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Extracellular signal-regulated kinases 1 and 2 regulate neuromuscular junction and myofiber phenotypes in mammalian skeletal muscle

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Abstract

The neuromuscular junction is the synapse between a motor neuron of the spinal cord and a skeletal muscle fiber in the periphery. Reciprocal interactions between these excitable cells, and between them and others cell types present within the muscle tissue, shape the development, homeostasis and plasticity of skeletal muscle. An important aim in the field is to understand the molecular mechanisms underlying these cellular interactions, which include identifying the nature of the signals and receptors involved but also of the downstream intracellular signaling cascades elicited by them. This review focuses on work that shows that skeletal muscle fiber-derived extracellular signal-regulated kinases 1 and 2 (ERK1/2), ubiquitous and prototypical intracellular mitogen-activated protein kinases, have modulatory roles in the maintenance of the neuromuscular synapse and in the acquisition and preservation of fiber type identity in skeletal muscle.

Keywords

MAP kinase; ERK1/2; Agrin; neuromuscular junction; synapsespecific transcription

1. ERK1/2.

Mitogen-activated protein kinases (MAPKs) are part of intracellular signaling modules that control multiple cellular processes. MAPK modules consist of 3 core protein kinases. The most downstream enzyme, the actual MAPK, is the S/T (Ser/Thr) kinase that phosphorylates the transcription factors, cytoskeletal elements or other kinases, that are the targets of regulation by signaling pathways launched at the cell surface by activation of receptor tyrosine kinases, integrins and ion channels. A MAPK is activated by an upstream MAPK kinase (MAP2K), which in turn is activated by a MAP2K kinase (MAP3K). MAP3Ks generally integrate signals derived from small, monomeric GTPases such as the Ras family or from other more elaborate mechanisms [1]. In mammalian cells the prototypical MAPK

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module includes the MAPKs ERK1/2, the MAP2Ks MEK1/2 and the MAP3K Raf (Figure 1). ERK1/2 regulate normal cellular responses to numerous growth factors and cytokines in proliferation, differentiation and apoptosis [2].

2. ERK1/2 and Muscle Fiber Mass and Type.

In adult muscle, four major myofiber types can be distinguished based on the expression of myosin heavy chain (MyHC) protein isoforms: type 1 (expressing MyHC-β/slow encoded by *Myh7*), type 2A (MyHC-2A encoded by *Myh2*), type 2X (MyHC-2X encoded by *Myh1*) and type 2B (MyHC-2B encoded by *Myh4*) [3, 4]. The contractile properties of these myofiber types are controlled by different classes of motoneurons, which impose on them specific patterns of activity from the slow (type 1), to the fast fatigue resistant (type 2A) and fast fatigable (2B). These patterns are paired with different metabolic properties that match the strength and persistence of the contractile forces involved: type 1 and 2A are more oxidative while type 2B are more glycolytic.

Table 1 summarizes results of studies on regulation of fiber size and specification by ERK signaling covered in this review. Pharmacological inhibition of ERK signaling induced atrophy in myotubes derived from the mouse muscle cell line C2C12. This result suggested that ERK1/2 might have a role in maintaining myofiber mass *in vivo* [5]. Consistent with these results, developmental selective abrogation of *Erk1* and *Erk2* in myofibers induced muscle fiber loss and atrophy, with stronger effects in the type1-fiber-rich soleus (SOL) than in type 2-fiber-rich tibialis anterior (TA) and sternomastoid (STN) [6, 7]. However, sustained ERK activation via selective myofiber expression of an MEK1 constitutively active mutant [8] or a Ras mutant that only activates the MEK-ERK1/2 pathway and not the PI3K-Akt pathway [9], failed to induce fiber hypertrophy. Together, these results suggest that ERK1/2 signaling is necessary, but not sufficient, to maintain normal fiber size.

ERK1/2 have also been implicated in the acquisition and maintenance of fiber type identity. Seemingly paradoxical, both the type 1 and type 2 phenotypes have been reported to be regulated by ERK signaling (Table 1). Thus, Shi and colleagues concluded that ERK signaling was critical to promote the fast type 2 fiber phenotype [10] based on the effects of overexpressing (i) a constitutively active ERK2 in slow rat SOL muscle, which induced the fast fiber type program; and (ii) MAPK phosphatase 1 (MKP-1) to inactivate ERK in fast adult mouse and rat skeletal muscle, which induced the slow fiber program. However, MKP-1 not only inactivates ERK1/2 but actually shows substrate preference for other MAPKs such as JNK and p38 [11]. Thus, the *in vivo* effects on fiber type expression reported in this study could have been unspecific to the inactivation of ERK1/2. Nevertheless, recent studies in the rat continue to support a role for ERK signaling in the promotion of the fast fiber phenotype [12]. On the other hand, compelling evidence supports a role for ERK1/2 in the promotion of slow fiber development and maintenance. Thus, a constitutively active form of Ras that selectively activates ERK1/2 induced MyHC-β/slow expression in regenerating denervated rat SOL muscle, while a dominant-negative form of Ras had the opposite effect [9]. In the same study, a low frequency/high amount impulse pattern (20 Hz), typical of slow motoneurons that innervate type 1 fibers, elicited the highest increase in ERK phosphorylation when applied to adult rat denervated SOL muscle. Firing

