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Review

Brain-derived neurotrophic factor signaling in the neuromuscular junction during developmental axonal competition and synapse elimination

Josep Tomàs[*,#], Víctor Cilleros-Mañé, Laia Just-Borràs, Marta Balanyà-Segura, Aleksandra Polishchuk, Laura Nadal, Marta Tomàs, Carolina Silvera-Simón, Manel M. Santafé, Maria A. Lanuza[*,#]

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Abstract

During the development of the nervous system, there is an overproduction of neurons and synapses. Hebbian competition between neighboring nerve endings and synapses performing different activity levels leads to their elimination or strengthening. We have extensively studied the involvement of the brain-derived neurotrophic factor-Tropomyosin-related kinase B receptor neurotrophic retrograde pathway, at the neuromuscular junction, in the axonal development and synapse elimination process *versus* the synapse consolidation. The purpose of this review is to describe the neurotrophic influence on developmental synapse elimination, in relation to other molecular pathways that we and others have found to regulate this process. In particular, we summarize our published results based on transmitter release analysis and axonal counts to show the different involvement of the presynaptic acetylcholine muscarinic autoreceptors, coupled to downstream serine-threonine protein kinases A and C (PKA and PKC) and voltage-gated calcium channels, at different nerve endings in developmental competition. The dynamic changes that occur simultaneously in several nerve terminals and synapses converge across a postsynaptic site, influence each other, and require careful studies to individualize the mechanisms of specific endings. We describe an activity-dependent balance (related to the extent of transmitter release) between the presynaptic muscarinic subtypes and the neurotrophin-mediated TrkB/$p75^{NTR}$ pathways that can influence the timing and fate of the competitive interactions between the different axon terminals. The downstream displacement of the PKA/PKC activity ratio to lower values, both in competing nerve terminals and at postsynaptic sites, plays a relevant role in controlling the elimination of supernumerary synapses. Finally, calcium entry through L- and P/Q- subtypes of voltage-gated calcium channels (both channels are present, together with the N-type channel in developing nerve terminals) contributes to reduce transmitter release and promote withdrawal of the most unfavorable nerve terminals during elimination (the weakest in acetylcholine release and those that have already become silent). The main findings contribute to a better understanding of punishment-rewarding interactions between nerve endings during development. Identifying the molecular targets and signaling pathways that allow synapse consolidation or withdrawal of synapses in different situations is important for potential therapies in neurodegenerative diseases.

Key Words: acetylcholine release; adenosine receptors; axonal competition; brain-derived neurotrophic factor; calcium channels; motor end-plate; muscarinic acetylcholine receptors; postnatal synapse elimination; serine kinases; tropomyosin-related kinase receptorB

Introduction

During the development of the nervous system, there is an overproduction of synaptic connections. Hebbian competition between adjacent nerve terminals with different activities leads to the elimination of some synapses and the strengthening of others (Fields and Nelson, 1992; Sanes and Lichtman, 1999; Mennerick and Zorumski, 2000). This developmental synaptic elimination is a fundamental mechanism in neurogenesis that allows the final configuration and performance specification of the neural circuits.

Unitat d'Histologia i Neurobiologia (UHNeurob), Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Reus, Spain
***Correspondence to:** Maria A. Lanuza, PhD, mariaangel.lanuza@urv.cat; Josep Tomàs, MD, PhD, josepmaria.tomas@urv.cat.
https://orcid.org/0000-0003-4795-4103 (Maria A. Lanuza); https://orcid.org/0000-0002-0406-0006 (Josep Tomàs)
#Both authors contributed equally to this work.

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Developing skeletal muscle cells are also initially polyinnervated, but after postnatal nerve terminal competition, individual neuromuscular junctions (NMJ) become innervated by only one axon at their motor endplate. Several presynaptic metabotropic receptors, such as muscarinic acetylcholine autoreceptors (mAChR), adenosine autoreceptors (AR), and tropomyosin-related kinase B receptor (TrkB) among others, allow motor nerve terminals to communicate directly themselves, or through the postsynaptic or Schwann cell synaptic components. In particular, presynaptic mAChR subtypes allow direct competitive interaction between multiple nerve endings through differential activity-dependent acetylcholine (ACh) release because the more active endings may directly punish the less active ones or reward themselves (Nadal et al., 2016; Tomàs et al., 2023). At the postsynaptic site, the brain-derived neurotrophic factor (BDNF)/TrkB pathway serves as retrograde muscle-to-nerve feedback to influence the presynaptic site during NMJ activity and differentially modulate transmitter release from the multiple developing nerve terminals (Saini et al., 2021; Rentería et al., 2022). The different axonal inputs to a given NMJ are generally intermingled during development, and indeed they often share the same postsynaptic gutter, especially in the first half of the elimination process. These nerve terminals can compete by generating activity-mediated signals to directly destabilize synaptic sites associated with other inputs. In this limited space, target-derived neurotrophic factors such as BDNF (and others such as NT-3) or their absence, may contribute to the final functional suppression of some nerve terminals and neuromuscular synapses during development.

