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Muscarinic Receptors in Developmental Axonal Competition at the Neuromuscular Junction

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Abstract

In recent years, we have studied by immunohistochemistry, intracellular recording, and western blotting the role of the muscarinic acetylcholine receptors (mAChRs; M_1, M_2, and M_4 subtypes) in the mammalian neuromuscular junction (NMJ) during development and in the adult. Here, we evaluate our published data to emphasize the mAChRs' relevance in developmental synaptic elimination and their crosstalk with other metabotropic receptors, downstream kinases, and voltage-gated calcium channels (VGCCs). The presence of mAChRs in the presynaptic membrane of motor nerve terminals allows an autocrine mechanism in which the secreted acetylcholine influences the cell itself in feedback. mAChR subtypes are coupled to different downstream pathways, so their feedback can move in a broad range between positive and negative. Moreover, mAChRs allow direct activity-dependent interaction through ACh release between the multiple competing axons during development. Additional regulation from pre- and postsynaptic sites (including neurotrophic retrograde control), the agonistic and antagonistic contributions of adenosine receptors (AR; A_1 and A_{2A}), and the tropomyosin-related kinase B receptor (TrkB) cooperate with mAChRs in the axonal competitive interactions which lead to supernumerary synapse elimination that achieves the optimized monoinnervation of musculoskeletal cells. The metabotropic receptor-driven balance between downstream PKA and PKC activities, coupled to developmentally regulated VGCC, explains much of how nerve terminals with different activities finally progress to their withdrawal or strengthening.

Keywords Postnatal synapse elimination · Axonal competition · Acetylcholine release · Muscarinic acetylcholine receptors · Serine kinases · Calcium channels

Introduction

During the nervous system development, an overproduction of neurons and synapses generates an extensive connectivity that is corrected by an activity-dependent reduction that

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refines the specificity of the neuronal circuit [1–4]. The final specificity is attributable to the appropriate matching between the origin and the target of the nerve fibers. Thus, Hebbian competition between nerve endings with different activities leads to the elimination or strengthening of their synapses [5–7]. This developmental synaptic elimination occurs throughout the nervous system, representing a basic mechanism of sinaptogenesis [2, 8–14].

During development, skeletal myocytes start polyinnervated by several axons [15–17], but after their competitive interactions, neuromuscular junctions (NMJ) finally become innervated by only one axon [6, 13, 14, 18]. There are many reviews about synapse elimination mainly focused on the NMJ [1, 18–27]. Several relevant cues of the molecular and cellular mechanisms involved in the elimination of supernumerary nerve terminals have been investigated and collected in the cited reviews. The non-competitive reduction in the number of nerve endings which a motoneuron can support has been studied and characterized. Moreover, it seems also that there is an initial and continuous exchange of the appropriate nerve terminals to produce homogenous fiber-type motor units. At this motorneuron level, [22, 28] show that neuromuscular synapse elimination was accelerated in mutant mice lacking connexin 40, a developmentally regulated gap junction protein, expressed in motor and other spinal neurons, to facilitate electrical coupling.

A major factor, however, is the level of activity of each nerve terminal in a poliinnervated NMJ, and, on the whole, the axonal loss is retarded at low levels of activity and accelerated at increased levels. During the axonal competition, the various nerve endings in a NMJ have a mutual influence on one another and on the postsynaptic muscle cell and the terminal Schwann cell. In adults, terminal Schwann cells sense the release of ACh and ATP from the nerve through M_1 and A_1 receptors and in turn influence transmitter release [29, 30]. Terminal Schwann cells have been involved also in axonal competition during development. Glial activity induces synaptic potentiation (through presynaptic adenosine 2_A receptors) of strong input in dual junctions but not in weak terminals [31]. Roche et al., [32] using mice lacking Nfasc155, a glial protein detected a delay in postnatal synapse elimination at the NMJ. Moreover, neuregulin-1 signaling between terminal axons and glia during development influences glial cell activation and interposition between the terminal and muscle [23] affecting axon loss. Recently, Jung et al. [33] proposed a model that reproduced synapse elimination showing that synapse elimination is accelerated by increased areas of teloglial cell vacancies. Activity-induced changes in the stability of the muscle cell postsynaptic nAChRs can contribute to reduce the synaptic efficacy of ruled-out nerve terminals [34–36]. Several pre- (calcitonin gene-related peptide, CGRP, [37]) and postsynaptic-derived signals (BDNF, [38, 39]) can also influence supernumerary axonal loss by rewarding or punishing certain nerve terminals. Je et al. [40] using genetic manipulations and pharmacological studies show the involvement of endogenous proBDNF and mBDNF in synapse elimination.

Several presynaptic receptors—mainly muscarinic acetylcholine (ACh) autoreceptors (mAChR), adenosine autoreceptors (AR), and tropomyosin-related kinase B receptor (TrkB)—allow the multiple developing nerve terminals to communicate in the competition that leads to synapse loss in the NMJ [41–43]. This communication can occur directly or with the intermediation of the postsynaptic or Schwann cell components of the tripartite synapse. In particular, mAChRs in the motor terminals seem to permit direct competitive interaction between multiple nerve endings through differential activity-dependent ACh release in the shared narrow developing synaptic cleft. The more active endings may directly punish the less active ones and reward themselves [24, 34, 43–45], and asynchronous activity seems to optimize this interaction to promote synapse elimination [46].

The presence of mAChRs in the motor nerve terminal presynaptic membrane is a clear example of an autocrine mechanism in which a secreted product of a cell can externally influence itself as a feedback modulation both during development and in the adult. Interleukin-2 is another example, being produced by and acting on T lymphocytes themselves (in addition of its paracrine action on target macrophages and other immune cells) [47, 48]. In the case of the cholinergic autoreceptors, the presence of several muscarinic subtype molecules coupled to different downstream pathways can move the autoregulation of the neurosecretion in a broad range between positive and negative effects [49–53]. Because of all these reasons, mAChRs are a relevant component of the complex regulation through pre- and postsynaptic activities of the supernumerary synapse elimination conducting to the optimized monoinnervation of the musculoskeletal system.

