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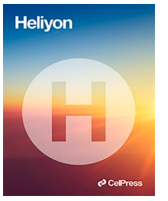

Research article

Adenosine A2A receptor activation is necessary to gate the TrkB-dependent intramuscular nerve sprouting during muscle reinnervation after a nerve crush

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ABSTRACT

Compelling evidence has demonstrated that rehabilitation through physical exercise, a non-invasive and non-surgical intervention, enhances muscle reinnervation and motor recovery after peripheral nerve injury (PNI) by increasing muscle-derived brain-derived neurotrophic factor (BDNF) expression and triggering TrkB-dependent axonal plasticity. Adenosine has been widely acknowledged to trigger TrkB via A2A receptor (A2AR). Since motor nerve terminals co-express TrkBs and A2ARs and depolarizing conditions increase muscle release of BDNF and adenosine, we examined whether A2ARs activation could recapitulate the functional recovery benefits of intermittent exercise after a nerve crush. Immunohistochemical and in situ proximity ligation assay (isPLA) analyses were used to localize A2ARs and A2A-TrkB heteroreceptor complexes at the neuromuscular level in undenervated animals. The reinnervation process of the soleus muscle was examined in both sedentary and trained animals ten days following a nerve crush injury. The effects of A2A and TrkB interplay on muscle fiber multiple-innervation was assessed using a functional approach. We confirmed that A2A immunoreactivity is mainly localized at the axonal level and provided evidence that A2ARs may form heteroreceptor complexes with TrKb at muscle plasmalemma. The pharmacological activation of either TrkBs or A2ARs mirrored the effect of motor activity on target muscle reinnervation after a nerve crush. Furthermore, the block of A2ARs abolished the effect of TrkBs agonism on nerve endings sprouting. Our results demonstrated that activation of adenosine A2ARs is required to gate the activity-related TrkB-dependent enhancement of axon sprouting during the reinnervation process after a nerve crush. Moreover, our isPLA data suggest that A2ARs can physically interact with TrkBs at the muscle plasmalemma.

1. Introduction

Peripheral nerve injuries (PNIs) pose a major community health challenge, with a rising number of cases each year, leading to considerable reductions in quality of life [1,2]. While it is widely known that damaged peripheral nerves can regenerate upon PNI, this

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process is typically sluggish, and complete functional recovery is rarely achieved [3,4]. In fact, complete functional recovery remains the most difficult issue in PNI treatment and rehabilitation despite the continuous progress in surgical methods for peripheral nerve repair, coupled with an expanding knowledge of the peripheral nervous system's pathophysiology [2]. Non-surgical methods that complement surgery and promote recovery and reinnervation have garnered considerable interest in recent years [2]. Among non-surgical treatments for PNI, several studies have highlighted the effectiveness of 'activity-associated therapies' (AATs) such as muscle physical exercise, in improving functional recovery of the target muscle by enhancing either axonal regeneration and reinnervation process [5–8]. The usefulness of AATs in treating PNI are mainly related to the capacity to promote the production of neurotrophins by regenerating axons and skeletal muscle [6,10]. A large body of evidence, indeed, has highlighted the pivotal role of neurotrophins, such as brain-derived neurotrophic factor (BDNF), in functional recovery after PNI [5,6], and in this context, skeletal muscle, due to its endocrine role [11], can be considered as a significant source of different growth factors that strongly impact on muscle reinnervation [6]. In this regard, we showed that a moderate-intensity intermittent running activity could increase the muscle expression of BDNF, which, in turn, activates presynaptic Tropomyosin-related kinase B receptors (TrkBs) and stimulates nerve-terminal sprouting of regenerated axons, ultimately resulting in full muscle reinnervation and restored motor function in rats with crushed nerves [6,7]. Thus, we provided evidence that an intermittent exercise pattern may represent a promising clinically transferable AAT for treating PNI and, most importantly, that the BDNF/TrkB interplay is crucial in mediating these exercise-induced beneficial effects [6,7]. From a translational perspective, this latter finding is particularly intriguing as it indicates that a neurotrophin-based therapy, able to activate TrkBs during the reinnervation process, could be a crucial treatment to mitigate some common limiting factors associated with the use of exercise-based therapies in PNI patients, such as pain and immobility [12]. Nevertheless, it is worth mentioning that although neurotrophin-based therapies have demonstrated considerable therapeutic potential in various disease conditions, the development of these therapies remains highly challenging [13,14]. The primary concerns associated with neurotrophin-based therapies are the high cost of production and the short duration of these peptides in the bloodstream. These limitations have restricted the potential use of neurotrophins as a noninvasive and efficacious treatment for numerous diseases [14,15]. Considering these translational difficulties, modulating neurotrophin receptor signalling using small compounds that can transactivate tyrosine protein kinase receptors appears promising [15]. In this regard, adenosine, a ubiquitous neuromodulator, has been proven to be a suitable pharmacological tool to overcome the above-mentioned translational difficulties in developing neurotrophin-based therapies. Substantial evidence, indeed, has demonstrated that the activation of the adenosine A2A receptor (A2AR) led to autophosphorylation of the BDNF high-affinity TrkB receptor [16,17], paving the way to mimic the BDNF action in the nervous system using the crosstalk TrkB/A2A [14,15]. In addition, considering that activation of A2A receptors is crucial in shaping synaptogenesis [18,19], that motor nerve terminals co-express TrkB and A2A receptors [15], and that depolarizing conditions - such as those during motor activity - promote the release of both BDNF and adenosine from muscle [15], it is reasonable to hypothesize that activating TrkB via A2A receptor stimulation during the reinnervation process following nerve injury could mimic the beneficial effects on functional recovery achieved by intermittent exercise. To test this hypothesis, the present study investigates the role of the adenosine A2A receptor in the reinnervation of the adult rat soleus muscle following nerve crush, employing a functional approach in both sedentary and trained animals.

