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Cloning and Characterization of Glutamate Receptor Subunit 4 (GluA4) and Its Alternatively Spliced Isoforms in Turtle Brain

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Abstract

Ionotropic glutamate receptors sensitive to α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), GluAs, play an important role in neural development, synaptic plasticity, and neurodegeneration. Previous studies using an *in vitro* model of eyeblink classical conditioning in pond turtles suggested that acquisition of conditioning is associated with synaptic delivery of AMPA receptors containing GluA4 subunits. However, sequences of the GluA4 subunit, expression profile, and its alternatively spliced isoforms in turtle brain have not been previously determined. The sequence and domain structure of turtle GluA4 (tGluA4) and its splice variants was characterized. We found ten isoforms of tGluA4 including several previously unidentified truncated variants. Analysis of the nucleotide sequences of tGluA4 *flip/flop*, tGluA4c *flip/flop* and tGluA4s showed they are highly similar to known isoforms of the GluA4 subunit identified in chick. Examination of the relative abundance of mRNA expression for the tGluA4 variants showed that the *flip* and *flop* versions of tGluA4 and tGluA4c, and a novel truncated variant, tGluA4trc1, which is also expressed as protein, are major forms in the adult turtle brain. Identification of these alternatively spliced isoforms of tGluA4 will provide a unique opportunity to assess their role in synaptic plasticity through the application of short interfering RNAs (siRNAs).

Keywords

AMPA receptor; GluA4; turtle; *flip/flop* splice variants; alternative splicing

Introduction

Glutamate receptors that respond to AMPA (GluAs) underlie excitatory neurotransmission in the brain and play an important role in neural development, synaptic plasticity, and neurodegeneration. This class of glutamate receptors, AMPA receptors (AMPA receptors), contributes to fast synaptic transmission and in response to synaptic activity can be highly mobile through protein trafficking mechanisms. AMPARs consist of four subunits, designated GluA1–4, which combine to form tetramers (Derkach et al. 2007; Collingridge et al. 2009). The subunit composition of AMPARs varies in different brain regions but

generally they combine to form heteromers whose subunit composition confers specific functional properties to the receptor. Previous studies using an *in vitro* model of eyeblink classical conditioning in turtles suggested that acquisition of conditioned responses (CRs) is associated with synaptic insertion of AMPARs containing GluA4 subunits (Keifer 2001; Zheng and Keifer 2008; Zheng and Keifer 2009). In order to further investigate the role of GluA4 in conditioning we characterized the sequence and structure of the turtle GluA4 subunit and its alternatively spliced isoforms.

The typical AMPAR subunit is composed of about 900 amino acids and has a molecular weight of ~105 kDa. The GluA1–4 subunits share 68–74% amino acid sequence identity (Madden 2002). Standard GluA subunit structure consists of an amino (N)-terminal domain, a ligand-binding domain (LBD), four hydrophobic membrane-embedded domains, three of which are transmembrane domains (TM1-TM3), a fourth hydrophobic domain (M2) that forms a re-entrant pore loop, and a carboxy (C)-terminal domain (Hollmann et al. 1994; Kuusinen et al. 1995). The N-terminal domain is homologous to the leucine/isoleucine/valine-binding protein (LIVBP), one of the bacterial periplasmic binding proteins, and is designated as a LIVBP-like domain (Greger et al. 2007). The LBD is homologous to the glutamine binding protein QBP (Madden 2002) and is divided into S1 and S2 segments by a transmembrane component. The S1-S2 ligand-binding domain is formed by two sequences that share structural similarity with the glutamine-binding protein (Nakanishi et al. 1990; Hsiao et al. 1996; Armstrong et al. 1998; Madden 2002). The C-terminal part of S2 is not directly involved in agonist binding and, due to alternative RNA splicing, is expressed in two forms, *flip* and *flop*, that differ by only a few amino acids but which results in receptors with different desensitization and endoplasmic reticulum export kinetics (Sommer et al. 1990; Mosbacher et al. 1994; Audinat et al. 1996; Coleman et al. 2006). Additional functional diversity of AMPARs is provided by alternative splicing and by mRNA editing (Sommer et al. 1990, 1991). It has been shown that the different spliced and edited forms are selectively expressed across brain regions and developmental stages (Sommer et al. 1990; Monyer et al. 1991; Ravindranathan et al. 1997; Kawahara et al. 2004).

Cloning of the chick GluA4 AMPAR subunit has demonstrated conservation of *flip/flop* alternative splice variants with those of mammalian GluA4. Conservation of post-transcriptional modifications such as RNA editing (R/G sites) just before the *flip/flop* region was also shown (Ravindranathan et al. 1996, 1997). In addition, GluA4c, GluA4d and GluA4s splice variants were characterized in chick and GluA4c in mammals (Ravindranathan et al. 1997; Kawahara et al. 2004). In chick, GluA4c and GluA4d have 113 and 184 bp inserts in the C-terminus, respectively, whereas GluA4s is a shortened form that lacks the nominal third transmembrane domain as well as the *flip/flop* domains and shares a common C-terminal region with GluA4 (Ravindranathan et al. 1996, 1997). To date, there are at least seven identified splice variants of GluA4 expressed in chick brain (*flip* and *flop* forms of GluA4, GluA4c, GluA4d, and GluA4s which does not contain a *flip/flop* region). Although homologs of GluA4d and GluA4s have not been reported in mammalian brain, GluA4c *flop* has been described and there is also evidence for the existence of GluA4c *flip* (Gallo et al. 1992). In the present study, we identified and characterized the turtle GluA4 (tGluA4) AMPAR subunit and its alternatively spliced isoforms from brain tissue. Our

