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Research paper

The role of hypoxia on Alzheimer's disease-related APP and Tau mRNA formation

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ABSTRACT

The removal of introns from mRNA precursors (pre-mRNAs) is an essential step in eukaryotic gene expression. The splicing machinery heavily contributes to biological complexity and especially to the ability of cells to adapt to altered cellular conditions. Hypoxia also plays a key role in the pathophysiology of many diseases, including Alzheimer's disease (AD). In the presented study, we have examined the influence of cellular hypoxia on mRNA splice variant formation from Alzheimer's disease-related *Tau* and *APP* genes in brain cells. We have shown that the hypoxic microenvironment influenced the formation of Tau mRNA splice variants, but had no effect on *APP* mRNA splice variant formation. Additionally, our presented results indicate that splicing factor SRSF1 but not SRSF5 alters the formation of *Tau* cellular mRNA splice variants in hypoxic cells. Obtained results have also shown that hypoxic brain cells possess enhanced *CLK1-4* kinase mRNA levels. This study underlines that cellular hypoxia can influence disease development through changing pre-mRNA splicing.

1. Introduction

Alzheimer's disease (AD) is among the most wide-spread neurological disorders, characterized by progressive loss of memory and cognitive functions. There are several molecular hallmarks of the AD pathology: aggregation of extracellular β-amyloid (Aβ) peptides generated from amyloid precursor protein (APP) and accumulation of microtubule-associated protein Tau that forms neurofibrillary tangles (Selkoe, 2001; Kametani and Hasegawa, 2018; Laurent et al., 2018).

Recent studies have reported that oxygen deficiency (hypoxia) triggering disruption of cellular homeostasis in the brain is one of important risk factors in AD pathogenesis (Jha et al., 2018a). Cellular hypoxia promotes the production of proteins that mediate oxidative stress, inflammation, apoptosis, mitochondrial metabolism, metal homeostasis and synaptic transmission and cause neuronal damage leading to neurodegeneration (Merelli et al., 2018; Zhang et al., 2019). A cellular response to hypoxia induces the stabilization of hypoxia-inducible transcription factors (HIFs). HIFs activate the transcription of a network of genes involved in the ability of cells to adapt to the altered environmental conditions (Ruas and Poellinger, 2005). A number of reports describe hypoxia induced changes in alternative pre-mRNA splicing patterns that are needed for cell survival under unfavourable conditions (Sena et al., 2014; Nakayama and Kataoka, 2019).

Alternative pre-mRNA splicing (AS) process consists of intron removal from mRNA precursors (pre-mRNAs) and joining of exons in different combinations to form a mature mRNA. In humans more than 90% of pre-mRNAs are alternatively spliced allowing cells to produce different mRNA splice variants that encode proteins with distinct cellular functions (Izquierdo and Valcarcel, 2006; Will and Luhrmann, 2011; Dvinge, 2018). The splicing process is carried out by the spliceosome, a dynamic complex of five small nuclear ribonucleoproteins (U snRNPs), and by more than a few hundred auxiliary proteins (Nakayama and Kataoka, 2019). The spliceosome is assembled and activated through a series of ATP/GTP-dependent steps from complex E to complexes A, B, and C by RNA-RNA and RNA-protein interactions (Araki et al., 2015).

A prominent group of auxiliary splicing regulators is the family of serine / arginine rich (SR) proteins that play an important role in exon selection and also are implicated in mRNA metabolism, including export and localization (Bradley et al., 2015). SR protein family, consisting of 12 members, has a similar structure: one or two RNA binding domains at the N-terminus and a serine/arginine rich (RS) domain at the C-terminus

Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; Tau, microtubule-associated protein Tau; SRSF1, serine/arginine-rich splicing factor 1; SRSF5, serine/arginine-rich splicing factor 5; CLK, cdc-like kinases.

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(Jakubauskiene et al., 2015). SR proteins attract the elements of the splicing machinery through RS domain-mediated protein–protein interactions. Different SR proteins such as SRSF1 and SRSF5 bind to distinct enhancer/silencer sequences, and that the combination of SR proteins bound at a particular splice site and the position of their binding relative to the splice site, determines its strength or probability that it will be used during the processing of that pre-mRNA (Shepard and Hertel, 2009; Jeong, 2017).

The family of cdc2-like (CLKs) kinases has been shown to directly phosphorylate the RS domain of SR proteins *in vivo* (Giannakouros et al., 2011; Zhou and Fu, 2013). Mammalian cells express four CLKs (CLK1-4) having a significant impact on SR protein functionality, as it may affect their binding to target mRNAs, their interaction with other proteins and their intracellular localization (Naro and Sette, 2013; Jain et al., 2014).

APP is a type I transmembrane glycoprotein which is ubiquitously expressed, but is the most abundantly in the brain (Imbimbo et al., 2005). The *APP* gene is comprised of 18 exons that via alternative splicing mechanism are joined to produce 11 different mRNA isoforms. In neurons, there are three main isoforms that are important to the development of neurodegenerative diseases: APP770 produced by joining all 18 exons; APP751 – isoform lacking exon 8, which codes OX-2 antigen domain; and APP695 – isoform lacking exons 7 and 8 that code Kunitz-type protease inhibitor and OX-2 antigen protein domains (Nalivaeva and Turner, 2013). In the brain, APP is processed through proteolytic cleavage to produce Aβ peptide (Fragkouli et al., 2017). Different lengths of Aβ peptides exist, including Aβ42, which is a main pathogenic Aβ component. An increase level of Aβ42 peptide leads to the aggregation of oligomers and fibrils, that develop into amyloid plaques (Kametani and Hasegawa, 2018). It has been reported that in the brain of patients with Alzheimer's disease APP770 mRNA splice variant is increased and APP695 is reduced (Matsui et al., 2007). Alterations in *APP* pre-mRNA splicing may lead to aberrant Aβ formation that is associated with AD progression (Biamonti et al., 2019).

Tau is a microtubule-associated protein, predominantly expressed in the neurons, functions to stimulate and stabilize microtubule (MT) assembly (Hartmann et al., 2001). The human *Tau* pre-mRNA consists of 16 exons from which exons 9–12 encode the microtubule-binding domain of Tau. In adult human brain, exons 2, 3 and 10 are alternatively spliced to produce six different *Tau* splice variants. Exon 10 encodes the second repeat region for microtubule binding and its inclusion/exclussion has been identified as a relevant contributor to the development of neurodegeneration (Zhou et al., 2008). Exon 10 inclusion generates isoforms with four microtubule-binding repeats called four-repeat (4R) Tau and exon 10 skipping results in three-repeat (3R) Tau splice variant. The 4R Tau isoform, containing an additional microtubule-binding repeat, shows a higher activity in MT assembly than 3R Tau isoform (Wu et al., 2017). The 4R/3R mRNA ratio in normal adult brain is balanced at approximately equal levels, but alterations in isoform ratio results in neurodegeneration (D'Souza and Schellenberg, 2006; Liu and Gong, 2008a).

