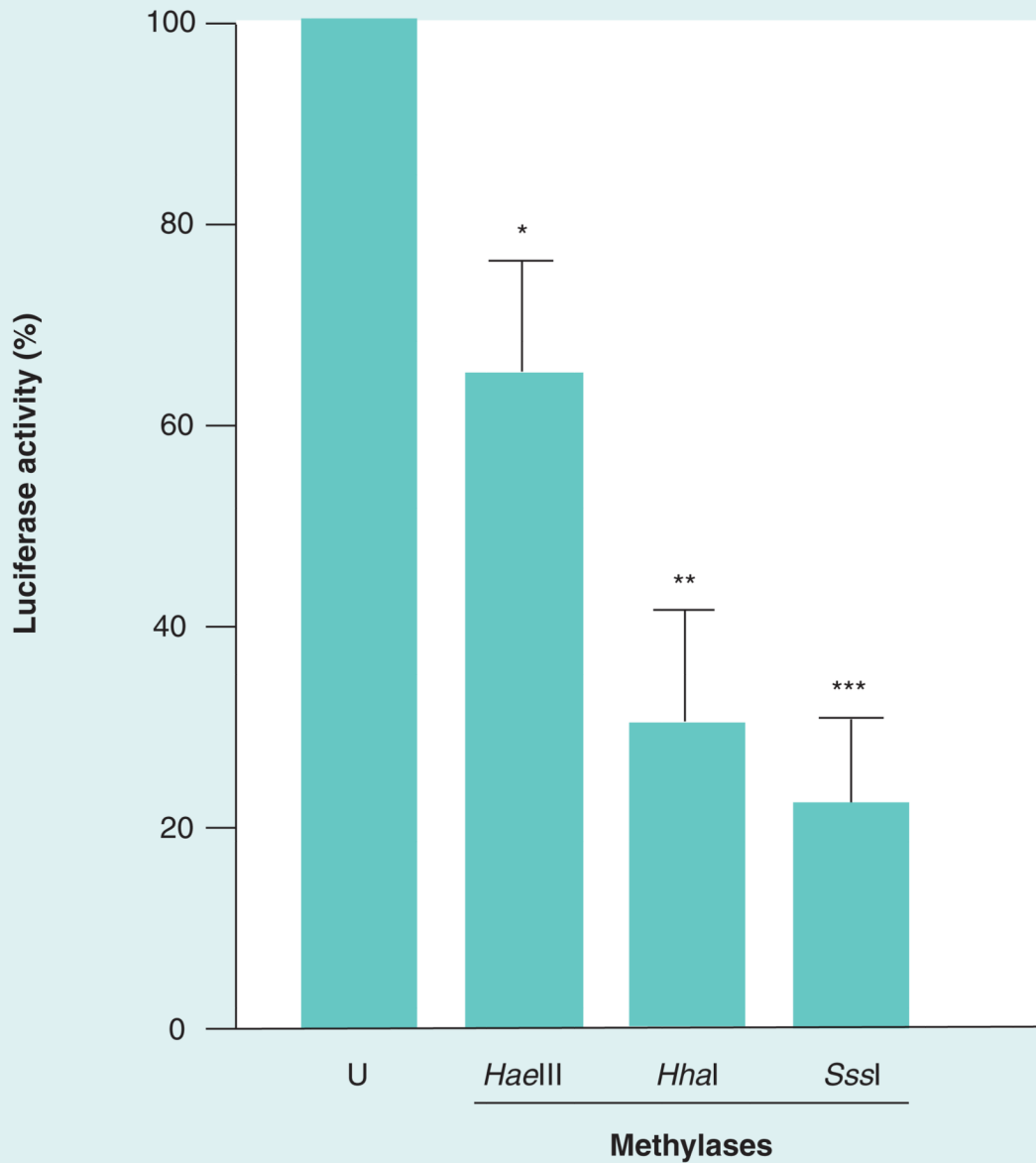MMI: Mean methylation index; Nt: Nucleotide; Str: Structure.

Figure 2. Promoter activity of *ISYNA1* can be suppressed by methylation
The minimal promoter of *ISYNA1* [20] in pGL3 luciferase reporter vector (Promega, WI, USA) was methylated *in vitro* using *Hae*III, *Hha*I and *Sss*I methylases. Equal amounts of the methylated and U promoter plasmids were transfected into SK-N-AS human neuroblastoma cells along with a control vector (pRL-TK) expressing *Renilla* luciferase and normalized luciferase activities were determined. The experiment was repeated three-times with duplicates and the results expressed as mean ± standard error of the mean. The reporter activity of the U parent plasmid was taken as 100%. Statistical significance was computed using the unpaired two-tailed Student's t-test using GraphPad software (GraphPad Software, Inc., CA, USA).