previous work suggests that synaptic incorporation of tGluA4-containing AMPAR subunits underlies acquisition of learned responses using an *in vitro* model of eyeblink classical conditioning (Keifer 2001; Zheng and Keifer 2009; Keifer et al. 2009). Molecular characterization of tGluA4 will allow selective manipulation of this subunit to assess its role in learning. Here, ten distinct isoforms of tGluA4 were identified. Analysis of the nucleotide sequences of tGluA4 *flip/flop* and tGluA4c *flip/flop* showed that they are highly similar to chick GluA4 subunits (94% identity) and that the alternatively spliced *flip/flop* exons are conserved. tGluA4 also appears to have potential sites for phosphorylation and palmitoylation similar to GluA4 subunits characterized in other species. In addition, we also identified a tGluA4s isoform, and several previously undescribed truncated variants of the tGluA4 subunit. The expression profile of tGluA4 mRNA and its alternatively spliced isoforms showed that the *flip* and *flop* versions of both tGluA4 and tGluA4c, and a novel truncated variant, tGluA4trc1, which is also expressed as protein, are major forms found in the adult turtle brain. In addition to providing information about AMPAR splice variants for comparison across species, these data will be used to generate short interfering RNAs (siRNAs) against the tGluA4 AMPAR isoforms to assess their specific role in synaptic plasticity.

Materials and Methods

RT-PCR

Freshwater pond turtles, *Pseudemys scripta elegans*, obtained from commercial suppliers were anesthetized by hypothermia and decapitated. Protocols involving the use of animals complied with the guidelines of the National Institutes of Health and the Institutional Animal Care and Use Committee. The brain stem was transected at the levels of the trochlear and glossopharyngeal nerves and the cerebellum was removed as described previously (Zheng and Keifer 2009). Therefore the preparation consisted of only the pons with the cerebellar circuitry removed. Prior to RNA isolation, brain stem preparations underwent pseudoconditioning using unpaired stimuli as described previously (Keifer, 2001; Zheng and Keifer 2008; Zheng and Keifer 2009). Total RNA was isolated from turtle brain stems using the RNeasy Mini kits (Qiagen). During the process of isolation, samples were treated by RNase-free DNase (Qiagen) to digest DNA contamination of the samples according to the manufacturer's protocol. RT-PCR was performed with the Strata Script One-Tube RT-PCR System (Stratagene). The following primers were used to amplify the coding sequences of tGluA4: forward primer, 5'-GCAAAAGAGAAGATG AGGATWATTTSC-3'; reverse primer, 5'-TTATGGTAGGTC CGATGCAATKACAG-3. PCR fragments were purified from agarose gels with a Zymoclean Gel DNA Recovery kit (Zymo Research), cloned into the pGEM-T Easy Vector (Promega) and sequenced (Iowa State University Sequencing Facility, Ames, IA). RT-PCR was also performed to determine the complete sequences of truncated variants of tGluA4. Different pairs of primers were used in these reactions; one of the primers was specific to the end of the truncated version (tGluA4tr) and the other primer was specific to the end of other known variants of tGluA4 (Table 2). Products of the PCR analysis were resolved on agarose gels and photographed under UV illumination.

Restriction enzyme analysis for *flip* and *flop* isoforms of tGluA4

RT-PCR was employed to amplify the region between TM2 and the C-terminus of tGluA4 with the following primers: 5'-TGGATTCGAAAGGCTATGG-3' and reverse 5'-TTATGGTAGGTCCGATGCAATKACAG-3'. HpaI digestion of PCR products was performed under standard conditions overnight using a final concentration of 5 units HpaI in a total reaction volume of 20 µl. The resulting fragments were resolved on agarose gels and photographed.

RNA ligase mediated rapid amplification of cDNA ends (RLM-RACE)

RLM-RACE reactions were performed with the FirstChoice RLM-RACE kit (Ambion). This kit is designed to amplify cDNA only from full-length, capped mRNA. Treatment of total RNA by calf intestine alkaline phosphatase (CIP) allows avoiding amplification of uncapped RNA or genomic DNA. Nested primers 5'-AGATGTGTACTTTGGAGGGCTGTCA-3' and 5'-CTTCTTCCAGCGCTGCATGAGTTTAGT-3' were used for 5' RLM-RACE. For 3' RLM-RACE, nested primers 5'-GATGGGGTACTTGTGATGGCTGAA-3' and 5'-ACCATGGGGCCAGGGGATTGA-3' were used (Figs. 3-5).

Confirmation of amino acid replacements by PCR with AccuPrime Pfx DNA Polymerase

Template cDNA was synthesized by the SuperScript First-Strand synthesis system (Invitrogen) using random primers and 5 µg of total RNA. The region of interest was amplified with the following primers: forward 5'-TGGATTCGAAAGGCTATGG-3' and reverse 5'-TTATGGTAGGTCCGATGCAATKACAG-3'. To minimize PCR errors, reactions were performed using AccuPrime Pfx DNA Polymerase (Invitrogen) which has proofreading 3' to 5' exonuclease activity. PCR reactions were performed according to manufacturer protocol.

