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Isolation and molecular characterization of Rem2 isoforms in the rainbow trout (*Oncorhynchus mykiss*): Tissue and central nervous system expression

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1. Introduction

The RAS superfamily of GTP binding proteins is composed of six subfamilies, the RAS, RAB, RHO, ARF, RAN, and RGK (REM, RAD, and GEM/KIR) subfamilies. The RGK subfamily of GTPases all share features that distinguish them from the other five RAS subfamilies. Such features include the lack of lipid modification for membrane anchorage (Del Villar et al., 1996), N-terminal and C-terminal extensions beyond the RAS core as well as a unique G3 motif (Finlin et al. 2000), and they are regulated at the transcriptional level (Maguire et al, 1994; Cohen et al., 1994; Vanhove et al., 1997; Leone et al., 2001; Suyama et al., 2003; Ohsugi et al., 2004; Warton et al, 2004; Finlin et al., 2005; Paradis et al., 2007). In mammals, among the RGK family, *REM2* was the first of the RGK family to be found at high expression levels in neural tissue (Finlin et al. 2000). Within neural tissue, REM2 plays a prominent role in very diverse functions including modulation of Ca²⁺ currents (Chen et al., 2005), excitatory and inhibitory synapse development (Paradis et al., 2007; Ghiretti and Paradis 2010), survival of embryonic stem cells (Edel et al., 2010a, 2010b), and regulation of dendrite morphology (Ghiretti and Paradis, 2010).

In mammals, REM2 has been well-characterized. The original clone of mammalian *REM2*, described in rat (Finlin et al., 2000), may be a shorter N-terminal splice variant as a long form was later identified (Church et al., 2009; thereafter, REM2 refers to the long form). There is a high level of conservation among the RAS core of REM2 with other members of the RGK subfamily. However, the extended N-terminus of REM2 appears distinct and may be responsible for functions unique from other RGK subfamily members (Seau and Pitt, 2006). REM2 appears conserved across vertebrate classes (Edel et al., 2010b). However, the zebrafish Rem2 protein appears to lack the extended N-terminus of mammalian REM2. Whether this difference is consistent across vertebrate classes or is species-specific is not known due to a lack of *rem2* clones from non-mammalian species. This can also be said for the C-terminal extension, as mammalian REM2 possesses an extended C-terminus distinct from the other RGK members as well (Seau and Pitt, 2006).

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In the non-mammalian vertebrates *Xenopus laevis*, *X. tropicalis*, and the zebrafish (*Danio rerio*) *rem2* has been isolated (Klein et al., 2002; Edel et al., 2010b), however its characterization including tissue specificity has been limited to zebrafish during embryological development (Edel et al., 2010b). *Rem2* has not been characterized in an adult teleost fish species. Unlike mammals, adult teleost fish have been shown to have an enormous capacity for neurogenesis and brain repair (Zupanc, 1999). In both mammals and fish, *REM2* has been shown to influence the central nervous system (CNS) regarding neural tissue development, synapse formation, cell proliferation and apoptosis, and stem cell survival (Paradis et al., 2007; Edel et al., 2010a, 2010b; Ghiretti and Paradis, 2010). Such mechanisms of neural plasticity, such as apoptosis and cell proliferation, have been found to be part of the response of the adult fish brain to injury that characterizes the tremendous regenerative properties of their CNS (Zupanc and Zupanc, 2006).

To begin understanding the role that *Rem2* could have in adult teleost neurogenesis and brain regenerative capabilities, this study characterized *Rem2* and examined its gene expression in the rainbow trout (*Oncorhynchus mykiss*). Rainbow trout are used extensively as a physiological model system because they are an experimentally tractable species of importance in aquaculture (Thorgaard et al., 2002). Furthermore, rainbow trout are distinctive in having a relatively recent tetraploid ancestry (Allendorf and Thorgaard, 1984; Moghadam et al., 2005; Shiina et al., 2005; Evans et al., 2008), which allows for examination of the process of evolution by gene duplication (Thorgaard et al., 2002). Most importantly, rainbow trout have become a species of interest in behavioral and physiological processes as it relates to neurogenesis and adult brain repair (Menuet et al., 2003, Sørensen et al., 2007, 2011).

The present study describes the first isolation of two alternate forms of *Rem2* in a non-mammalian species, the rainbow trout. We present the complete characterization of the cDNAs for both *rem2* genes. Furthermore, we compare the tissue specificity of both *rem2* genes as well as the expression profiles of both genes across major regions of the CNS.

2. Materials and methods

2.1 Animals

Rainbow trout (*Oncorhynchus mykiss*, Salmonidae) were obtained from the South Carolina Department of Natural Resources, Walhalla State Fish Hatchery (Mountain Rest, SC, USA). Trout were housed in 'living streams' (Frigid Units, Inc.) with capacities of 530 operating liters of dechlorinated water, which was kept at 10 °C. Animals were maintained on a diet of trout chow (Goldfin Slow Sinking Pellets 5.0 mm; Zeigler) and fed twice per day.

2.2 Tissue collection and RNA isolation

Trout were anesthetized using 2-phenoxyethanol (1ml/L H₂O) and then sacrificed by rapid decapitation. Tissues were collected, weighed, and immediately homogenized in cold (4°C) TRIzol® reagent for total RNA isolation using the TRIzol® Plus RNA Purification Kit (Invitrogen) in combination with the PureLink™ DNase Set (Invitrogen) to remove genomic DNA. For the isolation of the full-length *rem2* genes, whole brain tissue was collected from rainbow trout. For tissue specificity of the rainbow trout *rem2* genes, tissue was collected from each of five rainbow trout and included the brain, bulbus arteriosus, gill, head kidney, intestine, liver, spleen, stomach, trunk kidney, ventricle, and red and white muscle. For the regional distribution of gene expression of both *rem2* genes in the rainbow trout CNS, brains were removed from five rainbow trout and the olfactory bulb, cerebrum, midbrain, cerebellum, and hindbrain were isolated from each as was a portion of the spinal cord.