patterns more similar to those for type 2B or type 2A fibers failed to stimulate, or more modestly induced ERK activation, respectively [9]. Thus, these results suggested innervation-dependent, higher basal levels of ERK activation in type 1 than type 2 myofibers. Consistent with the above results, selective myofiber expression of a constitutively active MEK1 led to a fast-to-slow-fiber type switch both in adult skeletal muscle as well as during development [8]. A molecular mechanism that may underlie induction of MyHC-β/slow by ERK activation involves increases in intracellular Ca^{2+} downstream of motoneuron-elicited electrical activity, which activate ERK1/2. Active ERK1/2, in turn, is proposed to phosphorylate the transcriptional co-activator p300 within the nucleus, which acetylates the transcription factor nuclear factor of activated T cells c1 (NFATc1) stimulating its DNA binding activity and resulting in up-regulation of *Myh7* transcription [13]. Prior studies showed that the nuclear localization of NFATc1, which is regulated by the Ca^{2+}-dependent phosphatase calcineurin, is critical for the promotion and maintenance of the slow fiber phenotype *in vivo* [14–16]. Thus both the calcineurin-NFAT and the ERK1/2-NFAT pathways are thought as slow nerve activity sensors [17]. Developmental loss-of-function manipulation of *Erk1* and *Erk2* in myofibers failed to induce slow-to-fast fiber-type switching [6], however it yielded preferential dramatic atrophy of type 1 fibers, with reduction in *Myh7* mRNA expression consistent with a role for ERK signaling in the postnatal growth of slow fibers. The persistence of slow-fibers in ERK-deficient muscles, albeit atrophied, suggest that the calcineurin-NFAT signaling axis may still work in these muscles, and that consistent with results in C2C12 cells [13], it does not require active ERK *in vivo*, it is just potentiated by ERK activity. Although this possibility needs further investigation, it could explain the striking ability of gain-of-function manipulations of ERK signaling to induce a fast-to-slow fiber type switch [8, 9], and the inability of loss-of-function manipulations to generate a robust slow-to-fast fiber type switching. Thus although results are conflicting, perhaps due to particulars of experimental systems and methodologies, most *in vitro* and *in vivo* studies reviewed above favor an important role for ERK1/2 in type 1 fiber specification.

3. ERK1/2 at the Neuromuscular Junction.

3.1 Key Cellular and Molecular Players at the NMJ.

During embryonic development motor neurons send out axons that synapse onto skeletal muscle fibers to form the neuromuscular junction (NMJ) [18]. Three different cell types contribute the components of the NMJ. Synaptic vesicles containing the neurotransmitter acetylcholine (ACh) reside within the motoneuron's presynaptic axon terminal; nicotinic acetylcholine receptors (AChRs) cluster on the myofiber's postsynaptic sarcolemma, and the nerve terminal is capped by the non-myelinating glia terminal Schwann cells (tSCs), a.k.a. perisynaptic Schwann cells [19]. A narrow extracellular space, the synaptic cleft, filled with a specialized extracellular matrix (ECM), the synaptic basal lamina, separates pre- and postsynaptic components. Basal laminae also encapsulate tSCs and muscle fibers extrasynaptically. Cells called kranocytes, currently of unknown nature and function, that lie outside basal laminae but that cap the NMJ, have been proposed as a fourth cellular component of the NMJ [20]. Neuromuscular synaptic transmission is terminated by the hydrolysis of ACh by acetylcholinesterase (AChE), which is concentrated at the synaptic

