Stimulation-specific remodeling of the neuronal transcriptome via intron retention programs

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Summary

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The mammalian brain's ability to constantly integrate, sort, and respond to a plethora of environmental changes is particularly impressive. This adaptability is called plasticity and supports brain functions such as learning, acquisition of skills, or memory formation. Transcription of distinct sets of pre-mRNA upon specific neuronal stimuli is critical for appropriate plasticity event formation. Besides transcription, a growing body of studies supports that nuclear sequestration of transcripts plays a critical role in shaping activity-dependent gene expression programs. Recent studies have revealed the existence of stable intron-retaining transcripts sequestered in the nucleus where they cannot participate in protein synthesis. Importantly, certain stable intron-retaining transcripts can complete their splicing and join the cytoplasm upon various types of cellular signals, including neuronal activity. However, this mechanism was only shown for a couple of candidate transcripts. Thus, it is unknown whether nuclear sequestration and cytosolic release of stable intron-retaining transcripts is a general mechanism allowing global neuronal transcriptome remodeling. Moreover, the studies reporting stimulus-dependent regulation of these intron-retaining transcripts were performed in different cellular models or focusing on individual transcripts. Thereby, it is unclear whether stable and nuclear intron-retaining transcripts can be regulated in a stimulus-specific manner.

In my thesis, I investigated whether nuclear sequestration of stable intron-retaining transcripts is a general mechanism and whether these transcripts can be regulated in a neuronal stimulus-specific manner. First, we found that most stable intron-retaining transcripts are sequestered in the nucleus. Then we probed the response of these nuclear transcripts upon elevation of neuronal network activity or treatment with BDNF, a neurotrophin. We show that stable and nuclear intron-retaining transcripts can complete their splicing upon these two neuronal stimuli and undergo cytoplasmic export. Interestingly, we also identify activity-dependent retained isoform decrease corresponding most likely to nuclear degradation. Importantly, we show for the first time that elevation of network activity and BDNF treatment control stimulation-specific populations of stable intron-retaining transcripts. Finally, stimulation specificity of these populations arises from molecularly distinct signaling pathways. Thereby, my thesis supports cue-specific remodeling of the neuronal transcriptome via splicing completion and cytoplasmic export of pre-existing intron-retaining transcripts. As it does not rely on de novo transcription, this mechanism can support rapid mobilization of new mRNAs upon distinct environmental changes and we speculate that it can support different forms of plasticity.

1. Introduction

1.1 General Introduction

The brain is one of the most complex organs of the human body. It controls a large variety of processes ranging from motor control or breathing to higher brain functions such as cognition and complex behavioral responses. The correct operation of this highly organized machinery relies on a precise network of neuronal connections in and between different brain regions that are set up during development but also evolve during adulthood. The beginning of modern neuroscience is often attributed to the pioneering work of Santiago Ramón y Cajal which shed light on the main functional unit of the brain, the neuron. Besides his initial work on describing neuronal morphology, Ramón y Cajal also participated in developing the concept of brain plasticity. This refers to the capacity of neurons to modulate their properties and the nature of their connections in order to adapt to multiple types of environmental changes. Indeed, neurons have the fascinating ability to discriminate a plethora of distinct environmental changes and to produce the appropriate outcome in response.

One century of research has revealed important features of the brain's adaptation to environmental changes. We now know that the receptor cells detect physical or chemical changes in the environment and encode these changes via different patterns of electrical activity. Most of the time, these signals are then communicated in the central nervous system, to the thalamus that distributes them in the brain areas specialized in the treatment of precise types of sensory inputs (Rikhye et al., 2018). Here, the stimuli are decrypted and an adapted plastic response may be triggered. In the mammalian brain, the information exchange between neurons is mainly made at chemical synapses. Classically, synapses are places where two neurons are in close proximity and where biomolecules such as neurotransmitters may be liberated in response to depolarization of the presynaptic neuron. The binding of neurotransmitters to postsynaptic receptors changes the membrane potential of postsynaptic neurons which may trigger a plastic response. The nature of the neuronal spiking pattern represents is particularly important to encode environmental changes (Lee and Fields, 2021). Moreover, different types of neurotransmitters can be released and other molecules such as neuromodulators or growth factors are also secreted. This highlights how complex is the encoding of different signals in the brain and much work has been done to understand which plasticity events are modified in response to distinct types of stimuli as well as the nature of the molecular mechanisms controlling those cue-specific changes.

The precise regulation of gene expression programs is extremely important to develop cue-specific plasticity events. One of the most studied mechanisms encoding stimulation-specific neuronal responses is the synthesis of specific sets of new mRNAs. The molecular mechanisms of activity-dependent transcription have been strongly studied and its role in functional and structural plasticity formation is now widely accepted. (Yap and Greenberg, 2018). Besides activity-dependent transcription, other gene expression regulation mechanisms are now known to take place upon neuronal stimulation. For example, pre-mRNA maturation steps such as splicing or the usage of alternative splice sites known as alternative splicing (AS) also have an important impact in shaping the neuronal transcriptome upon activity (Furlanis and Scheiffele, 2018; Vuong et al., 2016). Alternatively, local translation of mRNA upon neuronal stimuli is associated with the formation of certain plasticity events (Aakalu et al., 2001; Kang and Schuman, 1996; Sutton and Schuman, 2006). Similarly, regulation of mRNA translation and stability by micro RNA (miRNA), a type of small non-coding RNA, has emerged as a strong modulator of neuronal gene expression upon environmental cues with an important impact on neuronal plasticity (Aksoy-Aksel et al., 2014; Khudayberdiev et al., 2009; Krol et al., 2010; Olde Loohuis et al., 2015; Schratt et al., 2006). Importantly, nuclear sequestration of RNAs is now is emerging as a critical regulator of gene expression programs (Dumbović et al., 2021; Mauger et al., 2016; Prasanth et al., 2005). Indeed, many transcripts that cannot leave the nucleus are ultimately degraded and represent a dead-end in the gene expression process.

A growing amount of work is focusing on immature transcripts that retain an intron and are sequestered in the nucleus without being degraded (Mauger et al., 2016; Naro et al., 2017; Ninomiya et al., 2011). These stable transcripts blocked in the nucleus constitute a reserve pool of RNAs able to participate in protein synthesis upon completion of splicing and export in the cytoplasm. Importantly, as they are pre-existing transcripts, there is no requirement for new transcription which is a time-limiting step in the gene expression process (Jonkers and Lis, 2015; Singh and Padgett, 2009). Interestingly, many work report populations of intron-retaining transcripts that are specifically regulated upon various types of external stimuli, including neuronal activity (Mauger et al., 2016; Mendel et al., 2021; Ni et al., 2016; Ninomiya et al., 2011; Park et al., 2017; Shalgi et al., 2014). Then control of activity-dependent gene expression programs via intron-retention and cellular compartmentalization represents a potential mechanism for cue-specific plasticity formation in neurons. It could thereby be an important regulator of complex adaptation processes and behavioral responses upon distinct neuronal cues. However, as these works were performed in various types of cells, it is unknown whether this specificity arises

from different cell types or different stimuli. Moreover, the stability and subcellular localization of intron-retaining transcripts are not systematically assessed and only a handful of stable intron-retaining transcripts were experimentally observed in the nucleus. Thus, it is unclear whether nuclear sequestration of stable intron-retaining transcripts is a general mechanism mediating global neuronal transcriptome remodeling or whether it is a rare mechanism affecting only a handful of transcripts.

During my PhD, I discovered that stable intron-retaining transcripts are strongly enriched in the nucleus and that distinct neuronal cues mobilize stimulation-specific populations of these nuclear RNAs. In order to introduce the context of my dissertation project, I will first discuss the concept of transcriptomic remodeling upon neuronal activity and the importance of activity-dependent transcription and splicing in this process. I will also discuss the recent work supporting the impact of nuclear sequestration on activity-dependent gene expression regulation in neurons. Secondly, I will describe the published literacy regarding stimulation-specificity in neuronal gene expression regulation and develop further the molecular mechanisms encoding this specificity. In the last step, I will examine the emergence of intron retention as a widespread and critical regulator of mammalian gene expression programs and how intron-retaining transcripts are regulated upon signals.

1.2 Transcriptomic reprograming upon neuronal activity

The most studied mechanism in activity-dependent gene expression regulation is de novo transcription. The last 30 years have demonstrated its importance in shaping the neuronal transcriptome in vivo and upon physiological stimuli and proper activity-dependent transcription is critical for shaping the synaptic strength and the structural properties of the neurons (Leslie and Nedivi, 2011; Yap and Greenberg, 2018). Similarly, much work has been performed to decipher the role of alternative splicing (AS) regulation upon neuronal activity. AS results in the production of distinct mRNA isoforms arising from a single transcriptional unit. Thereby, AS is able to increase transcriptome diversity but also regulate gene expression levels (Mauger and Scheiffele, 2017). Activity-dependent AS is involved in important neuronal functions such as synapse formation and specification, axon guidance but also synaptic transmission (Furlanis and Scheiffele, 2018). Interestingly, more recent works are exploring the importance of cellular compartmentalization, especially nuclear sequestration of synthesized RNAs, as a regulator of neuronal gene expression in development or upon environmental stimuli

(Mauger et al., 2016; Naro et al., 2017; Prasanth et al., 2005). As it does not require new transcription, the regulation of subcellular localization of the transcripts provides a temporally flexible mechanism to remodel the neuronal transcriptome upon environmental changes.

In the following parts, I will describe the importance of activity-dependent transcription for plasticity formation, its molecular mechanisms, and its limitations in neuronal gene expression regulation. I will then discuss the current knowledge about AS and how activity-dependent AS regulates many neuronal functions upon internal or external signals. Finally, I will introduce the recent work underscoring the importance of cellular compartmentalization and how it is regulated by signaling.

1.2.1 Activity-dependent transcription

Upon environmental stimuli, synaptic signals are conveyed to the nucleus to elicit new transcription. There is a first wave of transcription mainly composed of immediate-early genes (IEG) that are mostly transcription factors (TF). These TFs will support a second wave of transcription that codes for late-response genes (LRG) which are thought to be the main effectors of neuronal plasticity.

History of activity-dependent transcription

The first report of gene transcription upon cellular stimulation was published in 1984 by Ziff and Greenberg (Greenberg and Ziff, 1984) that showed an increase in the proto-oncogene *c-fos* mRNA upon growth factor treatment in a mouse cell line. Rapidly, this was also observed in neuronal cell lines (Greenberg et al., 1986, 1985). At the time, it was known that sensory stimulation triggers an increase in RNA synthesis and that various types of learning lead to long term changes at the synapses in an RNA and protein synthesis-dependent manner (Glassman, 1969; Kernell and Price Peterson, 1970; Schwartz et al., 1971). The discovery of rapid *c-fos* induction upon external signals provided a potential molecular mechanism to support the formation of different kinds of neuronal plasticity events thought to be the cellular basis of learning, memory formation, and various behavioral responses. Since this early discovery and thanks to the development of genome-wide tools, many genes have been identified as rapidly transcribed upon neuronal activity and the temporal dynamic of their production has been explored (Leslie and Nedivi, 2011). This has shed light on the importance of activity-

dependent transcription in the regulation of synaptic strength and neuronal structural properties during sensory experiences. Importantly, defect in factors involved in activity-dependent transcription is also associated with various pathologies such as autism spectrum disorders, schizophrenia, intellectual disability, or major depressive disorders (Ebert and Greenberg, 2013).

IEGs induction and regulation

Molecular signaling induced by neurotransmitters and growth factors binding to postsynaptic receptors leads to the first wave of transcription composed of IEGs, including c-fos (Minatohara et al., 2016; Sheng and Greenberg, 1990). Besides *c-fos*, many IEGs are now identified such as *Npas4*, which encodes for a TF, or *Arc*, which encodes for a protein involved in vesicular recycling of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Chowdhury et al., 2006; Lin et al., 2008). Large-scale studies have revealed multiple dozens of IEGs in different brain regions or cell types (Hrvatin et al., 2018; Tullai, 2007; Tyssowski et al., 2018). IEGs transcription rapidly takes place within minutes and usually peaks 30 minutes to 1 hour upon neuronal stimulation. IEGs are short and generally poised with RNA-polymerase II (RNA-pol II) in close proximity of their promoter region, which is favoring their rapid induction upon neuronal activity (Saha et al., 2011). Moreover, induction of IEGs does not require new protein synthesis indicating that they are under the control of pre-existing and stimulation-sensitive transcription factors binding to cis-acting DNA regulatory elements. Recruitment of TFs upon stimulation relies on post-translational modifications, especially phosphorylation. For example, neuronal depolarization triggers the phosphorylation of the cAMP response element-binding protein (CREB) within 5 minutes (Sheng and Greenberg, 1990). This enables CREB to bind on a calcium response element (CaRE) located -60 nucleotides upstream of the c-fos transcription start site (Sheng et al., 1988). Similarly, other pre-existing TFs such as the serum response factor (SRF), the myocyte enhancer factor-2 (MEF2) or the ETS Like-1 protein ELK-1 and others are modified post-translationally upon neuronal activity and trigger IEGs induction (Chawla et al., 1998; Flavell et al., 2006; Janknecht and Nordheim, 1992; Shalizi et al., 2006; West et al., 2002). Importantly, many DNA elements bound by pre-existing TFs upon neuronal activity have been identified. For example, more than 10000 enhancer regions are bound upon neuronal depolarization by the transcriptional co-activator CBP, which acts in close proximity with CREB (Kim et al., 2010; Malik et al., 2014).

Direct impact of IEGs on plasticity

Certain IEGs directly regulate neuronal functions. For example, there is a strong increase in *Arc* mRNA upon different forms of neuronal activity, *Arc* mRNAs are then transported in synapses and dendrites where they are locally translated (Link et al., 1995; Lyford et al., 1995; Steward et al., 1998). It was shown that in the post-synapse, ARC proteins associate with certain isoforms of DYNAMIN2 and ENDOPHILIN3 in early recycling endosomes and promote AMPA receptor endocytosis (Chowdhury et al., 2006). Rial-Verde et al demonstrated that upon elevation of network activity with Picrotoxin, an antagonist of $GABA_A$ receptors, there is a 7 to 10-fold increase in ARC protein level accompanied by a reduction of AMPA-mediated excitatory postsynaptic current (EPSC) amplitude. Importantly, ARC knockdown or depletion of a region in ARC responsible for the interaction with ENDOPHILIN3 restored AMPA-mediated EPSC amplitude (Rial Verde et al., 2006). In another study, ARC was shown to be necessary for homeostatic scaling of AMPA receptors and ARC knockout mice display impaired consolidation of fear and spatial memory. Moreover, even transient inhibition of Arc transcription during fear conditioning impaired fear memory formation (Nakayama et al., 2015; Ploski et al., 2008). These studies demonstrate that activity-dependent transcription of the IEG Arc is critical for proper regulation of synaptic transmission involved in long term changes on synaptic strength necessary memory consolidation.

IEGs acting as transcription factors, an amplification mechanism

Besides the direct role of certain IEGs in neuronal plasticity, many IEGs encode for new TFs such as FOS, NPAS4, or EGR1 that then support the second wave of transcription composed of so-called "late response genes" (LRG). Transcription of LRGs occurs several hours after neuronal stimulation and requires new protein synthesis (Tullai et al 2006; Tyssowski et al., 2018; Yap and Greenberg, 2018).

In the case of c-fos, brain-specific FOS knockout leads to decreased performance in the Morris water maze and in recalling contextual fear memory which indicates a deficit in spatial and associative memory (Fleischmann et al., 2003). Moreover, neurons exhibiting *c-fos* induction upon sensory stimuli were shown to encode contextual information compared to their *c-fos* negative neighbors (Tanaka et al., 2018). However, investigation of the FOS transcriptional network and associated neuronal functions is difficult because of the nature of FOS binding on DNA. Members of the FOS family associate with the JUN family of TFs to form the AP-1 heterodimer complex. AP-1 is composed of at least 7

functionally redundant proteins with the possibility of forming different heterodimers (Bejjani et al., 2019) (fig 1.1).

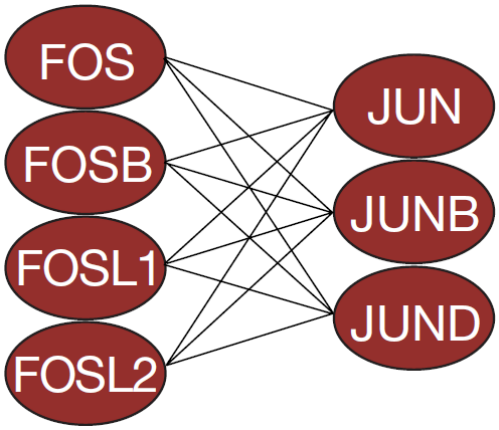

Figure 1.1: Representation of the possible combination in the AP-1 complex. Adapted from (yap et al., 2020).

Scheme representing the members FOS and JUN families and their possible combination in the AP-1 complex.

In this regard, a recent study from Yap and colleagues reported an increase in PV interneuron-mediated inhibition of CA1 pyramidal neurons that express c-fos upon mice exploration of a novel environment. Importantly, this was concomitant with a decrease of cholecystokinin (CCK) interneuron-mediated inhibition. This regulation of bidirectional inhibition of *c-fos* positive pyramidal neurons in CA1 upon environmental change was abolished upon deletion of the 3 most induced members of the AP1 complex in CA1 neurons. Using a combination of tools, the authors identified the genes whose expression was affected upon the triple knock out and, using shRNAs and a knock out mice, they identified the LRG Scg2 as critical for the regulation of PV and CCK-mediated inhibition of c-fos positive neurons in CA1 (Yap et al., 2020). This study illustrates the importance of IEGs acting as TFs and their regulatory network in the regulation of neuronal properties upon sensory experience. Recent studies using chromatin immunoprecipitation followed by DNA sequencing identified more than 104 binding sites for activity-dependent or pre-existing TFs and combination with RNA-sequencing led to an estimation of several hundred LRGs regulated by these TFs (Benito and Barco, 2015; Mardinly et al., 2016; Spiegel et al., 2014; Kim et al., 2010; Malik et al., 2014).

Late-response genes: the example of bdnf

LRGs are thought to be the main effectors of neuronal plasticity. However, only a few LRGs are functionally characterized. Probably one of the most studied LRGs is the brain-derived neurotrophic factor (BDNF). Upon sensory stimuli and neuronal depolarization, *bdnf* transcription occur within 2-4 hours and the inducible TF NPAS4 was shown to bind to the Bdnf locus upon mice exposed to an enriched environment (Bloodgood et al., 2013; Tao et al., 2002; Thoenen, 1991). BDNF is a neurotrophin that is secreted by neurons and was first studied for its role in cell survival, differentiation, neurite outgrowth, and synapse formation during nervous system development (Park and Poo, 2013). BDNF also has a key role in regulating mature neuronal circuits. The Bdnf gene harbors 9 different promoters (Pruunsild et al., 2007). A key advance in understanding the role of *bdnf* induction upon neuronal activity was the identification of promoter IV as mainly responsible for activity-dependent *bdnf* transcription. Disruption of this promoter leads to impaired inhibitory cortical circuits demonstrating a role for BDNF independently of its constitutive release (Hong et al., 2008). The BDNF protein is synthesized as a precursor peptide and is cleaved into pro-BDNF, which can be secreted before cleavage or can be cleaved intracellularly before release (Dieni et al., 2012; Lessmann et al., 2003; Matsumoto et al., 2008). Once released, BDNF binds the Tropomyosin receptor kinase B (TrkB) and regulates LTP formation (Minichiello et al., 2002) and structural dendritic and spine rearrangements (Tanaka et al., 2008). Importantly pro-BDNF binding to P75 receptors was shown to promote LTD formation and apoptosis which strongly contrasts with TrkB-mediated functions (Lee and Chao, 2001; Pang and Lu, 2004; Woo et al., 2005). TrkB-mediated regulation of neuronal plasticity can happen both pre and postsynaptically. For example, presynaptic TrkB activation modulates the synaptic release of neurotransmitters through an increase of cytoplasmic Ca^{2+} (Li et al., 1998) or phosphorylation of SYNAPSIN, a synaptic vesicle membrane protein (Jovanovic et al., 2000). On the other hand, postsynaptic TrKB activation modulates neurotransmitter receptors properties such as the surface expression of NMDA and AMPA receptor (Lin et al., 1998; Park and Poo, 2013; Suen et al., 1997). Additionally, postsynaptic TrkB activation is also associated with gene expression regulation. For example, postsynaptic TrkB activation triggers CREB phosphorylation via the Ca^{2+}/calmodulin-dependent protein kinase (CaMK) and mitogen-activated protein kinase (MAPK) pathways. This leads to transcription of activity-regulated genes (Gaiddon et al., 1996; Ginty et al., 1994; Je et al., 2006). These studies illustrate how a single LRG can control synaptic transmission and structural properties of neurons via both pre- and postsynaptic mechanisms including gene expression regulation.

Another important feature in LRG induction is their high selectivity for distinct neuronal cell types. The development of recent tools allowing to access precise neuronal cell types or even individual neurons supports a model where core IEGs are commonly induced in most neurons upon sensory stimuli. These IEGs then trigger cell type-specific LRG induction through their TF activity. For example, Spiegel and colleagues showed that cultures enriched in excitatory or inhibitory neurons expressed strongly overlapping sets of IEGs upon 1 hour of membrane depolarization while hundreds of LRGs were differentially expressed after 6 hours (Spiegel et al., 2014). Similar results were observed in more defined inhibitory cell types or even at the single-cell level in the mouse visual cortex (Hrvatin et al., 2018; Mardinly et al., 2016). These studies support that cell type-specific LRG programs are the main mediators of neural circuit plasticity. In the future, identification of stimulation- and cell-type-specific LRG programs will provide more insight into how neural circuits are regulated by the ever-changing environment.

In conclusion, many works have demonstrated the critical role of activity-dependent transcription in the formation of plasticity events and ultimately the complex brain responses to environmental changes. Importantly, activity-dependent transcription has been investigated in vivo upon physiological stimuli such as exposure to an enriched environment (Yap et al., 2020) or light stimulation (Hrvatin et al., 2018; Spiegel et al., 2014). However, the major drawback of de novo transcription upon neuronal activity lies in its poor temporal dynamic. Indeed, only a few IEGs were found to directly regulate neuronal functions and most of the changes in neuronal properties are thought to be controlled by LRGs. However, LRGs synthesis takes place several hours after neuronal activity and transcription is a time-limiting step in the gene expression process. Indeed, the elongation rate of the RNA polymerase II, which is responsible for the transcription of mRNAs, is estimated between 1 and 6 kb/minutes (Darzacq et al., 2007; Jonkers and Lis, 2015; Singh and Padgett, 2009). Thereby, in the case of very long genes; which are particularly enriched in neuronal cells (Gabel et al., 2015); new transcription can take several hours. This huge time requirement is not consistent with the rapid onset of certain plasticity events and does not allow rapid and flexible gene expression regulation upon neuronal activity.

1.2.2 Activity-dependent alternative splicing

Splicing consists in the removal of introns, parts of the pre-mRNA that are not used for the protein-coding function, and subsequent ligation of adjacent exons. Alternative splicing

(AS) consists in the removal or insertion of different sequences in the final mRNA representing a major gene expression regulation mechanism. Importantly, AS is regulated upon neuronal activity and is critical for important neuronal properties such as neuronal recognition, synapse formation and synaptic transmission (Furlanis and Scheiffele, 2018).

The splicing reaction

The splicing reaction is catalyzed by a dynamic and complex ribonucleoprotein machine called the spliceosome (Matera and Wang, 2014). There are two different spliceosomes, the major and the minor spliceosome. The major spliceosome is responsible for most splicing reactions (>99%) (Baumgartner et al., 2019). The major spliceosome is composed of five small nuclear ribonucleoprotein complexes (snRNPs) which are named after the small nuclear RNA (snRNA) they contain (U1, U2, U4, U5 and U6). There is a coordinated interplay between the different snRNPs and multiple accessory proteins that dynamically enter or leave the complex during the different steps of the splicing reaction. Briefly, the U1 and U2 snRNPs first bind to the 5' splice site (5' SS) and the branching point sequence respectively (Das et al., 2000; Du and Rosbash, 2002; Fox-Walsh et al., 2005; Sharma et al., 2008). Physical rearrangement of the complex then brings U1 and U2 in close proximity (De Conti et al., 2013). This allows the binding of U4, U5 and U6 as a tri-snRNPs to the existing complex. U1 and U4 are then released leaving the spliceosome in a catalytic competent state and promoting cleavage at the 5' SS and ligation of the 5' intronic extremity to the branching point (Raghunathan and Guthrie, 1998). In a last step, the 3' SS is cleaved and the adjacent exons are ligated. The product of this reaction is the liberation of a free lariat intron and a spliced RNA. In conclusion, the spliceosome is a highly dynamic complex that requires different proteins and RNAs at the different steps of the splicing reaction.

Alternative splicing

Importantly, alternative 5' and 3' SS can be chosen by the cell for a defined splicing event. This is called alternative splicing (AS) and, if AS was at first thought to be a rare mechanism, it is now known to happen in around 95% of mammalian genes (Pan et al., 2008; Wang et al., 2008). Importantly, AS is particularly prevalent in neuronal cells which may be explained by the high diversity of neuronal cell types and the plethora of different signals received by these neurons (Mazin et al., 2021; Yeo et al., 2004). Moreover, AS dysregulation is associated with various neurodevelopmental disorders such as autism-

spectrum disorder (Irimia et al., 2014; Quesnel-Vallières et al., 2016), amyotrophic lateral sclerosis (Polymenidou et al., 2011) or in cancer (Zhang et al., 2021).

There are multiple types of AS events. The most common type of alternative splicing represents the skipping or inclusion of a given alternative exon in the mRNA. Four other main types of AS exist: (i) intron retention, the persistence of an intronic sequence in an otherwise fully processed RNA, (ii) Mutually exclusive exons or (iii and iv) the usage of alternative 5' or 3' SS (Figure 1.2).

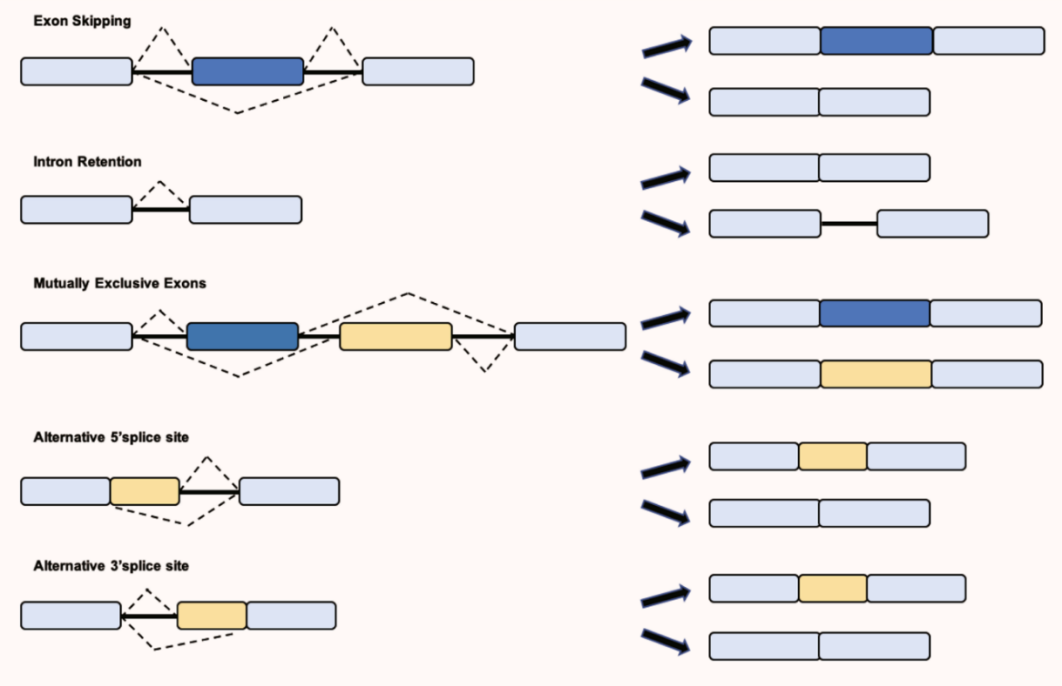

Figure 1.2: Representation of the different types of alternatives splicing. Adapted from (Zhang et al., 2021).

Exon skipping corresponds to the inclusion or exclusion of an alternative exon (dark blue). Intron retention corresponds to the persistence of an intronic sequence in an otherwise fully matured RNA. Mutually exclusive exons are exons whose inclusion will exclude per se another exon. In this scheme, the outcome is either inclusion of the dark blue or the yellow exon. Alternative 5' or 3' splice sites usage are events were one of the 2 splice sites is changed leading to longer or shorter exons.

Alternative splicing regulation

Besides classic RNA sequences controlling splicing (5' and 3' SS, branching point and poly-pyrimidine tract) other cis-acting elements influence the splicing choices. These sequences are exonic/intronic splicing enhancers or silencers. These cis-acting elements mainly act via recruitment of trans-acting factors that promote or disfavor splicing of given segments during both classic and alternative splicing (Busch and Hertel, 2012; Zhou and Fu, 2013).

Many RNA-binding proteins able to regulate splicing and AS have been identified. The best characterized families of such splicing factors are serine/arginine-rich (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNP). According to their binding site on the transcripts, these splicing factors can positively or negatively regulate splicing (Long and Caceres, 2009). The activity of SR proteins and hnRNPs is controlled by multiple post-translational modifications such as methylation, acetylation and phosphorylation (Choudhary et al., 2009; Roth et al., 1991; Zahler et al., 1992). The best understood post-translational modification of such splicing factors is through phosphorylation. If multiple kinases have been shown to control SR proteins phosphorylation in vitro, only the serine-arginine protein kinases (SRPK) and the cdc2-like kinase (CLK) were shown to phosphorylate SR proteins in living cells (Fukuhara et al., 2006; Hayes et al., 2006; Yomoda et al., 2008; Zhong et al., 2009).

Most of SR proteins and hnRNPs are ubiquitously expressed. Thereby, their regulation controls AS in large cell populations. However, other splicing factors exhibit restricted expression for specific cell types (Furlanis et al., 2019; Iijima et al., 2014; Traunmuller et al., 2016). For example, the KH-domain RNA-binding protein SLM2 is highly expressed in pyramidal cells of the mouse hippocampus but also in specific subsets of GABAergic neurons were it controls cell type-specific AS events (Traunmuller et al., 2016). Importantly, phosphorylation state and sub-cellular localization of splicing factors can be controlled upon external signal providing a way to rapidly modulate splicing and AS in response to environmental changes (Zhou et al., 2012). For example, hnRNPA1 is phosphorylated in response to osmotic stress triggering its transport in the nucleus (Allemand et al., 2005).This shows that the specific expression of particular splicing factors as well as their post-translational modifications and change in sub-cellular localization are critical factors to determine splicing choices. This offers multiple potential leverages for AS regulation in neurons upon environmental changes.