We have been working in developmental axonal competition and synapse elimination since the late 1970s (for instance [54, 55]), and the molecular mechanisms are far from being fully elucidated today though many questions have been answered [4, 6, 18]. In recent years, we have studied by immunohistochemistry, intracellular recording electrophysiology, western blotting, subcellular fractionation and co-immunoprecipitation, the involvement of the mAChR subtypes in the mammalian NMJ functionality during development and in the adult [3, 56–61]. In the adult, we characterized how M_1 and M_2 mAChRs regulate the PKA subunits (catalytic and regulatory), the PKC (PKCβI and ε isoforms), and their exocytotic targets (Munc18-1, SNAP-25, and MARCKS phosphorylation) showing a co-dependent balance between muscarinic auto receptors which orchestrates transmitter release regulation [62, 63]. We analysed also the involvement of altered metabotropic receptor

signaling in amyotrophic lateral sclerosis SOD1-G93A mice [64]. Here, we review previously published data to evaluate the relevance of mAChRs during development in synapse elimination and their crosstalk with other receptors, the downstream kinases, and the targeted voltage-gated calcium channels (VGCC).

Our contribution to the understanding of the synapse elimination process can be summarized in this review: (1) the characterization of the presumably most relevant membrane receptors involved, the mAChRs and adenosine receptor subtypes (both allowing a direct paracrine influence between activity-different neighbor competing nerve endings) and the trophic factor receptor TrKB allowing the retrograde influence through BDNF (2) the characterization of the changes in the downstream PKC/PKA ratio (cPKCβI and nPKCε favor axonal retraction) in the competing nerve terminals as a relevant point of the process, and (3) the specific involvement of several VGCC in determining the transmitter release capacity and the retraction of the nerve endings.

mAChR in Developing NMJ

mAChR Subtypes in Transmitter Release During Development

We studied the effect of mAChR subtype modulation—mainly in the rodent *Levator auris longus* muscle (LAL)—by comparing neurotransmission at the newborn stage (P7-P9) versus in the monoinnervated mature NMJs (P30). Axonal elimination almost occurs during the first 2 weeks after birth, although a residual 3% of multiply innervated synapses remains at the end of the first month. The period P7-P9 corresponds to the middle of the axonal loss process and the nerve terminal elimination coincides with the morphological maturation of the postsynaptic component on the NMJ. At birth, nearly all of the NMJs (96%) in the LAL muscle are innervated by more than one axon, and at the end of the first week, the percentage of polyinnervation is reduced to nearly 50%. Newborn P7-P9 neurotransmission study includes developing monoinnervated NMJ and developing polyinnervated ones, mainly dual junctions passing the last process of axonal competition. In the latter, strong and weak nerve terminals can be identified by their evoked endplate potentials (EPP), with the least quantal content originating from the weak terminal in dually-innervated junctions [39, 59, 65–68]. Intracellular recordings using muscarinic agonists and blockers show that some of the mAChR subtypes (M_1, M_2, and M_4) influence ACh release both in developing [58–61] and in adult NMJs [41, 69, 70]. Table 1 shows the list of substances used in the described experiments.

In the adult, the M_1 receptor increases ACh release because its selective block with pirenzepine [PIR] or muscarinic toxin-7 [MT-7] reduces it. On the contrary, the M_2 receptor reduces ACh release because its selective inhibition with methoctramine [MET] or AFX-116 increases it [41, 52, 58, 71]. The M_3 (4-DAMP) and M_4 (tropicamide [TRO] and muscarinic toxin-3 [MT-3]) blockers do not produce any effect in transmitter release. Thus, in the adult, M_1 and M_2 receptors influence ACh secretion in a positive and negative feedback, respectively.

However, during development, all the selective M_1 and M_2 blockers were tested to reduce the release both in the recently monoinnervated NMJ and in the strongest endings of dual junctions (Fig. 1) that are still in the competition at P7-P9. Interestingly, the effect of any M_1 antagonist was not additive with the effect of any M_2 antagonist, suggesting the operation of the same mechanism in both cases (i.e., the effect of PIR is completely prevented by a preincubation with MET and *viceversa*). This suggests the commitment of all muscarinic pathways to promote neurotransmission in these nerve endings during maturation. Nevertheless, in the weakest nerve contact in dual junctions, only the M_2 blockers reduce release whereas M_1 and M_4 blockers increase the EPP (Fig. 1) [39, 58–61]. We sequentially added PIR and TRO to the muscle and found that their respective effects on the weak nerve terminals were additive, showing that M_1- and M_4-mediated pathways are different in these endings. However, like in the strong and in the monoinnervated junctions, the effect of the M_1 and M_2 drugs is still mutually exclusive and not additive. Thus, the two additive M_4 and M_1 mechanisms result in a powerful inhibitory modulation that probably overcomes the M_2 mechanism, which enhances neurotransmission in all kinds of endings during development. Finally, as stated, in the adult monoinnervated NMJs, M_2 and M_1 receptors change their coupling to regulate neurotransmission by negative and positive feedback, respectively.

In summary (Fig. 1), an advantage of the more differentiated nerve terminals seems to be the commitment of all mAChR subtypes (M_1 and M_2 in this case) to enhance ACh release, and using this autocrine mechanism, the strongest endings may reinforce themselves to win the competition. However, the weakest axon may be negatively influenced by ACh release from the strongest axons through the M_1 and M_4 subtype pathways. The described evidence argues in favor of the relevance of local factors explaining, in fact, how an axon that fails at one muscle endplate can win the competition at another [72]. So, a developmentally regulated specific expression of the mAChR subtypes seems to be a relevant mechanism in synaptogenesis.