Here, we summarize our previously published results related to BDNF signaling evaluating the relevance of this neurotrophic pathway in the developmental synapse elimination and neuromuscular synaptogenesis (Nadal et al., 2017; Tomàs et al., 2017) by comparing the crosstalk effect of the BDNF pathway with our previous results on the autocrine muscarinic pathways (Tomàs et al., 2023). **Figure 1** shows a graphic representation of the components of these pathways that affect the different nerve endings in competition during synapse elimination and in adulthood.

Search Strategy

The references in this review were selected by reviewing our personal files and searches of PubMed for indexed publications between 1992 and 2023. The following combinations of words were used to select the articles: “motor end-plate,” “postnatal synapse elimination,” “axonal competition,” “acetylcholine release,” “muscarinic acetylcholine receptors,” “adenosine receptors,” “BDNF,” “TrkB,” “serine kinase,” “calcium channels.” The final list was determined by the novelty of the data and relevance to the field of mammalian neuromuscular development.

Brain-Derived Neurotrophic Factor Favors Acetylcholine Release in All Nerve Terminals That Are in Competition during Neuromuscular Junction Development

Neurotrophins (BDNF, NT-4 and NT-3) and their receptors ($p75^{NTR}$, TrkB isoforms: full length –FL- and truncated -T1, T2-, and TrkC) are expressed in muscle during both development and adulthood (Pitts et al., 2006). In both stages, immunohistology shows that the neurotrophins and receptors are present in the pre- and postsynaptic elements.

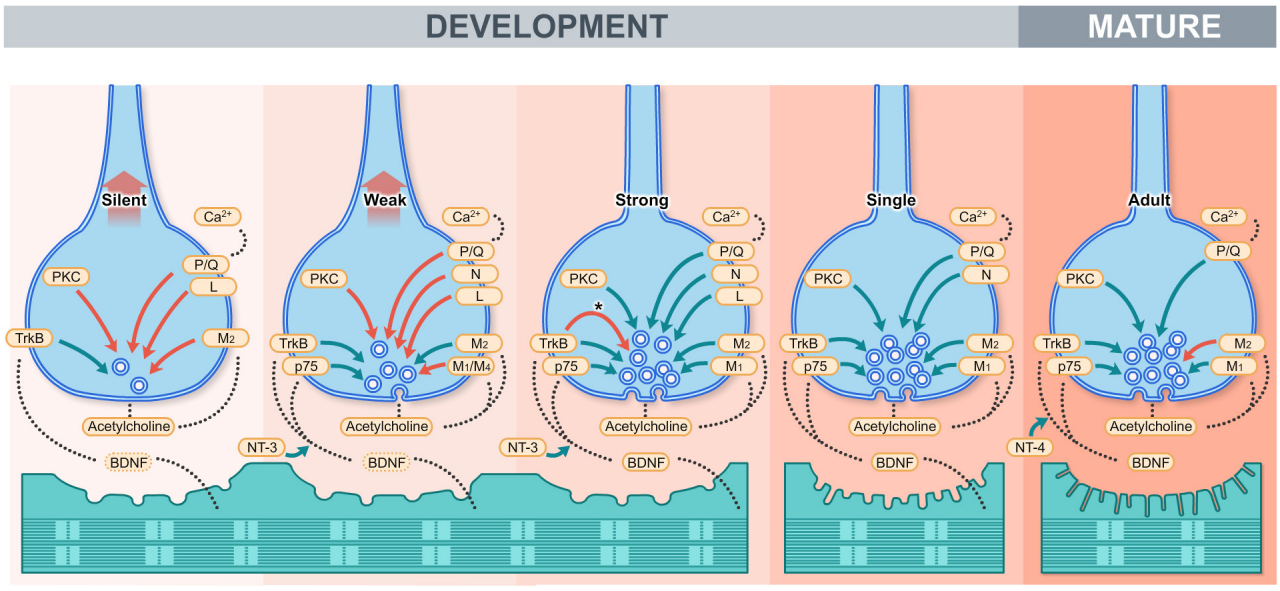

Figure 1 | Graphic representation of the neurotrophins BDNF, NT-4 and NT-3, and the receptors TrkB and $p75^{NTR}$ and the muscarinic pathways (M1, M2 and M4 presynaptic autoreceptors) affecting different nerve endings in competition during synapse elimination (P7–P9) and in the adult.