Consequences of alternative splicing

AS usage allows a formidable expansion of the genome coding power. This is reflected by the production of protein isoforms exhibiting different expression patterns, localization, and functions. For example, AS in the Dscam gene in drosophila can theoretically lead to up to 38000 different isoforms (Schmucker et al., 2000). From those, more than 18000 isoforms were experimentally detected and are differentially used by individual neurons (Sun et al., 2013). DSCAM is a surface receptor and neurites exhibiting similar DSCAM isoforms will repulse from each other providing a mechanism for neurite guidance and

neuronal recognition (Chen et al., 2006; Hattori et al., 2007; Wojtowicz et al., 2004). In mammals, the synaptic adhesion molecules NEUREXINs (NRXN) also exhibit a high degree of transcript diversity. There are 3 Neurexin genes (Nrxn1, Nrxn2, and Nrxn3) that are controlled by 2 alternative promoters and contain up to six alternatively spliced segments. This generates more than a thousand different isoforms as detected by long-read sequencing (Schreiner et al., 2014; Treutlein et al., 2014). Importantly, pull-down of NRXNs postsynaptic partners and analysis of associated proteins via mass spectrometry identified NRXNs bearing specific splice segments involved in trans-synaptic interactions (Schreiner et al., 2015). Different NRXN isoforms can interact with distinct postsynaptic partners and strongly influence synapse formation, specification, and function (Aoto et al., 2013; Nguyen et al., 2016; Traunmuller et al., 2016). These examples illustrate how AS can regulate neuronal functions through expansion of the proteome diversity.

Alternatively, AS can also control gene expression via the regulation of mRNA level. AS can be coupled with nonsense-mediated mRNA decay (NMD) in case of insertion of an exon containing a premature termination codon (PTC) before the last exon junction complex. NMD was initially thought to only act as a surveillance mechanism degrading immature or defective transcripts escaping the nucleus. It is now known that NMD is also used by the cells to regulate gene expression levels (Lykke-Andersen and Jensen, 2015; Traunmuller et al., 2014). In neurons, a substantial part of AS events has been found to introduce PTC-containing sequences (Yan et al., 2015). A classic example of this kind of mechanism is the inclusion of exon 18 in Psd95, which encodes an important postsynaptic scaffold protein. Exon 18 insertion introduces a PTC and results in transcript degradation via NMD. In non-neuronal cells, exon 18 is inserted in *Psd95* transcripts due to high expression of the RNA-binding proteins PTBP1/2, leading to reduction of PSD95 mRNA and protein levels. During neuronal differentiation, PTBP1/2 level decreases leading to exclusion of exon 18 and production of PSD95 proteins which are necessary at this developmental stage for glutamatergic synapse development (Zheng et al., 2012).

AS can also regulate transcript's sub-cellular localization. For example, Ciolli Mattioli and colleagues cultured and differentiated mouse embryonic stem cells into neurons on a device allowing physical separation of neurites and the soma. Using 3' mRNA-sequencing they were able to identify mRNA isoforms enriched in the soma or the neurites according to their 3'UTR. Changes in 3'UTRs can be due to alternative polyadenylation site or to the usage of an alternative last exon. In this study, the authors identified hundreds of transcripts whose alternative splicing in the last exon induced alternative localization, including the cell polarity gene cdc42 (Ciolli Mattioli et al., 2019).

These studies illustrate that AS can regulate neuronal gene expression via multiple molecular mechanisms ranging from an increase in transcript diversity, modulation of gene expression level, or even regulation of transcript sub-cellular localization.

Regulation of alternative splicing by neuronal activity

AS can select specific isoform expression and modulate gene expression levels as well as transcripts cellular localization. Thereby, modulation of AS represents a formidable tool for neurons to adapt to environmental changes. Activity-dependent AS was reported to affect many different neuronal functions such as synaptic transmission or synapse formation and specification.

For instance, the increase and decrease of neuronal activity control the insertion or exclusion of exon 22 in *Grin1* mRNA, a transcript encoding for an N-methyl-D-aspartate (NMDA) receptor subunit. Diminution of network activity induces exon 22 exclusion leading to the usage of another stop codon allowing the synthesis of a GRIN1 protein isoform harboring an endoplasmic reticulum (ER) export signal (C2' segment). Incorporation of GRIN1-C2' isoform in the NMDA receptor is shown to increase loading in ER vesicles ultimately upregulating NMDA receptor surface expression (Daoud et al., 1999; Mu et al., 2003; Zukin and Bennett, 1995) (Fig 1.3, A). Exons 5 and 21 of Grin1 are also modulated upon membrane depolarization. Interestingly, exclusion of exon 21 upon KCl treatment depends on calcium influx through NMDA receptors and CaMKIV activity but also on multiple UAGG elements and a CaMKIV-responsive element (CaRRE) present on the *Grin1* transcript (An and Grabowski, 2007; Lee et al., 2007). Similarly, AS of the stress axis hormone-regulated exon (STREX) in the Kcnma1 gene, which encodes for a potassium channel, is regulated upon neuronal depolarization (Xie and Black, 2001). Depolarization-dependent exclusion of the STREX exon relies on CaMKIV activity and subsequent hnRNPL phosphorylation and binding to a CaRRE present in the STREX exon (Liu et al., 2012; Xie, 2005). STREX inclusion ultimately increases potassium channel sensitivity to Ca^{2+} and affects neuronal burst firing (Liu et al., 2012). These examples illustrate the importance of activity-dependent AS in the control of the synaptic transmission. Interestingly, various studies also support a control of synapse formation and specification via activity-dependent AS. For example, the *Nrxn1* transcript undergoes depolarization-dependent exclusion of the AS4 segment resulting in a shift in postsynaptic NRXN partners in granule cells. AS of AS4 in response to depolarization was shown to rely on the RNA-binding protein SAM68 (Iijima et al., 2011).

Moreover, recent studies revealed large programs of activity-dependent alternative exons usage in neurons including a population of micro-exons (3-27nt). Most of the time, micro-exons preserve the open reading frames and insert new amino acid sequences in proteins involved in neurogenesis, axon guidance, and synaptic functions (Capponi et al., 2020; Gonatopoulos-Pournatzis et al., 2020; Irimia et al., 2014; Quesnel-Vallières et al., 2016a, 2019; Ustianenko et al., 2017). These examples show that signal-dependent AS modulate neuronal gene expression via the production of alternative mRNA isoforms which increase proteome diversity.

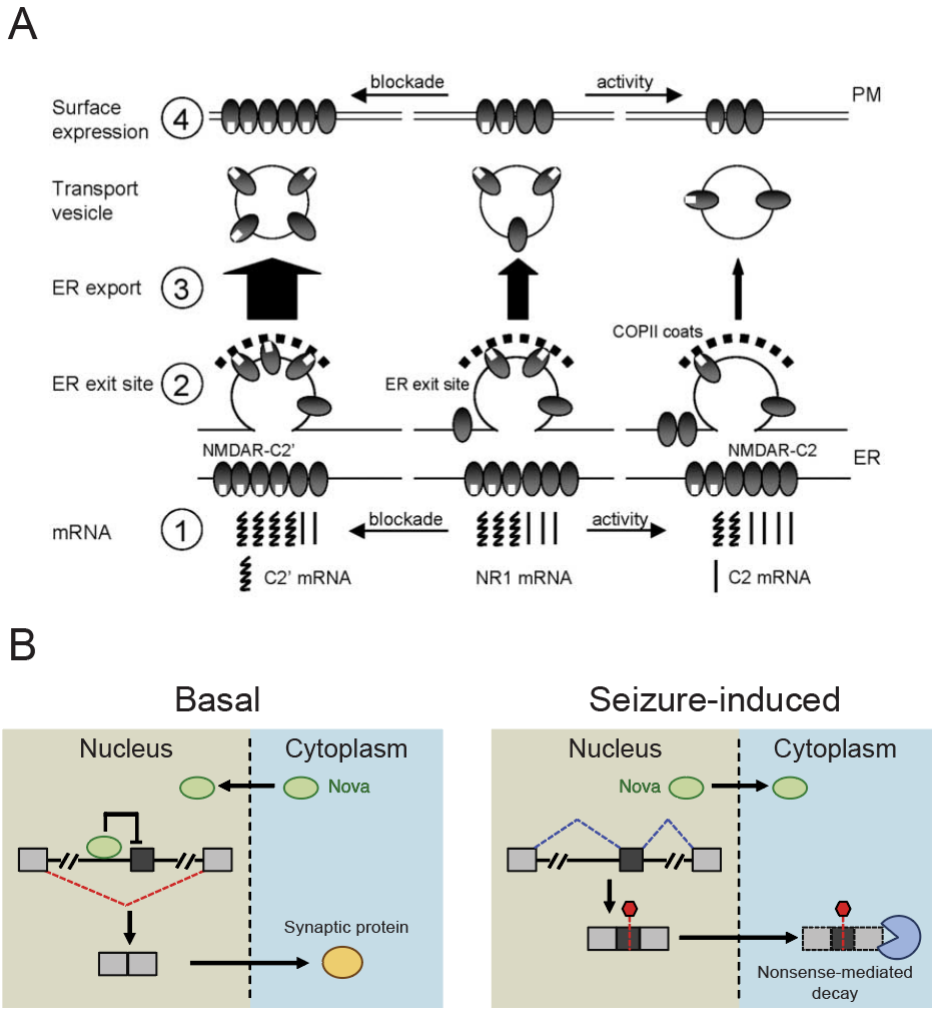

Figure 1.3: Illustration of activity-dependent AS expanding proteome diversity or regulating gene expression level. Adapted from (Mu et al., 2003) and (Raj and Blencowe, 2015).

A: Example of proteome expansion via activity-dependent AS. Grin1 exon 22 alternative splicing is regulated by neuronal activity. Blockade of neuronal activity promotes exon 22 exclusion leading to the production of C2' Grin1 mRNA. C2'-containing NMDARs exhibit an ER export signal interacting with the COPII coats leading to facilitated NMDAR trafficking to the plasma membrane (PM). In contrast, an increase in neuronal activity leads to the inclusion of exon 22 and ultimately ends up in a reduction of NMDA receptor surface expression. B: Example of gene expression level regulation by AS-coupled NMD upon neuronal stimulation. In basal condition, NOVA represses the

inclusion of a cryptic exon in the nucleus. Upon pilocarpine-induced seizure, NOVA RBPs leave the nucleus promoting the inclusion of a PTC-containing decoy exon leading to transcript degradation via NMD.

Importantly, activity-dependent AS can also regulate gene expression levels. For instance, mice treated with pilocarpine, a drug that induces acute electrical seizures, exhibit a clear change in NMD exons usage for multiple targets including transcripts encoding proteins that regulate synaptic biology or vesicular transport. Importantly, pilocarpine treatment triggers cytosol to nucleus transport of the NOVA proteins which promote the inclusion of PTC-containing sequences (Eom et al., 2013) (Fig 1.3, B).

In conclusion, regulation of AS upon neuronal activity strongly contributes to shaping the neuronal transcriptome. Generation of new isoforms to increase protein diversity but also control of gene expression level upon neuronal depolarization regulate synaptic transmission, synapse formation, and specification. Importantly, most splicing events happen co-transcriptionally or require transcription-related complexes (Ding et al., 2017; Luco et al., 2011). Thereby, activity-dependent splicing suffers from a similar temporal limitation as activity-dependent transcription even if the splicing reaction by itself happens in minute range (Drexler et al., 2020; Singh and Padgett, 2009).

1.2.3 Activity-dependent cellular compartmentalization

Cellular compartmentalization of RNAs is emerging as an important layer of gene expression regulation. Especially, nuclear storage and release of pre-existing stable RNAs is extremely well suited for rapid new mRNA mobilization and subsequent protein translation independently of de novo transcription. The regulation of nuclear sequestration by external signals is only starting to be explored but such a mechanism could support transcription-independent and stimulation-specific remodeling of neuronal transcription upon environmental changes.

Evidences for cellular compartmentalization

Neurons are highly polarized cells and precise spatiotemporal gene expression regulation is critical for proper neuronal function. Thus, regulation of mRNA localization is a strong modulator of gene expression upon environmental change. For example, mRNA targeting to neurites and synapses and activity-dependent translation provides rapid and on-site new protein synthesis in response to neuronal activity. Moreover, many studies support a

role for local translation in plasticity formation even though new tools are necessary to dissociate the functional impact of local protein synthesis from bulk activity-dependent translation (Sutton and Schuman, 2006).

Another example of transcript relocalization is the transport from the nucleus to the cytoplasm. This transport is strongly regulated as mRNAs are charged molecules with a high molecular weight which make them unable to passively diffuse through the nuclear pore complexes (Keminer and Peters, 1999; Mohr et al., 2009; Paine et al., 1975). In order to be exported, mRNAs need various export factors proteins that are recruited during transcription and maturation of pre-mRNAs (Wickramasinghe and Laskey, 2015). mRNAs failing their cytosolic export cannot participate in protein production and most of the time end up being degraded by the nuclear (Bresson and Tollervey, 2018).

Efforts in recent years have highlighted populations of RNAs experiencing nuclear sequestration in multiple cell types including neurons (Bahar Halpern et al., 2015; Benoit Bouvrette et al., 2018; Yeom et al., 2021). Nuclear transcript sequestration can be in the range of minutes and many mRNAs see their cytoplasmic export delayed after transcription (Bahar Halpern et al., 2015; Battich et al., 2020). Interestingly, some transcripts are retained in the nucleus for longer periods. For example, the CTN-RNA is stably retained in the nucleus until partial cleavage of its 3'UTR (Prasanth et al., 2005). Similarly, recent studies reported candidate intron-retaining transcripts sequestered in the nucleus and stable for hours or even days (Mauger et al., 2016; Naro et al., 2017; Ninomiya et al., 2011).

Putative mechanisms for nuclear sequestration

The molecular determinants leading to long-term nuclear sequestration are not fully known. Nevertheless, some potential regulatory mechanisms have been described. For example, retention of CTN-RNA in the nucleus was associated with ADAR enzyme binding and possible A-to-I editing in its 3'UTR (Prasanth et al., 2005). Alternatively, Yeom and colleagues reported polyadenylated transcripts enriched in the nucleus and associated with chromatin which may explain their nuclear sequestration (Yeom et al., 2021). Another mechanism, intron retention is known as a particularly strong determinant of stable nuclear sequestration in different cell types, including neurons (Mauger et al., 2016; Naro et al., 2017; Ninomiya et al., 2011). A recent study reported that the telomerase reverse transcriptase gene (TERT) and the taurine upregulated gene 1 (TUG1) encode for RNAs that are strongly enriched in the nucleus where they exhibit intron retention. These nuclear intron-retaining isoforms are stable for up to 4.5h as demonstrated upon transcription

inhibition, while in contrast the spliced isoforms present in the cytoplasm are slowly degraded. Importantly, splicing inhibition with an antisense oligonucleotide targeting the retained introns in these 2 transcripts leads to an increase in nuclear enrichment illustrating the causative role of intron retention in nuclear sequestration (Dumbović et al., 2021).

The molecular determinants of nuclear retention of incompletely spliced transcripts are not clearly defined, one possible explanation is the defective recruitment of export factors. An important platform for export factors binding is the exon junction complex, a set of proteins deposited close to the splice junction after splicing completion (Schlautmann and Gehring, 2020). In the case of retained introns, one or multiple EJCs are missing on the transcript which may disfavor export factors binding. Alternatively, retained introns may harbor regulatory sequences allowing binding of proteins favoring nuclear retention.

Functional role and regulation of nuclear transcript sequestration

The potential functional consequences of transcripts sequestration in the nucleus are intimately linked to the duration of this sequestration. It was shown that short nuclear retention of mRNAs upon transcription completion is used to confine transcriptional noise to the nucleus (Bahar Halpern et al., 2015; Battich et al., 2020). Indeed, minute range delay in mRNA export buffers the stochastic fluctuation induced by transcriptional burst.

On the other hand, transcripts can be stably retained in the nucleus to achieve a non-coding function. This is the case for certain long non-coding RNA (lncRNA) such as *Xist*. *Xist* is transcribed from one of the two X chromosomes in females. *Xist* is then recruited to one X chromosome through DNA-binding protein partners and recruit a set of RNA-binding proteins that will transcriptionally silence one copy of the X chromosome to ensure balanced gene dosage (Lu et al., 2017). Alternatively, it was shown that certain nuclear transcripts are architecturally involved in nuclear bodies formation via liquid-liquid phase separation (Shevtsov and Dundr, 2011).

Another particularly interesting mechanism is the on-demand release of nuclear transcripts. A long known example is the 3'UTR cleavage of CTN-RNA in paraspeckles upon cellular stress leading to the formation of translation competent mRNAs (Prasanth et al., 2005). Alternatively, acute external stimuli such as heat shock, osmotic stress, or elevation of neuronal network activity trigger splicing and cytoplasmic release of a handful of stable and nuclear intron-retaining transcripts (Mauger et al., 2016; Ninomiya et al., 2011). In a similar manner, splicing and cytosolic release of stable and nuclear intron-retaining transcripts, synthesized days before, is necessary for the late stage of

spermatogenesis, when transcription is off (Naro et al., 2017). These studies demonstrate the emerging role of cellular compartmentalization in gene expression regulation during development or upon acute external signals. Signal-dependent splicing and release of stable and nuclear intron-retaining transcripts represent the core mechanism of my dissertation project and will be discussed in more detail in chapter 1.4 of this dissertation.

Such a mechanism allows quite flexible and rapid new mRNA mobilization as it relies on splicing of stable nuclear RNA and cytoplasmic export rather than de novo transcription. Indeed, splicing is a minute range mechanism (Drexler et al., 2020) and nucleoplasmic diffusion of mRNAs to nuclear pore complexes only takes minutes and the transit itself is a millisecond range event (Ma et al., 2013; Mor et al., 2010; Siebrasse et al., 2012). However, regulation of mRNA cellular compartmentalization in response to environmental changes is only starting to be explored. Additional investigations will be necessary to uncover how largely this mechanism is used by different types of cells. Moreover, whether this mechanism represents a universal gene expression regulation program or whether different stimuli are able to differentially regulate RNA nuclear sequestration is unknown. Finally, the molecular signaling pathways controlling this mechanism and the functions associated with this type of gene expression regulation, notably in neurons, are yet to be explored.

1.3 Cue specificity of neuronal transcriptomic programs

Neurons can receive multiple types of stimuli that specifically change their functional properties. As the formation of plasticity events strongly relies on gene expression regulation, neurons must encode various stimuli into specific signals reaching the nucleus (Heinz and Bloodgood, 2020). At first, stimulation-specific gene expression regulation was investigated using candidate-based approaches. This type of assay uncovered cue-specific transcription and splicing events upon pharmacological treatments or direct electrical stimulation mimicking different types of neuronal activity or environmental stress (Bergkessel et al., 2011; Greer and Greenberg, 2008). Importantly, more recent studies also identified cue-specific gene expression regulation in-vivo upon distinct sensory experiences or behavioral paradigms (Mukherjee et al., 2018; Nakashima et al., 2019). Subsequent studies using genome-wide tools shed light on large populations of gene expression regulation events specifically regulated upon distinct neuronal stimuli (Lee and Fields, 2021; Tyssowski et al., 2018). The molecular mechanisms supporting stimulus-specific signal delivery to the nucleus have been strongly studied and involve the activation

of signaling pathways based on post-translational modifications. These signals ultimately trigger unique regulation of trans-acting binding factors that are the direct mediators of transcriptional and splicing events in the nucleus (Shin and Manley, 2004; Flavell and Greenberg, 2008). In the following paragraphs, I will describe pieces of evidence of stimulation-specific gene expression regulation gathered in-vitro and in-vivo using both candidate-based or genome-wide approaches. In the second part, I will discuss the different molecular mechanisms supporting stimulation-specific gene expression regulation. As these mechanisms have been mainly studied in the context of activity-dependent transcription, I will focus on the signaling pathways and subsequent regulation of transcription factors involved in this process. Finally, I will describe the molecular mechanisms that regulate splicing upon distinct stimuli and how these can encode cue-specific splicing events.

1.3.1 Evidences of cue specificity in the neuronal transcriptome

Stimulus specificity in candidate-based approaches

Early studies investigating stimulation-specific gene expression regulation mainly focused on particular transcript candidates. A striking example is a differential expression of *c-fos* and *nur/77*, two IEGs, in response to different depolarizing firing patterns applied onto dorsal root ganglion neurons (Sheng et al., 1993). Applying 6 depolarizing impulses per minute for 2 minutes triggers strong *c-fos* transcription while 12 impulses per minute do not. On the other hand, *nur/77* expression was induced upon both depolarizing patterns. Alternatively, the use of pharmacological treatments mimicking the binding of different neurotransmitters or growth factors to their target receptors revealed specific gene expression regulation events in neurons. For example, it was demonstrated that nerve growth factor (NGF) and epidermal growth factor (EGF) treatments trigger the transcription of *c-jun* in PC-12 cells while KCl-induced depolarization did not (Bartel et al., 1989). Similarly, transcription of the IEG Npas4 was shown to happen specifically upon membrane depolarization in cultured hippocampal neurons in contrast to growth factor treatments, including neurotrophins (Lin et al., 2008). A similar type of treatment was used to identify stimulation-specific AS events. For example, the cyclin *Ania6* can be produced in two distinct isoforms in striatal neurons. The short *Ania6* isoform code for a protein containing a domain rich in arginine and serine (RS domain) and is localized at nuclear speckles. This protein interacts with CDK11, a protein known to regulate pre-mRNA

splicing (Dickinson et al., 2002; Hu et al., 2003). The long *Ania6* isoform encodes a shorter protein lacking the RS domain and is not enriched in the nucleus. The long *Ania6* is induced upon glutamate treatment while KCl-induced depolarization favors the short *Ania6* isoform (Berke et al., 2001). Interestingly, BDNF and dopaminergic agonist treatments lead to the upregulation of both isoforms (Berke et al., 2001; Sgambato et al., 2004). More recently, different studies investigated cue-specific gene expression regulation upon more physiologically relevant paradigms. For example, Nakashima and colleagues showed that olfactory sensory neurons expressing different olfactory receptors also exhibit different patterns of Ca^{2+} influx during brain development. Using optogenetically modified olfactory sensory neurons, they could mimic the Ca^{2+} influx received during development and induce the expression of distinct axon-guidance cues (Nakashima et al., 2019). Additionally, different behavioral paradigms can lead to distinct transcriptomic signatures (Guzowski et al., 2001). For example, positive and negative experiences with addictive properties induce specific IEG expression patterns in mice (Mukherjee et al., 2018).

Stimulus specificity transcriptome wide

The development of next-generation sequencing methods allowed the identification of large and stimulus-specific populations of gene expression events. For example, Lee and colleagues stimulated dorsal root ganglion neurons with 2 different patterns of depolarization yielding the same number of action potentials but different inter-burst intervals. They then identified large populations of genes differentially regulated upon these 2 different depolarization patterns (Lee et al., 2017). Similarly, a recent study identified differential gene expression programs induced by different durations of neuronal depolarization. Sustained depolarization induced by KCl treatment in mouse cortical neurons triggered 3 transcriptional waves. On the other hand, transient depolarization (1-5 minutes) triggered only the first transcriptional wave which is mainly composed of IEGs. Importantly, similar results were observed in the visual cortex of dark-housed mice stimulated with sustained or brief light exposure (Tyssowski et al., 2018). These studies indicate that different temporal patterns of neuronal activity induce stimulus-specific and large gene expression regulation programs that may mediate changes in neuronal properties. However, investigation of stimulation-specific gene expression events in-vivo lags behind because most behavioral paradigms recruit only sub-populations of neurons. Thereby, a single-cell resolution is necessary to capture cue-specific events in-vivo and recent development in single-cell sequencing will be of great use in this purpose. Moreover, the stimulation-specificity of gene expression programs has been mainly

investigated for transcriptional events and more work will be necessary to identify large populations of stimulation-specific AS events.

1.3.2 Molecular mechanisms of neuronal cue specificity

At the molecular level, neurons can encode different signals via the release of various neurotransmitters, neuromodulators or growth factors. Additionally, a single neurotransmitter can bind to different types of receptors. For example, glutamate, the main excitatory neurotransmitter in the adult mammalian brain, can bind to ionotropic and metabotropic glutamate receptors that trigger different signals with distinct kinetics in neurons (Reiner and Levitz, 2018). In the nucleus, stimulus-specific gene expression events are directly controlled by the combinatorial effect of various trans-acting binding factors such as transcription factors (TF) in the context of activity-dependent transcription. Recruitment of these transcription factors is mediated by various signaling pathways that transport the signal from the synapse to the nucleus upon distinct types of neuronal activity. In contrast, stimulation specificity of splicing events is less studied. Activity-dependent splicing events are controlled by similar signaling pathways that result in splicing factor regulation and splicing modulation of target RNAs.

Stimulus specificity encoded by transcription factor regulation

In the case of activity-dependent transcription, an array of TF have been identified and individual TF have been associated with different transcriptional events (Chawla et al., 1998; Flavell et al., 2006; Janknecht and Nordheim, 1992; Shalizi et al., 2006; West et al., 2002). For example, *bdnf* transcription in cortical neurons is dependent on CaRF, a TF that binds to a calcium regulatory element on the bdnf transcript upon depolarization-induced Ca^{2+} increase but not cAMP level increase (Tao et al., 2002). This illustrates how certain TFs can be specific for a particular type of neuronal stimulus.

Interestingly, multiple stimuli can converge on a single TF. Both KCl- and glutamate-induced depolarization trigger CREB phosphorylation at ser133 and ser142 but BDNF treatment only triggers phosphorylation at ser133. Importantly, both ser133 and 142 phosphorylation were necessary for CREB-dependent transcriptional regulation of a c-fos reporter suggesting that stimulus-specific post-translational modification of a TF can lead to distinct transcriptional events (Kornhauser et al., 2002). Another layer of regulation lies in the combinatorial effect of various TFs on a single transcriptional unit. For example, the

c-fos gene is controlled by a promoter and five different enhancers and CHIP-sequencing revealed differential binding of the TFs CREB, SRF, CBP, and NPAS4 on these regulatory DNA elements (Kim et al., 2010). KCl and BNDF treatment in mouse cortical neurons both induce *c-fos* expression via both common and specific enhancers activation as measured by the induction of different enhancer RNAs. KCl treatment specifically triggers the activation of the enhancer 2 while BDNF treatment specifically triggers the activation of enhancer 4. Moreover, knockdown of specific TFs upon KCl or BDNF treatment revealed that CREB and MEF2A are necessary for KCl-induced enhancer 2 activation and optimal *c-fos* expression but not SRF. On the other hand, only MEF2A was necessary for BDNF-induced enhancer 4 activation and *c-fos* expression (Joo et al., 2016). These studies underscore the complexity of the TFs combinatorial effects to mediate stimulation-specific transcriptional regulation in neurons.

Interestingly, more recent studies shed light on the complex cooperation of trans-acting DNA binding factors to support stimulus-specific transcriptional events. For example, the nuclear receptor corepressor 2 (NCoR2) is associated with the Aryl hydrocarbon receptor nuclear translocator 2 (ARNT2) and suppresses the expression of depolarization-sensitive genes in absence of depolarization. NCoR2 leaves the complex upon depolarization and ARNT2 recruits other factors such as NPAS4 which favors the transcription of the target genes (Sharma et al., 2019). This type of mechanism suggests a competition between repressors and activators that can be controlled by the nuclear concentration of such proteins and fine-tune the transcription of target genes upon distinct stimuli. Additionally, a recent study from the Bloodgood laboratory describes a mechanism based on dendritic translation and nuclear transport of NPAS4 and ARNT1 to encode synaptic depolarization input in CA1 neurons. NPAS4 and ARNT1 form a heterodimer and bind specific promoters and enhancers while antidromic action potential leads to de novo Npas4 transcription and somatic interaction with ARNT2 resulting in differential binding of this heterodimer on the genome (Brigidi et al., 2019). This type of mechanism allows the integration of different type of depolarization and show that local translation can be used as a mechanism for nuclear signaling and stimulus-specific transcriptional programs.

Signaling pathways from synapse to nucleus

In order to regulate transcription factors activity, neurons need to transport the stimulus-specific signals integrated at the synapses to the nucleus. Upon neuronal depolarization, such signaling pathways are highly dependent on calcium influx through NMDA receptors and voltage-gated calcium channels (VGCC) (Cole et al., 1989; Murphy et al., 1991). Ca^{2+}

can also be released from the endoplasmic reticulum (ER) stock (Verkhratsky, 2002) or through AMPA receptors lacking the GluA2 subunit (Burnashev et al., 1992; Geiger et al., 1995). Elevation of intracellular Ca^{2+} is sensed by a panel of Ca^{2+}-binding proteins in close proximity to the Ca^{2+} channel (Husi et al., 2000), such as Calmodulin, that are the starting points of signaling pathways ending up in the nucleus, besides their eventual local action (e.g at the synapse). Ca^{2+}-binding proteins then recruit diverse kinases and phosphatases upon neuronal depolarization including the CaMKs. Phosphorylation cascade between members of the CaMK family culminates in phosphorylation of nuclear CaMKI or IV and subsequent CREB phosphorylation which is necessary for c-fos induction (Bito et al., 1996; Sheng et al., 1991). Additionally, CaMKII can shuttle from cytoplasm to nucleus upon Ca^{2+} influx through L-type VGCCs and deliver Ca^{2+}-bound calmodulin to trigger CaMKI/IV phosphorylation (Ma et al., 2014). Importantly, the entry route for calcium is a major determinant of gene expression specificity. For example, Ca^{2+} influx through L-type VGCCs upon neuronal depolarization lead to a strong induction of *bdnf* mRNA while this induction is weaker upon Ca^{2+} influx through NMDA receptors (Ghosh et al., 1994).

Another widely studied signaling pathway in the context of neuronal activity is the mitogen-activated protein kinase (MAPK) pathway. This pathway has been associated with different types of stimulus including neuronal depolarization (Dolmetsch et al, 2001) and neurotrophin binding to tyrosine kinase receptors (Bonni et al., 1999). For example, neurotrophin treatment triggers phosphorylation of the GTP-binding protein RAS which is the starting point of the MAPK pathway ending up with ERK translocation in the nucleus and phosphorylation of ribosomal S6 kinase (RSK) and mitogen and stress-activated protein kinase (MSK) families which trigger CREB phosphorylation (Bito et al., 1996; Ginty et al., 1994; Xing et al., 1996, 1998). These studies illustrate how different neuronal stimuli can trigger various type of nuclear signals ending up in gene expression regulation in the nucleus.

