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AGE AND GENDER-DEPENDENT ALTERNATIVE SPLICING OF P/Q-TYPE CALCIUM CHANNEL EF-HAND

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Abstract—$Ca_v2.1$ Ca^{2+} channels (P/Q-type), which participate in various key roles in the CNS by mediating calcium influx, are extensively spliced. One of its alternatively-spliced exons is 37, which forms part of the EF hand. The expression of exon 37a (EFa form), but not exon 37b (EFb form), confers the channel an activity-dependent enhancement of channel opening known as Ca^{2+}-dependent facilitation (CDF). In this study, we analyzed the trend of EF hand splice variant distributions in mouse, rat and human brain tissues. We observed a developmental switch in rodents, as well as an age and gender bias in human brain tissues, suggestive of a possible role of these EF hand splice variants in neurophysiological specialization. A parallel study performed on rodent brains showed that the data drawn from human and rodent tissues may not necessarily correlate in the process of aging.

Key words: calcium channel, EF hand, mutually-exclusive exons, developmental switch, aging.

$Ca_v2.1$ (P/Q-type) voltage-gated calcium channels (VGCCs) play key roles in the trigger of neurotransmitter release in the central and peripheral nervous systems (Takahashi and Momiyama, 1993; Dunlap et al., 1995) regulation of the neuro-architecture of developing cerebellar Purkinje cells (PCs) (Miyazaki et al., 2004), and optimization of presynaptic function (Piedras-Renteria et al., 2004). Mutations in the principal α_{1A} subunit have been associated with a number of neurological conditions including cerebellar or episodic ataxia, familial hemiplegic migraine (FHM-1) (Ophoff et al., 1996; Zhuchenko et al., 1997), epilepsy (Fletcher et al., 1996) while auto-antibodies directed against the same channel lead to myasthenic syndrome with particular features (Flink and Atchison, 2003). The α_{1A} subunit is highly expressed in the brain, particularly in the soma and dendrites of the Purkinje neurons of the cerebellum (Stea et al., 1994), and the presynaptic terminals of synapses. Conceivably, this class of channels plays a major role in determining the electrical excitability of these neurons as well as regulating neurotransmitter release.

The α_{1A} subunit is subjected to extensive alternative splicing (Mori et al., 1991; Starr et al., 1991; Ophoff et al., 1996; Zhuchenko et al., 1997; Bourinet et al., 1999; Soong et al., 2002; Tsunemi et al., 2002), in which seven and nine loci of variation have been characterized to date in human (Soong et al., 2002) and in rat cerebellum (Kanumilli et al., 2006) respectively. Likewise, $Ca_v2.1$ channel splice variants had also been reported in mice (Vigues et al., 1998; Tsunemi et al., 2002), including some novel C-termini that were detected in mutant mouse (Fletcher et al., 1996).

At the carboxyl terminal, exons 36 and 37 encode the EF hand-like domain. Exon 37 is encoded by two mutually-exclusive exons 37a and 37b, hence producing two variants of the EF hand, EFa and EFb. Channels lacking exon 37 had also been reported in rodents (Ligon et al., 1998; Vigues et al., 1998). The EF hand plays a role in the transduction of the IQ-motif-bound Ca^{2+}/calmodulin (CaM) into channel modulations (Peterson et al., 2000; Kim et al., 2004). CaM exerts two opposing effects, calcium-dependent facilitation (CDF), an activity-dependent enhancement of channel opening and a subsequent slower process, calcium-dependent inactivation (CDI), a process of channel inactivation following Ca^{2+} influx. Interestingly, in both heterologous systems expressing recombinant channels and in cerebellar Purkinje neurons, the alternative splicing of exon 37 acts as a molecular switch for CDF without affecting CDI (Chaudhuri et al., 2004, 2005). EFa expression confers this facilitative property to the channel. We also demonstrated a developmental activation of CDF due to enhanced postnatal EFa expression (Chaudhuri et al., 2005). This correlates with the differential expression of the two exons previously reported in development (Vigues et al., 2002; Chaudhuri et al., 2004) or in different regions of the human brain (Chaudhuri et al., 2004). Distinct and separate subcellular localization of the two splice variants observed in PCs strongly suggested specialized neurophysiological roles such as synaptic plasticity, gene tran-

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Abbreviations: CaM, calmodulin; CDF, calcium-dependent facilitation; CDI, calcium-dependent inactivation; cDNA, complementary DNA; CF, climbing fiber; dNTP, deoxynucleotide triphosphate; P, postnatal day; PC, Purkinje cell; PCR, polymerase chain reaction; RT, reverse transcription.

