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Localization of *rem2* in the central nervous system of the adult rainbow trout (*Oncorhynchus mykiss*)

Anna G. Downs, Katie R. Scholles, and David M. Hollis^{†,a}

Furman University, Greenville, SC, USA

Abstract

Rem2 is member of the RGK (Rem, Rad, and Gem/Kir) subfamily of the Ras superfamily of GTP binding proteins known to influence Ca^{2+} entry into the cell. In addition, Rem2, which is found at high levels in the vertebrate brain, is also implicated in cell proliferation and synapse formation. Though the specific, regional localization of Rem2 in the adult mammalian central nervous system has been well-described, such information is lacking in other vertebrates. We sought to localize *rem2* gene expression in the central nervous system. Because Rem2 is involved in neuronal processes where differences in the capacities between adults of the different vertebrate classes exist, we sought to localize the *rem2* gene in the central nervous system of an adult anamniotic vertebrate, the rainbow trout (*Oncorhynchus mykiss*). *In situ* hybridization using a digoxigenin (DIG)-labeled RNA probe was used to identify the regional distribution of *rem2* expression throughout the trout central nervous system, while real-time polymerase chain reaction (rtPCR) further supported these findings. Based on *in situ* hybridization, the regional distribution of *rem2* occurred within each major subdivision of the brain and included large populations of *rem2* expressing cells in the dorsal telencephalon of the cerebrum, the internal cellular layer of the olfactory bulb, and the optic tectum of the midbrain. In contrast, no *rem2* expressing cells were resolved within the cerebellum. These results were corroborated by rtPCR, where differential *rem2* expression occurred between the major subdivisions assayed with the highest levels being found in the cerebrum, while it was nearly absent in the cerebellum. These data indicate that *rem2* gene expression is broadly distributed and likely influences diverse functions in the adult fish central nervous system.

Keywords

rem2; rainbow trout; central nervous system; *in situ* hybridization

[†]Corresponding author. Tel: 864-294-2306; Fax: 864-294-2058.

^aCurrent address: Furman University, 3300 Poinsett Highway, Department of Biology, Greenville, SC 29613, USA. david.hollis@furman.edu

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1. Introduction

The protein Rem2 belongs to the RGK (Rem, Rad, and Gem/Kir) subfamily of small molecule GTPases of the Ras superfamily of GTP binding proteins. Like other RGK members, Rem2 is implicated in Ca^{2+} current modulation (Chen et al., 2005; Finlin et al., 2005; Seu and Pitt, 2006; Leyris et al., 2009; Yang et al., 2012), as well as changes in cytoskeletal reorganization (Béguin et al., 2005; Correll et al., 2008).

Rem2 has been well-characterized and appears conserved as it has been isolated in different classes of vertebrates including mammals and fish (Klein et al., 2002; Edel et al., 2010b; Hollis et al., 2012; DeRocher et al. 2014). Because Rem2 regulates voltage-gated calcium channels (VGCCs), it enables Ca^{2+} to influence neurotransmitter release. Thus, it also influences cell division, differentiation, migration, and death, of which, tremendous discrepancies between the mammalian and fish brain exist (Zupanc, 1999; Ferretti, 2011). Rem2, unlike other RGK members, is characterized by having high expression levels in neural tissue relative to other tissues, which is conserved between mammals and fish, as well as amphibians (Finlin et al., 2000; Hollis et al. 2012; DeRocher et al., 2014). However, comparative data regarding the specific regional distribution of *rem2* in the central nervous system between the vertebrate classes is lacking. In mammals, expression of *rem2* was described in the forebrain (Becker et al., 2008), however, recent work has shown that, not only does the distribution of *rem2* occur throughout the major subdivisions of the mammalian central nervous system, but there are profound differences in its level of expression between specific brain areas (Liput et al., 2016). While the distribution of *rem2* in the major subdivisions of the central nervous system has been examined in both developing and adult fish (Edel et al, 2010b; Hollis et al., 2012), as well as in an adult amphibian (DeRocher et al., 2014), the specific regional localization of *rem2* throughout the adult anamniotic vertebrate central nervous system has yet to be examined.