Table 1 Substances list. Muscarinic, purinergic, TrkB, PKC, PKA substances, calcium channel modulators, calcium ions modulators, and their targets. Stock solutions were prepared using PBS or DMSO in accordance with the commercial product information after preparing the working solution

Substance list					
Muscarinic substances	Abbreviations	Function	Product information	Stock solution	Working solution
Pirenzepine dihydrochloride (PIR)	PIR	M1 antagonist	1071, Tocris Bio	10 mM	10 μM
Methoctramine	MET	M2 antagonist	M105, Sigma- Aldrich	1 mM	1 μM
Muscarinic toxin 3	MT-3	M1 and M4 antagonist	M-140, Alomone	50 μM	100 nM
Muscarinic toxin 7	MT-7	M1 antagonist	Peptides International	50 μM	100 nM
1,1-dimethyl-4-diphenylacetoxypiperidinium iodide	4-DAMP	M3 antagonist	0482, Tocris Bio	100 mM	1 μM
Tropicamide	TRO	M4 antagonist	0909, Tocris Bio	10 mM	1 μM
Purinergic substances					
8-cyclopentyl-1,3-dipropylxanthine	DPCPX	A_1 R antagonist	C101, Sigma-Aldrich	50 mM	100 nM
2-(2-furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1, 2, 4] triazolo[1,5-c] pyrimidin-5-amine (SCH-58261)	SCH-58261	A_{2A} R antagonist	2270, Tocris Bio	100 mM	50 nM
TrkB substance					
Recombinant human trkB/Fc Chimera	trkB-Fc	TrkB receptor-related substance	688-TK, R&D Systems	100 μg/ml	5 μg/m
PKC substances					
Chelerytrine	Che	PKCs antagonist	C-400, Alomone	10 mM	1 μM
Calphostin C	CaC	PKCs antagonist	C6303, Sigma-Aldrich	2.5 mM	200 nM
Peptide βIV_{5-3}	βIV_{5-3}	PKCβI selective antagonist	Mochly Rosen, Standford University	10 mM	10 μM
Peptide εV_{1-2}	εV_{1-2}	PKCε selective antagonist	539,522, Calbiochem	1 mM	10 μM
Bryostatine-1	BRY	PKCs agonist	2283, Tocris Bio	10 μM	1 nM
Phorbol 12-myristate 13-acetate	PMA	PKCs agonist	P1585, Sigma	10 mM	10 nM
12-deoxyphorbol-13-phenylacetate-20-acetate	dPPA	PKCβI selective activator	BML-PE-182–0001, Enzo	1 mg/mL	0.2 μg/mL
2-((2-Pentylcyclopropyl) methyl) cyclopropaneoctanoic acid	FR236924	PKCε selective activator	3091, Tocris Bio	100 mM	100 nM
PKA substances					
N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride	H89	PKA antagonist	19–141, Millipore-Merck	5 mM	5 μM
8-Bromoadenosine-3′,5′ cyclic monophosphorothioate	Rp8	RI-PKA selective antagonist	129,735–00-8, Biolog	5 mM	100 μM
Adenosine-3′,5′-cyclic monophosphorothioate	Rp	RII-PKA selective antagonist	A002S, Biolog	5 mM	100 μM
Adenosine 3',5'-cyclic monophosphorothioate,8-bromo-Sp-isomer	Sp8Br	PKA agonist	116,818 Calbiochem	5 mM	10 μM
Calcium channel modulators					
Nitrendipine (NT)	NT	L-type channel blocker	N144, Sigma-Aldrich	50 mM	1 μM
ω-conotoxin-GVIA	ω-CON	N-type channel blocker	C9915, Calbiochem	1 mM	1 μM
ω-Agatoxin IVA	ω-AGA	P/Q-type channel blocker	STA-500, Alomone	100 nM	100 nM

Table 1 (continued)

Substance list					
Muscarinic substances	Abbreviations	Function	Product information	Stock solution	Working solution
1,4-Dihydro-2,6-dimethyl-5-nitro-4- (2-trifluoro-methylphenyl) pyridine-3-carboxylic acid methyl ester	Bay-K8644	L- type calcium channel agonist	B-350, Alomone	50 mM	5 µM
(2R)-2-[(6-{[(5-Methyl-thiophen-2-yl) methyl] amino}-9-propyl-9H-purin-2-yl)amino]butan-1-ol	GV-58	CaV2.2 and CaV2.1 Ca^{2+} Channels activator	G-140, Alomone	20 mM	20 µM
Calcium ions modulators					
1,2-Bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid tetrakis acetoxymethyl ester	BAPTA-AM	Ca^{2+} chelator	Ab120503, Abcam	10 mM	5 µM

