Investigating the Impact of Alternative Splicing on Behavioural Plasticity in Animals

Bachelor thesis Biology

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Preface

Presented before you is the bachelor thesis entitled “Investigating the Impact of Alternative Splicing on Behavioural Plasticity in Animals”. This thesis has been written to fulfill the final requirements for my pre-master’s program Biology at the University of Groningen.

After finishing the course ‘Genes and Behaviour’, I knew I wanted Jean-Christoph to be my supervisor. His profound expertise in genetics, a research field that aligned with my academic passions, made him an ideal supervisor. In my previous Biotechnology bachelor’s degree, I also did an internship in the University Medical Centre Groningen (UMCG) at the department of pediatrics, section molecular genetics, which I found fascinating.

When Jean-Christoph and I had a conversation about potential topics for this thesis, the subject alternative splicing intrigued me. Later we decided that it was going to be about how alternative splicing could influence behavioural plasticity. Jean-Christoph told me that he did not read articles about this subject yet, which made me extra motivated. It also gave me chances to develop my own opinion about the subject as well. This was also quite challenging for me, since I have only written a thesis about my own experiments, which is in my opinion more straightforward.

I would like to thank Jean-Christoph for supporting me where necessary. In the period between starting the thesis, and now finalizing it, many things have happened such as the bachelor project. I am grateful that I have gotten this space. There was a point where I was a bit stuck within writing, but then there was Jean-Christoph who gave me some practical tips.

I hope you enjoy reading my thesis.

Britt Martijn

Hoogeveen, May 25, 2024

Summary

Alternative splicing (AS) is a post-translational process in which exons from the same gene are joined in different combinations leading to different mature mRNA strands. AS is the mechanism behind the discrepancy between the 25,000 genes recorded in humans and the 100,000 proteins that our species actually makes. Most genes in humans generate a minimum of two alternative transcripts. These alternative spliced mature mRNA strands are finally translated into many different proteins with different structures and functions. A higher level of alternative splicing leads to more functionally distinct protein isoforms which increases organism complexity. However, the role of alternative splicing in phenotype complexity remains quite unclear. Behaviour is the most complex phenotype observed and changes within different environments. Therefore, it is unlikely that behaviour is only determined by an animal's genotype. So, could it be that behavioural plasticity is driven by the mechanism of alternative splicing?

This thesis showed multiple examples of how alternative splicing is related to a certain behaviours or behavioural phenotypes observed within a disease such as sensitization, circadian rhythm, reproduction, sex differentiation, aggressiveness, learning, autism-like behaviour, schizophrenia, depression, and anxiety. These examples indicate that alternative splicing could potentially be an important molecular mechanism influencing behaviour and physiological differences between individuals. Alternative splicing could thus contribute to proteome diversification which is associated with behavioural plasticity.

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1 Introduction

Alternative splicing (AS) is a post-translational process where exons from the same gene are rearranged in various combinations leading to diverse mature mRNA strands (figure 1). The process of AS was first identified as a mechanism for removing viral sequences from a pre-mRNA sequence (Berget, Moore, & Sharp, 1977). The remaining sequences were joined together and led to mature mRNA strands. From initial observation in bacteria, the phenomenon 'alternative splicing' was found to be widespread and explain previous paradoxes. For instance, AS is the mechanism behind the discrepancy between the 25,000 genes recorded in humans and the 100,000 proteins that our species makes (International Human Genome Sequencing Consortium, 2004). There needed to be a process responsible for this major diversity in protein sequences, which was later acknowledged to be through alternative splicing. Researchers discovered later that more than 95% of the human genes undergo alternative splicing (Nilsen & Graveley, 2010).

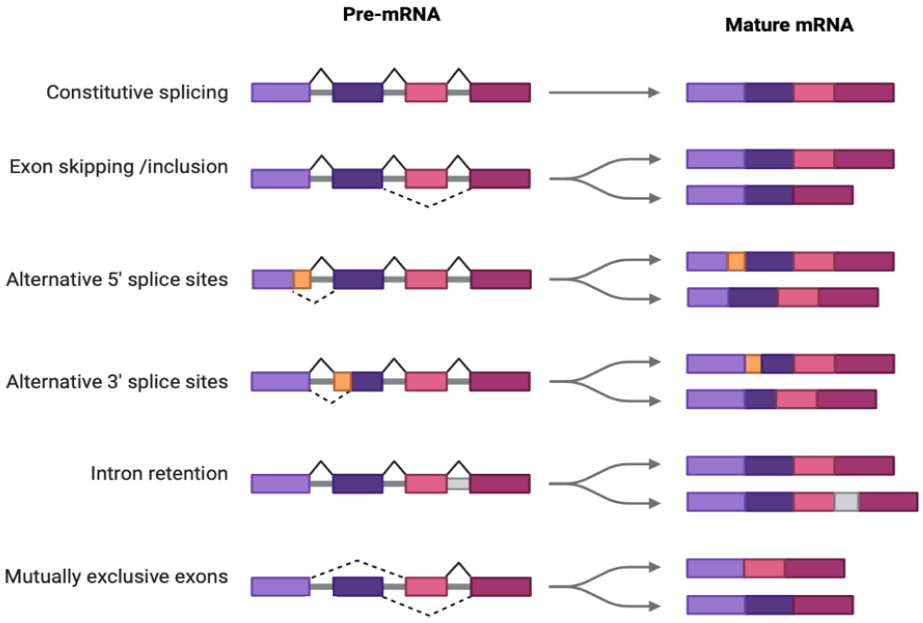

Figure 1. Representation of splicing methods. *Constitutive splicing is the general splicing method and exon skipping, alternative 5' and 3' splice sites, intron retention, and mutually exclusive exons are alternative splicing methods.*

Currently, there are six known types of splicing, including constitutive splicing, which is the regular type of splicing, and alternative types such as, exon skipping, alternative 5' splice site (SS), alternative 3' splice site, intron retention, and mutually exclusive exons (figure 1) (Bhadra, Howell, Dutta, Heintz, & Mair, 2020). Most genes in humans generate a minimum of two alternative transcripts. The mature mRNA strands resulting from AS are ultimately translated into many different proteins with distinct structures and functions.

