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Regulation of Gene Expression at the Neuromuscular Junction

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Abstract

Gene expression in skeletal muscle is profoundly changed upon innervation. 50 years of research on the neuromuscular system have greatly increased our understanding of the mechanisms underlying these changes. By controlling the expression and the activity of key transcription factors, nerve-evoked electrical activity in the muscle fiber positively and negatively regulates the expression of hundreds of genes. Innervation also compartmentalizes gene expression into synaptic and extra-synaptic regions of muscle fibers. In addition, electrically-evoked, release of several factors (e.g. Agrin, Neuregulin, Wnt ligands) induce the clustering of synaptic proteins and of a few muscle nuclei. The sub-synaptic nuclei acquire a particular chromatin organization and develop a specific gene expression program dedicated to building and maintaining a functional neuromuscular synapse.

Deciphering synapse-specific, transcriptional regulation started with the identification of the N-box, a six base pair element present in the promoters of the acetylcholine δ and ϵ subunits. Most genes with synapse-specific expression turned out to contain at least one N-box in their promoters. The N-box is a response element for the synaptic signals Agrin and Neuregulins as well as a binding site for transcription factors of the Ets family. The Ets transcription factors GABP and Erm are implicated in the activation of post-synaptic genes via the N-box. In muscle fibers, Erm expression is restricted to the NMJ whereas GABP is expressed in all muscle nuclei but phosphorylated and activated by the JNK and ERK signalling pathways in response to Agrin and Neuregulins. Post-synaptic gene expression also correlates with chromatin modifications at the genomic level as evidenced by the strong enrichment of decondensed chromatin and acetylated histones in sub-synaptic nuclei. Here we discuss these transcriptional pathways for synaptic specialization at NMJs.

Keywords

Neuromuscular Junction, synapse, compartmentalization of gene expression, Ets transcription factors, N-box, chromatin.

1. Introduction

Skeletal muscles are attached to bones by tendons and their primary function is to provide movement to the skeleton. This is achieved by the contraction of terminally differentiated muscle cells called muscle fibers. Muscle fibers are multinucleated cells resulting from the fusion of mononucleated progenitors. Muscle fibers typically contain several hundreds of nuclei that share the same cytoplasm. Their contraction is initiated by motor innervation through a chemical, cholinergic synapse formed between the extremity of a motor neuron and the central area of a muscle fiber. Action potentials in motor axons trigger the release of acetylcholine by nerve terminals. Acetylcholine released in the synaptic cleft binds acetylcholine receptors (AChRs) present in the post-synaptic muscle membrane. The AChR is a heteropentameric, ligand-gated, ion channel that allows entry of cations in the muscle fiber upon acetylcholine binding. Sodium entry at the neuromuscular junction (NMJ) through the AChR creates a local depolarization triggering a muscle action potential that will ultimately induce muscle contraction via a calcium-dependent excitation-contraction coupling [1,2].

Myasthenia is the typical disease of NMJ dysfunction. Most congenital forms of myasthenia are caused by mutation that affect AChR function. However, to function correctly, neurotransmission at the NMJ also requires sufficient expression levels of AChR genes. Insufficient AChR expression at the NMJ reduces the efficiency of neurotransmission and causes myasthenic syndromes [3,4].

To achieve sufficient AChR density at the NMJ, two mechanisms are involved. The first is post-translational and results from clustering and stabilization in the sarcolemma of AChR molecules at the NMJ via a complex post-synaptic protein scaffold [5]. The second results from the specialization of (a few) sub-synaptic nuclei (Fig. 1) to allow expression of genes coding for NMJ components (so called synaptic genes) including AChR subunits [6]. Interestingly these mechanisms, which were initially thought to act via distinct pathways, have been shown by gene inactivation approaches in mice, to converge on the Agrin/LRP4/MuSK pathway [7,8].