2.3 Molecular cloning and sequencing of full-length *rem2* genes

One microgram of total RNA from rainbow trout whole brain tissue was used for reverse transcription using BD PowerScript Reverse Transcriptase (BD Biosciences). The synthesized first strand cDNA was then used as a template in 3' and 5' RACE assays using the RNA ligase-mediated Rapid Amplification of cDNA Ends (RLM-RACE) Amplification kit (BD Biosciences) according to the manufacturer's protocol. Initial *rem2* 5'-RACE and 3'-RACE primer design was based on the analysis of multiple sequence alignments (ClustalW2) between members of the mammalian RGK subfamily and a single 922 bp fragment of a *rem2* gene transcript isolated from rainbow trout brain tissue in preliminary differential display assays (GeneFishing™; SeeGene Inc.) using primers supplied by the manufacturer's kit (Table 1; Hollis unpublished results). From this alignment, four gene-specific primers, two forward and two reverse primers (primary and nested primers for each), for nested RLM-RACE PCR were designed, and a full-length *rem2* gene (*rem2a*) was isolated using these primers (see Table 1). Sequence analysis (described below) during the isolation of this *rem2* gene indicated the presence of a second possible *rem2* gene transcript. From this transcript, a second set of gene-specific primers were designed for nested RLM-RACE and a second full-length *rem2* gene (*rem2b*) was isolated (see Table 1). The cycling conditions for RLM-RACE for both *rem2* genes were 94 °C (3 min) followed by 35 cycles of 94 °C (30 s), 59.5 °C (30 s), 72 °C (1 min), followed by 72 °C (7 min). Verification of the full-length open reading frames (ORFs) for both transcripts was performed using primers specific for the extreme 5'- and 3'-ends of each *rem2* gene ORF (see Table 1) with the exception of the reverse primer, which was degenerate in 2 of its 24 base pairs. The cycling conditions for this standard PCR reaction were 94 °C (5 min) followed by 35 cycles of 94 °C (30 s), 60 °C (30 s), 72 °C (1 min), followed by 72 °C (3 min).

Products from all PCR were separated on agarose gels, visualized under UV light and the appropriate sized band was cut and cloned into TOPO pCR 2.1 (Invitrogen). Plasmid preparations were made from randomly selected positive colonies and sequenced using the dideoxy chain termination method with "Big Dye Terminator" (Applied Biosystems) and vector primers. Sequencing reactions were precipitated and resuspended in "Hi-Di Formamide with EDTA" (Applied Biosystems) and run on an ABI Prism 3730 automated sequencer (Applied Biosystems). Each product was cloned and sequenced a minimum of 20 times. Four to five additional sequences were also obtained by directly sequencing DNA from PCR reactions. The DNA was purified directly from the PCR reaction (if only one transcript was present in gel electrophoresis analysis) using the ExoSAP-It kit (USB/Affymetrix) following the manufacturers' instructions. If multiple transcripts were present in gel electrophoresis analysis, the appropriate sized band was cut and gel purified using the QIAquick Gel Extraction kit (QIAGEN) following the manufacturers' instructions. Direct sequencing of the isolated DNA was performed on an automated sequencer (ABI 3730) at the Arizona State University DNA Laboratory (Tempe, AZ, USA).

Sequence chromatogram files were trimmed for quality using phred (<http://www.phrap.org/phrap.docs/phred.html>), vector screened using cross match (<http://www.phrap.org/phrap.docs/phrap.html>) and analyzed locally using the Basic Local Alignment Search Tool (BLAST: <http://blast.ncbi.nlm.nih.gov/Blast.cgi>), specifically blastx against the NCBI nonredundant (nr) protein database and blastn against the NCBI nucleotide (nt) database. Nucleotide alignments were performed using an online ClustalW2 alignment program (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). Deduced amino acid sequences also used the ClustalW2 alignment program as well as manual alignments.

2.4 Gene expression analysis

Gene-specific primers were used in combination with isoform-specific probes used for real-time TaqMan® PCR (see Table 1). Real-time TaqMan® PCR was carried out following the manufacturer's instructions for the SYBR Green One-Step RT-PCR method (Applied Biosystems). TaqMan® probes were labeled with the FAM (6-carboxyfluorescein) fluorophore. A total of 250 ng of total RNA was used per reaction. Reverse transcription was carried out using MultiScribe Reverse Transcriptase (Applied Biosystems) and the generated cDNA was used as a template for PCR amplification. The cycling conditions were, for the reverse transcription, 48 °C (30 min), which was then followed by PCR consisting of 95 °C (10 min), then 40 cycles of 95 °C (15 s), and 60 °C (1 min). Reverse transcription and PCR amplification were performed on a DNA Engine Opticon®2 (BIO-RAD) thermal cycler and analyzed using Opticon® Monitor 3 software (BIO-RAD). For tissue specificity and CNS distribution, measurements were taken from five individual rainbow trout. Gene expression was normalized using a 101 bp fragment of the *elongation factor 1 alpha (eef1a1)* gene using gene-specific primers following the methods of Verleih et al. (2010; see Table 1). Expression of *eef1a1* was performed using real-time PCR following the manufacturer's instructions for the SYBR Green One-Step RT-PCR method (Applied Biosystems). Total RNA from rainbow trout brain tissue was used to produce standard curves (ranging from 15 to 2000 ng; in triplicate) which were used to compute expression values for all genes analyzed. Data are calibrated to the lowest expressing tissue (red muscle). The cycling conditions for *eef1a1* were as those for *rem2*. Statistical significance for overall expression pattern between the *rem2* genes was evaluated by two-way ANOVA ($P < 0.05$), while differences in expression levels among the individual tissues was evaluated by t-test ($P < 0.05$). Differential expression across tissues and CNS regions within a single *rem2* gene was evaluated by one-way ANOVA ($P < 0.05$) followed with Tukey's multiple comparison test ($P < 0.05$). All statistics were performed using GraphPad Prism software.