2. Methods

2.1. Surgical procedure and experimental design

The study adhered to Italian regulations on animal experimentation (D.lgs 26/2014) and was conducted under authorization No. 149/2018-PR, granted by the Italian Ministry of Health. A total of 48 male Sprague-Dawley rats, aged 1.5 months, were obtained from Charles River Laboratory. The animals were housed under a 12-h light/dark cycle and provided with unrestricted access to food and water. Every effort was taken to minimize animal distress and to use the smallest number of animals necessary. The animals were anesthetized via intraperitoneal injection of sodium thiopental (45 mg/kg body weight). The soleus branch of the left tibial nerve was compressed to induce a nerve-crush lesion 4 mm proximal to its entry into the muscle, facilitating the formation of initial neuromuscular synaptic contacts within 3–4 days following denervation [20]. The function of the soleus muscle is functionally redundant with that of the gastrocnemius. Consequently, the animal's ability to locomotion is unaffected by its denervation [6,7]. Therefore, this denervation model is appropriate for implementing motor activity protocols after the injury. Thus, starting four days after the nerve crush injury, the rats performed treadmill activity twice daily for six consecutive days. Following this period, the animals were euthanized for experimental analysis. This endpoint of 10 days after nerve crush was selected based on prior research showing that the most significant changes in muscle reinnervation between trained and control groups occurred at this point [6,7]. Undenervated controls ($n = 4$), consisting of rats that did not undergo surgery and were housed in standard cages, were included exclusively for immunohistochemistry analyses. Conversely, the denervated animals were allocated into two experimental groups: the Trained group, consisting of nerve-crushed rats that underwent treadmill training until the electrophysiological assessment ($n = 18$), and the Sedentary group, comprising nerve-crushed rats maintained in standard cages without training until the electrophysiological evaluation ($n = 26$). Trained animals were further divided into Runners controls (RUN; $n = 8$), exercised without treatment and Runners treated with either a potent and selective antagonist of the A2A receptors (ZM; n = 5) or TrkB receptors (ANA; $n = 5$). All trained animals engaged in a 30-min exercise session of intermittent moderate-intensity activity. This involved alternating 5-min intervals of running and rest, with each running period structured as 4 min of continuous acceleration (ranging from 0 to 27 m/min) followed by 1 min at the maximum speed of 27 m/min [6]. This exercise pattern was chosen based on previously published data [7], showing that an intermittent moderate-intensity activity represents the more suitable exercise protocol to enhance muscle innervation upon a nerve

crush. Sedentary animals were further divided into Sedentary controls without treatment (SED; $n = 7$), treated with either a potent and selective agonist of A2A receptors (CGS; $n = 5$) or TrkB receptors (DHF; $n = 10$) and treated with the A2A receptors antagonist followed by the TrkB agonist (ZM + DHF; $n = 4$). Fig. 1 provides a schematic overview of the experimental design and exercise protocol employed in the study.