Exon skipping is most prevalent method of AS in animals. This happened to be through a two-step process. Initially, exon skipping in early animals occurred with greater frequency for preserving the reading frame. Following that, there was a significant surge in exon skipping occurrences among bilateral ancestors. This was probably because of a genetic shift towards more exon-definition and frame-preserving exon skipping events to diversify their proteome (Grau-Bové, Ruiz-Trillo, & Irimia, 2018). Plants show a low level of alternative splicing. In plants, intron retention is the most common form of alternative splicing (approx. 50%) and exon skipping the least common type (Kim, Goren, & Ast, 2008). There are two main hypotheses explaining the lower alternative splicing in plants compared to animals. First, the analysis of alternative splicing is at a considerably earlier phase in plants in contrast to humans and rats (Zhang, Yang, & Yang, 2016). Another hypotheses dates back to 1994 when Hughes proposed that a gene duplication possibly leads to two different copies of the gene which results in two alternative splicing isoforms. This principle is called 'functional-sharing' (Hughes, 1994). Intron retention is also common in protozoa and fungi (Edgell, Belfort, & Shub, 2000) (Yokobori, et al., 2009). Until today, there is no reported alternative splicing happening in bacteria and archaea. In prokaryotes, transcription and translation happens simultaneously, which makes it impossible to perform alternative splicing (Rex, Surin, Besse, Schneppe, & McCarthy, 1994). Despite that there is not AS in archaea, there is evidence of intron splicing

and pre-RNA processing, but further links between alternative splicing and archaea are not known (Tang, et al., 2002).

The spliceosome is an important molecule for splicing: it removes introns from pre-mRNA. To remove introns, introns are defined by (1) the 5' splice donor site (SDS), (2) branch point adenosine, and (3) 3' splice acceptor site (SAS). The spliceosome is formed from 5 small nuclear RNAs (snRNAs): U1, U2, U4, U5, and U6. Each snRNA binds to a particular set of proteins, giving rise to a small nuclear ribonucleoprotein (snRNP) particle. Inside the spliceosome, the snRNAs play crucial roles in both catalysis and recognizing substrates. The snRNAs and snRNP particles together form the spliceosome (figure 2) (Will & Lührmann, 2011).

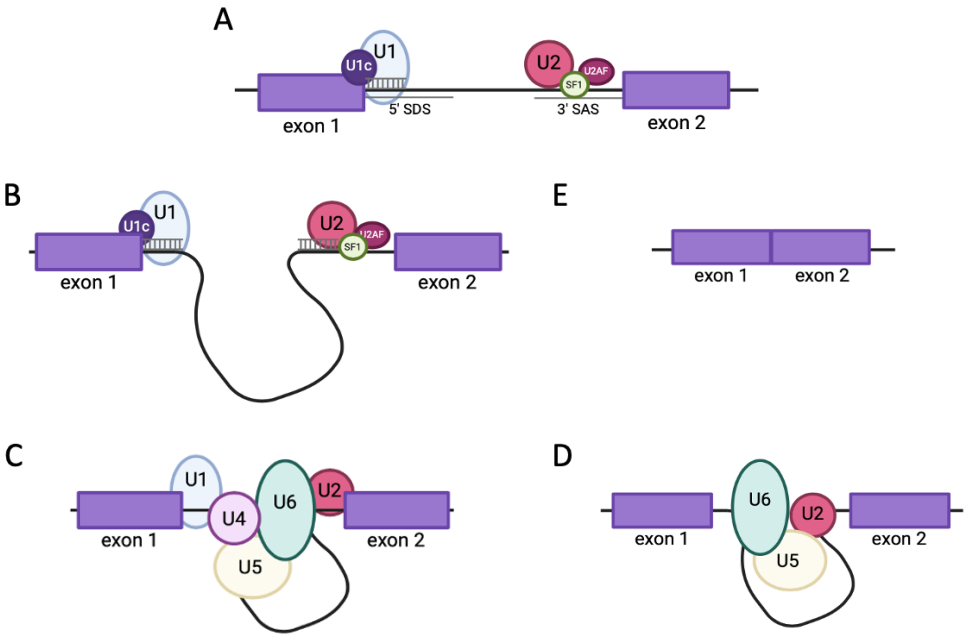

Figure 2. Representation of the splicing process. *(A) U1 snRNP identifies the splice donor site and U2 small nuclear RNAs (snRNP) identifies the splice acceptor side to form the spliceosome complex. (B) U2 identifies the adenosine at the branch-site. (C) The U4/U6/U5 tri-snRNP connects the spliceosome. (D) U4 and U1 are released, and splicing reaction is performed. (E) Exons are connected.*

Alternative splicing evolves through either *cis*- or *trans*-mutations (Singh & Ahi, 2022). New isoforms in *cis*-mutations are produced through a mutation in a gene. For example, in intron retention, a mutation causes an intron to become an exon and would therefore be included

in the mature mRNA sequence (Ast, 2004). Normally, the introduction of a new exon in a gene would lead to a frameshift mutation. However, negative selection pressure against the expression of a new alternative isoform is tempered since the ancestral isoform is continuously expressed at regular levels. This is possible when only one allele is modified leading to the alternative isoform. When the new isoform has an adaptive value, the allele frequency across the population might increase (Singh & Ahi, 2022). Mutations in *trans*-acting factors affects the specificity of splice site recognition, which is crucial for splicing in multiple exons genes. When mutations happen in *trans*-acting factor, this might cause differential gene expression (Sterne-Weiler & Sanford, 2014).