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scription and remodeling (Chaudhuri et al., 2005). To date, the $Ca_v2.1$ (P/Q-type) calcium channels are the only channels in the Ca_v1–2 family to exhibit CDF (Liang et al., 2003; Inchauspe et al., 2004), although CaM kinase-mediated facilitation had been earlier established in $Ca_v1.2$ (L-type) calcium channels (Dzhura et al., 2000). With these physiological bases, this report investigates the splice variant distribution within individual mouse and rat brains during development. Parallel analyses of human cerebellar samples provided valuable information on age and gender bias of EF hand expression. For a broader perspective, we also obtained data from other human brain regions.

EXPERIMENTAL PROCEDURES

RNA extraction and complementary DNA (cDNA) synthesis

Swiss albino mice, F344 rats of 1–30 months of age (NIA, Bethesda, MD, USA), early postnatal Wistar rats were killed by anesthesia and subsequent cervical dislocation, and the cerebellar/whole brain tissues were harvested and immediately stored in RNAlater reagent [Ambion® (Europe) Ltd., Cambridgeshire, UK]. Two to five brains from rodents of each time point (the minimum number necessary for statistical significance) were pooled together for analyses, except for the three individual female mouse brains shown in Fig. 2C. Postmortem human brain samples were obtained from the brain tissue repository of the Johns Hopkins Medical Institutions, and the National Neuroscience Institute. Prior to RNA extraction by the RNAeasy kit (Qiagen GmbH, Hilden, Germany), the RNAlater reagent was removed and homogenization was performed. The protocols used in this study conformed to our institution's IACUC and IRB regulations and guidelines (NUS-IRB 05-034E; IACUC 859/05). All efforts were made to minimize the number of animals used and their suffering.

Reverse transcription (RT) was performed using 5 μg of total RNA, Superscript II™ reverse transcriptase (Invitrogen, Carlsbad, CA, USA), 0.5 mM deoxynucleotide triphosphates (dNTPs), 1×1st strand buffer (Invitrogen) and 0.1 M dithiothreitol (DTT) (Invitrogen). For mouse brain tissues, mouse exon 38 reverse primer (5′-TATCAAGGGCGGTTCGGATCA-3′) was used for cDNA synthesis. RT was performed in the following sequence: a 5-min denaturation step at 80 °C and rapid cooling, a 75-min 42 °C RT step, and a final 15-min inactivation step at 70 °C. RT was performed similarly with extracted rat and human brain RNA except for the use of 18-mer oligo-dT in place of a specific reverse primer and a human exon 38 reverse primer (5′-GCTGTGCGGATCAGA-3′) respectively.

Screening using polymerase chain reaction (PCR)

As a control, DNA of full-length human EFa and EFb clones (Soong et al., 2002) was linearized by *Hind*III (New England Biolabs, Inc., Ipswich, MA, USA), quantified by spectrophotometry and the following ratios of each were mixed: 1:10; 1:1; 10:1. After serial dilutions, 10 ng of these DNA mixtures were used for subsequent PCR and colony screening.