Rem2 is known to play a role in diverse functions in neural tissue including excitatory and inhibitory synapse development (Paradis et al., 2007; Ghiretti and Paradis, 2011), survival of embryonic stem cells (Edel et al., 2010a), and regulation of dendrite morphology (Ghiretti and Paradis, 2011; Ghiretti et al., 2014). Where such functions occur regionally under the influence of Rem2 throughout the adult central nervous system of fish is unknown, however, localization would identify specific areas of interest. Therefore, we sought to regionally localize *rem2* gene expression in the rainbow trout (*Oncorhynchus mykiss*) brain and spinal cord using *in situ* hybridization in addition to real-time PCR (rtPCR) to examine its sphere of influence in the adult fish central nervous system.

2. Material and methods

2.1 Animals

Animal husbandry, anesthesia, and sacrifice were all performed under the guidelines set by the Furman University Institutional Animal Care and Use Committee (IACUC). Adult rainbow trout were obtained from the South Carolina Department of Natural Resources Walhalla State Fish Hatchery (Mountain Rest, SC). Trout were housed in “living streams” (Frigid Units, Inc., Toledo, OH) with capacities of 530 operating liters (dimensions; L =

213cm, W = 61cm, H = 56cm) of de-chlorinated water, which was kept at 10°C and kept on a 12:12 light-dark cycle. Animals were maintained on a diet of trout chow (Goldfin Slow Sinking Pellets 5.0mm; Zeigler, Gardners, PA) 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. Brain tissue was collected, weighed, and immediately homogenized in QIAzol® Reagent (QIAGEN) for total RNA isolation using the QIAzol® RNeasy Lipid Tissue Mini Kit (QIAGEN) along with the RNase-Free DNase Set (QIAGEN) to remove genomic DNA. Whole brain tissue was collected for use in standard curve assays for rtPCR, as well as for the isolation of the trout *rem2* gene fragment to serve as a cRNA probe template for *in situ* hybridization. For the relative expression levels of the *rem2* gene in the major subdivisions of the trout central nervous system, brains were dissected from six individual animals and the olfactory bulb, cerebrum, diencephalon and midbrain, cerebellum, and hindbrain of each were isolated, as was a portion of the anterior region of the spinal cord.

2.3 Gene expression analysis

Gene-specific primers were used for rtPCR (Table 1). Primers were designed from the full-length trout *rem2* gene, of which there are currently two catalogued isoforms (accession no. JN175327 and JN175328) that share the same patterns of brain expression (Hollis et al. 2012). Thus, the primers were designed from regions of the isoforms that share 100% identity to ensure the amplification of both so as to identify total *rem2* expression. The isolated fragment (128bp) was within the open reading frame of both as well. The rtPCR was carried out following the manufacturer's instructions for the iTaQ Universal SYBR®Green One-Step kit reaction method (BioRad). A total of 60ng of total RNA was used per 12µl reaction (5ng/µl per reaction). All reactions were performed in triplicate. Reverse transcription was carried out using iScript Reverse Transcriptase (BioRad) and the generated cDNA was used as a template for PCR amplification. The cycling conditions were, for the reverse transcription, 50°C (10min), which was then followed by PCR consisting of 95°C (1min), then 40 cycles of 95°C (15sec), and 60°C (1min). Reverse transcription and PCR amplification were performed on an Eppendorf Mastercycler® ep realplex² thermal cycler. Gene expression was normalized using a 101bp fragment of the *elongation factor 1 alpha (eef1a1)* gene using gene-specific primers following the methods of Verleih et al. (2010; see Table 1). Standard curves used to compute expression values for both *rem2* and *eef1a1* were generated from total RNA of trout whole brain tissue with individual 12µl reactions, performed in triplicate, ranging from 7.8ng to 1000.0ng in total RNA (0.65ng/µl to 83.33ng/µl).