Next to *cis*- or *trans*-mutation in introns or exons, alternative splicing can also arise from *cis*- or *trans*-regulatory mutations (Ule & Blencowe, 2019). *Cis*-regulatory mutations mainly affects a single gene by changing splice-sites of RNA-elements, whereas *trans*-regulatory mutations affect splicing factors or spliceosome proteins, leading to large scale effects on multiple genes (Kornblihtt, et al., 2013). By altering spliceosome proteins, the splice-site recognition is modified leading to novel splice forms (Ast, 2004).

The level of alternative splicing varies across species. A higher level of alternative splicing leads to more functionally distinct protein isoforms which increases organism complexity (Nilsen & Graveley, 2010). There are some examples in which alternative splicing seems to have a role in generating phenotypic diversity such as reproductive behaviour in *Drosophila melanogaster*. Courtship behaviour in *D. melanogaster* males requires proteins encoded by one gene that has different sex-specific isoforms through alternative splicing (Salvemini, Polito, & Saccone, 2010). In this example, sex-specific alternative splicing has a direct effect on a phenotypic behaviour, which will be discussed more in-depth later. However, the role of alternative splicing in phenotype complexity remains quite unclear (Verta & Jacobs, 2022). Besides the level of alternative splicing, the type also varies across species.

Almost all organisms show some type of alternative splicing even though it might be very limited. This fact suggests its prominent role for the mechanisms in evolutionary mechanisms. Chen *et al*. demonstrated that the number of alternative spliced genes in eukaryotic species has moderately increased over the last 1,400 million years (Chen, Bush, Tovar-Corona, Castillo-Morales, & Urrutia, 2014). Since then, research showed a correlation between splice variations and ecological adaptations. For example, in deer mice (genus *Peromyscus*), multiple alternative isoforms are expressed from the pigmentation gene *Agouti*. Deer mice who live on the Nebraska Sand Hill have locally adapted to light-coloured populations in the last 10,000 years (Mallarino, Linden, Linnen, & Hoekstra, 2017). These mice increased a single *Agouti* isoform which led to the light-coloured skin camouflaging them in the sandy environment. The colour of the mice is an important mechanism for camouflage and is used to avoid predation (Dice, 2004).

Behaviour is the most complex phenotype observed. Animals can continuously change their behaviour in changing environments, which is also called behavioural plasticity. Therefore, it is not possible that all behaviour observed in animals is only controlled by an animal's genotype. So, could it be that behavioural plasticity is driven by the mechanism of alternative splicing?

To investigate this, this thesis will dive into on the effect of alternative splicing on receptors, channels, and transcription factor transcripts and how this eventually influences behaviour in different animals. These proteins are of specific interest because they are crucial in obtaining and transmitting signals from the environment. Besides this, by activating receptors and ion channels, transcription factors can be selectively activated leading to differential gene expression (figure 3).

