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Protective effects of grape seed procyanidin extract on neurotrophic and muscarinic signaling pathways in the aging neuromuscular junction†

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At the neuromuscular junction (NMJ), which coordinates movement, postsynaptic-derived neurotrophic factors have neuroprotective functions and retrogradely regulate the exocytotic machinery involved in neurotransmitter release. In parallel, presynaptic autocrine muscarinic signaling plays a fundamental modulatory role in this synapse. We previously found that these signaling pathways are impaired in the aged neuromuscular system. In this follow-up study, we investigated an anti-aging strategy using grape seed procyanidin extract (GSPE), a common dietary antioxidant known for its neuroprotective properties in various pathologies, but its effects on the aged neuromuscular system remain unexplored. This study analyses whether GSPE can mitigate age-associated impairments in neurotrophic and muscarinic signaling within the neuromuscular system. We assessed the expression (protein levels) and activation (phosphorylation) of the key proteins in the brain-derived-neurotrophic-factor (BDNF)/neurotrophin 4 (NT-4) and muscarinic pathways in the *extensor digitorum longus* (EDL) muscles of aged rats, with comparisons to GSPE-treated aged rats and young controls. The results demonstrate that GSPE treatment prevents the most relevant aging-induced changes in neurotrophic and muscarinic receptor isoforms, downstream protein kinases, and their targets in the neurotransmitter exocytotic machinery. Nevertheless, GSPE was less effective at preventing alterations in some other proteins within these pathways, such as calcium channels, and did not modify several other molecules involved in these pathways, which remain unchanged during aging. Overall, this study highlights the neuroprotective potential of GSPE in preventing fundamental age-related molecular changes at the NMJ, which helps improve functionality and may increase the quality of life and lifespan in aged individuals.

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Introduction

Aging is associated with progressive muscle mass and strength loss and a decline in neurophysiological functions. The NMJ plays a key role in this musculoskeletal impairment that occurs with aging. Its integrity is a major determinant of how a muscle responds to age-related perturbations. Aged NMJs lose their stability, and the bidirectional communication between nerves and muscles, which is fundamental for their health, is partially disrupted. Thus, aging provides a molecular cue that triggers the NMJ to undergo morphological, functional, and molecular changes, ultimately leading to degeneration.[1–4]

Although the molecular mechanisms behind its age-associated deregulation remain largely unknown, several signaling pathways that control neurotransmission are involved. The BDNF and NT-4 acting through their receptors, tropomyosin-related kinase-B (TrkB) and neurotrophic receptor p75 ($p75^{NTR}$), are well known for their neuroprotective functions, maintaining neurons and synapses in the brain[5] and the neuromuscular system[6,7] and contributing to neuromuscular neurotransmission. This signaling pathway enhances presyn-

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aptic downstream serine-threonine protein kinases (mitogen-activated protein kinase (MAPK), protein kinase C (PKC) and protein kinase A (PKA) isozymes) to maintain and regulate presynaptic soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE)-Sec1/Munc like (SM) exocytotic proteins such as synaptosomal-associated protein 25 (SNAP-25) and mammalian uncoordinated-18-1 (Munc18-1)[7–10] and therefore transmitter release. The full BDNF-NT4/TrkB-p75^{NTR}/PKC-PKA/SNARE-SM pathway is essential to preserve the stability and functionality of the neuromuscular synapse[11] and is perturbed with aging.[3,12,13]

Interacting with the retrograde neurotrophic regulation, neurotransmission at the NMJ is also modulated by presynaptic muscarinic receptors (M1 and M2).[8,14,15] This autocrine pathway is crucial for balancing neuronal signaling since M1 and M2 can enhance and decrease, respectively, acetylcholine (ACh) release by regulating the phosphorylation of synaptic targets present in the SNARE-SM complex, which control synaptic vesicle docking and fusion with the membrane.[16,17] Furthermore, two other important factors that regulate neurotransmission are voltage-gated calcium channels (VGCC) and the ACh reuptake mechanism, which, together, complete the synaptic vesicle cycle. These processes are essential for replenishing ACh reservoirs inside vesicles and for collecting and maintaining these vesicles near the presynaptic membrane. Recently, we have shown that aging has an impact on these pathways.[3] Therefore, therapeutic strategies for recovering these signaling pathways should improve NMJ functionality, and this may increase the life quality and lifespan of aged individuals.

Increased oxidative stress levels are observed in aged muscle, and reactive oxygen species (ROS) accumulation has been suggested to play a role in muscle changes and sarcopenia.[18–20] It is well documented that the interaction of ROS with proteins results in their oxidative modification, leading to structural alterations, loss of physiological function, and the accumulation and aggregation of damaged proteins.[21,22] Therefore, strategies that counteract oxidation would be useful in reducing the molecular deterioration caused by aging.[23–29] With increasing evidence of dietary influences on healthy functional living in aging,[23,30–33] specific dietary nutrients have been shown to be beneficial in preventing or attenuating the age-related decline in muscle and physical function through ROS buffering.[34,35] Polyphenols have been suggested to have a therapeutic effect in neurodegenerative diseases[36–38] and aging.[23,32,39,40] Recently, it has been demonstrated that procyanidins from GSPE as dietary supplements act as satiating agents in young healthy[41] and aged rats,[39] resulting in a decreased body weight after treatment. Therefore, apart from what was previously mentioned, its beneficial effects might be related to its caloric restriction mimetic effect.

The most abundant polyphenols are found in grapes, apples, red grape juice, red wine and chocolate.[42,43] GSPE contains a mix of polyphenols and has been widely studied because of its protective effects in different pathologies.[43–45] However, its effectiveness on aging processes in the neuromuscular system has not been proven.

The current study analyses whether procyanidins from GSPE can prevent the previously described age-associated neurotrophic and muscarinic signaling impairment in the neuromuscular system. The study of the relationship between nutrition and neuromuscular system health is an innovative area that is poorly explored but offers promising and emerging anti-aging therapeutic strategies.

Materials and methods

Animal model

Female Wistar rats obtained from Envigo (Barcelona, Spain) were used for this study.

We evaluated the EDL muscle from 24 month-old rats separated into two groups, one treated with GSPE and the other administered vehicular tap water. Aged rats from both groups weighed 350–400 grams with no significant difference between groups. They were housed individually at a room temperature of 23 °C with a standard 12 h light–dark cycle, ventilation and *ad libitum* access to a standard chow diet and tap water. The selection of female rats was based on methodological consistency and scientific rationale. While some studies have reported sex-based differences in NMJ morphology, these variations are muscle-specific and not consistently observed across all muscle types.[46,47] Our previous research has not identified significant sex-related differences in the molecular pathways regulating neurotransmission.[16] Additionally, to adhere to the ethical 'four Rs' principles, Reduction, Refinement, Replacement and Responsibility,[48] the use of female rats aligns with our collaboration with another research group specializing in metabolic pathways, where female rodents are routinely used due to their stronger adiposity response to dietary interventions. This procedure was approved by the Experimental Animal Ethics Committee of the Generalitat de Catalunya, Spain (Department of Territory and Sustainability, General Directorate for Environmental and Natural Policy, project authorization code: 10183). For each type of experimental condition, at least three animals ($n \geq 3$) were used as a biological iteration.

Video processing for rat behavior analysis

The video monitoring of rats' behavior was performed for one hour per day for 7 days. Two sessions of recording were conducted: morning video monitoring (between 8 a.m. and 10 a.m.) and afternoon video monitoring (between 12 p.m. and 14 p.m.). We used a video camera, Sony Handycam HD, for recording the rats' activity. It allowed the monitoring of several cages of rats at the same time. To estimate the activity of each group of rats, the zoom function was used for each frame cage. To differentiate the types of activity, 6 patterns of behavior were assigned: sitting, sleeping, actively moving, eating/drinking, smelling, and grooming.

Proanthocyanidin extract

GSPE was obtained from Les Dérivés Résiniques et Terpéniques (Dax, France). According to the manufacturer, the GSPE used in this study (lot 207100) had a total proanthocyanidin content of 76.9% consisting of a mixture flavan-3-ol monomers (23.1%), dimers (21.7%), trimers (21.6%), tetramers (22.2%) and pentamers (11.4%).