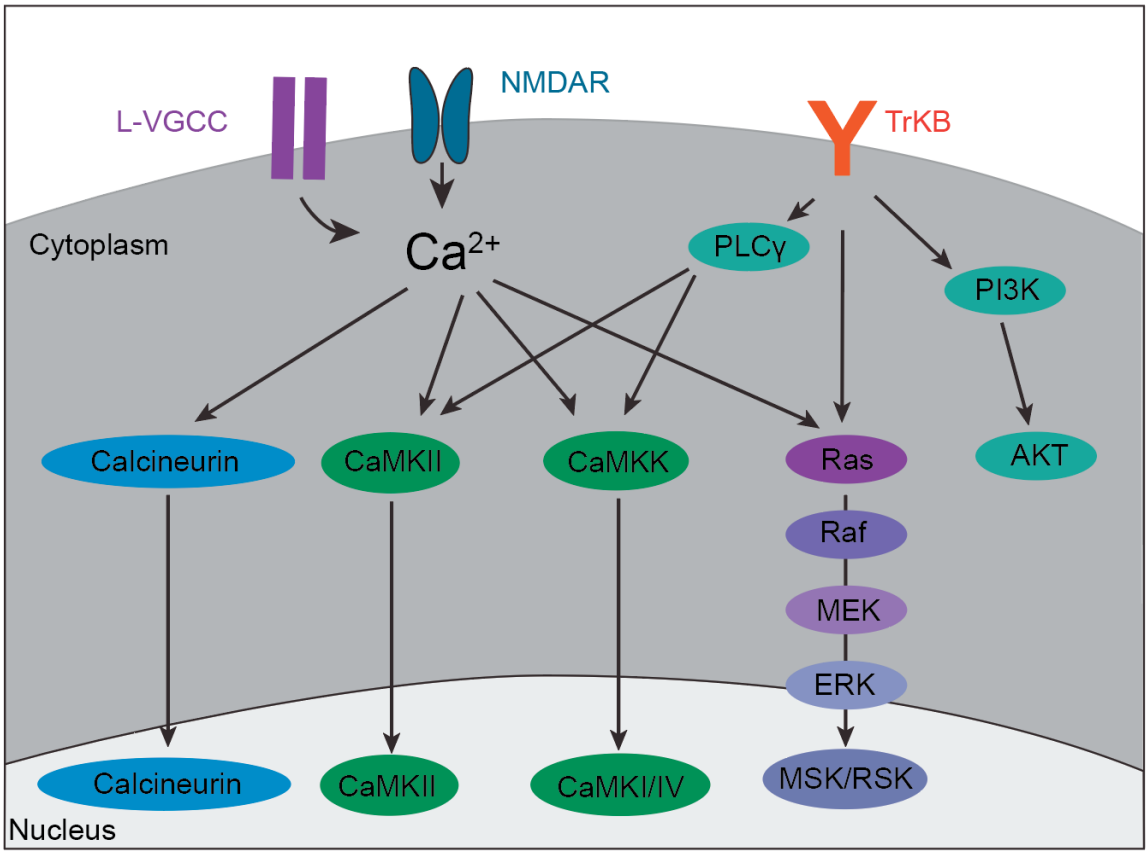

Figure 1.4: Main neuronal signaling pathways in action upon neuronal depolarization or TrKB activation. Adapted from (Yap and Greenberg, 2018).

Schematic representation of the main signaling pathways activated upon neuronal depolarization and TrKB activation leading to nuclear signals. Ca^{2+} entry through NMDA receptors or L-VGCCs and association with various calcium-binding proteins trigger the activation of various phosphatases and kinases such as Calcineurin and the CaMK pathway or the activation of the MAPK pathway. The phospholipase Cγ is directly phosphorylated by the TrKB receptor which promotes inositol-1,4,5-trisphosphate formation and Ca^{2+} release from ER stock and subsequent CaMK activation. Through various adaptor proteins, the TrKB receptor activates Ras and subsequently the MAPK pathway. The TrKB receptor activates the protein kinase B (AKT) via phosphatidylinositol 3-kinase recruitment; AKT activation mediates neurotrophin's effects on neuronal survival by antagonizing the action of the Bcl2 protein family (Minichiello, 2009). Important AKT targets include the Glycogen synthase kinase 3 GSK3 and mTORC1 (Manning and Toker, 2017).

However, these early works also underscore the strong overlap between different signaling pathways upon neuronal stimuli leading to distinct gene expression programs (Bartel et al., 1989; Lin et al., 2008). This supports that other properties of these signals must encode part of the stimulus specificity. More recent studies strongly support that the specificity of a nuclear signal is supported by the intensity and temporal pattern of signaling pathways activity. For example, sustained or transient activation of the MAPK pathway upon BDNF treatment of cultured neurons leads to differential gene expression programs. Sustained

MAPK activity trigger Arc and Homer transcription while transient MAPK activation does not (Ji et al., 2010). As the signaling pathways providing the nuclear signal strongly rely on phosphorylation cascades, the role of various phosphatases is critical in shaping the temporal dynamics of these pathways. For example, Ca^{2+} influx through NMDA receptors activates the phosphatase CALCINEURIN which dephosphorylates and activates the striatal-enriched protein tyrosine phosphatase STEP which directly dephosphorylates ERK and inhibits its nuclear export. This restricts MAPK activation upon elevation of neuronal network activity and leads to only transient activation of this pathway (Paul et al., 2003). The development of experimental procedures allowing concomitant measurement or visualization of Ca^{2+} level, phosphorylation events, and transcription of new mRNA will be of great interest to understand better how different stimuli trigger distinct gene expression programs

Molecular determinants of stimulus-specific alternative splicing

Stimulus-specific regulation of neuronal gene expression has been mainly studied in the context of activity-dependent transcription. However, the neuronal pathways signaling to the nucleus and involved in transcription regulation have been found to also regulate activity-dependent splicing. Activity-dependent splicing upon neuronal depolarization has been strongly linked to Ca^{2+}-dependent pathways. For example, exclusion of the STEX exon in the Kcnma1 transcripts upon neuronal depolarization depends on Ca^{2+} influx through L-VGCC and CaMKIV phosphorylation (Xie, 2005; Xie and Black, 2001). Similarly, exclusion of exon 20 in *Nrxn1* upon KCl-induced depolarization in cerebellar neurons is abolished upon pharmacological inhibition of CaMK but not MAPK pathway (Iijima et al., 2011). Importantly, the CaMK pathway is also associated with activity-dependent post-transcriptional splicing events. Splicing completion of stable intron-retaining transcripts upon elevation of network activity in cortical culture was abolished upon inhibition of the CaMK pathway (Mauger et al., 2016). On the other hand, in non-neuronal cells, alternative splicing of exon5 in the cell surface adhesion molecule *Cd44* is regulated by the MAPK pathway. Transfection of an active form of RAS in T-lymphoma cells triggers the inclusion of exon 5 (Konig et al., 1998). Additionally, in rat primary striatal neurons, *Ania6* splicing is regulated by multiple types of neuronal stimuli, including glutamate and BDNF treatments. Interestingly, MAPK inhibition prior to BDNF treatment blocked BDNF-induced short *Ania6* isoform induction. Importantly, induction of the same *Ania6* isoform upon KCl treatment was sensitive to CaMK but not MAPK pathway inhibition (Berke et al., 2001; Sgambato et al., 2004). These data indicate that the signaling pathways reported to control

transcriptional regulation also regulate activity-dependent AS. Moreover, these signaling pathways seem to control stimulation-specific AS events in neurons.

In order to modulate splicing choice in a stimulus-specific manner, neuronal signals must control precise spicing factors binding on particular RNA elements. In this regard, it was shown that CaMKIV interacts directly with hnRNP-L and triggers its phosphorylation at ser513. Upon phosphorylation, hnRNP-L binds to an RNA element on *Kcnma1* near the 3'SS of the intron upstream of the STREX exon which prevents binding of the splicing factor U2AF65 and favor inclusion of the STEX exon (Liu et al., 2012; Xie and Black, 2001). Similarly, SAM68, a member of the signal transduction and activation of RNA (STAR) protein family was shown to mediate *Nrxn1* AS upon neuronal depolarization. Intronic regions surrounding the exon 20 in *Nrxn1* contain AU-rich regions exhibiting binding sites for SAM68. Moreover, exon 20 splicing in a reporter gene was abolished upon SAM68 point mutation that inhibits its RNA binding properties (Chawla et al., 2009; Lin et al., 1997) or upon inactivation of SAM68 nuclear localization signal (Paronetto et al., 2007) supporting a nuclear translocation of SAM68 upon neuronal depolarization resulting in *Nrxn1* exon 20 exclusion (Iijima et al., 2011). Importantly, SAM68 was also reported to mediate MAPK-induced AS of *Cd44* exon 5 via binding to exonic motifs. Additional assays supported SAM68 phosphorylation in a MAPK-dependent manner upon T-lymphoma cell activation (Matter et al., 2002). Similarly, treatment with the external growth factor EGF triggers serine/arginine protein kinases (SRPKs) autophosphorylation, nuclear translocation, and the phosphorylation of SR- proteins. EGF-induced SRPK autophosphorylation was dependent on protein kinase B (AKT) activation. Inhibition of this signaling pathway abolished a large population of EGF-induced splicing events (Zhou et al., 2012). These studies indicate that cellular signaling pathways control various splicing factors activity that mediate activity-dependent AS events, including in neurons. Differential control of splicing factors upon distinct external stimuli was only poorly studied and stimulus-specific recruitment of splicing factors in the same cellular model was not reported. However, similarly to activity-dependent transcription control, cue-specific splicing seems to be controlled by the combinatorial action of multiple trans-acting factors that, in this case, favor or disfavor the inclusion of particular RNA segment and appear to be controlled by various neuronal signaling pathways.

1.4 Regulation of neuronal gene expression via intron retention

Intron retention (IR) is defined as the persistence of an intronic sequence in an otherwise fully processed RNA. IR is a type of alternative splicing that has been understudied for a long time because it was thought to be the result of spliceosome malfunction disrupting the gene expression process (Monteuuis et al., 2019). Indeed, many incompletely spliced RNAs cannot leave the nucleus and are ultimately degraded (Palazzo and Lee, 2018). Additionally, quantitative analysis of IR is not trivial and specific tools had to be developed to study it with accuracy (Broseus and Ritchie, 2020). Using these tools, researchers defined IRs as a widespread type of AS in the mammalian genome. This new status has led to strong investigations of IR molecular determinants which appear to be complex and multifactorial.

Research in the last decade shed light on various populations of IRs regulating the gene expression process via various molecular mechanisms according to their subcellular localization and the location of the retained intron in the RNA sequence. Additionally, there is a growing body of evidence of IR regulation during development but also upon more acute stimuli that require rapid gene expression modulation (Jacob and Smith, 2017). Importantly, IR regulation can happen co- or post-transcriptionally with a profound impact on gene expression kinetic (Mauger et al., 2016; Ni et al., 2016; Ninomiya et al., 2011; Park et al., 2017; Shalgi et al., 2014).

Intron retention regulation represents the core of my dissertation project and in the following paragraphs, I will discuss in detail the genome-wide mapping of IRs in various organisms as well as the different sequence features and trans-acting factors that are thought to cause IR. I will also describe the different populations of IR and how they regulate gene expression at the molecular level. Finally, I will summarize the work reporting IR regulation during development but also upon external signals.

1.4.1 Intron retention : a widespread class of alternative splicing

Intron retention distribution across species and tissues

The emergence of next-generation sequencing methods gave the first hint at the distribution of intron retention (IR) genome-wide in various organisms. These first

pioneering reports indicated that IR is a major alternative splicing (AS) event in plants, viruses, and unicellular organisms (Ner-Gaon et al., 2004; Rekosh and Hammarskjold, 2018). In Arabidopsis thaliana, IR account for 30% to 60% of all AS events depending on growth conditions (Filichkin et al., 2010; Kalyna et al., 2012; Marquez et al., 2012). In contrast, only 5% of human AS events were recognized as IR events (Galante, 2004; Wang et al., 2008). However, IR is a type of AS event that is particularly difficult to assess due to overlapping intronic features such as snRNAs, unannotated exons or antisense RNA, and repetitive regions with low read mappability (Broseus and Ritchie, 2020). The development of IR-specialized bioinformatics pipelines and the use of higher RNA-sequencing read coverage completely revised the early estimation of IR events in mammals. In a more recent study, Braunschweig and colleagues reported that 35% of human and mouse multiexonic genes harbor one or more retained intron in at least 50% of their transcripts. The number of IR-containing genes even rises to 77% when including retained introns with a percentage of intron retention (PIR) of at least 10% (Braunschweig et al., 2014). Another recent study assessed IR in more than 2000 human samples and found that around 80% of human protein-coding genes are affected by IR (Middleton et al., 2017). Notably, assessment of IR across different tissues in human and mouse indicate a strong enrichment in the brain. Moreover, brain-associated IR events show a high degree of evolutionary conservation compared to other tissues (Braunschweig et al., 2014). This suggests an important role for IR in the brain.

These studies have shed light on the importance of IR in mammalian genomes. Moreover, using these new quantification tools, IR was associated with multiple pathologies such as cancer (Dvinge and Bradley, 2015), various neurodevelopmental disorders (Jeromin and Bowser, 2017; Sznajder et al., 2018), and Alzheimer disease (Xu et al., 2018). Consequently, the revised position of IR as a widespread AS event motivated the exploration of the molecular determinants and functions of IR in mammals.

Molecular determinants of intron retention

In order to understand the role of IR in physiology and disease, numerous studies attempted to identify its molecular determinants. Certain sequence features are associated with the likelihood for an intron to be retained. First, retained introns generally harbor weaker 5' and 3' splice sites (SS) (Galante, 2004; Sakabe and de Souza, 2007; Stamm et al., 2000). Splice site strength is calculated as the degree of similarity of splice site sequences to consensus sequence and reflects the probability for a SS to be used by the splicing machinery (Yeo et al., 2004). Mutation of suboptimal IR splice site, resulting in a

sequence more similar to the consensus sequence, was shown to restore splicing in exogenous reporters (Dirksen et al., 1995; Romano, 2001). Retained introns also exhibit high guanosine/cytosine content (GC content). Notably, such GC sequences are enriched near the splice sites and are predicted to form stable RNA secondary structures such as hairpins or G quadruplexes (Ciesiolka et al., 2017; Handa et al., 2005; Park et al., 2015; Sznajder et al., 2018). These secondary structures may disfavor trans-acting factors binding and contribute to IR. Finally, retained introns are shorter than canonically spliced introns (Braunschweig et al., 2014; Sakabe and de Souza, 2007; Schmitz et al., 2017). Besides these sequence features, retained introns are enriched for cis-acting elements bound by certain splicing factors such as SR protein (Middleton et al., 2017); ENCODE project consortium 2012), hnRNPs, or others RNA-binding proteins (RBP) that affect their splicing efficiency (Charlet-B. et al., 2002; Cho et al., 2014). Finally, methylation recently emerged as a strong regulator of IR. Interestingly, reduction of DNA methylation at CpGs negatively regulates the binding of MeCP2 which is known to recruit various splicing factors including TRA2B, resulting in increased IR (Long et al., 2011; Wong et al., 2017; Young et al., 2005). Alternatively, RNA methylation has been shown to increase IR. In C.elegans, methylation of the 3' SS in the intron 2 of the S-adenosylmethionine synthase *sams-3* (ortholog of human *Mat2a*) disfavor U2AF recruitment and leads to intron 2 retention (Mendel et al., 2021).

In conclusion, IR has been identified as a widespread AS event. Moreover, IR appears to have many molecular determinants and to be rather multifactorial. These multiple strategies for a cell to regulate IR suggest that it may be used to modulate mammalian gene expression in a particularly flexible way.

1.4.2 Impact of intron retention in gene expression programs

IR is now accepted as a widespread type of AS in mammals, particularly in the brain where retained introns are highly conserved (Braunschweig et al., 2014). This has recently attracted a strong interest in the role of IR in gene expression regulation. Studies in the last decades revealed that IR events result in a heterogeneous population of transcripts that can be regulated via different molecular mechanisms (Fig 1.4) (Jacob and Smith, 2017).

IR-induced nuclear or cytoplasmic degradation

One molecular consequence of IR is the nuclear sequestration of intron-retaining isoforms. Intron-retaining transcripts (intron-retaining transcript) sequestrated in the nucleus cannot participate in protein production and are ultimately degraded. This population of intron-retaining transcripts is unaffected by nonsense-mediated decay (NMD) inhibition and is stabilized upon knockdown of certain nuclear exosome components (Bergeron et al., 2015; Yap et al., 2012). These studies describe a gene expression regulation mechanism where intron-retaining transcripts are retained in the nucleus and ultimately degraded by the nuclear exosome.

Alternatively, intron-retaining transcripts can be exported in the cytoplasm. Generally, retention of an intron leads to the introduction of a premature stop codon (PTC), making these transcripts classical targets for the NMD machinery. Multiple studies reported stabilization of certain intron-retaining transcripts upon translation inhibition or caffeine treatment, two treatments that lead to NMD inhibition (Eom et al., 2013; Wong et al., 2013). Additionally, knock-down of key NMD components such as the ATP-dependent RNA helicase Up-frameshift 1 (UPF1) also resulted in intron-retaining transcripts stabilization (Lareau et al., 2007). These results indicate the cytoplasmic export of a population of intron-retaining transcripts and their degradation via NMD.

These works have described two populations of rather unstable IR events. Intron-retaining transcripts are either sequestered in the nucleus and degraded by the nuclear exosome, either exported in the cytosol and degraded via NMD. These mechanisms allow the cells to fine-tune their gene expression level by targeting transcripts to degradation via IR.

Intron retention to increase proteome diversity

Alternatively, when the retained intron is in the coding region, IR can expand the proteome diversity via translation of alternative protein isoforms (Hossain et al., 2016; Li et al., 2016; Marquez et al., 2015). Interestingly, translation of a new protein isoform via IR can be coupled to NMD to precisely control the protein level. For instance, the *Robo3* RNA, which encode a Slit receptor, is present in two isoforms. *Robo3.1* is spliced from e26 to e27 while *Robo3.2* retains i26. While *Robo3.1* is necessary for commissural neurons axon guidance to and across the spinal cord ventral midline, *Robo3.2* potentiates midline repulsion in post-crossing axons. Importantly, fine control of *Robo3.2* protein level by NMD is thought to adjust the degree of repulsion from the midline (Chen et al., 2008; Colak et al., 2013). This example shows that the translation of intron-retaining transcripts is important for

correct axon pathfinding in the developing nervous system. However, intron retained in the coding region and maintaining a coding frame without introducing a PTC are rare and there are only a few reports of proteins encoded by intron-retaining transcripts. Thereby IR rather acts in regulating gene expression levels than via expanding the proteomic diversity.

Retained introns as a platform for trans-acting binding factors

IR can regulate other aspects of the gene expression process via the recruitment of trans-acting factors. In these cases, the retained intron can be used as a platform for RBPs which regulate transcript sub-cellular localization (Bell et al., 2008; Buckley et al., 2011; Ortiz et al., 2017; Sharangdhar et al., 2017), translation efficiency (Tahmasebi et al., 2016) or stability (Thiele et al., 2006). A good example is the dendritic targeting of the *Calm3* transcript, which encodes for an important Ca^{2+}-binding protein that is involved in synapse-to-nucleus signaling upon neuronal activity. *Calm3* isoforms retaining an intron in their 3' UTR were found in neuronal dendrites by RNA FISH. This dendritic localization is significantly reduced upon knockdown of the RBP STAUFEN2. In this case, the retained intron is located in the 3'UTR thereby not altering the coding frame of the transcript. It could then support the local translation of intron-retaining transcripts (Sharangdhar et al., 2017). In other cases, the retained intron can be located in the coding region. For example, the intron 16 in the *CaMKIIα* transcript is retained and bound by STAUFEN2 which enhances its dendritic localization (Ortiz et al., 2017). Another study underscores the role of ID elements, a class of short interspersed repetitive elements, in dendritic targeting of a group of neuronal intron-retaining transcripts (Buckley et al., 2011). In this case, intron-retaining transcripts could produce alternative protein isoforms or have non-coding functions. For example, it has been hypothesized that these transcripts could be used as a sponge for miRNA, thus reducing the number of miRNAs free to bind on their target mRNAs. However, this type of mechanism remains to be experimentally validated.

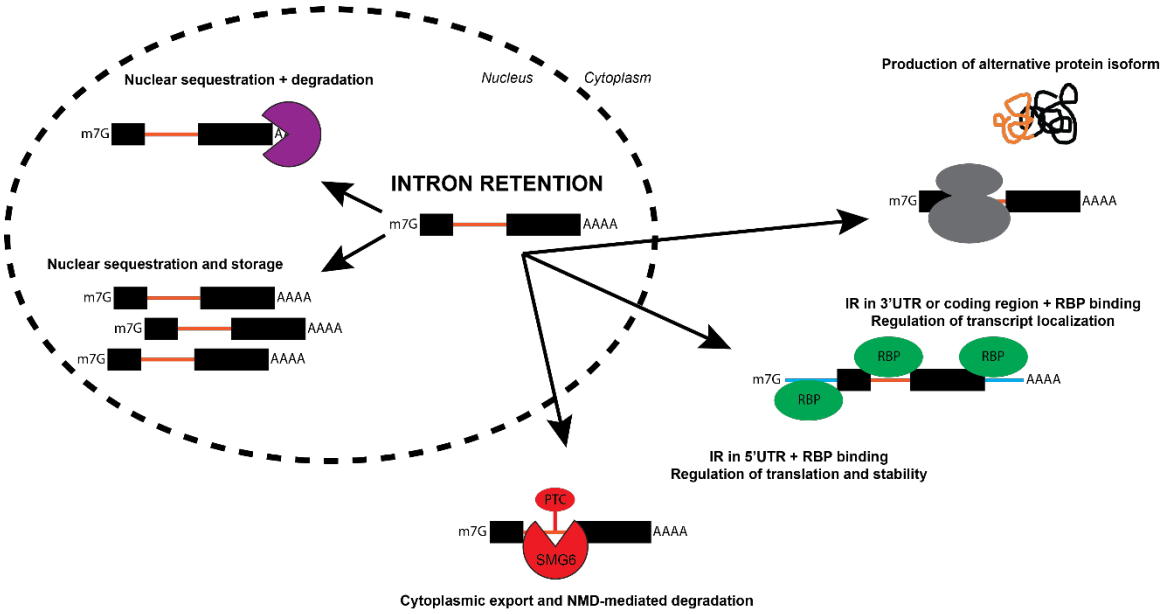

Figure 1.5: Representation of different intron-retaining transcript populations.

Intron-retaining transcripts can be sequestered in the nucleus and be degraded by the nuclear exosome. Nuclear intron-retaining transcripts can also be stably stored. Alternatively, intron-containing transcripts can be exported in the cytoplasm where they can encode for alternative protein isoforms or be degraded by the NMD machinery. Finally, retained intron located in UTR's can recruit RNA-binding protein (RBP) that will affect translation efficiency, stability, or transcript sub-cellular localization.

Nuclear storage and release of intron-retaining transcripts

Finally, multiple studies described a population of intron-retaining transcripts that are localized in the nucleus but remain stable. These intron-retaining transcripts were first reported to have a half-life of 30 minutes to 1 hour (Boutz et al., 2015; Ninomiya et al., 2011) and are sometimes termed "detained introns". Interestingly, more recent studies have described an even longer half-life. Mauger and colleagues assessed IR genome-wide in cultured cortical neurons and revealed that 50% of all intron-retaining transcripts detected exhibited less than 20% change in expression level 2 hours after inhibition of transcription. Surprisingly, certain intron-retaining transcripts were even stable for up to 6 hours after transcription inhibition (Mauger et al., 2016). Similarly, Naro and colleagues followed intron-retaining transcripts fate during germ cell differentiation into spermatids. Spermatogenesis comprises 3 main steps: a mitotic phase and a meitotic phase, when transcription is active, and the last phase called spermiogenesis when transcription is inactive. They injected 5-ethynyl uridine (EU), a chemically modified nucleotide that will integrate into nascent RNA, in living mice at the end of the meiotic phase. EU-labelled RNA was collected from testis 1 and 9 days later. Analysis of IR profiles revealed that 50% of intron-retaining transcripts were still present after 24h and up to 10% after 9 days. In

contrast, properly spliced transcripts were absent from this fraction 9 days after EU labeling (Naro et al., 2017). This indicates that these transcripts remained stable for days after transcription. Some of these very stable intron-retaining transcripts were found enriched in the nucleus. Usage of cellular fractionation revealed that the intron-retaining isoforms are stably sequestered in the nucleus while the spliced isoforms are enriched in the cytoplasm. Studies reporting stable and nuclear IR events also reported that these transcripts can complete their splicing post-transcriptionally upon additional development of environmental signals (Mauger et al., 2016; Naro et al., 2017; Ninomiya et al., 2011).

These works report nuclear intron-retaining transcripts that remain stable rather than being degraded by the nuclear exosome. Only a few papers describe this population in detail and a systematic sub-cellular mapping of these stable IRs is missing. Moreover, more work is needed to understand how these transcripts escape nuclear degradation. Such a population of IRs constitutes a reserve pool of RNA that could be mobilized for protein production in particular conditions. This would represent a temporally flexible way to regulate gene expression as it relies on pre-existing transcript splicing rather than de novo transcription.

1.4.3 Regulation of intron retention upon stimulus

Different populations of IR events are regulated by various types of stimuli. These cues range from developmental signals, that usually take place over days, to acute environmental stimuli. Importantly, certain studies report candidate intron-retaining transcripts stably enriched in the nucleus and that are post-transcriptionally spliced upon an acute stimulus.

Intron retention regulation during development

Interestingly, probing of IR profiles during differentiation of hematopoietic cells (Edwards et al., 2016; Pimentel et al., 2016; Wong et al., 2013), breast epithelial cells (Gascard et al., 2015), muscles cells (Llorian et al., 2016) or even neurons (Braunschweig et al., 2014; Yap et al., 2012; Yeom et al., 2021) shows an increase of IR during cell differentiation. This increase of IR levels is associated with degradation in the nucleus (Edwards et al., 2016; Pimentel et al., 2016; Yap et al., 2012) or in the cytoplasm through NMD (Wong et al., 2013) and leads to a decrease in protein level. To note, a population of genes involved in vesicle exocytosis, a key mechanism in neuronal communication, exhibited an opposite

pattern as IR level in these genes was reduced during brain development (Yap et al., 2012). These studies indicate that regulation of large IR populations during development is critical for proper cell differentiation. Cellular differentiation is a process occurring over days and most IR events regulated during this process probably arise co-transcriptionally. However, post-transcriptional IR regulation sometimes happens to generate proteins needed during certain developmental processes. For example, intron-retaining transcripts synthesized during early spermatogenesis are spliced days later, when transcription is inactive, exported in the cytoplasm, and found enriched on polysomal fraction which supports their use for protein synthesis (Naro et al., 2017).

Co-transcriptional intron retention regulation upon acute environmental stimuli

Besides its regulation by developmental signals, IR profiles were also probed upon a vast panel of external stimuli. For example, worms growing in medium rich in nutrients exhibit higher methylation at the 3' SS of intron 2 in *sams-3* transcripts which favors its retention and causes SAMS3 protein level decrease. As SAMS3 promotes the synthesis of S-adénosylméthionine (SAM), the major methyl donor in cells, this creates a feedback regulatory loop controlling methylation homeostasis (Mendel et al., 2021) (Fig 1.5, A). Regulation of methylation homeostasis by IR has been studied in mammals where the *Mat2a* transcript (ortholog of *sams-3* in mammals) exhibits a decrease in intron 8 retention and increase in MAT2A protein in response to methionine depletion (Pendleton et al., 2017). Importantly, methionine depletion in presence of flavopiridol, a transcription inhibitor, does not lead to an increase in *Mat2a* mRNA. However, a decrease of *Mat2a* intron-retaining isoforms is observed upon 2h and 6h hours of transcription inhibition (Pendleton et al., 2018). This supports that *Mat2a* IR regulation upon methionine depletion happens co-transcriptionally on newly synthesized transcripts. Moreover, *Mat2a* intron-retaining isoforms are fated to degradation, representing a dead-end transcript. Similarly, Park et al reported that IR of intron 4 in the *Ogt* (O-linked N-acetylglucosamine transferase) transcript is dependent on the cellular level of β-N-acetylglucosamine (O-GlcNAc), a post-translational protein modification whose homeostasis perturbation is associated with diabetes, cardiovascular diseases, and Alzheimer disease (Bond and Hanover, 2013; Yuzwa et al., 2012). High O-GlcNAc level leads to intron 4 retention and decrease in OGT protein, the enzyme that promotes O-GlcNAc deposition in cells. Importantly, diminution of O-GlcNAc levels leads to *Ogt* splicing and an increase in OGT protein (Park et al., 2017). In this study, transcriptional dependency was not experimentally demonstrated but the long response time (minimum 6 hours) for spliced transcript increase suggests that new

transcription is involved. Moreover, another study observed that *Ogt* splicing upon OSMI-1, a drug abolishing the enzymatic activity of OGT, was abolished in presence of a transcription inhibitor (Pendleton et al., 2018). This supports that *Ogt* splicing also happens co-transcriptionally. Other studies have used next-generation sequencing to report the regulation of large retained intron populations upon acute external stimulation. For example, Shalgi and colleagues probed AS genome-wide upon 2h heat shock in mouse fibroblasts. They found that 53% of IR were regulated upon heat shock, which was the most affected type of AS. These represent a large population of around 1700 IRs events of which 74% showed an increased intron inclusion and 26% showed a decrease. Importantly, intron-retaining transcripts were localized in the nucleus as probed by cellular fractionation and RNA FISH. Moreover, IR increase upon heat shock resulted in reduced ribosomal occupancy for these transcripts (Shalgi et al., 2014). Similar regulation of IR was observed upon T cell activation where hundreds of IR events exhibit decreased retention resulting in an increase in spliced mRNA. Importantly, these IR events are present in genes encoding for proteins enriched for the proteasome complex which is critical for T cell proliferation and cytokine release (Ni et al., 2016). This study illustrates that large IR programs can be regulated upon an acute stimulus such as heat shock. Importantly, the authors did not experimentally assess the transcriptional dependency of IR events regulation. As heat shock represents a transient and acute form of stimulation, one could imagine that it requires rapid transcriptome remodeling ideally independently of de novo transcription. However, whether these intron-retaining transcripts represent pre-existing and stable RNA that quickly spliced upon signals or whether transcription of new transcripts is necessary for IR regulation is unclear. Thereby, defining whether an IR regulation event happens co- or post-transcriptionally will be particularly critical to understanding the importance of IR regulation upon acute external stimuli. Interestingly, another recent study reported sensory experience-dependent IR regulation in drosophila. The authors reported the splicing of a retained intron in the *Orb2a* transcript upon appetitive associative olfactory training and male courtship suppression training (Gill et al., 2017). However, whether *Orb2a* splicing happens co- or post-transcriptionally is unknown. Moreover, the authors could not draw a direct link between Orb2a splicing and long term memory formation taking place upon these behavioral paradigms.