Sequence comparison and deposition

Nucleotide sequences of tGluA4 isoforms were aligned and assembled. Sequences of tGluA4 and its isoforms were compared with sequences of *Gallus gallus* GluA4 *flip*, GluA4 *flop*, GluA4c, GluA4s, GluA4d variants and mouse GluA4 *flip* and *flop* variants (GenBank numbers: NM_001113186.1, NM_205214.2, U65992, U65991.1, U65993.1, NM_019691, NM_001113180, respectively). Nucleotide sequences of tGluA4 and its isoforms were translated into amino acid sequences and compared with *Gallus gallus* GluA4 *flip*, GluA4 *flop*, GluA4c, GluA4s, GluA4d and also with human, mouse and rat GluA4 isoform 3 precursors (GenBank numbers: NP_001106657.1, NP_990545.1, AAC34247.1, AAC34246.1, AAC34248.1, NP_001106283, NP_001106652, NP_001106656, respectively). Alignment and comparison of sequences were performed by the DNASTAR software package. Sequences for tGluA4 variants described here were deposited in GenBank under accession numbers: HM209319, HM209320, HM209321, HM209322, GU079942, GU079943, GU079944, GU079945, GU079946, and GU079947 for full length cDNA of tGluA4*flip*, tGluA4*flop*, tGluA4c*flip* and tGluA4c*flop*, tGluA4s, tGluA4trc1-3 and tGluA4trn1-2 transcripts, respectively.

Real-Time RT-PCR

Total RNA was isolated from the turtle brain stem using the TRIzol reagent (Invitrogen) and purified by the RNeasy Mini kit (Qiagen). During purification samples were treated by RNase-free DNase (Qiagen) as described above. Real-time RT-PCR was performed using 50 ng total RNA per reaction. RNA was combined with primer/probe sets and TaqMan Gold RT-PCR Master Mix (Applied Biosystems, Inc., Foster City, CA) which contains MultiScribe Reverse Transcriptase and RNase Inhibitor. Gene-specific primers and probes were created for *P. scripta elegans* to total tGluA4, tGluA4 *flip*, tGluA4 *flop*, tGluA4c *flip*, tGluA4c *flop*, tGluA4s, tGluA4trc1-trc3, tGluA4trn1-trn2, and actin (Table 3), using the Primer Express Software (Applied Biosystems). Target specificity of each primer set was confirmed by performing real-time RT-PCR reactions with clones of all identified tGluA4 variants. Each primer/probe set was specific only for its target tGluA4 variant. Efficiency of reactions for each set of primers was close to 100% (actin 96.5%, tGluA4 98.9%, tGluA4 *flip* 97.6%, tGluA4 *flop* 97.6%, tGluA4c *flip* 99.4%, tGluA4c *flop* 96.2%, tGluA4s 94.9, tGluA4trc1 98.03%), except for the tGluA4trc2-3 and tGluA4trn1-2 variants which were expressed at very low levels. Efficiency of reactions was measured using the CT slope method. Briefly, serial dilutions of samples were generated and real-time RT-PCR reactions were performed on each dilution. The CT values were then plotted versus the log of the dilution and a linear regression was performed. Efficiency = $(10^{-1/\text{slope}} - 1) \times 100\%$ (Pfaffl 2001). Real-time RT-PCR assays were run on an ABI PRISM 7000 analyzer (Applied Biosystems). The profile consisted of one cycle at 48 °C for 30 min and 95 °C for 10 min, followed by 55 cycles at 95 °C for 15 s and 60 °C for 1 min. All reactions were performed twice. Real-time RT-PCR data were normalized to turtle actin and analyzed by the comparative CT method. Samples were confirmed to be free of DNA contamination by performing reactions without reverse transcriptase. Total RNA from five animals was used. Data were analyzed using StatView software by ANOVA.

Expression of recombinant tGluA4trc1 protein and Western blot analysis

Human neuroblastoma SH-SY5Y cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 ug/ml streptomycin at 37°C and 5% CO₂ atmosphere. Sequences corresponding to full-length tGluA4trc1 were inserted into a pcDNA3.1/V5-HisTOPO TA expression vector and cells at 70% confluence were transfected with 15 ug of vector. Plasmids (15 ug) were diluted in 100 ul of Opti-MEM I Reduced Serum Medium and transfection of cells was performed using Lipofectamine LTX Reagent (25 ul; Invitrogen). After 48 h incubation, cells were harvested. Protease inhibitors were added to the cell lysates to a final concentration of 10 ug/ml leupeptin and 0.4 mM PMSF. Lysates of SH-SY5Y cells transfected with a tGluA4trc1 expression plasmid (containing recombinant tGluA4trc1) were used as a positive control for Western blot analysis. Lysates of SH-SY5Y cells transfected with an empty plasmid or untransfected SH-SY5Y cells were used as negative controls. Brain stem preparations were homogenized in NP-40 lysis buffer (150 mM NaCl₂, 1% NP-40, 50 mM Tris, pH 8). Brain stem lysates (50 ug) or control lysates (50 ug) were subjected to SDS/PAGE followed by Western blot analysis using goat polyclonal antibodies against the N-terminus of GluA4 (Abnova; cat# PAB11410).