GSPE treatment

The protocols of GSPE administration have been extensively tested and published.[39,49–52]

After a week of adaptation to the environment and another week of adaptation to oral gavage, rats were weighed and divided into three experimental groups: young, aged, and aged GSPE groups.

To assess the long-term effects of GSPE in aged rats, it was orally administered for 10 days at a dose of 500 mg kg^{-1}. During these 10 days, aged animals (aged GSPE group) were fasted starting from 15:00 h. The GSPE was dissolved in tap water and orally gavaged to the aged GSPE animals at a dose of 500 mg GSPE per kg of body weight at 18:00 h, one hour before the onset of darkness. Animals in the other group (aged) received an equivalent volume of tap water at the same time points. The diet was administered at the onset of darkness (19:00 h), and the intake was measured after 20 h, the next day at 15:00 h, when the animals were fasted again. At the beginning and end of the 10-day treatment, the rats were weighed. After the treatment, animals were maintained for 75 more days on a chow diet, and body weights were recorded (Table 1).

The dose of 500 mg GSPE per kg was chosen due to its effects on the modulation of the enteroendocrine system observed after acute treatments and in standard-fed rats.[53,54] This dose corresponds to 81 mg per kg bw in adult humans, when considering the body surface area according to Reagan-Shaw *et al.*[55] This is a dose achievable through supplements.

Sample processing

Whole cell lysate. At the end of the study, the animals were fasted for 12 h and euthanized by decapitation, and then, EDL muscles were extracted and deep-frozen using liquid nitrogen. To perform the western blot technique, muscles were homogenized using a VWR VDI 12 homogenizer in ice-cold lysis buffer (in mM: NaCl 150, Tris-HCl (pH 7.4) 50, ethylenediaminetetraacetic acid (EDTA) 1, sodium fluoride (NaF) 50, phenylmethylsulfonyl fluoride (PMSF) 1, sodium orthovanadate 1; NP-40 1%, Triton X-100 0.1%, and protease inhibitor cocktail 1%) (Sigma-Aldrich, Saint Louis, MO, USA). Protein lysates were obtained by collecting supernatants after removing insoluble materials by centrifugation at 4 °C, and aliquots were stored at −80 °C. Protein concentrations were determined using the DC protein assay (Bio-Rad, Hercules, CA, USA).

Western blot. Protein samples of 30 μg were separated by electrophoresis using an 8% or 12% SDS-polyacrylamide gel and electro-transferred to a polyvinylidene difluoride (PVDF) or a nitrocellulose membrane. Membranes were blocked for an hour and then incubated with the primary antibody overnight. Finally, the membranes were incubated with the corresponding secondary horseradish peroxidase-conjugated antibody for one hour. Since each primary antibody has its own specifications regarding the membrane, blocking solution, concentration and secondary antibody, these are summarized in Table 2.

Membranes were revealed using the Bio-Rad ECL kit on the ChemiDoc XRS+ machine (Bio-Rad, Hercules, CA, USA). The integrated optical density of the bands was normalized with respect to (1) the background values and (2) the total protein transferred onto PVDF membranes, measured by total protein analysis (Sypro Ruby protein blot stain, Bio-Rad). To calculate the fold change in protein expression, we first quantified the intensity of the target protein bands using ImageJ software. These intensities were then normalized to the total protein signal in each lane to correct for any variations in loading and transfer efficiency. Finally, we compared the normalized signal intensities between the groups to determine the fold change in expression. Data were taken from densitometry measurements made in at least three separate western blots. Although the comparison between young and aged rats has already been published[3] and discussed, in the current study, we included a dotted line in the graphs representing the mean of the young rat results for a better understanding of the comparison between the aged and aged GSPE groups. Samples from young, aged and aged GSPE groups were analysed and quantified on the same membrane for western blot, allowing us to refer to the values of young rats represented as a dotted line in the graphs.

Statistical analysis. All values are presented as mean ± standard deviation (SD) within each group, and each dot represents the value of one animal to visualize its distribution. Statistical significance of the differences between means was evaluated using the Mann–Whitney test (GraphPad Prism software, San Diego, CA, USA). The criterion for statistical mean significance was: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ when comparing aged and aged GSPE groups, and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ when comparing the young group with the aged or aged GSPE group.

Table 1 Summary of the three experimental groups

		Week											
Group	Pretreatment	0	1	2	3	4	5	6	7	8	9	10	11	Sacrifice
1. Aged	Vehicle (10 days)	Normal diet												24-month-old
2. Aged GSPE	GSPE, 500 mg kg^{-1} (10 days)	Normal diet												

Table 2 Antibody summary

Target	kDa	Antibody origin	Reference	Dilution	Blocking solution	Membrane	Family	
VGCC P/Q-Type CaV2.1	250	Rabbit polyclonal	ACC-001	1/1000	Milk	PVDF	Calcium channels	
BDNF	28	Rabbit polyclonal	28205-1-AP	1/1000	Milk	PVDF	Neurotrophins	
NT4	28	Rabbit polyclonal	sc-365444	1/500	Milk	PVDF		
p75NTR	75	Rabbit polyclonal	07-476	1/1000	Milk	PVDF		
pTrkB (Y816)	145	Rabbit polyclonal	Novus NBF1-03499	1/1000	BSA	PVDF	Neurotrophic receptors	
TrkB	95/145	Rabbit polyclonal	4603S 80E3	1/1000	BSA	PVDF		
PLCβ	155	Mouse monoclonal	sc-5291	1/1000	BSA	PVDF	PLCs	
pPLCy1 (Y783)	155	Rabbit polyclonal	2821S CST	1/800	BSA	Nitrocellulose		
PLCy1	155	Mouse monoclonal	sc-7290	1/1000	Milk	PVDF		
M1 mAChR	100	Rabbit polyclonal	AMR-001	1/1000	Milk	PVDF	Muscarinics	
M2 mAChR	90	Rabbit polyclonal	AMR-002	1/1000	Milk	PVDF		
pRafC (S259)	74	Rabbit polyclonal	9421 CST	1/1000	BSA	PVDF	MAPK pathway	
pRafC (S338)	74	Rabbit monoclonal	9427 CST	1/1000	BSA	Nitrocellulose		
RafC	65–75	Rabbit monoclonal	9422 CST	1/1000	BSA	PVDF		
pMAPK/ERK (T202/204)	42	Rabbit polyclonal	9101 CST	1/1000	BSA	PVDF		
MAPK/ERK	42	Rabbit polyclonal	9102 CST	1/1000	BSA	PVDF		
pPDK1 (S241)	58–68	Rabbit polyclonal	CST (3061)	1/1000	BSA	PVDF	PDK	
PDK1	58–68	Mouse monoclonal	sc-17765	1/1000	BSA	Nitrocellulose		
pPKCβ1 (T642)	78	Rabbit polyclonal	ab5782	1/1000	BSA	PVDF	PKCs	
PKCβ1	78	Mouse monoclonal	sc-8049	1/1000	Milk	PVDF		
pPKCε (S729)	90	Rabbit polyclonal	sc-12355	1/1000	BSA	PVDF		
PKCε	90	Rabbit polyclonal	sc-214	1/1000	Milk	PVDF		
PKA Cα	40	Mouse monoclonal	sc-28315	1/1000	Milk	PVDF	PKAs	
PKA Cβ	40	Rabbit polyclonal	sc-904	1/1000	Milk	PVDF		
PKA RIα	48	Mouse monoclonal	sc-136231	1/1000	Milk	PVDF		
PKA RIβ	51	Rabbit polyclonal	sc-907	1/800	Milk	Nitrocellulose		
PKA RIIα	50	Rabbit polyclonal	sc-909	1/1000	Milk	PVDF		
PKA RIIβ	50	Rabbit polyclonal	ABS-14	1/800	Milk	Nitrocellulose		
Adenylate cyclase	160	Rabbit polyclonal	PA5-35382	1/1000	BSA	Nitrocellulose	AC	
pMunc18-1 (S241)	68	Rabbit polyclonal	Ab183484	1/1000–1/700	BSA	PVDF	Target of MAPK pathway	Munc18-1 (SM)
pMunc18-1 (S313)	68	Rabbit polyclonal	ab138687	1/1000	p-Block	PVDF	Target of PKA	
Munc18-1	68	Rabbit polyclonal	CST (D406 V)	1/1000	Milk	PVDF		
pSNAP-25 (S187)	28	Rabbit polyclonal	ab169871	1/1000	BSA	PVDF	Target of PKC	SNAP-25 (SNARE)
pSNAP-25 (T138)	28	Rabbit polyclonal	orb163730	1/1000	BSA	PVDF	Target of PKA	
SNAP-25	28	Rabbit polyclonal	CST (5309)	1/1000	BSA	PVDF		
pCREB (S133)	45	Rabbit polyclonal	CST (9191S)	1/1000	BSA	PVDF	Target of PKA, p90RSK, MSK, CaMKIV, and MAPK-2	CREB (transcription factor that activates target genes)
CREB	45	Rabbit polyclonal	CST (9192)	1/1000	Milk	PVDF		
CHAT	48	Rabbit polyclonal	207471AP	1/1200	Milk	Nitrocellulose	Synaptic vesicle cycle	
AChE	68	Goat polyclonal	ab31276	1/1000	BSA	Nitrocellulose		
VAChT	68	Rabbit polyclonal	SAB4200559	1/300	BSA	Nitrocellulose		
ChT	68	Rabbit polyclonal	ABN458	1/2000	milk	Nitrocellulose		
Secondary antibody		Donkey polyclonal	711-035-152	1/10 000	—	—		
Secondary antibody		Rabbit polyclonal	A9044	1/10 000	—	—		