3. Results

3.1 Molecular cloning and sequencing of full-length *rem2* genes

The two full-length *rem2* genes were isolated by cDNA cloning of PCR products from 5'-RACE and 3'-RACE. The ORF of each gene was 912 bp and within the ORF, the two nucleotide sequences were identical in all but 72 base pairs (92% identity). From these nucleotide differences, 29 amino acid differences resulted (Fig. 1). BLAST searches (blastx) in the GenBank database resulted in deduced amino acid sequences of both genes having highest identity (64%) with that of the zebrafish Rem2 protein. Thus, the two genes might represent Rem2 isoforms and therefore, were named *O. mykiss rem2a* (GenBank accession number [JN175327](#)) and *O. mykiss rem2b* ([JN175328](#)). The full-length cDNA sequence of *rem2a* was 1459 bp, including a 107 bp 5' untranslated region (UTR), a 912 bp ORF, and a 426 bp 3' UTR (excluding the poly(A)⁺ tail). The full-length cDNA sequence of *rem2b* was 1186 bp, including an 87 bp 5' untranslated region (UTR), a 912 bp ORF, and an 174 bp 3' UTR (excluding the poly(A)⁺ tail). There was less identity between the UTRs than between the ORFs. The *rem2a* gene had both a longer 5' UTR (20 additional nucleotides) and a longer 3' UTR (239 additional nucleotides) than *rem2b*. Excluding gaps in the 5' UTR (86 positions), *rem2a* and *rem2b* shared 56% identity. In the 3' UTR, excluding gaps, there were 174 positions where both genes possessed nucleotides, sharing 79 % identity (excluding the poly(A)⁺ tail).

3.2 Deduced amino acid sequence alignment

The rainbow trout Rem2a and Rem2b proteins each consist of 304 deduced amino acids with 90.5% identity between the two based on our alignments (Fig. 2). The deduced amino

acid alignment indicates that the rainbow trout Rem2 proteins have highest identity (72%) with that of an unnamed protein of the pufferfish (*Tetraodon nigroviridis*), which had been identified in BLAST (tblastx) searches, followed closely by the zebrafish Rem2 protein (Rem2a, 61%; Rem2b, 62%). When aligned with all four of the mammalian RGK subfamily members, both rainbow trout isoforms aligned most closely with that of REM2 (43%), as compared to REM (36%), RAD (35%), or GEM (32%). The rainbow trout Rem2a and Rem2b proteins share conservation with the RAS superfamily G1, G3, G4, and G5 GTP binding sites, but like the RGK subfamily, lack the threonine residue of the G2 GTP binding domain that is conserved throughout the rest of the RAS superfamily (Thr³⁵ in H-RAS; Colicelli, 2004). Also, mammalian RGK subfamily members are distinct from the RAS superfamily in that they possess altered residues (Glu⁸⁴ in GEM, Pro⁸⁹ in REM and Pro¹⁰⁰ in RAD, and Ser¹²³ in REM2) of the residue equivalent to the highly conserved Gly¹² in H-RAS within the G1 domain (Colicelli, 2004; Correll et al. 2008). Consistent with this, rather than a glycine residue, both rainbow trout Rem2 isoforms possess an asparagine (Asn⁹⁹) in this position, as does the zebrafish Rem2 protein (Asn⁸³) and the unnamed pufferfish protein (Asn²⁰⁴). Furthermore, rainbow trout Rem2a and Rem2b share the conserved DXWE G3 motif with the rest of the RGK subfamily members, which is divergent from the DTAGQ motif found in the rest of the RAS superfamily. Finally, within the N-terminal extension of both rainbow trout Rem2 proteins, the amino acids Met²²Thr²³Leu²⁴ were manually aligned with the conserved amino acids Met¹Thr²Leu³ found in REM, RAD, and GEM as this may represent homology. One residue deviation from the conserved RAS superfamily G1–G5 binding motifs was observed in Rem2a, which possessed a threonine (Thr²²⁵) in a position typically occupied by an alanine (Ala²²⁵ of Rem2b) in the G5 motif.

3.3 Tissue specificity of rainbow trout rem2 genes

The overall pattern of expression for the two rainbow trout *rem2* genes was very similar, with the brain showing significantly greater levels of expression than any other tissue for both isoforms (Fig. 3). After the brain, the spleen consistently showed high levels of expression. This was followed by the kidney and bulbus arteriosus. The lowest levels of expression were typically seen in regions associated with the digestive system, as well as the red and white skeletal muscle. With the exception of the brain and head kidney, the *rem2a* gene showed greater levels of expression than that of the *rem2b* gene in every tissue, with significantly greater expression levels in the liver, kidney, intestine, stomach, spleen, ventricle, bulbus arteriosus, gill, white muscle and red muscle.

3.4 Central nervous system distribution of rainbow trout rem2 genes

The level of expression for the two *rem2* genes in the CNS was similar, with the cerebrum showing high levels of expression (Fig. 4). Along with the cerebrum, the olfactory bulb and the midbrain also had relatively high levels of expression. The cerebellum, hindbrain, and the spinal cord all had relatively low levels of *rem2* gene expression. In fact, for each *rem2* gene, the olfactory bulb, cerebrum, and midbrain all had significantly higher expression levels than the cerebellum and hindbrain. The cerebrum had significantly higher levels of both *rem2* genes than the spinal cord as well. The cerebellum consistently had the lowest levels of expression for both genes. There was a significantly higher level of relative expression of *rem2a* than *rem2b* in the spinal cord. Furthermore, though not significant, the *rem2a* mean levels of expression were consistently higher than *rem2b* with the exception of the olfactory bulb.