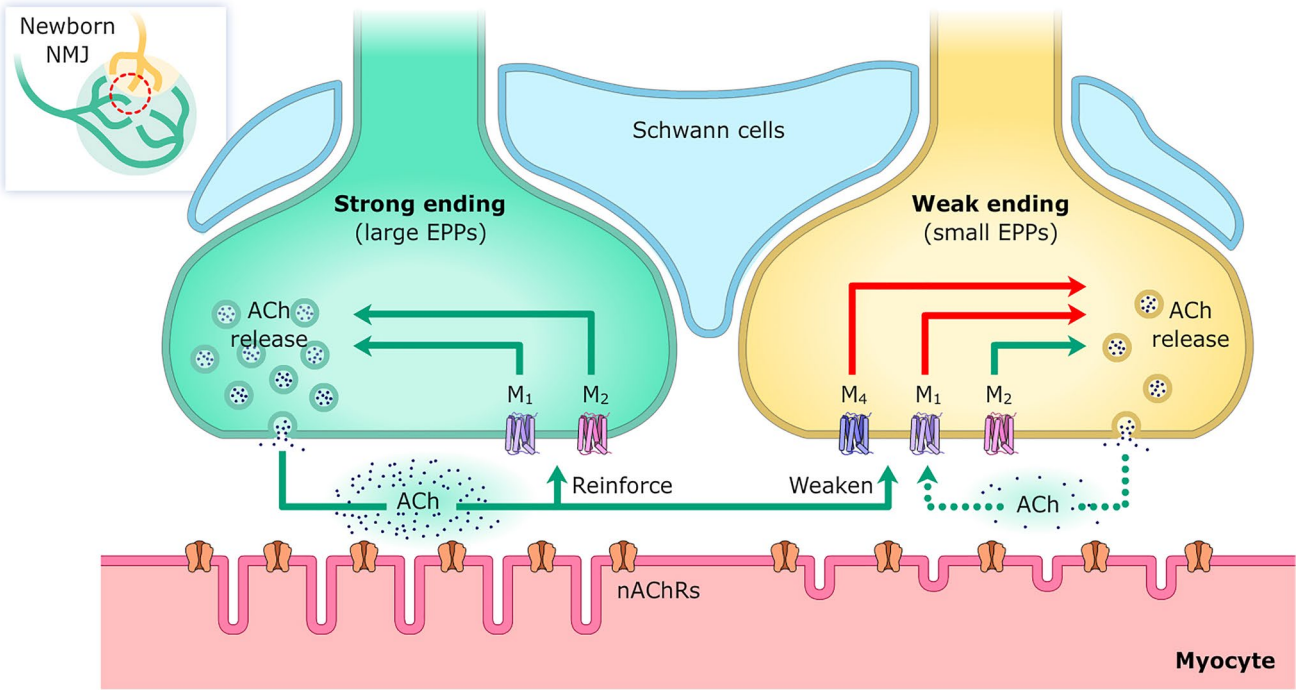

Fig. 1 Representation of two neighboring axon terminals in a dually innervated NMJ during development in the common endplate. The strong ending (that evokes the largest EPP) is in green and the weak one (that evokes the smallest EPP) is in yellow. In these nerve endings, muscarinic receptor subtypes and their coupling to ACh release behave differently. In the strong ending (and also in the single endings at the end of the competitive process), both M_1 and M_2 enhance ACh release. Using this autocrine mechanism, the strongest endings may reinforce themselves to win the competition. In the weak endings, only M_2 stimulates the release, whereas M_1, with the additional involvement of M_4, reduces the EPP size. Thus, the weakest nerve endings may be negatively influenced by the ACh release from the strongest axons. Inside the nerve terminals, green arrows indicate transmitter release potentiation, and red arrows depression. The effects of M_1 and M_4 in weak endings are additive whereas the effects of M_1 and M_2 in the strong ones are not. Nicotinic ACh receptors (nAChRs) are represented in the postsynaptic membrane

mAChR in the Physical Withdrawal of Supernumerary Axons

How the described evidence of the developmental muscarinic modulation of ACh release can be related with supernumerary axon elimination from the NMJ? We favor the hypothesis of the final strengthening and consolidation of the strongest endings in dual junctions because their molecular and functional similarity with the solitary endings in the most mature NMJ. Thus, both M_1 and M_2 receptors are coupled to potentiate release in these endings along with the expression in these nerve terminals of a more differentiated VGCC stoichiometry and serine-threonine kinases coupling to ACh release (see later). To investigate this, we made 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 [43]. Muscles were processed to detect also the postsynaptic nicotinic acetylcholine receptors (nAChRs) with TRITC-α-BTX (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 the considered muscarinic substance [3, 56, 57].

In P7 mice, we observed that when M_1 or M_4 receptors are selectively blocked by PIR or MT3, axonal loss is accelerated (but not when M_2 is blocked with MET). Thus, at P7 (considering the effect on neurotransmission of the muscarinic receptors, see Fig. 1), M_2 favors ACh release and possibly the competitive force, related with more transmitter release, in all axons although not affecting the axonal elimination rate. However, M_1 increases release in the strong axon and decreases it in the weak one (together with M4 in this case), and these tonic effects resulting in a delay in axon loss (evidenced by a conspicuous acceleration when M_1 or M_4 receptors are selectively blocked) [43]. We do not know which one can be the prevalence of any of these muscarinic receptors in the different nerve endings at this period, but it is conceivable that mAChR subtypes would participate and even be involved in determining competitive interactions rather than speeding up axonal elimination around P7 [3]. Thus, it appears that at P7, mAChRs-mediated competitive axonal interactions (and also interactions mediated by AR and TrkB receptors—see later) are taking place with the result of an initial delay in synapse elimination because effective axon loss is not yet occurring in most synapses at this moment.

Two days later (P9), the continued exposition to PIR or MET (but not to the M_4 blocker MT3) for 4 days results in a clear delay in axon loss. This indicates that both M_1 and M_2 receptors acquire during this period the role of promoting the full sequence of axonal elimination. Interestingly, MET has a greater ability than PIR to delay the final monoinnervation, indicating the powerful effect of the M_2 on axon loss at P9, probably potentiating the strongest nerve endings [43]. Joint inhibition of M_2 and M_1 (MET + PIR) pathways show that their effect on axonal elimination is not additive, suggesting a shared downstream mechanism at this developmental stage (see below) and the commitment of all muscarinic receptors to promote axon elimination. In the adult, mAChRs show some G protein promiscuity [73, 74] suggesting that M_2–M_1 shared developmental mechanism may relate with G protein sharing. The effect on the ACh release of these two receptors may reinforce the increasingly stronger endings and be detrimental to the weak ones. The presence of the M_4 mAChR subtype in the weakest ending at P7 and their functional disappearance at P9 along with the shift of the M_1 function (from ACh release reduction in the weakest endings to favoring ACh release in the endings that become stronger) can be important changes in synapse elimination. Interestingly, the progressive change in M_4 and M_1 function during NMJ maturation coincides with the slightly later shift of the M_2 function that will change to negatively modulate ACh release around P15 and for the rest of the adult stage [43]. Therefore, the functional shift of all mAChR types during the maturation of the neuromuscular synapses argues in favor of their relevance in this process. The shift mechanism must be able to explain how M2 changes from positive to negative action on ACh release whereas M_1 changes reciprocally. The possibility that a change in the expression (protein level) of the M_1 and M_2 mAChRs themselves may contribute to explain their functional change needs to be seriously considered. However, the movement of both M_1 and M_2 receptors between the extreme positive and negative influences on ACh release during maturation raises some concerns about the possible relevance of the protein level change to *fully* explain these extreme changes in their downstream coupling. Alternatively, as stated, these metabotropic receptors are GPCR, and a developmentally regulated displacement of their coupling between Gs and Gi proteins seems to be an attractive hypothesis. This mechanism may be facilitated because several GPCR function within lipid raft plasma membrane microdomains, which may be important for regulating their signal transduction. In a previous study in the mature NMJ, however [75], we show that the disruption of lipid rafts (methyl-beta-cyclodextrin, 2%) does not change the normal coupling and mutual relations of adenosine receptors and mAChRs on ACh release.

Relation of the mAChR with AR (A_1, and A_{2A}) and TrkB Receptor on Developmental Axonal Elimination

Even the continued application of M_1 and M_2 inhibitors cannot stop axonal loss, which is completed around P15 [43].