Statistical significance for differences in relative expression levels across the major subdivisions of the central nervous system was evaluated by One-Way ANOVA ($P < 0.05$) followed with Tukey's multiple comparison test ($P < 0.05$). Statistics were performed using GraphPad Prism software.

2.4 In situ hybridization

A *rem2* cDNA fragment of 408bp was isolated from cDNA of rainbow trout brain tissue using gene specific primers (see Table 1), which were designed from the full-length trout *rem2* gene isoforms from regions that shared 100% identity resulting in a heterogeneous probe that recognized both isoforms so as to identify all *rem2* regional localization. The isolated fragment was verified as belonging to the trout *rem2* gene via sequencing (see below). The cDNA fragment was transformed into a plasmid (pCR[®]II TOPO[®] 4.0; ThermoFisher Scientific[™]) and amplified in *E. coli* using the TOPO[®] TA Cloning[®] Kit for Sequencing (ThermoFisher Scientific[™]). Large scale plasmid-preps were performed by alkaline lysis using the PureLink[®]HiPure Plasmid DNA Maxiprep Kit (ThermoFisher Scientific[™], Waltham, MA, USA). From the plasmid preps, reactions were assembled for sequencing to validate the fragment as *rem2* 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) at the Arizona State University DNA Laboratory (Tempe, AZ). The plasmid was then linearized with either *Spe I* or *Not I* restriction enzymes. The linearized plasmid was used as a template in an *in vitro* transcription reaction to produce the digoxigenin (DIG)-labeled RNA probe using the RNA polymerases T7 or Sp6 to yield sense and anti-sense probes, respectively. *In vitro* transcription was performed using the DIG RNA Labeling Kit; Roche) and probe synthesis was verified on formaldehyde gel following the methods of Zimmerman et al. (2013). Probes were extracted with 3M Sodium Acetate (pH = 5.2) with 10mg/ml tRNA and ethanol precipitation (–80°C overnight) and then re-suspended in hybridization buffer (50% deionized formamide, 10% dextran sulfate, 5X SSC, 1X Denhardt’s solution, 2mM EDTA, and 100µg/ml calf thymus). Probe concentrations were determined using dot blot assay, also following the methods of Zimmerman et al. (2013). The *in situ* hybridization technique was based on the methods of Dijkman et al. (1995) and Zimmerman et al. (2013), with modifications for optimization for the trout brain tissue. Anesthetized animals were sacrificed by rapid decapitation and brains were removed, embedded in Histoprep Frozen Tissue Embedding Medium (ThermoFisher Scientific), and immediately stored at –80°C until sectioning. Whole brains were cut into frontal sections at a thickness of 20µm at –30°C using a cryostat. Sections were thaw-mounted (on a slide warmer at 37°C for 1min) on Superfrost Plus[®] positive charged microscope slides (Shandon, Inc.), and stored at –80°C until use. The mounted tissue slices were prepared for fixation and prehybridization washes by allowing them to thaw at room temperature for 2min. Tissue RNA was then baked to the tissue on a slide warmer at 50°C for 30sec. The tissue was then allowed to air dry for 20min at room temperature. Next, the tissue slices were delipidated in chloroform for 5min and allowed to air dry for 5min to allow the chloroform to evaporate before fixation. The tissue was then fixed for 7min in 4% paraformaldehyde (in 1X phosphate buffered saline; PBS) followed by one rinse in 1X PBS (pH = 7.2–7.4) for 3min then two rinses in 2X standard saline citrate (SSC, pH = 7.0) for 5min each. All rinses occurred on an orbital shaker (15–20rpm). For prehybridization, prewarmed (52°C) hybridization buffer was placed on the tissue (600µl/slide). Slides were then placed in a humidified chamber (Boekel Slide Moat) and incubated at 52°C for 1hr. After 1hr, the hybridization buffer was poured off and replaced with 300µl of hybridization solution, which was hybridization buffer warmed to