Two different cell lines derived from human brain were used in our studies: glioblastoma (U-87) and neuroblastoma SK-N-Be(2). There is little data on how different brain cells respond to hypoxia. As currently precise mechanistic role played by hypoxia in mediating key processes of the brain remains unclear it is important to understand how different cells, derived from brain, respond to hypoxic conditions. In the current study we have analyzed and provided experimental evidence on the influence of cellular hypoxic microenvironment on *Tau* and *APP* alternative pre-mRNA splicing. We have investigated the influence of SRSF1 and SRSF5 factors in the regulation of alternative *Tau* exon 10 splicing in hypoxic cells comparing them to normoxic ones and also have shown that SR protein kinase expression levels are enhanced in hypoxic brain cells. Our results, for the first time, have shown that hypoxic conditions influence *Tau* but not *APP* pre-mRNA splicing. The expression of CLK family kinases is increased in hypoxic cells.

2. Materials and methods

2.1. Cell lines

Experiments described in this paper were performed with glioblastoma U-87 MG and neuroblastoma SK-N-Be(2) cells obtained from ATCC. U-87 cells were cultivated in EMEM (#11095080, Gibco, Thermofisher Scientific), whereas SK-N-Be(2) cells were maintained in 1:1 mixture of EMEM:F12 (#11095080 and #11765054, Gibco, Thermofisher Scientific) medium, containing 10% fetal bovine serum (#S0615, Biochrom, Merck), 100 U/ml penicillin and 100 μg/ml streptomycin (#P4333, Biochrom, Merck) at 37 °C in a humidified 5% CO_2 conditions. U-87 and SK-N-Be(2) cells were subcultured every 5 and 7 days, respectively. The cells were cultivated for no more than 30 passages, discarded and then a lower passage number stock was thawed. Cell cultures were routinely tested for Mycoplasma.

2.2. Construction of plasmids and transfections

The expression plasmids pSRSF1 and pSRSF5 were constructed by insertion of *SRSF1* and *SRSF5* coding DNA sequences obtained by PCR from cDNA (see method 2.3) using respective primers (Supplementary Table 1). PCR products and vector were digested using either HindIII/BamHI or HindIII/EcoRI restriction endonucleases (Thermofisher Scientific), purified using GeneJet Gel extraction Kit (Thermofisher Scientific) from 1% TAE agarose gel and ligated into the pcDNA3 (Invitrogen, Thermofisher Scientific) vector using T4 DNA ligase (Thermofisher scientific) following the manufacturer's protocol. Ligation reactions were transformed to competent *E. coli* (DH10B) cells using heat-shock transformation method and plated on LB-Agar (Sigma-Aldrich, Merck) plates containing ampicillin. Obtained colonies were screened using PCR with gene specific primers (Supplementary Table 1). Positive colonies were propagated in liquid LB media and plasmid DNA was extracted using ZymoPUREII Maxiprep Kit (Zymo research). Gene coding sequences were confirmed by DNA sequence analysis.

U-87 MG or SK-N-Be(2) cells were seeded in 6-well plates at 2×10^5 cells/cm^2 density in complete medium and grown to ~70% of confluence. Then, cells were transfected with 2 μg of corresponding expression or empty pcDNA3 plasmid DNA using jetPRIME transfection reagent (Polyplus transfection) following manufacturer's instructions. After transfections cells were allowed to recover for 24 h before subjecting them to hypoxia. In experiments using hypoxia, cells were cultured in *Invivo200* workstation (Ruskin Technologies) at 1% O_2 for 24 h prior to collecting. All experiments were repeated at least 3 times.

2.3. RNA isolation and RT-PCR

Total RNA from cell lines was isolated using Quick-RNA™ MiniPrep kit (Zymo Research) and cDNA synthesis was carried out using SensiFast cDNA Synthesis Kit (Bioline). PCR from the obtained cDNA were performed using specific primers (Supplementary Table 1). The RT-PCR products were separated on a 1.5% agarose gel in TBE buffer. PCR products were quantified using MultiGauge analysis software (Fuji). Results were expressed as a separate mRNA isoform relative ratio (%), when both mRNA isoforms expression was set as 100%. The bar plots represent means ± SD from at least 3 independent experiments.

2.4. Reduction of cellular protein levels using CRISPR/Cas9 system

Cas9 expression vector (pSpCas9(BB)-2A-Puro (PX459)) was obtained from Addgene (Addgene no 62988). Synthesized oligos for single guide RNA (sgRNA) expression were denaturated at 95 °C for 5 min and annealed at room temperature before being cloned between two BpiI sites of linearized Cas9-sgDNA expression vector. Specific target sequences for *SRSF1* and *SRSF5* genes editing were selected by using CRISPR Design Tool (http://tools.genome-engineering.org). The oligo

sequences used for sgDNA synthesis are listed in Supplementary Table 1.

All CRISPR/Cas9 targeting experiments were performed in human glioblastoma U-87 MG and neuroblastoma SK-N-Be (2) cell lines. For targeting individual genes, cells were seeded at 8×10^4 cells per well of 12-well plate in complete medium as described earlier and transfected with constructed Cas9-sgDNA or empty Cas9 plasmid (containing no sgDNA). Cells were treated with puromycin (2 μg/ml) 72 h post transfection, diluted, and plated in to 96 well plates (1 cell/well). Cells were incubated for 2–3 weeks. Reduced target protein expression containing single cell colonies were identified by Western blotting and confirmed by DNA sequence analysis.

2.5. Western blotting

Cells were collected in a modified RIPA buffer (150 mm NaCl, 50 mm Tris-Cl pH 8.7, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mm dithiothreitol, 1 mm phenylmethylsulfonyl fluoride and 0.2 mm N-ethylmaleimide) containing a protease inhibitor mixture (Roche Applied Science). Proteins were separated on 12% SDS-polyacrylamide gel and transferred onto the nitrocellulose membrane (GE Healthcare Life science). The membrane was incubated with appropriate primary antibody: anti-SRSF1 (ThermoFisher Scientific), anti-SRSF5 (ThermoFisher Scientific), anti-β-actin (Abcam) and secondary mouse or rabbit (Agilent – Dako) antibodies conjugated with HRP. The used antibodies and used dilutions are indicated in the Supplementary Table 1. TMB reagent (ThermoFisher Scientific) was used for protein detection. β-actin was used as a loading control. The data was quantified using MultiGauge software (Fuji).

2.6. Statistical analysis

Statistical significance of all experiments was tested with a Mann Whitney U non-parametric test using R (version 3.5.0) statistical software based on a minimum of 3 independent experiments. A value of $^{**}p < 0.01$; $^{*}p < 0.05$ was considered statistically significant.