4. Discussion

4.1 Molecular cloning and sequencing of full-length rem2 genes

In this study, we isolated, cloned, and characterized two forms of the *rem2* gene, designated *O. mykiss rem2a* and *rem2b*, from the adult rainbow trout. The presence of Rem2 isoforms in rainbow trout is not unexpected as salmonids are well-known to possess isoforms implicated as the result of gene duplication (Moore et al., 1999; Doyon et al., 2003; Englesma et al., 2003; Richards et al., 2003; Koskinen et al., 2004; Gabillard et al., 2006; Quezada et al., 2006; Gharbi et al., 2007; Negler et al., 2007; Ostbye et al., 2007; Saito et al., 2007; Zakhartsev et al., 2007; Chondrou et al., 2008; Hansson and Hahn, 2008; Guo et al., 2009; Østergaard et al., 2009; Morash et al., 2010). REM2 was first described in mammals (Finlin et al., 2000), and has since been isolated in fish (Edel et al. 2010b). Isoforms of REM2 have been previously identified in mammals in the form of splice variants including a short form (Finlin et al., 2000), as well as a long form (Church et al., 2009). It is possible that trout *rem2a* and/or *rem2b* RNAs could be alternatively spliced. This subject merits further investigation. This study is the first report of Rem2 isoforms arising from gene duplication, rather than splice variants.

4.2 The deduced amino acid sequences of the rainbow trout Rem2 isoforms

Within the ORF, rainbow trout *rem2a* and *rem2b* genes are highly conserved at both the nucleotide and protein level. The only residue deviation from the conserved binding motifs of not only the RGK family, but the RAS superfamily as a whole, was with regard to Rem2a. Within the G5 motif of Rem2a, a threonine (Thr²²⁵) is in a position typically occupied by an alanine (Ala²²⁵ of Rem2B), though a threonine also resides in this position in RAS superfamily member RASL11A (Colicelli, 2004). This difference has been suggested to indicate that the universal switch mechanism of small GTPases may be structurally different for RASL11A and members of its subfamily (Louro et al., 2004). This may be the case for Rem2a as well. Additionally, zebrafish Rem2 also shows a deviation from this highly conserved alanine and instead, has a valine (Val²¹³). This is similar to members of the NKIRAS branch of the RAS superfamily (Colicelli, 2004). Therefore, the deviation seen in the Rem2a G5 motif, though uncommon, is not completely unique.

Like other members of the RGK subfamily of proteins, mammalian REM2 possesses N-terminal and C-terminal extensions that flank its RAS core (Finlin et al., 2000). It is speculated that the effects of mammalian REM2 upon Ca²⁺ channel kinetics is due to its extended N-terminus and C-terminus (Seu and Pitt, 2006). Thus, the N-terminal and C-terminal extensions are likely a source of other functional distinctions among RGK proteins. Both rainbow trout Rem2a and Rem2b possess the N-terminal and C-terminal extensions. As a result of the relatively large phylogenetic distance between species used in our alignment, ClustalW2 alignments overlooked certain biologically relevant regions (such as the 5' 14-3-3 protein binding site). Therefore, additional manual alignments were made to reflect this conservation. In past studies as well as this study, deduced amino acid alignments of the RGK subfamily N-terminal and C-terminal extensions show much less conservation between mammalian REM2 with the rest of the RGK subfamily (Colicelli, 2004; Seu and Pitt, 2006; Spingard et al., 2007). Our deduced amino acid alignments indicate large discrepancies between not only the vertebrate classes, but between different species within the same vertebrate classes as well. The mammalian REM2 N-terminal extension is unique compared to that of other RGK subfamily members in that it is extended with signal transduction binding sites, which likely allows REM2 to be a transcription regulator as is speculated in its ability to hold human embryonic stem cells in a state of pleuropotency by blocking p53 and cyclinD1 transcription (Edel et al., 2010a). The N-terminal extensions of the rainbow trout Rem2a and Rem2b isoforms appears to lack a large

portion of the mammalian REM2 N-terminal extension as there are only 29 amino acids in the rainbow trout isoforms within the first 63 amino acid residues of the mammalian REM2 protein (human Met¹ to Pro⁶³). This occurs just upstream of much more highly conserved regions. However, based on manual alignments, the rainbow trout Rem2 isoforms and the pufferfish unnamed protein do in fact indicate potential homology with mammalian REM2 within the first 29 amino acids (human REM2, Met¹ to Pro²⁹). Typically in Clustal alignments, Met¹¹ of mammalian REM2 (human Met¹¹) has been aligned to indicate conservation with the initial methionine (Met¹) of RAD, REM, and GEM (Seu and Pitt, 2006; Spingard et al., 2007). However, it may be, as indicated in our alignment, that the initial methionine of RAD, REM, and GEM (Met¹ of each), plus the following threonine (Thr² of each) and leucine (Leu³ of each) are not only conserved among all three of these proteins, but with both the rainbow trout Rem2 isoforms as well (rainbow trout Rem2a and Rem2b Met²², Thr²³, Leu²⁴; see Figure 2). The fact that mammalian REM2 lacks identity with these three highly conserved residues may indicate a derived distinction in function from both its homologs as well as its fellow RGK subfamily members. With regard to the C-terminal extension, a distinct extended region of mammalian REM2 (human REM2 Gly²⁸³-Gln²⁹⁰; Seu and Pitt, 2006) appears to be, for the most part, lacking in rainbow trout Rem2. However, a glycine residue in rainbow trout Rem2 (rainbow trout Rem2a and Rem2b Gly²⁴⁴) within this extended C-terminus region appears to be conserved. It is possible that this conserved glycine could be of functional significance. While this alignment is speculative, it does maintain a fairly conserved region between all fish Rem2 proteins and mammalian REM2 regarding the adjacent, downstream amino acids (from rainbow trout Rem2a and Rem2b Ser²⁴⁶-Gly²⁵¹) that encompass the Src homology 3 (SH3) binding motif (Spingard et al. 2007; see Figure 2). While the rainbow trout Rem2a and Rem2b proteins offer further insight into conservation within the distinct N-terminal and C-terminal extensions of REM2, further comparative studies, particularly involving additional species of amphibians, non-avian reptiles, and birds would offer even greater perspective into putative functional domains within these extensions.