Results

24 month-old aged rats treated or not with GSPE were compared to analyze the neurotrophic and muscarinic signaling pathways. We analyzed the total and phosphorylated protein levels of the BDNF/TrkB downstream signaling pathway including BDNF and NT4; their receptors TrkB and $p75^{NTR}$; the muscarinic pathway that includes two presynaptic muscarinic receptors; then downstream proteins such as adenyl cyclase (AC), phospholipase (PLC)β and γ; two PKCs (βI and ε) and their priming kinase, phosphoinositide-dependent kinase 1 (PDK1); different PKA subunits; MAPK-related proteins; two PKC and PKA targets related to neurotransmitter release (Munc18-1 and SNAP-25); presynaptic P/Q-type VGCC; vesicle

recycling-related proteins (vesicular membrane acetylcholine transporter (VAChT), choline acetyltransferase (ChAT), choline transporter (ChT) and acetylcholinesterase (AChE)); and the transcription factor cyclic AMP response element-binding protein (CREB).

The age-induced values were referred to as the values from the young group (6 month-old rats), represented as a dotted line in the graphs, to better illustrate the effect of GSPE treatment on preventing age-related changes (see also Balanyà-Segura *et al.*, 2024[3]).

Metrics of the weights and activity of the animals and the EDL muscle

First, before the molecular analysis, aged rats (24 months) with and without GSPE treatment were compared to analyze their weight and motility (Fig. 1). Fig. 1A shows that there is no difference in the ratio of EDL muscle weight to total animal weight between aged and aged GSPE animals. The spider graphs in Fig. 1B show that aged GSPE rats spent less time sleeping during the morning and more time engaged in active movements, grooming and smelling behaviors. Fig. 1C shows no significant differences in the activity patterns observed throughout the day.

BDNF and NT-4 neurotrophins

The postsynaptic-derived neurotrophic factors BDNF and NT-4 trigger their neurotrophic pathways. Thus, the protein level of BDNF and NT-4 was analyzed by WB in aged and GSPE-treated aged animals (Fig. 2). The results show that in aged animals treated with GSPE, the age-related changes in the stoichiometry of BDNF and NT-4 persist, with no significant differences between the two aged groups, indicating that GSPE treatment does not prevent the age-induced alterations in neurotrophin levels.

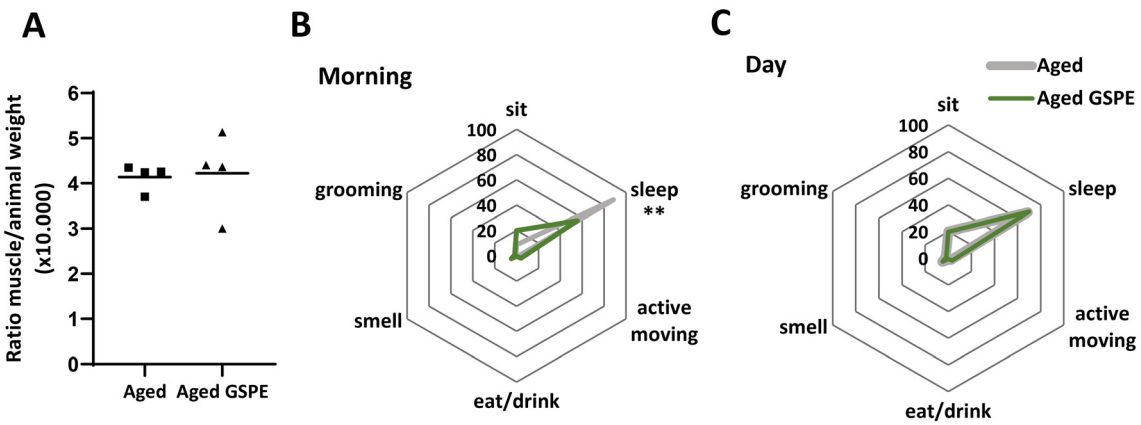

Fig. 1 Activity of rats. (A) Graph showing the ratio of the muscle weight to animal weight, multiplied by 10 000. There are no differences between the groups. (B and C) Spider graphs showing activity parameters during morning and afternoon time (s) for aged and aged GSPE rats. Aged rats without treatment tend to sleep more during the morning than the GSPE-treated rats. Data information: n = 4 rats for each group. Data are presented as means ± SD. Statistical significance was determined using a two-tailed unpaired Welch's t-test (**p < 0.01).

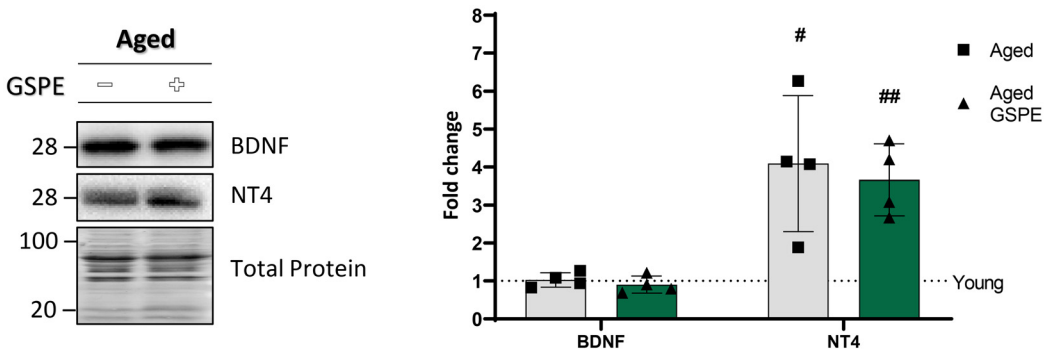

Fig. 2 Neurotrophic factor protein levels of aged and aged GSPE EDL muscles. Western blot analysis of neurotrophins shows that BDNF protein levels do not change, but there is a significant increase of NT-4 protein levels in both conditions compared with the young group and no difference between aged and GSPE-treated animals. Data information: n = 4 rats for each group. Data are presented as means ± SD. Statistical significance was determined using a non-parametric Mann–Whitney's t-test ($^{\#}p$ < 0.05; $^{\#\#}p$ < 0.01, significance when comparing the young group with the aged or aged GSPE group). The dotted line in the graphs represents the mean value for the young group previously published in Balanyà-Segura *et al.* (2024).[3]