2. Extracellular proteins and membrane receptors involved in synaptic gene activation

2.1. *Agrin*

Agrin is a heparan sulfate proteoglycan with a protein core of ~400kDa that is decorated with carbohydrate side chains. Agrin was first biochemically purified from the basal lamina of the electric organ of *Torpedo californica* by assaying fractionated proteins for AChR aggregation in cultured myotubes [9]. Different alternatively-spliced isoforms of Agrin are produced by muscles or motoneurons and only the isoform produced by motoneurons efficiently induces AChR clustering [10,11]. The generation of mice with a targeted deletion of the *Agrin* gene

helped demonstrate that Agrin was not only required for AChR clustering but also to activate synaptic gene expression in sub-synaptic nuclei [12–14]. Like AChRs, the muscle-specific receptor tyrosine kinase (MuSK) is found at a high density in the sub-synaptic membrane; a clue to its synaptic function. Both Agrin and MuSK KO mice die perinatally of their inability to breathe due to the absence of neuromuscular junctions [15–16]. The phenotype of MuSK knock-out (KO) mice was so reminiscent of the phenotype of Agrin KO mice, that MuSK was proposed to be the muscle receptor for Agrin. Though MuSK was shown to be required for Agrin-dependent AChR clustering [15,16], no direct interaction between Agrin and MuSK could be demonstrated. The explanation came from the identification of LRP4, a member of the LDLR family (Low-density lipoprotein receptor), as the actual Agrin binding protein that associates with MuSK inducing its phosphorylation subsequent to binding Agrin [17–19]. Ectopic expression of Agrin or of a constitutively active mutant of MuSK, in extra-synaptic regions of muscle fibers, is sufficient to induce the formation of post-synaptic-like structures with underlying nuclei similar to sub-synaptic nuclei [20–22]. Altogether, Agrin secreted by the nerve terminal binds LRP4 in the post-synaptic muscle membrane to activate the tyrosine kinase MuSK. MuSK activation shapes the post-synaptic domain by clustering and stabilizing synaptic proteins via the recruitment Rapsyn, Dystroglycan and Utrophin among other proteins, as well as by activating intracellular signaling cascades that induce the formation of sub-synaptic nuclei expressing synaptic genes (Fig. 2).

2.2. Neuregulins

Neuregulins (NRGs) were first called ARIA (Acetylcholine Receptor Inducing Activity) and were identified as trophic factors purified from chicken brain that stimulated AChR expression in cultured myotubes [23]. There are 4 *Neuregulin* genes (*Neuregulin-1* to *-4*) that are expressed largely in the nervous system. ARIA or Neuregulin-1 (also known as Heregulin β 1, GGF or NDF) is encoded by the *Neuregulin-1* gene that codes for 15 alternatively-spliced isoforms [24]. Neuregulin-1 (Nrg1) is produced by a variety of cells including motoneurons and muscle cells [25,26]. Neuregulin-2 is produced by terminal Schwann cells and motoneurons [27]. Neuregulin-3 and Neuregulin-4 have less structural similarity with Neuregulin-1 than Neuregulin-2 and their role on muscle remain to be characterized.

Neuregulins signal via the activation of their receptor tyrosine kinases ErbB2, ErbB3, and ErbB4, which are present in the post-synaptic membrane of NMJs and in terminal Schwann cells [27,28]. The precise contribution of Neuregulins and their receptors to the activation of transcription in sub-synaptic nuclei has been hampered by the diversity and probable redundancy of Neuregulin receptors and ligands present in the microenvironment of the NMJ.

Because of their synaptic localization and their ability to activate the transcription of synaptic genes in cultured myotubes, Neuregulins (NRGs) and their receptors were initially proposed to be involved in the activation of synaptic genes transcription (for reviews see [6,24]). Neither Nrg1, nor ErbB2 or ErbB4 KO mice allowed to demonstrate the importance of these factors due to embryonic lethality [29–31]. Nrg1 KO heterozygous mice showed a 50% reduction in post-synaptic AChR density and exhibited a myasthenic phenotype. This finding strongly supported the idea that Nrg1 was involved in the activation of *AChR* genes expression in sub-synaptic nuclei [25].

The finding that Neuregulins and their ErbB2 and ErbB3 receptors were crucial for Schwann cells survival and therefore for NMJ formation and maintenance, further complexified the interpretation of the initial gene inactivation approaches [32–34].

To circumvent these problems conditional KO mice were produced to specifically inactivate Neuregulins receptors in skeletal muscle. Surprisingly, muscle fiber specific inactivation of ErbB receptors with a catalytic domain (i.e. ErbB2 and ErbB4, ErbB3 being deprived of kinase activity) did not perturb NMJ formation and maintenance, indicating that Neuregulin signaling in muscle fibers was dispensable [35]. Later on, ErbB2/ErbB4 muscle-specific KO mice were followed postnatally and shown to exhibit several structural and functional defects at the NMJ involving defects of phosphorylation of α -dystrobrevin 1 [36].