4.3 Tissue specificity of rainbow trout rem2 genes

In mammals, *REM2* is unique among the RGK subfamily members in that it expressed at relatively high levels in neural tissue (Finlin et al., 2000; Becker et al., 2008). Consistent with this, early embryos of zebrafish also show high levels of *rem2* in neural tissue, and in fact, it is exclusively in the brain and eye (Edel et al., 2010b). The adult rainbow trout expressed both *rem2a* and *rem2b* in all tissues examined and is consistent with previous work in that relative expression of *rem2* in general, was significantly higher in the brain than every other tissue examined. The pattern of expression of both *rem2* genes was very similar except that *rem2a* was significantly higher in most tissues. Differential expression of isoforms has been observed in salmonid fish, specifically with somatostatin in rainbow trout (Moore et al., 1999), as well as myostatin in brook trout (Roberts and Goetz, 2001). It has been suggested that the different isoforms of myostatin possess different functional roles (Ostbye et al., 2007). Whether the observed expression differences between *rem2a* and *rem2b* in the different tissues represent functional differences in the rainbow trout is unknown and the subject for future examination.

Expression of *rem2a* and *rem2b* was detected in all tissues assayed. The detection of *rem2* in the bulbous arteriosus is the first description of *rem2* expression in this tissue, which mainly consists of connective tissue and smooth muscle (Heidal et al., 1997). Furthermore, this is the first description of *rem2* gene expression in the gills and head kidney as well. The spleen of the rainbow trout showed levels of *rem2a* and *rem2b* expression that were higher than all other tissues except the brain. The high expression of *rem2* in the spleen of the rainbow trout contrasts sharply with that of *rem2* expression in the spleen of mammals (Finlin et al., 2000;

Becker et al., 2008). In rats, *Rem2* is found at relatively high levels in the kidney (Finlin et al., 2000), which contrasts with the relatively low expression levels of the *rem2* genes in the rainbow trout kidney as well as that of the mouse from previous work (Becker et al., 2008). Finally, based on this study and that from previous work in rodents (Finlin et al., 2000; Becker et al., 2008), *Rem2* appears to be at low expression levels across vertebrates in tissues associated with the alimentary canal as well as in skeletal muscle, whereas there appear to be discrepancies between different species with regard to the liver and respiratory organs. If expression levels bear any reflection on functional Rem2 protein levels, the significance and role of Rem2 in the different tissues may be species-specific.

4.4 The expression of rainbow trout *rem2* genes in the CNS

The relative expression levels of rainbow trout *rem2a* and *rem2b* in the different brain regions were very similar with no significant differences between the two genes. The only significant expression difference between the two genes in the CNS occurred outside of the brain, in the spinal cord. These findings are the first such description of relative regional brain expression of *rem2* in an adult non-mammalian vertebrate as far as we know. In mice, *Rem2* does not appear to be ubiquitous, but it does exhibit strong expression levels in certain regions of the telencephalon (Becker et al., 2008). Expression of *rem2* has also been described in the brain of the zebrafish embryo at different stages of development (between 24 hours and 5 days) and was found to be expressed exclusively in the tectum (Edel et al., 2010b). These findings in zebrafish embryos contrast sharply with those of this study, where both *rem2* genes were expressed in the olfactory bulb, telencephalon, mesencephalon, cerebellum, hindbrain and spinal cord. Thus, there likely exists a developmental shift in *rem2* gene expression from embryo to adult in the fish brain. However, there exists the possibility for species-specific differences as well, or some combination of both. An examination of *rem2* regional gene expression in the adult zebrafish brain, as well as those of rainbow trout embryos, would offer significant insight into this question.

The question as to the role of Rem2 in the developing embryonic fish versus that of the adult fish is of particular interest due to the exceptional ability of adult fish, unlike adult mammals, to retain brain neurogenesis and regeneration capabilities (Zupanc, 1999; Zupanc and Zupanc, 2006; Alvarado and Tsonis, 2006; Chapouton et al., 2007). The adult teleost fish brain has many zones of new cell proliferation including the olfactory bulb, dorsal telencephalon, and regions of the diencephalon, mesencephalon, rhombencephalon, and cerebellum (Zupanc et al., 2005). These new cells have survival times up to nearly ¼ of the entire lifespan of the fish. Furthermore, in the cerebellum of adult brown ghost (*Apteronotus leptorhynchus*), new cells survive for nearly ½ of the animals' adult life span (Ott et al., 1997; Zupanc et al., 2006). Also observed in the brown ghost, those cells that do not reach their targets, about 50% of new cells, undergo apoptosis (Soutchek and Zupanc, 1996). It seems quite likely that the prevalence of *rem2* expression throughout the adult rainbow trout brain may reflect a possible role for Rem2 in the persistence of newly generated cells. The cerebellum of the adult rainbow trout showed extremely low relative expression of both *rem2a* and *rem2b*. In adult zebrafish, the cerebellum exhibits the greatest number of mitotic cells (Zupanc et al., 2005). The relatively high number of mitotic cells in the cerebellum appears to be consistent in teleost fish (Kranz and Richter, 1970; Zupanc and Horshke, 1995; Zikopoulos et al., 2000; Ekström et al., 2001). The low levels of *rem2* gene expression in the rainbow trout cerebellum could possibly point toward a regulatory role for Rem2 regarding mitotic activity in the adult fish brain. However, in mammals, REM2 holds human embryonic stem cells in a state of pluripotency and accelerates the cell cycle (Edel et al., 2010a). REM2 is also known to promote endothelial cell proliferation in angiogenesis (Bierings et al., 2008). Thus, the low levels of *rem2* gene expression we found in the rainbow trout cerebellum would seem to be inconsistent with the idea of Rem2 as having a