This suggests the complex involvement of multiple other pathways including postsynaptic-derived factors [39, 43, 56, 57]. We found that, in addition to the presynaptic mAChR M_1, M_2, and M_4, at least adenosine receptors (AR; A_1 and A_{2A}) and the tropomyosin-related kinase B receptor (TrkB), cooperate in synapse elimination [56, 57]. It seems that this multiple signaling would define the conditioning factors of the axonal competition and thus, the final fate of the individual nerve terminals. However, the achievement of the monoinnervated NMJ may be constitutively regulated.

Concerning to transmitter release, AR is present in the motor terminals of the newborn and adult NMJs [76, 77] and, during development, released adenosine from different components of the synapse may activate both A_1R and $A_{2A}R$ and have a facilitatory action on ACh release. Unfortunately, we do not know the specific involvement of these receptors in the strong and weak nerve endings. Neurotrophins and their receptors are also expressed in both development and adulthood [78–83]. Low doses of BDNF rapidly induce a TrkB-dependent potentiation at developing NMJs in culture [84] and, in ex vivo developing NMJ, BDNF increases ACh release in both the weak and strong endings around P7 [68]).

Concerning to developmental axonal elimination, specific inhibitors reveal that both AR delay axonal loss at P7 but accelerate it at P9. This effect is similar to that of mAChRs. The BDNF-TrkB pathway also plays a biphasic role because BDNF initially delays elimination and subsequently accelerates it at P9 [3]. Thus, several metabotropic receptors overlap and share the common function of modulating a major mechanism of synaptogenesis as can be the definition of the final matching of the synaptic partners. Interestingly, for all receptors, an initial delay in axonal elimination observed at P7 is followed by the acceleration at P9, pointing to the existence of a multifactorial and redundant mechanism aimed at ensuring the specific NMJ monoinnervation. For instance, all receptors (except M_4) directly accelerate axonal loss at P9. Ranked according to their importance (from more to less), these are M_2-M_1-A_1-A_{2A}-TrkB [3].

Given the observed downstream shared effect of these receptors, we simultaneously applied two selective antagonists to reveal the cooperation between mAChR, AR, and TrkB receptors and the possible additive (synergistic) or occlusive (antagonic) crosstalk between them (Fig. 2) [4, 43, 56, 57]. Studying the adult NMJ, we identified several links between purinergic receptors and mAChRs and

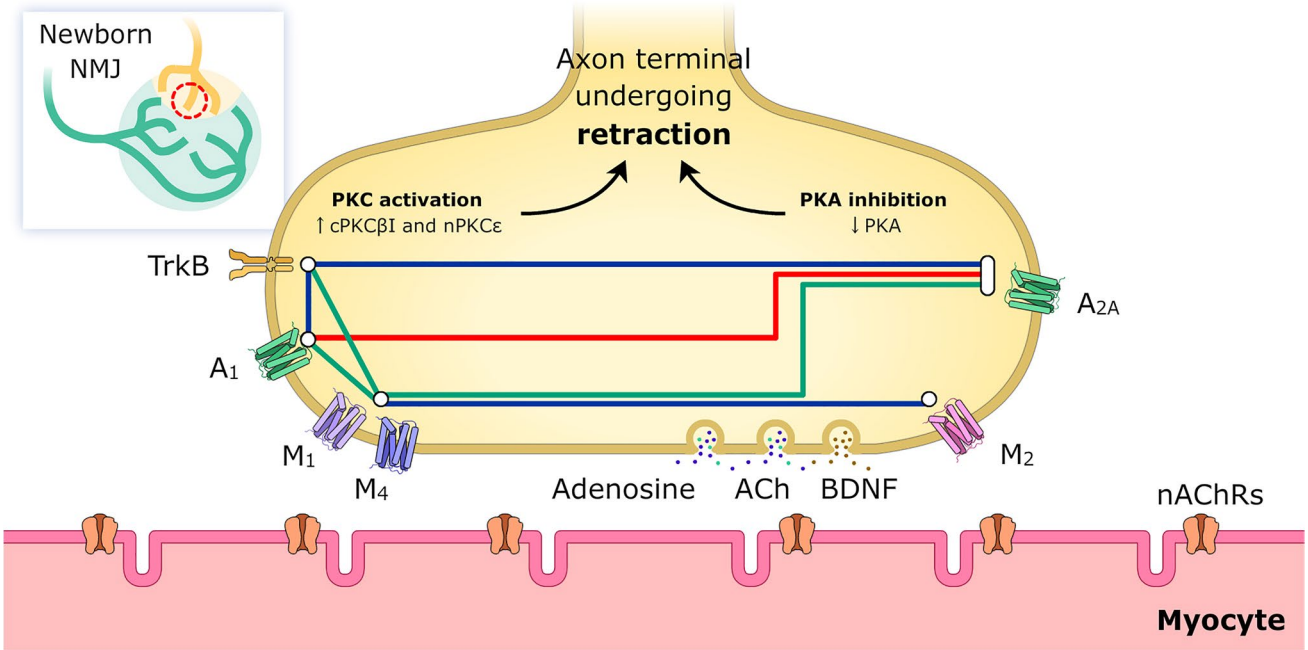

Fig. 2 Retracting axon terminal around P9. Metabotropic receptors at the left have downstream pathways linked to PKC activation (M_1, A_1, and TrkB; M_4 is included here because its effect is similar to the M_1 effect, see the text). The PKC isoforms in the NMJ presynaptic component are cPKCβI and nPKCε. Receptors at the right are linked with PKA inhibition (M_2 and A_{2A}). The individual action of all these six receptors (M_4 indirectly) promotes or accelerates axonal retraction and loss. Thus, a metabotropic receptor-driven balance between PKA and PKC activities regulates axonal withdrawal. Synergistic or antagonistic crosstalk between mAChR, AR, and TrkB can be revealed by inhibiting two receptors at a time. In the figure, the receptors that are related with a blue link (M_1/M_4 with M_2, A_1 with TrkB, and TrkB with A_{2A}) seem to share the same pathway because their dual inhibition produces the same effect as their individual inhibition over axon loss. On the other hand, M_1/M_4 show a synergistic additive behavior (green link) with TrkB, A_1, and A_{2A}. Finally, both AR, A_1, and A_{2A} show an antagonistic relationship and are mutually occlusive (red link)