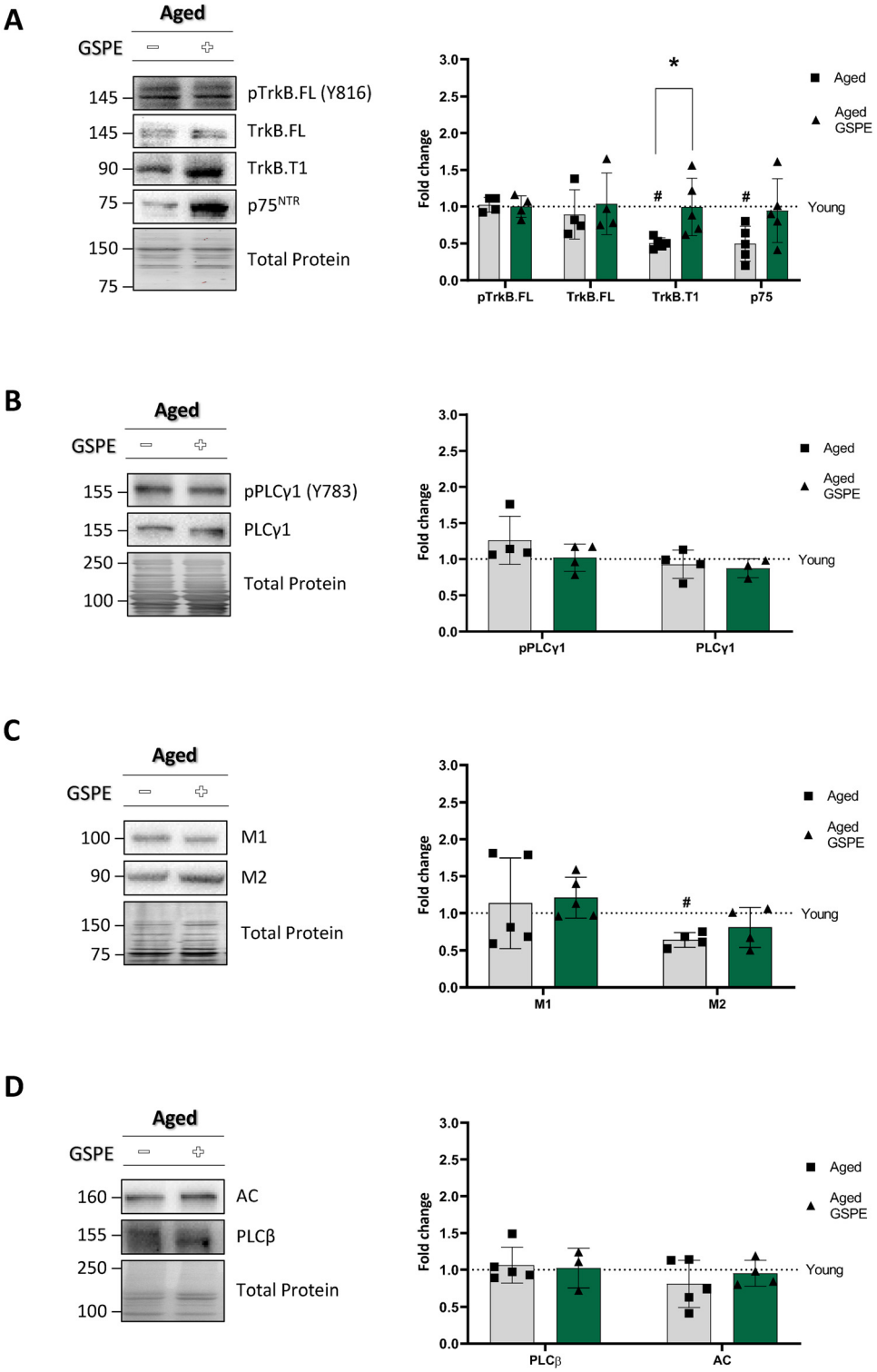

Fig. 3 Neurotrophic factor receptors and muscarinic receptor protein levels of aged and aged GSPE EDL muscles. (A) Western blot analysis of neurotrophic receptors showing that TrkB-FL and its phosphorylated form, pTrkB-FL, do not change in both conditions compared with the young group. However, there is a significant decrease in the TrkB-T1 isoform and $p75^{NTR}$ receptor in aged EDL compared with the young group, with the aged GSPE group recovering these values. (B) Western blot analysis of PLCγ1 and p PLCγ1 showing no differences between aged and aged GSPE muscles. (C) Western blot analysis of muscarinic receptors, M1 and M2, showing a decrease in M2 with respect to the young group, which recovers in the aged GSPE group. (D) Western blot analysis of PLCβ and AC, which show no change among groups. Data information: n = 3–5 rats for each group. Data are presented as means ± SD. Statistical significance was determined using a non-parametric Mann–Whitney's t-test (*$p < 0.05$, significance when comparing aged and aged GSPE; $^{\#}p < 0.05$ significance when comparing the young group with the aged or aged GSPE group). The dotted line in the graphs represents the mean value for the young group previously published in Balanyà-Segura *et al.* (2024).[3]

Neurotrophic factor receptors

Next, we analyzed the protein level and phosphorylation of neurotrophin receptors (Fig. 3). The results show that GSPE treatment prevents the age-related downregulation of TrkB.T1 and $p75^{NTR}$ in the aged animals (Fig. 3A). However, GSPE treatment does not alter the levels of TrkB.FL, pTrkB.FL, PLCγ and pPLCγ compared to untreated aged animals, which had already been unaffected by aging (Fig. 3A and B).

Muscarinic receptors

In addition to the neurotrophic pathway, other proteins that allow autocrine regulation of neurotransmission at the NMJ are muscarinic receptors (mAChRs). The results show that GSPE treatment prevents the age-related downregulation of M2 in the aged animals (Fig. 3C). This finding suggests that GSPE may contribute to maintaining the autocrine regulation of neurotransmission at the NMJ by preserving M2 mAChR levels during aging. GSPE treatment does not alter the levels of M1 and the muscarinic receptor downstream transducers PLCβ and AC compared to untreated aged animals, which had already been unaffected by aging (Fig. 3C and D).

Protein kinases

The main downstream signalling for the TrkB-FL receptor and mAChRs is mediated by some presynaptic isoforms of the serine-threonine kinase PKC.[11] Fig. 4 shows that GSPE did not affect the exclusively presynaptic isoforms cPKCβI and nPKCε nor the priming kinase PDK1 (and their respective phosphorylated active forms).[16,53]

However, GSPE improved the age-related downregulation of PKA functionality, as represented by the significant increase in the RIα regulatory subunit (Fig. 5). Indeed, GSPE treatment resulted in a significant rise in the Cα subunit of PKA, accompanied by an even greater increase in the RIα regulatory subunit (Fig. 5), which suggests a compensatory regulation of PKA activity. These changes may be associated with the preservation of pCREB protein levels, a key PKA target, whose age-related reduction in untreated aged animals was effectively prevented by GSPE (Fig. 5B). The maintenance of pCREB levels indicates that GSPE treatment may help sustain transcriptional regulation associated with PKA signaling, despite age-associated alterations.

Regarding other kinases regulated by neurotrophic and muscarinic pathways, such as rapidly accelerated fibrosarcoma C (RafC) (S259/338) and MAPK 42/44, no significant changes were observed due to aging or GSPE treatment, indicating that these pathways remain stable under the experimental conditions (Fig. 5C and D).

Kinase targets in the SNARE-SM ACh release complex

The kinases considered in this study regulate the presynaptic SNARE-SM ACh release complex. To assess whether GSPE treatment affects the ACh release machinery in aged rats, we analyzed the protein levels and phosphorylation of two key components of the SNARE-SM complex: Munc18-1 and SNAP-25 (Fig. 6).

The protein levels of Munc18-1 and its phosphorylated forms (S241 and S313), as well as SNAP-25 and its phosphorylation at PKA-dependent T138 and PKC-dependent S187, are preserved in GSPE-treated aged animals. This contrasts with untreated aged animals, where changes in these proteins and their phosphorylation states are observed.

The prevention of these age-associated alterations by GSPE highlights its potential role in maintaining the integrity and function of the ACh release machinery at the NMJ during aging. This suggests that GSPE may help stabilize critical presynaptic mechanisms involved in neurotransmission.