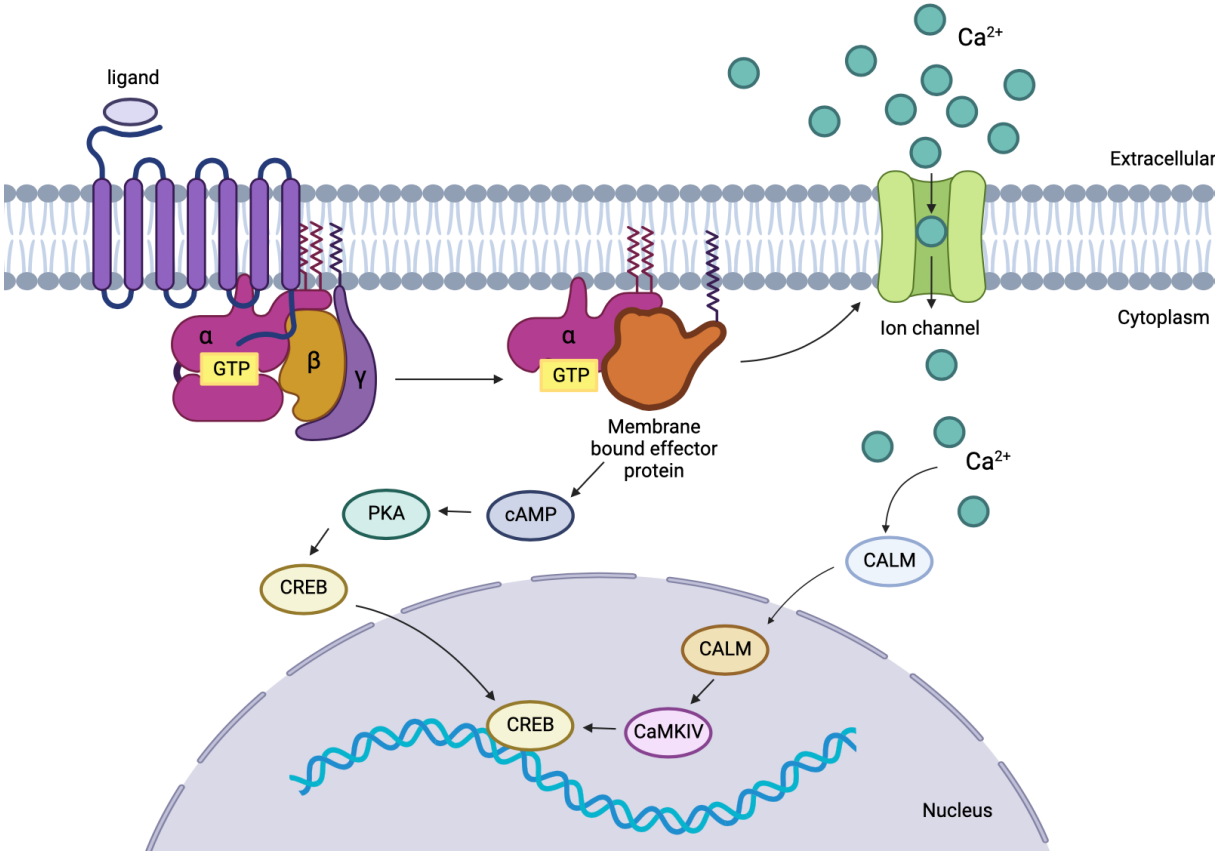

***Figure 3. Example of signaling transduction pathway**. When a ligand binds the G-coupled protein receptor (GCPR), this leads to the activation of membrane bound effector protein. This activates a signaling cascade which finally activates a transcription factor, e.g. CREB. The transcription factor then moves in the nucleus and enhances gene expression. When an ion channel gets activated and opens, ions can enter the cytosol, e.g. Ca^{2+}. Through a signaling pathway this could also lead to the activation of a transcription factor, e.g. CREB.*

2 Receptors

Cellular receptors are proteins that are present in the inside or the surface of cells. Receptors bind specific ligand which lead to the activation of the receptor and thereby a cellular response such as altering gene transcription or translation (Heldin, Lu, Evans, & Gutkind, 2016). There are different types of receptors such as channel-linked receptors, internal receptors, G-protein coupled receptors (GPCR), and enzyme-linked receptors (Purves, et al., 2001).

In *Caenorhabditis elegans* (*C. elegans*) learning behaviour is depended on the cell-surface insulin receptor (IR) *daf-2* which is alternatively spliced (Tomioka, Naito, Kuroyanagi, & Iino, 2016). This organism contains only 302 neurons which consist of 60 sensory neurons (White, Southgate, Thomson, & Brenner, 1986). *C. elegans* uses sensory neurons present in the tip of the nose to learn behaviour responses and avoid unfavorable conditions. Each amphid includes 12 sensory neurons that are important sensors for environmental chemicals and temperature. The insulin receptor is an important signaler in the taste receptor neuron ASE right (ASER) that plays a crucial role in taste-avoidance learning. During this learning, *C. elegans* learns to avoid salt concentrations during starvation conditions (Tomioka, et al., 2006) (Ohno, et al., 2014).

C. elegans expresses two isoforms of the insulin receptor: DAF-2a and DAF-2c which differ in the inclusion or skipping of exon 11.5 (figure 4) (Ohno, et al., 2014). The alternative splicing of daf-2c (exon 11.5+) is regulated by the RNA binding alternative splice factors

RBFOX, CELF, and PTB proteins. These alternative splice factor proteins are restricted to neurons where *daf-2* exon 11.5 inclusion splice variants are present.

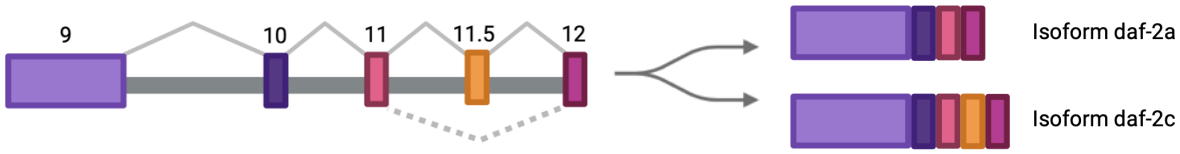

Figure 4. Schematic representation of alternative splicing mechanisms of insulin receptor (IR) pre-mRNA in C. elegans. *Isoform daf-2a splices out exon 11.5 and isoform daf-2c includes exon 11.5.*

Tomioka *et al*. showed that alternative spliced *daf-2* exon 11.5 inclusion is essential for taste-avoidance learning (Tomioka, Naito, Kuroyanagi, & Iino, 2016). In specific, splice factor PTB seems to be a critical determinant for exon 11.5 inclusion since mutations in PTB regions lead to defects in taste-avoidance learning. In mammals, RBFOX, CELF and PTB families are also alternative splicing factors that function in the brain. RBFOX transcription factor binds to UGCAUG sequence which is present in the intron downstream of exon 11.5. Surprisingly, humans and mouse IR genes have the RBFOX-binding motive near the 5' splice site in the intron downstream exon 11 (Ohno, et al., 2014). This evidence shows the biological importance of alternative splicing of the IR pre-mRNA strand regulated by RBFOX. Besides this, mammalian CELF family proteins together with other splicing factors, including PTB, regulate alternative splicing in a cell-type specific manner (Gromak, Matlin, Cooper, & Smith, 2003). This raises the possibility that CELF, RBFOX, and PTB might cooperatively control AS in mammalian insulin receptor genes, analogous to the alternative splicing of exon 11.5 in *C. elegans*. Dysfunction of neuronal IR signaling is linked to various neurological diseases, such as Alzheimer's disease (AD) (Blázquez, Velázquez, Hurtado-Carneiro, & Ruiz-Albusac, 2014).