regulatory role regarding mitotic activity in the fish brain. However, newly generated cells in the adult zebrafish cerebellum also migrate long distances within the cerebellum, from the molecular layers to the granule cell layers, and differentiate (Zupanc et al., 2005). It is well-established that during cell differentiation, changes in dendritic morphology occur as synapses are formed with cells in the cerebellum, including granule cells (Duffy and Rakic, 1983). Knockdown of *Rem2* in cultured mammalian hippocampal neurons alters their dendritic morphology by increasing the number of dendritic branches (Ghiretti and Paradis, 2010). The low levels of *rem2* gene expression we found in the rainbow trout cerebellum could be reflective of the fact that low levels of *rem2* previously observed in the developing fish brain are associated with conditions of cell differentiation previously described (Edel et al., 2010b). Thus, the regional, differential expression of the *rem2* genes observed across brain regions may reflect overall regional differences in new cell proliferation, migration, and differentiation.

4.5 Conclusion

Among the different GTP-binding proteins, REM2 is unique due to its regulatory role in different neural pathways including neural development and synapse formation. In this study, we isolated and identified, for the first time, Rem2 in an adult teleost fish, and report the first known incidence of Rem2 isoforms that are the result of gene duplication, Rem2a and Rem2b. The pattern of tissue specificity of each of the rainbow trout *rem2* genes shows similarity to mammals, yet, there are several distinctions such as relatively high levels in the spleen and relatively low levels in the kidney. Furthermore, this is the first report of *rem2* in the gills of a fish, as well as the bulbus arteriosus and head kidney. Teleost fish, such as the rainbow trout, are recognized as unique models of adult neurogenesis and, as tetraploid organisms, they provide the opportunity to examine gene evolution, particularly if functional distinctions exist. The isolation and characterization of rainbow trout *rem2* will allow further investigation into understanding the role of this GTP-binding protein in CNS pathways relevant not only to neural development, but likely to adult vertebrate neurogenesis and brain repair as well.