basal lamina. The formation and maintenance of the NMJ involves reciprocal interactions among these cell types. The best characterized molecular signaling pathway in NMJ formation and maintenance is the Agrin-Lrp4/MuSK-Dok7-Rapsyn cascade responsible for the clustering of AChRs on the postsynaptic apparatus [21]. The fundamental role of these proteins in the formation of the NMJ is underscored by the absence of synapses in loss-of-function mutant mice for all their corresponding coding genes [22–26]. Conditional gene targeting and siRNA knockdown have also demonstrated an essential role for Agrin, Lrp4 and MuSK in NMJ maintenance [27–30]. Agrin was purified from extracts of the electric organ of *Torpedo californica*, a source rich in NMJ proteins, based on its ability to induce aggregates or clusters of AChRs on the membrane of cultured myotubes [31]. Agrin is a heparan sulfate proteoglycan that localizes to the basal lamina. It is encoded by a large gene that through alternative splicing and differential promoter usage generates multiple isoforms with differential subcellular localization and biological activity [32, 33]. Binding of Agrin to basal lamina is mediated by an N-terminal domain [34], while the C-terminal domain harbors sequences encoded by exon Z (so named in mammals), of either 8, 11, or 19 amino acids, which are critical for Agrin's AChR clustering activity [35]. Agrin is expressed by motoneurons, skeletal muscle fibers and tSCs, but only motoneurons express and secrete Z+ Agrin, the isoforms able to cluster AChRs (and many other synaptic proteins [36, 37]). Z- Agrin isoforms are inactive in AChR clustering assays. Although it was later found in other tissues, notably brain [38], the Muscle-Specific Kinase (MuSK) is a receptor tyrosine kinase first cloned from *Torpedo* electric organ [39], but shortly after shown to be highly concentrated at mammalian NMJs, induced by muscle denervation [40] and essential for neuromuscular synaptogenesis [24]. Albeit it does not bind MuSK, Z+ Agrin acts through MuSK by inducing its tyrosine phosphorylation [41]. The Low Density Lipoprotein (LDL)-receptor-related protein 4 (Lrp4) associates with MuSK and selectively binds Z+ Agrin [42, 43]. It is required for the assemply of the postsynaptic apparatus but has also been proposed as an early retrograde signal for presynaptic differentiation [44]. Downstream of tyrosine kinase 7 (Dok7) was identified as a member of the Dok family of phosphotyrosine-binding adaptor proteins that selectively binds to the tyrosine-phosphorylated NPXY (Gln-Pro-Any Amino acid-Tyr) intracellular motif of MuSK. Tyrosine-phosphorylation of this MuSK domain is induced by Z+ Agrin and is critical for Agrin's AChR clustering activity [45, 46]. Thus, Dok7 binds and stabilizes Agrin-induced NPXY MuSK phosphorylation, is concentrated at synaptic sites and is essential for NMJ formation [25]. The AChR-associated protein at the synapse (Rapsyn) was also purified from Torpedo, even before Agrin, as a 43 kDa peripheral membrane protein that binds to intracellular domains of the AChR [47]. Although it is critical for AChR clustering *in vivo* [26], exactly how it is involved in the process is still a mystery. Initially proposed as a purely structural anchoring link between AChR clusters and the underlying synaptic cytoskeleton [48], recent findings indicate that Rapsyn is also endowed with enzymatic activity, specifically neddylation [49], that seems important for AChR clustering.

3.2 Experiments that Initially Suggested a Role for ERK Signaling at the NMJ.

The high concentration of AChRs at the NMJ is the result of both their clustering at the postsynaptic sarcolemma and their localized synthesis, which is mainly driven by the high rate of transcription for their coding genes at the synaptic myonuclei relative to that at

extrasynaptic nuclei [50]. Synapse-specific transcription extends to the genes encoding many other synaptic proteins (e.g. *MuSK*) [18] and its physiological significance is underscored by the cases of human congenital myasthenias caused by mutation of the specific cis-regulatory promoter elements involved [51, 52]. Nerve-derived inductive signals were traditionally thought as responsible for clustering and synapse-specific transcription of synaptic proteins and their encoding genes. However, until around the turn of the century, separate signals, and signaling pathways, were believed to be involved. While Z+ Agrin was the clustering signal [53], the favorite nerve-derived signal for synapse-specific transcription was ARIA, or acetylcholine receptor-inducing activity [54]. Whereas soluble Z+ Agrin was unable to stimulate AChR gene transcription in cultured muscle cells [55]. ARIA was capable of inducing AChR synthesis without causing AChR clustering in cultured myotubes. Molecular cloning of the ARIA gene revealed that ARIA belongs to the family of ligands of the *neu* protooncogene. This gene was later renamed the Neuregulin 1 gene. It encodes multiple isoforms differentially expressed by motoneurons, muscle fibers and tSCs. Neuregulin-1 (Nrg-1) is a growth factor-like protein, that acts via tyrosine phosphorylation of the EGF-like receptors ErbB2, -3 and -4 [56]. Several labs showed that Nrg-1-induced AChR gene transcription *in vitro* was mediated, at least in part, by ERK signaling, which were among the first experiments that suggested a possible role for ERK1/2 at the NMJ [57–60]. Although multiple lines of evidence supported a critical role for Nrg-1 as the key neural signal driving synapse-specific AChR transcription (reviewed in [61]), ultimately loss- and gain-of-function gene targeting experiments showed that Nrg-1 was mostly dispensable for synapse-specific AChR gene transcription *in vivo* [62–64].

Because ACh and ATP are co-released from synaptic vesicles at the NMJ, and ATP can stimulate AChR- and AChE gene transcription in cultured myotubes via purinergic receptors P2RY1 and P2RY2 and downstream ERK activation [65], ATP was also investigated as a candidate for a neural signal for synapse-specific transcription. However, in *P2ry1*$^{-/-}$ mice, which also showed a large reduction in P2RY2 expression, AChE mRNA levels were very modestly decreased and an expected reduction in AChR gene transcription, consistent with prior *in vitro* experiments [65], was not even reported [66].

Genetic approaches surprisingly demonstrated that Z+ Agrin, acting through Lrp4/MuSK-Dok7, was necessary and apparently sufficient to mediate both AChR clustering and synapse-specific gene expression *in vivo* [22–25, 36, 67]. Rapsyn was found essential for AChR clustering but dispensable for synapse-specific transcription [26]. Because Z+ Agrin could induce the aggregation of muscle-derived Nrg-1 and its ErbB receptors *in vivo* [67, 68], a tantalizing mechanism by which Agrin could induce AChR gene transcription was to aggregate an autocrine Nrg-1/ErbB receptor signaling complex on the sarcolemma [69, 70], which would have implicated local downstream activation of ERK signaling. However, this potential mechanism was also shown unlikely *in vivo* by the aforementioned Nrg-1/ErbB mouse genetics experiments [62, 63].