3. Results

3.1. Alternative Tau and APP pre-mRNA splicing in hypoxic U-87 and SK-N-Be(2) cells

Human glioblastoma (U-87) and neuroblastoma [SK-N-Be(2)] cells cultivated under normoxic or hypoxic conditions were used for cellular endogenous *Tau* and *APP* pre-mRNAs' alternative splicing studies (Fig. 1A and 2A). Initially we have analyzed cellular hypoxia's influence to 4R and 3R *Tau* mRNA splice variant formation. RT-PCR analysis showed an increase in 4R mRNA splice variant formation in both cell lines cultivated under hypoxic conditions compared to *Tau* 4R isoform formation in normoxic cells (Fig. 1B and D). The inclusion of exon 10 into forming mRNA differs ~2-fold between normoxic and hypoxic cells (Fig. 1B and D, lanes 1, 2).

Next, we have compared the formation of three major *APP* mRNA splice variants in normoxic and hypoxic U-87 and SK-N-Be(2) cells. Our studies have revealed that hypoxia did not have any influence on the formation of APP770, APP751 and APP695 mRNA splice variants in tested cell lines (Fig. 2B–E).

These results indicate that in U-87 and SK-N-Be(2) cells alternative Tau pre-mRNA splicing is regulated by cellular hypoxia in contrast to *APP* pre-mRNA splicing; therefore, in our further attempts to elucidate the role of individual splicing factors we have concentrated on hypoxia

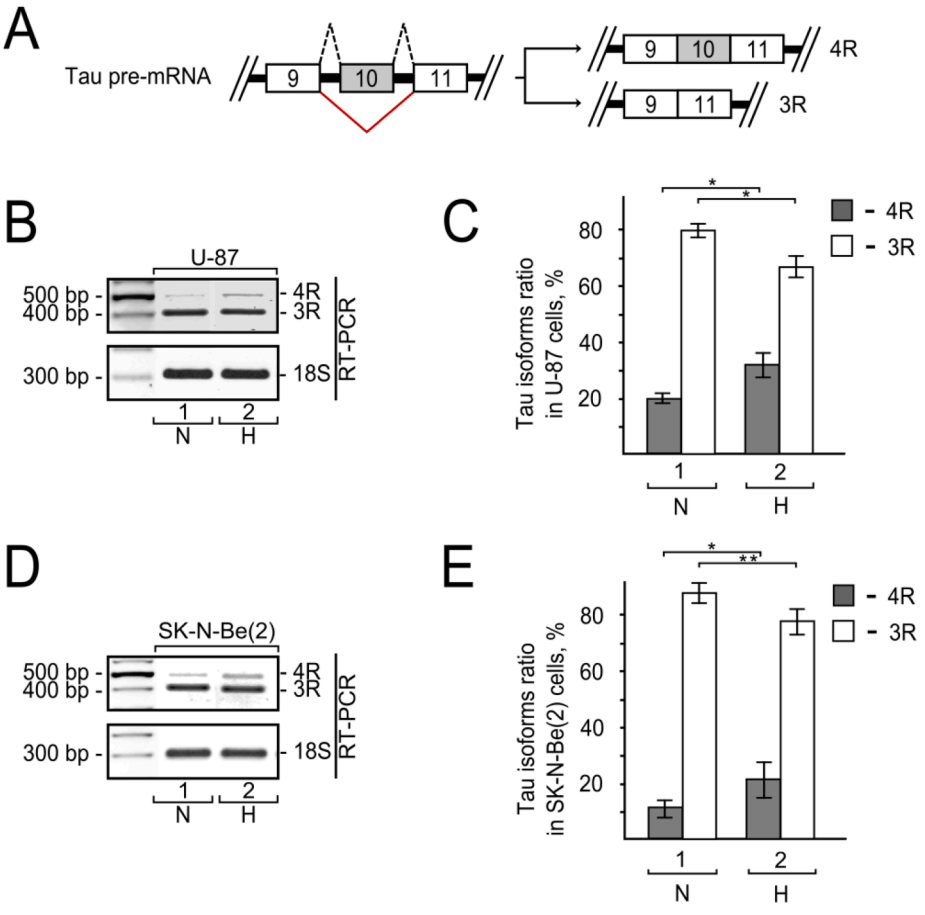

Fig. 1. *Tau* pre-mRNA alternative splicing. (A) Schematic representation of *Tau* pre-mRNA and alternatively spliced 4R and 3R mRNA splice variants. (B), (D) Reverse transcriptase PCR (RT-PCR) results of 4R and 3R isoform mRNA profiles in U-87 and SK-N-Be(2) cells cultivated under normoxic (N) or hypoxic (H) conditions. 18S RNA was used as loading control. (C), (E) Quantification of the 4R and 3R mRNA splice variant relative ratio from normoxic or hypoxic U-87 and SK-N-Be(2) cells. Total Tau (4R + 3R) pre-mRNA was accounted as 100%. The bar plots represent means ± SD from five independent experiments (n = 5), $^{**}p < 0.01$; $^{*}p < 0.05$.