Alzheimer's disease is a neurodegenerative disease in which patients suffer from memory loss, cognitive impairment, and behavioural changes. AD is characterized by the accumulation of amyloid-β (Aβ) and tau proteins both leading to the degeneration of neurons (DeTure & Dickson, 2019). Another pathology of Alzheimer's disease is that the glucose

metabolism is significantly decreased. Glucose metabolism is crucial for sustained brain activity and somehow brains of AD patients become 'resistant' to insulin (Arnold, et al., 2018). In humans, the IR has two types of isoforms through alternative splicing: IR-A and IR-B (figure 5) (Belfiore, et al., 2017). They both show the same affinity to insulin; however, IR-A has a better affinity for insulin-like growth factor (IGF)-2 and proinsulin (Yamaguchi, Flier, Benecke, Ransil, & Moller, 1993).

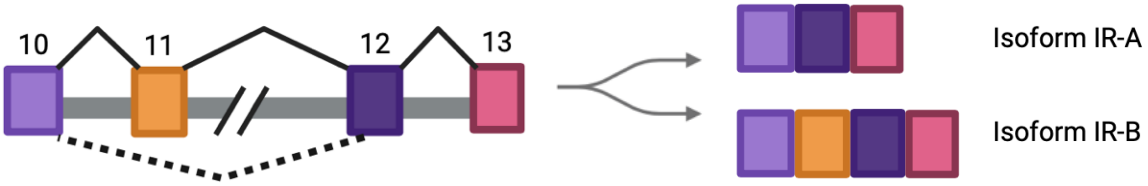

Figure 5. Schematic representation of alternative splicing mechanisms of insulin receptor (IR) pre-mRNA in humans and mice*. Isoform IR-A splices out exon 11 and isoform IR-B includes exon 11.*

In the brains of mice, the most prominent expression of IR is found in the olfactory bulb, hippocampus, hypothalamus, cerebellum, and cerebral cortex (Lee, Javedan, & Bondy, 1992). AD patients suffer from reduced brain IR sensitivity and show decreased levels of IR and IGF-1R. Leclerc *et al*. showed that in healthy human and murine IR-B is present in high density in micro vessels instead of the parenchyma (Leclerc, et al., 2023). However, subjects diagnosed with AD show lower vascular concentrations of the long IR-B isoform in the parietal cortex. Decreased IR-B isoforms are positively correlated with lower cognitive scores and consistent with insulin resistance in Alzheimer's disease brain. Besides this, the loss of insulin receptors is also associated with a lower clearance and higher production of Aβ (Leclerc, et al., 2023). The mechanism behind this phenomenon is not clear but is assumed to be caused by either amyloid-β inducing lysosomal degradation of IR in capillaries of the brain or Aβ acting as a ligand to the insulin receptor and thus contributing to insulin resistance (Gali, et al., 2019) (De Felice, 2013). These data represent how defects in the alternatively spliced IR-B isoform can

contribute to brain insulin resistance and how this might be associated with amyloid-β pathologies in Alzheimer's disease.

3 Ion channels

Ion channels are membrane-bound signaling protein that have many important functions in health and disease. Ion channels are a large superfamily of channels that is divides into two types: ligand- and voltage-gated ion channels. Ligand-gated ion channels (or channel-linked receptors) are primarily located at synapses, bind neurotransmitters, and thereby control synaptic transmission between neurons. However, voltage-gated ion channels are responsible for the generation of action potential in an excitable tissue, such as neurons. Together, they fulfill many physiological processes such as muscle contraction or cognition and memory in the central nervous system (CNS). There are many different voltage-gated channels that are for instance selective to Cl^-, Ca^{2+}, Na^+ and K^+.

Voltage-gated calcium channels are the key players in depolarization-mediated Ca^{2+} entry into excitable cells. There are only 10 genes, of which 9 are expressed in the nervous system, that encode for calcium channels. These genes can contain to approximately 50 exons generating up to 1,000 isoforms through alternative splicing. Alternative splicing happens at sites that control channel activity, and it creates thereby a diversity of functionally distinct calcium channels so that each neuron can optimize channel activity for a specific task.

Neurons in the brain contain specialized structures in the postsynaptic terminal, called the dendritic spine. These spines have crucial roles in neuronal plasticity and memory formation in mammals. Disorders in the CNS such as AD, Parkinson's Disease (PD), autism spectrum disorder (ASD), and schizophrenia are often associated with morphological changes and changes in the number of spines (Sala & Segal, 2014). Dendritic spike morphology and function is primarily depended on the concentration of calcium (Yuste, Majewska, & Holthoff, 2000). Calcium entry is regulated by the NMDA and calcium-permeable receptors as well as voltage-gated calcium channels (Yasuda, Sabatini, & Svoboda, 2003). The dendritic spines in the brain express L-type calcium channels $Ca_v1.2$ and $Ca_v1.3$. $Ca_V1.3$ channels activate more negative

membrane potentials than $Ca_V1.2$ and thereby control the excitability potential of neurons, neuronal development, and disease (Koschak, et al., 2001)

$Ca_V1.3$ can be alternatively spliced which eventually led to a long $Ca_V1.3_{42}$ isoform and short C-terminal splice isoform $Ca_V1.3_{42A}$ (Bock, et al., 2011). Isoform $Ca_V1.3_{42A}$ contains a stop codon in exon 42 (figure 6) (Bock, et al., 2011). $Ca_V1.3_{42A}$ has shown that this short splice isoform has an increased calcium influx. Calcium influx is necessary for dopamine (DA) release by the dopamine neurons (Surmeier, Guzman, Sanchez-Padilla, & Schumacker, 2011). However, constitutive influx might cause metabolic stress and mitochondrial damage leading to cell death. Besides this, $Ca_V1.3_{42A}$ channels are associated with a modified dendritic spine morphology of dopamine reduction which is linked to Parkinson's Disease phenotype (Verma & Ravindranath, 2020).