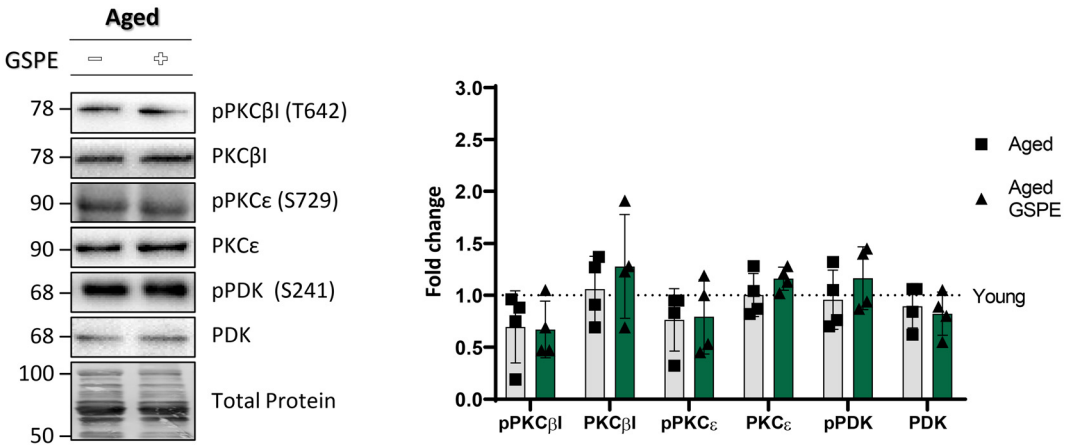

Fig. 4 Protein kinase C protein levels of aged and aged GSPE EDL muscles. Western blot analysis of different protein kinases and their phosphorylated forms. Quantification analysis shows that cPKCβI, nPKCε and PDK1 protein levels (and their respective phosphorylated active forms) do not change in any group. Data information: n = 4 rats for each group. Data are presented as means ± SD. Statistical significance was determined using a non-parametric Mann–Whitney's t-test. The dotted line in the graphs represents the mean value for the young group previously published in Balanyà-Segura *et al.* (2024).[3]

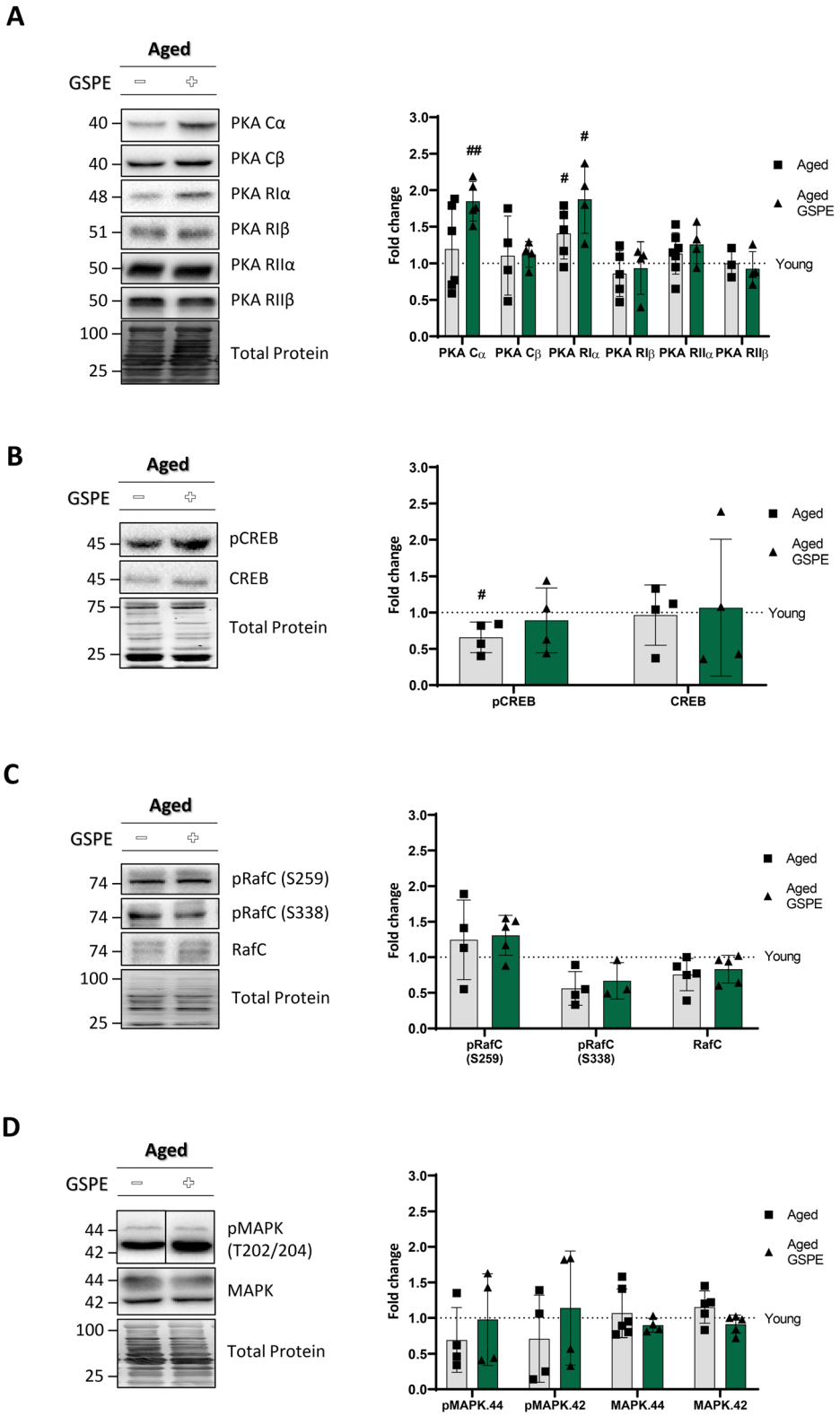

Fig. 5 Protein kinase A, CREB, RafC and MAPK protein levels of aged and aged GSPE EDL muscles. (A) Western blot analysis of different PKA subunits, showing an increase in the Cα subunit in aged GSPE muscles compared with the young group, and an increase in RIα in both aged groups compared with the young group. (B) Western blot analysis of CREB and its phosphorylated form, showing a reduction in pCREB in aged muscles, which is maintained in aged GSPE muscles. (C and D) Western blot analysis of RAfC and MAPK proteins, showing no change in the protein levels in both groups. Data information: n = 3–7 rats for each group. Data are presented as means ± SD. Statistical significance was determined using a non-parametric Mann–Whitney's t-test ($^{\#}p < 0.05$; $^{\#\#}p < 0.01$, significance when comparing the young group with the aged or aged GSPE group). The dotted line in the graphs represents the mean value for the young group previously published in Balanyà-Segura *et al.* (2024).[3]

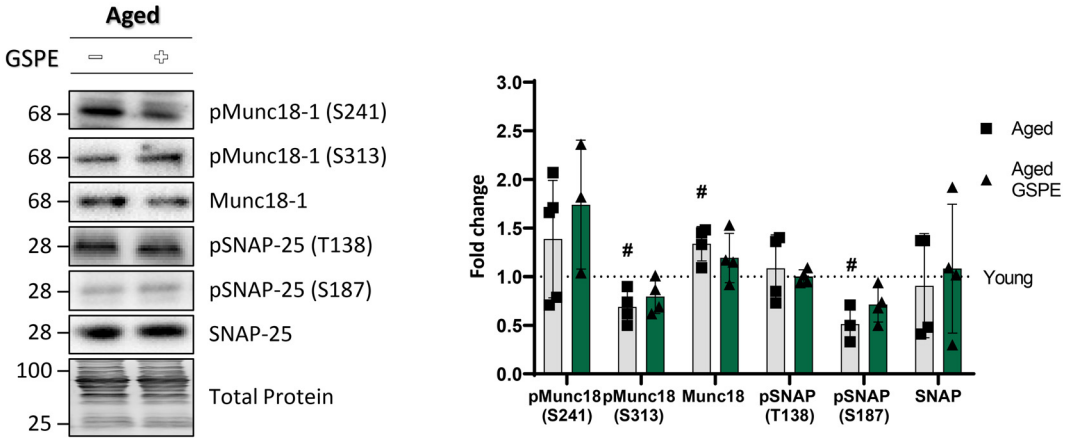

Fig. 6 Protein levels of key molecules related to the SNARE-SM ACh release complex of aged and aged GSPE EDL muscles. Western blot analysis of different protein kinase targets and their phosphorylated forms. Quantification analysis shows a decrease in phosphorylated Munc18-1 (S313) and an increase in Munc18-1 protein levels in aged muscles compared with young muscles. SNAP-25 protein level does not change, nor does PKA-dependent pSNAP-25 (T138). However, the cPKCβI- and nPKCε-dependent pSNAP-25 (S187) is greatly reduced in aging compared to the young group. We observed that all these changes that occur in aged animals are prevented by GSPE treatment. Data information: n = 3–5 rats for each group. Data are presented as means ± SD. Statistical significance was determined using a non-parametric Mann–Whitney's t-test ($^{\#}p < 0.05$, significance when comparing the young group with the aged or aged GSPE group). The dotted line in the graphs represents the mean value for the young group previously published in Balanyà-Segura *et al.* (2024).[3]