Results

Identification of tGluA4, tGluA4c and their flip and flop variants

To identify coding sequences of turtle GluA4 we designed degenerated primers based on the sequences of known GluA4 subunits from mammals and birds. Following RT-PCR we obtained a 2707 bp PCR product that was cloned and sequenced. Analysis of this sequence by BLAST showed that the *flop* isoform of tGluA4 was identified and is 94% identical to the chick GluA4 *flop* variant as shown in Fig. 1 and Table 1. Analysis of mouse and chick GluA4 *flip/flop* and tGluA4 *flop* showed that the *flop* variant has a cleavage site for the HpaI restriction endonuclease while the *flip* variant does not (Fig. 1). A schematic diagram of the predicted exon structure of tGluA4*flip* mRNA is also shown (Fig. 2) and is compared to mouse GluA4*flip* mRNA sequences. The presence of the HpaI cleavage site for tGluA4*flop* gave us the opportunity to identify turtle GluA4, GluA4c, GluA4d and their *flip* and *flop* variants by amplification of the region between TM2 and the C-terminus followed by digestion of PCR products by HpaI. Restriction analysis showed that both tGluA4 and tGluA4c isoforms were expressed as *flip* and *flop* variants in turtle brain (Fig. 3). However, no evidence for the existence of tGluA4d in turtle brain was found. PCR products specific to these variants were cloned and sequenced, and analysis confirmed that the *flip* and *flop* variants of tGluA4 and tGluA4c were identified. tGluR4 *flip/flop* and tGluR4c *flip/flop* have > 93% identity with chick (Table 1). Similar to mammals and chick, tGluA4c mRNA has a 113 bp insert containing a stop codon resulting in a shortened C-terminus (Fig. 4). Comparison of *flip* and *flop* variants of tGluA4 and tGluA4c also showed that tGluA4c contains an additional exon compared to tGluA4 (Fig. 2, 4), similar to chick and human (Ravindranathan et al. 1997; Kawahara et al. 2004). Since the tGluA4 clones are cDNA clones representing the mature mRNA, we can not verify that the glycine present in the R/G site of tGluA4 is the result of RNA editing.

Identification tGluA4s and truncated tGluA4 (tGluA4tr) variants

In order to predict the splice sites of tGluA4 we used the NetGene2 server (<http://www.cbs.dtu.dk/services/NetGene2/>) to analyze the nucleotide sequence of tGluA4 cDNA. Analysis showed the possibility of additional variants of tGluA4. To identify all variants we designed primers and performed both 5' - and 3' - ligase-mediated rapid amplification of cDNA ends (RLM-RACE; Figs. 4, 5). Products of 5' - and 3' - RLM-RACE were cloned and sequenced. Analysis of these sequences showed that a tGluA4s isoform and two groups of truncated variants of tGluA4 (tGluA4tr) were identified. tGluA4s lacks the *flip/flop* exon number 15 and exon 16 (Fig. 2) immediately following the *flip/flop* exon (exon structure of tGluA4 was assigned by comparison to mouse GluA4). tGluA4s also contains a novel 3' terminal exon compared to tGluA4 (Fig. 4). For the truncated tGluA4 variants, one group is truncated on the 3' terminus (tGluA4trc) and other on the 5' terminus (tGluA4trn). Three transcripts were identified for tGluA4trc variants (tGluA4trc1-3) and two for tGluA4trn variants (tGluA4trn1-2). All of the tGluA4tr variants contain start and stop codons, polyadenylation signals AAUAAA or AUUAAA, and poly-A tails as indicated in Fig. 5. However, we initially sequenced only the 5' and 3' ends of these truncated variants and were unable to predict whether these isoforms were truncated versions of tGluA4, tGluA4c or tGluA4s. To resolve this issue, RT-PCR reactions were performed to determine the

complete sequences of these truncated variants (Fig. 6; Table 2). PCR products of the predicted length were observed for tGluA4trc1-3 indicating that all tGluA4trc are truncated variants of tGluA4 (Fig. 6). Strikingly, we found that the tGluA4trn are truncated isoforms of tGluA4s (Fig. 6; see also Fig. 8).

The cDNA sequences of the truncated variants of tGluA4 were analyzed. This showed that tGluA4trn1-2 variants are missing exons 2-5 (Fig. 2) at the 5' end and contain a novel 5' terminal exon compared to tGluA4 (Fig. 5). These exons are different between tGluA4trn1 and tGluA4trn2. Interestingly, no homology with other species was found for the 5' terminal exons of the tGluA4trn1-2 variants by BLAST search. Similar to tGluA4s, tGluA4trn1-2 variants lack the *flip/flop* exon 15 and exon 16, and both contain a novel 3' terminal exon compared to tGluA4 (Figs. 2, 4, 5). tGluA4trc1-3 transcripts are missing several coding exons at the 3' end: tGluA4trc1 is missing exons 10-16, tGluA4trc2 11-16, and tGluA4trc3 12-16 (Figs. 5, 2). All of the tGluA4trc transcripts contain a novel 3' terminal exon compared to tGluA4 that are again unique among the tGluA4trc1-3 variants (Fig. 5). Surprisingly, none of the variants of tGluA4 identified by RLM-RACE were predicted by the NetGene2 server. Recently, we sequenced *flip* and *flop* isoforms of turtle GluA1 (unpublished). These sequences were also analyzed by the NetGene2 server. Analysis showed the possibility of additional variants of tGluA1, however, none of the predicted variants were identified by RLM-RACE. The NetGene2 server is designed to predict variants in genomic DNA largely from humans and may have limited usefulness for analysis of cDNA from less well characterized species.

