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DNA methylation in a sea lamprey vasotocin receptor gene promoter correlates with tissue- and life-stage-specific mRNA expression

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ABSTRACT

The jawless vertebrate sea lamprey (*Petromyzon marinus*) genome has a different structure from both invertebrates and jawed vertebrates featuring high guanine–cytosine (GC) content. This raises the question of whether DNA methylation of cytosine–guanine (CpG) dinucleotides could function to regulate lamprey gene transcription. We previously characterized a lamprey arginine vasotocin (AVT) receptor gene (Pm807) possessing characteristics of both arginine vasopressin (AVP) V1A and oxytocin (OXT) receptor genes of jawed vertebrates. Lamprey Pm807 mRNA is highly expressed in adult heart and larval liver but not expressed in adult liver. Using high-resolution melt (HRM) PCR on bisulfite-converted DNA, we pinpointed a region with tissue-specific differences in DNA melt characteristics, indicating differences in methylation level. Sequencing revealed a pattern of methylation at specific CpGs at consistently higher levels in adult heart and larval liver than adult liver. These CpGs are associated with putative transcription factor binding sequences organized similarly to functional OXTR promoters in mammals, suggesting functional similarity in lamprey gene transcription regulation.

1. Introduction

Whole-genome bisulfite sequencing is frequently used to characterize cytosine–guanine dinucleotide (CpG) methylation patterning throughout mammalian genomes. Mammalian genomes exhibit a global methylation pattern; almost all CpGs are methylated within gene bodies, whereas CpG islands, where CpG dinucleotides occur at a higher density than the expected probability, are often associated with gene promoter regions and remain mostly unmethylated (Okamura et al., 2010). Methylation in the promoter region of a gene is a highly studied epigenetic modification that is generally associated with transcriptional repression in mammals (Hu et al., 2013). More genomes of non-mammalian animals are now being studied with whole-genome bisulfite sequencing. A wide variety of methylation patterning has been observed, but the genome-wide pattern in general transitions from mosaic in invertebrates to global methylation in vertebrate genomes (Okamura et al., 2010; Zhang et al., 2015). The sea lamprey (*Petromyzon marinus*) occupies a pivotal position in the evolutionary transition from invertebrate to vertebrate physiology. The guanine–cytosine (GC) content of the lamprey genome overall is high, and the genome structure differs from both vertebrates and invertebrates (Smith et al., 2013), raising the question of whether CpG methylation can be a functional regulator of lamprey gene transcription. The methylation pattern in sea lampreys is global (occurring throughout the genome) as in vertebrates, but at a lower percentage of methylated CpGs (Tweedie et al., 1997). However, little is known about how methylation patterning and specific epigenetic events in lamprey gene promoters relates to transcription of individual genes.

The arginine vasopressin (AVP)/oxytocin (OXT) hormone receptor system in mammals mediates multiple functions including social behavior, reproductive physiology, and metabolism. The promoter regions of these genes in humans have been well studied (Dhakar et al., 2013), since single–nucleotide polymorphisms (SNPs), microsatellites, and epigenetic modifications have been implicated in behavioral variation and disease. CpG islands are associated with gene transcription regulation in both the human V1AR and OXTR gene promoter regions (Kusui et al., 2001). Additionally, in mouse OXTR promoter DNA, a pattern of methylation was observed at specific CpGs, and the pattern differed among tissues (Mamrut et al., 2013). Further, these specific CpGs occurred within known transcription factor consensus binding sequences, and the level of methylation was correlated with levels of OXTR mRNA expression (Harony-Nicolas et al., 2014).

We previously characterized the sea lamprey arginine vasotocin (AVT) homologs of the mammalian AVP/OXT hormone receptors (Mayasich and Clarke, 2016). We determined through phylogenetic and syntenic analyses that the AVT receptor found on scaffold 807 is most likely orthologous to an OXTR but comprises characteristics of both the V1A and the OXT receptors. Messenger RNA from the Pm807 AVTR gene (Genbank Accession no. **KC731437.1**) was found to be expressed in several adult and parasitic-phase lamprey tissues

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including brain, gills, gonads, heart, and kidneys but not liver (Mayasich and Clarke, 2016).