Of note, MuSK was shown to be able to activate the MAP kinases pathway that stimulates AChR expression in response to Neuregulins [37]. Therefore, the possibility remains that forcing NMJ to form in the absence of Neuregulin signaling induces a compensatory mechanism mediated by MuSK. Other studies propose that Neuregulins act as second messengers of Agrin that would activate the expression of the *AChR ϵ* subunit in cultured myotubes in an ErbB2-dependent manner [38,39]. In addition, AChR clusters induced by Agrin or by forced expression of constitutively active MuSK also contain ErbB 2 and 3 receptors, indicating that ErbB receptor accumulation in the post-synaptic membrane is initiated by the activation of MuSK by Agrin [40].

2.3. *Wnts*

MuSK contains a cysteine rich domain reminiscent of Frizzled family of the Wnt receptors. Indeed, Wnt factors were shown to bind MuSK and to be involved in NMJ specification in zebrafish [41]. Since then, there have been several reports describing a role for Wnt factors at mammalian NMJs [42–44] ; or for a review see [38–40] where Wnt factors have emerged as regulators of MuSK activity. Wnt9a, Wnt9b, Wnt10b, Wnt11, Wnt16 and Wnt4 enhance AChR

clustering in a dose-dependent manner, even in the absence of Agrin [42–44]. In addition, Wnt4 and Wnt11 were shown to activate the expression of the *AChR* subunits in cultured myotubes [48]. Wnt4, Wnt9a and Wnt11 directly bind to the extracellular domain of MuSK to induce MuSK dimerization and activation. Moreover, deletion of the Frizzled-like domain of MuSK alters NMJ development and maintenance [43,44]. Altogether, these results suggest that Wnt factors participate to the regulation of AChR genes expression. However, another study has shown that Wnt4 and Wnt11 were not required for synapse formation in mice, and that the Frizzled-like domain of MuSK was dispensable for synapse formation and function in mice [49]. Differences in genetic background of the mouse models could explain these discrepancies [8,48].

Finally, Wnt3a, Wnt7a and Wnt8a have been shown to antagonize Agrin-induced AChR clustering [46], indicating that different Wnt factors may have different roles at the NMJ.

As for Neuregulins, the diversity of Wnt ligands and receptors, their redundancy and the numerous biological processes they regulate, complexifies the study of their specific role at the NMJ and further studies will be needed to precisely understand their synaptic function(s).

3. Signaling pathways involved in synaptic activation of transcription

3.1. Neuregulins and ErbB pathways

The binding of Neuregulins to ErbB monomers (ErbB2 and ErbB4) activates homodimers or heterodimers (ErbB2/ErbB3, ErbB2/ErbB4; ErbB3/ErbB4, ErbB4/Erb4) and induces a cascade of intracellular signals that activate synaptic gene expression [50,51]. In cultured myotubes as well as *in vivo*, ErbB phosphorylation activates the MAP kinases ERK (Extracellular-signal Regulated Kinase) and c-Jun N-terminal kinase (JNK) [52–55]. The dissection of these pathways with specific dominant negative mutants, constitutively-active mutants and small molecule inhibitors showed that the MAP kinase pathway is essential for the Neuregulin-induced activation of *AChR* ϵ subunit expression that is key to formation of a mature AChR [52–55]. Neuregulins also activate the PI3K pathway and promote Akt phosphorylation [52,56] but there have been conflicting results on the participation of this pathway in synaptic gene expression [52,53,55].

The cyclin-dependent kinases 5 (Cdk5) was reported to mediate Neuregulin signaling at the NMJ. ErbB receptors bind the Cdk5/p35 complex, which is involved in ErbB2/3 phosphorylation and kinase activity [56–58]. In C2C12 myotubes, Cdk5 participates in the activation of *AChR* gene expression by Neuregulins [56], while *in vivo* Cdk5 regulates AChR clustering [57,58]. Indeed, in Cdk5 cKO mice *AChR* subunits transcription was unchanged although ErbB

receptor activation was reduced. A compensatory mechanism was proposed to take place *in vivo* in the absence of Cdk5, which could involve redundancy with the Agrin/MuSK pathway.