Post-transcriptional regulation of intron retention upon acute environmental stimuli

Importantly, other studies reported a post-transcriptional control of IR upon various acute external stimuli. Ninomiya and colleagues described an increase of the Cdc2-like kinase

Clk1 mRNA upon heat shock, osmotic stress, or treatment with the CLK kinase inhibitor TG003. This increase was accompanied by a decrease in nuclear *Clk1* intron-retaining isoform supporting a regulation at the splicing level. Importantly, *Clk1* splicing was maintained after inhibition of transcription with α-amanitin showing that this regulation occurs in pre-existing transcripts that have finished their transcription (Ninomiya et al., 2011). Similarly, Boutz and colleagues detected a diminution in *Clk1* intron-retaining isoform upon CB19 treatment, an inhibitor of CLK kinase activity. This effect was maintained upon inhibition of ongoing transcription. Moreover, sub-populations of retained introns that were enriched in the nucleus and exhibited a half-life of approximately 30min were regulated upon CB19 treatment. The remaining introns in the host transcripts of the majority of these retained introns did not exhibit an increase in read coverage, suggesting that this regulation was not due to transcription regulation. However, the transcription independence was experimentally assessed for only 2 IR events, including the retained introns in *Clk1* (Boutz et al., 2015). In an effort to characterize the regulation of stable intron-retaining transcripts genome-wide in neurons, Mauger and colleagues found that a subpopulation of stable IR events is regulated upon an elevation of neuronal network activity. Genome-wide analysis upon transcription inhibition revealed more than 3000 stable intron-retaining transcripts in mouse neocortical neurons. Of which approximately 300 exhibited increase or decrease of IR level upon bicuculline treatment, a $GABA_A$ receptor antagonist that induced an increase of neuronal network activity (Mauger et al., 2016) (Fig 1.5, B). This study also used cellular fractionation and immunoprecipitation of ribosome-associated RNAs to confirm for certain candidates that the intron-retaining isoform is located in the nucleus and that upon activity-dependent splicing, the newly spliced mRNAs are exported in the cytoplasm and loaded onto the ribosomal machinery. Importantly, sample collection at different time points revealed that certain stable intron-retaining transcripts can excise their intron as soon as 15 minutes upon neuronal stimulation.

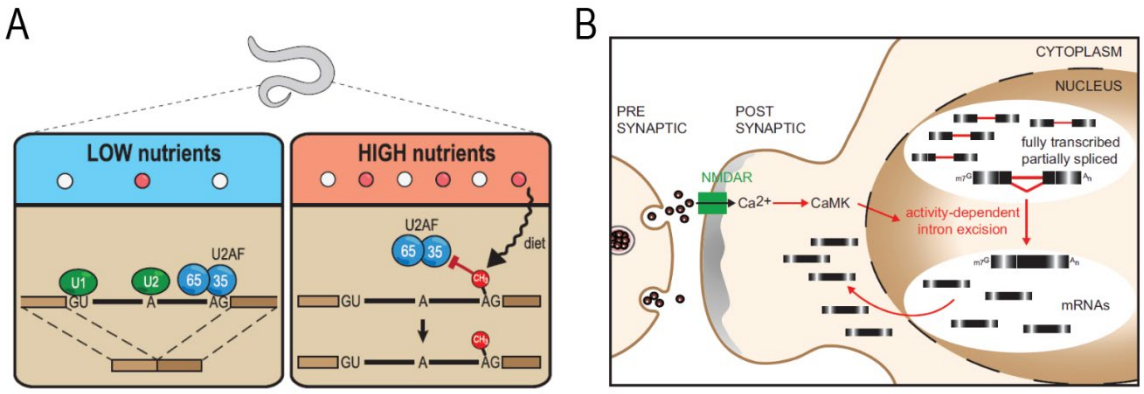

Figure 1.6: Illustration of intron retention regulation upon external signals via co-transcriptional or post-transcriptional mechanism. Adapted from (Mendel et al., 2021) and (Mauger et al., 2016).

A: High nutrient diet in C.elegans leads to sams-3 methylation at the 3' SS intron 2. This methylation disfavors U2AF binding and induces intron 2 retention. B: Increase in neuronal network activity triggers splicing completion in a population of pre-existing and nuclear intron-retaining transcripts. Newly spliced transcripts are then exported in the cytoplasm where they are associated with the ribosomal machinery. This mechanism is dependent on Ca^{2+} influx through NMDA receptors and the CaMK pathway.

These studies uncovered the post-transcriptional regulation of IR upon various external stimuli such as heat shock, osmotic stress, or even neuronal activity. Reduction of IR in pre-existing transcripts leads to the rapid mobilization of new mRNA independently of de novo transcription. However, activity-dependent splicing of stable and nuclear intron-retaining transcripts was demonstrated only for a handful of candidates. Thus, it is unknown whether there is a large population of stable intron-retaining transcripts in the nucleus ready to complete their splicing. Importantly, studies assessing IR regulation co- or post-transcriptionally genome-wide report that only subpopulations of IR events were affected by a given stimulus (Boutz et al., 2015; Mauger et al., 2016; Ni et al., 2016; Shalgi et al., 2014). This raises questions regarding whether unaffected IR events are insensitive to any kind of stimulus and exhibit a very slow degradation rate or whether they could be regulated by other stimuli. The large range of acute stimuli controlling IRs, including neuronal activity, suggests that regulation of stable and nuclear intron-retaining transcripts allows stimulus-specific regulation of neuronal gene expression. However, more work in this direction will be necessary as studies reporting activity-dependent IR regulation were performed in different cellular models. Thereby, whether distinct stimuli can trigger IR regulation in the same cellular model and whether different IR event populations respond to these stimuli is yet to be explored.

1.5 The dissertation project

The work of many laboratories over the last 30 years has shed light on the mechanisms supporting precise gene expression regulation in response to specific neuronal stimuli. These studies have identified activity-dependent transcription and AS as critical regulators of structural and functional neuronal properties. Moreover, in vitro studies using pharmacological treatments described important molecular mechanisms supporting the stimulation specificity of de novo transcription and AS programs upon distinct types of neuronal activity, including membrane depolarization and growth factors signaling. Thanks to the development of new tools, stimulation-specific transcriptional regulation is now demonstrated in vivo and the following years will most probably provide more insight into physiologically relevant regulation of AS upon sensory experience.

As they mainly happen concomitantly, de novo transcription and AS exhibit similar temporal kinetic that depend on gene length and elongation rate of the RNA polymerase. For this reason, more and more attention is given to molecular mechanisms allowing new mRNA mobilization upon neuronal activity independently of de novo transcription. In this direction, recent studies have started to underscore the potential of nuclear sequestration in shaping neuronal gene expression programs upon activity. Precisely, stable nuclear retention of intron-retaining transcripts and their cytoplasmic liberation after splicing was reported upon different types of stimuli, including elevation of neuronal network activity. However, only a handful of stable intron-retaining transcripts were experimentally observed in the nucleus. Thus, it is unknown whether this is a general mechanism allowing global neuronal transcriptome remodeling. Moreover, as various cellular models were used to investigate the stimulus sensitivity of these transcripts, it is unclear whether there are stimulation-specific populations of stable and nuclear intron-retaining transcripts.

My thesis project aims at investigating stimulation-specific gene expression programs controlled by nuclear sequestration and the release of stable intron-retaining transcripts upon splicing. In this regard, we used a combination of cell fractionation, deep RNA sequencing, and state-of-the-art bioinformatics pipelines to identify and locate stable and nuclear intron-retaining transcripts in neurons and characterize their responses upon elevation of neuronal network activity and growth factors exposure. Our results show a clear nuclear enrichment of stably retained introns and uncover specific populations responding to an elevation of network activity or growth factor treatment. These results are here presented in the form of a manuscript.

2. Results

2.1 Preface

The following result chapter, in form of a manuscript, represents the work of my PhD, carried out in close collaboration with several people. Below I will illustrate the individual contributions of each person involved in my PhD thesis project. Supplementary experiments that are not represented in the manuscript will be mentioned and put into perspective in the discussion part.

This project was supervised by Dr Oriane Mauger who was involved in writing the manuscript. The work was supported by the Ambizione fellowship and the manuscript prepared in collaboration with Prof. Dr Peter Scheiffele.

Cue-specific remodeling of the neuronal transcriptome through intron retention programs.

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[2]Equal contribution

In preparation

In this project, I performed the mouse primary neocortical cultures, pharmacological treatments, cell lysis and RNA isolations, RT-PCRs, RT-qPCRs, western blots and immunostainings to assess differential regulation of stable IR events upon distinct stimuli and identification of the involved signaling pathways. I generated all samples for RNA-sequencing and assessed their quality.

Dr Oriane Mauger performed the cellular fractionation coupled to RNA-sequencing experiment and subsequent western blot validations.

RNA-sequencing library preparation was performed by Philippe Demougin.

RNA-sequencing raw read alignment was performed in collaboration with the company Genosplice. Specific analysis of intron retention was performed jointly by Dr Oriane Mauger and myself.

2.2 Cue-specific remodeling of neuronal transcriptome through intron retention programs

ABSTRACT

Sub-cellular compartmentalization through the nuclear envelope has for a long time been primarily considered a physical barrier that separates nuclear and cytosolic contents. More recently, nuclear compartmentalization has emerged to harbor key regulatory functions in gene expression. A sizeable proportion of protein-coding mRNAs is more prevalent in the nucleus than in the cytosol reflecting the existence of mechanisms to control mRNA release into the cytosol. However, the biological relevance of the nuclear retention of mRNAs remains unclear. Here, we provide a comprehensive map of the subcellular localization of mRNAs in mature neurons and reveal that transcripts stably retaining introns are broadly targeted for nuclear retention. We systematically probed these transcripts upon neuronal stimulation and found that sub-populations of nuclear-retained transcripts are bi-directionally regulated in response to cues: some appear targeted for degradation while others undergo splicing completion to generate fully mature mRNAs which are exported to the cytosol to increase functional gene expression. Remarkably, different forms of stimulation mobilize distinct groups of intron-retaining transcripts and this selectivity arises from the activation of specific signaling pathways. Overall, our findings uncover cue-specific control of intron retention as a major regulator of acute remodeling of the neuronal transcriptome.

INTRODUCTION

Transcriptome remodeling plays a major role in cellular differentiation and plasticity. Modifications in RNA repertoires are highly specific to the cues received by cells. In development, regional signals and transcription factors direct transcriptomic programs that specify cell types. However, even in post-mitotic cells, transcriptomes remain dynamic to drive structural and functional changes for plasticity. In particular, mature neurons – that integrate numerous and diverse cues - have developed cue-specific pathways for transcriptome remodeling to support various forms of plasticity (Greer and Greenberg, 2008). For example neuronal activity or growth factor signaling each trigger specific programs of de novo transcription resulting in the up-regulation of highly selective and specific sets of genes that modify neuronal wiring and function (Lambert et al., 2013; Mardinly et al., 2016; Russek et al., 2019; Spiegel et al., 2014).

More recent studies revealed that subcellular compartmentalization, in particular nuclear retention of mRNAs, represents another major mechanism to control functional gene expression. In non-neuronal cells, nuclear compartmentalization plays a substantial role in transcription noise buffering which prevents stochastic mRNA fluctuations in the cytosol (Bahar Halpern et al., 2015; Battich et al., 2015). Furthermore, active nuclear retention of mRNAs is emerging as a novel form of post-transcriptional gene regulation. Notably, nuclear retention of RNAs can be regulated through long-lasting processes such as neuronal differentiation (Yeom et al., 2021). Also, candidate gene approaches in several systems revealed that some stored and nuclear transcripts can be released into the cytosol upon acute signals, thereby rapidly increasing mRNAs' availability for translation (Boutz et al., 2015; Mauger et al., 2016; Naro et al., 2017; Ninomiya et al., 2011; Prasanth et al., 2005). While further work is required to know whether this affects only rare RNAs or if this is a widespread mechanism, this discovery has generated considerable attention because it enhances functional gene expression independently of de novo transcription, a time-limiting step due to the finite processivity of the RNA polymerase II (Darzacq et al., 2007; Fuchs et al., 2014; Singh and Padgett, 2009; Tennyson et al., 1995; Veloso et al., 2014). The examination of transcript compartmentalization control is only emerging and further investigations are required to decipher its comprehensive potential in neuronal transcriptome remodeling. Notably, it remains unexplored whether this regulated cellular compartmentalization can also selectively remodel the transcriptome upon distinct signals.

Regulated intron retention (IR) has recently emerged as one candidate mechanism for nuclear retention and signaling-induced release of mRNAs. IR is a unique form of alternative splicing and consists of the persistence of a complete intron in otherwise fully synthetized mRNAs. IRs are highly prevalent and tightly regulated during development and in response to environmental signals supporting their major role in gene expression control (Jacob and Smith, 2017). IRs are a heterogeneous class of alternative splicing events that can direct their host mRNAs to multiple fates. A minority of intron retaining transcripts (IR-transcripts) are protein coding and exported to the cytoplasm where they generate protein isoforms (Grabski et al., 2021; Marquez et al., 2015). In other cases, IR elicits the degradation of the transcript either in the nucleus or the cytoplasm (Braunschweig et al., 2014; Wong et al., 2013; Yap et al., 2012). More recent studies shed light on IR as a mechanism for regulating transcriptome dynamics (Boutz et al., 2015; Gill et al., 2017; Mauger et al., 2016; Naro et al., 2017; Ninomiya et al., 2011; Park et al., 2017; Pendleton et al., 2017, 2018). Some IR-transcripts are initially targeted for nuclear retention where they remain stored in the nucleus for many hours and several days

(Mauger et al., 2016; Naro et al., 2017). These transcripts form a reservoir of RNAs that can be released into the cytosol upon signals through splicing completion independently of new transcription. Interestingly, intron retention programs appear to target different sets of transcripts in different cellular systems. In neurons, an elevation of network activity and calcium influx has been shown to rapidly lead to the splicing completion of transcripts encoding proteins involved in cytoskeletal regulation and signaling pathways (Mauger et al., 2016). By contrast, in male gametes, some mRNAs coding for proteins implicated in spermatogenesis are subject to splicing completion in the latest stage of gametogenesis (Naro et al., 2017). The difference in the identity of regulated transcripts likely reflect cell class-specific IR programs. However, it remains unexplored whether stimuli trigger splicing completion of IR-transcripts by releasing a common brake of intron excision or whether there are cue-specific IR programs controlled through dedicated signaling pathways. Noteworthy, in yeast, different cellular stresses modify the splicing kinetics of distinct sets of constitutively spliced introns (Bergkessel et al., 2011). Moreover multiple signaling pathways have been implicated in regulation of alternative exon choices in response to external stimuli (Matter et al., 2002; Shin and Manley, 2004; Zhou et al., 2012). This raises the possibility that distinct cues may target select IR programs in other systems including mature neurons.

In the present study we systemically mapped the subcellular localization of neuronal IR-transcripts and their response to neuronal stimuli. We found that the majority of transcripts that stably retain introns are subject to nuclear retention. After neuronal stimulation, the vast majority of transcripts that complete splicing are exported to the cytosol indicating that IR is a widespread mechanism to control storage and on-demand release of mRNAs from the nucleus. Remarkably, stimulation with brain-derived neurotrophic factor *versus* a brief elevation of neuronal network activity mobilizes distinct pools of IR-transcripts. This cue-specificity of IR programs arises from the engagement of distinct signaling pathways that convey specific messages to the neuronal nucleus. Overall, we conclude that IR programs allow a rapid, transcription-independent and cue-specific remodeling of neuronal transcriptome during plasticity.

RESULTS

The majority of stable intron-retaining transcripts are localized in the nucleus

To systematically assess sub-cellular localization of transcripts with stable intron retentions (IR), we developed a dedicated experimental workflow. We performed biochemical cell fractionation (Suzuki et al., 2010) and separated nuclear and cytosolic RNAs of mature mouse primary neocortical cells **(Figure 1A)**. To solely analyze the population of stable intron retaining transcripts (IR-transcripts) rather than transcripts containing transient IRs, we pharmacologically blocked transcription for 3 hours before collecting cells **(figure supplement 1A)**. For each sample: whole-cell extract, nuclear-enriched (designated as “Nucleus”) and cytosolic-enriched (designated as “Cytosol”) compartments, polyadenylated (poly(A)+) RNAs were isolated from three biological replicates and spike-in RNAs were added to assess the absolute nuclear-to-cytosol ratio of expressed transcripts (see Materials and Methods). Samples were sequenced at high depth (ca. 100 million reads per sample, 100-mer reads). Ribosomal RNAs represented ca. 1% of the mapped reads **(Table 1)**, indicating that the enrichment of poly(A)+ RNAs was highly efficient.

For every intron of the mouse genome, we analyzed the percentage of intron retention (PIR) with a previously established and validated pipeline (Mauger et al., 2016) **(see Materials and Methods, Figure 1B – figure supplement 1B)**. Introns were considered as retained if the PIR value was higher than 20 in whole-cell-extract. Similar to a previous analysis (Mauger et al., 2016), we identified 1465 stable IRs arising from 903 genes - among 10894 genes expressed in primary neocortical neurons. We then probed the distribution of intron retention levels for these events in each subcellular compartment **(Figure 1B)**. We found that PIR values were overall higher in the “Nucleus” than in the “Cytosol” (mean $PIR_{Nucleus}$= 66; mean $PIR_{Cytosol}$=48) suggesting that stable IR-transcripts are predominantly localized to the nucleus. To confirm this, we compared the expression of intron-retaining isoforms in the “Nucleus” and “Cytosol” samples **(Figure 1C and D)**. To calculate an absolute nuclear-to-cytosol ratio, we used the spike-in RNAs for normalizing the nuclear and cytosolic reads (see Materials and Methods). Among the 1465 IR-transcript isoforms, 820 (56%) were strongly enriched in the “Nucleus” (nucleus-to-cytosol ratio>2), while 274 (19%) were more abundant in the “Cytosol” (nucleus-to-cytosol ratio<0.5); the remaining isoforms (25%) were similarly detected in the “Nuclear” and the “Cytosolic” fractions. By contrast, only a minority of fully spliced transcripts is enriched in the “Nucleus” (4%) and the majority of them is highly enriched in the “Cytoplasm” (52%).

This indicates that the prevalence of IR-transcripts in the “Nucleus” samples is not a consequence of an inefficient biochemical fractionation **(Figure 1D)**.

To conclude, our data reveal an unprecedented large population of stable IR-transcripts predominantly localized in the nucleus and thus highlight that stable IR-transcripts are largely targeted for nuclear retention.

Stable nuclear IRs share features with canonical spliced introns

We hypothesize that the nuclear localization of stable IR-transcripts is intronically encoded. To test this hypothesis, we examined whether the nuclear IRs harbor specific sequence properties. Nuclear retained introns exhibit weak 5’ and 3’ splice sites compared to canonically spliced introns - a general property of IRs (Boutz et al., 2015; Braunschweig et al., 2014; Mauger et al., 2016; Ullrich and Guigó, 2020; Yeom et al., 2021). However, the splice site strength of stable nuclear retained introns is indistinguishable from the one of cytosolic retained introns **(Figure 1E and F)**. Thus, the splice site sequences themselves are not dictating the nuclear localization of IR-transcripts.

However, stable nuclear retained introns display specific features in terms of length and GC content: while nuclear retained introns remain shorter than spliced introns, they are markedly longer than cytosolic retained introns **(Figure 1G)**. Similarly, GC content of nuclear retained introns is lower than the one of cytosolic retained introns and comparable to GC content of canonical spliced introns **(Figure 1H)**.

To conclude, in respect to several sequence features, stable nuclear IRs resemble canonically spliced introns and retention can be regulated by trans-acting factors. Thus, we hypothesize that a substantial proportion of stable nuclear retained introns preserves the ability to be excised through splicing; but as opposed to canonical spliced introns, enhancing cues may be required to promote their removal.

Nuclear intron-retaining transcripts are regulated by several forms of neuronal stimulation

Intron retention rates have been reported to be regulated over days of neuronal differentiation (Braunschweig et al., 2014; Yap et al., 2012; Yeom et al., 2021) or in mature neurons in response to elevation of neuronal network activity (Mauger et al., 2016). Mature neurons exhibit specific forms of plasticity in response to specific plasticity cues. To explore whether such cues can acutely target subsets of IRs for rapid transcriptome

remodeling, we first probed whether different stimuli can regulate IRs in mature neocortical neurons. We used two modes of neuronal stimulation. Mouse primary neocortical cultures were treated for one hour with i) bicuculline, an antagonist of $GABA_A$ receptors which induces a robust increase in neuronal network activity, or with ii) the brain-derived neurotropic factor (BDNF) which is specifically released during forms of synaptic plasticity and facilitates long-term potentiation (Gottmann et al., 2009; Harward et al., 2016). In each condition, cells were treated with a transcription inhibitor to solely focus on IR-transcripts that are stable in unstimulated neocortical cells **(figure supplement 2A)**. Given that this study exclusively focuses on such stable IR-transcripts, we will simply designate them as "IR-transcripts" in the remainder of the manuscript. Both, bicuculline and BDNF stimulation induced a robust increase of ERK phosphorylation in nearly all neurons, indicating that both treatments stimulated the vast majority of neurons in culture **(Figure 2A and B – figure supplement 2B and C)**. Interestingly, we found that both bicuculline and BDNF stimulation regulate a sizeable set of IR-transcripts in the absence of de novo transcription. Upon bicuculline treatment, the expression level of 430 IR-transcripts was altered (fold-change>20% and |z-score|>1.5); 382 IR-transcripts exhibit a lower expression upon stimulation (resulting from induced splicing or degradation, **see Figure 3**) and 48 transcripts were up-regulated (resulting from inhibition of basal splicing or enhanced stabilization, **see Figure 3**) **(Figure 2C – figure supplement 2D)**. As for BDNF stimulation, it regulated the expression of 385 IR-transcripts (243 down-regulated and 142 up-regulated) **(Figure 2D – figure supplement 2D)**. Importantly, on average regulated IR-transcripts are expressed at a similar level as unregulated IR-transcripts. Thus, these mRNAs constitute a major transcript pool rather than a lowly expressed subpopulation **(figure supplement 2E)**. The vast majority of regulated IR-transcripts were localized in the nucleus (nucleus-to-cytosol ratio>2; 84% and 79% of bicuculline- and BDNF-sensitive IR-transcripts respectively) **(Figure 2E and F)**. Remarkably, the population of regulated transcripts is even more enriched in the nucleus than the overall population of IR-transcripts (two-sided Mann-Whitney test, p-value<10^{-5} for both bicuculline- and BDNF-regulated transcripts) **(figure supplement 2F)**.

Thus, our data reveals that multiple plasticity cues target nuclear IR-transcripts. We hypothesize that a large population of IRs can be regulated through nuclear processes including splicing.

Neuronal stimulation regulates intron-retaining transcripts through splicing and degradation processes

The fate of IR-transcripts and their contribution to protein production is determined by the rates of splicing, degradation, and nuclear export. Each of these processes could be targeted for establishing specific transcriptome modifications in response to distinct forms of neuronal stimulation. Hence, before thoroughly probing the cue-specificity of IR programs, we dissected the contribution of degradation and splicing for the two forms of neuronal stimulation. We performed a pairwise comparison of the expression regulation of IR-transcripts and their counterpart spliced transcripts. Upon splicing, the decrease of intron-retaining isoforms is accompanied by an increase of the spliced isoforms. Applying stringent criteria to select IRs that follow this scheme **(see Materials and Methods)**, we found that 83 and 46 IR-transcripts underwent splicing upon neuronal stimulation with bicuculline and BDNF, respectively **(Figure 3A and B – figure supplement 3A and B)**. We performed targeted validations using semi-quantitative PCR and real-time quantitative PCR assays for several IR-transcripts. Notably, the transcripts encoding the AMPA receptor subunit GRIA3 and the transcription factor TCF25 exhibit a concomitant decrease of the intron-retaining isoforms and an increase of the spliced isoforms confirming their regulation through splicing induction **(Figure 3C – figure supplement 3C)**. We further validated a decrease of the intron-retaining isoforms and concomitant increase of the spliced isoforms of transcripts encoding the cytoskeletal regulator FNBP4 and the transcript arising from the microRNA containing gene Mirg **(Figure 3D – figure supplement 3D)**. Interestingly, we also identified IR-transcripts that showed increased retention and reduced levels of the spliced isoforms upon neuronal stimulation (2 for bicuculline, 7 for BDNF). This indicates that intron excision can also be slowed-down in response to signaling. Note that the apparent low number of IR-transcripts that undergo reduced splicing results from the fact we focused our analysis on IR-transcripts that were stable before stimulation; i.e., those associated with IRs whose retention level remains higher than 20% after transcription inhibition.

Remarkably, our data also reveal that a substantial population of regulated IRs cannot readily be explained by a splicing mechanism. Many IRs were associated with spliced and intron-retaining isoforms regulated in the same direction. They were either both increased or decreased suggesting a respective overall stabilization or degradation not instructed by IRs **(Figure 3A and B)**. We also found a sizeable set of regulated IR-transcripts whose spliced counterpart was not regulated (see Materials and Methods) suggesting that induced-degradation/stabilization process was specifically targeting the IR-transcript isoforms **(Figure 3A and B – figure supplement 3A and B)**. Note that in some cases, these events could also arise from a splicing reaction targeting other splice sites; however, our pipelines did not detect examples for such cases. Notably, 46 and 29

transcripts were destabilized upon stimulation with bicuculline and BDNF respectively. Interestingly, while our analysis focused on IR-transcripts that were stable in unstimulated conditions, we also found that a few (4 and 13) IR-transcripts were even more stable upon neuronal stimulation with bicuculline or BDNF. PCR assays confirmed the reliable identification of such regulation by degradation. For example, the transcript encoding the DNA double strand regulator CCDC136 exhibits a consistent decrease of intron-retaining isoforms but no change in the spliced isoforms in response to bicuculline stimulation **(Figure 3E – figure supplement 3C)**. Similar regulation was observed for the transcripts encoding the brain-specific actin regulator KLHL17 upon BDNF application **(Figure 3F – figure supplement 3D)**.

In aggregate, our data support that in mature neocortical neurons, neuronal signaling not only drives transcription-independent modifications of the neuronal transcriptome through splicing completion but also through transcript-specific degradation.

Activity-dependent splicing of intron-retaining transcripts promotes cytosolic export of fully spliced transcripts

For a small number of selected IR-transcripts the neuronal activity-dependent splicing completion was shown to be followed by nuclear export and translation (Mauger et al., 2016). However, it remains unknown whether the release of nuclear retention of mRNAs upon splicing completion is a general mechanism. To address this question at a transcriptome-wide scale, we systematically probed the localization of IR-transcripts and their spliced mRNA counterparts in the nucleus and the cytosol 1-hour after bicuculline-mediated elevation of neuronal network activity. First, we mapped the total cellular repertoire of IRs and regulated IR-transcripts (whole cell extract samples, same criteria than in previous figures, see Materials and Methods). Nearly all regulated IR-transcripts were predominantly localized to the nucleus **(Figure supplement 4A)**. As expected, IR-transcripts regulated through splicing (based on whole cell extract data) were significantly less abundant in the nucleus in response to bicuculline stimulation (consistent with splicing being a nuclear process) **(Figure 4, left panel)**. A concomitant increase of the spliced isoforms was also observed in the nucleus while it was not the case for transcripts degraded upon stimulation **(Figure 4, middle panel)**. Remarkably, we found that the spliced transcripts were also significantly enriched in the cytosol one hour after bicuculline application indicating the newly spliced transcripts were exported to the cytosol after splicing completion **(Figure 4, right panel)**. By contrast, the spliced transcripts associated

with IR-transcripts regulated through degradation were almost unchanged upon stimulation.

This suggests, that IR enables the nuclear compartmentalization of transcripts; furthermore, intron removal through splicing completion represents a widely-used mechanism for stimulus-dependent release of transcripts into the cytosol, thereby rapidly making them available for translation. Importantly, this major form of gene regulation occurs in the absence of alterations in total transcript levels **(Figure supplement 4B)** and, thus, is not detectable with conventional transcriptomic methods.

Stimulus-specific regulation of sub-populations of intron retentions

Neurons undergo distinct forms of plasticity in response to specific cues. Thus, we then wondered whether the regulation of IR programs exhibits cue-specific mobilization of specific transcript pools. We compared the regulation of IR-transcripts upon stimulations with bicuculline and BDNF **(Figure 5A and B)**. This analysis clearly revealed three major categories of IR-transcripts: i) IR-transcripts that are regulated by both bicuculline and BDNF stimulation, ii) IR-transcripts that are solely regulated by bicuculline stimulation, and iii) IR-transcripts only affected by BDNF stimulation.

For the category of commonly regulated IR-transcripts, 113 and 29 IR-transcripts were respectively down- and up regulated by both bicuculline and BDNF stimulations **(Figure 5B)**. For instance, the transcript encoding the metabolic enzyme NDST3 associated with schizophrenia is regulated upon both bicuculline and BDNF stimulation **(Figure 5C – figure supplement 5A and B)**. Nevertheless, *Ndst3* regulation harbors specificity as another stimulus (the group I metabotropic glutamate receptor agonist DHPG) did not impact its IR profile **(Figure 5C)**. Amongst commonly regulated transcripts, we could identify with high confidence 12 IR-transcripts regulated by splicing and 4 transcripts regulated by degradation upon both stimuli. Note that because we used very stringent criteria to identify transcripts regulated through splicing *versus* degradation, we could not confidently assign many of regulated IR-transcripts - identified in Figure 3 - to splicing or degradation.

Remarkably, a sizeable population of IR-transcripts were specifically regulated by only one mode of stimulation **(Figure 5A and B)**. Using stringent criteria (fold-change < 5% for unregulated events, see Materials and Methods), we confidently identified 48 and 34 IR-transcripts solely regulated upon bicuculline or BDNF stimulation, respectively. Notably, the IR-transcripts encoding the AMPA receptor subunit GRIA3 and the transcription factor

TCF25 are only regulated upon bicuculline stimulation but do not exhibit any change in response to upon BDNF application **(Figure 5D – figure supplement 5A and B)**. To further probe the selective regulation of these targets we used the group I mGluR agonist DHPG and similarly found no change in IR in these transcripts **(Figure 5D)**. By contrast, the transcripts arising from the miRNA-containing gene Mirg and the transcripts encoding the brain-specific actin regulator KLHL17 are exclusively regulated by BDNF stimulation but not upon stimulation with bicuculline or DHPG **(Figure 5E – figure supplement 5A and B)**.

Another striking category of specific IRs is associated with IR-transcripts that are bi-directionally regulated upon bicuculline *versus* BDNF stimulation. More precisely, some cue-specific IR-transcripts are regulated upon both bicuculline and BDNF stimulations but in opposite directions **(Figure 5E)**. For instance, the IR-transcripts encoding the splicing factor SRSF2 undergo degradation upon stimulation with bicuculline, while BDNF signal stabilized them **(Figure 5F – figure supplement 5B)**.