Figure 6. Schematic representation of alternative splicing mechanisms of calcium channel 1.3 pre-mRNA in humans and mice. *Isoform $Ca_V1.3_{42}$ splices out exon 42A and isoform $Ca_V1.3_{42A}$ includes exon 42A which contains a stop codon.*

Research in PD mice showed that short variants $Ca_V1.3_{42A}$ were expressed in the substantia nigra (SN) (Verma & Ravindranath, 2020). The SN is an area in the brain that is characterized by its high density of dopaminergic neurons. The substantia nigra is mainly affected in PD through the loss of DA neurons (Villalba & Smith, 2018). Surprisingly, exon 42A-containing transcripts were most abundant in the substantia nigra of Parkinson's Disease rats than other brain areas. Opposite to $Ca_V1.3_{42}$, which was more expressed in the cortex compared to the midbrain. Together, these results indicate that isoform $Ca_V1.3_{42A}$ contributes to the calcium overload in the midbrain DA neurons in the substantia nigra. This indication is strengthened by the fact that calcium density is approximately 2.5 times higher through

$Ca_V1.3_{42A}$ than $Ca_V1.3_{42}$. Later, Parkinson's Disease mice were treated with MPTP[1] and expression of long and short calcium channels were measures. After 14 days of the MPTP treatment, no difference in $Ca_V1.3_{42}$ and $Ca_V1.3_{42A}$ mRNA were observed compared to the vehicle. It might be that the DA neurons overproduce $Ca_V1.3$ L-type calcium channels to attempt to maintain their activity in surviving neurons (Verma & Ravindranath, 2020).

All taken together, a large influx of calcium through $Ca_V1.3_{42A}$ might lead to the degeneration of dopaminergic neurons in MPTP mouse models of Parkinson's Disease.

[1] MPTP causes degeneration of midbrain DA neurons leads to PD-like phenotype in animals.

4 Transcription factors

Transcription factors are proteins that recognize specific DNA sequences which are responsible for controlling transcription. Regulation of genes is crucial to development and cellular differentiation. By binding to specific DNA sequences, transcription factors can either downregulated or upregulated the expression of genes which is essential for response to environmental stimuli (Lambert, et al., 2018).

4.1 CP2 transcription factor in honeybees

In eusocial[2] insects, such as honeybees (*Apis mellifera*), the production of daughters is often restricted to queens whereby the workers are often functionally sterile. This phenomenon is explained by kin selection theory (Jarosch, Stolle, Crewe, & Moritz, 2011). However, sometimes workers counter this control and become social parasites by activating their ovaries, producing diploid female offspring, and producing queen-like amount of queen pheromones (Ratnieks, 1988) (Winstron & Slessor, 1998). All these traits are influenced by a single locus on chromosome 13 named *thelytoky* (*th*) which consist of 15 genes that code for transcription factors ATF2 (XM_393896) and CP2 (XM_001121158) (Lattorff, Moritz, Crewe, & Solignac, 2007). Workers that are homozygous for the *th* allele are workers that exhibit the social parasite traits (Anderson, 1962) (Lattorff, Moritz, Crewe, & Solignac, 2007). Jarosch *et al*. first looked at whether there are differences in ATF2 and CP2 isoform expression between worker bees and queens (Jarosch, Stolle, Crewe, & Moritz, 2011). The transcription factor ATF2 did not show differential isoform expression between workers and queens in honeybees. However, the CP2 transcription factor did show differential mRNA isoform expression. Comparison between the

[2] Social group where members are working integrated together in raising the offspring.

gemini splice patterns showed that laying and non-laying workers both produce two different isoforms at exon 5 and exon 7 in the CP2 gene. Exon 5 contains either the full transcript or a shorter mRNA transcript with a 78 bp deletion. Exon 7 contains two alternative 5' splice sites. The first splice product has a 59 bp deletion with a shift in the reading frame causing a stop codon in exon 8, resulting in an incomplete DNA-binding domain. This transcript has never been identified due to its low abundance. The second splice transcript has a deletion of 24 bp and lost 8 aa in the DNA-binding domain from exon 7 to exon 9.

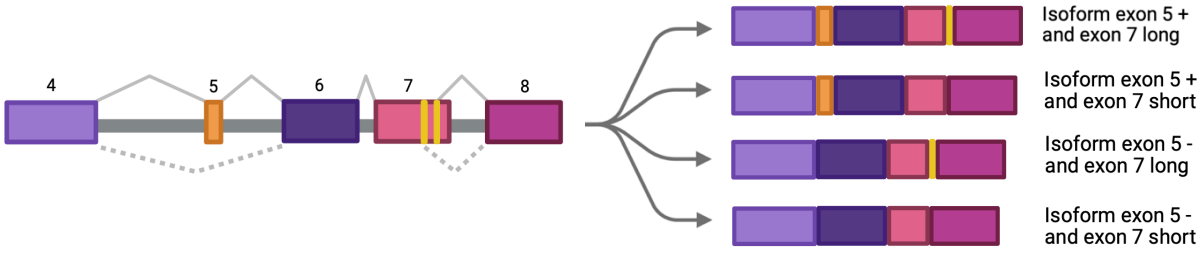

Figure 7. Schematic representation of alternative splicing mechanisms of CP2 pre-mRNA in honeybee Apis mellifera. *The alternative splicing happens in exon 5 and exon 7. Exon 5 can be included or spliced out in the mature mRNA. Exon 5 has two alternative 5' splice sites. By these splicing mechanisms, four isoforms are created. (+) long; (-) short isoform.*