3.2. The Agrin / LRP4 / MuSK pathway

MuSK/LRP4 activation by Agrin triggers several kinases (e.g Src, Crk, Abl, CK2 or Pak1) and intracellular signaling pathways (for review [8]). Most of them are dedicated to post-translational organization of the post-synaptic domain but some have been shown to regulate synaptic gene expression. In particular, MuSK was shown to activate the JNK kinase and MKK7 mutants were shown to prevent Agrin-induced gene expression [37]. Two ways, both downstream of Agrin and MuSK, might therefore co-exist at the NMJ to activate the expression of synaptic genes via JNK. One pathway directly downstream of MuSK via MKK7 and a second, indirect, as a result of the clustering of ErbB receptors in the post-synaptic membrane in response to MuSK activation by Agrin. MuSK also activates the serine/threonine kinase Pak1 (p21-Activated Kinase) [59]. In addition to its action on Actin and its participation in Wnt signaling, Pak1 was shown to participate in the re-expression of AChR subunits in extra-synaptic nuclei in response to denervation via the exclusion of the transcriptional repressor CtBP from the nucleus [60]. It would be interesting to determine if Pak1 also neutralizes transcriptional corepressors at the NMJ.

3.3. Wnt signaling

Wnt factors can activate several pathways including the canonical signaling pathway Wnt/ β -catenin and two non-canonical pathways, the planar cell polarity (PCP) and the Wnt/Ca²⁺ pathway [61]. Dvl1 is a central effector of Wnt signaling and interacts with MuSK to regulate AChR clustering [59,62]. The presence of APC and β -catenin at the NMJ further indicates that the Wnt canonical pathway is active at the NMJ [63,64]. Indeed, NMJs are perturbed in muscle specific β -catenin KO and β -catenin action requires its transcriptional domain but not its cell adhesion domain. The main defects of β -catenin inactivation in muscle being pre-synaptic, β -catenin was proposed to regulate the expression of a retrograde signal. Consistently, β -catenin was shown to regulate the expression of Slit2 [65] ; and reference therein. Another argument in favour of the involvement of the canonical Wnt pathway in regulating gene expression at the NMJ came from the observation that NMJ defects caused by the deletion of the Wnt-binding

domain of MuSK could be compensated by lithium chloride, an inhibitor of GSK3 that activates the Wnt canonical pathway by preventing β -catenin degradation [44].

3.4. Hippo pathway

Recent studies indicate a role at NMJs of the Hippo pathway and of the transcriptional co-activator YAP. The Hippo pathway negatively regulates the transcriptional co-activators YAP and TAZ. Active YAP/TAZ translocates to the nucleus and interacts with TEAD transcription factors to increase transcription [66,67]. Agrin was recently shown to inhibit the Hippo pathway through LRP4, MuSK and Pak1 and thus promote transcriptional activation by YAP [68]. Moreover, the conditional knockout of YAP resulted in disrupted NMJ integrity [69]. Interestingly, there is crosstalk between the Hippo and Wnt pathways and stimulating the Wnt/ β Catenin pathway improves the phenotype of YAP conditional KO [69]. Together, these studies suggest that mechanical inputs at the NMJ could influence gene expression in sub-synaptic nuclei. It will be interesting to further characterize the nature of the mechanical signals and the transcriptional targets of this pathway at the NMJ.

4. Transcription factors and regulatory elements

Two classes of transcription factors play key roles in the regulation of gene expression during muscle development: MRFs (Myogenic Regulatory Factors) and MEF2 (Myocyte Enhancer Factor 2) family members. MRFs and MEF2, bind respectively to E-boxes or MEF2 binding sites in the promoters of muscle genes. Many synaptic genes contain E-boxes in their promoters and are activated by MRFs. This is the case for the 4 subunits of the embryonic form of the acetylcholine receptor (α , β , γ , δ).