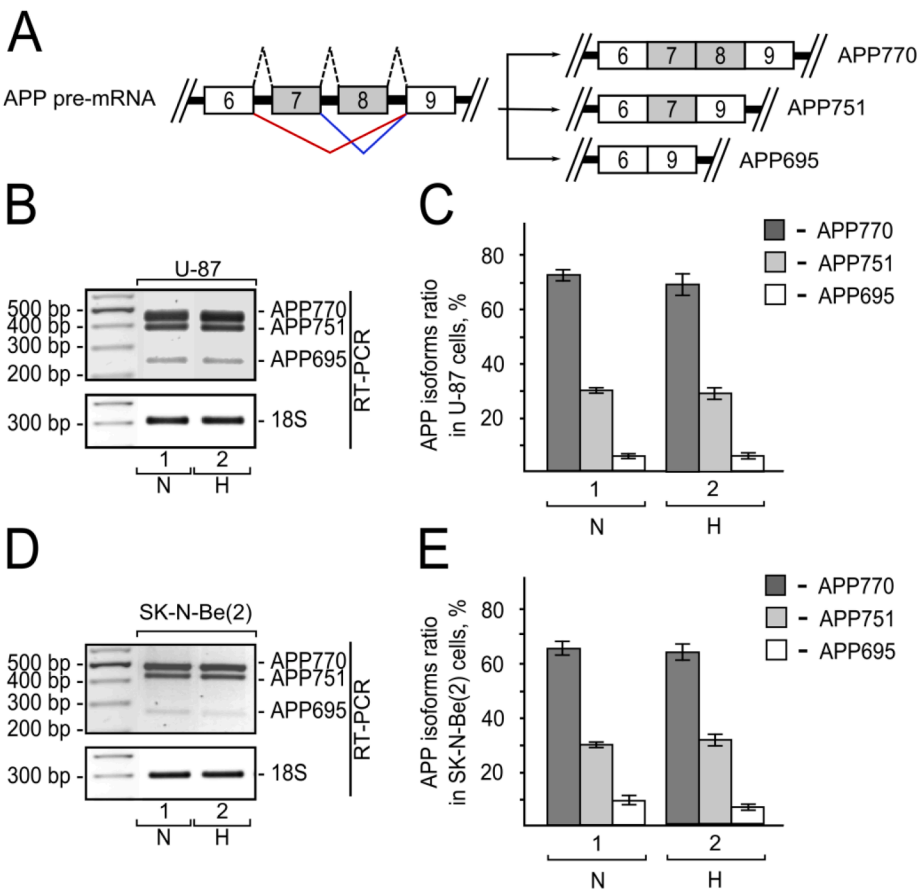

Fig. 2. *APP* pre-mRNA alternative splicing. (A) Schematic representation of *APP* pre-mRNA and alternatively spliced three major mRNA splice variants. (B), (D) RT-PCR results of APP770, APP751 and APP695 isoform mRNA profiles in U-87 cells and SK-N-Be(2) cells cultivated under normoxic (N) or hypoxic (H) conditions. 18S RNA was used as loading control. (C), (E) Quantification of the *APP* mRNA splice variant relative ratio from normoxic or hypoxic U-87 and SK-N-Be(2) cells. Total APP (APP770 + APP751 + APP695) pre-mRNA was accounted as 100%. The bar plots represent means ± SD from five independent experiments (n = 5).

regulated Tau alternative splicing.

3.2. The effects of SRSF1 or SRSF5 overexpression on Tau exon 10 inclusion/exclusion into mRNA in normoxic and hypoxic cells

SRSF1 has been identified as a regulator of *Tau* exon 10 inclusion/exclusion into *Tau* mRNA under normoxic conditions in non-neuronal and neuronal cells (D'Souza and Schellenberg, 2006). However, no data was available on SRSF5 role on *Tau* splicing regulation in brain cells. Thus, further using brain cell lines we have investigated whether splicing factors SRSF1 and SRSF5 can influence *Tau* exon 10 exclusion/inclusion into forming mRNA under hypoxic conditions.

Overexpression of SRSF1 in U-87 and SK-N-Be(2) cells cultured under normoxic conditions resulted in enhanced 4R *Tau* mRNA splice variant formation (Fig. 3A–D, lane 2). Our data revealed that SRSF1 promotes exon 10 inclusion into 4R *Tau* mRNA splice variant by ~1,5-fold compared to control cells (Fig. 3A–D, lanes 1, 2). Overexpressed SRSF1 in cells under hypoxic conditions also showed ~1.5-fold increase in exon 10 inclusion into *Tau* mRNA (Fig. 3A–D, lanes 4, 5). Overexpression of splicing factor SRSF5 in U-87 and SK-N-Be(2) cells showed that this factor does not have any significant influence on exon 10 inclusion/exclusion into forming *Tau* mRNA in cells cultivated under either normoxic or hypoxic conditions (Fig. 3A–D lanes 1, 3, 4, 6).

Comparison of SRSF1 and SRSF5 protein expression analysis showed similar amounts for SRSF1 and SRSF5 proteins in normoxic and hypoxic cellular microenvironment (Supplemental Fig. 3A, B, lanes 1, 3).

Our data has revealed that the overexpression of SRSF1, but not SRSF5, promotes *Tau* 4R mRNA formation in hypoxic U-87 and SK-N-Be (2) cells. We have not observed any significant effects of hypoxia on SRSF1 and SRSF5 protein expression levels.

3.3. The effects of reduced SRSF1 or SRSF5 protein cellular levels on Tau 4R and 3R mRNA splice variant formation in normoxic and hypoxic cells

Further we have investigated whether the reduction of SRSF1 and SRSF5 cellular protein expression would have any influence on Tau pre-mRNA alternative splicing in normoxic and hypoxic cells. To reduce endogenous SRSF1 and SRSF5 protein cellular levels in U-87 and SK-N-Be(2) cells a CRISPR/Cas9 system was used (Supplemental Fig. 4). Reduction of SRSF1 protein expression in cells cultivated under normoxic conditions caused up to 2-fold decrease in 4R mRNA splice variant formation (Fig. 4A–D, lane 1, 2). Under hypoxic conditions, reduction of SRSF1 in both cell lines showed only a slight decrease in 4R Tau mRNA splice variant formation (Fig. 4A–D, lane 4, 5), however this change is also significant regarding that hypoxia by itself increases the 4R splice variant formation.

Reduction of splicing factor SRSF5 cellular protein level did not have any significant effect on Tau 3R/4R mRNA splice variant formation ratio in both tested cell lines cultured under either normoxic or hypoxic conditions (Fig. 4A, D and B, E, lanes 3, 6). Therefore we conclude that SRSF5 is not involved in *Tau* mRNA exon 10 inclusion/exclusion regulation.

In our studies under hypoxic conditions we have determined that reduction of SRSF1 cellular protein level has only a slight effect on *Tau* 4R/3R mRNA formation ratio. We did not find any influence of SRSF5 on *Tau* exon 10 inclusion/exclusion into forming mRNA. The complexity of the cellular hypoxic response gives a strong reason to assume that alternative *Tau* exon 10 splicing could be regulated by SRSF1 together with a currently unidentified splicing factor.