For the current study, we used high-resolution melt (HRM) polymerase chain reaction (PCR) and sequencing of bisulfite-modified genomic DNA to investigate the relationship of CpG methylation to mRNA expression at the individual gene level. Our hypothesis is that 1) DNA methylation in the promoter region of the Pm807 AVTR may, as in mammals, correlate with tissue-specific transcription in lampreys, and 2) for such methylation-regulated transcription to occur, we would expect to find relevant transcription factor consensus binding sequences in the vicinity of CpG sites in the promoter region. Further, we tested whether the Pm807 AVTR gene is expressed in the liver of larval lampreys to determine the relationship between methylation level, mRNA expression, and life stage.

2. Materials and methods

2.1. Animal treatment and tissue collection

Sea lamprey larvae and adult upstream migrants were obtained from USGS Hammond Bay Biological Station (HBBS) (Millersburg, MI). Adult animals (which no longer feed) were held in a 100-gal stock tank at 15 °C for a maximum of 2 weeks after receipt. Adults were euthanized in 2 g/l tricaine methanesulfonate (MS-222, Western Chemical, Inc., Ferndale, WA), pH 7.4 and stored at −20 °C prior to removing the heart and liver from each animal. A section of approximately 100 mg of each tissue was placed in a sterile Petri dish and minced with a scalpel; 25 mg of the minced tissue was weighed into a 1.5 ml microcentrifuge tube for DNA isolation. The larvae (approximately 100–110 mm long) were maintained at the University of Minnesota–Duluth in a sand-lined 2-gal aquarium in dechlorinated tap water at a temperature of 10 °C within a circulating water bath. A 24-h feeding of 5 g of moist cake yeast slurry was provided prior to euthanizing in 1 g/l MS-222 in dechlorinated tap water. Whole livers (approximately 15–20 mg each) were immediately harvested, placed in 1.5 ml microcentrifuge tubes, and stored at −70 °C for DNA and RNA isolation. Animal treatment conformed to University of Minnesota animal care standards (IACUC protocol number 1305–30612A).

2.2. Nucleic acid isolation and treatment

Heart and liver tissues from four adult lampreys and livers from four larval lampreys were used in the HRM PCR screening experiments. The tissues were crushed and homogenized in individual microcentrifuge tubes on dry ice using a sterile Teflon pestle. The DNeasy kit (Qiagen, Germantown, MD) was used to isolate genomic DNA following the manufacturer's protocol, with a 3-h incubation at 56 °C to lyse the tissue. DNA was eluted with AE buffer. Nucleic acid concentration was quantified by Nanodrop spectrophotometry, and a Qubit® dsDNA BR assay kit (Life Technologies, Carlsbad, CA) was used with a Qubit® fluorometer to determine the specific DNA concentration. These results were subsequently used to normalize DNA to 1 μg for methylation and 2 μg for bisulfite conversion reactions. DNA quality was checked on a 1% agarose gel showing strong bands at 23 kilobases (kb) (Suppl. Fig. 1). Fully methylated reference DNA was created in vitro by incubating 1 μg DNA for 2 h at 37 °C with SssI methylase and S-adenosyl methionine substrate according to the manufacturer's instructions (New England Biosciences, Ipswich, MA). Each 20 μl reaction was stopped by heating at 65 °C for 20 min, and three reactions were pooled, precipitated in a final volume of 75% ethanol, and resuspended in 25 μl nuclease-free water.

Bisulfite conversion of unmethylated cytosines in the methylated and untreated DNA samples was conducted with 2 μg of DNA per reaction. The EpiTect® Fast DNA Bisulfite Kit (Qiagen) was used according to the manufacturer's instructions for a 140 μl reaction. Thermal cycler conditions included two cycles of denaturation for 5 min at 95 °C and incubation for 15 min at 60 °C. The converted DNA was stored at 4 °C if used within 24 h, thereafter at −20 °C.