2.2. Drugs

All the drugs used to treat either Trained or Sedentary animals were injected intraperitoneally twice daily. Trained animals were treated 30 min before each exercise session. Sedentary animals of the ZM + DHF group were treated firstly with ZM and after 30 min with DHF. Both previously published papers (for details, see Table 1) and pilot trials carried out in our laboratory were utilized to determine the appropriate dose for each drug. All drug solutions were freshly prepared before each experiment.

2.3. Immunohistochemistry and in-situ proximity ligation assay

The soleus muscle from non-denervated control rats was used for these experiments. Animals were anesthetized via intraperitoneal injection of sodium thiopental (45 mg/kg body weight) and euthanized using a terminal dose of the same anesthetic. The soleus muscles were carefully excised and immediately fixed in a 4 % paraformaldehyde solution prepared in 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 1 h at room temperature, followed by rinsing in PBS. The fixed muscles were then sectioned longitudinally into slices of 50 μm thickness using a freezing microtome. These sections were mounted onto slides coated with 0.5 % gelatin to ensure adherence during subsequent staining. Neuromuscular junctions (NMJs) were visualized using fluorescent α-bungarotoxin (α-BTX Alexa 555), which labels acetylcholine receptors (AChRs), and a mouse-derived anti-A2AR monoclonal antibody, as described previously [6]. Specific information regarding antibodies and their dilutions can be found in Table 2. After 3 h of washing, slides were mounted in Vectashield (Vector). To detect A2AR-TrkB heteroreceptor complexes, some sections were further processed using the in-situ Proximity Ligation Assay (isPLA), following established methods [26]. In this procedure, immunohistochemical steps were carried out up to the primary antibody incubation stage. The sections were then incubated with species-specific primary antibodies: rabbit-derived polyclonal anti-TrkB and mouse-derived monoclonal anti-A2AR, both diluted appropriately in blocking solution and incubated overnight at 4 °C (details in Table 2). The isPLA protocol was then completed according to the manufacturer's instructions using the Duolink in situ PLA detection kit (Sigma-Aldrich, Italy), with probes tailored to rabbit and mouse antibodies. Negative controls were included by omitting the primary antibodies during both the immunohistochemistry and isPLA procedures. Qualitative analysis of A2AR immunoreactivity and isPLA signals was performed using a Leica TCS SP5 confocal microscope.