found that the functional integrity of mAChRs coupling to the neurotransmission depends on normal purinergic receptors operation. This indicates the clear interaction between both receptor families in the adult [75]. In the newborn, the main results show a synergistic role of the M_1 mAChR, which potentiates the effect of both AR (A_1, 58% and A_{2A} 36%) and TrkB (25%) on axonal elimination. On the other hand, though the M_4 subtype is not directly involved in axonal loss as previously stated, it strongly potentiates the effect of AR (A_1, 33% and A_{2A} 32%) and TrkB (23%) thus acting similarly as to the M_1 receptor. Interestingly, a comparable effect of M_1 and M_4 is observed on the ACh release capacity of the weakest nerve terminals in dual junctions as shown above (see also Fig. 1). However, as previously stated, M_2 has the most powerful effect on axon loss and the inhibition of both AR or TrkB receptor does not affect their function. When the TrkB inhibitor TrkB-Fc is associated with one of the AR inhibitors (DPCPX for A_1, or SCH58261 for A_{2A}), the final effect is just the same as the individual effect of one of them on axon loss. When both ARs are blocked simultaneously, occlusion is complete, and the final result is no different from that of the untreated control [57].

Thus, taking these data into consideration, we represented in Fig. 2 the observed relations of the considered receptors that modulate developmental supernumerary axonal loss. In Fig. 2, the receptors that are related with a blue link (M_1/M_4 with M_2, A_1 with TrkB, and TrkB with A_{2A}) seem to share the same pathway because their dual inhibition produces the same effect as their individual inhibition over axon loss. On the other hand, M_1/M_4 show a synergistic additive behavior (green link) with TrkB, A_1, and A_{2A}. Finally, both AR, A_1, and A_{2A} show an antagonistic relationship and are mutually occlusive (red link). All these receptors are involved in promoting supernumerary axonal elimination, and we investigate their downstream links.

mAChR Coupling to Serine Kinases During Synapse Elimination

The mAChR downstream signaling converges in intracellular effector kinases, mainly the serine-threonine protein kinases A and C (PKA and PKC), which phosphorylate targets involved in synaptic function and axon loss. Receptors and kinases may coordinately regulate the developmental synapse elimination. Generally, M_1 operates by stimulating PKC whereas M_2 and M_4 inhibit PKA. It is known that, in most cells, A_1, M_1, and TrkB operate mainly by stimulating the phospholipase C (PLC) and, therefore, PKC and the inositol triphosphate (IP_3) pathway, whereas A_{2A}, M_2, and M_4 inhibit the adenyl cyclase (AC) and PKA pathway [50, 51, 85, 86].

At the left of Fig. 2, we represented the receptors coupled to activate PKC (M_1, A_1, and TrkB—we added M_4 due to a connection with M_1—), and on the right, those that downregulate PKA (M_2 and A_{2A}), and we investigated the hypothesis of the reciprocal involvement of these kinases in synapse elimination. In dually innervated, developing NMJs, the block of PKC (for instance, with calphostin C or chelerytrine) increases ACh release from the weakest nerve terminals (roughly 80%) but does not change ACh release from the strong nerve terminal or even from the more mature monoinnervated junctions. Moreover, after blocking PKC, the mean number of functional axon terminals per synapse increases by about 50%, indicating some recruitment of silent synapses at this time that are probably in the process of disconnection [39, 58–60, 67, 87]. Thus, PKC is involved in reducing neurotransmission in certain weak nerve endings which may facilitate axonal elimination. Recently, we found [88] that PKC favors axon loss through cPKCβI and nPKCε isoform activity (as judging by the effect of their general [Bry-1 or PMA] and specific activators [dPPA, FR236924] and inhibitors [βIV_{5-3} and εV_{1-2}] respectively) whereas PKA-I and II activity (as judging by the effect of their specific blockers [H-89, Rp8-Br, and Rp-cAMPs] and activator [Sp8Br], respectively) delay axonal loss in P9 mice. Furthermore, no significant differences exist between the effects of PKA activators and PKC inhibitors, or between PKA inhibitors and PKC activators, on changing axon loss rate [25]. Moreover, a similar level of PKA inhibition and PKC potentiation (mainly of the cPKCβI and nPKCε isoforms that are strictly localized on the presynaptic site [89–91]) seems to be required to advance in axonal loss, clearly suggesting the complementarity of these kinases. On the contrary, the increase of the PKA activity, the reduction of the PKC activity, or, in most cases, both situations simultaneously can reduce synapse elimination [57]. Thus, a metabotropic receptor-driven balance between PKA and PKC activities seems to be involved in synapse elimination and axonal withdrawal as represented in Fig. 2.

It is known that reduction of the postsynaptic activity or contraction results in a delay in synapse elimination during NMJ development [18, 92–95]. In line with this, we made experiments blocking the muscle cell's contractile activity with μ-conotoxin GIIIB which blocks muscle cell sodium channel but preserves neurotransmission because does not influence the nAChR [26]. Accordingly, incubation with μ-conotoxin GIIIB also results in a delay in axon loss. Thus, a contractile activity-related retrograde influence from the postsynaptic site may contribute to the synapse elimination. The simultaneous application of one presynaptic cPKCβI or nPKCε activator and μ-conotoxin GIIIB fully prevents the postsynaptic contraction block effect on axon loss. Thus, the axonal loss can be altered by acting directly in presynaptic targets (and receptors like mAChRs). Possibly, the

above-cited presynaptic PKCs may be modulated by retrograde control (for instance, through BDNF production). This argues in favor of a complex regulation through pre- and post-synaptic activity of the serine-threonine kinases as mediators of the synapse elimination. The regulation of these kinases by mAChR and neurotrophic receptors affecting their phosphorylating activity on targets of the exocytotic vesicular release apparatus (as synapsin I and the SNARE/SM proteins Munc18-1 and SNAP-25) has been described by us in the adult NMJ [96, 97].

mAChR Coupling to Calcium Channels During Synapse Elimination

mAChR and Calcium Channels in Transmitter Release During Development

During development, P/Q, N, and α1D-L subtypes of the VGCC are present in the nerve terminals on the LAL muscle. The protein expression of all these channels increases during the development P5-P7 time period. Western blots at P30 show that the P/Q level is at its highest whereas α1D-L and N channel proteins stabilize at a lower level [26].