Conceptual translation products of tGluA4 variants

Analysis of the sequences of tGluA4 *flip* and *flop* showed they encode long-tailed proteins comprised of 901 amino acids (Fig. 7). Comparison of *flip* and *flop* isoforms of tGluA4 with protein from the GenBank database revealed that they are most similar to chick GluA4 *flip* and *flop* isoforms (98% identity). However, the sequence of turtle tGluA4 *flip* (Figs. 1, 7) predicts two amino acid replacements (Pro to Leu and Pro to Ala) outside and before the *flip/flop* region. To verify that these replacements are real and not errors in PCR, amplification of this region was performed. It has been shown that using DNA polymerases with 3' to 5' proofreading exonuclease activity and minimizing the number of PCR cycles will decrease the frequency of PCR errors (Eckert and Kunkel 1991; Cummings et al. 2009). To minimize such errors, the amplification reaction was performed using AccuPrime Pfx DNA Polymerase (Invitrogen), which has proofreading 3' to 5' exonuclease activity, and was executed using only twenty two PCR cycles. The product of this amplification was digested by a HpaI restriction endonuclease and the resulting fragments were resolved on agarose gels. The band for the tGluA4 *flip* variant was cloned (Fig. 3) and six of these clones were sequenced from both directions. Analysis of the nucleotide sequences of these clones showed that the two amino acid replacements are real and are not errors introduced by PCR or sequencing. The domain structure of tGluA4 protein was assigned with reference to mammalian and chick GluA4 (Paperna et al. 1996) and this comparison showed tGluA4 to have a similar domain architecture as other known GluA4 subunits (Figs. 7, 8).

tGluA4c *flip/flop* variants are predicted to encode 883 amino acid short-tailed proteins. These variants have a postsynaptic density-95/discs large/zona occludens-1 (PDZ) domain binding motif on the C-termini (Fig. 5; Kawahara et al. 2004). The PDZ domain binding motif of AMPARs is important for synaptic scaffolding and interaction with regulatory proteins to target and cluster AMPARs to specific subcellular domains. tGluA4c *flip/flop* variants were found to have the PDZ domain binding motif, similar to human, rat and chick GluA4c (Kawahara et al. 2004), while the other variants of tGluA4 do not have a PDZ domain binding motif (Fig. 7). tGluA4s encodes a 767 amino acid protein which lacks part of the S2 segment containing the *flip/flop* region, and the TM3 and C-terminal domains (Figs. 7, 8). tGluA4trc transcripts encode truncated isoforms of tGluA4 at the C-terminus. tGluA4trc1 encodes a 392 amino acid protein which consists only of the LIVBP-like domain. tGluA4trc2 is a slightly longer 432 amino acid variant with a short S1 segment. tGluA4trc3 encodes a 504 amino acid protein that includes the LIVBP-like domain and a nearly complete S1 segment (Figs. 7, 8). Finally, the tGluA4trn1-2 GluA4 isoforms are predicted to encode the same 515 amino acid protein. The tGluA4trn protein lacks part of LIVBP-like domain at the N-terminus, part of the S2 segment containing the *flip/flop* region, TM3, and the C-terminal domain (Figs. 7, 8). tGluA4trn protein lacks the signal peptide which is present for the other tGluA4 variants and is therefore predicted to be a non-secretory protein.

Expression of tGluA4 mRNA and its alternatively spliced isoforms in turtle brain

To examine the abundance of tGluA4 variants relative to total tGluA4 mRNA in turtle brain, real-time RT-PCR was performed for total tGluA4 and all of its isoforms (Table 3). Total RNA was isolated from five adult turtle brain stems. These data showed that the alternatively spliced isoforms of tGluA4 are differentially expressed in turtle brain (Fig. 9). tGluA4 *flip/flop* was by far the most abundantly expressed variant (55% of total tGluA4). Expression level of tGluA4c *flip/flop* mRNA was two-fold lower than tGluA4 which was significant (26% of total tGluA4; $P < 0.0001$). There were significant differences between the expression profiles of the *flip* and *flop* alternatively sliced isoforms. Expression of the *flop* version of tGluA4 was significantly higher than the *flip* version ($P = 0.003$), and tGluA4 *flop* was expressed in the greatest amounts of all the variants in turtle brain. The level of expression of the *flip* variant of tGluA4c was significantly greater than *flop* ($P < 0.001$). Unexpectedly, tGluA4trc1 was also expressed at high levels in brain (15% of total GluA4). Levels of expression of the other truncated isoforms of tGluA4 were undetectable.

Identification of tGluA4 and tGluA4trc1 proteins

In order to establish that the novel tGluA4trc1 variant is expressed at the protein level, Western blot analysis of turtle brain stem lysates was performed using antibodies against the N-terminus of goat GluA4 (Fig. 10). Lysates of SH-SY5Y cells transfected with tGluA4trc1 expression plasmid (recombinant tGluA4trc1) was used as a positive control. Lysates of SH-SY5Y cells transfected with empty plasmid or untransfected SH-SY5Y cells were used as negative controls. tGluA4 and tGluA4trc1 bands were identified in brain stem lysates at ~105 and ~42 kDa, respectively. The presence of tGluA4trc1 in brain stem was supported by expression of the recombinant protein which had a similar molecular weight. An additional band was observed in brain stem lysates with an approximate size of ~88 kDa and

may correspond to tGluA4c (Fig. 10). This band is unlikely to be tGluR4s because the mRNA for this variant is expressed at very low levels. These bands correspond well with the predicted molecular weights of the tGluA4 variants, especially when allowing for post-translational modifications that typically occur *in situ* (Table 4).