52°C plus either sense or anti-sense probe to a final concentration of 200ng/ml. Before being added to the hybridization buffer, probes were denatured for 10min at 80°C, then immediately placed on ice for 5min before being added to the hybridization buffer. After hybridization solution was placed on the slides, the slides were cover-slipped with Hybrislips™ (Grace Bio-Labs) and placed back in the humidified chamber for 20hr at 52°C. After 20hr, the Hybrislips™ were removed by placing the slides in Pyrex® dishes filled with 2X SSC (room temperature), which was gently agitated on an orbital shaker until the Hybrislips™ floated off (between 10–20min). The tissue was then placed in 2XSSC (52°C) for 5min followed by three subsequent rinses in 60% formamide/0.2X SSC (52°C). This was then followed by two rinses in 2X SSC (room temperature) for 5min each while gently agitated on an orbital shaker.

Immunological detection was preceded by rinsing the tissue in buffer 1 (100mM Tris-HCl, 150mM NaCl, pH = 7.5) for 5min followed by a 30min incubation in 2% blocking solution (buffer 1 with Blocking Reagent; Roche) at room temperature. The tissue was then incubated in antibody solution (anti- DIG-alkaline phosphatase Fab fragments; Roche) at 1:200 in 2% blocking solution for 2hr at room temperature. The tissue was then rinsed twice in buffer 1 for 5min followed by a buffer 3 (100mM Tris-HCl, 100mM NaCl, 50mM MgCl₂, pH = 9.5) for 10min. Finally, the signal was visualized by placing the tissue in 4-Nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP; Roche) in buffer 3 with 1mM levamisole for 16hr. After 16hr, the tissue was washed in distilled water for 5min and allowed to air dry for 2–3min before mounting coverslips with glycerol gelatin aqueous slide mounting medium (preheated to 58°C). The tissue sections were analyzed using a Nikon OptiPhot light microscope and microphotographs captured with a Jenoptik ProgResR Gryphax Subra HD USB3 digital camera (Jena, Germany). The microphotographs were analyzed using GRYPHAX V1.0.2.50 software (Jenoptik Optical Systems; Jena, Germany). Nomenclature used to refer to neuroanatomical regions was based on the work of Billard and Peter (1982), Piñuela et al. (1992a; 1992b), Vecino et al. (1992), Vetillard et al. (2002), Castro et al. (2003), Folgueira et al. (2004a; 2004b), and Kinoshita et al. (2006).

3. Results and discussion

Our results from both the rtPCR and the DIG *in situ* hybridization show that the *rem2* gene is found throughout much of the central nervous system of the adult rainbow trout. Despite being found throughout the central nervous system, rtPCR data showed that there is tremendous discrepancy in the relative expression levels of *rem2* between each the major subdivisions of the brain and the spinal cord (Fig. 1). The highest relative levels of *rem2* were located more anterior in the central nervous system, within the cerebrum, olfactory bulb, as well as the midbrain/diencephalon, however, the cerebrum was significantly higher than all other regions. This contrasted with the more posterior regions, where low levels of relative *rem2* expression were observed in the cerebellum, hindbrain and spinal cord. The cerebellum, in particular, was nearly lacking in detectable *rem2* expression levels. This pattern is consistent with the patterns of expression of *rem2* isoforms in the trout using Taqman probes (Hollis et al., 2012). The significantly high levels of *rem2* seen in the cerebrum, as well as the near absence of observed *rem2* expression in the cerebellum are consistent with the pattern seen in the brain of an amphibian (DeRocher et al., 2014). Thus,

the overall pattern of relative expression levels of *rem2* across the major subdivisions within the anamniotic central nervous system appears to be conserved.