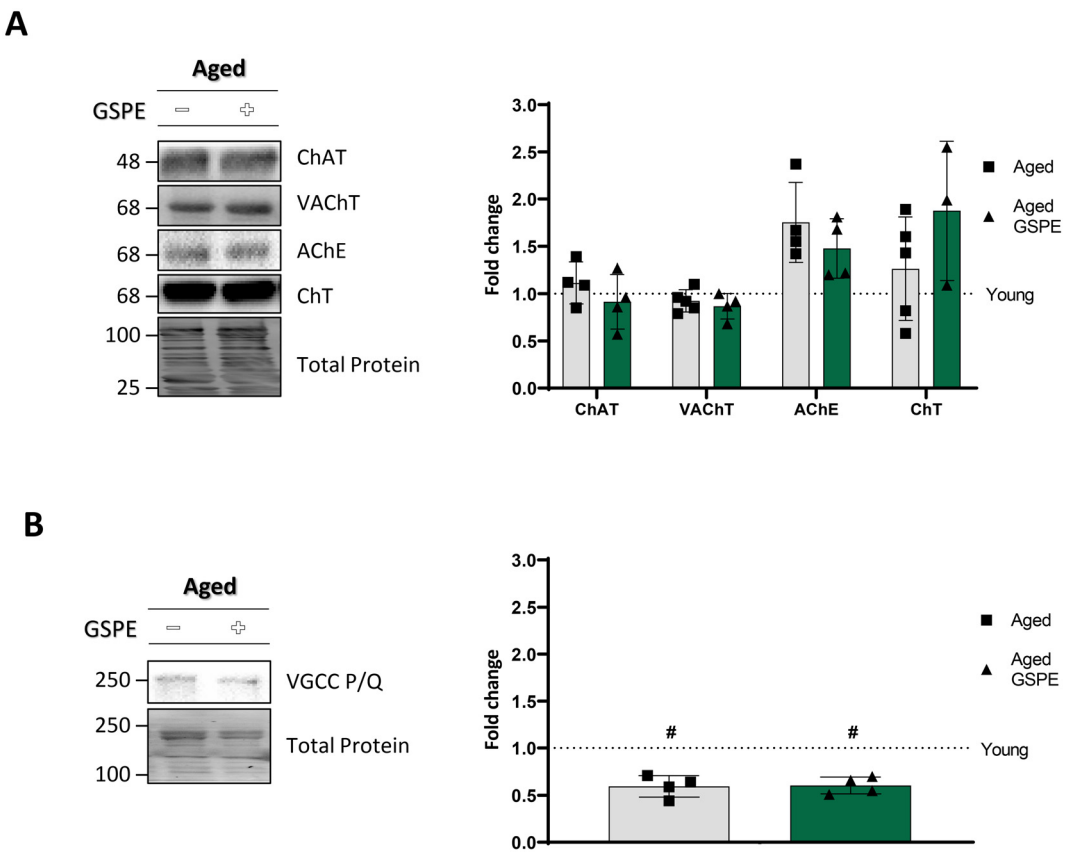

Fig. 7 Synaptic vesicles and calcium channels levels of aged and aged GSPE EDL muscles. (A) Western blot analysis of different key presynaptic molecules related to synaptic vesicles. Note that no change is found in any protein analyzed. (B) Western blot analysis of the P/Q-type VGCC showing a significant decrease in the protein levels between young and both aged groups. Data information: n = 3–5 rats for each group. Data are presented as means ± SD. Statistical significance was determined using a non-parametric Mann–Whitney's t-test ($^{\#}p < 0.05$, significance when comparing the young group with the aged or aged GSPE group). The dotted line in the graphs represents the mean value for the young group previously published in Balanyà-Segura *et al.* (2024).[3]

Acetylcholine cycle proteins and calcium channels

Finally, to assess the potential impact of GSPE treatment on the synaptic vesicle cycle, we analyzed the protein levels of key molecules involved in ACh availability and recycling, as well as VGCCs (Fig. 7). In GSPE-treated aged animals, proteins related to ACh management, including VAChT, ChAT, ChT and AChE, remained unchanged, consistent with their stability across all groups. However, GSPE treatment did not prevent the age-related reduction in P/Q-type VGCC levels observed in aged animals.

In summary, GSPE treatment prevents several age-associated changes at the NMJ. Specifically, it maintains the stoichiometry of BDNF/NT-4 receptors and preserves the M1/M2 mAChRs ratio. Additionally, GSPE stabilizes the phosphorylation states of key proteins in the SNARE-SM ACh release complex and ensures normal levels of Ach management molecules. However, GSPE does not prevent the age-related reduction in P/Q-type VGCC levels observed in aged animals. Notably, the treatment increases the PKA Cα subunit and further enhances RIα subunit levels, suggesting a compensatory mechanism in PKA signaling (Fig. 8). These findings