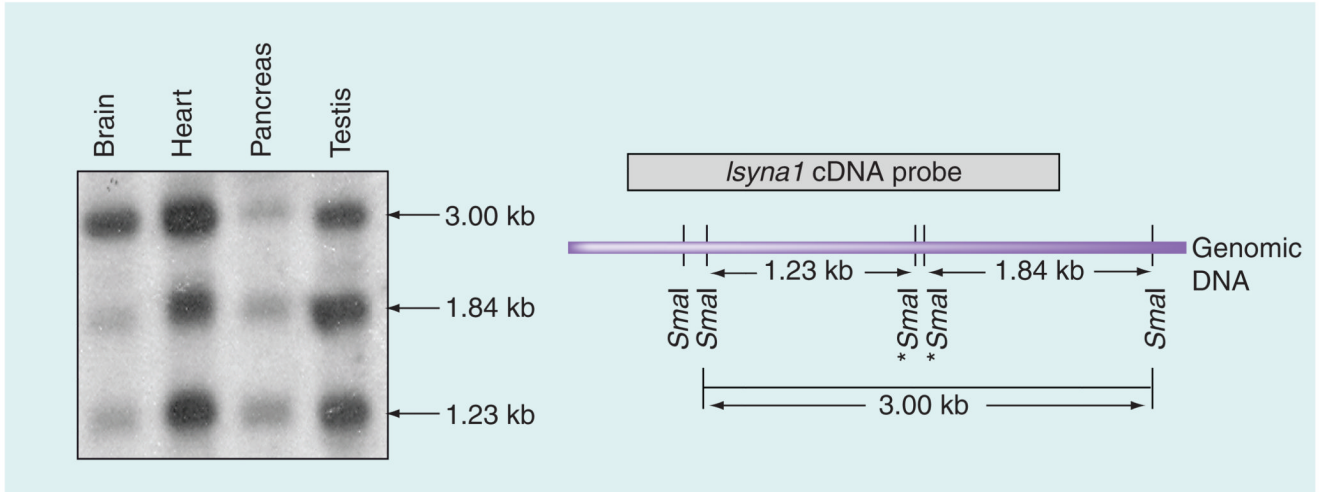*p < 0.05; **p < 0.005; ***p < 0.001.
U: Unmethylated.

