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Identification and characterization of two novel alternatively spliced E2F1 transcripts in the rat CNS

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ABSTRACT

E2F1 is a transcription factor classically known to regulate G_0/G_1 to S phase progression in the cell cycle. In addition, E2F1 also regulates a wide range of apoptotic genes and thus has been well studied in the context of neuronal death and neurodegenerative diseases. However, its function and regulation in the mature central nervous system are not well understood. Alternative splicing is a well-conserved post-transcriptional mechanism common in cells of the CNS and is necessary to generate diverse functional modifications to RNA or protein products from genes. Heretofore, physiologically significant alternatively spliced E2F1 transcripts have not been reported. In the present study, we report the identification of two novel alternatively spliced E2F1 transcripts: E2F1b, an E2F1 transcript retaining intron 5, and E2F1c, an E2F1 transcript excluding exon 6. These alternatively spliced transcripts are observed in the brain and neural cell types including neurons, astrocytes, and undifferentiated oligodendrocytes. The expression of these E2F1 transcripts is distinct during maturation of primary hippocampal neuroglial cells. Pharmacologically-induced global translation inhibition with cycloheximide, anisomycin or thapsigargin lead to significantly reduced expression of E2F1a, E2F1b and E2F1c. Conversely, increasing neuronal activity by elevating the concentration of potassium chloride selectively increased the expression of E2F1b. Furthermore, experiments expressing these variants in vitro show the transcripts can be translated to generate a protein product. Taken together, our data suggest that the alternatively spliced E2F1 transcript behave differently than the E2F1a transcript, and our results provide a foundation for future investigation of the function of E2F1 splice variants in the CNS.

1. Introduction

Alternative splicing is an evolutionarily conserved mechanism allowing cells to generate diverse functional RNA transcripts through combinatorial pairing of different splice sites (Nilsen and Graveley, 2010). Through the coordinated expression of various splicing regulatory factors, different cell types can generate a vast number of different proteins and functional modifications necessary for their own physiologic development from an identical genome. Tissue-specific alternatively spliced RNA transcripts are particularly common during the development of the central nervous system (CNS) as neural cells enter their fully differentiated state (Grabowski, 2011). A recent example of this developmentally controlled splicing of RNA transcripts is the gene encoding the neuronal per-arnt-sim domain protein 3 (NPAS3), such that the splice variant is only expressed in the ventricular zone during brain development (Shin and Kim, 2013). Disruption of the tight regulation of RNA splicing can induce developmental defects in the CNS as evidenced by deletion of Nova2, a regulator of splicing, which leads to aberrantly spliced RNA transcripts of disabled-1 protein that results in defects in neuronal migration (Yano et al., 2010). Additionally, alternative splicing leading to the introduction of a premature stop codon in the mRNA can be coupled with the nonsense-mediated mRNA decay (NMD) pathway to temporally regulate the expression of classically generated protein products during brain development. For example, *DLG4*, encoding postsynaptic protein 95, which is crucial in synapse maturation, is alternatively spliced into a transcript containing a premature stop codon that is degraded via NMD during embryonic development. However, loss of the splicing regulators polypyrimidine tract binding protein (PTBP) 1 and 2 during brain maturation results in aberrant expression of the full length PSD-95 transcript and thus,

Abbreviations: CNS, central nervous system; NPAS3, neuronal per-arnt-sim domain protein 3; NMD, nonsense-mediated mRNA decay; PTBP, polypyrimidine tract binding proteins; PSD-95, postsynaptic density-95; TDP-43, TAR DNA-binding protein-43; qPCR, quantitative real-time PCR; TBP, TATA-box binding protein; HRP, horseradish peroxidase

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initiates synapse formation earlier than in healthy brains (Zheng et al., 2012). Indeed, disruptions in the splicing machinery can also result in neurodegeneration, as seen in Amyotrophic Lateral Sclerosis with the dysregulation of TAR DNA-binding protein (TDP)-43 (Polymenidou et al., 2011).

E2F1 classically functions as a transcription factor regulating the G_0/G_1 to S phase transition in cycling cells, yet it also has an important role in regulating cell death (Blais and Dynlacht, 2004; Giacinti and Giordano, 2006; Iaquinta and Lees, 2007). E2F1 can induce apoptosis via indirect up-regulation of p53 expression or by direct up-regulation of pro-apoptotic target genes, including APAF1, PUMA, NOXA, Smac/Diablo, and caspases (Hershko and Ginsberg, 2004; Moroni et al., 2001; Nahle et al., 2002; Xie et al., 2006). Given this capacity to regulate cell death, the role of E2F1 in neuronal death is well characterized in a number of different in vitro models of toxicity (Giovanni et al., 2000, 1999; O'Hare et al., 2000; Park et al., 2000). Aberrant E2F1 expression has been reported in a number of different neurodegenerative diseases, including Alzheimer's, Huntington's, Parkinson's, and amyotrophic lateral sclerosis (Höglinger et al., 2007; Jordan-Sciutto et al., 2001, 2002; Ranganathan et al., 2001; Lopez-Sanchez et al., 2017). However, despite the well-characterized anomalies in these diseases, little is known about E2F1 function or regulation in the CNS. We have previously reported data showing that E2F1 has a physiologic role in the CNS, as transgenic mice lacking functional E2F1 exhibit age-dependent synaptic disruption and behavioral deficits (Ting et al., 2014). In the present study, we explored alternative splicing as a potential mechanism for E2F1 regulation and identified two novel E2F1 splice variants prominently observed in the brain. We did not detect these alternatively spliced transcripts in other tissues, organs, or fibroblast cell lines despite ubiquitous expression of the E2F1a transcript. Furthermore, we characterized the expression pattern of the E2F1 splice variants: during maturation of neuroglial cells, in response to pharmacologically-induced NMD inhibition, and increased neuronal activity. Taken together, our results show that E2F1 is indeed alternatively spliced into two novel transcript variants in the CNS, which are distinct from the originally identified E2F1a mRNA.