For the brain samples, the synthesized cDNA was used for PCR amplification of the EF hand. A control reaction consisting of all the components but omitting the reverse transcriptase was also run in parallel. The mouse exon 35 forward primer (5′-CGGCAGACTGCGGCAACGAG-3′) (5′-GGGAAACCGTGTGATAAGAA-3′) and exon 38 primer (for sequence see section 2.1) were used for the amplification of a 384-bp fragment from the mouse brain samples. The reaction mix consisted of 200 μM dNTPs, 100 nM of primers, 2.5 U of *Taq*DNA polymerase (Promega Corporation, Madison, WI, USA), 1× Mg^{2+}-free buffer (Promega Corporation), Mg^{2+} concentration of 1.5 mM and 2 μl of cDNA in a final volume of 25 μl. The cycling profile was set as follows: initial denaturation of template DNA at 94 °C for 2 min, five touch-down cycles of denaturation at 94 °C for 30 s, annealing at 62 °C to 58 °C for 40 s, and extension at 72 °C for 1 min, followed by 30 cycles in which the annealing temperature was maintained at 57 °C. The final extension was performed at 72 °C for 10 min. Control reactions in which water and product from the control RT experiment were used in place of the cDNA were included. For rat tissues, the exon 35 primer (5′-CACAGGGGAAGCGTGGCAC-3′) and exon 38 primer (5′-GGGCGGTTCGGATCAG-3′) were used to amplify a 351-bp fragment. A final annealing temperature of 50 °C was used. For human templates, the exon 35 primer (5′-GGGAAACCGTGTGATAAGAA-3′) and exon 38 primer (5′-GCTGTGCGGATCAGA-3′) were used to amplify a 410-bp fragment. The PCR reaction mix required a Mg^{2+} concentration of 2 mM, and a final annealing temperature of 53 °C was used.

These amplicons represented the pool of both EFa and EFb cDNA forms. The fragment was gel-purified (Qiagen GmbH), cloned into pDrive (Qiagen GmbH) or pGEM T Easy (Promega Corporation) and transformed into *E. coli* DH10B cells by electroporation. The clones, each carrying an insert of either splice variant, were identified by a blue–white selection and cultured in Luria-Bertani broth with 100 μg/ml of ampicillin in 96-well round-bottomed microtiter plates. A minimum of two plates of white transformants was collected from each sample.

Mouse EF hand screening was performed with primer pairs, exon 35 primer and exon 37a primer (5′-GTACATGTCCTTATAGTGAA-3′) or exon 37b primer (5′-ATACATGTCCGGGTAAGGCA-3′), both generating amplicon sizes of 224 bp. The reaction mix consisted of 50 μM of dNTPs, 50 nM of each primer, 0.3 U of *Taq* polymerase (Promega Corporation), 1× PCR Buffer (Promega Corporation), a Mg^{2+} concentration of 1 μM and 1 μl of culture in a final volume of 12.5 μl. Similar touch-down cycling profiles were used in which the final annealing temperature was maintained at 51 °C (for EFa screening) or 54 °C (for EFb screening). Rat EFa screening was performed with primer pairs, exon 35 primer (5′-CACAGGGGAAGCGTGGCAC-3′) and exon 37a primer (5′-GCAAGCAACCCTATGAGGAC-3′), while EFb screening was performed with exon 35 primer (5′-TCCAAAAACCAGAGTGTG-3′) and exon 37b primer (5′-CATGTGTCTCAGCATCTGA-3′). The amplicon sizes were 367 bp and 248 bp respectively. The reaction mix was similar to that for mouse EF screening, except that a Mg^{2+} concentration of 2 μM was used and that a final annealing temperature of 50 °C was adopted. Human nested PCR was performed directly on the clones using an exon 36 primer (5′-CGTCATCATGGACAACTT-3′) and either the exon 37a primer (5′-ATATTACTCGTAATAAACTG-3′), or the exon 37b primer (5′-GGGCGGAGACATGTGTCTCA-3′). The amplicons were 154 bp and 175 bp respectively. The reaction mix was similar except that the Mg^{2+} concentration used was 2 μM (for EFa screening) or 1 μM (for EFb screening). The final annealing temperature was maintained at 54 °C for both EFa and EFb screening. Appropriate controls were performed for all samples at both the RT and PCR steps, as mentioned previously.

All PCR products were visualized in 1.5% agarose gel. Only clones that are positive for EFa and negative for EFb, and vice versa, were taken to be confirmative results. All ambiguous results were disregarded. We analyzed at least 100 clones per tissue sample to ensure unbiased quantification. For confirmation, identified clones were randomly selected for DNA sequencing.

Quantitative restriction analyses

Equal amounts of PCR amplicons from each set of RT reactions were pooled together and digested with *Not*I (Roche Diagnostics GmbH, Mannheim, Germany) and *Sph*I (Roche Diagnostics