We studied the strong and weak endings in dual junctions (defined by the size of the EPP that each of them can evoke), and the almost eliminated silent endings that, however, can be transitorily recovered to release ACh. The solitary nerve terminal that wins the competition and the mature nerve endings in the adult (P30) are also discussed. The inhibitory (red arrows) and stimulatory effects (green arrows) of several downstream molecules have been also considered (PKC isoforms, PKA, and VGCC subtypes). Created with Adobe tools software. BDNF: Brain-derived neurotrophic factor; L: L-type; N: N-type; NT: neurotrophin; PKA: protein kinases A; PKC: protein kinases C.

Exogenously applied neurotrophins

At the adult NMJ, BDNF reduces synaptic depression during repetitive stimulation, significantly increases quantal release at all fiber types, and enhances synaptic vesicle replenishment and cycling (Mantilla et al., 2023). Low doses of BDNF rapidly induce a TrkB-dependent potentiation at developing NMJs in culture (Poo, 2001). At the NMJ of the levator ani muscle (LAL) of developing rodents, exogenously applied BDNF (10 nM for 3 hours or 50 nM for 0.5–1 hour) increases ACh release in all nerve terminals that are in competition (also in the most mature already singly innervated NMJ and in the adult) (**Figure 1**; Garcia et al., 2010). The effect of BDNF has also been studied in dually innervated synapses, the most workable polyinnervation situation (around P7). In those synapses, exogenous BDNF increases ACh release both in the weak and strong endings, which are defined according to the size of the end-plate potentials (EPP) that are able to evoke when the nerve is stimulated (Garcia et al., 2010). This effect is specific for BDNF because NT-4 does not modulate the release at P7 (although it does in the adult). Blocking one of its two receptors (TrkB or $p75^{NTR}$ with K-252a or anti-p75-192-IgG, respectively), prevents the BDNF effect (Garcia et al., 2010). NT-3 (100 ng/mL for 0.5–1 hour), also potentiates the evoked release from the weak (70%) and strong (50%) nerve endings but not in the singly innervated or adult synapses (Garcia et al., 2010).

In summary, the BDNF pathway favors transmitter release in all nerve terminals during developmental synaptic competition (with the involvement of NT-3, but not NT-4) until only one ending persists in the mature NMJ.

Endogenous neurotrophins

Blocking neurotrophin receptors demonstrates their tonic involvement in ACh release mediated by endogenous signaling, transactivation, or related mechanisms. Direct receptor blockage at P7 reveal complex signaling during developmental synapse elimination (Garcia et al., 2010). Endogenous BDNF acts tonically through $p75^{NTR}$ to potentiate ACh release in all developing nerve terminals, as the anti-p75-192-IgG antibody directly reduces release in all of them. Surprisingly, blocking the TrkB receptor (K-252a) reveals a TrkB-mediated inhibition of ACh release only in the strong endings at double junctions (asterisk in the red arrow indicates the strong ending in **Figure 1**). These results indicates a balance between an endogenously produced BDNF-induced $p75^{NTR}$-mediated ACh release potentiating mechanism that does not discriminate nerve terminals (a similar effect that exogenously applied BDNF as shown above) and a more specific TrkB-mediated release inhibitory mechanism on the most mature endings still in competition. Interestingly, this is further supported by the fact that after neutralization of endogenous BDNF with a TrkB-IgG fusion protein, inhibition of ACh release occurs only at strong endings of the dual innervation junction(Tomàs et al., 2017). Due to the complexity of signaling events that simultaneously affect several nerve terminals in a developing NMJ, several interpretations for this complex situation would be formulated. It may be that in the absence of endogenous BDNF or TrkB coupling, a neurotrophin such as NT-3 influences $p75^{NTR}$ to favor ACh release in all developing nerve terminals at P7. Also, it could be that endogenous BDNF, acting through TrkB, tonically inhibits specifically ACh release only in the strong ending. If the latter is true, it could be hypothesized that a reduction in the release capacity of the strong nerve terminals may favor a prolongation of the competition between all endings and thus a delay in in the elimination of the weakest endings. In accordance with this, endogenous BDNF effectively delays synapse elimination at P7.

Why do exogenous and endogenous BDNF, produced locally in the complex architecture of the multiinnervated NMJ, show such functional differences? Exogenous BDNF probably activates signaling pathways that are not equally active in all endings during polyinnervation. Endogenously produced BDNF may activate differently developing nerve endings with stoichiometry of different receptor types and even with different developmentally or activity-dependent regulated downstream coupling. This is true for differences in mAChR and VGCC subtypes in the different nerve terminals in competition (Garcia et al., 2022; Tomàs et al., 2023). Also, the accessibility of the postsynaptically derived BDNF to the presynaptic endings would be different in various developing nerve terminals in competition according to their position and surface occupation of the common synaptic gutter. All these differences may contribute to the specific coupling of the neurotrophins to the molecular machinery of the different nerve endings during developmental synapse disconnection.