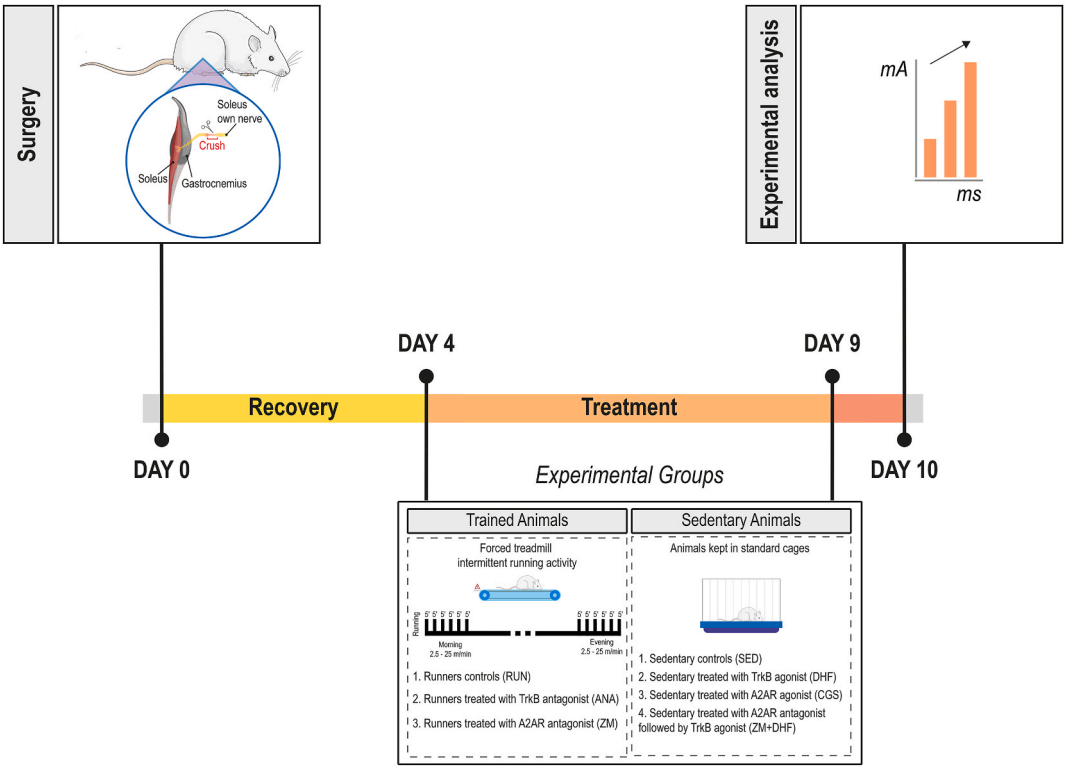

Fig. 1. Schematic illustration of the experimental design and the exercise protocol utilized in the study.

Table 1
Chemicals.

Chemicals					
Reagent	Dilution	AR	Source	Characterization	Identifier
ANA 12	0.5 mg/kg	IP	Tocris	[21]	Cat # 4781
ZM 241385	3 mg/kg	IP	Tocris	[22]	Cat # 1036
CGS 21680	1 mg/kg	IP	Tocris	[23]	Cat # 1063
DHF	10 mg/kg	IP	Tocris	[24]	Cat # 3826
d-tubocurarine	$10^{-6} - 10^{-7}$ g/ml	N.A.	Tocris	[25]	Cat # 2820

Abbreviations: AR, administration route; IP, intraperitoneal.

Table 2
Primary and secondary antibodies.

Primary antibodies					
Antibodies	Host	Dilution	Source	Characterization	Identifier
TrkB	R	1:100 (isPLA)	Abcam	[9]	Cat # ab18987, RRID: AB_444716
A2AR	M	1:200 (IF) 1:100 (isPLA)	Millipore	[27,28]	Cat # 05–717, RRID: AB_11213750
Secondary antibodies Conjugated to	**React**	**Dilution**	**Source**		**Identifier**
α-BTX Alexa Fluor 555®	N/A	1:200 (IF)	Thermo Fisher Scientific		Cat #B35451, RRID: AB_2617152
Alexa Fluor 488®	M	1:250 (IF)	Jackson ImmunoResearch		Cat # 115-545-003, RRID: AB_2338840

Abbreviations: IF, immunofluorescence; isPLA, in situ Proximity Ligation Assay; M, mouse; R; rabbit.

2.4. Electrophysiology

Ten days after the nerve crush, the animals were euthanized with an overdose of sodium thiopental. The soleus muscles, along with a portion of their associated nerve, were then carefully removed and placed in a recording chamber containing Ringer's solution, oxygenated with a 95 % O2 and 5 % CO2 mixture, to perform intracellular muscle recordings, as outlined in previous studies [6,7]. In brief, intracellular recordings of synaptic transmission events, specifically excitatory postsynaptic potentials (EPSPs), were obtained following nerve stimulation using a borosilicate electrode containing 3 M KCl. Recordings were made with a DUO 773 amplifier (WPI, United States) and analyzed with WinWCP software (Strathclyde V 5.2.7, United Kingdom; RRID: SCR_014713). To prevent the generation of action potentials and muscle contractions induced by stimulation, neuromuscular junctions were partially blocked by adding d-tubocurarine to the perfusion bath (refer to Table 1 for details). Muscle cells were considered innervated by multiple inputs if two or more EPSPs were observed with different thresholds (the stimulus intensity required to evoke the EPSP) or latencies (the time delay between the stimulus artefact and the EPSP, as stimulus strength was gradually increased). The percentage of multiple innervation was quantified as an axon sprouting index for each muscle, following previously described methods [6,7]. A minimum of 50 cells per muscle were analyzed.