Figure 3. Southern blot analysis of *Sma*I-digested *Isyna1* genomic DNA in different rat tissues
A total of 20 µg of genomic DNA from four rat tissues (brain cortex, heart, pancreas and testis) was first digested with *Eco*RI and *Hind*III, followed by *Sma*I, resolved on 1% agarose gels, Southern blotted onto a Zeta-Probe GT membrane (BioRad, CA, USA) and probed with a P[32]-labeled *Isyna1* cDNA. The *Sma*I restriction map of the rat *Isyna1* genome is shown on the right. The 1.23-kb and 1.84-kb bands represent completely digested *Sma*I fragments while the 3.0-kb fragment is due to partial digestion resulting when one or both internal *Sma*I sites (marked by asterisks) are methylated. The probe detects 1.23-, 1.84- and the 3.00-kb fragments. Differential methylation was inferred by comparing the intensity of the 3.00 kb fragment with that of the other two restriction digest fragments on the blot. Note the striking difference in band intensities between the brain cortex (more methylated) and testis (less methylated).
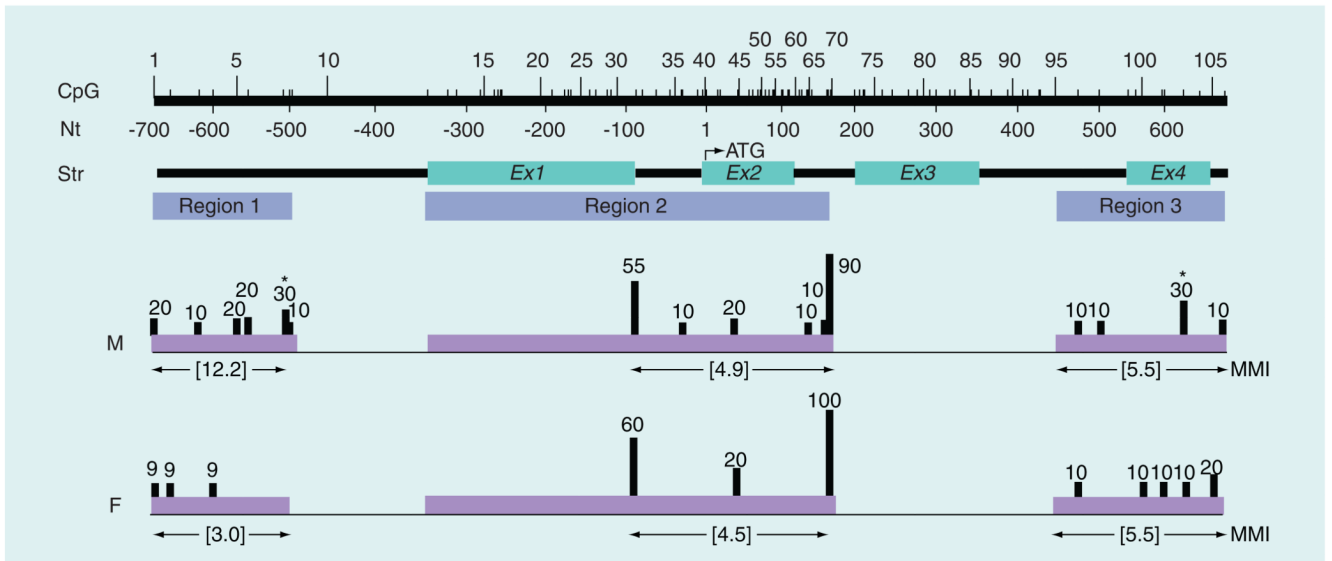
Figure 4. Comparison of *Isyna1* methylation profiles between male and female rat brain cortex
Details are the same as in Figure 1. Methylation levels were relatively higher in region 1 in
the male compared with the female, with no significant differences in regions 2 or 3. The
male brain cortex also harbored two significantly methylated CpG residues (asterisks) at
CpG8 and CpG103 (each 30%). CpGs31 and 70, although highly methylated, exhibited no
major sex-specific differences. The CpG profile for the male brain cortex is derived from
Figure 1.
F: Female; M: Male; MMI: Mean methylation index; Nt: Nucleotide; Str: Structure.
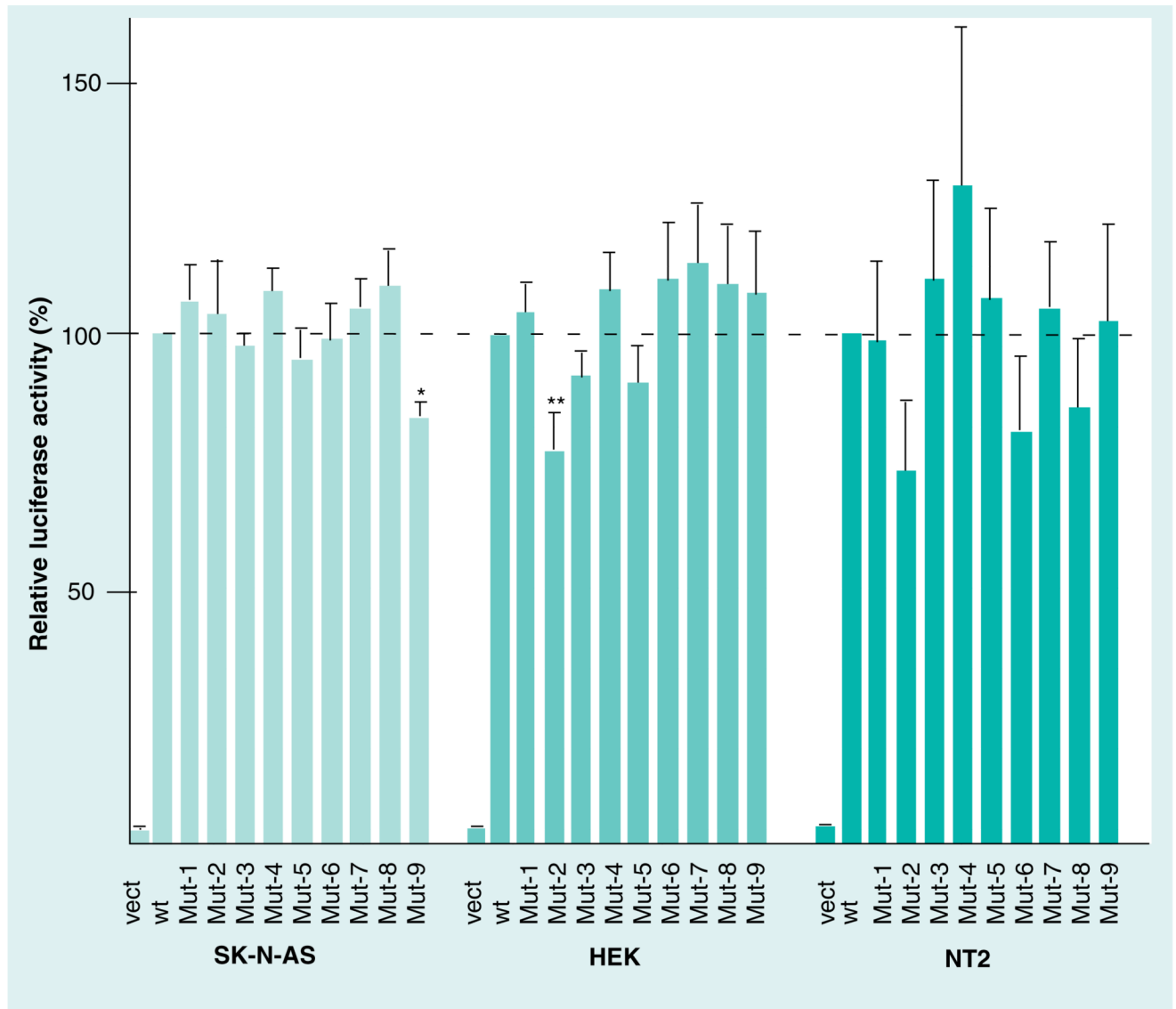
Figure 5. Mutation analysis of CpG residues in region 1 (−700 bp– −500 bp) of rat *Isyna1*
A 758-bp PCR fragment from the 5' flanking region of *Isyna1* containing CpG residues 1–9 of region 1 was cloned into the pFRL2 vector [22]. CpG residues were mutated (CG>AG) to yield the cognate single mutants, Mut-1–9. The mutants, the wt parent and the empty pFRL2 vect were transfected into SK-N-AS (human neuroblastoma), HEK and NT2 cell lines and their relative luciferase activities were determined after normalization with *Renilla* luciferase. The normalized promoter activity of the parent vector was set at 100% (dotted line). Mut-9 in SK-N-AS and Mut-2 in HEK cell lines decreased promoter activity to 83 and 77%, respectively. The mutations did not affect the promoter activity in the NT2 cell line. Transfections were carried out at least three-times using duplicate wells. Values are expressed as mean ± standard error of the mean. Statistical significance was computed using the unpaired two-tailed student's t-test using GraphPad software (GraphPad Software, Inc., CA, USA).
*p = 0.002; **p = 0.01.