BDNF transiently recovers ACh release in the nerve terminals that have already become silent during the elimination process

Exogenous BDNF (50 nM) transiently recruits in minutes functionally depressed silent nerve terminals (observed as new EPP and, therefore, neurotrophin increases the release of ACh in these endings), and this effect seems to be mediated only by TrkB (**Figure 1**). Using intracellular recording, we counted the number of functional inputs (clearly separated on successive stimulations, by the excitability threshold, the latency, or both) for a large number of neuromuscular synapses in P7 LAL muscles and calculated their mean value or polyinnervation index (PI) of the muscle. In these developing muscles at this time, the PI was 1.63 with almost 48% of monoinnervated junctions (Lanuza et al., 2001). Exogenously applied BDNF increases PI to almost 2.1 (and thus the number of functional nerve endings in many NMJs), which is a value equivalent to that observed in normal, untreated P2–P3 animals with no more than 15% of monoinnervated NMJs. Incubation with NT-3, NT-4 or glial-derived neurotrophic factor does not produce this effect. The newly recruited nerve terminals are uncovered in ~20 minutes after BDNF had been added to the bath. Importantly however, after 3 hours in the presence of 50 nM BDNF, the distribution of the number of inputs completely returns to the control P7 values (Garcia et al., 2010). Thus, BDNF-induced recruitment is a brief transient effect that delays but does not impair the ongoing process of synapse elimination. We probably observed synapses that became transiently silent before they completely retracted during the stereotyped developmental process that eliminates some nerve endings. Similarly, mammalian synapses have been observed to appear and disappear rapidly in tissue culture (Haydon and Drapeau, 1995).

We also investigated the possible involvement of endogenous BDNF and their receptors in the ACh release capabilities of the silent endings. As stated, exogenous BDNF increases PI, while no endogenous BDNF inhibitor can reduce it below the control value at P7 (including the TrkB inhibitor K-252a, the BDNF chelator TrkB-IgG chimera, the anti $p75^{NTR}$ antibody anti-p75-192-IgG, and the $p75^{NTR}$ inhibitor Pep-5). Therefore, a putative neurotrophin-mediated mechanism, activated by endogenous BDNF *in vivo* in the silent nerve terminals, coupled to favoring the transmitter release mechanism, and being opposed to axonal elimination, appears to be functionally irrelevant because only stimulation with exogenously applied BDNF recruits silent inputs and increases PI (Garcia et al., 2010). Thus, a tonic effect of endogenous BDNF does not occur in these unfavorable nerve terminals. These observations suggest that exogenous BDNF only indicates the presence of an ineffective signaling in the silent nerve terminals. This pathway may have stopped working because the low activity-dependent production of BDNF near the poorly active or silent nerve endings could not counteract the synapse silencing and elimination mechanism.

In summary, the BDNF pathway (stimulated with exogenous neurotrophins) enhances ACh release in all nerve terminals during development and in the mature NMJ. NT-3 acts more specifically only in axon terminals still in competition. The $p75^{NTR}$ and TrkB receptors are involved in the neurotrophic effect, though the role of the endogenous BDNF and the TrkB receptor in axons even in competition is not entirely understood. It could be that the small amount of BDNF production near the weakest or even close to the already silent nerve endings made this signaling ineffective allowing the full operativity of the withdrawal signals in these nerve endings.

Relation of the Brain-Derived Neurotrophic Factor–Tropomyosin-Related Kinase B Receptor and the Muscarinic Subtype Pathways

Intracellular recording using subtype unselective (Oxotremorine T and M, and atropine) and selective (methoctramine, pirenzepine, tropicamide, 4-DAMP, muscarinic toxins 3 and 7) mAChR agonists and antagonist show that the subtypes M1, M2 and M4 can influence ACh release both in developing and adult NMJ (Santafé et al., 2009). In the adult, presynaptic M1 and M2 receptors regulate neurotransmission by positive and negative feedback, respectively (Santafé et al., 2009) and recruit the same downstream kinases as BDNF-TrkB (Cilleros-Mañé et al., 2021; Hurtado et al., 2017). During the initial stage of development (P7), a switch of the mAChR signaling is observed and both M1 and M2 receptors favor release in the recently monoinnervated NMJs (single) and in the strongest endings in dual junctions. However, in the weakest nerve ending, M2 favors ACh release whereas M1 and M4 reduce it (**Figure 1**; Santafé et al., 2009). Thus, M2 couples to enhance neurotransmission in all nerve terminals during developmental competition. It can be suggested that the strongest endings by using this ACh autocrine mechanism, may reinforce themselves and their release capacity to win the competition. On the other hand, the weakest axons may be negatively influenced by ACh release from the strongest ones through M1 and M4 pathways (Tomàs et al., 2023).