The rtPCR results were corroborated with *in situ* hybridization. Based on *in situ* hybridization using the species-specific DIG-labeled RNA probe, *rem2* gene expression was widely distributed within the central nervous system of the trout and consistent within each animal assayed. Tissue incubated with the anti-sense DIG labeled probe showed populations of *rem2* cells (Fig. 2A–K), while tissue incubated with the sense DIG-labeled probe was devoid of any labeling (Fig. 2 Sense (+)). Positively labeled *rem2* cells were either found in distinct populations or in populations not confined by neuroanatomical boundaries both rostro-caudally and dorso-ventrally. The general distribution pattern of *rem2* mRNA-labeled cells in the trout central nervous system was higher population density and greater labeling intensity in the more rostral areas of the brain, such as the olfactory bulb, cerebrum, and midbrain, as opposed to smaller, more confined populations with less intense labeling of individual cells occurring in the more caudal regions of the hindbrain and spinal cord (Fig. 2A–K).

The specific localization of *rem2* mRNA showed very distinct, densely labeled cells in the granular layer of the olfactory bulb (gr) that occurred from the more rostral (Fig. 2A) to the more caudal parts of this region (Fig. 2B). Some of the most intensely, distinctly-labeled cells were observed in the dorsal region plus dorsolateral region of the dorsal telencephalic area (dd+dl–d), the medial area of the dorsal telencephalon (dm), and the central area of the dorsal telencephalon (dc) (Fig. 2C–E). The labeling of *rem2* mRNA expressing cells in the dorsal telencephalon persisted caudally into the posterior area of the dorsal telencephalon (dp) (Fig. 2F). In the ventral telencephalon, *rem2* mRNA-labeled cells also continued throughout the cerebrum with labeled cells occurring in the dorsal area of the ventral telencephalon (vd), the ventral area of the ventral telencephalon (vv), the intermediate region of the vv (vv–i), the lateral area of the ventral telencephalon (vl), and in the commissural nucleus of the ventral telencephalon (vc) (Fig. 2C–E). Like the dorsal telencephalon, individual *rem2* mRNA-labeled cells were distinct, though the intensity often appeared less than that of the cells of the dorsal telencephalon. In the diencephalon, cells of various intensity were observed in the parvocellular preoptic area (pp) (Fig. 2F). In the mesencephalon, the distributions of *rem2* mRNA-labeled cell populations were observed in the habenula (ha), optic tectum (ot), nucleus anterioris periventriculus (nap–v), and the nucleus recessus lateralis (nrl) (Figs. 2G–H). In the cerebellum, there were no *rem2* mRNA-labeled cells observed (Fig 2I). This was consistent with the rtPCR results, where the amplification of *rem2* within the cerebellum was nearly lacking. Thus *rem2*, though present in the cerebellum of the trout, is at such low levels that our *in situ* hybridization technique was unable to resolve any positively labeled cells. In the hindbrain, light *rem2* mRNA-labeling was observed lateral to Mauthner's fiber (fM) and in the medial longitudinal fasciculus (flm) (Fig. 2I), while more caudally, similar light labeling was observed in the nucleus fasciculi solitarii (nufsol (Fig. 2J). Finally, in the spinal cord, *rem2* mRNA-labeling was observed laterally to both Mauthner's fiber (fM) and in the medial longitudinal fasciculus (flm) (Fig. 2K).

These data are the first report of the specific regional localization of the *rem2* gene throughout the central nervous system of an adult anamniotic vertebrate. The high levels of *rem2* expression in areas of the forebrain of the rainbow trout is similar to that seen in the mouse (Liput et al. 2016). Like the mouse, where rtPCR data indicated the highest relative *rem2* expression levels in the striatum and nucleus accumbens, large populations of densely labeled *rem2* mRNA expressing cells were observed in the subpallial regions of the vd and vc. These regions are considered to be homologous to the striatal formations of mammals, with the vd in particular, evidenced to be a partial putative homolog to the nucleus accumbens (reviewed in O'Connell and Hofmann, 2011). Furthermore, *in situ* hybridization showed that, in addition to the vd and vc, the high levels of *rem2* expression seen in the cerebrum by way of rtPCR is also due to the high large population of *rem2* mRNA-labeled cells in regions homologous to the vertebrate medial pallium, or mammalian hippocampus (Ganz et al., 2015). This included the dp, dl-v, and dl-d (homologous to the dentate gyrus). In the mouse hippocampus, specifically hippocampal formation CA3, there is a very high level of *rem2* expression relative to other brain regions (Liput et al., 2016). Another contributing factor to the high levels of *rem2* expression seen in the rainbow trout cerebrum would be the large population of *rem2* mRNA-labeled cells observed in the dm, which is considered homologous to the mammalian amygdala (Ganz et al., 2015). In the mouse, the amygdala also has a significant level of *rem2* expression (Liput et al., 2016).