The current data suggest the following scenario. Once innervated, acetylcholine released by the nerve terminal binds the acetylcholine receptor, thus triggering ion entry in the post-synaptic zone. This local depolarization initiates electrical activity and excitation/contraction coupling in muscle fibers. Electrical activity strongly affects gene expression in muscle, mainly by repressing the expression of the MRFs, leading to the loss of *AChR* mRNA subunits expression in muscle nuclei (Fig. 2). Loss of MRF expression in innervated muscle fibers involves both calcium influx through L-type channels and release from the sarcoplasmic reticulum [70,71]. These calcium fluxes activate, protein kinase C (PKC) and PKB/Akt kinases which in turn modulate the expression and localization of the transcriptional repressors Dach2, HDAC4 and HDAC9 [72–76].

Altogether, acetylcholine release at the NMJ leads to the loss of the expression of its receptor in extra-synaptic nuclei. Consequently, after innervation only a few nuclei located directly beneath the nerve terminal strongly express the adult form of the AChR (composed of α , β , δ , ϵ AChR subunits) [77,78]. Expression of AChR subunits in these nuclei do not rely on myogenic factors and do not involve E-boxes but rather relies on the specific sub-synaptic mechanisms described in the following sections. This mosaicism of expression by muscle nuclei contributes to the sustained expression of AChRs at the synapse. In contrast, loss of innervation in adult muscle alleviates the repression of gene expression by electrical activity and thus induces the re-expression of MRFs, who in turn activate the expression of embryonic AChR along muscle fibers. This phenomenon is responsible for the hypersensitivity to acetylcholine of denervated muscles [6].

4.1. The N-box: a neural response element

Mechanisms for compartmentalized expression in muscle fibers have been extensively investigated for *AChR* subunits genes. *In vivo* DNA injection of reporter genes placed under the control of wild type (WT) or mutated promoters of the *AChR* δ and ϵ subunits, allowed the identification of a 6 base pair element required to target gene expression to the sub-synaptic area. This element defined by the sequence CCGGAA was named the “N-box” [79,80]. The importance of the N-box for synaptic expression was further confirmed in a knock-in transgenic mouse model with a reporter gene controlled by the promoter of the *AChR* δ subunit [81]. This mouse model also demonstrated that the N-box was not required for the up-regulation of the transgene expression after denervation, indicating that the N-box was not involved in the control of muscle gene expression by electrical activity [81]. The definitive proof of the importance of the N-box for *AChR* expression came from teams studying the physiopathology of genetic disorders of the NMJ: G to A mutations were identified in the N-box of the *AChR* ϵ promoter of several patients with congenital myasthenic syndromes [82–84].

The subsequent demonstration of the requirement of the N-box for the synaptic expression of other synaptic genes (*MuSK*, *Utrophin*, *Acetylcholine esterase*) indicated that the N-box was present in the promoters of many synaptic genes to activate their transcription in sub-synaptic nuclei [37,85–87].

4.2. Transcription factors involved in activation of sub-synaptic transcription via the N-box

The sequence of the N-box corresponds to a binding site for transcription factors of the Ets family. The N-box binding activity present in extracts of cultured myotubes and mouse muscles turned out to be the Ets transcription factor GABP (GA binding Protein), also known as NRF2 or E4TF1. GABP is a heterodimer composed of two polypeptides of 58 and 43 kDa, corresponding to the GABP α and GABP β subunits, respectively [81,86,88]. GABP α contains the conserved Ets DNA-binding domain while GABP β has no DNA-binding activity but reinforces DNA binding of the complex and contains the transcriptional activation domain required for GABP activity. Three forms of GABP β exist, two alternatively spliced forms encoded by the *Gabp β 1* (GABP β 1L and GABP β 1S), and GABP β 2 encoded by the *Gabp β 2* genes. GABP β 2 and the long form of GABP β 1 (GABP β 1L) contain a leucine zipper that allows the formation of α 2 β 2 tetramers (for a review see [89]). Dominant negative mutants of GABP α or GABP β were shown to block N-box-mediated transcriptional activation in cultured myotubes [88]. Dominant negative mutants of transcription factors are often obtained by removal of the activation domain to generate inactive mutants that can still bind DNA. When overexpressed, such mutants can theoretically prevent the binding of other transcription factors that recognize the same sequence by occupying their binding site. Since GABP β does not bind DNA, the GABP β mutant that was used did not fall in this category. Rather, it deprived GABP of its transcriptional activation domain without blocking its binding site. The same GABP β mutant was used *in vivo* to show that GABP was required for the activation of the expression of the *AChR ϵ* subunit gene by Agrin. The GABP β mutant was also shown to inhibit the formation of Agrin-induced AChR clusters in muscle fibers as well as the accumulation at these clusters of Acetylcholinesterase and membrane cytoskeletal protein, Utrophin [81,90].