mAChRs are also involved in regulating transmitter release from silent nerve terminals. Thus, an increase in PI (similar to that seen after BDNF stimulation) can also be observed after the unspecific muscarinic block with atropine or the specific block of the M2-type mAChR with methoctramine, resulting also in no more than a 15% of monoinnervated NMJs because of the recovering of silent endings in many NMJs. This effect is not observed after M1 (muscarinic toxin 7, MT-7) or M4 (muscarinic toxin 3, MT-3) subtypes block. Altogether, these observations indicate that the normal function of the M2 muscarinic receptor favors the silent endings disconnection, which is the contrary effect of exogenous BDNF. This downregulating action of M2 in silent endings appears to be the last switch of their signaling, achieving the final downregulating action similar to the adult nerve terminals. Interestingly, the average PI of a muscle cannot be reduced below the control value by activating all mAChRs with Oxotremorine M or T at P7. This suggests that the muscarinic mechanism that represses ACh release in the silent nerve endings may operate at their maximum efficiency. However, a significant acceleration of axonal elimination can be done in the presence of the non-specific muscarinic agonist Oxotremorine T as the three- and four-input junctions are quickly reduced (though on average the PI does not change). This indicates that the repressive mechanism that reduces ACh release and contributes to disconnecting synapses may be additionally stimulated in this case and the withdrawal of supernumerary axon terminals can be accelerated by M2 stimulation.

In summary, mAChR subtypes are strongly involved in the competitive relations between axon terminals during development, regulate the transmitter release on the mature NMJ, and undergo several shifts of their coupling to stimulate or inhibit ACh release (**Figure 1**). This functional versatility, probably linked to developmental expression changes and coupling to the different G proteins (Jakubík et al., 2011) results in an M1/M2 balance that enhances transmitter release in the strongest nerve terminals and decreases the release in the weakest and silent nerve endings. Thus, the comparison between the neurotrophin and muscarinic pathways acting on transmitter release during development indicates that a low neurotrophin stimulation associated with a specific M1- and M2-mediated transmitter release reduction results in the ineffectiveness of the weakest synapses in competition. BDNF separately may stimulate synapse generation, for instance as it does in the visual cortex (Cabelli et al., 1995). In poorly active nerve endings, the low activity-dependent production of endogenous BDNF is not enough to counteract the mAChR inhibitory mechanism.

How is related the above-described influence of the retrograde (BDNF-TrkB/p75NTR pathway) and the autocrine (mAChR) signaling on transmitter release at different competing nerve terminals with the physical axon loss? We used confocal microscopy and quantitative morphological analysis to investigate this question. We performed axonal counts in confocal LAL preparations from B6.Cg-Tg (Thy1-YFP)16 Jrs/J

mice that express spectral variants of GFP (yellow-YFP) at high levels in motor neurons and axons are brightly fluorescent (Nadal et al., 2016). Muscles were processed to detect also the postsynaptic nicotinic acetylcholine receptors (nAChRs) with TRITC-α-Bungarotoxin. We counted the percentage of singly-, dually- and triply- (or more) innervated synapses at P7, P9 and P15 after 2 (days 5, 6), 4 (days 5–8) and 10 (days 5–14) subcutaneous applications over the LAL muscle surface of several substances acting on the neurotrophin (TrkB-IgG fusion protein) and muscarinic pathways (subtype-selective antagonists) (Nadal et al., 2017, 2016). The results showed that both the coupling of the mAChRs (M1 and M4) and the endogenous BDNF effect initially resulted at P7 in a clear delay of synapse elimination (a delay in the transition 3-to-2-to-1 nerve terminals in the NMJ). However, at P9, the role of M1, M2 (not M4), and endogenous BDNF become similarly involved in strongly accelerated axon loss (Nadal et al., 2016). These data reinforce the previous suggestion that some activity-dependent balance (related to transmitter release) between the muscarinic subtypes and the neurotrophin BDNF pathway can influence the timing and fate of the interactions between axon terminals in competition.

Interestingly, specific inhibitors of the adenosine receptors (for instance DPCPX for the A1R and SCH-58261 for the A2AR) show that these two receptors also delay axonal loss initially at P7 but their continued presence accelerates it at P9, therefore resulting in a similar effect to the neurotrophin and muscarinic pathways. At P9, the order of the receptors' ability to finally accelerate nerve terminal elimination (from more to less) is M2-M1-A1-A2A-TrkB (Nadal et al., 2016). The strong effect of mAChRs in supernumerary axonal withdrawal seems to be complemented by a moderate involvement of the BDNF-TrkB pathway. Thus, several presynaptic metabotropic receptors overlap and share the common function of regulating the relevant mechanism of synaptogenesis that allows the matching of the synaptic partners at the NMJ. In all cases, each considered receptor initially (P7) delays axonal elimination (probably contributing to the establishment of competition conditions), which finally results in axonal loss acceleration at P9.