2.5. Statistical analysis

The data were analyzed with GraphPad Prism 9 (GraphPad Software, United States; RRID: SCR_002798). To detect and exclude outliers, the ROUT method (Q = 1 %) [29] was applied to each dataset before statistical analysis and visualization. The distribution of the data was assessed using the Shapiro-Wilk test, while Levene's test was used to verify the equality of variances. To determine group differences, one-way ANOVA was performed, followed by Tukey's post hoc test for multiple comparisons. A p-value of less than 0.05 was considered statistically significant. The statistical approach for the experimental analysis is described in the corresponding figure legend.

3. Results

3.1. A2A receptor monomers and A2AR-TrkB complexes are distinctly located at the neuromuscular level

Since we hypothesize that the adenosine A2A receptors could enhance the intramuscular nerve sprouting during muscle reinnervation after a nerve crush by transactivating the TrkBs, we first examined the A2ARs distribution at the neuromuscular level. To this end, we used a series of immunohistochemical analyses to localize A2ARs at the soleus NMJs in undenervated animals. We confirmed [30,31], by double-labelling A2A receptors and nAChRs, that A2A immunoreactivity is mainly localized at the axonal bundles

(Fig. 2A), along with terminal and pre-terminal axons approaching to the nAChR cluster establishing the end-plate area (Fig. 2B). Furthermore, it is important to highlight that the A2ARs distribution pattern found here is similar to the TrkB receptors localization pattern found in our previous work [6]. Beyond the 'transactivation process' involving the A2A and TrkB monomers [16], compelling evidence showed that these two receptors can physically interact, forming heterocomplexes [26,32]. In this regard, using the isPLA approach, we provide the first evidence of proximity, namely less than 40 nm of distance, between A2AR and TrkB receptors at the plasmalemma of soleus muscle in undenervated controls (Fig. 2C). Collectively, these findings suggest that adenosine may affect the BDNF action at the neuromuscular level by two distinct pathways, namely the well-characterized transactivation of TrkB via A2A [16] may take place predominantly at the axonal and end-plate level. At the same time, a novel allosteric modulation of TrkB by a potential physical interaction with A2A receptors may occur at the muscular level.

3.2. Adenosine A2A receptors are necessary to trigger the TrkB-dependent intramuscular nerve sprouting during muscle reinnervation in nerve-crushed rats

To explore whether A2ARs affect the TrkB-dependent intramuscular nerve sprouting during muscle reinnervation, we performed a series of electrophysiological recordings 10 days after soleus denervation to assess the percentage of multiple innervation (Fig. 3A), which is commonly regarded as an indicator of axonal sprouting [6,7]. Consistent with our prior research [6,7], we confirmed that an activity-related enhancement of axon sprouting occurs during the reinnervation process. Indeed, a mid-intensity running activity induced a significantly higher percentage of multiple innervation (RUN; $M = 28.50$ %, SEM = 3.99) compared to sedentary condition (SED; $M = 9.50$ %, SEM = 1.70; Fig. 3B). In addition, an increased axon sprouting, comparable to RUN controls, was induced by treating sedentary controls with a TrkB receptor agonist (DHF; $M = 23.40$ %, SEM = 2.45). Conversely, trained animals treated with a TrkB receptor antagonist (ANA) showed a significant decrease in the percentage of multiple innervation ($M = 6.54$ %, SEM = 0.43) compared to the RUN group displaying similar values to those collected in sedentary controls (Fig. 3B). As previously reported [6,7], these findings demonstrated that the activity-related enhancement of axon sprouting during the reinnervation process found here is dependent on TrkB receptors. Surprisingly, the effect of motor activity on the axon sprouting was mirrored in sedentary animals treated with a selective adenosine A2A receptor agonist (CGS; $M = 24.54$ %, SEM = 2.53) and abolished, with a residual percentage of multiple innervation close to SED controls, by blocking A2ARs using a potent antagonist (ZM; $M = 7.6$ %, SEM = 1.73) in trained animals. The data obtained so far showed that pharmacological activation of either TrkB or A2A receptors led to recapitulating, almost identically, the motor activity effect on the reinnervation of target muscle after a nerve crush. Therefore, we decided to treat sedentary animals with an A2ARs antagonist followed by a TrkB receptors agonist (ZM + DHF) to gain insight into the molecular underpinnings of A2A-TrkB interplay in modulating nerve-terminal sprouting. In the ZM + DHF group, the A2ARs antagonism completely prevented the TrkB activation induced by DHF, thus, with comparable values to those obtained in the SED group, significantly reduced the percentage of multiple innervation ($M = 10.50$ %, SEM = 1.04) compared to CGS, DHF and RUN animals (Fig. 3B). This latter finding provided evidence that A2ARs activation is required to gate the TrkB-dependent intramuscular nerve sprouting during muscle reinnervation in nerve-crushed rats.