The fact that GABP is expressed all along muscle fibers raised the question of how it could precisely activate the transcription of synaptic genes at the NMJ. The activity of Ets transcription factors is often regulated by phosphorylation. Accordingly, in cultured myotubes GABP is phosphorylated and activated by JNK in response to Agrin [37] and by ERK and JNK in response to Neuregulins, and both GABP subunits are phosphorylated [81,88,91,92] (Fig. 2).

Interestingly, GABP activity is negatively regulated by the Hippo pathway, and Hippo pathway inhibition allows GABP to activate the expression of YAP [93]. Since Agrin inhibits the Hippo pathway and since YAP is involved in NMJ integrity and is linked to Wnt signaling [68,69], it is tempting to imagine coordination between the MAPK, Wnt and Hippo pathways allowing concerted action of GABP, β Catenin and YAP transcription factors at the NMJ. GABP can also

cooperate with NFAT and the coactivator PGC-1 α to activate the expression of synaptic genes. This was initially demonstrated for Utrophin [94]. The cooperation between GABP and PGC-1 α was further shown to participate in the activation of the expression of the *AChR* δ and ϵ subunits [95]. Interestingly, Neuregulins were shown to induce PGC-1 α phosphorylation, which favours its interaction with GABP [95].

Mouse KO models of GABP α have not been very informative of the role of GABP at the NMJ. Two studies were published, only one of them describing rather mild NMJ alterations [96,97]. This suggested that either another transcription factor was acting *in vivo* to activate transcription via the N-box or that other mechanisms, possibly mediated by other members of the Ets family, compensated for the absence of GABP α . In contrast, gene inactivation by homologous recombination in mice of the Ets factor *Erm* proved to be much more informative. *Erm* KO mice presented severe NMJ deficits reminiscent of congenital myasthenic syndromes [98]. Analysis of gene expression in *Erm* KO mice demonstrated that the expression of almost half of the synaptic genes was downregulated in the absence of *Erm* [98]. Possible cooperation between *Erm* and GABP was not investigated. Unlike GABP, *Erm* expression is restricted to sub-synaptic nuclei. Thus while clearly important in regulation of sub-synaptic transcription these data do not reveal how compartmentalization of gene expression is generated in muscle fibers and how expression of *Erm* is specifically activated in sub-synaptic nuclei remains to be determined. It would be interesting to investigate if N-boxes are present in the promoter of *Erm*, and if *Erm* sub-synaptic expression involves GABP.

A recent study on mutations causing ALS (amyotrophic lateral sclerosis) identified FUS as an actor of *AChR* expression. FUS is an RNA binding protein in which mutations cause severe forms of ALS. Picchiarelli et al. recently showed that NMJs were strongly perturbed in a knock-in model of a mutation of FUS causing ALS [99]. They further showed that FUS was enriched in sub-synaptic nuclei, interacted with *Erm* in an RNA dependent manner and was required for the activation of the expression of *AChR* subunits by *Erm* [99]. Altogether this suggests that FUS is involved in synapse-specific expression of *AChR* genes (Fig. 2).

4.3. Immediate early genes

Immediate early genes are known to participate in the response to neural activity and to neural factors [100]. Analysis of transcriptional activation of *AChR* expression by Neuregulins showed that it is a multistep process requiring initial activation of JNK kinase and expression of the immediate early genes *Fos* and *Jun* [55]. Since Agrin also activates JNK, it probably also activates *Fos* and *Jun* expression.

Egr transcription factors were also shown to participate in transcriptional activation by Neuregulins. Egr-3 mediates Neuregulin response in intrafusal muscle fibers and activates *AChR* expression ([101] and references therein). Neuregulins were also shown to activate Egr-1 expression in myotubes and an Egr binding site was identified in the *AChR* ϵ promoter [102]. Interestingly, Egr-1 was recently shown to negatively regulate *Agrin* expression [103].