Discussion

In this report we have identified a diversity of alternatively spliced isoforms of the GluA4 AMPAR subunit in turtle brain. At least ten GluA4 splice variants expressed in brain were identified, tGluA4 *flip/flop*, tGluA4c *flip/flop*, tGluA4s, and several novel isoforms truncated at the 3' terminus, tGluA4trc1-3, and at the 5' terminus, tGluA4trn1-2. Significantly, tGluA4trc1 mRNA is expressed in relatively high levels in turtle brain, and as protein, suggesting some as yet uncharacterized function. Analysis of the identified sequences and domain architecture of the tGluA4 *flip/flop*, tGluA4c *flip/flop*, and tGluA4s AMPAR isoforms showed they are highly similar to those identified in chick and mammals. Moreover, there is considerable conservation of the nucleotide sequence of the alternatively spliced *flip/flop* exons. For example, the *flop* exon of tGluA4 has 100% similarity to chick while the *flip* exon has 94% similarity. This high level of conservation extended to the post-transcriptional processing and post-translational modification sites present in the GluA4 subunit. Analysis of the amino acid sequences suggested that tGluA4 *flip* and *flop* have potential phosphorylation sites at Ser842 and Thr830 shown to be phosphorylated *in vitro* by PKA, PKC and CaMKII, and PKC, respectively (Carvalho et al. 1999). Additionally, tGluA4 isoforms, with the exception of the truncated variants, contain predicted sites for palmitoylation at Cys591 and Cys817 (Hayashi et al. 2005).

Two groups of truncated variants of tGluA4 (tGluA4tr) were identified. One group is truncated on the 3' terminus (tGluA4trc) and other on the 5' terminus (tGluA4trn). The domain structure of tGluA4trc1-3 isoforms lack the intracellular C-terminus, the S2 segment, the TM domains, and part or all of the S1 segment. Therefore, they contain predominantly the LIVBP-like domain alone (Fig. 8). The truncated 3' terminus splice isoforms of GluA4 have also been characterized in human, mouse and rat (GenBank numbers: NP_001106283, NP_001106652, and NP_001106656, respectively). These isoforms have a domain structure most similar to tGluA4trc2 described here. It has been shown that the LIVBP-like domain of the GluA4 subunit forms dimers in solution, most likely related to a role in receptor assembly, and fails to bind ligand (Kuusinen et al. 1999). Furthermore, the LIVBP-like domain appears to be important for glutamate receptor subtype-specific assembly, that is, recognition of AMPAR versus kainate subunits (Leuschner and Hoch 1999). It is possible that tGluA4trc variants are able to associate with "normal" AMPAR subunits and form non-functional receptors. Whether this potential for assembly has any physiological consequences in brain is unknown. However, this may be of significance since one of the isoforms, tGluA4trc1, is present in brain in relatively substantial amounts. The function of these truncated subunits, if any, requires further study.

The N-terminal truncated variants, tGluA4trn, are lacking a small part of the LIVBP-like domain, part of the S2 segment containing the *flip/flop* region, TM3, and the C-terminal domain (Fig. 8). Moreover, they lack the signal peptide. Basically, they appear to be a non-

secreted version of tGluA4s. The function of the GluA4s isoform has not been well investigated, however, a truncated variant similar in structure to tGluA4s was characterized for GluA1 isolated from rat hippocampus and chick retinal cultures (Gomes et al. 2008). This GluA1 transcript lacked the *flip/flop* cassette, TM3, and the intracellular C-terminus. It was shown that this truncated GluA1 may associate with full-length GluA1 and exert a dominant negative effect by forming receptors that pass less current than full-length receptors. Furthermore, homomeric receptors made up of the truncated variant do not bind AMPA, while heteromeric receptors do. Interestingly, transfection of hippocampal neurons with truncated GluA1 resulted in a significant reduction in apoptotic cell death triggered by toxic concentrations of glutamate. These data indicate that the N-terminal truncated variants of GluA1, similar to tGluA4s, are able to assemble with full-length AMPAR subunits and play a functional, possibly neuroprotective, role. Although it was not reported whether the truncated GluA1 described previously has a signal peptide (Gomes et al. 2008), the N-terminal truncated variant of tGluA4 is identical to tGluA4s except that it lacks the signal peptide and is unlikely to be incorporated into receptors. Consistent with this, tGluA4s and tGluA4trn1-2 mRNA is expressed at negligible levels in turtle brain.

Analysis of the expression of tGluA4 AMPAR subunit mRNA and its alternatively spliced isoforms showed that the *flip* and *flop* versions have a major representation in the adult turtle brain. Comparison of expression levels indicated that tGluA4 mRNA was the most abundant isoform followed by tGluA4c. tGluA4 *flop* had the highest level of expression compared to all isoforms. In chick cerebellar granule cells, GluA4c mRNA was found to be expressed at about half that of GluA4, and GluA4s was less than one tenth of GluA4 (Ravindranathan et al. 1997). These values are comparable to turtle brain except that tGluA4s expression was negligible in turtle. Surprisingly, the tGluA4trc1 isoform showed a relatively high level of expression in turtle brain, similar to values for tGluA4c. To confirm that this novel splice variant was expressed as protein, Western blot analysis was performed on turtle brain tissue and compared to cultured cells transfected with a tGluA4trc1 expression plasmid. Using the recombinant protein as a marker, tGluA4trc1 was identified in turtle brain at ~42 kDa. While the function of this protein is unknown, our preliminary data show that it is significantly upregulated during *in vitro* classical conditioning (Sabirzhanov et al. 2009) indicating that it is regulated in an activity-dependent manner.

Our ongoing line of research (Keifer 2001; Zheng and Keifer 2008; Zheng and Keifer 2009) suggests that acquisition of *in vitro* eyeblink classical conditioning in turtle is associated with synaptic insertion of AMPARs containing tGluA4 subunits. This hypothesis can be directly tested using an siRNA approach. The sequencing and identification of tGluA4 and its alternatively spliced isoforms gives us the opportunity to design tGluA4 specific siRNA oligonucleotides to selectively inhibit the function of tGluA4-containing AMPARs. The data obtained here will provide a valuable tool to examine the function of tGluA4 and its alternatively spliced isoforms to understand their biological significance and potential role in synaptic plasticity mechanisms.