Though the regional distribution of *rem2* mRNA-labeled cells in the cerebrum appears conserved with the mammalian (mouse) brain, a notable difference was observed in the dc, which is considered homologous to the mammalian cortex (Ganz et al., 2015). In the rainbow trout, the dc possessed a large population of *rem2* mRNA-labeled cells, which were some of the most intensely labeled in the entire central nervous system. In contrast, the cortex of the mouse indicates an exceptionally low level of *rem2* expression (Liput et al., 2016). This discrepancy may either be due to different roles of *rem2* across vertebrates within the region or due to functional differences between the homologous regions. For example, unlike mammals, in adult fish the dc is known to possess cell proliferative activity (Zupanc et al., 2005; Zupanc, 2006). Because adult fish possess the ability to retain brain neurogenesis and regeneration capabilities (Zupanc, 1999; Alvarado and Tsonis, 2006; Zupanc and Zupanc, 2006; Chapouton et al., 2007), it is possible that the prevalence of *rem2* expression in the dc, as well as throughout much of the adult rainbow trout brain, may reflect a role for Rem2 in the persistence of newly generated cells. Rem2 is known to hold embryonic stem cells in a state of pleuropotency and accelerate the cell cycle (Edel et al., 2010a). It is also known to promote endothelial cell proliferation in angiogenesis (Bierings et al., 2008). Many *rem2* cell populations in the rainbow trout brain were found in known proliferation zones of other teleost fish (Zupanc et al., 2005; Zupanc, 2006). In these teleost species, proliferation zones where *rem2* was localized in the rainbow trout, include the internal cellular layer (granular layer) of the olfactory bulb, regions of the dorsal and ventral telencephalon, the parvocellular preoptic nucleus, habenula, and optic tectum. This may also include areas homologous to the mammalian reticular formation as *rem2* mRNA-labeled cells were found laterally to fM. Because Rem2 regulates VGCCs, specifically Ca_v1.2, Ca_v1.3 (Finlin et al., 2005; Pang et al. 2010), as well as Ca_v2.2 (Chen et al., 2005), it could potentially influence cell proliferation by controlling intracellular Ca²⁺ levels. For example,

Ca²⁺-dependent calpains have recently been found to block neural stem cell proliferation in the two known regions of cell proliferation in the mammalian brain, the subventricular zone and dentate gyrus (Machado et al. 2015). Thus, Rem2 could potentially regulate cell proliferative activity indirectly by influencing calpain protease activity.

Despite populations of *rem2* mRNA-labeled cells occurring in known regions of cell proliferation, there were two notable exceptions to this pattern, which was the lack of *rem2* mRNA-labeled cells in the glomerular layer of the olfactory bulb and the cerebellum. The glomerular layer is well-established as an area of cell proliferation in the adult teleost fish brain, including the rainbow trout (Alonso et al., 1989; Zupanc and Horschke, 1995; Byrd and Brunjes, 2001; Zupanc et al., 2005). Furthermore, the cerebellum has consistently been found to be the brain area richest in mitotic cells in adult teleost fish (Kranz and Richter, 1970; Zupanc and Horschke, 1995; Ekström et al., 2001; Zikopoulos et al., 2000; Zupanc et al., 2005). As discussed in Hollis et al. (2012), these data appear inconsistent with the idea of Rem2 as having a mitosis-enhancing role in the adult fish brain. But low Rem2 levels are also associated with conditions of cell differentiation (Edel et al., 2010b), as well as increases in dendritic branching (Ghiretti and Paradis, 2011). In the cerebellum, new cells not only differentiate (Zupanc et al., 2005), but form new synapses with changes in morphology (Duffy and Rakic, 1983). Therefore, the contrasting lack of *rem2* mRNA-labeled cells in the glomerular layer of the olfactory bulb and cerebellum versus other regions of known cell proliferation may reflect regional differences as to whether Rem2 has an intrinsic role in new cell proliferation or differentiation.