5. Epigenetic regulation at the NMJ

Regulation of transcription is not restricted to the activity of DNA-binding transcription factors. In eukaryotes, DNA is wrapped around histone octamers to form nucleosomes, which spatially organize the DNA to generate more or less compact chromatin. Chromatin compaction, in turn, regulates the accessibility of promoter elements to DNA-binding transcription factors and RNA polymerase plays a central role in the regulation of gene expression. Interestingly, sub-synaptic nuclei have a different architecture than extra-synaptic nuclei (Fig. 1). This had been noticed in the 19th century by Louis-Antoine Ranvier who named these nuclei “fundamental nuclei” [104]. Subsequent visualization of condensed and relaxed chromatin in nuclei by electron microscopy allowed one to see that sub-synaptic nuclei are enriched in relaxed chromatin and usually contain a large cluster of heterochromatin (a chromocenter), whereas extra-synaptic nuclei contain more electron-dense heterochromatin and several chromocenters (Fig. 1A, B, D).

The N-terminal tails of histones protrude from nucleosomes and are subject to a large number of dynamic and reversible post-translational modifications such as methylation, acetylation, phosphorylation, ubiquitination, and SUMOylation. These modifications can facilitate or repress transcription, DNA repair, DNA replication, DNA condensation and chromosome segregation [105]. For example, methylation of histone H3 lysine 9 (H3K9me), histone H3 lysine 27 (H3K27me), and histone H4 lysine 20 (H4K20me) are associated with transcriptional repression. Conversely, the presence of H3S10 phosphorylation, H3K9 acetylation and H3K4 di- and tri-methylation is linked to active transcription [106,107]. Sub-synaptic chromatin have been shown to be enriched in acetylated histone H3 and *Agrin* was shown to be sufficient to increase histone H3 acetylation in nuclei recruited at *Agrin*-induced *AChR* clusters [108]. In addition, chromatin immunoprecipitation experiments showed that Neuregulins induced histone H3 acetylation and phosphorylation on *AChR* promoters. It was proposed that this acetylation took place in sub-synaptic nuclei via the recruitment of the histone acetyl transferase p300 on N-boxes by the active (phosphorylated) form of GABP. Accordingly, p300 inhibition reduced *AChR* ϵ subunit expression at the NMJ [108]. In the absence of Neuregulins, GABP recruits the histone deacetylase 1 (HDAC1) rather than p300 on the N box [108]. The

presence of HDAC1 on the promoters of *AChR* probably participates in the silencing of their expression in extra-synaptic nuclei (Fig. 2). Finally, Neuregulins were shown to induce a decrease of HDAC1 levels in myotubes [108].

Other histone deacetylases are regulated by innervation and limit histone acetylation in extra-synaptic nuclei. Histone H3 and H4 acetylation is strongly increased in these nuclei after denervation [72,75]. In innervated muscles, MITR, a splice variant of HDAC9, represses myogenin expression in part via the inhibition of transactivation by MEF2 and the recruitment of HDAC1 and HDAC3 (Fig 2). In denervated fibers, *MITR* expression is downregulated, thus alleviating its repressive action on MEF2 activity and *myogenin* expression. MITR overexpression in muscle fibers is sufficient to prevent *myogenin* and *MyoD* up-regulation and the global increase of histone acetylation following denervation [72]. Interestingly, *HDAC9* expression was shown to be activated by MEF2, thereby creating a negative feedback loop between HDAC9 and MEF2 [109]. On the other hand, *HDAC4* expression is upregulated after denervation. HDAC4 represses both the expression of *MITR* and the myogenin transcription inhibitor *Dach2*, thereby participating in the up-regulation of *myogenin* expression [73,74,76]. HDAC4 also seems to play a role at the NMJ. HDAC4 accumulates at the NMJ [76] and it was recently shown to participate to the maintenance of the post-synaptic structure after denervation. In denervated fibers, Akt is activated and phosphorylates HDAC4 which is then translocated into the nucleus where it activates *AChR* subunits expression, most likely via myogenin up-regulation. The activation of *AChR* expression in denervated fibers maintains AChR turnover in the post-synaptic region thus contributing to its stability [75].