3.4. The role of hypoxia on CLK family expression

The reduction of SRSF1 in hypoxic cells had only a slight effect on

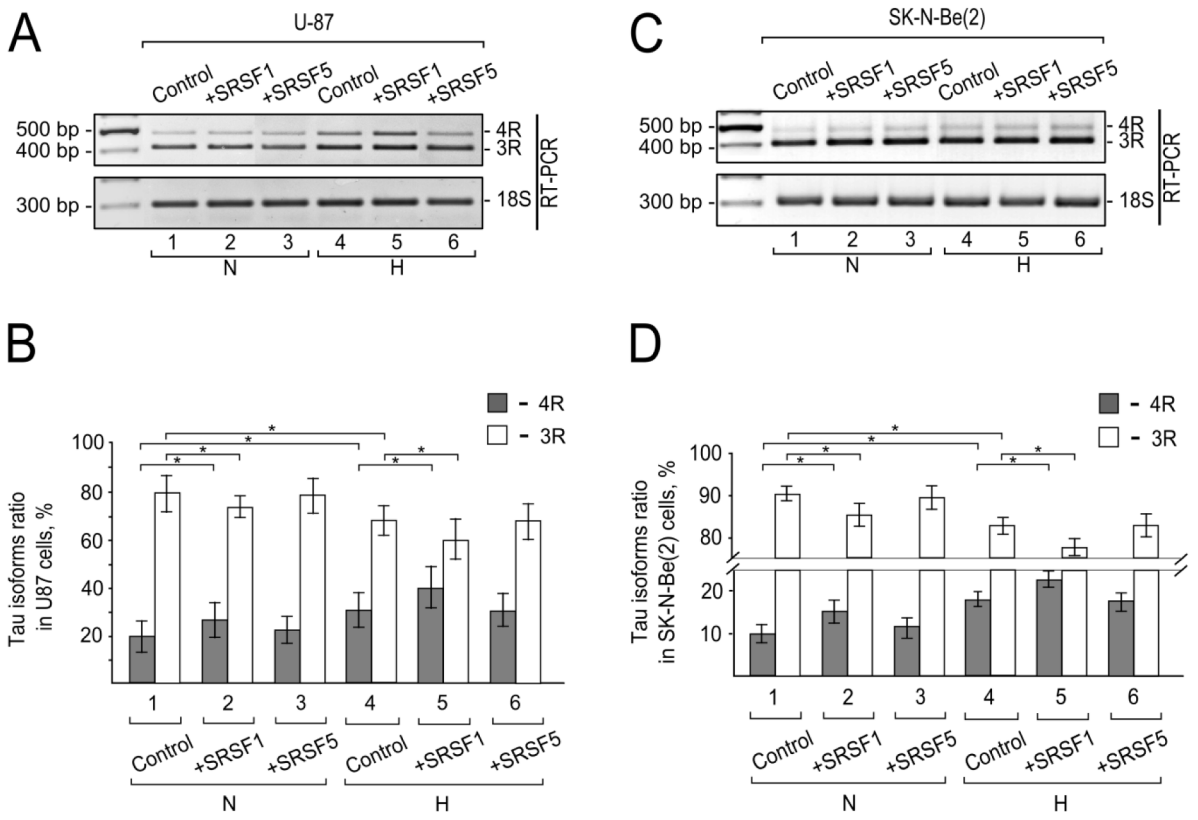

Fig. 3. The influence of splicing factor SRSF1 and SRSF5 overexpression to Tau mRNA splice variant formation profiles. (A), (C) RT-PCR results showing 4R and 3R isoform mRNA profiles in U-87 and SK-N-Be(2) cells cultivated under normoxic (N) or hypoxic (H) conditions. Lanes 1 and 4 – controls; lanes 2 and 5 – SRSF1 overexpression; lanes 3 and 6 – SRSF5 overexpression. 18S RNA was used as loading control. (B), (D) Quantification of the 4R and 3R mRNA splice variant relative ratio changes in control and SRSF1 or SRSF5 transfected U-87 and SK-N-Be(2) cells. Total Tau 4R and 3R mRNA isoforms ratio was accounted as 100%. The bar plots represents means ± SD from 3 independent experiments (n = 3), *p < 0.05.

Tau 4R and 3R mRNAs isoform formation (Fig. 4B and D, lane 5). Previously, we and others showed that expression levels of SR protein kinases (CLK1, SRPK1 and SRPK2) are enhanced in cervical and prostate cell lines cultivated under hypoxic conditions. It has also been shown that SR proteins are hyper-phosphorylated in hypoxic cells changing their activity (Jakubauskiene et al., 2015; Bowler et al., 2018). In this context, we have further investigated the expression of four proteins from the CLK kinase family (CLK1-4) in hypoxic cells. We have found that all of tested CLK kinase (CLK1, CLK2, CLK3, CLK4) mRNAs' levels were increased under hypoxic conditions in U-87 and SK-N-Be(2) cells (Fig. 5A and C).

4. Discussion

Alternative splicing is an essential step of gene expression that increases transcriptomic and proteomic diversity in eukaryotic cells (Lareau et al., 2004; McManus and Graveley, 2011). It has been shown that splicing machinery heavily contributes to cells' adaptability to altered microenvironment – hypoxia (Jakubauskiene et al., 2015; Kanopka, 2017). Cellular response to the reduction of oxygen levels results in stabilization of HIFs that activate transcription of genes involved in angiogenesis, erythropoiesis, neovascularization, iron metabolism, glucose metabolism, cell proliferation, apoptosis and cell cycle control (Lee et al., 2019). Hypoxia induces cell survival and promotes the initiation and the progression of various human diseases, including neurodegenerative disorders (Sharp and Bernaudin, 2004; Jha et al., 2018b). However, the precise mechanistic role played by hypoxia in mediating key processes of the brain and in triggering pathological signals remains unclear (Jha et al., 2018a).

One of the principal players involved in Alzheimer's disease is the presence of extracellular amyloid deposits in the brain, which is derived from the β-amyloid precursor protein (APP). It is still an open question whether hypoxia affects *APP* alternative pre-mRNA splicing. In the present study performed in brain cell lines, we showed that hypoxic cellular microenvironment does not influence exon 7–8 inclusion/exclusion into forming *APP* mRNA. More recently published study reported that hypoxia increased amyloid-β formation is due to altered expression levels of enzymes involved in the cleavage of the APP protein and dysregulation of calcium homeostasis in glial and neuronal cells (Lall et al., 2019).

The formation of insoluble intraneuronal aggregates composed of hyperphosphorylated tau proteins constitutes a major defining characteristic of Alzheimer's disease. Our results, obtained in this study, show that cellular hypoxic conditions promote exon 10 inclusion into forming *Tau* mRNA i.e. 4R Tau isoform formation. It is reported that dysregulation of Tau 3R /4R mRNA splice variant balance in the brain is sufficient to cause neurodegeneration and dementia (Liu and Gong, 2008b). Thus, for the first time, we show that cellular hypoxia, via changes in 3R/4R *Tau* mRNA formation ratio, influences the development of neurodegenerative diseases. Our results demonstrate the importance of pre-mRNA splicing in neurodegenerative diseases' development pathway.

It is known that not all cells respond to hypoxia in an identical fashion during physiological and pathological adaptations. Due to variable baseline situations and responses, different tissues cells respond to hypoxia individually (Chi et al., 2006). Therefore, cell lines derived from two: glial and neuronal brain tissue, were chosen for *APP* and *Tau* alternative pre-mRNA splicing studies in response to hypoxia. Our results revealed that the effect of hypoxia on *APP* and *Tau* alternative pre-mRNA splicing are characteristic not only glial but to neuronal cells as