Soluble Z+ Agrin was shown to activate ERK1/2 phosphorylation in myotubes derived from the C2 mouse muscle cell line [45, 71]. Within 5-10 min after application, Z+ Agrin induced a rapid and transient ERK1/2 activation in wild-type cells that was absent in myotubes from muscle cell lines genetically lacking either the Lrp4 or MuSK genes [71]. Beyond 30 min

after application, Z+ Agrin-treated cultures exhibited ERK1/2 phosphorylation levels below baseline. This latter response was observed in WT cells and in the Lrp4- or MuSK-deficient myotubes [71]. Thus, Z+ Agrin induces both Lrp4/MuSK-dependent and –independent changes in ERK1/2 activation in the muscle cell lines studied thus far. Z+ Agrin-induced Lrp4/MuSK-dependent ERK1/2 activation is not necessary for Z+ Agrin-induced AChR clustering on C2 myotubes. Actually, pharmacological inhibition of ERK1/2 activation had either no effect [45] or potentiated [71] Z+ Agrin induced AChR clustering in these cells. Experiments that test the effects of soluble or substrate-bound Z+ Agrin treatment on ERK1/2 activation, and its role in AChR clustering or gene expression in the more physiological primary myotubes have yet to be done. In this context, it is important to mention here that soluble Z+ Agrin can stimulate ERK1/2 phosphorylation in myeloid cells [72] and primary cardiomyocytes [73]. In both settings, ERK1/2 activation is dependent on Agrin binding to α-dystroglycan, a receptor for both Z+ and Z− Agrin [74]. Dystroglycan is a transmembrane α–β heterodimer glycoprotein, β-dystroglycan appears to be a scaffold for MEK2 and ERK1 signaling [75], and interacts with the adaptor protein Grb2 [76], which usually links plasma membrane receptors to the canonical RAF-MEK-ERK pathway [2]. Duchene muscular dystrophy (DMD) is caused by loss of dystrophin protein due to mutation of the gene encoding it. Dystrophin depletion also leads to the loss of the dystrophin glycoprotein complex (DGC) from the sarcolemma, which contains dystroglycan and other proteins and contributes to the dystrophic phenotype. The DGC links the cytoskeleton to the ECM and provides mechanical stability to the sarcolemma during forceful contraction or stretch. At the NMJ, the dystrophin homolog utrophin binds AChRs and links them to the DGC [77]. Dystrophin is also concentrated at the NMJ but does not interact directly with AChRs [78]. Possible roles for α– and β–dystroglycan in the maintenance of the NMJ have been documented (e.g. [79, 80]).

3.3 Recent Experiments that Implicate ERK Signaling at the NMJ.

In a seminal study, William Snider and collaborators were the first to use gene targeting to study the developmental role of ERK1/2 signaling in Schwann cells, motor and sensory neurons [81]. Abrogation of *Erk1* and *Erk2* in Schwann cells or their precursors revealed essential roles for ERK1/2 signaling in glial cell survival and their ability to support and myelinate axons, by acting downstream of the Nrg1-ErbB2/3 interaction. On the other hand, loss of ERK signaling in motoneurons resulted surprisingly dispensable for the motor axons to grow out and form NMJs during embryonic development [81]. Potential roles for motoneuronal ERK signaling in the maintenance of the NMJ after birth or in the adult are yet to be studied using mouse genetics. It is not currently possible to perform gene targeting experiments selectively on tSCs without also perturbing Schwann cells that myelinate axons. Thus, mouse genetic experiments that specifically test the role of tSC ERK signaling in NMJ differentiation and maintenance remain to be done. Mice with selective developmental abrogation of *Erk1* and *Erk2* in skeletal muscle fibers formed NMJs as embryos but displayed postnatal defects in NMJ maintenance. Lack of ERK1/2 in myofibers caused reduction in mRNA and protein expression of *Chrne*, the gene encoding the ε-subunit of the pentameric AChR, typical of mature NMJs [82], specifically transcribed at synaptic myonuclei [50]. This effect was fiber-type independent and only required the abrogation of *Erk2* to be observed [6, 7]. This result is consistent with prior experiments by Lin Mei and

colleagues, who showed that dominant negative mutants forms of Ras, Raf, and MEK1 selectively inhibited synapse-specific expression of *Chrne*-luciferase reporters that were expressed in adult TA muscle following plasmid DNA injection [60]. These findings suggest that *in vivo* ERK signaling regulates AChRε expression at the transcriptional level.