In dually innervated fibers during NMJ maturation (P7-P9), the block of any of these three VGCC reduces about 2/3 of the EPP produced by the strongest ending [65, 66], indicating the multichannel dependence of calcium entry to promote ACh release in these endings. In the early monoinnervated endplates, the P/Q-type channel blocker ω-Aga-IVA and the N-type blocker ω-CgTx-GVIA still reduce the EPP amplitude (~ 80% and ~ 60%, respectively) whereas the L-type blocker nitrendipine does not anymore. Finally, in the adult (at P30), only the P/Q-type VGCC functionally persists being the only one that, when blocked, strongly inhibits ACh release [69]. However, in the weak ending of dual NMJs, the block of any VGCC channel results in an increase of the size of the evoked EPP, indicating that a part of the calcium entry through all channels can negatively influence transmitter release and even may contribute to disconnect these endings [65, 66].

Figure 3 shows that in the strongest endings, there is a differential coupling of the calcium channels with the M_1 and the M_2 receptors. M_1 receptors need the P/Q- and the L-types VGCC whereas the M_2 effect needs the P/Q- and

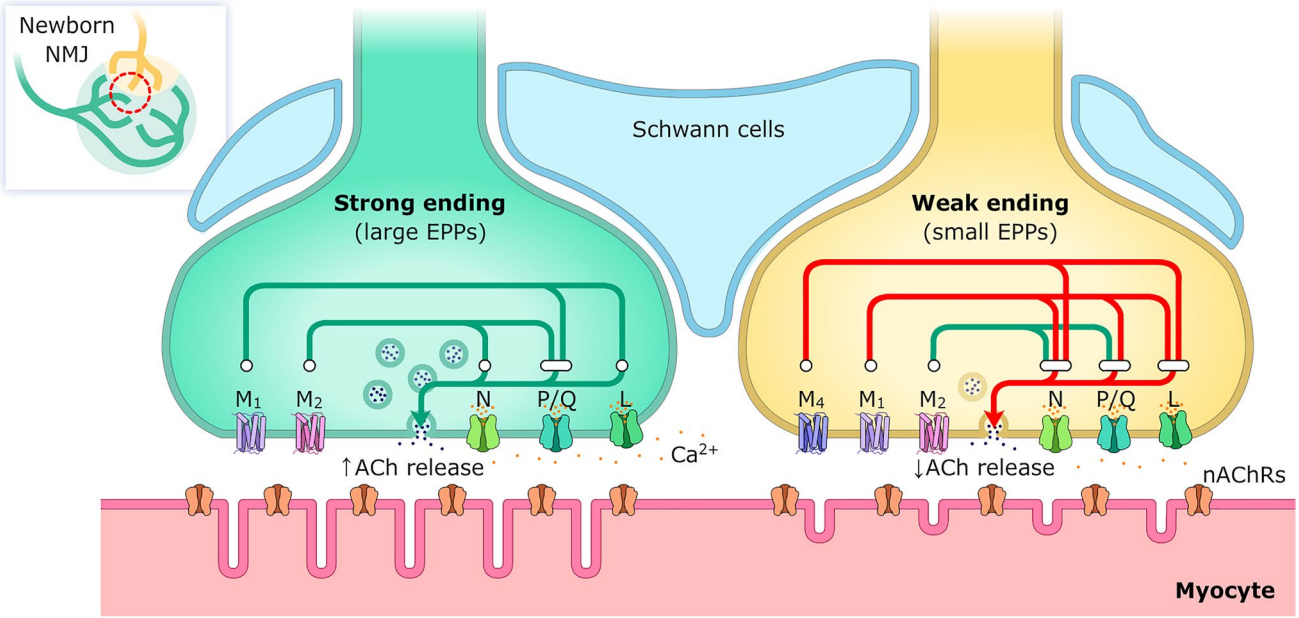

Fig. 3 Differential coupling of VGCC and mAChRs in the strong and weak endings in a common endplate. The effect of the mAChR and VGCC on ACh release is shown in green (potentiation) or red (depression). The links between receptors and channels indicate the mutual dependence between these molecules to produce the final effect. In the strongest nerve terminal (shadowed in green), M_1 receptors need the P/Q- and the L-type VGCC, whereas the M_2 need the P/Q- and N-type VGCC to potentiate ACh release. The weak nerve ending (in yellow) is represented with the mAChR (M_1, M_2, and M_4) and the VGCC (P/Q, N, and L) subtypes that are operative in these endings. The M_2 function (also potentiation in these endings), similarly depends on P/Q- and N-type channels, but not on the L-type channel. A leading role for the L channel in axonal loss seems to emerge because of their unique coupling to M_1 and M_4 mAChRs, which clearly reduces neurotransmission in the weak nerve terminals presumably in process of elimination. Moreover, the coupling of all muscarinic and metabotropic receptors studied to promote axonal elimination at P9 had a multichannel dependence (P/Q- and L-type) with a relevant role of the L channel

N-types VGCC. As previously stated, in these strongest ending in dually-innervated synapses, both M_1 and M_2 mAChR have an ACh release potentiating effect (see Fig. 1). In the monoinnervated junctions, the ACh release potentiating effect of both M_1 and M_2 mAChR relies only on the P/Q-type VDCC because the effect of the receptors is occluded only when this channel is inhibited, even in the presence of high Ca^{2+} concentration [59]. However, in the weak endings, the function of tropicamide-sensitive M_4 mAChRs did not depend on the P/Q-type VDCCs, although it did depend on the normal function of the L- and N-type channel [39, 58, 59, 61]. In the same weak endings, the pirenzepine-sensitive M_1 mAChRs function had multichannel dependence (P/Q-, N-, and L-types), and the methoctramine-sensitive M_2 function also had a multichannel dependence (P/Q- and N-type channels but not the L-type channel). As previously stated, in the weakest nerve contact in dual junctions, only the M_2 has an ACh release potentiating effect whereas M_1 and M_4 reduce release (Fig. 1).