All this argues in favor of a multifactorial mechanism to ensure specific monoinnervation of the motor endplates. Trying to understand the relation existing between transmitter release and the final withdrawal of certain nerve endings, a look at **Figure 1** can facilitate some interpretation. After an early onset of the competitive interactions at P7, the unbalance of the neurotrophic and muscarinic pathways may favour the ACh release inefficiency and loss of certain less active nerve terminals (the weakest and silent).

Coupling of Serine-threonine Kinases to Developmental Axonal Loss

It is surprising that such different receptors coupled to different developmentally regulated downstream pathways are so coordinated to support axonal competition and achieve the final goal of excessive synapse withdrawal. Even more, this receptor coordination results in a common stereotyped action that allows the transition from an initial delay in synapse elimination to the final acceleration of the axon loss at the end of the 1st week postnatal. Analyzing the possible mechanism of the receptors' common function, it can be observed that the intracellular signaling of the six considered receptors mainly couples to modulate downstream serine-threonine kinases (Nadal et al., 2017; Tomàs et al., 2017; Garcia et al., 2019, 2021). TrkB, A1 and M1 receptors are linked to the protein kinase C (PKC) pathway whereas A2A, M2 and M4 mainly operate through the protein kinase A (PKA) pathway (Nadal et al., 2017). M1 and TrkB operate through the phospholipase Cβ) and gamma (PLCγ), respectively—and therefore the PKC pathway and the inositol triphosphate (IP3) pathway—whereas A2A, M2, and M4 are coupled to the adenylyl cyclase (AC) and the PKA pathway. Some receptors such as A1, M2, and M4 are negatively coupled to the AC signal-transduction pathway (through a Gi protein) and signal via phospholipase C (through a pertussis toxin-sensitive Go protein). Additionally, G protein-coupled receptors can directly modify effector targets. On the other hand, TrkB also acts through the kinase mammalian target of rapamycin (mTOR) at the NMJ (Delezie et al., 2019; Liu et al., 2021).

Related to PKC (**Figure 1**), its activity (modulated by several agonists and antagonists, for instance, see Table 1 for a list in a stufy by Garcia et al. (2021) and in (Tomàs et al., 2023)) couples to promote transmitter release at all developing and mature nerve terminals, except at the weakest of the polyinnervated synapses, in which PKC couples to reduce the release (Tomàs et al., 2017). Also, blocking of all PKC isoforms (Calphostin C, Staurosporine) results in a recovery of the ACh release in many silent endings, which is comparable to the previously described effect of M2 mAChR block or stimulation with exogenous BDNF to allow the uncovering of these nerve terminals. These observations put in context PKC as a downstream key element for inducing synapse inefficacy and withdrawal.

Because of the functional relation between PKC and PKA, we also investigated the involvement of PKA and PKC in axonal loss. By using many selective activators and blockers of the PKA and PKC isoforms (Tomàs et al., 2023), we found that PKA activity stabilizes multiinervation by delaying both axonal elimination and postsynaptic nAChR pretzel-like cluster differentiation at P9. Contrarily, PKC activity promotes both axonal loss (through cPKCβI and nPKCε isoforms) and postsynaptic nAChR cluster maturation (a possible role for PKCθ; Garcia et al., 2019).

Thus, the phosphorylation of pre- and postsynaptic PKA and PKC targets involved in nerve terminal stability and neurotransmission seems to be a relevant step in the molecular mechanism of developmental synapse loss. We have statistically compared the effect of PKA activators with the effect of PKC inhibitors (both situations delayed synaptic maturation) and found no significant difference in either the level of multiinnervation of the NMJ or in the postsynaptic maturation (in this case only when isoform unselective PKC inhibitors like Calphostin C or Chelerythrine are used). The block of particular PKC isoforms as cPKCβI or nPKCε, with specific inhibitory peptides (βIV5–3 and εV1–2 respectively), results in no postsynaptic alteration, indicating the specific

presynaptic action of these PKC isoforms (Garcia et al., 2021). Conversely, when PKA inhibitors are compared with PKC activators (both situations accelerate synapse elimination and maturation), no difference can be observed in axonal loss, but there is a difference in the maturation of the postsynaptic receptor cluster since non-selective PKC activators such as PMA strongly accelerates postsynaptic maturation, suggesting the involvement of another PKC isoform at this site.