NMJs in myofibers lacking ERK1/2 tended to be fragmented, with discontinuous domains of AChRs resembling endplates in aged muscles [83, 84], or in muscles from mammalian models of DMD [85, 86]. Synaptic fragmentation in DMD muscle is linked to persistent cycles of degeneration/regeneration of muscle fibers due to contraction-induced damage. Unlike normal fibers that have myonuclei in their periphery, close to the sarcolemma, regenerating fibers accumulate myonuclei in the center of the fiber, so called central nuclei [87]. Thus, central myonuclei are a hallmark for regenerating muscle tissue that has undergone damage. Sternomastoid muscle in mice lacking myofiber ERK2 exhibited NMJs as fragmented as those in muscles lacking both ERK1 and ERK2. However, the fraction of fibers with central nuclei in the former was no different than in WT control (1-2%), and in the double knockout *Erk1/2* mutant the percentage of fragmented endplates (~70%) was much higher that the percentage of myofibers with central nuclei (~4%) [7]. Thus, synaptic fragmentation in ERK-deficient muscle is not tightly linked to muscle damage and is, perhaps, a consequence of disruption of local mechanisms that control the expression of synaptic components such as the AChRε subunit. Although this was not examined quantitatively, dystrophin is present on the surface of myofibers lacking ERK1/2 both synaptically and extrasynaptically [7]. This result suggests that α-dystroglycan and the rest of the DGC are also likely to be present on ERK1/2-less fibers. Whether the NMJ maintenance phenotype in ERK1/2-deficient fibers stems from DGC defects on composition, assembly or downstream signaling remains an open question.

In addition to synaptic fragmentation, evidence of at least partial denervation was also observed on a muscle group-dependent manner in the *Erk1/2* double knockout mice [6, 7]. The SOL and STN muscles, but not the TA, displayed cellular and/or molecular signs of denervation such as terminal nerve sprouting and induction of denervation markers such as *Musk*, *Chrng*, the gene encoding the γ-subunit of the AChR, typical of fetal and denervated NMJs [88], the transcription factors Runxl [89] and myogenin [90], and the embryonic myosin heavy chain Myh3 [89]. Mitochondrial biogenesis and functional defects, consistent with denervation, were also observed in the ERK-deficient SOL [6]. In addition, in the mutant SOL a defective AChR γ/ε-subunit switch at innervated endplates preceded muscle fiber atrophy. This seemingly synaptic maturation defect was most prominent in type 1 than in type 2 muscle fibers [6]. This dramatic phenotype is unlikely the result of a simple reduction in AChRε expression and upregulation and persistence of AChRγ expression. Mice harboring a deletion of *Chrne* show persistent postnatal AChRγ expression and some synaptic fragmentation but were not reported to undergo bona fide denervation or muscle atrophy [91, 92]. It will be important to figure out what are the molecular mechanisms by which anomalies in synaptic maturation apparently lead to denervation and subsequent muscle atrophy in ERK1/2-deficient myofibers of specific muscle groups.

4. ERK1/2 in Satellite Cells and Potential Role in Synaptic Regeneration after Nerve Injury and Age-related NMJ Degeneration.

Following muscle damage, satellite cells (SCs), the resident myogenic stem cells in skeletal muscle [93], are activated to divide and give rise to myoblasts and these, in turn, fuse to produce myotubes that then become myofibers. Satellite cells are thus the principal source of myofiber nuclei during regeneration of adult muscle. Recent studies also revealed detectable levels of continuous SC activation, differentiation, fusion and turnover even in noninjured adult muscles [94]. Genetic depletion of SCs in young adults (3-6 month-old) [95] does not lead to synaptic myonuclei loss. Reduction of synaptic myonuclei in such SC-depleted muscles only is observed following transient denervation, which suggests that SCs have a direct role in regeneration of NMJs after nerve injury [95]. Thus from these data SCs are not required for normal NMJ homeostasis, at least in young mice. However, if SCs are depleted in young muscle and tissue examined at middle age [96], NMJs from SC-depleted animals show much more age-induced-like degeneration than controls. Thus, onset of age-related degeneration at NMJs seems accelerated by depletion of SCs. Age-related loss of NMJ myonuclei and NMJ integrity was rescued by SC-specific overexpression of *Spry-1*, a suppressor of receptor tyrosine kinase/FGF (RTK/FGF)-induced ERK signaling [96]. Conversely, *Spry-1* deletion in SCs, which presumably raises levels of ERK activation, is linked to the loss of the SC pool in aged muscles [97]. Together, these results suggest that reducing ERK activity in SCs may lead to a delay in age-related degeneration of NMJs. Elevated ERK activity has been reported in muscle biopsies from old sedentary individuals, which presumably have associated NMJ degeneration [98]. This finding would be consistent with higher ERK signaling in SCs with aging. However, it is unclear from these data whether extrafusal fibers also contribute to this increase. Furthermore, as described above developmental deletion of *Erk1/2* in extrafusal fibers leads to postnatal aging-like NMJ fragmentation and even denervation depending on the muscle group examined [6, 7], which suggest that reduced extrafusal ERK signaling can cause NMJ degeneration. The literature regarding changes in basal ERK phosphorylation with aging in rodent muscles is highly variable, with reports of increases, no changes or decreases depending on the muscle group studied [99]. Additional studies are needed to confirm and further characterize a role for SC- and myofiber-ERK signaling both in NMJ regeneration after nerve injury and in age-related synaptic degeneration.