Conclusion

Since the characterization of the promoters of *AChR* subunit genes and the identification of the N-box 25 years ago, a lot has been accomplished to expand our understanding of the cascade of events leading from pre-synaptic signals to the transcriptional activation of synaptic genes. A global picture has gradually emerged with actors such as Wnt factors, Hippo/YAP signaling and the RNA binding protein FUS more recent additions that have broadened the landscape of transcriptional regulation at the NMJ. Many genes involved in congenital myasthenic syndromes (CMS) are still unknown, suggesting that many genes required for NMJ formation and function remain to be discovered. Hopefully, systematic sequencing of CMS patients will allow the identification of such genes.

However, the wealth of genetic and biological information available to date still gives little insight into how a single neural factor, Agrin, can induce a complete remodelling of the nuclear architecture and organization, including genome-wide modifications of chromatin post-

translational modifications and compaction. The chromatin remodelling machineries involved are not yet known, neither is the precise description of the differential spatial organisation of chromatin in sub-synaptic and extra-synaptic nuclei.

New genomic methods have unravelled unexpected features of the 3D organization of the genome. Distant genomic sequences are preferentially colocalized in the nucleus to form Topologically Associated Domains (TADs) in which gene expression is coordinated. Other domains called Lamin Associated domains (LADs) regroup genomic regions associated with lamins in the periphery of the nucleus in which gene expression is usually low and that participate to genome 3D organization. With the discovery of these domains also came the notion that genome organization in the nucleus was more dynamic than expected and that it changed during development [110].

Hopefully in the future, the isolation of sub-synaptic nuclei or the reconstitution of highly differentiated neuromuscular junctions *in vitro* will give access to such information and to a genome-wide description of sub-synaptic chromatin organization with the description of Lamina Associated Domains (LADs) and Topologically Associated Domains (TADs) in sub-synaptic nuclei.

Fig. 1: Representative structure of extra- and sub-synaptic nuclei at the NMJ of mice. (A-B) Confocal image of an 8 weeks old mouse neuro-muscular junction. White arrows identify sub-synaptic nuclei with their characteristic round shape and their chromocenter (generally one or two large spots of heterochromatin). Orange arrows identify some representative myonuclei with their oval shape and several small heterochromatin foci. (A) Merge with Desmin/Thy1-YFP/DAPI staining. Desmin stained intermediate filament in red; Thy1-YFP stained motor neurons (green); DAPI for nuclei staining (blue) (B); Scale bar = 10µm. (C-D) Ultrastructure of muscle nuclei by transmission electron microscopy. Sub-synaptic nucleus (C) and extra-synaptic nucleus (D). (nerve terminal (NT), muscle fiber (M), and terminal Schwann cell (SC); scale bar 1µm).

Fig. 2: A model of the transcriptional regulation of synaptic genes in sub-synaptic and extra-synaptic nuclei. In sub-synaptic nuclei, transcriptional regulation is initiated by the release of neural Agrin, which binds to LRP4 to activate the tyrosine kinase receptor MuSK. In addition, Wnt factors can directly bind and activate MuSK. MuSK activation leads to the recruitment of synaptic proteins including Pak1, Dvl and the Neuregulin receptors ErbB, Dok7 as well as AChR via Rapsyn. Activated MuSK triggers the JNK kinase and activated ErbB

receptors trigger both ERK and JNK kinases, as well as the Cdk5/p35 complex. JNK and ERK kinases phosphorylate α and β subunits of the transcription factor GABP to activate the transcription of synaptic genes via the N-box. Neuregulins also phosphorylate the co-activator PGC-1 α that cooperates with GABP to activate transcription via the N-box. The Ets factor Erm is specifically expressed in sub-synaptic nuclei and also binds the N-box to activate the expression of synaptic genes. The RNA binding protein FUS interacts with Erm and is required for transcriptional activation by Erm. GABP α interacts with the co-activator p300, leading to chromatin hyperacetylation.

In extra-synaptic nuclei, GABP recruits HDAC1 rather than p300 on the N-box. In parallel, nerve-evoked electrical activity triggers Ca²⁺ entry which activates serine/threonine kinases that will ultimately inhibit the transcription of Myogenin. Myogenin expression is inhibited by Dach2 and by the HDAC1/3/MITR complex via MEF2 inhibition. HDAC4 is an inhibitor of Dach2 and MITR expression. In innervated muscle fibers, HDAC4 is mainly cytoplasmic and expressed at low levels, thereby allowing inhibitory action of Dach2 and MITR.

Declaration of Competing Interest

The authors declare no competing financial or non-financial interests.

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