4. Discussion

Here, we provide the first evidence that axonal adenosine A2A receptor activation is required to gate the activity-related TrkB-dependent enhancement of axon sprouting during the reinnervation process after a nerve crush. In addition, we showed that A2AR monomers may physically interact with TrkBs, forming heteroreceptor complexes at the muscle plasmalemma, thus demonstrating that A2A-TrkB interplay may account for two different molecular cascades at the neuromuscular level.

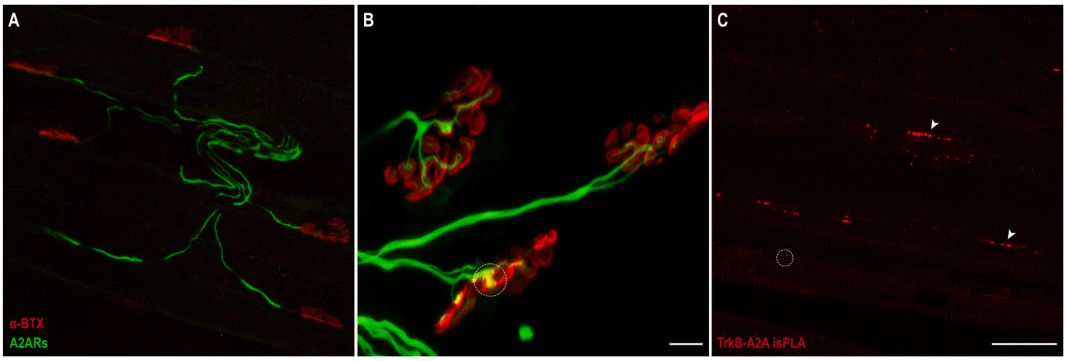

Fig. 2. Localization of the adenosine A2A receptor and A2AR-TrkB heteroreceptor complexes immunoreactivity at the soleus muscle. (A) Representative confocal image showing axonal and presynaptic (B) adenosine A2A receptor distribution at the soleus muscle of an undenervated control. The dashed circle highlights an A2AR-labeled axon (Green) contacting a neuromuscular junction (Red). (C) Representative confocal image of isPLA products (Red) showing the existence of A2AR and TrkB proximity at the plasmalemma of the soleus muscle of an undenervated control. Arrowheads point to positive isPLA clusters; single isPLA puncta were also detected (dashed circle). Scale bar: A-C, 40 μm; B, 10 μm.