Reverse-transcriptase polymerase chain reaction (RT-PCR) results showed that workers with undeveloped ovaries only produced the full transcript of exon 5 and workers that would lay eggs exclusively produce the splice product lacking exon 5. Both workers would produce the long splice variant of exon 7 even though laying workers would produce a higher level. Exon 7 is part of the CP2 DNA-binding domain and the deletion of 8 aa might influence the DNA-binding capacity of the alternative protein (Yoon, Li, & Roeder, 1994). However, since exon 5 is not part of the DNA-binding domain, a different mechanism must control the molecular switch to the onset of egg laying. An interesting result is that all honeybee workers share a common 9 bp deletion (named *thelytoky associated element 1* (*tae1*)) in the flanking intron of exon 5 which controls ovary activation that is differentially spliced in egg-laying honeybees. This *tae1* sequence is high in purines and is short in length which is similar to other intronic splice enhancer (ISE) motifs that control alternative splicing (Hastings, Wilson, &

Munroe, 2001). Thus, by having this 9 bp deletion in exon 5 it could have major consequences on the splice-site recognition and could not only affect exon 5 switch but also the splicing of exon 7. If the 9 bp deletion in exon 5 would activate ovaries and parasitic honeybees start egg-laying, it would only take as little as 9 bp to turn an altruistic worker into a social parasite.

4.2 Dsx and Fru transcription factor in fruit flies

Another group of insects are the hymenopteran insects (ants, bees, sawflies, and wasps) and dipteran insects (flies) (Bull, 1985). The sex determination in insects is determined by a cascade of genes. *Doublesex* (*Dsx*) is a gene that has been identified in many insects and is the master switching gene that give rise to either female of male offspring (Rideout, Dornan, Neville, Eadie, & Goodwin, 2010). The *dsx* gene codes for a transcription factor that is an important downstream regulator for sex differentiation. It is transcribed in both sexes but differs in splicing isoforms (Verhulst & Van de Zande, 2015). Each of these isoforms code for a functional protein: DSX^M and DSX^F. Specifically in *Drosophila melanogaster* (fruit fly), male specific isoform of transcription factor *Dsx* does not contain exon 4, whereas female specific isoform of transcription factor *Dsx* does contain exon 4 (figure 8) (Nagoshi & Baker, 1990).

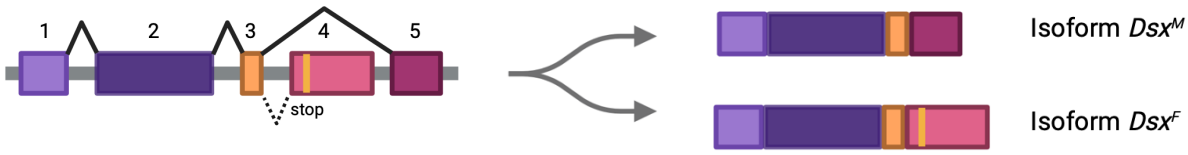

Figure 8. Schematic representation of alternative splicing mechanisms of dsx pre-mRNA in fruit fly Drosophila melanogaster. *The alternative splicing happens in exon 4 which contains a stop codon. Exon 4 can be included or spliced out in the mature mRNA, resulting in the male-specific DSX mature mRNA. Adult mRNA containing exon 4 is female-specific.*

Besides *doublesex* being important in sex differentiation, the *fruitless* (*fru*) gene, which is again sex-specifically spliced, also plays an important role. The sex-specific splicing of transcription factor *Fru* involves alternative 5' splice sites which is only used in males. The regular splice site is used in females (figure 9) (Demir & Dickson, 2005).

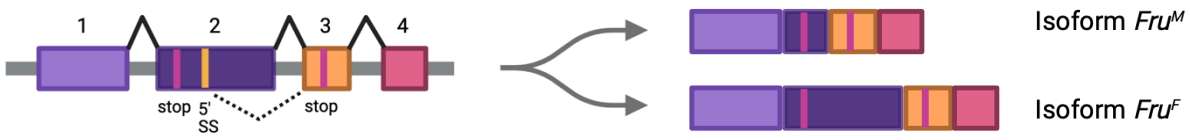

Figure 9. Schematic representation of alternative splicing mechanisms of Fru pre-mRNA in fruit fly Drosophila melanogaster. *The alternative splicing happens in exon 4 by an alternative 5' splice site (SS). Isoform DSX^F, present in females, contain the full-length of exon 2, whereas isoform DSX^M, present in males, contain a minor length of exon 2.*

Both *Fru* and *Dsx* control morphological and behavioural sexual dimorphism in *D. melanogaster*. The splicing of *Dsx* and *Fru* pre-mRNA is depended on the splicing factor *transformer* (*tra*). Similar to *Dsx* and *Fru*, the pre-mRNA of *tra* is sex-specifically spliced (Sosnowski, Belote, & McKeown, 1989). In males, Tra^M mature mRNA includes a premature stop codon, leading to a truncated, non-functional TRA protein (figure 10). In females, Tra^F mature mRNA contains the full-length TRA protein that promotes female specific splicing of *Dsx* and *Fru*.