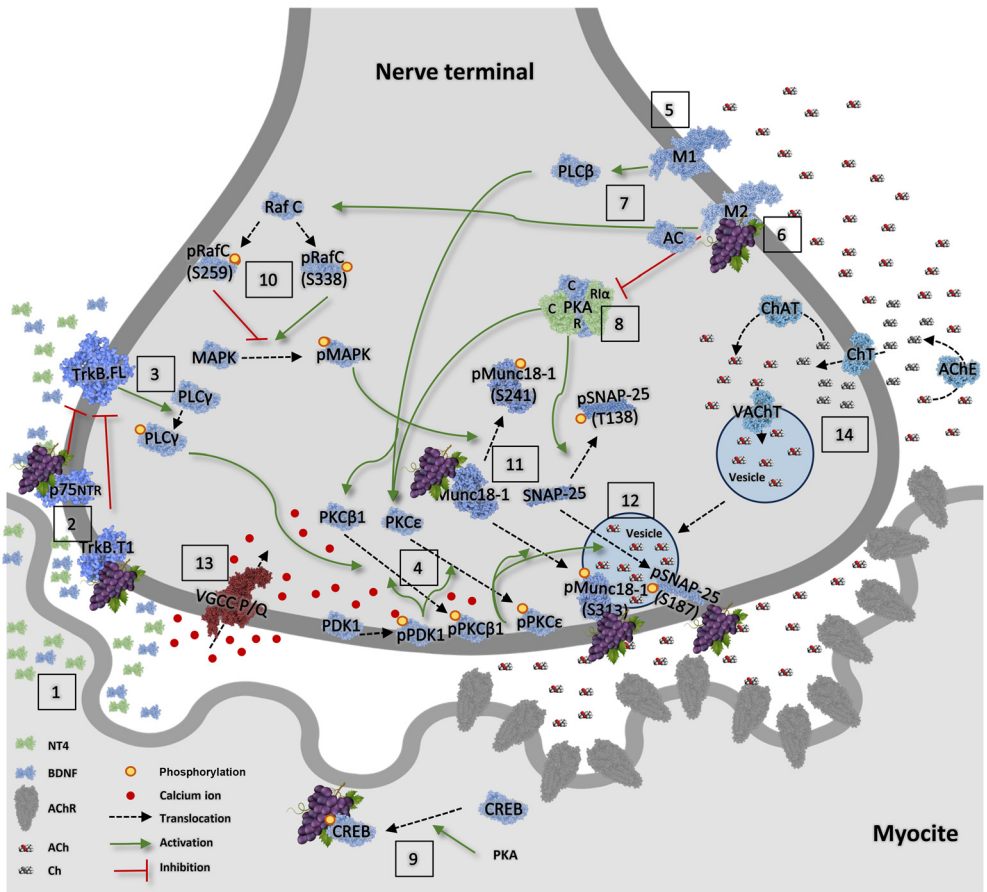

Fig. 8 Overview of the main molecular elements of neurotransmission signalling that are altered in aged EDL muscles. Protein levels of molecules represented in (1) green are increased in aged GSPE muscles, (2) red are decreased in aged GSPE muscles and (3) blue are maintained in aged GSPE muscles compared with young muscles. Molecules shown with a bunch of grapes are the ones that changed due to the GSPE effect, and the ones with a bunch of grapes and a cross are those that changed between young and aged, but GSPE did not recover their protein levels. Regarding the neurotrophic pathway, #1, NT-4 levels are increased in aged and aged GSPE muscles compared to young muscles. #2, TrkB.T1 and $p75^{NTR}$ protein levels do not change between aged and aged GSPE muscles, but under GSPE conditions, the TrkB.FL/T1 ratio is recovered. #3, TrkB.FL direct downstream transducer PLCγ and its phosphorylated form do not change in aged GSPE EDL muscles. #4, the protein levels of the presynaptic phosphorylated downstream PDK and PKCβI and PKCε isoforms are maintained in aged and aged GSPE muscles. #5, protein levels of M1 and M2 muscarinic receptors are maintained under GSPE conditions. #6, the levels of the M1 mAChR downstream transducer PLCβ and M2 mAChR downstream transducer adenylyl cyclase are maintained. #7, related to their downstream kinase PKA, there is an increase in PKA RIα and PKA Cα subunit protein levels, but there is no change in other regulatory or catalytic subunits. #8, upregulation of these subunits may be linked to the recovery of pCREB in the postsynaptic site of aged GSPE muscles. #9 and #10, RafC and MAPK, kinases regulated by neurotrophic and muscarinic pathways, are unaffected by GSPE. Both PKC and PKA regulate the SNARE-SM ACh release complex. #11, the imbalance of the SNARE-SM complex proteins Munc18-1 and SNAP-25 (and their phosphorylated forms) is recovered by GSPE. #12, SNAP-25 and PKA-dependent pSNAP-25 (T138) protein levels do not change, but cPKCβI- and nPKCε-dependent pSNAP-25 (S187) recover their protein levels in GSPE muscles. Finally, although there is a strong diminution of the P/Q-type VGCC in aging as well as in GSPE treatment, #13, all proteins related to ACh and recycling synaptic vesicles (acetylcholinesterase, choline transporter in the plasmalemma, choline acetyl transferase and vesicular acetylcholine transporter) are not modified in aged GSPE muscles, #14.

highlight GSPE's potential to mitigate age-related changes in NMJ function despite some persistent alterations.

Discussion

The relationship between nutrition and the preservation of the neuromuscular system is an innovative area that is still poorly explored. However, growing evidence highlights the strong impact that dietary components have on neuromuscular health. For instance, polyphenols limit demyelination by blocking neural inflammation and damage, making them promising therapeutic targets for immunodegenerative diseases, such as multiple sclerosis.[38] Resveratrol, a well-known antioxidant from the polyphenol family, increases the number of postsynaptic sites on myotubes, exhibiting a more youthful architecture.[56] In this context, we have analyzed the potential of the antioxidant GSPE to prevent age-related alterations in the molecular machinery underlying neuromuscular synapse stability and function. Positive findings would have significant implications for improving the quality of life and survival of aged people.

In particular, in this study, we have conducted a comprehensive analysis of the entire muscarinic and neurotrophic signaling pathways to gain a comprehensive understanding of how GSPE influences neuromuscular function during aging. These pathways are crucial for neuromuscular junction functionality and have been shown to adapt dynamically to optimize neurotransmission efficiency. While our previous study[3] suggested that some proteins are not significantly altered with aging, we hypothesized that GSPE might still impact these pathways, even in the absence of age-related changes. Specifically, we focused on the presynaptic effects of GSPE at the NMJ, analyzing key proteins such as PKCβI, PKCε, SNAP-25, and Munc18-1, which are integral to synaptic vesicle dynamics and are exclusively localized to the presynaptic terminal. Our previous studies demonstrated that PKCβI and PKCε are absent in extrajunctional areas and are significantly reduced after denervation, confirming their presynaptic specificity.[57,58] The effects of GSPE on these PKC isoforms and their downstream pathways likely reflect presynaptic remodeling, offering a potential mechanism for maintaining NMJ integrity with aging. Additionally, we studied the PKA subunit dynamics in relation to changes in presynaptic Synapsin-1, further emphasizing GSPE's impact on presynaptic signaling.

First, we analyzed body and EDL muscle weights, as well as activity levels, to assess potential physical differences between groups. Our results revealed that both groups exhibited the same weight characteristics and distribution. However, we found that GSPE-treated aged rats were more likely to sleep less during the morning period compared to the aged group. This suggests a potential positive effect of GSPE on global health, as rodents typically exhibit higher activity levels during the early morning. It has been found that antioxidants, by protecting against oxidative damage, have a good impact on physical activity by improving muscle performance and physical recovery.[59–61] This could explain the increased morning activity observed in GSPE-treated animals, pointing to a possible role for GSPE in supporting physical vitality during aging.

Regarding the molecular study, firstly we analyzed the BDNF/NT-4/TrkB pathway, which is the main retrograde signaling mechanism involved in NMJ stability and essential for regulating neurotransmission.[62–68] We have previously demonstrated that aging induces several alterations in this signaling pathway.[3]

When animals were treated with GSPE, the age-induced downregulation of TrkB.T1 and $p75^{NTR}$ was effectively prevented. This goes in line with findings from other studies suggesting that GSPE could restore the balance of the pro nerve growth factor (NGF)/NGF levels, as observed in the bladder of diabetic rats and in PC12 cells.[69–72]

Although the exact mechanism of action of GSPE on these receptors remains unclear, it has been shown that 7,8-dihydroxyflavone (7,8-DHF), a polyphenol, acts as a BDNF-mimetic compound with an agonistic effect on tropomyosin receptor kinase receptors.[73] One of the main effects attributed to GSPE is its antioxidant benefits, which suggests that GSPE could directly impact the BDNF pathway by mimicking its action and balancing the levels of its receptors at the aged NMJ, thereby improving the oxidative environment. This would prevent the downregulation of $p75^{NTR}$ and TrkB.T1, observed during aging, favoring a more complete exposure of TrkB.FL to neurotrophins and enhancing receptor signaling.

Neurotrophin signaling not only plays a major role in neuronal survival but also actively participates in redox mechanisms. A recent report indicates that the BDNF induces calcium (Ca^{2+}) release from ryanodine receptor channels and stimulates ROS production in the brain.[74] Furthermore, TrkB signaling, specifically through the TrkB/phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway, promotes the activation and nuclear translocation of nuclear factor erythroid 2-related factor 2 (Nrf2), which maintains redox homeostasis and, thus, confers neuroprotection against oxidative stress.[75,76] Therefore, another hypothesis is that the GSPE-associated recovery of neurotrophic signaling at the NMJ could include Nrf2 signaling to improve the redox environment. However, additional experiments are needed to test these hypotheses.

Muscarinic receptors are functionally linked to TrkB receptors, performing a cooperative mechanism that controls ACh release.[8,77] M1 and M2 muscarinic receptor subtypes exert opposing effects on ACh release at the NMJ, with M1 increasing the end-plate potential and M2 decreasing it.[78,79] GSPE treatment prevents the age-related M1/M2 unbalance, recovering M2 protein levels.

Regardless of the fact that direct evidence linking GSPE to muscarinic receptor modulation is limited, several studies have highlighted the positive effects of polyphenols in conditions such as cardiac hypertension,[80–82] cognitive impairment,[83,84] and intestinal smooth muscle dysfunction.[85,86] These studies suggest that polyphenols may exert their beneficial effects through a mimetic action on ACh. Additionally, the binding affinities of ACh and flavonoids to muscarinic receptors are similar, supporting the idea that flavonoids might influence muscarinic receptor activity. Thus, based on existing literature, which suggests that GSPE may balance the muscarinic system,

we hypothesize that the influence of GSPE, directly or indirectly, may make unnecessary any adaptive age-induced downregulation of M2. However, additional experiments are necessary to explore the physiological implications of muscarinic receptor rebalancing and its broader effects on neuromuscular function.