Comparatively, the lack of observable *rem2* mRNA-labeled cells in the rainbow trout cerebellum, in addition to its near lack expression via rtPCR, is consistent with another anamniotic vertebrate, the bullfrog (DeRocher et al. 2014). This is in sharp contrast to the mouse cerebellum, which has higher levels of relative *rem2* expression than the midbrain, brainstem, and spinal cord (Liput et al., 2016). As mentioned, the adult fish brain cerebellum is a region with high levels of cell proliferative activity, as well as cell migration and differentiation. Proliferative cell activity is also found in the cerebellum of adult amphibians (Raucci et al. 2006; D'Amico et al., 2013). The discrepancy in relative levels of *rem2* in the cerebellum between the anamniotes and the mouse may reflect either differences in cell proliferation capacity and/or related functions such as migration and differentiation.

As is the case with known proliferative regions, *rem2* mRNA-labeled cells were localized in the rainbow trout brain in regional patterns similar to certain neuronal systems with known relevance to Rem2 function. For example, *rem2* mRNA-labeling was observed where GABAergic cell populations are also distributed in the adult rainbow trout brain (Anglade et al., 1999). This includes regions homologous to the mammalian hippocampus from which Rem2 has been found to influence GABAergic neuron synapse development (Paradis et al., 2007). Further, a striking pattern similarity in the olfactory bulb occurs, where in the granular layer, *rem2* mRNA-labeled cells and GAD mRNA-expressing cells are both densely labeled and yet, noticeably absent from the glomerular layer. But like the absence of *rem2* mRNA-labeled cells in certain known regions of cell proliferation, there are also notable exceptions with known rainbow trout brain GABAergic regions as well, specifically the torus semicircularis and once again, the cerebellum. Therefore, while Rem2 may be involved

in inhibitory synapse development in the rainbow trout brain, its distribution may again reflect regional differences as to whether its role is ubiquitously intrinsic.

3.1 Conclusion

In the adult rainbow trout, the *rem2* gene is found throughout the central nervous system, but is differentially expressed with the highest expression levels being found in the cerebrum, which is attributable to large populations of *rem2* expressing cells in regions of the dorsal and ventral telencephalon. While this pattern is similar to mammals, discrepancies in relative expression levels of *rem2* between certain regions indicates that the influence of Rem2 within the central nervous system of different vertebrate groups likely varies. Rem2 has a diverse set of known functions, and based on its widespread localization in the rainbow trout brain, as well as its functional role in modulating calcium signal homeostasis, likely influences a diverse set of functions in the anamniotic vertebrate brain. The localization of *rem2* mRNA-labeled cell in the adult rainbow trout brain has revealed regions in which to investigate putative functional influences of Rem2 in the fish central nervous system.

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Abbreviations

ccb	corpus cerebelli
crcb	crista cerebellaris
dc	central area of the dorsal telencephalon
dd+dl-d	dorsal region plus dorsolateral region of the dorsal telencephalon
dl-v	ventrolateral region of the dorsal telencephalon
dm	medial area of the dorsal telencephalon
dp	posterior area of the dorsal telencephalon
flm	medial longitudinal fasciculus
fM	Mauthner's fiber
gl	glomerular layer of the olfactory bulb
gr	granular layer of the olfactory bulb
ha	habenula
ml	mitral layer of the olfactory bulb
nap-v	nucleus anterioris periventriculus