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	5'		
rem2a	AATCATGTGGCTTTGAGGTAGAGCGCGGAGGAGTGAGTGGGAGGCGGGAACGCTCGGAGTAG-GTAGTGGATAGTCACGGGTG	93	
rem2b	-ATATA-----TCAGAGAGCGAGATTGGATAGGACATC-----CAGGAGGAC-----CGAATTACTGTACAGGAGGACACCTG-TGG	76	
rem2a	ATTTCACATTGACAGATG CAG ACA GAT GCG CTC ATA GAC ATT ATC ANT ATC GAG GAA ACA CTG GCT AAG TGT GAC	167	
rem2b	GGGAGAGAA--AGATG CAG ACA GAT GCG CTC AAA GAC AGT ATC AGT ATC GAG GAA ACA CTG GCT AAG TGT GAT	147	
	M Q/P T D A L I/K D I/S I N/S I E E T L A/V K C D	20	
rem2a	AGT ATG ACT CTG TCC AGT GCC CCA CCT TTA CGC AGA GGA AGT ACC CCT CTG CCA ATC AAA CAC CAG CTC AGA	239	
rem2b	AGT ATG ACT CTG TCC AGT GCA CCG CCT TTA CGC AGA GGA AGT ACC CCT CTG CCA ATC AAA CAC CAG CTC AGA	219	
	S M T L S S A P P L R R G S T P V/L P I K H Q C L R	44	
rem2a	CGA GAG GAA GCA GTG CAT GAT GAT TGT GAT TGG ACA TCA GGG TTG GCT GGA CCT TCT CCT CCT CCA ATC AGT	311	
rem2b	CGG GAA GAA GCA GTT CAT GAT GAT TGT GAT TGG ACA TCA GGG TTG GCT GGA CCT TCT CCT CCT CCA ATC AGT	291	
	R E E A V H D D C D W T S G L A G P S P P P P I S	68	
rem2a	TAC AGC AGC GCG AAG GAT GAG ACC ATC ACC TCA CGC GGA GAG GGG AGA CCC ACC TCT GAA GGG CCC TTT CGA	383	
rem2b	TTC AGC CCG GCG AAG GAT GAG ACC ATC ACC TCA CGC GGA GAG GGG AGA CCC ACC TCT GAA GGG CCC TTT CGA	363	
	Y/F S T/L A K D E T I A/T S A A/V E G R P P/S S E G P F F R	92	
rem2a	ATT GCC CTG CTG GGG CAA AAT GGG GTG GGC AAG TCA AGC CTT GCT ATC GCC TTG GCT GGA GAT TTG GAA AGA	455	
rem2b	ATT GCC CTG CTG GGT CAG AAT GGG GTG GGC AAG TCA AGC CTT GCT ATC GCC TTG GCT GGA GAT TTG GAT GGA	435	
	I A L L G G V G K S S/C L A I A L A G D L E/D R	116	
rem2a	ACT GCT TCT GTG GAT TCT GAA GGA GAG GGC TAT GTA CAG ACT GTT ACC GTG GAC GAG GAG GAC TGC ACC ATC	527	
rem2b	ACT GCA TCT GTG GAT TCT GAA GGG GAG GGC TAT GTG CAG ACT GTT ACC GTG GAC GAG GAG GAC TGC ACC ATC	507	
	T A S V D S E G D/E G Y V R/H T V T V D D E D C T I	140	
rem2a	CTT ATC TAC GAC AAC TGG AGG CAG GAC CTG TCT GGT CTG CAG TGT GAG GTG TGT GTG GTG TTC TCT GTC	599	
rem2b	CTT ATC TAC GAC AAC TGG AGG CAG GAC CTG TCT GAT CTG CAG TGT GAG GTG TGT GTG GTG TTC TCT GTC	579	
	L I Y D N W R Q D L S A/D L Q C E V C V L V F S V	164	
rem2a	ACG GAC AGG CGT AGT TTC CAC CGC ACC GCC CAA CTT CGC CTC CTC CTC AGG GAG ACG CAG CAA ACT CCC	671	
rem2b	ACG GAC GGG CGG AGT TTC CAC CGC ACC GCC CAA CTT CGC CTC CTC CTC AGG GAG ACG CAA CCG CAA ACT CCC	651	
	T D R R S F H R T A Q L R L L L R E T Q P Q T P	188	
rem2a	ATC ATC CTC GTG GGC AAC AAG AGT GAC CTG GTC CGT TCG CGG GAG GTC AGC ACT CAA GAG GCC TTC ACC AGT	743	
rem2b	ATC ATC CTC GTG GGC AAC AAG AGC GAC CTG GTC CGT TCA CGG GAG GTC AGC ACT CAA GAG GCC TTC ACC AGC	723	
	I I L V G N K S D L V R S R E V S A/T Q E A F/L T S	212	
rem2a	GCT ACT CTG TTT GAC TGT CTG TAT GTG GAA ATG TCA ACC TCT CTG GAC CAC CGC ACG GTG GAG CTC CTG GAG	815	
rem2b	GCT ACT CTG TTT GAC TGT CTA TAT GTA GAG ATG TCA ACC TCT CTG GAT CAC CGC ACG GGA GAG CTC CTG GAG	795	
	A T/S L F D C L Y V E M S T/A S L D H R T V/A E L L E	236	
rem2a	TGT GCT GTG AGG TTA GCC AGA GGC CTC AGT CCG GTG CCG CCA GGG GCC TAT GGG CAC GTC ATG GTC GGC GGC	887	
rem2b	TAT GCT GTG AGG TTA GCC AGG GGC CTC AGC CCG GTG CCG CCA GGG GCC TGT GGG CAC GAT ACT GTC GGC GGC	867	
	S/Y A V R L A R G L S P V P P G A Y/C G H V/D M A G G	260	
rem2a	CTG GAG AAC ATC ACC CAC CGG GCT AAG COT TTT CTC ACT AGC CTG GTC CCA CGC TAC CCC CGC GAG AGG GAA	959	
rem2b	CGG GAG AGC ATC ACC CAC CGT GCT AAG COT TTT CTC ACT AGC CTG GTC CCA CGC TAC CCC CGC GAG AGG GAA	939	
	L/R E N/S I T H R A K R F L A/T S L V P R Y P R E/D R E	284	
rem2a	CGA GAG GGG GGC AAG TTC TTT AGA CAG AAG TCC CGG TCC TGC CAC GAC CTG GGA GCC CTG TGA TCAGGGATGA	1032	
rem2b	CGA GAG GGG GGC AAG TTC TTT AGA CAG AAG TCC CGG TCC TGC CAT GAC CTG GGT GCC CTG TGA TCAGGGATGA	1012	
	R D/E G G K F F R Q K S R S C H D L G A L	304	
rem2a	GGGAGGATAGAAATGAGGGATGTATAGAAAGAGAGAGGGGTGGCTAGAGAGCGAGCAAGAGGGAATAGAAATGTAAGCAATTGCAAAATAGAGA	1126	
rem2b	TGGAGGG-----ATAGAAAGAGAGAGGGTGGCTAGAGAGAG-----AGAGGTGGCTAGAA-----AGA	1069	
rem2a	GAGGGGTTCAAAGAGGTAAATATGTGTGAAGAGAAAAGTGAATGTAAAGTGAGTGATGAAGAGGATACAGTAATGGGGATGACACATGGAT	1220	
rem2b	GAGGGGT-----	1076	
rem2a	GAGCAGAGAGAGCCAGAGAGAGAGATTAAGGAGCGATGACGTATCTGGGTGATTCATGTAGAAATGTTGTAGTAGAGAACATA	1314	
rem2b	-----GCCAGAAAGAGAGT-----GTTGCT-----AGAAA-----GAAAAAAA	1113	
rem2a	GAGAATAGAGATTAGCTTTGACCCCTGTAATAACAACCTAAGCATCATACTGCAGTAGTTTGACTGATTCCTCACTCCATCCCATGTG	1408	
rem2b	AAAAATAGATGTAGCATTTGCA-----AAATAGAG-----AG-----	1150	
rem2a	ATTTCATTGAGGGCCAAATAAAATACTCTGAGAACTCAAAAAAAAAAAAAA	1459	
rem2b	GGTTCAAAGAGG-----TAAATTAT-----GCCAAAAAAAAAAAAA	1186	
	3'		

Fig. 1.

Nucleotide and deduced amino acid sequences of rainbow trout *rem2a* and *rem2b*. The nucleotide sequences are aligned and shown in the 5' to 3' direction and numbered to the right. The deduced amino acid sequences are in the single letter amino acid code and numbered to the right in italics. Where amino acids differences occur due to codon differences, the Rem2a amino acid is listed first, followed by a slash (/), followed by the Rem2b amino acid. The start and stop codons are boxed. Nucleotides highlighted in gray (A) indicate where trout *rem2a* and *rem2b* differ. The location of the primers and probes used in gene expression assays are underlined.