Both neurotrophic and muscarinic receptors trigger signaling pathways that converge on several kinases to modulate neurotransmitter release. A key family of kinases involved in this process is PKCs, which are activated by PDK1.[7,87] Of particular interest are cPKCβI and nPKCε, which are exclusive to the presynaptic nerve terminal at the NMJ and essential for ACh release.[7,58,88] Other kinase families involved in neurotransmission regulation, including PKA, RafC (S259/338), and MAPK 42/44, are also mainly regulated by TrkB and muscarinic receptors at the NMJ.[16,89] In the current study, most of the kinases analyzed were not altered by GSPE treatment, yet they were not altered with aging.[3] However, GSPE treatment notably increased the PKA Cα subunit, along with an even greater elevation in the PKA RIα subunit. Furthermore, GSPE effectively prevented the significant reduction in pCREB, a key PKA target, that was observed in our previous results on aging.[3] Other research suggests that the management of kinases, especially PKA, is closely linked to mitochondrial function, which often becomes impaired with aging, leading to an excess of ROS. Polyphenols and other antioxidants could help improve such imbalances.[86]

Related to this, a study showed that extremely low-frequency magnetic field treatment increased levels of PKA, PKCβ, and intracellular Ca^{2+}, while decreasing PKCα and BDNF levels in mice hippocampus.[90] However, oral administration of procyanidins from lotus seedpod significantly reversed these changes, restoring levels to normal.[90] The authors suggest that the effect of procyanidins could be possible *via* Ca^{2+} signaling. Consistent with this, our study suggests that GSPE would affect PKA levels and Ca^{2+} signaling (see later for the reduction in P/Q-type VGCC levels). However, our results show that GSPE does not affect PKCβ or PKCα levels. Other studies showed that procyanidins (50 and 100 mg per kg body weight, administered intragastrically) increased CREB phosphorylation by increasing PKA, calcium-/calmodulin-dependent protein kinase IV (CaMKIV), or MAPK phosphorylation in the hippocampus and cerebral cortex of cognitively impaired aged rats.[91] Procyanidins also affected SIRT1 transcription and enhanced CREB-dependent transcription, ultimately improving cognition in Alzheimer's disease. These studies are in concordance with our results as procyanidins from grape seeds increase the levels of certain PKA subunits and prevent the age-associated reduction of pCREB.

Some of the PKC, PKA, and MAPK targets are proteins of the exocytotic machinery that control neurotransmission at the NMJ, such as Munc18-1 and SNAP-25.[92,93] Here, we found that GSPE treatment prevents the age-related imbalance in SNARE-SM complex proteins, including Munc18-1 and SNAP-25. While there is limited research on the impact of polyphenols on vesicle SNARE-SM complexes, some studies have suggested that SNARE complexes are common targets of many polyphenols. For example, Yang *et al.* (2010) observed that some polyphenols intercalate into the SNARE complex (synapsin-1 (Syn1)/SNAP-25/vesicle-associated membrane protein 2 (VAMP2)), leading to the inhibition of neurotransmitter release from neuronal cells.[94] However, our results indicate that GSPE prevents the age-associated decrease in specific proteins of this complex. We hypothesize that this protective effect is related to the normalized level of the neurotrophin and muscarinic receptors observed in GSPE-treated animals. Nevertheless, further research is necessary to fully understand the impact of polyphenols on these specific molecules of the vesicle release machinery under both healthy and aging conditions.

Concerning the calcium channels, we observed that GSPE treatment was ineffective in preventing the strong diminution of P/Q-type VGCC in aging. There are studies linking ROS and calcium signaling, hypothesizing that increased ROS during aging might be responsible for neurodegeneration due to calcium disruption, or *vice versa*.[95–97] Moreover, it has been shown that polyphenols exert a beneficial effect on spastic movements of the smooth muscle and reduce arrhythmia in cardiac muscles through calcium blockade.[98–100] Despite that, our results suggest that the administration of GSPE may not be sufficient to reverse the age-associated downregulation of P/Q-type VGCC in EDL muscle. This could indicate that, while GSPE has a positive impact on certain signaling pathways, its effects on calcium channel regulation at the NMJ may be more limited, particularly in the context of aging.

In the brain, certain flavonoids act directly as competitive or noncompetitive inhibitors of AChE and indirectly by inducing changes in gene expression or modulating the activity of AChE inhibitors.[38,101,102] Additionally, resveratrol has been reported to improve the activity of AChE, ChAT, and VAChT, potentially contributing to some improvement in cognitive function. However, we found that the studied presynaptic molecules involved in synaptic vesicle management in the cholinergic vesicular system (AChE, ChAT, VChAT, and ChT) were unaffected by GSPE, as they were during the aging process. This suggests that, although GSPE may modulate other aspects of neuromuscular function, it does not appear to significantly influence the regulation of these cholinergic components in the context of the aging process.

In conclusion, our results demonstrate that GSPE treatment prevents important age-associated changes in the molecular organization of the NMJ. These include modifications in the stoichiometry of neurotrophin receptors, the imbalance of the M1/M2 mAChRs ratio, and alterations in the phosphorylation of relevant proteins in the SNARE-SM complex. In addition, GSPE treatment led to an increase in pCREB levels, which can be related to the enhanced expression of the PKA Cα subunit, accompanied by an even further increase in the PKA RIα subunit. However, GSPE treatment does not modify several other molecules involved in these signaling and neurotransmission pathways, which remain unchanged during aging. These include the neurotrophin BDNF, the coupling enzymes

PLCβ, AC, and PDK1, the downstream kinases PKCβI, PKCε, RafC and MAPK, and finally, the ACh management molecules, such as AChE, ChAT, VChAT and ChT. Interestingly, GSPE treatment does not prevent the age-associated increase in the neurotrophin NT-4 levels or the reduction in P/Q-type VGCC protein levels. Together, these results indicate that GSPE treatment, by modulating key signaling pathways and maintaining the integrity of important proteins at the NMJ, may mitigate age-related disruptions, potentially preserving neuromuscular function in aging individuals.

Overall, although further research is needed to fully elucidate the impact and mechanisms of action of grape seed procyanidins, they show promise as a nutraceutical for preventing or delaying some age-related changes, particularly those impairing the NMJ molecular pathways, which are crucial for skeletal muscle function.

Abbreviations

AC	Adenylyl cyclase
ACh	Acetylcholine
AChE	Acetylcholinesterase
AR	Adenosine autoreceptors
BDNF	Brain-derived neurotrophic factor
ChAT	Choline acetyltransferase
ChT	Choline transporter
CREB	Cyclic AMP response element-binding protein
EDL	*Extensor digitorum longus*
EDTA	Ethylenediaminetetraacetic acid
EPP	Evoked endplate potentials
GSPE	Grape seed procyanidins extract
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
MAPK	Mitogen-activated protein kinase
Munc18-1	Mammalian uncoordinated-18-1
NaF	Sodium fluoride
NMJ	Neuromuscular junction
NT-4	Neurotrophin 4
PDK	Phosphoinositide-dependent kinase
PLC	Phospholipase C
PKA	Protein kinase A
PKC	Protein kinase C
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene fluoride
RafC	Rapidly accelerated fibrosarcoma C
ROS	Reactive oxygen species
SD	Standard deviation
SNAP-25	Synaptosomal-associated protein 25
SNARE	Soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor
TrkB	Tropomyosin-related kinase B receptor
VAChT	Vesicular membrane acetylcholine transporter
VGCC	Voltage-gated calcium channels
WB	Western blot

Author contributions

Conceptualization, M. A. L., J. T. and E. H.; methodology, A. A., M. P., M. T., E. H. and M. B.-S.; software, M. B.-S.; formal analysis, M. B.-S. and E. H.; investigation, M. B.-S., A. P., L. J.-B., M. J.-E., C. S. and V. C.-M.; resources, A. A., J. T. and M. A. L.; data curation, M. B.-S.; writing—original draft preparation, E. H., J. T., A. P., L. J.-B., V. C.-M. and M. B.-S.; writing—review and editing, J. T., M. A. L. and E. H.; supervision, E. H., M. A. L. and J. T.; project administration, M. A. L.; and funding acquisition, M. A. L., J. T. and A. A. All authors have read and agreed to the published version of the manuscript.

Ethics approval

The rats 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. This procedure was approved by the Experimental Animal Ethics Committee of the Generalitat de Catalunya, Spain (Department of Territory and Sustainability, General Directorate for Environmental and Natural Policy, project authorisation code: 10183).

Data availability

The data supporting this article have been included as part of the ESI.†

Conflicts of interest

The authors declare no conflicting financial interests.

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