Thus, results indicate that the nerve ending that becomes strong during competition uses a specific coupling (different from the adult) of the M_1 and M_2 mAChR with a broad (the three VGCC are involved), well-defined VGCC stoichiometry that favors ACh release. This configuration courses with the maintenance of these axon terminals. On the contrary, it seems that in the weakest nerve ending, the M_2 release potentiating effect (linked to P/Q and N VGCC) is surpassed by the M_1/M_4 effect (linked to the three VGCCs) that depresses ACh release and favors axon elimination.

mAChR and Calcium Channels in the Withdrawal of Supernumerary Axons

The L and P/Q-type (but not the N-type) channels tonically enhance synapse elimination because their block prolongs the multiinnervation of the developing NMJ, whereas their exogenous stimulation (Bay-K8644 for L channel and GV-58 for P/Q channels) results in a significant acceleration [26]. Moreover, the delaying effect on axon loss of the L and P/Q channels block is equal to that produced by intracellular calcium sequestration with BAPTA-AM. Thus, the calcium entry through these operative channels present in immature nerve endings results in their final loss. The $[Ca^{2+}]_i$ increase contributes both to transmitter release reduction in certain weak axons (as shown above) as well as nerve terminal loss, and this coincidence argues in favor of a shared mechanism relating transmitter release and axonal competition. It is tempting to speculate about the involvement in the neurite retraction or growth during axonal competition of the differentially expressed Ca(2+) sensor proteins [NCS-1, Ca(2+)/calmodulin and several neuro-specific calmodulin-like Ca(2+) sensor proteins as CaBP1] [98–100]. There is a clear relation between PKA, PKC, and VGCC for developmental axonal loss and synapse elimination. The result after the block of the L-channel (and also after intracellular calcium sequestration) is the same as the inhibition of cPKCβI [25] and from stimulation of PKA [88]. However, the inhibition of the nPKCε produces a greater delay in synapse elimination than the L or P/Q channel block or calcium sequestration. This suggests a VGCC-independent component of the PKC-induced enhancement of axonal withdrawal. In adult NMJ, we have seen that nPKCε promotes the phosphorylation of SNARE/SM proteins Munc18-1 and SNAP-25 in an activity-dependent manner [96, 97]. Similar to the block of L channels, the block of P/Q-type channels produces retardation in axonal loss similar to that observed after cPKCβI inhibition. However, the effect of the P/Q block in delaying axonal loss is smaller than PKA activation, suggesting the relevant involvement of the PKA activity in axonal stabilization [25].

Concluding Remarks

Axonal competition for synaptic sites is a basic development process that is regulated to achieve optimal connectivity during neurogenesis. In the NMJ, supernumerary axon loss leads to the optimized monoinnervation of the voluntary muscle cells. This process involves activity-dependent autocrine, paracrine (between neighbor nerve terminals), and retrograde (from muscle cells) signalings impacting on the competing nerve terminals. The metabotropic mAChR (M_1, M_4, and M_2 subtypes), purinergic receptors (A_1 and A_{2A}), and TrkB receptors ensure downstream changes in the balance between PKA (favors axonal strengthening) and presynaptic PKC isoforms (cPKCβI and nPKCε favor axonal retraction) activities. mAChRs and kinases pathways differentially couple to P/Q, N, and L subtypes of the VGCC to differently modulate ACh release in the developing nerve terminals (for instance, the strong and weak endings in a dual junction at a given moment during competition). Moreover, calcium inflow through L- and P/Q-type channels could affect nerve terminals depending on their activity, leading to their final withdrawal or strengthening.

Beyond the analyzed competitive interactions between the multiple axons, several uncertainties persist in the understanding of developmental synapse elimination as, for instance, the mechanism of functionality shifting of muscarinic receptors (and other receptors such as adenosine receptors) or the mechanism of calcium-induced retraction of the ruled-out axons along with the molecular mechanism of rewarding strong endings. The described findings contribute to understanding several aspects of the punishment-rewarding interactions between nerve endings and the contribution of postsynaptic retrograde involvement. It can be stated, however, that the inhibition of any one of these

pathways only changes the rate of axonal elimination that finally is completed about 2–3 weeks postnatal indicating the complex multifactorial nature of the process. We think that the high number of molecules and different pathways in the cholinergic peripheral NMJ that is directed to the common objective of supernumerary synapse elimination suggests that some of these molecules may contribute to the same function in other neuronal systems. It seems that the multifactorial mechanism works with precision, though an alteration in many possible points may allow malfunctioning of receptors signaling, kinases ratio, or calcium channel balance resulting in the persistence of multiinnervation. In fact, this alteration has been suggested and even has been shown in a number of diseases such as autistic spectrum disorder (ASD) [101–104].

Abbreviations *ACh*: Acetylcholine; *AR*: Adenosine autoreceptors; *αBTX*: α-BungarotoxinBDNF, brain-derived neurotrophic factor; *EPP*: Evoked endplate potentials; *LAL*: Levator auris longus muscle; *nAChRs*: Nicotinic acetylcholine receptors; *mAChR*: Muscarinic acetylcholine receptor; M_1: M_1-type muscarinic acetylcholine receptor; M_2: M_2-type muscarinic acetylcholine receptor; M_4: M_4-type muscarinic acetylcholine receptor; *MET*: Methoctramine; *NMJ*: Neuromuscular junction; *P7*: Postnatal day 7; *P9*: Postnatal day 9; *P15*: Postnatal day 15; *PIR*: Pirenzepine; *PLC*: Phospholipase C; *PKA*: Protein kinase A; *PKC*: Protein kinase C; *TrkB*: Tropomyosin-related kinase B receptor; *TrkB-Fc*: Inhibitor recombinant human TrkB-Fc Chimera; *TRO*: Tropicamide; *VGCC*: Voltage-gated calcium channels

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Data Availability We believe that our data are not appropriate for the repository databases available in neuroscience.

Declarations

Ethics Approval The mice were cared for in accordance with the guidelines of the European Community's Council Directive of 24 November 1986 (86/609/EEC) for the humane treatment of laboratory animals. All experiments on animals have been reviewed and approved by the Animal Research Committee of the Universitat Rovira i Virgili (Reference Number: 0233).

Consent to Participate Not applicable to that section.

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