2. Materials and methods

2.1. Cell culture and transfection

Primary cortical or hippocampal neuroglial cultures were isolated from the brains of embryonic day 17 Sprague Dawley rats, as previously described (Wilcox et al., 1994). Dissociated cells were seeded onto poly-L-lysine coated plates and maintained in neurobasal media containing B27 supplement. Pure neuronal cultures were generated by treating neuroglial cultures with 10 μm Ara-C, 48 h after plating. Purity of cultures was confirmed by the absence of staining for the glial marker, glial fibrillary acidic protein (GFAP). Pure astrocyte cultures were generated by first culturing neuroglial cells in DMEM supplemented with FBS for 7–10 days, at which point glial cells constitute approximately 90% of the cells. The cells were manually shaken off and sub-cultured in another flask, this process was repeated twice to generate pure cultures for RNA collection (Akay et al., 2014). Pure oligodendrocyte cultures were prepared as previously described (Reid et al., 2012). Briefly, neuroglial cells were isolated from neonatal rat brain and cultured in neurobasal media supplemented with B27, 10 ng/ml basic fibroblast growth factor, 2 ng/ml platelet-derived growth factor (R&D Systems) and 1 ng/ml neurotrophin-3 (Peprotech). To differentiate cells into oligodendrocytes, cultures were subsequently maintained in differentiation media containing 50% DMEM, 50% Ham's F12 with 50 μg/ml transferrin, 5 μg/ml putrescine, 3 ng/ml progesterone, 2.6 ng/ml selenium, 12.5 μg/ml insulin, 0.4 μg/ml T4, 0.3% glucose, 2 mM glutamine, and 10 ng/ml biotin (R&D Systems). HEK293T and Rat2 cells were maintained in DMEM supplemented with fetal bovine serum (FBS). PC-12 cells were maintained in RPMI-1640 media supplemented with horse serum and FBS. All cells were cultured at 37 °C with 5% CO_2. Rat2 fibroblasts were arrested in S phase via double thymidine block, M phase by thymidine-nocodazole block, and G_0/G_1 phase by serum starvation, as previously described (Whitfield et al., 2002). Lipofectamine 2000 was used for transfection experiments in HEK293T and PC12 according to manufacturer protocols (Life Technologies). Transfection mixture was added to the cells for 2 h and subsequently replaced with the growth media.

2.2. RNA extraction and PCR

Tissues harvested from Sprague Dawley rats were snap frozen in liquid nitrogen and subsequently stored at −80 °C or used immediately after freezing for RNA extraction. Total RNA was extracted from tissues and cells using TRIzol reagent (Life Technologies) and subsequently purified using the RNeasy Plus Mini Kit, according to manufacturer recommendations (Qiagen). Total RNA was treated with DNase prior to cDNA synthesis using TURBO DNase (Thermo Fisher). cDNA was synthesized from 1 μg of total RNA (as quantified by Nanodrop) using superscript IV reverse transcriptase and oligo dT primers (Invitrogen), according to the manufacturers' recommendations. Following the cDNA synthesis reaction, all samples were digested with RNase H, according to the superscript IV manufacturers' recommendations. PCR reactions were performed using 2 μl of cDNA with the AccuPrime GC-rich DNA polymerase kit and specified primers (Table 1 and Supplemental Fig. S1) (Invitrogen), according to the manufacturers recommendations. To confirm PCR results with the AccuPrime GC reagents, most PCR reactions were also performed using OneTaq DNA polymerase, with the GC-rich buffer supplied in the kit (New England BioLabs). For all E2F1 PCR reactions, annealing temperatures were optimized to the specific primer sets. All E2F1 PCR reactions were performed with the following conditions: an initial melt of 95 °C for 2 min, then by cycling through 95 °C for 30 s, annealing for 30 s, and extension at 72 °C for 30–90 s (depending on product size) for 40 cycles, followed by a final extension of 10 min. PCR products were visualized by separation on 0.8% agarose gel containing ethidium bromide. Gels were imaged using a ChemiDoc MP system (Bio-Rad Laboratories, Inc.). For DNA sequencing of PCR products, DNA was gel extracted using the Qiagen DNA Gel Extraction Kit (Qiagen) according to the manufacturers' recommendations. DNA was submitted for sequencing (with primers specific to E2F1) by the Penn Genomics Analysis Core, which employs an ABI 3730 for Sanger sequencing. For sequencing alignments and primer design, rat E2F1 sequences were downloaded from the UCSC genome browser, rn6 assembly (Kent et al., 2002). Nicastrin-Δ3 isoform mRNA expression was quantified as in Confaloni et al., 2005.

2.3. Quantitative real-time PCR

Equal amounts of cDNA were loaded in technical triplicate for quantitative PCR (qPCR) using the Fast SYBR green master mix and 7500 Fast real time PCR systems according to the manufacturers protocol (Applied Biosystems). Primers used in these experiments are listed in Table 1 and shown in Supplemental Fig. S1. All qPCR primers were designed to similar amplification efficiency. Primers targeting specific E2F1 splice variants were also analyzed to ensure specificity to that variant. To determine E2F1 splice variant expression levels, qPCR results were calculated using the $2^{-\Delta\Delta CT}$ method, whereby the mean cycle threshold was first normalized to the endogenous control, TATA-box binding protein (TBP), and fold-change was subsequently calculated relative to the proper experimental control. All qPCR experiments were replicated biologically a minimum of four times and independently three times.

2.4. Antibodies and reagents

The following antibodies were purchased from the indicated