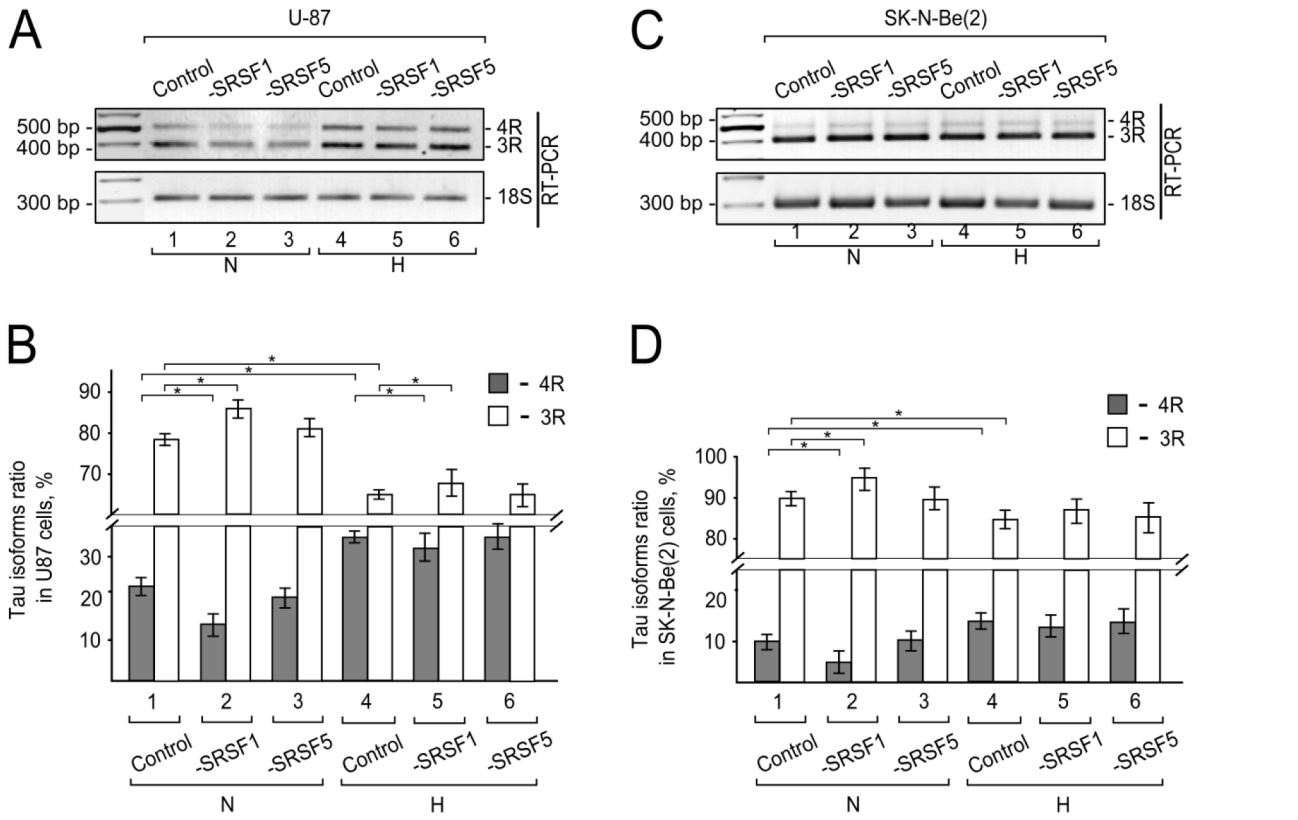

Fig. 4. The influence of reduced SRSF1 and SRSF5 cellular expression to *Tau* mRNA splice variant formation profiles. (A), (C) RT-PCR results showing 4R and 3R isoform mRNA profiles in normoxic (N) or hypoxic (H) conditions U-87 and SK-N-Be(2) cells, respectively. Lanes 1 and 4 – controls; lanes 2 and 5 – CRISPR-SRSF1 treated cells; lanes 3 and 6 – CRISPR-SRSF5 treated cells. 18S RNA was used as loading control. (B), (D) Quantification of the 4R and 3R mRNA splice variant relative ratio changes in control and CRISPR-SRSF1 or CRISPR-SRSF5 treated U-87 and SK-N-Be(2) cells, respectively. Total Tau 4R and 3R mRNA isoforms ratio was accounted as 100%. The bar plots represent means ± SD from three independent experiments (n = 3), *p < 0.05.

well.

Regulation of alternative splicing is a complex process in which cis-acting elements (exonic/intronic splicing enhancers and silencers) and trans-acting splicing factors are involved (Wang et al., 2015). One of the trans-acting players is a family of serine/arginine-rich (SR) proteins that have a diverse role in alternative splicing (Long and Caceres, 2009; Jeong, 2017). Splicing factor SRSF1 has been identified as an essential regulator of *Tau* exon 10 splicing in non-neuronal HeLa and neuronal PC12 cells (D'Souza and Schellenberg, 2006); however, very little is known about the influence of splicing factor SRSF5 on Tau 4R/3R mRNA splice variant formation in brain cells (Qian and Liu, 2014).

Our results revealed that SRSF1, but not SRSF5, in cells cultivated under normoxic conditions is involved in exon 10 inclusion/exclusion regulation into forming *Tau* mRNA. Recently in literature it has been shown that splicing factors, which are involved in alternative splicing regulation in normoxic cells, do not regulate the alternative splicing of the same pre-mRNAs in cells cultured under hypoxic conditions (Peciuliene et al., 2019). Thus, it was unclear if the role of SRSF1 and SRSF5 factors in *Tau* 3R/4R mRNA splice variant formation regulation in hypoxic cells is the same as in cells cultivated under normoxic conditions. Our investigations using brain cell lines cultivated under hypoxic conditions showed that enhanced SRSF1 cellular expression level promotes *Tau* exon 10 inclusion, whereas reduction of SRSF1 slightly suppressed it. Our results also show that altered SRSF1 cellular expression level changes the balance between *Tau* 3R/4R mRNA splice variants in hypoxic cells. It is known from previous studies that SR proteins are hyper-phosphorylated, changing their activity, in hypoxic cells (Jakubauskiene et al., 2015; Bowler et al., 2018). There is a high possibility that changes in SRSF1 splicing regulation ability in hypoxic cells are due to differential protein modification or because of participation of a currently unidentified splicing factor together with SRSF1.

We and other researchers have previously shown that cellular hypoxia enhances SR protein kinases' expression levels in cervical and prostate cell lines (Jakubauskiene et al., 2015; Bowler and Ladomery, 2019). In the present studies we demonstrate that the expression levels of CLK1-4 kinases are enhanced in hypoxic U-87 and SK-N-Be(2) cell lines. However, Hartmann et al. reported that overexpression of mouse CLK1-4 protein kinases in HEK293 cells promotes exon 10 skipping in *Tau* minigene construct (Hartmann et al., 2001). Despite enhanced kinase expression levels in hypoxic cells, our data shows that exon 10 inclusion is promoted into forming *Tau* mRNA content. Such discrepancy can be explained by the fact that in our studies we have used endogenous mRNA, while a minigene construct, mouse derived proteins and different cell lines were used to obtain results described in the literature.

To sum up, our studies show that *Tau* but not *APP* pre-mRNA's alternative splicing is dependent on cellular hypoxic microenvironment. Cellular hypoxia changes Tau 3R/4R mRNA splice variant ratio what contributes to AD progression. We have demonstrated that in hypoxic cells SRSF1 alters the formation ratio between *Tau* 4R and 3R mRNA splice variants. SRSF5 does not influence exon 10 inclusion/exclusion into *Tau* mRNA in neither normoxic nor hypoxic cells. We also show that hypoxic brain cells possess enhanced *CLK1-4* kinase mRNA levels compared to cells cultivated under normoxic conditions. Of course, the precise enhanced CLK kinases expression role in the regulation of Tau exon 10 exclusion/inclusion into forming mRNA will be determined in the future. Our studies for the first time show the contribution of hypoxia and hypoxia-mediated pathways to neurodegeneration.