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Figure 1. Comparison of cDNA sequences of turtle, chick and mouse GluA4 *flip* and *flop* variants. GenBank numbers of chick and mouse GluA4 *flip* and *flop* variants are NM_001113186.1, NM_205214.2, NM_019691 and NM_001113180, respectively. Turtle and chick GluA4 *flip* sequences are shown complete. Mouse GluA4 sequences are shown from 467 to 4150 bp. Exons of tGluA4 were assigned by comparison to mouse GluA4. Exon 1 of mouse GluA4 is not shown, exon 2 and 16 are partially shown. Differences between turtle, chick and mouse GluA4 *flip* variants are indicated by highlighting. Differences in GluA4 *flop* variants are indicated only where they differ from *flip* variants by underlining. Sites for HpaI restriction endonucleases are underlined and bold. Alignment was performed using the MegAlign program (DNASTAR). The start and stop codons are shown in bold. Polyadenylation signals (PAS) are shown in italics and underlined. A presumed R/G RNA editing site in chick GluA4 is marked by +.

Figure 2.

Schematic diagram of the predicted exon structure of tGluA4^{flip} mRNA. Mouse GluA4^{flip} mRNA sequences are shown for comparison. Exons of tGluA4 were assigned by comparison to mouse GluA4.

Figure 3.

Agarose gel electrophoresis of PCR products of the tGluA4/GluA4c *flip/flop* spliced segment. Lane 1, the PCR products. Lane 2, the PCR products after HpaI digestion.

Figure 4. Comparison of cDNA sequences of tGluA4 *flip* and *flop*, tGluA4c *flip* and *flop* and tGluA4s variants. The sequence of the tGluA4 *flip* variant is shown complete. Other variants are shown only where they differ from the tGluA4 *flip* variant and these differences are indicated by underlining. tGluA4c *flop* variants are shown only where they differ from tGluA4c *flip* variants and these differences are underlined. Alignment was performed using the MegAlign program (DNASTAR). The start and stop codons are bold. Polyadenylation signals (PAS) are shown in italics and underlined. Sites for RLM-RACE primers and real-time PCR primers and probes are shown in bold. Exon structure of tGluA4 was assigned by comparison to the mouse GluA4.

Figure 5. Comparison of cDNA sequences of tGluA4 *flip*, GluA4trc1-3 and GluA4trn1-2 variants. Sequence of the tGluA4 *flip* variant is shown through 2889 bp. Other variants of tGluA4 are shown only where they differ from the GluA4 *flip* variant and these differences are underlined. Alignment was performed using the MegAlign program (DNASTAR). The start and stop codons are bold. Polyadenylation signals (PAS) are shown in italics and underlined. Sites for RLM-RACE primers and real-time PCR primers and probes are shown in bold. Exon structure of tGluA4 was assigned by comparison to the mouse GluA4.

Figure 6.
Agarose gel electrophoresis of PCR products for truncated isoforms of tGluA4 (tGluA4tr).

Figure 7.

Comparison of amino acid sequences of chick GluA4 and turtle GluA4 variants. Chick GluA4 *flip* and tGluA4 *flip* sequences are shown complete. Differences between chick and turtle GluA4 *flip* variants are shown by highlighting. Sequences of other GluA4 variants are shown only where they differ from flip variants and these differences are shown by underlining. Alignment was performed using the MegAlign program (DNASTAR). First mature amino acid is marked by an asterisk. Coordinates are shown relative to the initial methionine. A presumed R/G RNA editing site of chick GluA4 is marked by +. PDZ domain binding motif is circled. Sites of potential phosphorylation and palmitoylation are shown by arrows. The LIVBP-like domain, TM1-TM3, M2 domain, and *flip/flop* region are indicated by lines of different styles. Domains of tGluA4 were assigned by comparison to mouse and chick GluA4.

Figure 8. Schematic depiction of the domain architectures of chick GluA4 and its alternatively spliced isoforms.

Figure 9.

Expression of total tGluA4 mRNA and its alternatively spliced isoforms in turtle brain stem. Expression is shown as the percent of total tGluA4 mRNA ($n = 5$). Error bars indicate standard deviation. The asterisks indicate significant differences.

Figure 10.

Western blot analysis of the tGluA4trc1 variant. Lysates of turtle brain stem, SH-SY5Y cells transfected with tGluA4trc1 expression plasmid (recombinant tGluA4trc1), SH-SY5Y cells transfected with empty plasmid and untransfected SH-SY5Y cells were subjected to SDS/PAGE followed by Western blot analysis using antibodies against the N-terminus of GluA4.

Table 1

Results of analysis of cds of tGluA4 and its isoforms by BLAST.

species	% of identity			
	tGluA4 flip	tGluA4 flop	tGluA4c flip	tGluA4c flop
human	89	91	89	90
rat	87	88	87	87
mouse	87	87	87	87
chick	93	94	94	93

Table 2

Primers used to determinate complete sequences of truncated variants of tGluA4.