HEK: Human embryonic kidney; Mut: Mutation; NT2: NTerra-2 human testicular embryonic carcinoma; vect: Vector; wt: Wild-type.

Table 1

PCR primers used for CpG methylation profiling of rat *Isyna1*.

Region	Primer name	CpG[†]	Sequence[‡]
Region 1	rM-Isyn 313	1–9	GATGAGTTTAGTTGGGTTGG
	rM-Isyn 553		ACCTCCCCCTAAACAA
Region 2[§]	rM-Isyn 611	11–31	ATTAGGAGTAGGTAGGATAAT
	rM-Isyn 930		CTTACCRACTAAAAATTAAATCAAAC
	rM-Isyn 905	31–43	GTTTGATTTAATTTTTAGTY
	rM-Isyn 1046		RAACTATCCACCAAAATCTC
	rM-Isyn 1027	44–70	GAGATTTTGGTGGATAGTTY
	rM-Isyn 1203		AAAAATAAATAAAAAATAAATCR
Region 3	rM-Isyn 1415	89–106	GTGGTTGTTAGGAGGA
	rM-Isyn 1770		AACACCAAACCCATCC

[†]Refers to the position of CpG residues in the 1.35-kb fragment (see Figure 1).

[‡]Primers used on bisulfite-modified DNA.

[§]CpG profile for region 2 was derived from three overlapping amplicons.

R: A/G; Y: C/T.

Table 2

Distribution of methylated CpG residues in *Isyna1*.

Tissue	Total methylated sites[†]	Region 1				Region 2				Region 3			
		Total	Significant sites[‡]	MMI	Total	Significant sites	MMI	Total	Significant sites	MMI	Total	Significant sites	MMI
Brain M[§]	16	6	CpG8	12.2	6	CpG31, 70	4.9	4	CpG103	5.5			
Brain F[§]	11	3		3.0	3	CpG31, 70	4.5	5		5.5			
Heart	8	1		1.1	6	CpG31, 45, 70	5.5	1		1.8			
Pancreas	7	0		0	4	CpG31, 45, 70	5.0	3		3.6			
Spleen	16	3		4.4	4	CpG31, 45, 70	4.8	7[¶]	CpG96, 103, 105, 106	18.2			
Testis	6	1		1.1	4	CpG31, 45, 70	4.9	1		1.0			

[†] Number of methylated CpG sites identified in the analyzed 1.35-kb region: regions 1–3.

[‡] CpG residues with ≥30% methylation.

[§] Brain cortex.

[¶] Nine CpGs, if the longer amplicon is considered (see Figure 1).

F: Female; M: Male; MMI: Mean methylation index.