Large quantities of RNA were present in the DNeasy eluate of the larval liver tissue. RNA was then further isolated by simply treating with DNase I (DNA-*free*, Ambion, Austin, TX) to remove genomic DNA according to the manufacturer's standard protocol in a 50 μl reaction. The larval liver RNA quality was checked on a 1% agarose gel, showing removal of the 23 kb genomic DNA band (Suppl. Fig. 1). RNA concentration was again measured by Nanodrop and 1 μg of RNA was reverse transcribed to cDNA using ThermoScript™ RT (Invitrogen, Carlsbad, CA) in a 20 μl reaction. A mock reaction with the same RNA concentration and other conditions but without the RT enzyme served as a negative control to demonstrate the lack of DNA contamination.

2.3. Expression of Pm807 AVTR mRNA

Polymerase chain reaction (PCR) assays were conducted on larval liver cDNA using primers for a 285-bp segment of the Pm807 coding region on the cDNA and the non-reverse-transcribed mock cDNA. Primers for a 280-bp segment of the lamprey β-actin gene were used to amplify the cDNA as a positive reference. Primer sequences are as follows: Pm807 forward AAGGCGTACATCACCTGGATGACA, reverse GACTGACC ACATCTGCACGAAGAA; β-actin forward ATCATGTTCGAGACCTTCAACA CGC, reverse TCTCCTTGATGTCACGCACGATCT. GoTaq Master-Mix (Promega, Madison, WI) was used in a 25 μl reaction with 1 μl cDNA; thermocycler profile was 2 min at 94 °C followed by 34 cycles of 30 s at 94 °C, 30 s at 60 °C, and 60 s at 72 °C, with a 5 min elongation step at 72 °C.

2.4. High-resolution melt PCR screening of putative promoter region DNA

Locations of CpG islands were determined in the region from the transcription start site (TSS; see Mayasich and Clarke, 2016) to approximately 1600 base pairs (bp) upstream and primers were designed using the MethPrimer program (Li and Dahiya, 2002). Discrete, generally overlapping segments of the promoter region (Fig. 1) were then screened using HRM PCR. Primer sequences and segment locations, sizes, CpG number, and reaction characteristics for adult heart and liver HRM PCR are shown in Table 1. Full genomic sequences of the segments are shown in Supplementary Table 1. The 25 μl HRM reactions were performed using the EpiTect® HRM™ PCR kit (Qiagen), with duplicate reactions (except where indicated) of livers ($n = 8$) and hearts ($n = 8$) for each of the four animals and a 100% methylated reference sample. Based on DNA input to the bisulfite reactions as well as post-conversion Nanodrop quantification checks of 10% of the samples, DNA template concentrations used in the HRM reactions were 25–30 ng for amplicons under 200 bp and 50–60 ng for longer amplicons. Reaction conditions followed the manufacturer's instructions of an initial activation step of 5 min at 95 °C, and cycles of 10 s denaturation at 95 °C, 30 s annealing at 55 °C, and extension at 72 °C with duration determined by amplicon length (generally, 10 s for products up to 150 bp, 8 s per 100 bp for larger products). Segment A was pre-amplified using TaKaRa EpiTaq™ HS polymerase kit (Clontech, Madison, WI; see conditions for first-round PCR) for bisulfite-treated DNA followed by semi-nested HRM reactions as noted in Table 1. High-resolution melting was conducted in 2 s, 0.1 °C increments from 65 °C to 95 °C, or 5 °C below to 5 °C above the temperature required to melt half of the PCR-produced DNA molecules (Tm), when the Tm was known from preliminary trials.

DNA isolated from livers of larval-stage animals were tested along with adults in the target region (Segments F and G), and downstream Segment E. Unmethylated reference DNA was also produced from adult heart and liver samples by first conducting standard PCR to cause the loss of methylation in the amplified product. Primers were designed for an 840 bp length of the native genomic DNA spanning just upstream and downstream of the target segments (Table 1) as follows: Segment G forward TGGTTGGAATCGGCTCTAATG, Segment F reverse