ndli	nucleus diffuses lobi inferioris
ndtl	nucleus diffuses tori lateralis
ne	nucleus entoptenduncularis
nlt	nucleus lateralis tuberi
npo-m	nucleus preopticus pars magnocellularis
npo-p	nucleus preopticus pars parvicellularis
npt	nucleus posterioris tuberi
nvm	nucleus ventromedialis thalami
nrl	nucleus recessus lateralis
nufsol	nucleus fasciculi solitarii
ot	optic tectum
pp	parvocellular preoptic nucleus
tbc	tractus tectobulbaris rectus
teg	tegmentum
ts	torus semicircularis
vcb	valvula cerebelli
vc	commissural nucleus of the ventral telencephalon
vd	dorsal area of the ventral telencephalon
vl	lateral area of the ventral telencephalon
vv	ventral area of the ventral telencephalon
vv-i	intermediate region of the vv
vv-v	ventral region of the vv

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Highlights

- The *rem2* gene is differentially expressed between brain subdivisions of the trout.
- Large populations of *rem2* labeled cells are in the dorsal and ventral telencephalon.
- Discrepancies exist in *rem2* brain cell localization between the trout and mammals.

Figure 1.

Regionalization of relative *rem2* expression in the trout CNS using rtPCR. The schematic of the trout brain in dorsal view below the figure indicates each region assayed. The trout *rem2* gene showed differential expression in the central nervous system (One-way ANOVA; $P < 0.0001$). The relative expression of *rem2* in the cerebrum was significantly greater than its expression in all other areas assayed (* = Tukey's multiple comparison; $P < 0.001$).

Abbreviations: OB = olfactory bulb, CR = cerebrum, DI= diencephalon, MB= midbrain, CB= cerebellum, HB = hindbrain, SC= spinal cord.

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Figure 2.

Representative photomicrographs depicting the distribution of *rem2* *in situ* hybridization labeling in the brain of the adult trout (n = 4). Brain schematic at top indicates corresponding level of each photomicrograph. **Top Panel, Sense (+):** Photomicrographs showing no labeling of cells in the cerebrum (Level E; see brain schematic) from a brain tissue section incubated with the *rem2* sense (+) DIG-labeled riboprobe. **A–J Anti-Sense (–):** Photomicrographs of labeled cells in brain tissue sections incubated with the *rem2* anti-sense (–) DIG-labeled riboprobe. On the left, low magnification (25×) photomicrographs of entire right-side frontal sections (ventral at bottom) of brain level mirrored by line drawings indicating major neuroanatomical areas. Boxes in low magnification photomicrographs indicate region with intermediate magnification (100×) in the box to the immediate right. Boxes in intermediate magnification photomicrographs indicate region with high magnification (400×) in the box to the far right. Scale bars for each panel are located below each photomicrograph. The region of the photomicrograph is indicated by abbreviations in each box. Abbreviations accompanied by arrows indicate location of labeling relative to that region with arrows pointing left indicating the region as being medial to the labeling and arrows pointing right indicating the region as being lateral to the labeling.

Table 1

Primers and the assays in which they were used.

Assay (Usage)	Primer sets	Primer sequences
rtPCR (Relative gene expression levels)		
	<i>rem2</i> (128bp)	
	<i>Gene specific primer (forward)</i>	5' – CCTTGCTATCGCCTTGGCT – 3'
	<i>Gene specific primer (reverse)</i>	5' – GCCTCCAGTTGTCGTAGATAAGGA – 3'
	<i>eef1a1</i> (101bp)*	
	<i>Gene specific primer (forward)</i>	5' – TGATCTACAAGTGCGGAGGCA – 3'
	<i>Gene specific primer (reverse)</i>	5' – CAGCACCCAGGCATACTTGA – 3'
End point PCR (Isolate DIG probe template)		
	<i>rem2</i> (408bp)	
	<i>Gene specific primer (forward)</i>	5' – TGGACCTTCTCCTCCTCCAATCAG – 3'
	<i>Gene specific primer (reverse)</i>	5' – CTCTTGTTGCCCACGAGGATGATG – 3'

* From Verleih et al. (2010)