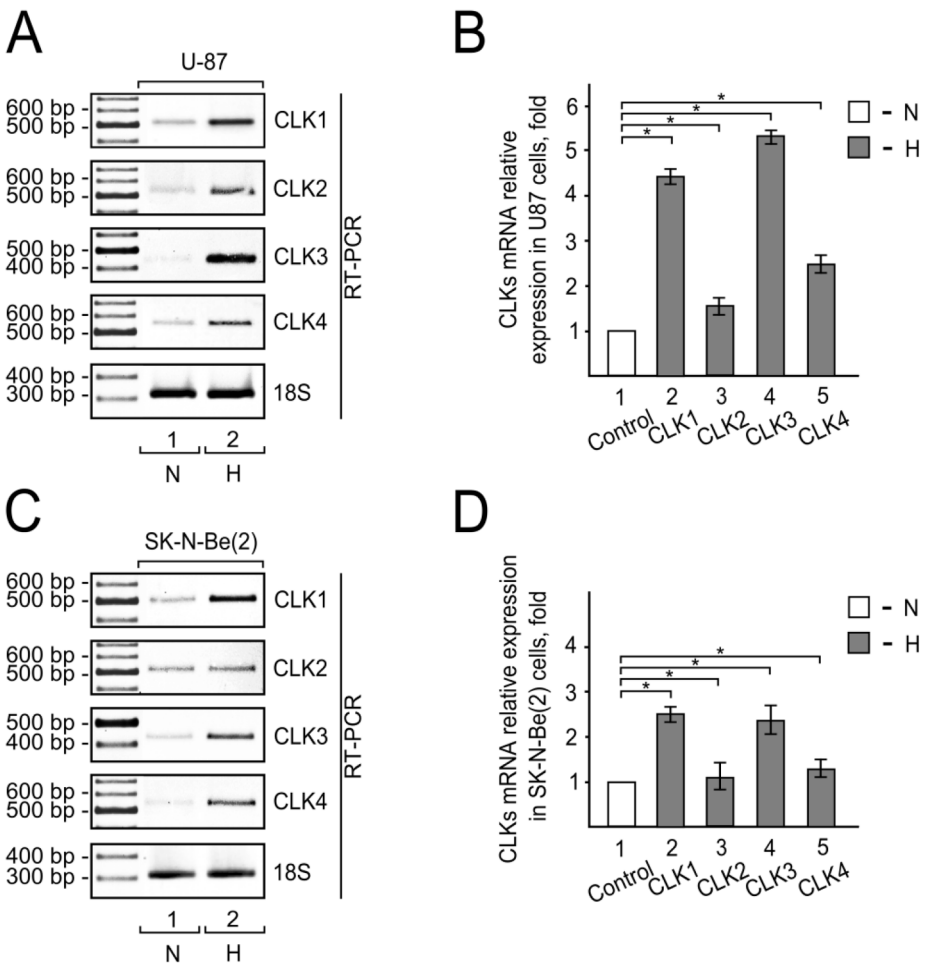

Fig. 5. Hypoxia induces changes in the expression of CLK family kinases. (A), (C) The expression of four CLKs at mRNA level in normoxic (1) or hypoxic (2) U-87 and SK-N-Be(2) cell lines, respectively. 18S RNA was used as loading control. (B), (D) Quantification of four CLKs mRNA expression levels between normoxic and hypoxic U-87 and SK-N-Be(2) cells, respectively. Normoxic expression levels of each CLK mRNA were set to 1 (lane 1 – control) and are represented in the figure by a single column for simplicity; lanes 2, 3, 4, 5 – represent CLK1, CLK2, CLK3, CLK4 mRNA expression fold changes in hypoxia treated cells compared to the control. The bar plots represent means ± SD from five independent experiments (n = 5) performed in triplicates, * $p < 0.05$.

Author contributions

EJ conducted RNA experiments, analyzed and interpreted the data, wrote the manuscript. LV contributed to the plasmid vector construction and performed the transfections experiments. IP helped in interpreting the results. AK is supervision of EJ and revised the manuscript. All authors read and approved the final manuscript.

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CRediT authorship contribution statement

Eglė Jakubauskienė: Conceptualization, Investigation, Methodology, Writing - review & editing, Visualization. **Laurynas Vilys:** Methodology, Writing - review & editing. **Inga Pečiulienė:** Conceptualization, Writing - review & editing. **Arvydas Kanopka:** Supervision, Project administration, Funding acquisition, Conceptualization, Writing - original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2020.145146.

References

Araki, S., Dairiki, R., Nakayama, Y., Murai, A., Miyashita, R., Iwatani, M., Nomura, T., Nakanishi, O., 2015. Inhibitors of CLK protein kinases suppress cell growth and induce apoptosis by modulating pre-mRNA splicing. PLoS One 10, e0116929.

Biamonti, G., Amato, A., Belloni, E., Di Matteo, A., Infantino, L., Pradella, D., Ghigna, C., 2019. Alternative splicing in Alzheimer's disease. Aging Clin. Exp. Res.

Bowler, E., Ladomery, M.R., 2019. Working with hypoxia. Methods Mol. Biol. 1990, 109–133.

Bowler, E., Porazinski, S., Uzor, S., Thibault, P., Durand, M., Lapointe, E., Rouschop, K. M.A., Hancock, J., Wilson, I., Ladomery, M., 2018. Hypoxia leads to significant changes in alternative splicing and elevated expression of CLK splice factor kinases in PC3 prostate cancer cells. BMC Cancer 18, 355.

Bradley, T., Cook, M.E., Blanchette, M., 2015. SR proteins control a complex network of RNA-processing events. RNA 21, 75–92.

Chi, J.T., Wang, Z., Nuyten, D.S., Rodriguez, E.H., Schaner, M.E., Salim, A., Wang, Y., Kristensen, G.B., Helland, A., Borresen-Dale, A.L., Giaccia, A., Longaker, M.T., Hastie, T., Yang, G.P., van de Vijver, M.J., Brown, P.O., 2006. Gene expression programs in response to hypoxia: cell type specificity and prognostic significance in human cancers. PLoS Med. 3, e47.

D'Souza, I., Schellenberg, G.D., 2006. Arginine/serine-rich protein interaction domain-dependent modulation of a tau exon 10 splicing enhancer: altered interactions and mechanisms for functionally antagonistic FTDP-17 mutations Delta280K AND N279K. J. Biol. Chem. 281, 2460–2469.

Dvinge, H., 2018. Regulation of alternative mRNA splicing: old players and new perspectives. FEBS Lett. 592, 2987–3006.

Fragkouli, A., Koukouraki, P., Vlachos, I.S., Paraskevopoulou, M.D., Hatzigeorgiou, A.G., Doxakis, E., 2017. Neuronal ELAVL proteins utilize AUF-1 as a co-partner to induce neuron-specific alternative splicing of APP. Sci. Rep. 7, 44507.