Thus, during NMJ synaptogenesis, there is a balance between PKC and PKA activities being PKC more active than PKA, which favors the elimination of the excessive axon terminals. The postsynaptic cluster maturation follows a similar PKA/PKC interaction but without the involvement of cPKCβI and nPKCε isoforms. Thus, overall, there is a reciprocal involvement of PKA and PKC, although the question is still open as to exactly how this is done at the various competing axon terminals (Garcia et al., 2019, 2021). All the results relating to transmitter release capacity and axonal withdrawal, favor the involvement of PKC activity as a key step in supernumerary axons disconnection during development. Thus, an activity-dependent shift of the PKA/PKC activity ratio to lower values at both nerve endings and postsynaptic sites plays a critical role in developmental synapse elimination. The prevalence of PKC-mediated mechanisms in some nerve terminals may favor their destabilization and withdrawal (cPKCβI and nPKCε isoforms are both necessary, sufficient, and interdependent for PKC involvement in axon loss). This prevalence seems to be regulated by the activity of presynaptic receptors, which varies with the pattern of nerve activation of each terminal. In particular, the PKA/PKC ratio is finally set to lower values in the adult NMJ, where PKC activity needs an external stimulation from membrane receptors to become active and coupled to ACh release (Santafé et al., 2009; Cilleros-Mañé et al., 2021).

In this context, we observed that the block of the muscle contraction (with μ-conotoxin GIIIB, which does not affect presynaptic neurotransmission) results in a delay in axon loss. This uncovers a retrograde influence from the postsynaptic site to the synapse elimination that can be mediated by the neurotrophic control. In fact, this neurotrophic effect might be mediated by the ratio PKA/PKC, because in mature NMJs muscle contraction per se retrogradely modulates TrkB isoforms, PKC and PKA (Hurtado et al., 2017; Polishchuk et al., 2023). The simultaneous application of PKC activators (dPPA for cPKCβI or FR236924 for nPKCε) and μ-conotoxin GIIIB, fully prevents the postsynaptic contraction block effect on axon loss, indicating the capital position of the presynaptic PKC's in determining axonal withdrawal (Garcia et al., 2022).

The Involvement of Calcium and Calcium Channels in Elimination

Relevant downstream mediators of the considered receptors and kinases are calcium ions and calcium channels. Specifically, TrkB links to PLCγ-PKC-IP3 pathway, which is closely related to cytosolic calcium movements. VGCCs are targets of PKC and the influence of PKC on ACh release depends on the P/Q-type VGCC (Garcia et al., 2022), and PKC can regulate N-type (Yokoyama et al., 2005) and L-type channels (Arenson & Evans, 2001).

Our studies show that there is a progressive change in how VGCC subtypes participate in transmitter release during NMJ development and synaptic maturation (Santafé et al., 2009; Tomàs et al., 2017). The block of any of the three VGCC subtypes that are present in nerve terminals during NMJ development (P/Q, N and L) reduces about 2/3 the EPP produced by the strongest ending of the dual NMJ (Garcia et al., 2022). Nevertheless, at weakest endings, it results in an increase in the size of the evoked EPP, indicating that some calcium entry through the channels negatively influences transmitter release and even contributes to disconnecting these weak endings (Garcia et al., 2022). Finally, in the adult NMJ, only the P/Q-type functionally persists to induce ACh release.

We previously described that blockade of PKC (but not PKA), blockade of M2-type mAChR (but not M1, M3, and M4 subtypes), and exogenous BDNF transiently restore transmitter release from silent nerve terminals that are about to be eliminated during development of the NMJ (and thus, the PI of the muscle increases). Interestingly, a similar increase in PI can be observed also after the specific block of calcium entry through L- (with Nitrendipine) and P/Q-type (with ω-agatoxin-IVA; but not N-type, blocked with ω-conotoxin-GVIA) VGCC, or high magnesium-mediated non-specific calcium inflow reduction. Thus, at least calcium entry through L- and P/Q-type VGCC contributes to reduce transmitter release in the most unfavored weak and silent nerve terminals during synapse elimination (**Figure 1**). We also investigated the additive or the mutually occlusive effects between several substances affecting calcium entry, calcium channels, PKC and the BDNF pathway on PI to show their link. The individual effects of two VGCC blockers, or of a channel blocker with high magnesium in the bath were not additive on PI. However, CaC increases the PI more than high magnesium but not more than any VGCC block, suggesting that PKC modulation of the VGCC-dependent calcium inflow is involved in ACh release reduction in the weak and silent nerve terminals (Tomàs et al., 2023). Interestingly, similar to the observed effect of CaC, BDNF can extend the effect of high magnesium and greatly increase PI. On the other hand, a previous incubation with nitrendipine or CaC completely impairs any additional effect of BDNF. These results indicate that incubation with exogenous BDNF has the same effect as PKC and VGCC block. Moreover, the M2 blocker methoctramine recovers no silent endings when it acts after a VGCC block, which indicates that the M2-mediated effect shares the same mechanism. These results indicate that the BDNF-TrkB and the ACh-mAChRs pathways coupled to a PKC-VGCC signaling influence calcium entry in the weakest nerve terminals in competition, leading to supernumerary synapse disconnection.