In sum, our data reveals that regulated IR-transcripts can be subdivided in several classes: some IRs are commonly regulated by bicuculline and BDNF stimulation whereas others are cue-specific. Thus, targeting IRs represents a way to specifically remodel the neuronal transcriptome upon distinct forms of neuronal stimulation.

Stimulation-specificity of intron retention programs is conveyed by distinct signaling pathways

To obtain insight into the mechanism of neuronal cue-specific regulation of IR, we probed the signaling pathways involved in stimulated intron excision. Neuronal activity-dependent signaling elicited by elevation of network activity by bicuculline treatment relies on calcium signaling either through NMDA receptors or voltage-dependent calcium channels. As for BNDF-signaling events, they largely depend on the mitogen-activated protein kinase (MAPK) pathway. We thus wondered whether the stimulation-specificity of IR programs is conveyed by the differential activation of calcium signaling and MAPK pathways.

Remarkably, the application of the selective NMDA receptor antagonist AP5 impaired the bicuculline-dependent intron excision of *Tcf25* and *Gria3* transcripts **(Figure 6A – figure supplement 6A)**. Intron excision in these transcripts was also suppressed by pharmacological inhibition of calcium^{2+}/calmodulin-dependent protein kinase (CaMK) - a downstream pathway activated by NMDAR-dependent calcium entry. By contrast, the

MAPK antagonist U0126 did not impact splicing induction of bicuculline-sensitive transcripts. Conversely, for BDNF-sensitive introns, we found that the MAPK pathways is essential for the splicing of *Mirg* and the stabilization of *Srsf2* IR-transcripts upon BDNF stimulation **(Figure 6B – figure supplement 6B)**. However, the pharmacological inhibition of NMDA receptors and CaMK pathways did not preclude their regulation upon BDNF stimulation. Overall, these results uncover a signaling-pathway specificity of IR programs.

To obtain deeper insight into this cue-specific regulation, we focused on kinases previously implicated in signaling-dependent alternative splicing control (Shin and Manley, 2004). SR-protein kinases (SRPK) and the CDC2-like kinases (CLK) families have previously been shown to link external cues and alternative splicing regulation in non-neuronal cells (Ninomiya et al., 2011; Zhou et al., 2012). These kinase families both regulate the phosphorylation status - and consequently the activity - of SR proteins, the main family of splicing factors. Interestingly, SRPIN340, a pharmacological inhibitor of SRPK impeded intron excision of *Tcf25* and *Gria3* transcripts upon bicuculline treatment **(Figure 6C)**. However, SPRK inhibition had no effect on the BDNF-dependent splicing of *Mirg* or the BDNF-dependent regulation of *Srsf2* transcripts **(Figure 6D)**. Interestingly, KH-CB19, a pharmacological blocker of CLK kinase activity had no impact on neither bicuculline- or BDNF-dependent introns. Thus, these results further support that the cue-specificity of IR programs strongly relies on the activation of selective signaling pathways transduced to the nucleus to convey the mobilization of distinct sets of IR-transcripts.

In summary, this work identifies that selective IR programs elicited upon distinct forms of neuronal stimulation. The activation of distinct signaling pathways drives the remodeling of the neuronal transcriptome in a cue-specific manner **(Figure 6E)**.

DISCUSSION

In this work, we revealed that regulated intron retention is a widespread, cue-specific mechanism for neuronal transcriptome remodeling. We performed a comprehensive mapping of the subcellular localization of mRNAs in mature neurons and revealed that transcripts that stably retain introns are broadly targeted for nuclear retention. We systematically probed these transcripts upon neuronal stimulation and found that sub-populations of nuclear-retained transcripts are bi-directionally regulated in response to several cues: some appear targeted for degradation while others undergo splicing completion to generate fully mature mRNAs. This latter set of transcripts is

exported to the cytosol to increase functional gene expression. Remarkably, distinct groups of IR-transcripts are regulated depending on the form of stimulation and this selectivity arises from the activation of specific signaling pathways. Overall, our data identifies reversible IR as a major regulator of nuclear mRNA retention and cue-specific mobilization

Targeting intron retentions is a widespread mechanism to acutely regulate subcellular compartmentalization of transcripts upon cellular signals

In the present study, we show that retention of select introns in mRNAs is a widespread mechanism to control transcript localization. The nuclear localization of transcripts that stably retain select introns makes them unavailable for protein synthesis **(Figure 1)**. Previous work localized instable IR-transcripts to the nucleus (Braunschweig et al., 2014). Here, we systemically mapped the subcellular localization of stable IR-transcripts, and find this population to be abundant in the nucleus of mature neurons **(Figure 1)**. This is consistent with previous candidate gene studies in other cell classes, including fibroblastic cells, stem cells and male gametes, which reported nuclear localization of IR-transcripts. (Boutz et al., 2015; Mauger et al., 2016; Naro et al., 2017; Ninomiya et al., 2011).

We hypothesized that controlling intron retention and excision patterns represents a general mechanism to mobilize specific mRNA pools for functional gene expression. In line with this, we shed light on the extensive export of transcripts that undergo splicing completion in response to neuronal stimulation **(Figure 4)**. Other studies also revealed a link between IRs and the nucleo-cytosolic localization of their host transcripts (Boutz et al., 2015; Mauger et al., 2016; Naro et al., 2017; Ninomiya et al., 2011; Yeom et al., 2021). Though, in most of the cases, it was unclear i) whether existing transcripts repurposed their fate and localization through splicing completion or ii) whether co-transcriptional production of spliced isoforms is required for their cytosolic expression. Indeed, the fate and nuclear localization of some IR-transcripts can be irreversibly set up from their transcription (Pendleton et al., 2018). By contrast, we show here that a sizeable population of IR-transcripts can repurpose their fate upon environmental signals and promote their release into the cytosol. Such a transcription-independent mechanism likely evolved as it facilitates rapid remodeling of the transcriptome, independently of transcription which is time-limiting due to the finite processivity of the RNA-polymerase II (Darzacq et al., 2007; Fuchs et al., 2014; Singh and Padgett, 2009; Tennyson et al., 1995; Veloso et al., 2014)

Intron retention programs remodel the neuronal transcriptome in a cue-specific manner

Previous studies showed that IR profiles across tissues and cell types are modified by numerous signals including cellular differentiation, neuronal stimulation, metabolic homeostasis and cellular stress (Boutz et al., 2015; Green et al., 2020; Haltenhof et al., 2020; Mauger et al., 2016; Naro et al., 2017; Park et al., 2017; Parra et al., 2018; Pendleton et al., 2018; Pimentel et al., 2016; Quesnel-Vallières et al., 2016; Wong et al., 2013). In the present work, we unveil that in mature neurons, IR programs are cue-specific and that selectivity arises from the activation of distinct neuronal signaling pathways **(Figure 5 and 6)**. Rather than constituting a universal program, distinct subsets of IR-transcripts are regulated in response to specific neuronal stimuli **(Figure 5)**. This specificity of IR programs in neocortical cells suggests that they contribute to exquisite neuronal plasticity events. Interestingly, in Drosophila, learning paradigms elevate the spliced isoform of *Orb2A* - encoding a protein involved in memory consolidation - over an unspliced isoform that is predominant in naive flies (Gill et al., 2017). The novel insights into the signaling mechanisms of neuronal IR regulation uncovered here will pave the way to probing the contribution of IR programs in transcription-independent plasticity events *in vivo*.

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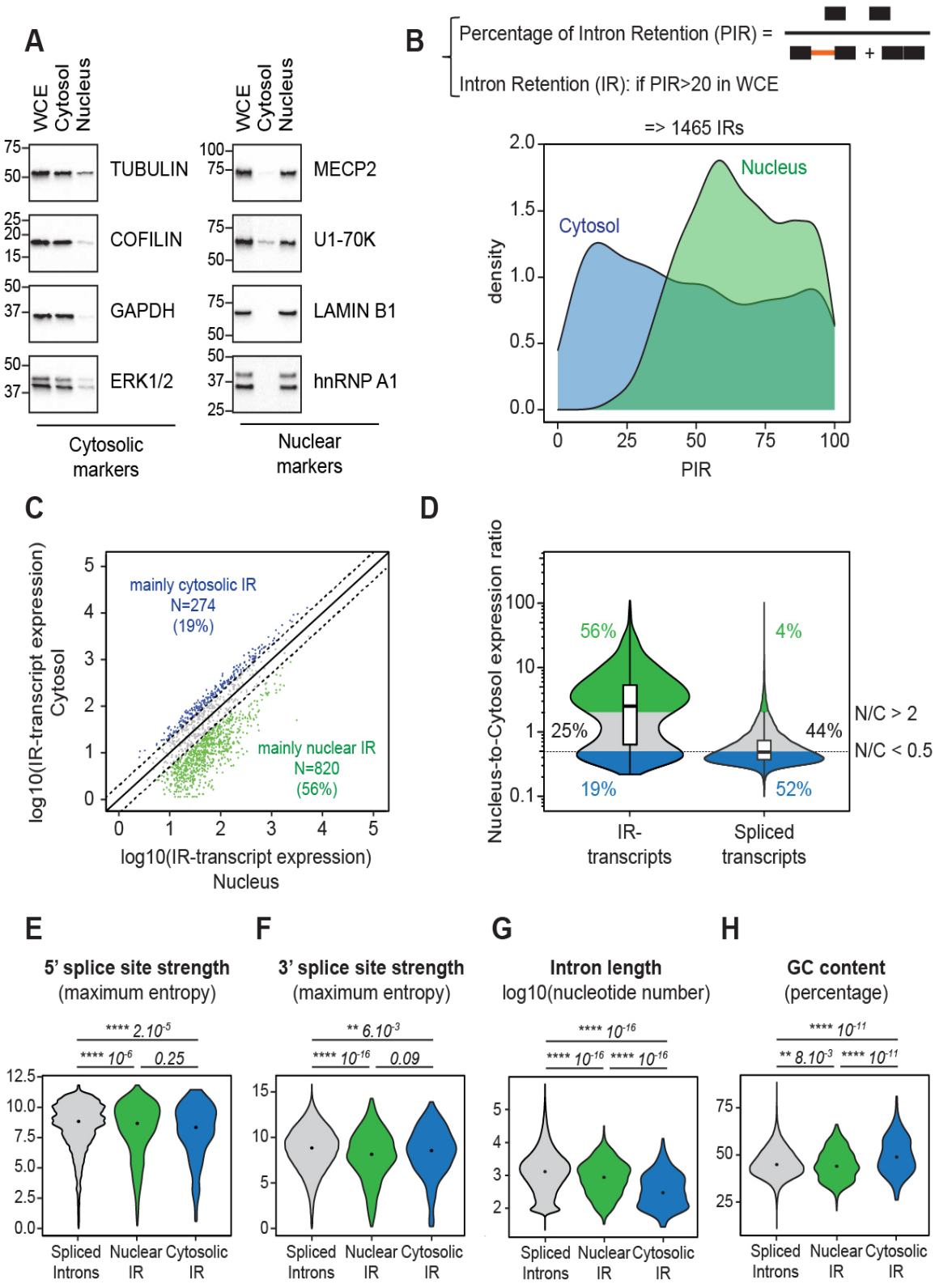
AUTHOR CONTRIBUTIONS

M.M. and O.M. conducted the experiments and performed the computational analysis. O.M., M.M. and P.S. designed the experiments and wrote the paper.

COMPETING INTERESTS

None competing interest to declare.

Figure 1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ERK1/2
MECP2
U1-70K
LAMIN B1
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Cytosolic markers
Nuclear markers
B
Percentage of Intron Retention (PIR) =
Intron Retention (IR): if PIR>20 in WCE
=> 1465 IRs
Nucleus
Cytosol
density
PIR
C
mainly cytosolic IR
N=274
(19%)
mainly nuclear IR
N=820
(56%)
log10(IR-transcript expression) Cytosol
log10(IR-transcript expression) Nucleus
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Nucleus-to-Cytosol expression ratio
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25%
44%
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(maximum entropy)
F
3' splice site strength
(maximum entropy)
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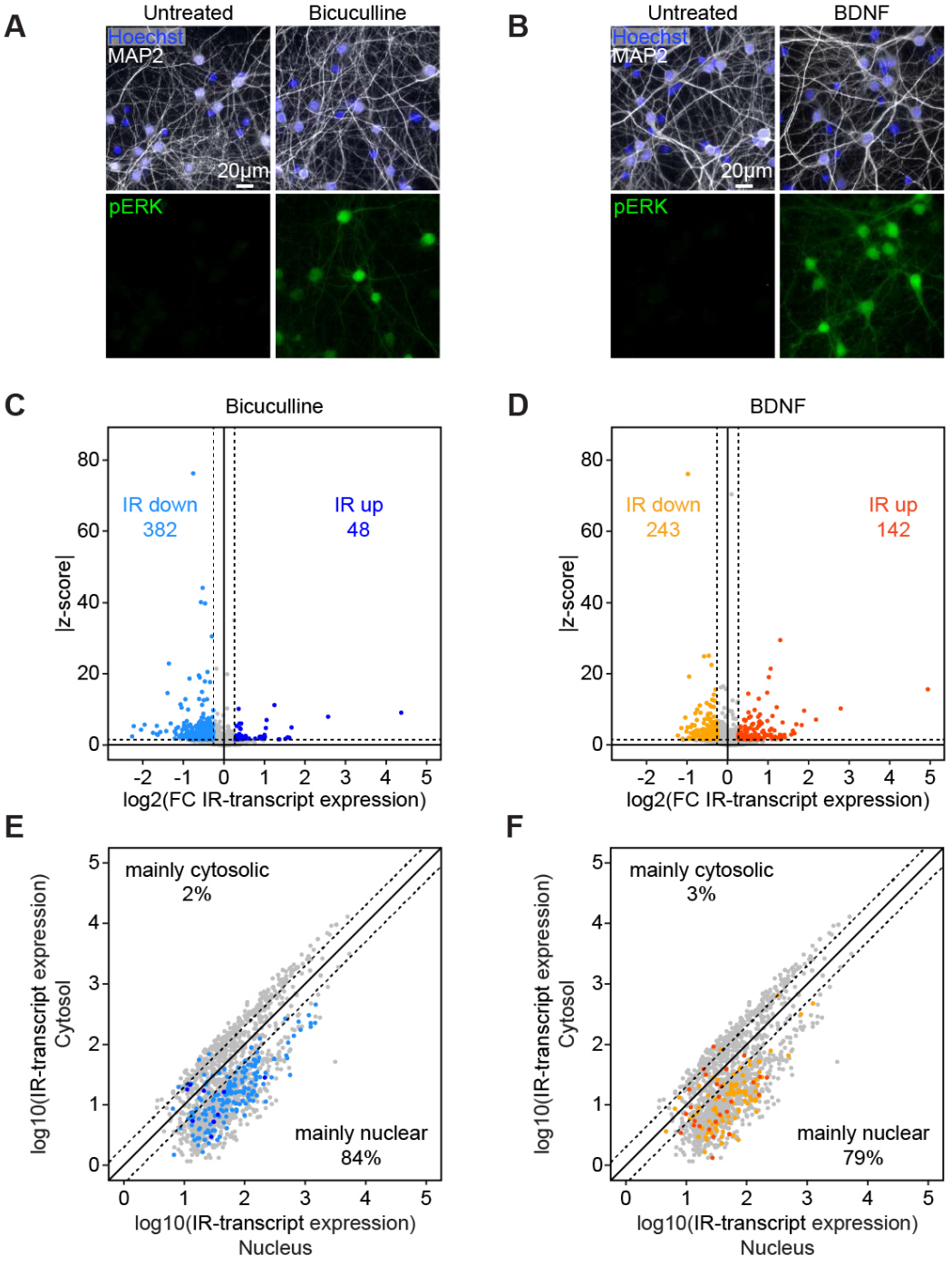
Intron length
log10(nucleotide number)
H
GC content
(percentage)
Spliced Introns
Nuclear IR
Cytosolic IR

Figure 1 : The majority of stable intron-retaining transcripts are localized in the nucleus

(A) Quality of the cell fractionation assays was controlled by western blot by assessing the distribution of nuclear and cytosolic markers in the whole cell extract (WCE), the cytosol and the nucleus. Protein lysates were isolated from mouse primary neocortical cells (14 days in culture) treated for 3hrs with the transcription inhibitor triptolide (1µM). (four independent cultures). **(B)** Top: percentage of intron retention (PIR) is assessed as the ratio between the IR-transcript expression and the total transcript expression (sum of intron-retaining and spliced transcripts). Intron are considered retained if minPIR ≥ 20 in WCE (see Materials and Methods); 1465 IRs were then identified. Bottom: Density plot displaying the PIR distribution in cytosol (blue) and nucleus (green) of the 1465 retained introns. (three independent cultures). **(C)** Pairwise comparison of the expression of the 1465 intron-retaining isoforms (IR) in nuclear *versus* cytosolic fractions. IR-transcripts enriched in the nucleus are labeled in green (nuclear-to-cytosol expression ratio ≥ 2); IR-transcripts enriched in the cytosol are labeled in blue (nuclear-to-cytosol expression ratio ≤ 0.5). **(D)** Violin plots displaying the nucleus-to-cytosol expression ratio of the 1465 intron-retaining isoforms (left) and all expressed spliced transcripts (right). Percentage of nuclear-enriched (green) and cytosolic-enriched (blue) transcripts are indicated on the panel. **(E)** to **(H)** Violin plots displaying 5' splice site strength (E), 3' splice site strength (F), intron length (G) and GC content (H) of canonically spliced introns (grey), nuclear IRs (green) and cytosolic IRs (blue). The p-values calculated with a two-sided Mann-Whitney test are indicated on the top of each panel.

Figure 2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Cytosol
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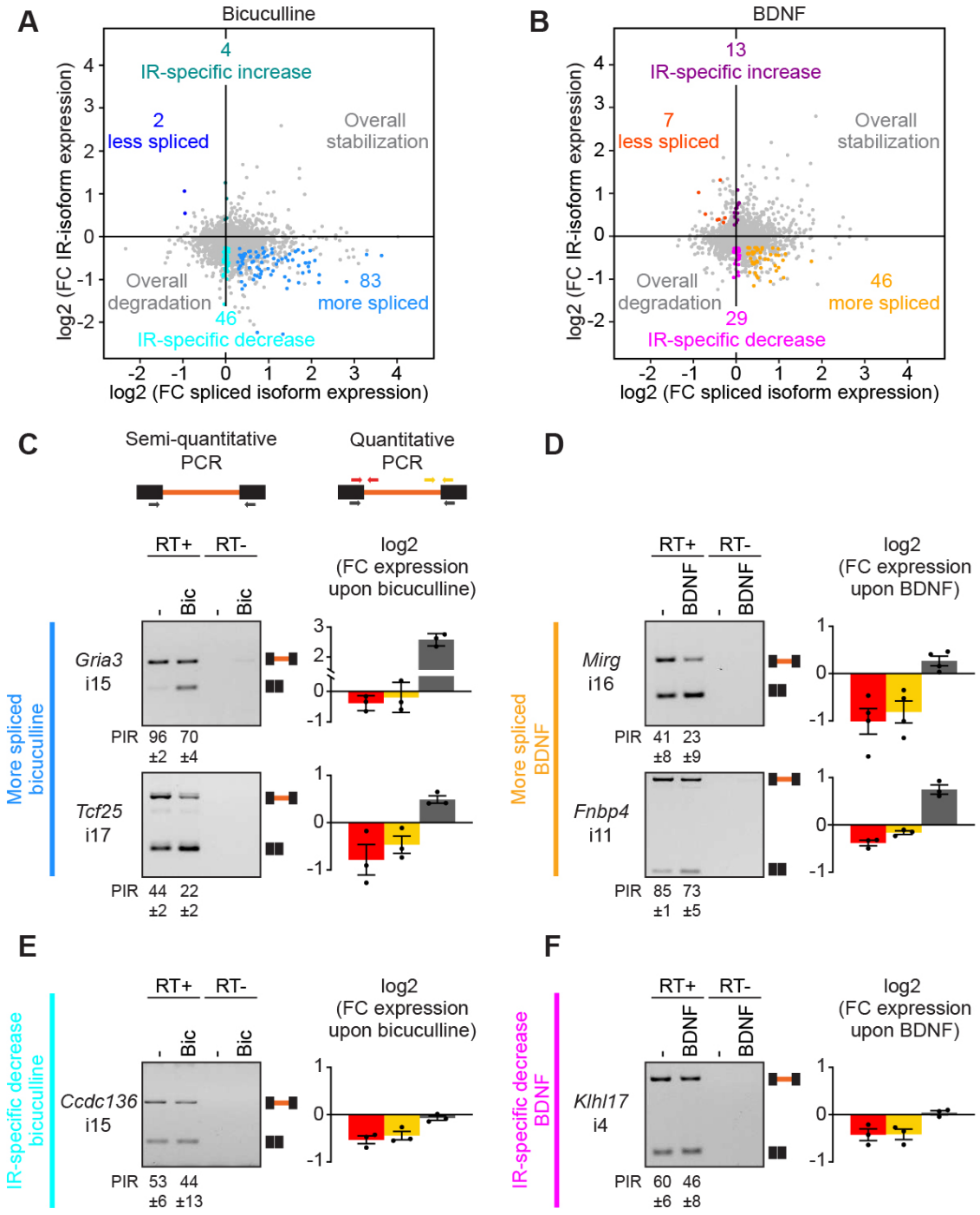
Nucleus
F
mainly cytosolic
3%
mainly nuclear
79%
log10(IR-transcript expression)
Cytosol
log10(IR-transcript expression)
Nucleus

Figure 2: Nuclear intron-retaining transcripts are regulated by several forms of neuronal stimulation

(A) and **(B)** Efficiencies of bicuculline (A) and BDNF (B) stimulations were assessed by controlling the induction of ERK phosphorylation by immunostaining. Mouse primary neocortical cells (14 days in culture) were stimulated with bicuculline (20μM) or BDNF (50ng/mL) for 5min. Cells were stained with anti-phospho-ERK (green, bottom) and anti-MAP2 (white, top) antibodies and counterstained with Hoechst (blue, top). (four independent cultures) **(C)** and **(D)** Volcano plots displaying the expression fold change of IR-transcripts and the corresponding z-score (absolute value) upon bicuculline (C) and BDNF (D) stimulations. DRB (50μM) was applied to mouse primary neocortical cells for 2hrs; 1hr before cell collection, bicuculline (20μM) or BDNF (50ng/mL) were applied or not (control). Every transcript retaining an intron (minPIR ≥ 20%) in unstimulated condition were plotted. IR-transcripts were considered downregulated (light blue or orange) or upregulated (dark blue or red) if the following applied: fold change of IR-transcript expression ≥ 20% and |z-score| ≥ 1.5. (three independent cultures) **(E)** and **(F)** Pairwise comparison of the expression of regulated IR-transcripts in the nuclear and the cytosolic fractions of unstimulated mouse primary neocortical cells. Every transcript retaining an intron (minPIR ≥ 20%) in whole cell extract were plotted. Intron retaining transcripts down- or up-regulated upon bicuculline stimulation (E) are labeled in light blue and dark blue respectively and those down- or up-regulated upon BDNF stimulation (F) are labelled in orange and red respectively. (three independent cultures).

Figure 3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Bicuculline
4
IR-specific increase
2
less spliced
Overall
stabilization
Overall
degradation
83
more spliced
46
IR-specific decrease
log2 (FC IR-isoform expression)
log2 (FC spliced isoform expression)
B
BDNF
13
IR-specific increase
7
less spliced
Overall
stabilization
Overall
degradation
46
more spliced
29
IR-specific decrease
log2 (FC IR-isoform expression)
log2 (FC spliced isoform expression)
C
Semi-quantitative PCR
Quantitative PCR
RT+
RT-
Bic
log2 (FC expression upon bicuculline)
More spliced bicuculline
Gria3 i15
PIR 96 ±2 70 ±4
Tcf25 i17
PIR 44 ±2 22 ±2
D
BDNF
log2 (FC expression upon BDNF)
More spliced BDNF
Mirg i16
PIR 41 ±8 23 ±9
Fnbp4 i11
PIR 85 ±1 73 ±5
E
IR-specific decrease bicuculline
Ccdc136 i15
PIR 53 ±6 44 ±13
F
IR-specific decrease BDNF
Klhl17 i4
PIR 60 ±6 46 ±8

Figure 3: Neuronal stimulation regulates intron-retaining transcripts through splicing and degradation processes

(A) and **(B)** Pairwise comparison of the expression fold change (FC) of intron-retaining isoforms (IR) and the spliced isoforms upon bicuculline (A) or BDNF (B) stimulation. In all conditions, DRB (50μM) was applied to mouse primary neocortical cells (14 days in culture) for 2hrs; 1hr before cell collection, bicuculline (20μM) or BDNF (50ng/mL) were applied or not (control). Every transcript containing a retained intron (minPIR>20%) in control condition are plotted. IR-transcripts are considered regulated through splicing if the following applied: (1) IR-isoform expression fold change ≥ 20% and |z-score| ≥ 1.5, (2) spliced isoform expression fold change ≥ 20% and |z-score| ≥ 1.5, (3) expression of the IR-isoforms and the spliced isoforms evolved in opposite directions. IR-transcripts are considered regulated through degradation (IR-transcript specific decrease) if the following applied: (1) IR expression fold change ≥ 20% and |z-score| ≥ 1.5, (2) spliced expression fold change ≤ 5% and |z-score| ≤ 1. (three independent cultures) **(C)** to **(F)** RT-PCR validations of regulated IR-transcripts through splicing upon bicuculline stimulation (C) and BDNF stimulation (D) and through degradation upon bicuculline stimulation (E) and BDNF stimulation (F). Expression of the IR-isoforms and the spliced isoforms were analyzed by semi-quantitative PCR (left panels). Means and SEMs of PIR values are shown beneath each panel. In addition, fold changes in the expression of the IR-transcripts (red and orange) and spliced (dark grey) isoforms were assessed with real-time qPCR using three different primer sets, as represented in the top scheme. SEMs are displayed (three-four independent cultures). Note that the Gria3 spliced transcript (C) does not correspond to the canonical mRNAs and presumably arise from a first step of recursive splicing and thereby likely require splicing completion to generate fully mature Gria3 transcripts (Sibley et al., 2015).

Figure 4

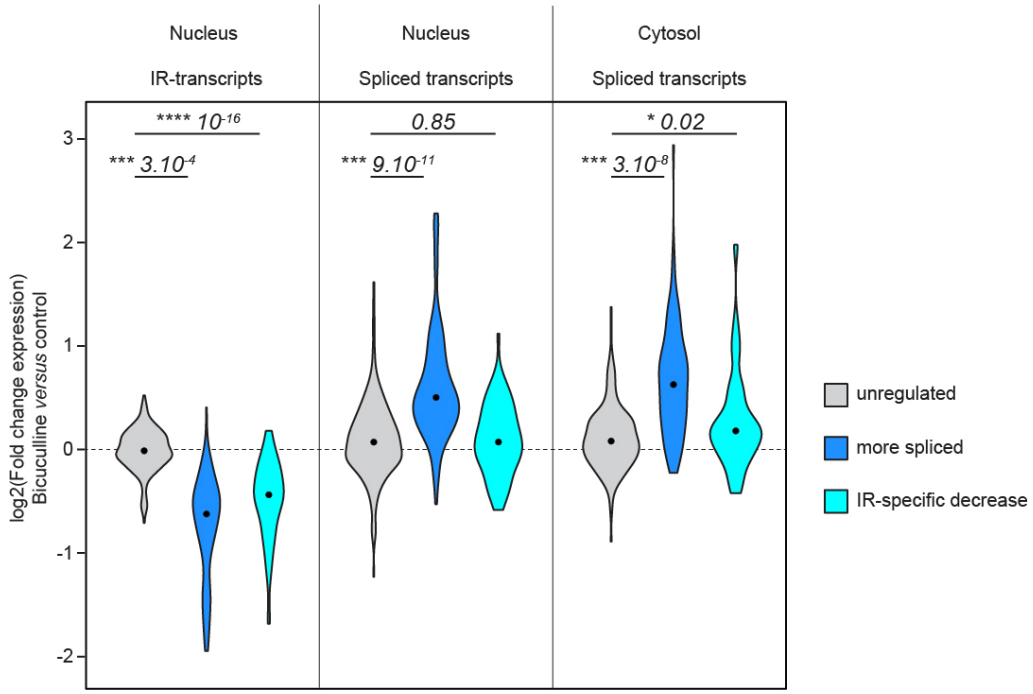

Nucleus
IR-transcripts
Nucleus
Spliced transcripts
Cytosol
Spliced transcripts
**** 10-16
*** 3.10-4
0.85
*** 9.10-11
* 0.02
*** 3.10-8
log2(Fold change expression)
Bicuculline versus control
3
2
1
0
-1
-2
unregulated
more spliced
IR-specific decrease

Figure 4: Activity-dependent splicing of intron-retaining transcripts promotes cytosolic export of fully spliced transcripts

Violin plots displaying expression fold change of IR-transcripts in the nucleus (left), spliced transcripts in the nucleus (middle) and spliced transcripts in the cytosol (right) upon bicuculline stimulation. Triptolide (10μM) was applied to mouse primary neocortical cells (14 days *in vitro*) for 3hrs; 1 hour before cell collection, bicuculline (20μM) was applied or not (control). Only transcripts that retained an intron in unstimulated and WCE conditions (minPIR ≥ 20%) were considered for the analysis. Transcripts were considered unregulated (grey) if the following applied: (1) fold change in the expression of the intron retaining transcripts ≤ 5% and |z-score| ≤ 1 in the WCE. Transcripts were considered regulated through splicing (dark blue) if the following applied: (1) IR-transcript expression fold change ≥ 20% and |z-score| ≥ 1.5 in the WCE, (2) Spliced isoform expression fold change ≥ 20% and |z-score| ≥ 1.5 in the WCE, (3) expression of the IR- and the spliced isoforms evolved in opposite directions in the WCE. Transcripts were considered regulated through degradation (IR-specific decrease, light blue) if the following applied: (1) IR-transcript expression fold change ≥ 20% and |z-score| ≥ 1.5 in WCE, (2) spliced expression fold change ≤ 5% and |z-score| ≤ 1 in WCE. The p-values calculated with a two-sided Mann-Whitney test are indicated on the top of the panel. (three independent cultures).