5. Conclusions and Future Directions.

The initial *in vitro* studies that suggested a role for ERK signaling in AChR gene transcription seem now borne out by the more recent *in vivo* studies that detected a decrease in *Chrne* expression in myofibers lacking ERK1/2 genetically. Two major mechanistic questions remain open: what key molecules are downstream of ERK1/2 activation and what is the signal that activates ERK1/2 at the synapse *in vivo*. In regards to the first question, it will be important to investigate whether ERK1/2 can regulate the expression, localization and activity of Erm/ETV5, a E26 transformation-specific (ETS) transcription factor that is necessary for the synapse-specific expression of many postsynaptic genes, including *Chrne* and *Musk* [100]. This seems likely as transcription factors of the ETS family, such as Erm/

ETV5, are known mediators and effectors of MAPK signaling in other cells [101–104]. If ERK signaling were to regulate Erm/ETV5 at the NMJ, its influence would extend to the transcription of many other postsynaptic genes besides *Chrne*, which might explain the larger impact of muscle ERK1/2-deficiency on NMJ integrity. Regarding the second question, Z+ Agrin has been shown to induce ERK activation but this has only been done in muscle cell lines and under conditions where Z+ Agrin fails to activate transcription. Future experiments need to determine whether Z+ Agrin, applied on an experimental condition where it stimulates AChR gene transcription (i.e. bound to ECM), can activate ERK1/2 phosphorylation, and whether that activation is Lrp4/MuSK- and/or α-dystroglycan-dependent. ERK1/2 can also be activated by increases in intracellular Ca^{2+} [1], which is critical to transduce patterns of electrical activity at the plasma membrane into changes in gene expression in nuclei of excitable cells such as muscle fibers (e.g. type 1 slow fibers) and neurons. About 4.1% of the ACh-evoked current passing through the ε-subunit-containing AChR at adult synapses is carried by Ca^{2+} ions [105]. This localized Ca^{2+} flux has putative physiological significance in that it activates Ca^{2+}-dependent K^+ channels that accelerate endplate repolarization [106]. It is tempting to speculate that this ACh-dependent influx of Ca^{2+} could also support high transcriptional levels of *Chrne* at subsynaptic myonuclei by inducing localized ERK1/2 activation. Figure 2 summarizes potential mechanisms by which ERK1/2 could regulate synapse-specific transcription of *Chrne* and perhaps additional postsynaptic genes. It is important to emphasize that regardless of the mechanism(s) ultimately involved, ERK1/2 activation is not essential for *Chrne* synapse-specific transcription. It just seems to enhance it perhaps to ensure the high rate of transcription required to maintain the high density of AChRs at the mature NMJ. Additional experimentation is also needed to account for the effects on NMJ integrity due to alterations of ERK activation in adult and aging muscle, which may as well involve transcriptional and posttranscriptional mechanisms.

The recent loss- and gain-of-function gene targeting experiments reviewed here [6–8] strongly support a role for ERK signaling in the determination and maintenance of the type 1 muscle fiber phenotype. Further experiments are needed to flesh out the mechanisms involved *in vivo*, in particular, the interactions of ERK-signaling with the Calcineurin-NFAT pathway, critical for type 1 muscle fiber identity. These studies may have translational relevance as the ability of constitutively active ERK1/2 to induce a fast-to-slow fiber switch has be invoked as a potential therapeutic approach to protect muscle fibers from the effects of lack of dystrophin [8]. Caution and further studies on ERK signaling in muscle are warranted though as excessive ERK activation is a hallmark of autosomal Emery Dreifuss muscular dystrophy (EDMD-A) [107], another muscular dystrophy due to mutation in the Lamin A/C encoding gene. Indeed, inhibition of ERK activation ameliorates skeletal and cardiac muscle symptoms of EDMD-A in a mouse model for the disease [108, 109].

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Highlights

- ERK1/2 signaling regulate neuromuscular synapse-specific transcription
- ERK1/2 signaling regulate acquisition and maintenance of slow myofiber phenotype
- ERK1/2 signaling may control muscle progenitor role after nerve injury and aging