In relation to the involvement of VGCC on axonal withdrawal, we found that the L- and P/Q-type channels are involved in favoring supernumerary synapse elimination because their specific block extends the period of multiinnervation and delays also the postsynaptic nAChR cluster maturation whereas their stimulation with specific channel activators (Garcia et al., 2022) results on the contrary effect. The effect of any channel block is the same as that observed after

intracellular calcium sequestration with BAPTA-AM. Thus, there is a clear relation between PKA, PKC and VGCC for synapse elimination. Our experiments show that the block of L or P/Q channels or the intracellular calcium sequestration with BAPTA-AM results in the same effect as the block of cPKCβI (and also PKA stimulation), although somewhat smaller than the effect of the nPKCε block in delaying axonal loss (Garcia et al., 2022).

In summary, the high calcium entry through the present and active VGCCs in immature nerve endings results in both transmitter release reduction in certain weak supernumerary axons and in nerve terminal loss. This unitary process is modulated by cPKCβI, nPKCε activity and PKA inhibition.

Concluding Remarks

We have extensively studied how muscarinic and neurotrophic signaling pathways regulate the postnatal synapse loss mechanism but the relationship between these two regulations has not been directly analyzed. Our results allow us to perform this analysis and, here, we try to understand the mutual influence between muscarinic and neurotrophic receptors on this process.

A complex molecular mechanism involving the neurotrophins BDNF and NT-3, the presynaptic metabotropic receptors (TrkB, p75NTR and mAChRs subtypes- other receptors such as adenosine receptors may be involved), PKA and PKC isoforms (presynaptic cPKCβI and nPKCε, and probably postsynaptic PKCθ), VGCC types (mainly P/Q and L) and calcium influx could be considered to determine ACh release and nerve terminal stability, which are in competition during developmental synapse elimination. Specifically, the weakest nerve endings can be discarded after being silenced by the coordinated action of this complex signaling.

The neurotrophin pathway (TrkB and p75NTR), turned on by exogenously applied BDNF, promotes transmitter release in all nerve terminals during synaptic competition, including the weakest and silent endings (with some involvement of NT-3, but not NT-4). However, endogenous BDNF does not affect the weak and silent synapses. Endogenous BDNF may differentially affect developing nerve endings with stoichiometry of different receptor types and with different downstream couplings. It could be that a small amount of BDNF production near the weakest or even near the already silent nerve endings renders this signaling ineffective, allowing the full efficacy of withdrawal signals in these nerve endings.

The low neurotrophin levels associated with a specific reduction in muscarinic release (mediated by M1 in the weakest endings and M2 in the already silent endings) can result in the ineffectiveness of these unfavorable synapses in competition. The low activity-dependent production of endogenous BDNF near the poorly active nerve endings cannot counteract the mAChR inhibitory mechanism. Thus, after an early onset of the competitive interactions at P7, the described imbalance between neurotrophic and muscarinic pathways may favor the inefficiency of ACh release and the loss of certain less active nerve terminals (the weakest and silent).

Why and how do such different receptors coupled to different pathways cooperate in axonal competition and synapse withdrawal? The intracellular signaling of all these pathways is mainly coupled to modulate downstream serine-threonine kinases. During NMJ synaptogenesis, results indicate the existence of a balance between PKC and PKA activity, with PKC being more active than PKA to favor the elimination of the excessive axon terminals. The prevalence of PKC-mediated mechanisms in some nerve terminals may promote their destabilization and withdrawal (cPKCβI and nPKCε isoforms are both necessary, sufficient, and interdependent for PKC involvement in axon loss). This prevalence appears to be regulated by the activity of the presynaptic receptor, which varies with the pattern of nerve activation at each terminal. The neurotrophin and muscarinic pathways coupled to the serine-threonine kinases and VGCCs that are present and active in immature nerve endings result in both the reduction of transmitter release from certain weak supernumerary axons and, ultimately, the nerve terminal loss.

The findings described here contribute to the understanding of the molecular signaling that regulates synapse consolidation or withdrawal during development. This developmental synaptic elimination is a fundamental mechanism in neurogenesis that allows the specific formation of neuronal circuits.

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