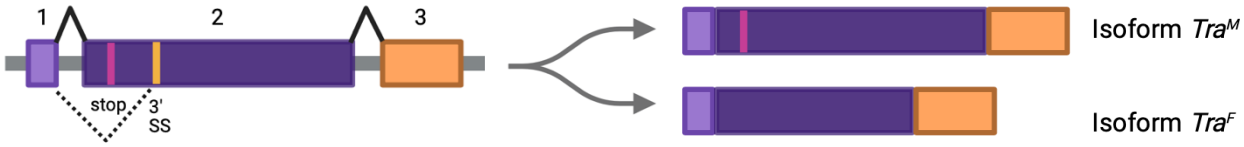

Figure 10. Schematic representation of alternative splicing mechanism of Tra pre-mRNA in fruit fly Drosophila *melanogaster. The alternative splicing happens in exon 2 containing a stop codon by an alternative 3' splice site (SS). Isoform Tra^M, present in males contain the full-length of exon 2. Isoform Tra^F, present in females, uses the 3' SS in exon 2 and therefore splice out the stop codon.*

Research in *D. melanogaster* showed that transgenic manipulations led to reverse typical sex-specific patterns of *fruitless* expression which led to phenotypes where male flies exhibited typical females behaviours. In females the opposite phenotype was observed: they behaved typical of males (Demir & Dickson, 2005) (Vrontou, Nilsen, Demir, Kravitz, & Dickson, 2006). These results indicated that *fruitless* alone was essential and capable of generating sex-specific behaviour. However, later research indicated that *fruitless* and *doublesex* are more likely to interact with each other and thereby creating sexually dimorphic features (Billeter, Rideout, Dornan, & Goodwin, 2006). This thought is strengthened by the fact

that neurons in the CNS express both *fruitless* and *doublesex* (Rideout, Billeter & Goodwin, 2007).

Courtship song is an important element of male courtship behaviour in *D. melanogaster* (Kyriacou & Hall, 1982). This enhances female receptivity to copulation. Regions in the CNS that are associated with song production, revealed co-expression of *fruitless* and *doublesex*. When researchers performed a double mutation in both *fruitless* and *doublesex* genes of males, they had a courtship index (CI) of 0 towards females and performed no song. The expression of either Dsx^M or Fru^M in females was also not sufficient for song production (Taylor, Villella, Ryner, Baker, & Hall, 1994). These results show that Dsx^M or Fru^M alone is not sufficient for performing courtship song (Rideout, Billeter & Goodwin, 2007).

5 Discussion

Until thus far, some examples of alternative spliced genes regarding receptors, channels, and transcription factors are explained. The first example illustrated how alternative splicing of the insulin receptor can (1) affect taste-avoidance learning in *C. elegans* and (2) contribute to brain insulin resistance in mice, which is associated with Alzheimer's Disease. The next example showed how alternative splicing of calcium ion channels might be related to degeneration of dopaminergic neurons in the substantia nigra, a morphology seen in Parkinson's disease. The final example demonstrated how alternative splicing of *gemini* gene can turn an altruistic honeybee worker into a social parasite. But how do these examples relate to behavioural plasticity?

The previous examples showed how alternative splicing influences a specific behaviour or a behavioural phenotype associated with a disease in animals. However, there are many other examples on how alternative splicing influences behaviours. Examples are sensitization (Krapacher, Fernández-Suárez, Andersson, Carrier-Ruiz, & Ibáñez, 2022), circadian rhythm (Sanchez, et al., 2010) (Foley, et al., 2019) (Martin Anduaga, et al., 2019), reproduction (Salvemini, Polito, & Saccone, 2010), sex determination (Planells, Gómez-Redondo, Pericuesta, Lonergan, & Gutiérrez-Adán, 2019), learning (Louadi, Oubounyt, Tayara, & Chong, 2019), and aggressiveness (Vu, et al., 2022) (Bresnahan, et al., 2023). Besides this, alternative splicing is of great importance in psychological diseases such as autism-like behaviour (Thacker, Sefyi, & Eng, 2020), schizophrenia (Zhang, Xiao, Zhang, Hu, & Li, 2022), depression (Le François, et al., 2018), and anxiety (Winter, et al., 2023).

When there would be more information available on how alternative splicing influences behavioural plasticity, scientist could also gain more information about when animals perform deviant behaviour. With this knowledge, these therapeutic targets could be modulated by a

treatment capable of altering the course of the disease. An example of a disturbed alternative splicing disorder is spinal muscular atrophy (SMA). Humans contain two nearly identical copies of survival motor neuron (SMN) gene: SMN1 and SMN2. Patients with SMA lack SMN1 gene due to an inactivating mutation (Bowerman, et al., 2017). The difference between SMN1 and SMN2 is the splicing pattern of the pre-mRNA. In most tissues, exon 7 in SMN2 is typically excluded from the adult mRNA which causes the production of a partially functional but unstable protein (Monani, et al., 1999) (Singh, Singh, Androphy, & Singh, 2006). The first therapeutic approval for SMA targets is nusinersen (Spinraza™), that blocks the removal of exon 7 on SMN2 pre-mRNA and therefore promote the formation of functional SMN protein (Chiriboga, et al., 2016). This therapy is called splice-switching therapy (Meijboom, Wood, & McClorey, 2017).

Despite that this thesis only focused on how alternative splicing influences behavioural plasticity in animals, it is important to mention that AS is also of extreme importance in physiological processes in plants. Examples are flowering time (Eckardt, 2002), DNA damage response (Nimeth, Riegler, & Kalyna, 2020), plant immunity (Yang, Tang, & Zhu, 2014), heat stress response (Rosenkranz, Ullrich, Löchli, Simm, & Fragkostefanakis, 2022), plant growth, and development (Chaudhary, Jabre, Reddy, Staiger, & Syed, 2019).

Seeing that there are only a few therapeutic treatments available against AS causing diseases, indicates that alternative splicing is still an understudied field. There are many researches listed in this thesis indicating that alternative splicing could potentially be an important molecular mechanism influencing behaviour and physiological differences between individuals (Grantham & Brisson, 2018). Alternative splicing could thus contribute to proteome diversification which is associated with behavioural plasticity.

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