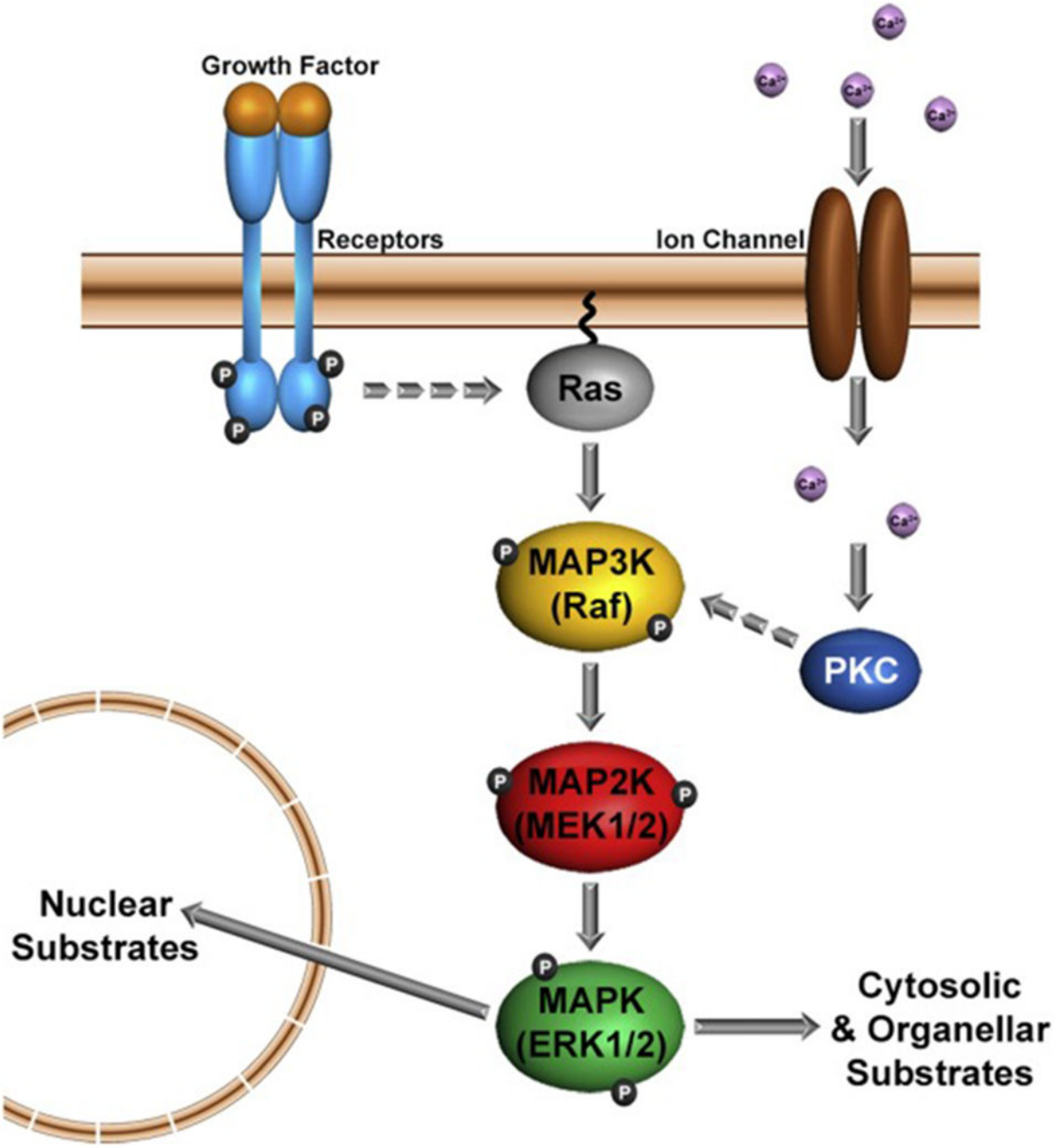

Figure 1. Schematic diagram of canonical ERK1/2 signaling.

Ligand activation of growth factor receptors at the plasma membrane leads to phosphorylation of their cytoplasmic domains. Adaptor proteins (not depicted) bind to these phosphorylated sites and transduce the signal to small GTP-binding proteins such as Ras (dashed arrow). GTP-Ras, activates the 3-tier MAP kinase module of Raf (MAP3K), MEK1/2 (MAP2K), ERK1/2 (MAPK). Active ERK1/2 (a.k.a. pERK1/2) regulate many cellular processes by phosphorylating target proteins in the nucleus, cytosol and subcellular organelles. A rise in intracellular Ca^{2+} (lavender spheres) through plasma membrane Ca^{2+}-

channels can also lead to activation of the ERK1/2-MAP kinase module via protein kinase C (PKC). For simplicity, other pathways for ERK1/2 activation via integrins or G-protein-coupled receptors are not presented.