Forward primer	Reverse primer	tGluA4 variant	Size of PCR products	Identified
ATGAGGATAAATTTCCAGACAGCT	TCAATAGACAGACAGACCCCTTTCAGATA	GluA4-trc1	1200	yes
ATGAGGATAAATTTCCAGACAGCT	CACATATGCAAAACAGGACAGGTAAA	GluA4-trc2	1750	yes
ATGAGGATAAATTTCCAGACAGCT	ATTCCGATTGTTTATTTTGTTCTTTG	GluA4-trc3	1590	yes
GCGTTTGCTGGCTTTTGATGA	TCAATAGACAGACAGACCCCTTTCAGATA	GluA4 trn1-trc1	626	no
GCGTTTGCTGGCTTTTGATGA	CACATATGCAAAACAGGACAGGTAAA	GluA4 trn1-trc2	1176	no
GCGTTTGCTGGCTTTTGATGA	ATTCCGATTGTTTATTTTGTTCTTTG	GluA4 trn1-trc3	1016	no
GCTTCACTCAGTTCTGTATCATTTCTCT	TCAATAGACAGACAGACCCCTTTCAGATA	GluA4 trn2-trc1	576	no
GCTTCACTCAGTTCTGTATCATTTCTCT	CACATATGCAAAACAGGACAGGTAAA	GluA4 trn2-trc2	1126	no
GCTTCACTCAGTTCTGTATCATTTCTCT	ATTCCGATTGTTTATTTTGTTCTTTG	GluA4 trn2-trc3	966	no
GCGTTTGCTGGCTTTTGATGA	CTGGCTTTGTTTCTTATGGCTTCA	GluA4-trn1	2104	no
GCGTTTGCTGGCTTTTGATGA	GAGGAAGTTGGATTAAAAAGTCTGTGC	GluA4c-trn1	1994	no
GCTTCACTCAGTTCTGTATCATTTCTCT	CTGGCTTTGTTTCTTATGGCTTCA	GluA4-trn2	2068	no
GCTTCACTCAGTTCTGTATCATTTCTCT	GAGGAAGTTGGATTAAAAAGTCTGTGC	GluA4c-trn2	1964	no
GCGTTTGCTGGCTTTTGATGA	CAACACAAAAAGAACTATATCAGGGACAT	GluA4s-trn1	1764	yes
GCTTCACTCAGTTCTGTATCATTTCTCT	CAACACAAAAAGAACTATATCAGGGACAT	GluA4s-trn2	1724	yes

Table 3

Primers and probes used for real-time RT-PCR.

target	Forward primer	Reverse primer	MGB probe
tGluA4	total tGluA4 forward CTATTATGGAAAAAGCGGGACAAA	total tGluA4 reverse CATTGGCTCCTCCATCGATA	total tGluA4 ACATCGTTGCAAACTGGGATTCAAAGATAT
tGluA4 flip	tGluA4/tGluA4c flip forward GGTGAAATGTGGAGCCAAAGGACT	tGluA4 flip/flop reverse TTCAGAAAAGGTCAACTTCATTGCGCTT	tGluA4/tGluA4c flip/flop CTTCTACATTCTGTTGGAGGCTTGGGCT
tGluA4 flop	tGluA4/tGluA4c flop forward GCAGCGGGGAGGTGAC	tGluA4 flip/flop reverse TTCAGAAAAGGTCAACTTCATTGCGCTT	tGluA4/tGluA4c flip/flop CTTCTACATTCTGTTGGAGGCTTGGGCT
tGluA4c flip	tGluA4/tGluA4c flip forward GGTGAAATGTGGAGCCAAAGGACT	tGluA4c flip/flop reverse GAGGAAAGTTGGATTAAAAGTCTGTGC	tGluA4/tGluA4c flip/flop CTTCTACATTCTGTTGGAGGCTTGGGCT
tGluA4c flop	tGluA4/tGluA4c flop forward GCAGCGGGGAGGTGAC	tGluA4c flip/flop reverse GAGGAAAGTTGGATTAAAAGTCTGTGC	tGluA4/tGluA4c flip/flop CTTCTACATTCTGTTGGAGGCTTGGGCT
tGluA4s	tGluA4s forward CTTCCCTCCTGGAGTCCACCAT	tGluA4s reverse CAACACAAAAAGAACTATATACAGGGACAT	tGluA4s probe CGAATACATTGAAACAGCGAAAGCCG
tGluA4trc1	tGluA4trc1 forward GGAAATGTTGAGTTTGATCATACTATGG	tGluA4trc1 reverse TCAATAGACAGACAGACCCCTTTCAGATA	tGluA4 probe TCGCAGAGTCAACTACACAAATGGATG
tGluA4trc2	tGluA4trc2 forward CCTGCAGTGTAGTAAACATTGAGCTAAT	tGluA4trc2 reverse CAGTGCCTGGCCATCCA	tGluA4trc2 probe TTCCCATATGGATTACTGTTC
tGluA4trc3	tGluA4trc3 forward GATGGTGGGAGAACTTGTATTATGG	tGluA4trc3 reverse GCCCCAGATCAAAATAAAATGCA	tGluA4trc3 probe AGCATACCAAATTTTG
tGluA4trn1	tGluA4trn1 forward GCGTTTGTCTGGCTTTGATGA	tGluA4trn1-2 reverse CATTTGGCTCCTCCATGCATA	tGluA4trn1-2 probe ACATCGTTGCAAACTGGGATTCAAAGATAT
tGluA4trn2	tGluA4trn2 forward GCTTCACCTCAGTTCTGTATCAATTCTCT	tGluA4trn1-2 reverse CATTTGGCTCCTCCATGCATA	tGluA4trn1-2 probe ACATCGTTGCAAACTGGGATTCAAAGATAT
actin	AGGGAAATCGTGCGTGACAT	GCGGCAGTGGCCATCTC	AAGCTGTGCTATGTTGC

Table 4

Predicted molecular weights of tGluA4 variants.

variant of tGluA4	kDa
tGluA4 mature	98.37
tGluA4c mature	96.79
tGluA4s mature	84.24
tGluA4trn	57.84
tGluA4trc3 mature	54.83
tGluA4trc2 mature	46.81
tGluA4trc1 mature	42.13