Fig. 2. Amino acid sequence alignment of the rainbow trout Rem2a (GenBank accession number **JN175327**) and Rem2b (**JN175328**) isoforms. Included in the alignment are the pufferfish unnamed protein (**CAG10942.1**), zebrafish Rem2 (**NM_001123046**), human REM2 (**NP_775798.2**), human RAD (**NP_004156.1**), human REM (**AAC33132.1**), and human GEM (**NP_005252.1**). Shades of gray indicate the corresponding level of identity across sequences with the darker shades indicating increased levels identity (■ >50%, ▨ > 75%, □ = 100%). The arrow (▼) indicates where the trout isoforms differ. Consensus sequences for GTP-binding regions (G1–G5) and the conserved C7 sequence motif are indicated below the alignments (Finlin et al., 2000). The G3 consensus shown is that which is unique to the R GK subfamily. The binding sites for the 14-3-3 protein are indicated with an *asterisk* (Correll et al., 2009). Bold lines below the alignment indicate N-terminal and C-terminal extensions (Splingard et al., 2007). The solid boxes indicate the mammalian REM2 extended N-terminus and C-terminus following Seu and Pitt (2006). The dashed box indicates the SH3 (Src homology 3) binding motif (Splingard et al., 2007). Underlined methionines (M) in Human REM2 (M¹¹), Rem (M¹) and GEM (M¹) indicate where these amino acids have been previously aligned (Seu and Pitt, 2006; Splingard et al., 2006). Amino acids are numbered to the right. Percent identity of Rem2a and Rem2b with each sequence is indicated at the end of the alignment.

Fig. 3.

Tissue specificity of rainbow trout *rem2a* and *rem2b* using Real-Time PCR with TaqMan® probes. The overall relative gene expression profile in the different tissues for each isoform was similar (Two-way ANOVA; not significant). There was a significant difference between the two isoforms regarding overall relative expression among the tissues (Two-way ANOVA; $P < 0.0001$). There was a significant difference in relative expression between all tissues for both isoforms (Two-way ANOVA; $P < 0.0001$). Significantly greater expression of a particular isoform in a tissue is indicated by an *asterisk* (t-test, $P < 0.05$). Each isoform showed differential expression between the different tissues (One-way ANOVA; $P < 0.0001$) and the relative expression in the brain of each isoform (indicated by “a” for *rem2a* and “b” for *rem2b*) was significantly greater than its respective expression in all other tissues (Tukey’s multiple comparison; $P < 0.01$).

Fig. 4.

Regionalization of *rem2a* and *rem2b* in the rainbow trout CNS using Real-Time PCR with TaqMan® probes. The overall pattern of relative gene expression in the CNS of the rainbow trout brain for each isoform was similar (Two-way ANOVA; not significant). There was no significant expression difference between the isoforms among the different regions. There was a significant difference in relative expression between all regions of the CNS assayed for both isoforms (Two-way ANOVA; $P < 0.0001$). Significantly greater expression of a particular isoform in a region is indicated by an *asterisk* (t-test, $P < 0.05$). Each isoform showed differential expression in the CNS (One-way ANOVA; *rem2a* and *rem2b*; $P < 0.0002$). The relative expression of each isoform in the cerebrum (indicated by “a¹” for *rem2a* and “b¹” for *rem2b*) was significantly greater than its respective expression in the cerebellum, hindbrain, and spinal cord, and in the case of *rem2a*, the olfactory bulb as well (Tukey’s multiple comparison; $P < 0.05$). Both the midbrain and the olfactory bulb had greater relative expression levels than the cerebellum (indicated by “a²” for *rem2a* and “b²” for *rem2b*), except *rem2a* relative expression in the midbrain was also significantly greater than its expression in the hindbrain as well ($P < 0.05$).

Table 1

Primers and the assays in which they were used.

Assay (Usage)	Primer Sets	Primer sequence
Gene-Fishing™		
Arbitrary primer 12		5'-GTCTACCAGGCAITCGCTTCATXXXXXXACCCTGGGACG-3'
dT-Arbitrary Control Primer 2		5'-CTGTGAATGCTGCGACTACGATXXXXXX(T)15-3'
Nested RLM-RACE PCR (Obtain full-length sequences)		
	<i>rem2a</i> 3' RACE (forward-outer)	5'-GATTGGACATCAGGGTTGGCTGGA-3'
	<i>rem2a</i> 3' RACE (forward-inner)	5'-GTGTTTCTCTGTACACGGACAGGCGT-3'
	<i>rem2a</i> 5' RACE (reverse-outer)	5'-ACGCTGTCCGTGACAGAGAACAC-3'
	<i>rem2a</i> 5' RACE (reverse-inner)	5'-AGCCCTCTCCTTCAGAATCCACAGA-3'
	<i>rem2b</i> 3' RACE (forward-outer)	5'-CGACCTGGTCCGTTACAGT-3'
	<i>rem2b</i> 3' RACE (forward-inner)	5'-TCACCCGACGGCAGAGC-3'
	<i>rem2b</i> 5' RACE (reverse-outer)	5'-TGAAAGTGTGATAGTAGACTCTGTCCAGTGCA-3'
	<i>rem2b</i> 5' RACE (reverse-inner)	5'-CGACAGATGCGCTCAAAGACAGTATCAG-3'
PCR (Obtain full-length ORFs)		
	<i>rem2a</i> (forward)	5'-ATGCAGACAGATGCGCTCATAGACATTATCAA-3'
	<i>rem2a</i> (reverse)	5'-TGCCACGACCTGGGAGGCCCTGTGA-3'
	<i>rem2b</i> (forward)	5'-ATGCCGACAGATGCGCTCAAAGACAGTATCAG-3'
	<i>rem2b</i> (reverse)	5'-TGCCACGACCTGGGAGGCCCTGTGA-3'
TaqMan PCR		
Gene-specific primers		
	<i>rem2</i> (forward)	5'-CCTTGCTATCGCCTTGGCT-3'
	<i>rem2</i> (reverse)	5'-TCCTTATCTACGACAACTGGAGGC-3'
Probes		
	<i>rem2a</i>	5'-AGAACTGCTTCTGTGGATTCTGAAGGAGAT-3'
	<i>rem2b</i>	5'-CGAACTGCATCTGTGGAATCTGAAGGGGAG-3'
Reference gene		
	<i>eef1a1</i> (forward)	5'-TGATCTACAAGTGGGAGGCA-3'
	<i>eef1a1</i> (reverse)	5'-CAGCACCCAGGCATACTTGA-3'