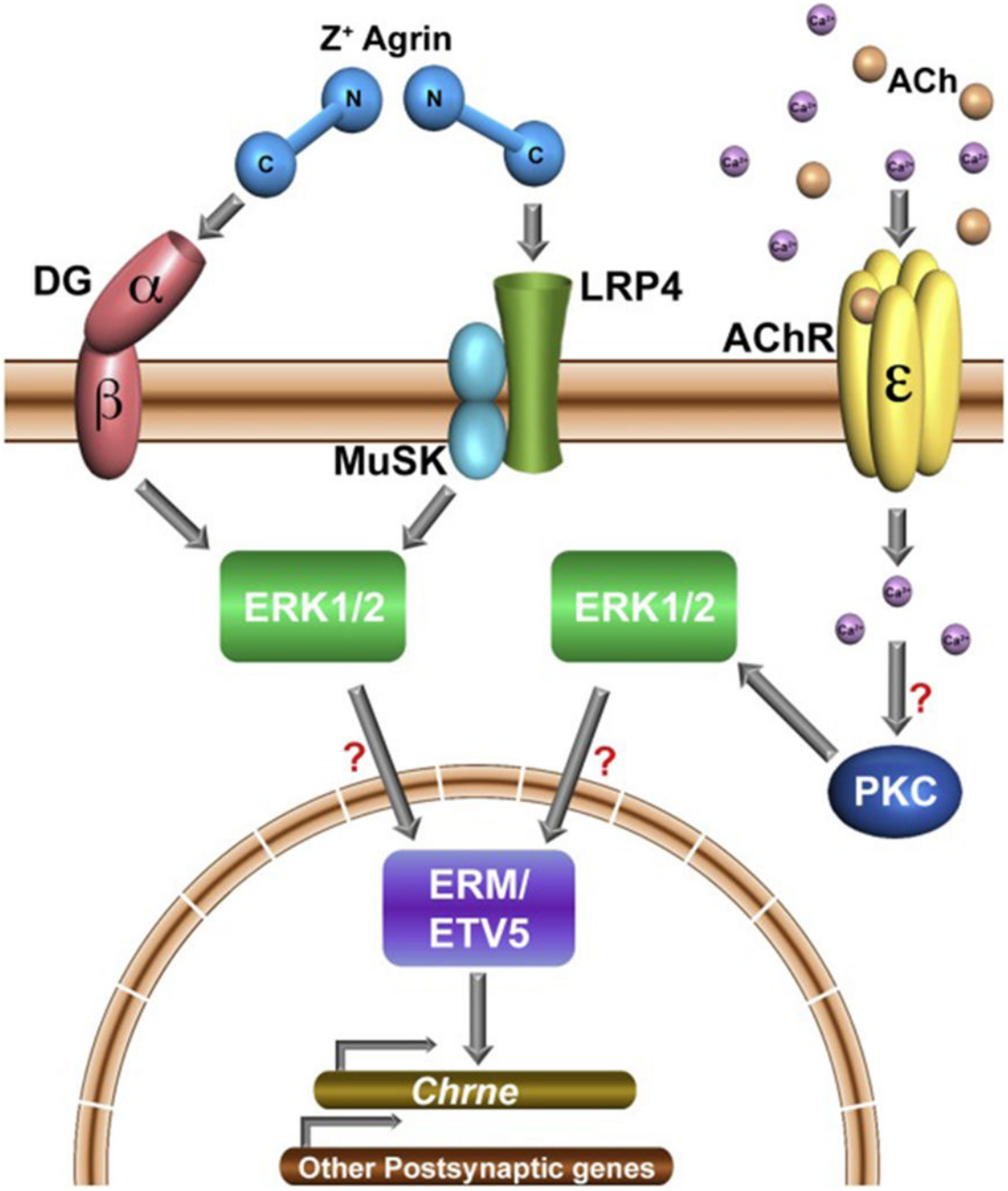

Figure 2. Potential mechanisms for ERK signaling involvement in synapse-specific transcription.

Z+ Agrin binding to either dystroglycan (DG) or to the Lrp4/MuSK complex can lead to ERK1/2 activation, which in turn would activate the Erm/ETV5 transcription factor. Erm/ETV5 would stimulate transcription of *Chrne* and other synapse-specific genes within synaptic myonuclei. ACh gating of the ε-subunit-containing AChR ($\alpha_2\beta\delta\varepsilon$ pentamer) would lead to Ca^{2+} entry that may activate PKC, which in turn would activate ERK1/2 and lead to

transcriptional stimulation of synapse specific genes via Erm/ETV5. (?): Steps without direct experimental evidence currently.

Table 1.

Summary of studies on ERK1/2 signaling and myofiber size and specification discussed in this review[a].

Study [Reference]	Experimental Model	Manipulation of ERK1/2 pathway	Major Findings
[5]	C2C12 cells	Pharmacological block of ERK1/2 activation	Reduced myotube size
[6]	Mouse	Muscle conditional, loss-of-function *Erk1/2* mutant. *Focus on slow fibers*	Dramatic fiber loss and atrophy. Marginal effect on fiber identity
[7]	Mouse	Muscle conditional, loss-of-function *Erk1/2* mutant. *Focus on fast fibers*	Mild muscle fiber loss and atrophy. Marginal effect on fiber identity
[8]	Mouse	Muscle conditional, gain-of-function *Mek1* mutant	No effect on fiber size. Fast-to-slow fiber switch
[9]	Rat	Electroporation of *Ras* constitutively-active (CA) & dominant-negative (DN) mutants Electrical stimulation of denervated SOL with slow/fast patterns	No effect on fiber size. MyHC-β/ slow expression: Induced with CARas; suppressed with DNRas ERK activation: strong with slow stimulation; weak with fast stimulation
[13]	C2C12 cells and mouse SOL	How ERK1/2 activation and calcineurin-NFATc1 regulate MyHC-β/ slow transcription	pERK1/2 enhances NFATc1 transcriptional activity
[10]	Rat and mouse	Electroporation of: CAERK2 MKP-1	Slow-to-fast fiber switch Fast-to-slow fiber switch
[12]	Rat	pERK1/2 levels *vis-a-vis* fiber type	High pERK1/2 linked to fast fibers

[a]For abbreviations see text