Figure 5

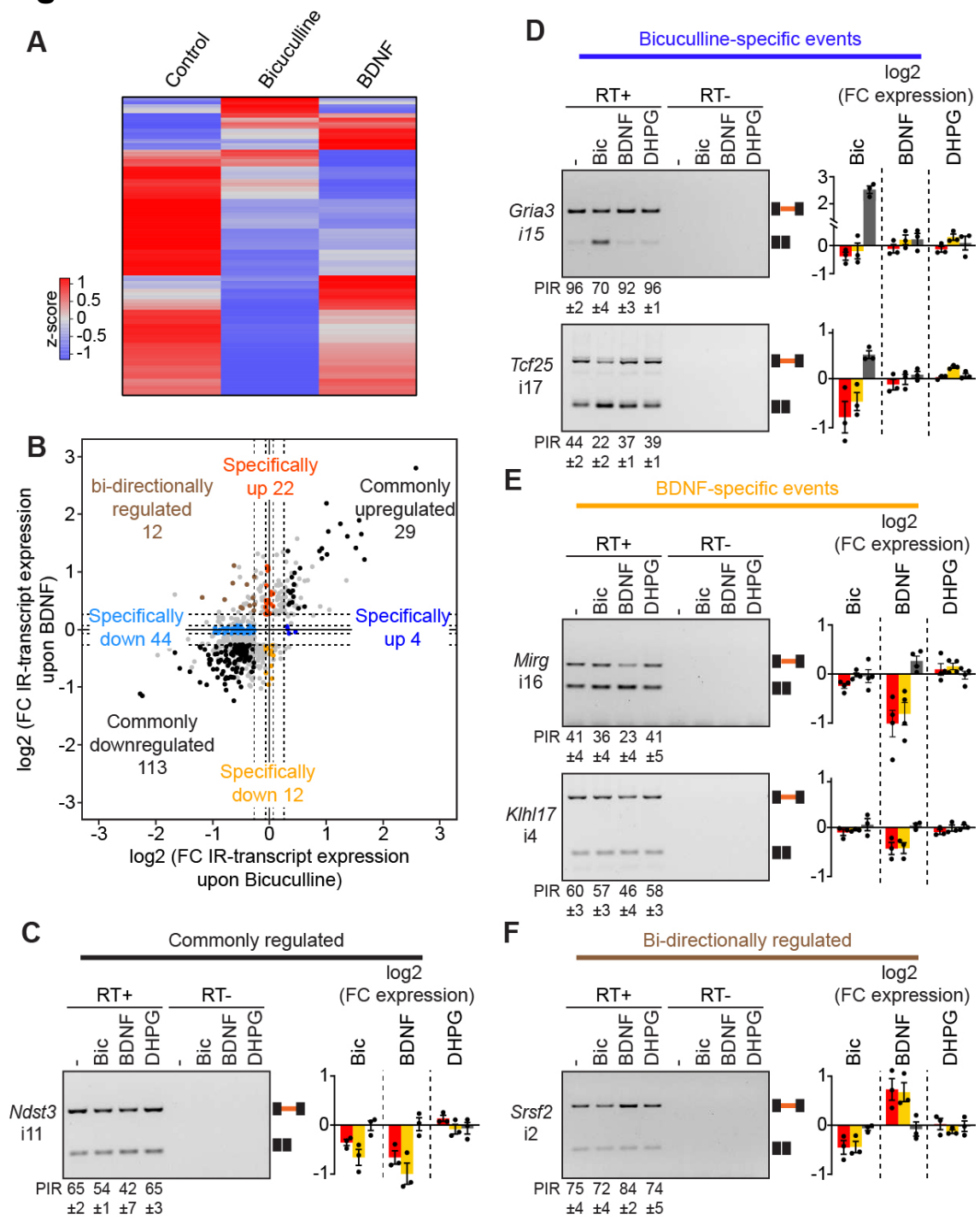

A
Control
Bicuculline
BDNF
z-score
1
0.5
0
-0.5
-1
B
bi-directionally regulated 12
Specifically up 22
Commonly upregulated 29
Specifically down 44
Specifically up 4
Commonly downregulated 113
Specifically down 12
log2 (FC IR-transcript expression upon BDNF)
log2 (FC IR-transcript expression upon Bicuculline)
C
Commonly regulated
RT+
RT-
- Bic BDNF DHPG - Bic BDNF DHPG
log2 (FC expression)
Bic BDNF DHPG
Ndst3 i11
PIR 65 54 42 65
±2 ±1 ±7 ±3
D
Bicuculline-specific events
Gria3 i15
PIR 96 70 92 96
±2 ±4 ±3 ±1
Tcf25 i17
PIR 44 22 37 39
±2 ±2 ±1 ±1
E
BDNF-specific events
Mirg i16
PIR 41 36 23 41
±4 ±4 ±4 ±5
Klhl17 i4
PIR 60 57 46 58
±3 ±3 ±4 ±3
F
Bi-directionally regulated
Srsf2 i2
PIR 75 72 84 74
±4 ±4 ±2 ±5

Figure 5: Stimulus-specific regulation of sub-population of intron retentions

(A) Heatmap of the IR-transcript expression values in control, bicuculline-stimulated and BDNF-stimulated conditions. IR-transcripts regulated in at least one condition are displayed (fold change of intron-retaining transcript expression ≥ 20% and |z-score| ≥ 1.5). **(B)** Pairwise comparison of the expression fold change (FC) of intron-retaining isoforms upon bicuculline and BDNF stimulation. Every transcript containing a retained intron (minPIR>20%) in control condition are plotted. IR-transcripts are considered regulated specifically upon bicuculline (light and dark blue) or (BDNF (orange and red)) stimulation if the following applied: (1) IR-transcript expression fold change ≥ 20% and |z-score| ≥ 1.5 upon bicuculline (or BDNF) stimulation (2) IR-transcript expression fold change ≤ 5% upon BDNF (or bicuculline) stimulation. IR-transcripts are considered commonly regulated upon bicuculline and BDNF stimulation (black) if the following applied: (1) IR-transcript expression fold change ≥ 20% and |z-score| ≥ 1.5 upon bicuculline stimulation (2) IR-transcript expression fold change ≥ 20% and |z-score| ≥ 1.5 upon BDNF stimulation (3) IR-transcripts evolved in the same direction upon bicuculline and BDNF stimulations. IR-transcripts are considered bi-directionally regulated upon bicuculline and BDNF stimulations (brown) if the following applied: (1) IR-transcript expression fold change ≥ 20% and |z-score| ≥ 1.5 upon bicuculline stimulation (2) IR-transcript expression fold change ≥ 20% and |z-score| ≥ 1.5 upon BDNF stimulation (3) IR-transcripts evolved in opposite directions upon bicuculline and BDNF stimulations. **(C)** to **(F)** RT-PCR validations of IR-transcripts commonly regulated (C), specifically regulated upon bicuculline stimulation (D), specifically regulated upon BDNF stimulation (E), and bi-directionally regulated (F). Expression of the IR-transcripts and the spliced isoforms were analyzed by semi-quantitative PCR (left panels). Means and SEMs of PIR values are shown beneath each panel. In addition, fold changes in the expression of the IR-transcripts (red and orange) and spliced isoforms (dark grey) were assessed with real-time qPCR. SEMs are displayed (three-four independent cultures).

Figure 6

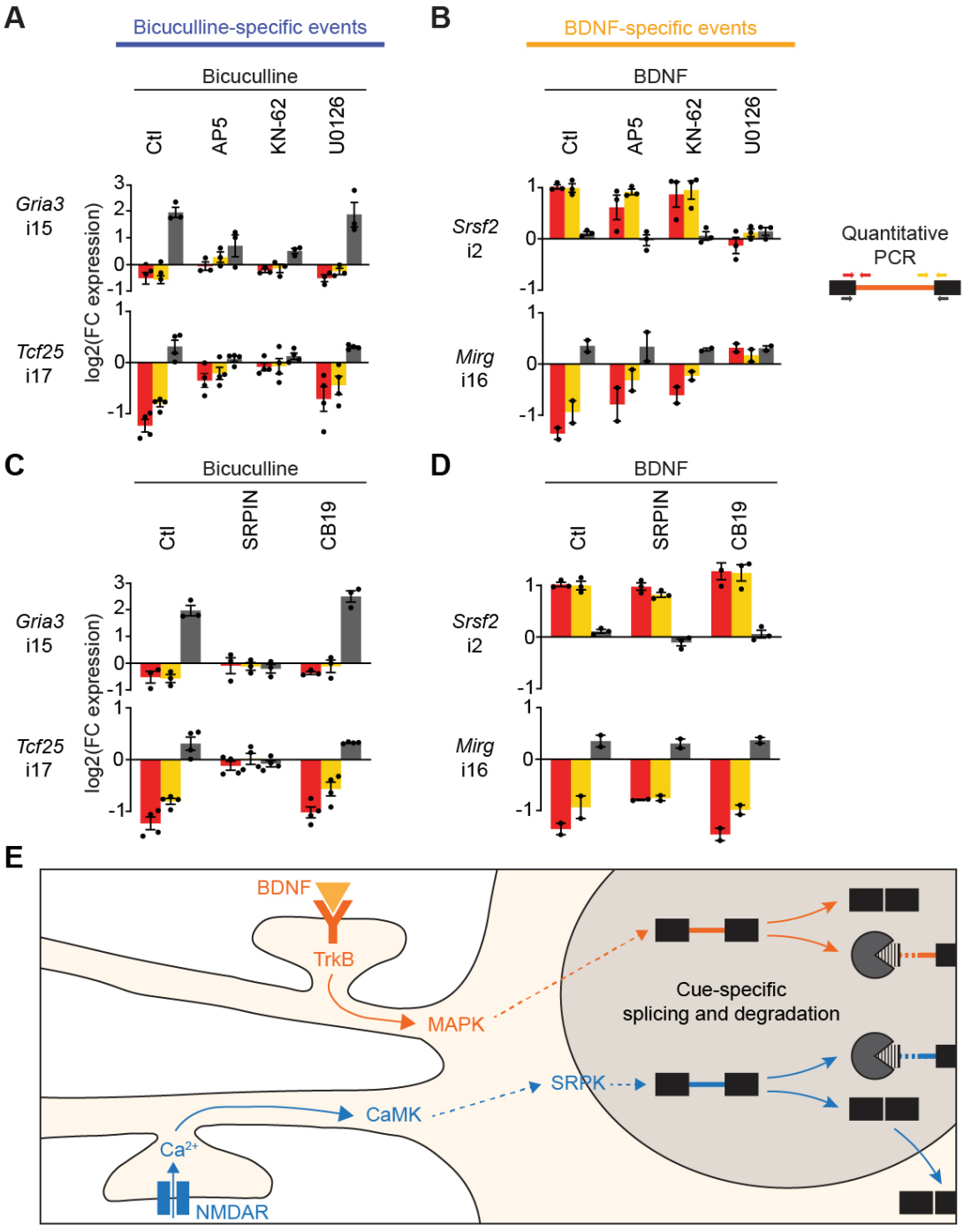

A
Bicuculline-specific events
Bicuculline
Ctl
AP5
KN-62
U0126
Gria3
i15
Tcf25
i17
log2(FC expression)
B
BDNF-specific events
BDNF
Srsf2
i2
Mirg
i16
Quantitative
PCR
C
Bicuculline
Ctl
SRPIN
CB19
D
BDNF
E
BDNF
TrkB
MAPK
CaMK
SRPK
Ca2+
NMDAR
Cue-specific
splicing and degradation

Figure 6: Stimulation-specificity of intron retention programs is conveyed by distinct signaling pathways

(A) and **(B)** Analysis of signaling pathways involvement in the regulation of IR-transcripts upon bicuculline (A) and BDNF (B) stimulations. In all conditions, DRB (50μM) was applied to mouse primary neocortical cells (14 days in culture) for 2hrs; 1hr before cell collection, bicuculline (Bic, 20μM) or BDNF (50ng/mL) was added to the cultures. 15min before bicuculline or BDNF stimulation, different pharmacological treatments were applied in order to block NMDA receptors with the antagonist AP5 (50μM) or the Calcium^{2+}/calmodulin-dependent protein kinases (CaMK) with KN-62 (10μM) or the mitogen-activated protein kinases (MAPK) with U0126 (10μM). Fold changes in the expression of the IR- (red and orange) and spliced (dark grey) isoforms were assessed with real-time qPCR using three different primer sets, as represented in the right scheme. SEMs are displayed (two-to-four independent cultures). **(C)** and **(D)** Analysis of SPRK and CLK involvement in the regulation of IR-transcripts upon bicuculline (C) and BDNF (D) stimulation. In all conditions, DRB (50μM) was applied to mouse primary neocortical cells (14 days in culture) for 2hrs; 1hr before cell collection, bicuculline (Bic, 20μM) or BDNF (50ng/mL) was added to the cultures. 15min before bicuculline or BDNF stimulation, different pharmacological treatments were applied in order to block SR-protein kinases (SRPK) with SRPIN340 (10μM) or CDC2-like kinases (CLK) with KH-CB19 (10μM). Fold changes in the expression of the IR- (red and orange) and spliced (dark grey) isoforms were assessed with real-time qPCR using three different primer sets, as represented in the right scheme. SEMs are displayed (two-to-four independent cultures). **(E)** Working model. Selective IR programs – elicited upon distinct forms of neuronal stimulations and the subsequent activation of signaling pathways – remodel neuronal transcriptome in a cue-specific manner.

Figure supplement 1

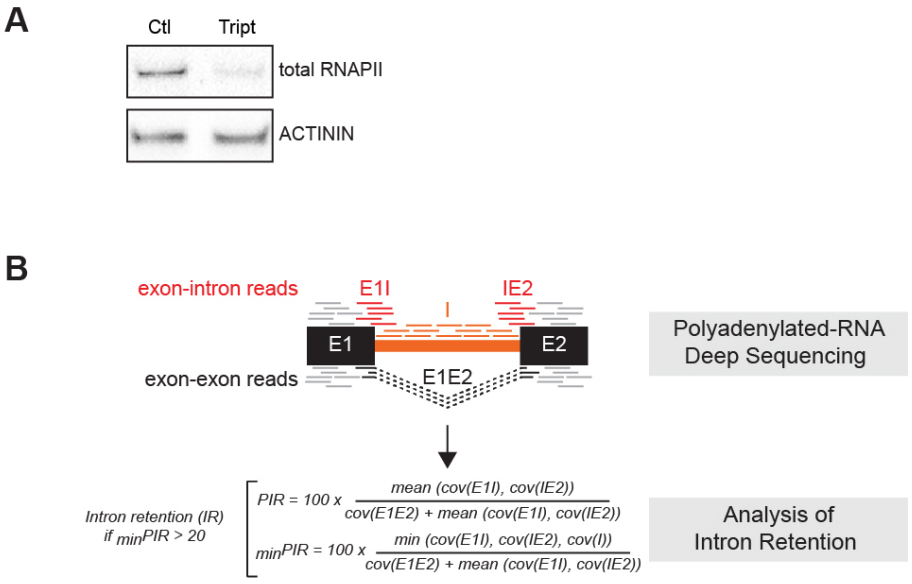


Figure supplement 1 – related to Figure 1

(A) Activity of the RNA polymerase II inhibitor triptolide (tript) treatment was probed by assessing global expression of RNA polymerase II. Triptolide results in transcription blocks as it induces a fast proteaosome-dependent degradation of the RNA-polymerase II subunit. Mouse primary neoortical cultures were treated for 2 hours with triptolide (1µM). ACTININ was used as a loading control. (four independent cultures). These controls replicate observations made in our previous work (Mauger et al., 2016). **(B)** Polyadenylated RNAs were isolated and sequenced. For each segment comprised of two consecutive exons and the intervening intron, coverage (cov) of reads spanning the 5' and 3' exon-intron junctions (E1I and IE2, respectively), the exon-exon junction (E1E2), and mapping the intron sequence (I) were calculated. Then, the percentage of intron retention (PIR) and the minimal PIR (minPIR) were evaluated as described in the formulas. Introns were considered as retained if PIR exceeded 20 and if there was a minimum (20%) sequence coverage across the entire intron.

Figure supplement 2

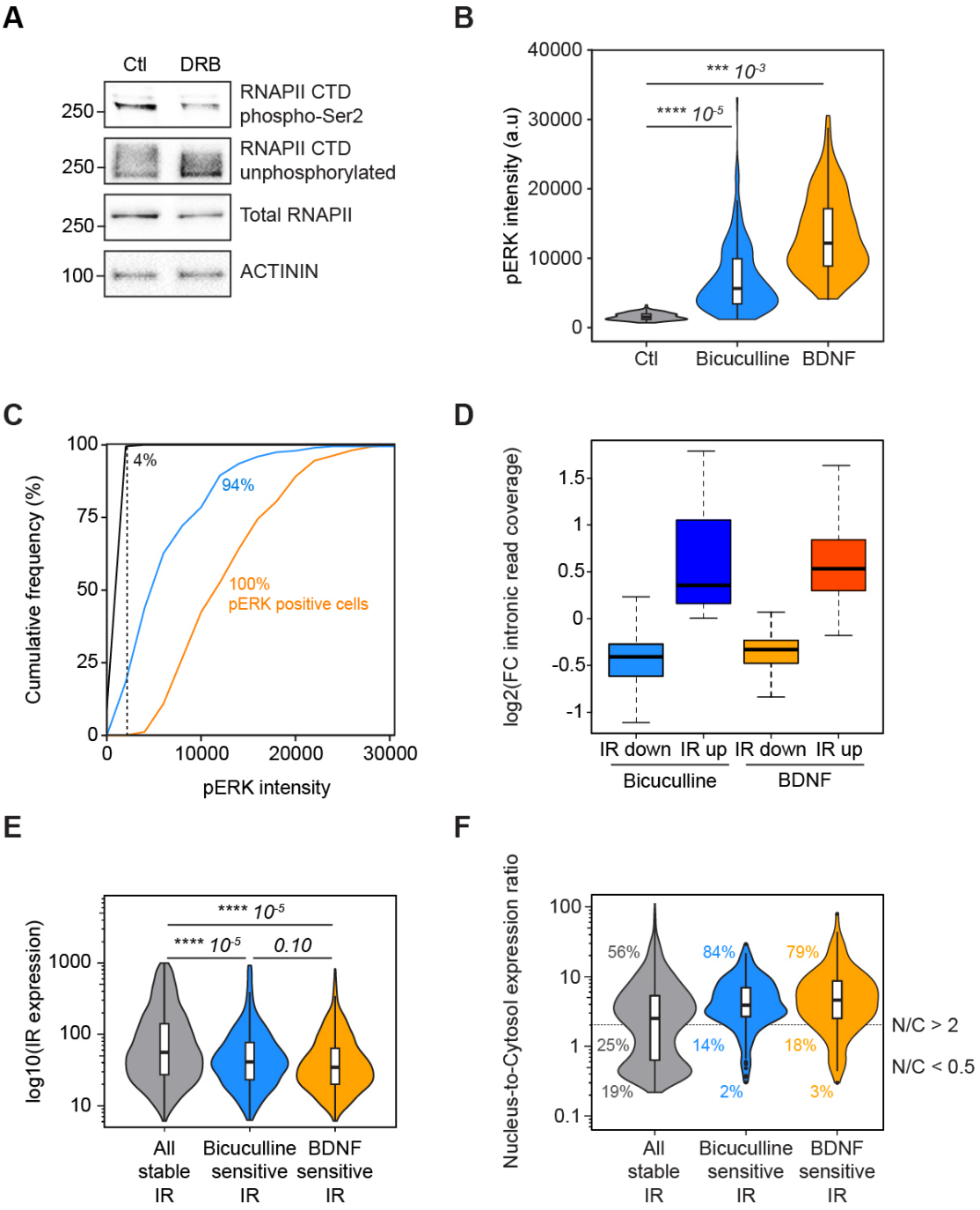


Figure supplement 2 – related to Figure 2

(A) Activity of RNA polymerase II inhibitor DRB treatment was probed by assessing C-terminal domain (CTD) phosphorylation and global expression of RNA polymerase II. DRB inhibits the CDK9 kinase and consequently prevent phosphorylation of the RNA polymerase II CTD (C-terminal domain). ACTININ was used as a loading control. These controls replicate observations made in our previous work (Mauger et al., 2016). **(B)** Violin plots displaying quantification of phospho-ERK signal in control (grey), bicuculline-stimulated (blue) and BDNF-stimulated mouse primary neocortical cells (orange). (four independent cultures; n=200 neurons (untreated), 201 neurons (bicuculline), 165 neurons (BDNF)) **(C)** Cumulative frequency plot displaying percentage of phospho-ERK-positive (pERK) neurons control (black), bicuculline-stimulated (blue) and BDNF-stimulated (orange) cultures. **(D)** Box plots displaying the fold change of read coverage along the whole retained intron of regulated transcripts. (three independent cultures) **(E)** Violin plots displaying the IR-transcript expression associated with stable IRs (grey), bicuculline-regulated IRs (blue) and BDNF-regulated IRs (orange). **(F)** Violin plots displaying the nucleus-to-cytosol expression ratio of IR-transcripts. All stable IR-transcripts (grey), bicuculline-regulated (blue) and BDNF-regulated transcripts are displayed. Percentage of nuclear-enriched (N/C>2) and cytosolic-enriched (N/C<0.5) transcripts are indicated on the panel. (three independent cultures). The p-values calculated with a two-sided Mann-Whitney test are indicated on the top of each panel (B and E).

Figure supplement 3

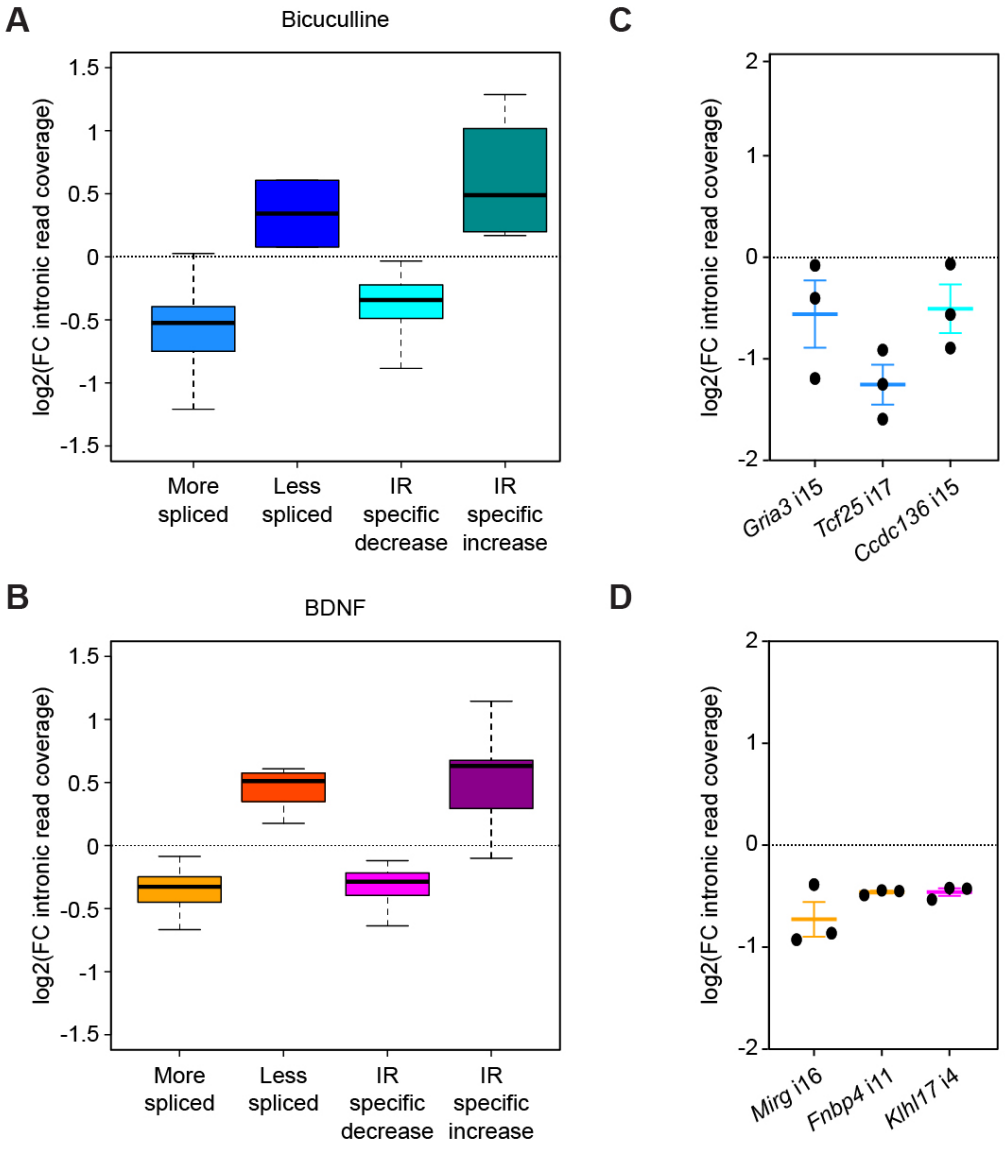
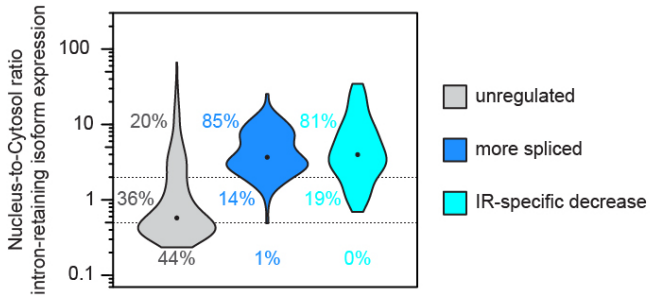


Figure supplement 3 – related to Figure 3

(A) and **(B)** Boxplot displaying the fold change of read coverage along the whole retained intron in the different categories of regulated IR-transcripts upon stimulation with bicuculline (A) or BDNF (B). **(C)** and **(D)** Boxplot displaying the fold change of read coverage along the whole retained intron of transcript candidates regulated upon stimulations with bicuculline (C) or BDNF (D). Displayed transcripts correspond to those assessed by PCRs in the main Figure 3. Means and SEMs are displayed.

Figure supplement 4

A



B

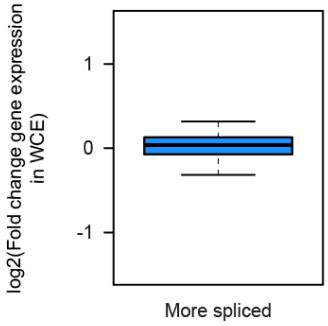


Figure supplement 4 – related to Figure 4

(A) Violin plots displaying the nucleus-to-cytosol expression ratio of IR-transcripts unregulated (grey), regulated through splicing (dark blue) or regulated through degradation (light blue). Percentage of nuclear-enriched (N/C>2) and cytosolic-enriched (N/C<0.5) transcripts are indicated on the panel. **(B)** Box plot displaying the fold change in the overall gene expression of gene containing a regulated retained intron upon bicuculline treatment.

Figure supplement 5

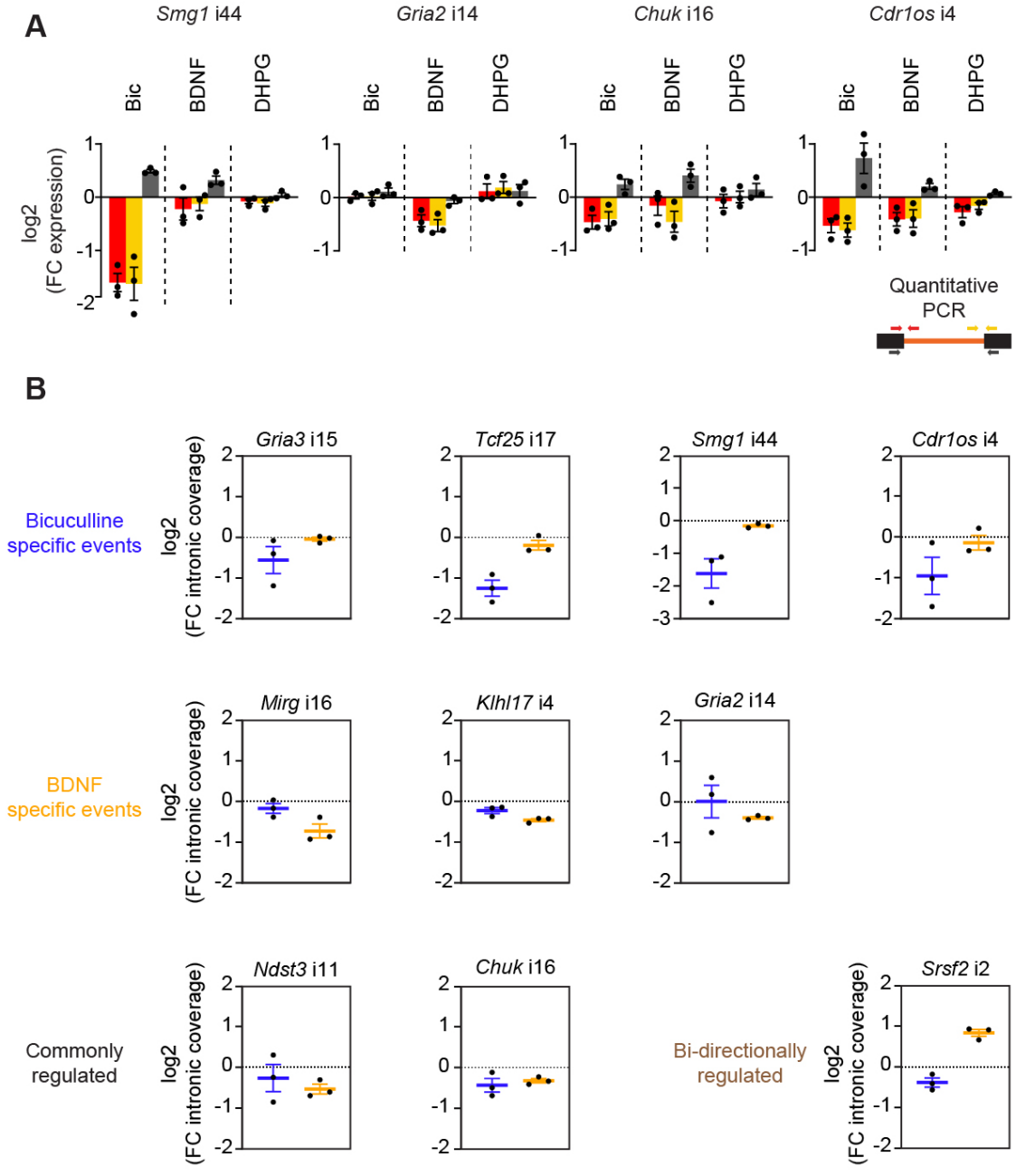


Figure supplement 5 – related to Figure 5

(A) RT-PCR validations of IR-transcripts specifically regulated upon bicuculline stimulation (*Smg1*), BDNF stimulation (*Gria2*), commonly regulated (*Chuk* and *Cdr1os*). Fold changes in the expression of the IR- (red and orange) and spliced (dark grey) isoforms were assessed with real-time qPCR using three different primer sets, as represented in the bottom right scheme. SEMs are displayed (four independent cultures). **(B)** Boxplot displaying the fold change of read coverage along the whole retained intron of transcript candidates regulated upon stimulations with bicuculline and BDNF. Displayed transcripts correspond to those assessed by PCR in the main Figure 5 and figure supplement 5A. Means and SEMs are displayed.