Giannakouros, T., Nikolakaki, E., Mylonis, I., Georgatsou, E., 2011. Serine-arginine protein kinases: a small protein kinase family with a large cellular presence. FEBS J. 278, 570–586.
Hartmann, A.M., Rujescu, D., Giannakouros, T., Nikolakaki, E., Goedert, M., Mandelkow, E.M., Gao, Q.S., Andreadis, A., Stamm, S., 2001. Regulation of alternative splicing of human tau exon 10 by phosphorylation of splicing factors. Mol. Cell. Neurosci. 18, 80–90.
Imbimbo, B.P., Lombard, J., Pomara, N., 2005. Pathophysiology of Alzheimer's disease. Neuroimaging Clin. N. Am. 15 (727–753), ix.
Izquierdo, J.M., Valcarcel, J., 2006. A simple principle to explain the evolution of pre-mRNA splicing. Genes Dev. 20, 1679–1684.
Jain, P., Karthikeyan, C., Moorthy, N.S., Waiker, D.K., Jain, A.K., Trivedi, P., 2014. Human CDC2-like kinase 1 (CLK1): a novel target for Alzheimer's disease. Curr. Drug Targets 15, 539–550.
Jakubauskiene, E., Vilys, L., Makino, Y., Poellinger, L., Kanopka, A., 2015. Increased serine-arginine (SR) protein phosphorylation changes pre-mRNA splicing in hypoxia. J. Biol. Chem. 290, 18079–18089.
Jeong, S., 2017. SR proteins: binders, regulators, and connectors of RNA. Mol. Cells 40, 1–9.
Jha, N.K., Jha, S.K., Sharma, R., Kumar, D., Ambasta, R.K., Kumar, P., 2018. Hypoxia-induced signaling activation in neurodegenerative diseases: targets for new therapeutic strategies. J. Alzheimer's Disease 62, 15–38.
Kametani, F., Hasegawa, M., 2018. Reconsideration of amyloid hypothesis and tau hypothesis in Alzheimer's disease. Front. Neurosci. 12, 25.
Kanopka, A., 2017. Cell survival: interplay between hypoxia and pre-mRNA splicing. Exp. Cell Res. 356, 187–191.
Lall, R., Mohammed, R., Ojha, U., 2019. What are the links between hypoxia and Alzheimer's disease? Neuropsychiatr. Dis. Treat. 15, 1343–1354.
Lareau, L.F., Green, R.E., Bhatnagar, R.S., Brenner, S.E., 2004. The evolving roles of alternative splicing. Curr. Opin. Struct. Biol. 14, 273–282.
Laurent, C., Buee, L., Blum, D., 2018. Tau and neuroinflammation: what impact for Alzheimer's disease and tauopathies? Biomed. J. 41, 21–33.
Lee, J.W., Ko, J., Ju, C., Eltzschig, H.K., 2019. Hypoxia signaling in human diseases and therapeutic targets. Exp. Mol. Med. 51, 68.
Liu, F., Gong, C.X., 2008a. Tau exon 10 alternative splicing and tauopathies. Mol. Neurodegener. 3.
Liu, F., Gong, C.X., 2008b. Tau exon 10 alternative splicing and tauopathies. Mol. Neurodegener. 3, 8.
Long, J.C., Caceres, J.F., 2009. The SR protein family of splicing factors: master regulators of gene expression. Biochem. J. 417, 15–27.
Matsui, T., Ingelsson, M., Fukumoto, H., Ramasamy, K., Kowa, H., Frosch, M.P., Irizarry, M.C., Hyman, B.T., 2007. Expression of APP pathway mRNAs and proteins in Alzheimer's disease. Brain Res. 1161, 116–123.
McManus, C.J., Graveley, B.R., 2011. RNA structure and the mechanisms of alternative splicing. Curr. Opin. Genet. Dev. 21, 373–379.
Merelli, A., Rodriguez, J.C.G., Folch, J., Regueiro, M.R., Camins, A., Lazarowski, A., 2018. Understanding the role of hypoxia inducible factor during neurodegeneration for new therapeutics opportunities. Curr. Neuropharmacol. 16, 1484–1498.
Nakayama, K., Kataoka, N., 2019. Regulation of gene expression under hypoxic conditions. Int. J. Mol. Sci. 20.
Nalivaeva, N.N., Turner, A.J., 2013. The amyloid precursor protein: a biochemical enigma in brain development, function and disease. FEBS Lett. 587, 2046–2054.
Naro, C., Sette, C., 2013. Phosphorylation-mediated regulation of alternative splicing in cancer. Int. J. Cell Biol. 2013, 151839.
Peciuliene, I., Vilys, L., Jakubauskiene, E., Zaliauskiene, L., Kanopka, A., 2019. Hypoxia alters splicing of the cancer associated Fas gene. Exp. Cell Res. 380, 29–35.
Qian, W., Liu, F., 2014. Regulation of alternative splicing of tau exon 10. Neurosci. Bull. 30, 367–377.
Ruas, J.L., Poellinger, L., 2005. Hypoxia-dependent activation of HIF into a transcriptional regulator. Semin. Cell Dev. Biol. 16, 514–522.
Selkoe, D.J., 2001. Alzheimer's disease: genes, proteins, and therapy. Physiol. Rev. 81, 741–766.
Sena, J.A., Wang, L., Heasley, L.E., Hu, C.J., 2014. Hypoxia regulates alternative splicing of HIF and non-HIF target genes. Mol. Cancer Res. 12, 1233–1243.
Sharp, F.R., Bernaudin, M., 2004. HIf1 and oxygen sensing in the brain. Nat. Rev. Neurosci. 5, 437–448.
Shepard, P.J., Hertel, K.J., 2009. The SR protein family. Genome Biol. 10, 242.
Wang, Y., Liu, J., Huang, B.O., Xu, Y.M., Li, J., Huang, L.F., Lin, J., Zhang, J., Min, Q.H., Yang, W.M., Wang, X.Z., 2015. Mechanism of alternative splicing and its regulation. Biomed. Rep. 3, 152–158.
Will, C.L., Luhrmann, R., 2011. Spliceosome structure and function. Cold Spring Harbor Perspect. Biol. 3.
Wu, X.L., Pina-Crespo, J., Zhang, Y.W., Chen, X.C., Xu, H.X., 2017. Tau-mediated neurodegeneration and potential implications in diagnosis and treatment of Alzheimer's disease. Chin. Med. J. 130, 2978–2990.
Zhang, F., Niu, L., Li, S., Le, W., 2019. Pathological impacts of chronic hypoxia on Alzheimer's disease. ACS Chem. Neurosci. 10, 902–909.
Zhou, J., Yu, Q., Zou, T., 2008. Alternative splicing of exon 10 in the tau gene as a target for treatment of tauopathies. BMC Neurosci. 9 (Suppl. 2), S10.
Zhou, Z., Fu, X.D., 2013. Regulation of splicing by SR proteins and SR protein-specific kinases. Chromosoma 122, 191–207.