Figure supplement 6

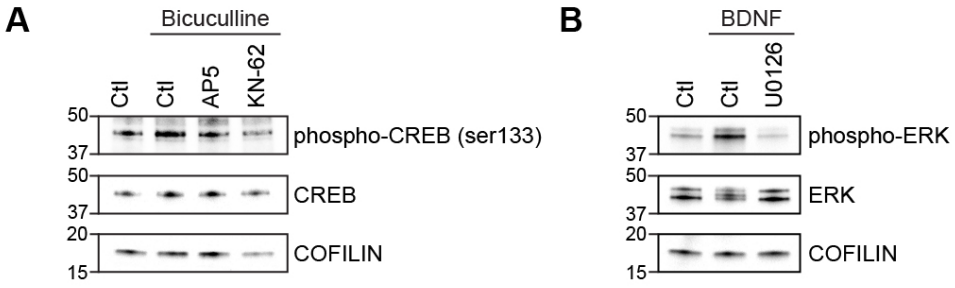


Figure supplement 6 – related to Figure 6

(A) Efficiency of the pharmacological compounds AP5 blocking NMDA receptors (NMDAR) and KN-62 targeting the Ca^{2+}/calmodulin-dependent protein kinases (CaMK) was controlled by assessing level of CREB serine 133 phosphorylation by western blot; COFILIN was used as a loading control. In all conditions, DRB (50µM) was applied to mouse primary neocortical cells (14 days in culture) for 2hrs; 1hr before cell collection, bicuculline (20µM) was added to the cultures. 15min before bicuculline stimulation, the NMDAR antagonist AP5 (50µM) or CaMK blocker KN-62 (10µM) were applied. (three independent cultures) **(B)** Efficiency of the pharmacological blocker U0126 of the mitogen-activated protein kinases (MAPK) was controlled by assessing level of ERK phosphorylation by western blot; COFILIN was used as a loading control. In all conditions, DRB (50µM) was applied to mouse primary neocortical cells (14 days in culture) for 2hrs; 1hr before cell collection, BDNF (50ng/mL) was added to the cultures. 15min before BDNF stimulation, the MAPK inhibitor U0126 (10µM) was applied. (three independent cultures).

Table 1

Table 1 - Summary of RNA sequencing read alignment

		Total Reads	Uniquely mapped reads	Mapped to too many loci	Mapped to multiple loci	Unmapped reads: too short	Unmapped reads: other	RIBOSOMAL	CODING	UTR	INTRONIC	INTERGENIC	mRNA
pooled samples during sequencing	Cytosol_Ctl_Rep1	102381005	87.31%	0.19%	7.40%	5.04%	0.07%	0.9%	41.0%	37.2%	3.3%	17.8%	78.1%
	Cytosol_Ctl_Rep2	99258948	87.81%	0.19%	7.25%	4.68%	0.07%	0.8%	43.6%	35.4%	3.1%	17.1%	79.0%
	Cytosol_Ctl_Rep3	101852925	86.03%	0.17%	8.02%	5.71%	0.07%	0.8%	41.4%	35.4%	3.1%	19.4%	76.8%
	Nucleus_Ctl_Rep1	92173824	87.25%	0.28%	6.69%	5.53%	0.25%	1.5%	35.4%	32.6%	10.3%	20.3%	68.0%
	Nucleus_Ctl_Rep2	96510469	87.87%	0.26%	6.41%	5.23%	0.23%	1.5%	37.7%	31.9%	9.7%	19.2%	69.6%
	Nucleus_Ctl_Rep3	94707116	86.22%	0.26%	7.04%	6.25%	0.23%	1.1%	36.9%	31.5%	9.9%	20.6%	68.4%
	WCE_Ctl_Rep1	108853793	87.62%	0.22%	6.89%	5.14%	0.14%	1.3%	39.8%	34.7%	6.1%	18.2%	74.5%
	WCE_Ctl_Rep2	100207337	87.11%	0.23%	6.60%	5.90%	0.15%	1.1%	41.6%	33.2%	6.6%	17.6%	74.8%
	WCE_Ctl_Rep3	95864215	86.70%	0.21%	7.61%	5.32%	0.15%	1.1%	38.9%	33.3%	6.6%	20.1%	72.2%
	Cytosol_Bic_Rep1	88611820	87.28%	0.20%	7.07%	5.37%	0.08%	0.8%	42.4%	36.2%	3.5%	17.1%	78.6%
	Cytosol_Bic_Rep2	105010780	87.69%	0.20%	6.96%	5.08%	0.07%	0.8%	44.8%	34.8%	3.1%	16.5%	79.6%
	Cytosol_Bic_Rep3	96224784	87.79%	0.19%	6.95%	4.99%	0.08%	0.7%	43.6%	35.8%	3.2%	16.8%	79.4%
	Nucleus_Bic_Rep1	103873601	88.72%	0.26%	6.17%	4.64%	0.21%	1.1%	37.8%	34.6%	8.3%	18.2%	72.5%
	Nucleus_Bic_Rep2	91452042	87.14%	0.27%	5.92%	6.44%	0.24%	1.2%	38.5%	32.1%	9.9%	18.4%	70.5%
	Nucleus_Bic_Rep3	104217750	87.36%	0.24%	6.79%	5.41%	0.19%	1.0%	39.5%	32.3%	8.0%	19.3%	71.8%
	WCE_Bic_Rep1	99460363	87.90%	0.23%	6.37%	5.37%	0.14%	1.0%	41.0%	35.3%	5.8%	16.9%	76.3%
	WCE_Bic_Rep2	108118242	87.77%	0.23%	6.44%	5.43%	0.14%	1.1%	43.0%	33.4%	5.7%	16.8%	76.5%
	WCE_Bic_Rep3	85634802	87.53%	0.22%	6.87%	5.27%	0.12%	0.9%	42.9%	33.8%	5.1%	17.4%	76.6%
pooled samples during sequencing	Ctl_Rep1	120787493	88.77%	0.25%	6.30%	4.60%	0.09%	0.9%	48.5%	31.7%	5.0%	13.9%	80.2%
	Ctl_Rep2	115636180	89.20%	0.23%	6.18%	4.29%	0.10%	0.7%	47.2%	31.8%	6.2%	14.1%	79.0%
	Ctl_Rep3	121220186	88.04%	0.23%	6.71%	4.92%	0.09%	0.7%	46.1%	32.8%	5.6%	14.9%	78.9%
	Bic_Rep1	133680600	88.60%	0.24%	6.65%	4.43%	0.09%	0.9%	47.4%	32.4%	4.7%	14.6%	79.7%
	Bic_Rep2	106630731	89.55%	0.23%	5.84%	4.26%	0.12%	0.6%	47.1%	31.4%	7.2%	13.8%	78.5%
	Bic_Rep3	168099149	87.95%	0.25%	7.17%	4.55%	0.09%	0.8%	45.9%	33.4%	4.6%	15.4%	79.3%
	BDNF_Rep1	108996997	88.77%	0.25%	6.28%	4.61%	0.09%	1.0%	48.6%	31.6%	5.0%	13.9%	80.2%
	BDNF_Rep2	186854844	89.56%	0.26%	5.81%	4.28%	0.10%	0.6%	48.2%	31.7%	6.3%	13.2%	79.9%
	BDNF_Rep3	142143812	88.69%	0.25%	6.23%	4.71%	0.11%	0.7%	47.5%	31.4%	6.3%	14.0%	79.0%

Table 2

Table 2- Primer sequences

EE = exon-exon junction
EI = 5' exon-intron junction
IE = 3' intron-exon junction

	Gene	Intron	Analysed junction	Forward/ Reverse	Sequences
real-time quantitative PCR primers	Hprt		EE	F	GATGAACCAGGTTATGACCTAGATTTGTTT
				R	ATGGCCTCCCATCTCCTTCAT
	Gria3	i15	EI	F	CAGAGCTACAGAAAGAACAGCAGGAG
				R	GACAGTGTGGTTCTACAACCTCTTCAA
			IE	F	CAGGTTGCCTGCAGTGTCTAAA
				R	CGGAGTCCTTGGCTCCACATT
			EE	F	CAGAGCTACAGAAAGAACAGCAGGAG
				R	CGGAGTCCTTGGCTCCACATT
	Tcf25	i17	EI	F	CACGGAAACACAATCGCCCTCTTCTT
				R	GCCCACACCACTCACCTTAGAATAG
			IE	F	CCCACTTAGCTGTGTCTCATTGTTCAG
				R	CTCCAGGTCGTTGAAGTGGAAGTT
			EE	F	CACGGAAACACAATCGCCCTCTTCTT
				R	TCCAGCCTCTCCCCCTCTGTGG
	Mirg	i16	EI	F	AGGTTGTCTGTGATGAGTTCGCTTTA
				R	GGAAGCCTTAGACAGGGACAAACA
			IE	F	CCTACGTGTGGTAAAGCGGAAACA
				R	ATAGGCAGGGTTCCTTGAACATCC
			EE	F	AGGTTGTCTGTGATGAGTTCGCTTTA
				R	ATAGGCAGGGTTCCTTGAACATCC
	Fnbp4	i11	EI	F	GCAGAAGTGAATGAAGAACAAGATTA
				R	AAAGAGCAAAGATTTCAAATAACGAAA
			IE	F	GTGTCTCTGTAGGAGAACAGATTT
				R	GACTCTCATCTCTAACTTCCTTTGT
			EE	F	GCAGAAGTGAATGAAGAACAAGATTA
				R	GACTCTCATCTCTAACTTCCTTTGT
	Ccdc136	i15	EI	F	GTGAGACCCTTCACAGGAGCTATG
				R	ACCTTCCCTGCCTGACCAATAA
			IE	F	CCAGAGGTGTAGAGTGTAGGACAGA
				R	CTCTTCCTGGCTCACTTGGTACAG
			EE	F	CCAGCAGCACAAGTGTGAGCTATAA
				R	ACTGTTTCCTCAAAGTGCTCCAAGTC
	Klhl17	i4	EI	F	CATTCGAGGATTTGCAGACACACAC
				R	CAAACTGGCATGTTACCTGCTTCAG
			IE	F	GCACATGCCCTCTGTCTGATACT
				R	CGTTCAGGCTATCACTAGAGACCAATTC
			EE	F	CATTCGAGGATTTGCAGACACACAC
				R	CCAATTCCAGCACCTGCTTCAG
	Smg1	i44	EI	F	GGGTGTAACTGGAGTAGAAGGTGTTT
				R	TCAGTAAACTCAACACAAGTTTCCA
			IE	F	CATATGGAAACTTGTGTTGAGTTTACTG
				R	TTCCATCTCTCGCTTACTCTGCTTG
			EE	F	TGTGAGCAGGTTCTCCACATCAT
				R	TTCCATCTCTCGCTTACTCTGCTTG
	Chuk	i16	EI	F	GAGCAGCGTGCCATTGATCTCTATAA
				R	TACCCACTTTGTTCAGTTAGGGTGTTT
			IE	F	GGAACGTCCTGTTTGGAAATTGATGTG
				R	GGTGACCAAACAGCTCCTTGAGAA
			EE	F	GAGCAGCGTGCCATTGATCTCTATAA
				R	GGTGACCAAACAGCTCCTTGAGAA
	Srsf2	i2	EI	F	CTCCAGAAGAAGAGGGAGCAGTTTC
				R	AGCATCACTCCCAAAGCTGAGTAA
			IE	F	GCTGTTTCATGCTGTTTGAGACCTATT
				R	CCTGGAGGATCAGCCAAATCAGTTA
			EE	F	CTGCCCGAAGATCCAAGTCCAAGTC
				R	CAGGAGACCGCAGCATTTTCTTAGGAAG
	Ndst3	i11	EI	F	AAATCCTTTGAGGAGGTACAGTTCTTT
				R	TGTGTGGCATGAATGTTATCTGTAGT
			IE	F	TCAGTCACCAGCATATAAACGTAAGGG
				R	GGAGCGTCCTCTGAATGGAAGTAA
			EE	F	AAATCCTTTGAGGAGGTACAGTTCTTT
				R	GGAGCGTCCTCTGAATGGAAGTAA
	Smg1	i44	EI	F	GGGTGTAACTGGAGTAGAAGGTGTTT
				R	TCAGTAAACTCAACACAAGTTTCCA
			IE	F	CATATGGAAACTTGTGTTGAGTTTACTG
				R	TTCCATCTCTCGCTTACTCTGCTTG
			EE	F	TGTGAGCAGGTTCTCCACATCAT
				R	TTCCATCTCTCGCTTACTCTGCTTG
	Gria2	i14	EI	F	GGAGTCACATTCAAGACACTGTTATTTGTT
				R	AATCTGAATCTTTGGGTAAGGTGGTAGAG
			IE	F	GCTCACCCTGTCTGACAAGTATGTT
				R	ACTCTCCTTTGTCGTACCACCATTT
			EE	F	TTGTTGTGGATAAATGCGGTTAACC
				R	CTCTCCTTTGTCGTACCACCATTTG
	Cdr1os (C230004 F18Rik)	i4	EI	F	ACATCGCTGTGGTCCATCTCTATTTAC
				R	ACTGGGATGGAGTAAAGGGTGAAAC
			IE	F	TCTATGTTGTGCAGAGTTACCTTATTACAC
				R	TGTATCTCTGCTGTAGGCCAGAATTG
			EE	F	ACATCGCTGTGGTCCATCTCTATTTAC
				R	TGTATCTCTGCTGTAGGCCAGAATTG
semi-quantitative PCR primers	Gria3	i15		F	CAGAGCTACAGAAAGAACAGCAGGAG
				R	CGGAGTCCTTGGCTCCACATT
				R	TTTCCCACCCTGTTCCACCAA
	Tcf25	i17		F	CTTCCGGTCCTTGTTGCCAAATTAC
				R	CTCCAGGTCGTTGAAGTGGAAGTT
	Mirg	i16		F	AGGTTGTCTGTGATGAGTTCGCTTTA
				R	ATAGGCAGGGTTCCTTGAACATCC
				R	GGAAGCCTTAGACAGGGACAAACA
	Fnbp4	i11		F	CCTCTGGAAGCAACTACTCCTGATTAAC
				R	TCGCCCGACTGTTCGTTTACATAG
	Ccdc136	i15		F	GTGAGACCCTTCACAGGAGCTATG
				R	CTCTTCCTGGCTCACTTGGTACAG
	Klhl17	i4		F	CATTCGAGGATTTGCAGACACACAC
				R	CGTTCAGGCTATCACTAGAGACCAATTC
	Hprt	EE		F	GATGAACCAGGTTATGACCTAGATTTGTTT
				R	ATGGCCTCCCATCTCCTTCAT
	Srsf2	i2		F	CTCCAGAAGAAGAGGGAGCAGTTTC
				R	CCTGGAGGATCAGCCAAATCAGTTA
	Ndst3	i11		F	AAATCCTTTGAGGAGGTACAGTTCTTT
				F	TTTGCACAGGAGCTAAGGTTTGATTATT
				R	GGAGCGTCCTCTGAATGGAAGTAA

3. Discussion and future directions

3.1 Conclusions

The main goal of my PhD was to investigate to which extend neurons use nuclear sequestration of stable intron-retaining transcripts and activity-dependent splicing and cytosolic release to remodel their transcriptome. The first question we wanted to answer was whether nuclear sequestration of stable intron-retaining transcripts is a general mechanism or whether it affects only a couple of candidate transcripts? Secondly, we asked whether there is a universal IR regulation program or whether sub-populations of stable and nuclear intron-retaining transcripts can be regulated in a neuronal stimulus-specific manner?

During my PhD, we used a combination of cell fractionation and deep RNA-sequencing to perform a systematic sub-cellular mapping of stable intron-retaining transcripts in neurons. This experiment revealed that stable intron-retaining transcripts are strongly enriched in the nucleus which indicates that large populations are targeted for nuclear sequestration. I then probed how these stable nuclear transcripts react to two distinct stimulation paradigms. Elevation of neuronal network activity, which stimulates synaptic glutamate release, and treatment with the neurotrophin BDNF are both able to regulate stable and nuclear intron-retaining transcripts. We identified large populations up or down-regulated upon both stimuli. Importantly, intron-retaining transcripts can be regulated via splicing or via possible degradation/stabilization. Importantly, we show that splicing regulation leads to an increase in cytosolic spliced mRNA, indicating export in the cytosol, where new mRNAs can be used for protein synthesis. For the first time, we characterized stimulation-specific populations of intron-retaining transcripts. Finally, I used pharmacological inhibitors to demonstrate that the stimulation-specificity of certain IRs populations arises from distinct molecular pathways. My results show that elevation of network activity requires Ca^{2+} influx through NMDA receptors and subsequent CaMKs pathway activation to control stable and nuclear intron-retaining transcripts. On the other hand, neurotrophin-specific events rely on MAPK pathway activity. Our results also support that the serine/arginine protein kinase (SRPK) family is necessary for IR regulation upon the elevation of neuronal network activity. These kinases are known to phosphorylate SR proteins, a class of well-known splicing factors, which may directly control stable intron-retaining transcripts splicing upon neuronal stimulation.

Taken together these results describe a mechanism allowing neurons to mobilize a large population of new mRNA in a cue-specific manner. Splicing completion and subsequent cytosolic export of stable and nuclear intron-retaining transcripts could then support rapid

modification of neuronal properties upon specific sensory inputs. In the following part, I will be developing the biological questions that are still open regarding activity-dependent intron excision. In the first part, I will discuss the possible molecular determinants of nuclear intron-retaining transcript's stability in the nucleus and how this can be linked to their sub-nuclear localization and their possible interactors. Secondly, I will describe a set of new tools that are necessary to assess the functional impact of activity-dependent IR regulation in pre-existing transcripts upon acute neuronal activity. Finally, I will describe potential experiences aiming at deciphering the functional impact of activity-dependent splicing and cytosolic release of stable intron-retaining transcripts.

3.2 Determinants of stability and interactome of stable intron-retaining transcripts

One remarkable feature of many nuclear intron-retaining transcripts is their stability and accumulation in the nucleus. How those intron–retaining transcripts escape the nuclear surveillance machinery is still an open question. The identification of the molecular determinants that mediate nuclear intron-retaining transcripts stability may provide an important step towards our understanding of the functional role of these RNAs in neurons. The stability of the nuclear intron-retaining transcripts may be linked to their sub-nuclear localization. The nucleus of eukaryotic cells contains various so-called ‘nuclear bodies’ that represent membrane-less sub-nuclear organelles (Staněk and Fox, 2017). The functional role of nuclear bodies is not entirely clear. However, some of them are known to be associated with nuclear-localized RNA, splicing factors, and components of the nuclear export machinery. For example, nuclear speckles are known to contain snRNPs (Morimoto and Boerkoel, 2013), certain SR proteins (Fu and Maniatis, 1990), CLK kinase proteins, and members of the EJC (Mintz et al.; Saitoh et al., 2004), suggesting their role in RNA splicing. Moreover, nuclear speckles were shown to be associated with polyadenylated RNAs (Carter et al., 1991) and were proposed as possible sites of post-transcriptional splicing (Girard et al., 2012). Another type of nuclear body, the paraspeckles, are strongly regulated upon different types of cellular stress and can retain certain polyadenylated RNAs and promote their processing and release (McCluggage and Fox, 2021; Prasanth et al., 2005). One can imagine that transcripts stably retaining an intron could be associated with certain nuclear bodies which may protect them from the nuclear degradation machinery. The dynamic nature of these nuclear structures and their enrichment for splicing factors and proteins associated with cytoplasmic RNA export

suggest that stable intron-retaining transcript might be processed and made ready for export on-site upon environmental signals. Interestingly, a growing number of studies describe the role of RNAs in the formation of nuclear bodies via liquid-liquid phase separation and stable intron-retaining transcripts could participate in this process (Clemson et al., 2009; Hondele et al., 2020; Wang et al., 2020).

Since the markers for many of these nuclear structures are well known (Morimoto and Boerkoel, 2013), their association with stable intron-retaining transcripts could be investigated via RNA-FISH. Recent developments in spatial transcriptomic allow the imaging of several thousands of RNAs simultaneously (Eng et al., 2019). Usage of intronic probes is possible for such large-scale RNA FISH experiments (Shah et al., 2018) and a study recently used a combination of DNA and RNA FISH along with immunostaining to simultaneously observe chromatin domains, nuclear bodies, and RNA localization (Takei et al., 2021). Implementation of these methods would provide great possibilities for large-scale subcellular localization studies of stable and nuclear intron-retaining transcripts.

An alternative approach to investigate the association of intron-retaining RNA with nuclear structures and proteins is the use of recently developed methods based on proximity-labeling by ascorbate peroxidase (APEX) (Chen and Perrimon, 2017) This methodology relies on a peroxidase fused to a protein of interest with a specific subcellular localization. The peroxidase will convert the exogenously supplied biotin-phenol and H2O2 to biotin phenyl radicals. These biotin phenyl radicals can react with certain amino acids as well as RNA molecules in proximity resulting in biotinylation of proteins and RNA in a radius of approximately 10-20 nm (Kaewsapsak et al., 2017; Padrón et al., 2019; Trinkle-Mulcahy, 2019). The application of this approach could be implemented to reveal populations of transcripts that stably retain an intron and are associated with certain cellular organelles or nuclear sub-compartments (Fazal et al., 2019). Additionally, APEX-based proximity labeling techniques have a high temporal resolution as biotin labeling can happen in minute range, thus providing a powerful tool to capture eventual splicing events and/or release of stable intron-retaining transcripts from particular nuclear domains under different physiological conditions. Proximity labeling can also be used to identify the cue-specific factors, which control the splicing of stable intron-retaining transcripts. In this regard, RNA-centric proximity labeling could be used to identify the interactome of an intron-retaining transcript of interest (Mukherjee et al., 2019; Ramanathan et al., 2018). Development of new techniques to target APEX enzyme to the RNA of interest, for example via a deactivated Cas, allows identification of endogenous RNA associated proteins (Trinkle-Mulcahy, 2019). Due to the excellent temporal resolution of proximity labeling technics, this approach could be used to capture interactome changes of a stable

intron-retaining transcript of interest. This could be done upon distinct types of neuronal stimuli and allow identification of cue-specific binding of RBPs to the RNAs of interest. Investigation of the sub-nuclear localization of stable intron-retaining transcripts as well as their dynamic interactome will bring important information regarding the regulation of such transcripts upon neuronal activity and provide potential leverage to interfere with their function.

3.3 New tools to interfere with activity-dependent intron retention regulation

Alternative splicing has been demonstrated to be crucial for a variety of cellular processes in neuronal cells including neuronal recognition, axon guidance, synapse formation, or even synaptic transmission (Furlanis and Scheiffele, 2018). Moreover, AS defect is associated with many pathological conditions such as spinal muscular atrophy, amyotrophic lateral sclerosis or early-onset Parkinson disease, and many others (Scotti and Swanson, 2016). This motivates the investigation of AS events to better understand their role under physiological and pathological conditions to potentially develop splicing-dedicated therapeutics. However, understanding the functional impact of an endogenous specific splicing event is not trivial. Whereas many tools are developed for specific loss of function at the gene level, the toolbox to manipulate splicing events is more restrained. Indeed, interference with a specific splicing event must not change the basal gene expression level. Importantly, in the context of events controlled by acute environmental changes, the desired manipulation of function must be performed within a particularly tight time window upon arrival of the stimuli. This makes the functional study of activity-dependent splicing, but also activity-dependent intron excision particularly difficult.

One promising tool to modulate a given splicing event is the use of antisense oligonucleotides (ASO). They are short (typically 8-50 nucleotides), synthetic, single-stranded oligonucleotides that are complementary for a sequence of interest on a particular RNA (Rinaldi and Wood, 2018). Target sequences are usually cis-acting regulatory elements affecting splicing such as 5' or 3'SS surrounding sequences, branchpoint sequences, poly-pyrimidine tract, or exonic and intronic splicing enhancer or silencers. Upon binding on those sequences, ASOs will prevent trans-acting factors from interaction and modulation of inclusion/exclusion of certain RNA segments. ASOs exhibit good diffusion in brain tissues, are easy to administrate, and have a long half-life which makes them ideal to interfere with splicing reactions in-vitro and in-vivo, in particular in the

central nervous system. ASOs cellular uptake in-vitro is reported as being in the hour range, which provides a way to specifically regulate single splicing events with an interesting temporal resolution (Crooke, 2017). Some ASO-based therapeutics targeting splicing reactions are now approved by the Food and Drug Administration agency (FDA). An excellent example is the treatment of SMA by an ASO which is favoring exon 7 inclusion in *Smn2* in order to compensate for SMN1 loss (Wood et al., 2017). One disadvantage of ASO-based regulation of AS is that it cannot be delivered in a cell type-specific manner. Also, ASOs are costly and multiple steps of screening with many different ASOs targeting adjacent sequences are necessary. Finally, more temporally sensitive systems would be necessary to study the AS regulation events upon acute stimulus such as neuronal depolarization. This type of temporally sensitive tool would be of great interest to interfere with activity-dependent IR regulation of pre-existing transcripts.

Other classes of tools based on the CRISPR-Cas system have been developed to modulate AS. The first type of these tools targets the DNA to induce modification that will affect splicing choices. For example, Yuan and colleagues used a deactivated Cas9 fused to the cytidine deaminase AID and targeted this construct to splice sites of interest with a specific guide RNA (gRNA). This leads to base editing in the splice site consensus sequence and using this approach, they could restore splicing in the Duchenne muscular dystrophy (DMD) gene (Yuan et al., 2018). However, modification at the DNA level will only affect splicing of newly transcribed RNA leaving unaffected the splicing of pre-existing transcripts. Moreover, DNA modification will trigger irreversible splicing changes. Alternatively, a catalytically inactive form of CasRx can be directly targeted to certain pre-mRNA. Utilizing this system, an increased exclusion of exon 10 of the *Mapt* pre-mRNA could be demonstrated (Konermann et al., 2018). Direct targeting of the pre-mRNA by CRISPR-based methodology provides the advantage that both co- and post-transcriptional splicing events could be manipulated. Additionally, the manipulation of splicing could be relieved upon Cas-protein clearance. Such CRISPR-based system has tissue or cell type specificity potential as they can be packed and delivered via viral vectors.

An interesting feature of CRISPR-based tools for splicing interference is the possibility to couple it with chemically or light-inducible systems, thus allowing precise temporal control of the desired manipulation. A recent study took advantage of the rapamycin-dependent dimerization of FK506 binding protein (FKBP) with an FKBP-rapamycin binding domain (FRB). In this study, the RNA recognition motif (RRM) of the splicing factor RBFOX1 was fused to a dCasRx-FKBP construct. dCasRx-FKBP is targeted to a specific region in intron 7 of an SMN2 minigene. Rapamycin treatment leads to FKBP-FRB dimerization thereby bringing RBFOX1 RRM to *Smn2* intron and induced exon 7 inclusion (Du et al., 2020). The

authors of this study assessed changes in exon 7 inclusion 24h after rapamycin treatment, however, FKBP-FRB dimerization was reported to happen already in minute range upon rapamycin treatment (Geda et al., 2008; Haruki et al., 2008). Due to its temporal precision and reversibility, this system has the great potential of triggering splicing modulation upon rapamycin treatment. It might represent a useful tool to assess the functional impact of stable intron-retaining transcript splicing upon neuronal activity. Another possibility is to couple splicing modulation with a light-inducible system. For example, in presence of phycocyanobilin (PCB), the plant phytochrome B (PhyB) and the phytochrome interacting factor (PIF) proteins can undergo dimerization if exposed to red light (Ni et al., 1999; Rockwell et al., 2006). One could imagine a system where PhyB is attached to a dCasRx and targeted to a specific RNA region via gRNA while a splicing factor would be attached to a PIF. Upon PCB treatment and red light illumination, the splicing factors would be brought to the RNA region of interest to modulate splicing. PhyB-PIF dimerization has been reported to happen as soon as 2 min upon red light illumination. Moreover, illumination with infrared light leads to dissociation of the complex (Di Ventura and Kuhlman, 2016). Thereby, such a system would potentially allow minute range splicing inhibition upon red light illumination and possible restoration of the initial splicing pattern upon infrared light. Such light-inducible systems but also usage of ASOs constitute powerful tools to investigate the functional impact of AS changes happening upon acute environmental signal, including investigation of activity-dependent intron excision of pre-existing transcripts.

3.4 Functional impact of activity-dependent intron excision in neurons

3.4.1 Probing the proteome regulation upon activity-dependent intron excision

An important step to better understand the functional impact of new mRNA mobilized upon activity-dependent intron excision is to investigate the effect of this mechanism on the proteome. Indeed, an increase of functional mRNA in the cytoplasm is expected to increase the protein level. In this direction, Naro et al measured a significant increase of 5-EU-labelled RNA associated with polysome fraction during the late phase of spermatogenesis. Importantly, transcription is inactive at this moment and 5-EU was injected in the mice 9 days earlier when transcription was active (Naro et al., 2017). Similarly, Mauger et al used mice harboring a tagged version of RPL22 allowing pull-down

of ribosome-associated RNA (Sanz et al., 2009) and reported an up to 5-fold change increase for the *Clk1* mRNA (Mauger et al., 2016).

However, direct report of protein level regulation upon IR regulation is not systematic. One reason for this lack of information of protein level variation upon activity-dependent IR regulation may be that the methods to assess large-scale proteome changes are way less sensitive than for transcriptomic regulation. Thereby, the capture of subtle proteomic changes induced by activity-dependent intron excision and cytosolic release by shotgun proteomics may be difficult. A possible way around this problem is the enrichment for newly synthesized protein using biorthogonal noncanonical amino acid tagging (Dieterich et al., 2006). This method relies on cell treatment with an azide-labeled amino acid analog upon neuronal stimulation. Newly-made proteins will integrate the azide-labeled analog in their sequence. After that, an alkyne-bearing tag is added to these labeled proteins by click chemistry and newly synthesized protein can be isolated. Another alternative lies in the recent progress in targeted mass spectrometry allowing measurement of many endogenous peptides (up to hundreds) in a single sample. These technical approaches can unravel potential large-scale proteome changes taking place upon activity-dependent splicing completion of stable intron retaining transcripts. Identification of proteins that strongly depend on intron-excision and cytosolic export for their regulation upon neuronal activity will provide a strong insight into the neuronal functions that rely on this mechanism.

Alternatively, certain studies focused on a single transcript candidate and reported protein changes upon signal-dependent intron retention regulation. Park and colleagues treated human cells with OSMI-1, an OGT inhibitor resulting in splicing of intron 4 in the *Ogt* transcript. They were able to detect an up to 2-fold change increase in protein level by western blot 6h after treatment. However, this increase in *Ogt* mature transcript was not confirmed to arise from pre-existing intron-retaining transcript splicing. Thereby, the OGT protein increase may come from newly transcribed mature *Ogt* (Park et al., 2017). This illustrates another hurdle in characterizing protein level change arising from splicing and cytosolic release of pre-existing intron-retaining transcripts. Protein level increase measured will reflect the translation of newly spliced mRNA as well as newly transcribed mRNA. Inhibition of splicing prior to neuronal stimulation would identify the protein level changes arising from the splicing of stable intron-retaining transcripts. However, the spliceosome is complex machinery composed of many RNAs and proteins and the drugs currently available to inhibit splicing effectively affect only a small proportion of splicing events with strong variability between the cellular models (Corrionero et al., 2011; Effenberger et al., 2017). Importantly, protein level changes caused by splicing and cytosolic export of intron-retaining transcripts will be resistant to transcription inhibition.

Thereby, transcription inhibition before neuronal stimulation will be an important requirement to investigate the proteome changes caused by activity-dependent splicing and export of pre-existing intron-retaining transcripts. As another possible bias, protein increase could also come from the increased translation of mRNA already present in the cytosol. Usage of drugs inhibiting mRNA export may be a solution to rule out any increase in translational efficiency. However, drugs inhibiting mRNA export exhibit a highly transcript-dependent efficiency and prior testing would be necessary to make sure that the export of RNAs of interest is indeed efficiently inhibited (Ferreira et al., 2020).

Whether activity-dependent nuclear storage and release of intron-retaining transcripts drive large changes on the neuronal proteome is yet to be explored. Identification of important protein level changes upon activity-dependent intron excision will give a hint on the neuronal functions regulated by this mechanism and will set the ground for a functional study.

3.4.2 Focus on CLK1

Cdc2-like protein kinase 1 (CLK1) is an SR protein kinase. The main CLK targets are SR proteins which are strongly involved in splicing regulation but also in many aspects of RNA processing from transcription to recruitment of export factors and translation regulation (Zhou and Fu, 2013). *Clk1* RNA is mainly present as an intron-retaining isoform (70%) that contains intron 3 and 4 and only 30% of the total RNA pool is completely spliced and exported in the cytosol. Importantly, *Clk1* intron-retaining transcripts are stably sequestrated in the nucleus and their splicing is regulated by several stimuli including heat shock, osmotic stress, and elevation of neuronal activity (Mauger et al., 2016; Ninomiya et al., 2011).

We assessed *Clk1* splicing upon elevation of neuronal activity in mouse primary neocortical neurons and found an up to 3-fold increase of *Clk1* spliced isoform (Figure 3.1 A). Moreover, we probed CLK1 protein level upon neuronal activity via targeted mass spectrometry and our data support an up to 2-fold increase (Figure 3.1 B). Importantly, the neurons were pre-treated with a transcription inhibitor to rule out protein production arising from newly synthesized transcripts. This preliminary result support that *Clk1* is an interesting candidate to investigate the functional impact of activity-dependent intron excision and cytosolic release in neurons.

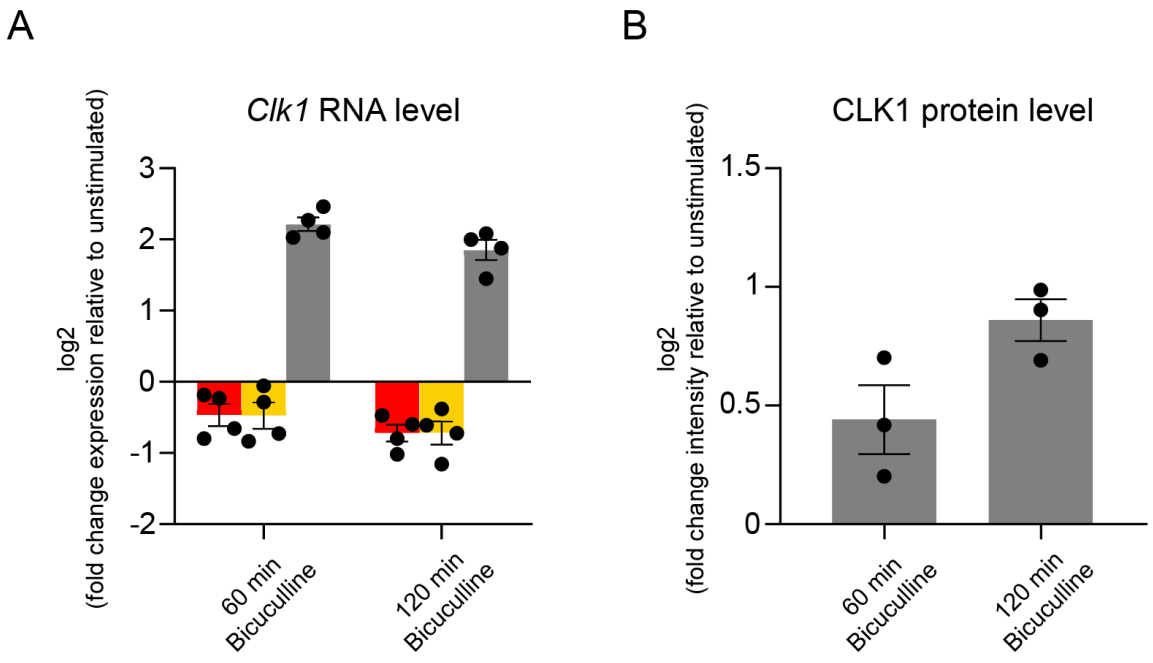


Figure 3.1: Clk1 mRNA and protein variation upon bicuculline.

Mouse primary cortical neurons (DIV14) were treated with DRB (50µM) for 2 hours and stimulated for the indicated time with bicuculline (20µM). Unstimulated condition corresponds to cells treated with DRB (50µM) for 6 hours. A: Retained isoform expression (red and orange) and spliced isoform expression (grey) were probed via RT-qPCR. N=4 independent cultures. SEM is displayed. B: CLK1 protein level was assessed by targeted mass spectrometry. Mean of 3 different peptides is displayed. SEM is displayed. N=3 independent cultures. SEM is displayed.

However, the functions of CLK proteins in neurons are yet to be understood. Indeed, the effect of CLK1 phosphorylation of SR protein on transcriptional or AS events has not been explored in neurons. More importantly, the molecular consequences of CLK1 protein increase upon neuronal activity are also unknown. It should be noted that CLK2 has been associated with SHANK3 deficient mouse model of ASD that exhibits a diminution in spine density. Phospho-proteomic analysis of SHANK3 deficient mice, a known risk factor in ASD, show downregulation of mTorc1 signaling resulting from hyperphosphorylation of the phosphatase 2A due to an increased steady-state activity level of CLK2. Importantly CLK2 inhibition restored the Shank3 defective synaptic phenotype (Bidinosti et al., 2016). This supports that CLKs proteins may exhibit other functions besides phosphorylation of SR protein in the nucleus and affect synaptic properties. In order to better understand the role of CLK1 induction upon neuronal activity, one would need to use tools with a precise temporal dynamic to interfere with *Clk1* splicing upon stimulation without affecting basal *Clk1* level or splicing pattern. Interestingly, a highly conserved region of *Clk1* is associated with intron 3 and 4 retention regulation upon CLK inhibition with TG003 (Ninomiya et al., 2011). Thereby ASO- or CRISPR-based system targeted to this highly conserved region

may interfere specifically with an activity-dependent increase in *Clk1* mRNA. Inhibition of *Clk1* post-transcriptional splicing upon neuronal activity followed by RNA-sequencing will reveal potential transcriptomic (gene expression or AS level) regulation controlled by CLK1 protein increase upon neuronal activity. We hypothesize that CLK1 protein would be produced quickly upon neuronal stimulation via intron-retaining transcript splicing and cytosolic export. CLK1 would then participate in the correct processing of newly transcribed RNA upon neuronal activity. In this model, activity-dependent splicing of pre-existing intron-retaining transcripts is critical for the correct formation of gene expression programs and subsequent modulation of neuronal functional and structural properties upon external stimuli.

3.4.3 Investigation of activity-dependent IR regulation in vivo

Splicing completion of stable and nuclear intron-retaining transcripts upon neuronal activity allows a rapid new mRNA mobilization. In my dissertation project, we show that distinct neuronal stimuli control specific populations of stable nuclear intron-retaining transcripts and that this specificity relies on different signaling pathways. However, these observations were made using pharmacological treatments mimicking neuronal stimuli in a primary neuron culture model. Thereby, there is a crucial need for in vivo characterization of such activity-dependent events upon more physiological stimuli to define what type of sensory input control activity-dependent IR regulation. One major limitation lies in the fact that only a subset of cells is recruited upon various sensory input or behavioral paradigms in the appropriate brain regions (Gallo et al., 2018; Guzowski et al., 2001; Josselyn and Tonegawa, 2020). Thereby, bulk RNA-sequencing is not suited to detect behavior-induced gene expression changes in associated brain regions. Important progress was made in this regard due to the development of single-cell RNA sequencing. However, low RNA capture rate from a single cell resulting in low sequencing depth makes splicing analysis via sgRNA-seq unreliable quantitatively. Interestingly, light exposure of dark-reared mice was shown to activate a high number of cells in the visual cortex (Kaminska et al., 1996; Spiegel et al., 2014). Thereby, visual cortex dissection and RNA isolation may allow detection of activity-dependent IR regulation. Importantly, using this physiological stimulus, a decrease in *Clk1* intron-retaining isoform was detected concomitantly with an increase in the spliced isoform as soon as 15 minutes upon light exposure (Oriane Mauger, unpublished data). This indicates that activity-dependent *Clk1* intron excision happens in vivo upon a physiological stimulus. RNA-sequencing of light-induced cortical neurons may then shed light on large activity-dependent IR regulation programs responding to visual

stimulation in vivo. However, *Clk1* exhibit a high level of IR in neurons and strong IR profile change upon neuronal activity. Moreover, *Clk1* expression is not limited to particular cell types in the mouse visual cortex (Hrvatin et al., 2018). Thereby, IR level changes in such genes may be easier to detect while intron-retaining transcripts exhibiting lower retention levels and sparse expression may not be detected. Another limitation is that, to ensure that spliced mRNA increase upon light exposure arise from pre-existing intron-retaining transcripts the animals must be treated with a transcription inhibitor to rule out any de novo RNA synthesis. In conclusion, these potential experiments may unravel the intron retention regulation programs taking place upon sensory input in vivo which represent a major step toward the identification of the neuronal function associated with this mechanism.

4. Materials and methods

4.1 Materials and Methods

Primary neocortical cultures and pharmacological treatments

All procedures related to animal experimentation were reviewed and approved by the Kantonales Veterinäramt Basel-Stadt. Dissociated cultures of neocortical cells were prepared from E16.5 mouse embryos (embryonic stage 16.5). Neocortices were dissociated by the addition of papain (130 units, Worthington Biochemical, LK003176) for 30 min at 37°C. Cells (45,000 cells/cm2) were maintained in neurobasal medium (Gibco, 21103-049) containing 2% B27 supplement (Gibco, 17504-044), 1% Glutamax (Gibco, 35050-61), and 1% penicillin/streptomycin (Bioconcept, 4-01F00-H) for 14 days. The following reagents for pharmacological treatments were used (at the indicated concentrations and from the stated sources): DRB (50 µM, Sigma, D1916), triptolide (1 µM, Sigma, T3652), bicuculline (20 µM, Tocris, 0130), BDNF (50 ng/mL, Sigma, B7395), DHPG (10 µM, Tocris, 0805), DL-AP5 (50 µM, Tocris, 3693), KN-62 (10 µM, Tocris, 1277), U0126 (10µM, Tocris, 1144), SRPIN340 (10 µM, Tocris, 5063) and KH-CB19 (10 µM, Tocris, 4262).

Cellular fractionation

Cell fractionation experiments were performed according the protocol from Suzuki et al. (Suzuki et al., 2010). Briefly, two million cells plated in a 10-cm^2 dish were collected in ice-cold PBS. After 10 sec-centrifugation, the supernatant was removed from each sample and the cell pellet was resuspended in 450 µL ice-cold 0.1% NP40 in PBS. One aliquot was collected as the whole-cell extract and then the leftover was spun for 10 sec. The supernatant was collected as the cytosolic-enriched fraction and the pellet (after one wash with 450 µL 0.1% NP40 in PBS) as the nuclear-enriched fraction.

Western blot and antibodies

Total proteins were separated by electrophoresis on 4%–20% gradient PAGE gels (Bio-Rad, 4561093) and transferred onto nitrocellulose membrane. The following antibodies were used: anti-phosphoCREB (Ser133) (Millipore, aa77-343), anti-CREB (clone 48H2, Cell signaling, 9197), anti-phosphoERK (Cell signaling, 4370S), anti-ERK (Cell signaling, 4695S), anti-phospho-CTD RNA polymerase II (Ser2) (Abcam, ab5095), anti-CTD RNA polymerase II (Abcam, ab817), anti-N-terminal RNA polymerase II A-10 (Santa Cruz,

sc17798), anti-ACTININ (Abcam, ab68194), anti-COFILIN (Abcam, ab54532), anti-GAPDH (clone D16H11, Cell Signaling, 5174), anti-MECP2 (Cell Signaling, 3456), anti-U1-70K (clone H111, Synaptic Systems, 203011), anti-LAMIN B1 (Abcam, ab133741), anti-hnRNPA1 (clone D21H11, Cell Signaling, 8443), anti-betaIII TUBULIN (Abcam, ab18207).

Immunocytochemistry and image analysis

For immunocytochemistry, mouse primary neocortical neurons were fixed with 4% PFA in 1X PBS for 15min. Cells were then permeabilized with ice-cold methanol for 10min and blocked (5% donkey serum, 0.03% Triton X-100 in 1X PBS) for 1hr at room temperature. Primary antibody incubation was performed overnight at 4°C in a humidified chamber. Secondary antibody incubation was then performed for 1hr at room temperature. The following antibodies were used: anti-phosphoERK (Cell signaling, 4370S), anti-MAP2 (Synaptic systems, 188004), Cy5-conjugated donkey anti-guinea pig (Jackson, 706-715-148) and Cy3-conjugated donkey anti-rabbit (Jackson, 711-165-152). Imaging was performed on a widefield microscope (FEI MORE) with a 40X objective (NA 0.95, air). Image analysis was performed on Fiji (Schindelin et al., 2012). Briefly, a mask for MAP2 signal was created and neuronal cell bodies were manually delimited. The mean of phospho-ERK signal intensity for each neuronal cell body was then measured.

RNA isolation and reverse transcription

Cells were lysed using Trizol reagent (Sigma, T9424). Total RNAs were isolated and DNase treated on columns (RNeasy micro kit, Qiagen, 74004) following the manufacturer's instructions. The cDNA libraries were built using between 100 and 200ng RNA reverse transcribed with SuperScript III reverse transcriptase (Thermo Fisher, 18080044), dNTPs (Sigma, D7295) and oligo$(dT)_{15}$ primer (Promega, C1101).

PCR

Semi-quantitative PCR was performed using Phusion High-Fidelity DNA Polymerase (New England Biolabs, M0530L) and revealed with GelRed (Biotium, 41003). For each PCR, the number of cycles necessary to end the amplification in its exponential phase was determined. In the case of long introns (>1,000–1,500 bp), multiplex PCRs were performed to amplify both the intron-retaining and the spliced transcript isoforms (i.e., using three

primers: one primer complementary to an internal region of the intron in addition to the primers mapping to each flanking exon).

Real-time quantitative PCRs were performed with FastStart Universal SYBR GreenMaster (Roche, 04-913-850-001). PCRs were carried out in a StepOnePlus qPCR system (Applied Biosystems) with the following thermal profile: 10 min at 95°C, 40 cycles of 15s at 95°C and 1 min at 60°C. Real-time quantitative PCR assays were analyzed with the StepOne software. The primers used for PCRs are listed in Table 2.

Deep RNA-sequencing

RNA samples were quality-checked on the TapeStation instrument (Agilent Technologies) using the RNA ScreenTape (Agilent, 5067-5576) and quantified by Fluorometry using the QuantiFluor RNA System (Promega, E3310). Library preparation was performed, starting from 200ng total RNAs, using the TruSeq Stranded mRNA Library Kit (Illumina, 20020595) and the TruSeq RNA UD Indexes (Illumina, 20022371). 15 cycles of PCR were performed. Libraries were quality-checked on the Fragment Analyzer (Advanced Analytical) using the Standard Sensitivity NGS Fragment Analysis Kit (Advanced Analytical, DNF-473) revealing an excellent quality of libraries (average concentration was 158±20 nmol/L and average library size was 351±6 base pairs). Samples were pooled to equal molarity. The pool was quantified by Fluorometry using the QuantiFluor ONE dsDNA System (Promega, E4871). Libraries were sequenced Paired-End 101 bases using the HiSeq 2500 or NovaSeq 6000 instrument (Illumina) and the S2 Flow-Cell loaded. Details on the number of sequenced reads for each sample are given in Table 1. Quality control of read sequences was performed in collaboration with the company GenoSplice technology (http://www.genosplice.com). Confidence score per base and per sequence, GC content, sequence length, adapter content and presence of overrepresented sequences was assessed using FAST-QC.

RNA-sequencing read alignment

For the RNA-sequencing data analysis, reads were aligned onto the mouse genome assembly mm10 using the STAR aligner version 2.4.0f1 (Dobin et al., 2013). The following parameters were used: –outFilterMismatchNmax 2 and –outFilterMultimapNmax 1 to filter out reads that have more than two mismatches and reads that map to multiple loci in the genome. Moreover, reads with <8 nt overhang for the splice junction on both sides were filtered out using –outSJFilterOverhangMin 30 8 8 8. Then, read alignment files (bam) were

processed with custom Perl scripts using the library Bio::DB::Sam. For each segment comprising a pair of consecutive exons (exon1 and exon2) and the intermediate intron, reads that mapped (1) exon1-intron junctions, (2) intron-exon2 junctions (3), exon1-exon2 junctions and (4) introns were counted. Note that the data discussed in this manuscript will be deposited in NCBI's GeneExpression Omnibus (Edgar et al., 2002) before publication.

PIR, intron-retaining isoform expression and spliced isoform expression

For each segment in the mouse genome, comprised of a pair of consecutive exons and the intervening intron annotated in FastDB (http://www.easana.com) (de la Grange et al., 2005), we assessed the expression level of transcripts retaining the intron (IR-isoforms) as the average number of reads mapping the 5' and 3' exon-intron junctions (= mean(cov(E1I), cov(IE2)). The expression level of the spliced isoform was estimated as the number of exon-exon junction reads (= cov(E1E2)). Given that coverage (cov) is by definition normalized by the length of the analyzed sequence, cov is corresponding to the absolute number of reads mapping a junction. All the read coverage values were normalized by the number of total mapped reads for each individual sample. Segments were considered as non-expressed and filtered out if (cov(E1E2) + mean(cov(E1I), cov(IE2)) < 10 in at least one sample. Note that, for the cell fractionation samples, the segment expression filter only applied to WCE condition. Also, segments whose spliced isoforms was not significantly expressed in any of the compared samples were filtered out: (cov(E1E2) < 10). Again, for cell fractionation samples, this filter only applied to WCE condition. The PIR was then calculated as the expression level of the IR-isoforms over the sum of the expression levels of the IR-isoforms and the spliced isoforms (PIR = mean(cov(E1I), cov(IE2))/(cov(E1E2) + mean(cov(E1I), cov(IE2))). We defined introns as retained if their PIR exceeded 20 and if they fulfilled the following criterion: the read coverage along the entire intron must represent at least 20% of the sum of the expression of the spliced and IR-isoforms (minPIR = (min(cov(E1I), cov(IE2), cov(I))/(cov(E1E2) + mean(cov(E1I), cov(IE2); min = minimum). In this way, we ensured that reads mapping the exon-intron junctions were indeed due to IR rather than other events, such as the usage of alternative 5' or 3' splice sites.

Analysis of intron-retaining transcript subcellular localization and spike-in RNAs

Because the quantity of RNAs in the "Nucleus" and the "Cytosol" fractions are not equal, the real enrichment of a given transcript in the nucleus *versus* the cytosol is lost during the

RNA sequencing library preparation. To overcome this hurdle, we added a defined amount of spike-in RNAs SIR-Set3 (Lexogen, Iso Mix E0/ERCC #051) in each fraction arising from a constant number cells before adjusting the RNA quantities during library preparation. Note that we put twice less spike-in RNAs for the nuclear samples. For our analysis, we only considered ERCC that are covered by >10 reads in every sample (18 ERCCs). We then calculated the cytosol-to-nucleus abundance ratio of each ERCCs. A correction factor was then assessed as the mean of cytosol-to-nucleus ratio for all ERCCs (1.03 ±0.07). Given that we introduce twice less spike-ins in the nuclear samples, the final correction factor used in this study is 0.515 (=1.03/2). We applied this factor to the RNA sequencing data to calculate a true nucleus-to-cytosol enrichment. IR-transcripts were considered enriched i) in the nucleus if (mean IR-isoform $expression_{nucleus}$ / mean IR-isoform $expression_{cytosol} \geq 2$) or ii) in the cytosol if (mean IR-isoform $expression_{nucleus}$ / mean IR-isoform $expression_{cytosol} \leq 0.5$). For the evaluation of splice site strength, maximum entropy scores for 9-bp 5' splice sites and 20-bp 3' splice sites were calculated using MaxEntScan (Yeo and Burge, 2004).

Analysis of stimulation-dependent intron-retaining transcripts

Intron-retaining transcripts were considered as regulated if the following criteria applied: (1) the fold change mean of IR-transcript expression (FC IR expression) ≥ 20%, and (2) |z-score| of IR-transcript expression ($|z\text{-score}|_{IR\ expression}$) ≥ 1.5, where z-score = ((mean IR $expression_{stimulated}$ – mean IR $expression_{unstimulated}$)/sqrt(paired variance IR expression)) and paired variance IR expression = ((variance IR $expression_{unstimulated}$ + variance IR $expression_{stimulated}$)-(2 x covariance IR expression)).

For all regulated intron-retaining transcripts, the stimulation-specificity was defined as follow: commonly regulated IRs: (1) FC IR expression ≥ 20% upon both bicuculline and BDNF stimulations, (2) IRs regulated in the same direction upon both bicuculline and BDNF stimulations, (3) $|z\text{-score}|_{IR\ expression} \geq 1.5$ upon both bicuculline and BDNF stimulations; differentially regulated IRs: (1) FC IR expression ≥ 20% upon both bicuculline and BDNF stimulations, (2) IRs regulated in opposite directions upon bicuculline and BDNF stimulations, (3) $|z\text{-score}|_{IR\ expression} \geq 1.5$ upon both bicuculline and BDNF stimulations; bicuculline-specific IRs: (1) FC IR expression ≥ 20% upon bicuculline stimulation, (2) $|z\text{-score}|_{IR\ expression} \geq 1.5$ upon bicuculline stimulation, (3) FC IR expression ≤ 5% upon BDNF stimulation; BDNF-specific IRs: (1) FC IR expression ≥ 20% upon BDNF stimulation, (2) $|z\text{-score}|_{IR\ expression} \geq 1.5$ upon BDNF stimulation, (3) FC IR expression ≤ 5% upon bicuculline stimulation. For regulated intron-retaining transcripts that do not belong

to one of these groups, we considered we could not confidently determine their stimulation-specificity.

To probe by which RNA process IR-transcripts are regulated, we used the following criteria for the spliced isoform: splicing: (1) fold change mean of spliced isoform expression (FC SI expression) ≥ 20%, (2) $|\text{z-score}|_{\text{SI expression}} \geq 1.5$, where z-score = ((mean SI $\text{expression}_{\text{stimulated}}$ – mean SI $\text{expression}_{\text{unstimulated}}$)/sqrt(paired variance SI expression)) and paired variance SI expression = ((variance SI $\text{expression}_{\text{unstimulated}}$ + variance SI $\text{expression}_{\text{stimulated}}$)-(2 x covariance SI expression)), (3) SI regulated in opposite directions compared to IR-isoforms; degradation/stabilization: (1) FC SI expression ≤ 5%, (2) $|\text{z-score}|_{\text{SI expression}} \leq 1$. For regulated intron-retaining transcripts that do not belong to one of these categories, we considered we could not confidently determine their type of regulation.

Targeted mass spectrometry assay

Mouse primary neocortical neurons were treated for 2 hours with DRB (50µM) and for the indicated time with bicuculline (20µM). 400000 cells were collected and lysed in 100 µl lysis buffer (1% sodium deoxycholate (SDC), 0.1 M TRIS, 10 mM TCEP, pH = 8.5) using strong ultra-sonication (10 cycles, Bioruptor, Diagnode). Protein concentration was determined by BCA assay (Thermo Fisher Scientific) using a small sample aliquot. Sample aliquots containing 50 µg of total proteins were reduced for 10 min at 95 °C and alkylated at 15 mM chloroacetamide for 30 min at 37 °C. Proteins were digested by incubation with sequencing-grade modified trypsin (1/50, w/w; Promega, Madison, Wisconsin) overnight at 37°C. Peptides were then cleaned up using iST cartridges (PreOmics, Munich) according to the manufacturer's instructions. To each peptide samples an aliquot of a heavy reference peptide mix containing chemically synthesized proteotypic peptides (Spike-Tides, JPT, Berlin, Germany) was spiked into each sample at a concentration of 4 fmol of heavy reference peptides per 1µg of total endogenous protein mass. Samples were dried under vacuum and stored at -80 °C until further use.

Chromatographic separation of peptides was carried out using an EASY nano-LC 1000 system (Thermo Fisher Scientific), equipped with a heated RP-HPLC column (75 µm x 30 cm) packed in-house with 1.9 µm C18 resin (Reprosil-AQ Pur, Dr. Maisch). Peptides were analysed per LC-MS/MS run using a linear gradient ranging from 95% solvent A (0.1% formic acid in water (v/v)) and 5% solvent B (80% acetonitrile, 19.9% water, 0.1% formic acid (v/v/v)) to 45% solvent B over 60 minutes at a flow rate of 200 nL*min-1. Mass spectrometry analysis was performed on Orbitrap Fusion Lumos mass

spectrometer (Thermo Fisher Scientific) operated with a default method for SureQuant acqusition.

For the quantitative analysis, the raw files were imported and processed using the Spectrodive software with the SureQuant default settings. For each target peptide, the total peak areas of the most intense transitions were exported. Only peptides with the Elution Group Q.Value <0.01 were considered for quantitative analysis. To control for variation in sample amounts, the total ion chromatogram (only comprising of peptide ions with two or more charges) of each sample was determined by Progenensis QI (version 2.0, Waters) and used for normalization. Protein level was assessed as the mean of at least 2 peptides for a target protein.

5. Appendix

Index of figures

Introduction

Results

Discussion and future directions

Index of abbreviations

3'SS = 3' splice site

3'UTR = 3' untranslated region

5'SS = 5' splice site

5'UTR = 5' untranslated region

ADAR = Adenosine deaminases acting on RNA

AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AP-1 = Activator protein 1

Arc = Activity-regulated cytoskeleton-associated protein

ARNT = Aryl Hydrocarbon Receptor Nuclear Translocator

AS = Alternative splicing

AS4 = Alternative segment 4

ASO = Antisense oligo nucleotide

BDNF = Brain-derived neurotrophic factor

CA1 = Cornu ammonis 1

Calm3 = Calmodulin 3

CaMK = Ca^{2+}/calmodulin-dependent protein kinase

cAMP = Cyclic adenosine monophosphate

CaRE = Calcium response element

CaRRE = CaMK IV-responsive RNA element

Cas = CRISPR-associated protein

CBP = CREB-binding protein

CCK = Cholecystokinine

CLK = Cdc2-like kinase

CopII = Coat protein complex II

Cpg15 = Candidate plasticity gene 15

CREB = cAMP response element-binding protein

CRISPR = Clustered Regularly Interspaced Short Palindromic Repeats

CTN-RNA = Cationic amino acid transporter 2 RNA

Dis3 = DIS3 Homolog, exosome endoribonuclease and 3'-5' exoribonuclease

DMD = Duchenne muscular dystrophy

DNA = Deoxyribonucleic acid

DSCAM = Down syndrome cell adhesion molecule

Egr1 = Early Growth Response 1

EJC = Exon junction complex

Elk-1 = ETS Like-1 protein

EPSC = Excitatory post-synaptic current

ER = Endoplasmic reticulum

EU = 5-ethynyluridine

Exosc9 = Exosome Component 9

FISH = Fluorescence in situ hybridization

FKBP = FK506 binding protein

FRB = FKBP-rapamycin binding domain

GABA = γ-aminobutyric acid

gRNA = guide RNA

hnRNP = Heterogeneous nuclear ribonucleoproteins

IEG = Immediate-early gene

IR = Intron retention

intron-retaining transcript = Intron-retaining transcripts

Kcnma1 = Potassium Calcium-Activated Channel Subfamily M Alpha 1

lncRNA = Long non coding RNA

LRG = Late response gene

LTD = Long-term depression

L-VGCC = L-type voltage-gated calcium channel

MAPK = Mitogen-activated protein kinase

Mat2a = Methionine adenosyltransferase 2A

MeCP2 = methyl CpG binding protein 2

MEF2 = Myocyte enhancer factor-2

miRNA = micro RNA

mRNA = Messenger ribonucleic acid

MTR4 = Exosome RNA Helicase

Ncam1 = Neural Cell Adhesion Molecule 1

NMD = Non sense-mediated decay

NMDA = N-methyl-D-aspartate

Npas4 = Neuronal PAS domain protein 4

NT-3 = Neurotrophin 3

NT-4 = Neurotrophin 4

O-GlcNAc = O-linked β-N-acetylglucosamine

Ogt = O-linked N-acetylglucosamine transferase

PCB = Phycocyanobilin

PhyB = Phytochrome B

PIF = Phytochrome interacting factor

PIR = Percentage of intron retention

PLC = Phospholipase C

Pre-mRNA = Precursor messenger RNA

PTBP = Polypyrimidine Tract Binding Protein

PTC = Premature termination codon

PV = Parvalbumin

RBFOX1 = RNA Binding Fox-1 Homolog 1

RBP = RNA-binding protein

RNA = Ribonucleic acid

ROBO = Roundabout guidance receptor

RPL22 = Ribosomal Protein L22

RRM = RNA recognition motif

SAM68 = SRC associated in mitosis of 68 kDa

Sams-3 = S-adenosylmethionine synthase 3

Scg2 = Secretogranin II

SHANK = SH3 and multiple ankyrin repeat domain

shRNA = Small hairpin RNA

siRNA = Small interfering RNA

SMA = Spinal muscular atrophy

SMN = Survival of motor neuron

snRNP = Small nuclear ribonucleoproteins

SR protein = Serine/arginine-rich protein

SRF = Serum response factor

SRPK = Serine/arginine-protein kinase

STREX = Stress-Axis Regulated Exon

TERT = Telomerase reverse transcriptase

TF = Transcription factor

TrK = Tropomyosin receptor kinase

TSS = Transcription start site

TUG1 = Taurine Up-Regulated 1

U2AF = U2 auxiliary factor

Xist = X Inactive Specific Transcript

6. References

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