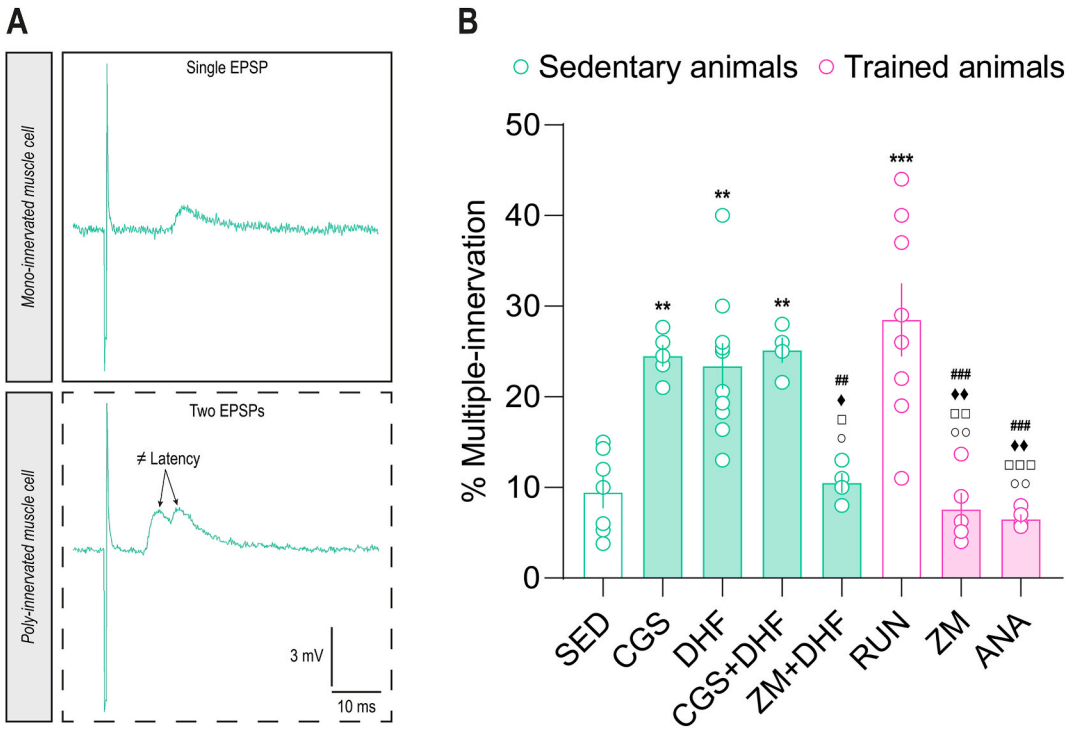

Fig. 3. Effect of the adenosine A2A on the TrkB-dependent nerve-terminal sprouting during reinnervation of the soleus muscle. (A) Representative recordings of excitatory postsynaptic potentials (EPSPs) obtained intracellularly in response to nerve stimulation. The top panel illustrates a single EPSP recording, indicative of a mono-innervated muscle fiber, while the bottom panel demonstrates two distinct EPSPs with different latencies observed during incremental nerve stimulation. (B) Effect of the adenosine A2A and TrkB receptor interplay on soleus muscle multiple-innervation 10 days after nerve crush. One-way ANOVA: F (7, 40) = 12,00, $p < 0.001$; Tukey's *post hoc* test: $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. SED; $^{\circ}p < 0.05$ and $^{\circ\circ}p < 0.01$ vs. CGS; $^{\square}p < 0.05$, $^{\square\square}p < 0.01$ and $^{\square\square\square}p < 0.001$ vs. DHF; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. RUN. Data are expressed as mean ± SEM (SED $n = 7$, CGS $n = 5$, DHF $n = 10$, ZM + DHF $n = 4$, RUN $n = 8$, ZM $n = 5$ and ANA $n = 5$).

The primary goal of PNI treatment and rehabilitation programs is to promote the functional recovery of target muscles, thus ameliorating the patient's quality of life [2]. In this regard, it has been widely acknowledged that non-surgical activity-related therapies based on specific exercise patterns can improve motor functions following PNIs [33]. Consistently we previously showed that an exercise pattern characterized by an intermittent mid-intensity running activity performed during the reinnervation of target muscle positively impacts functional recovery by enhancing axonal plasticity in rats subjected to nerve crush injury [6,7]. The impact of this running protocol on muscle reinnervation and functional recovery was mediated through the enhanced activity-dependent expression of muscle-derived BDNF and the involvement of presynaptic TrkB receptors. The induction of the TrkB-dependent intramuscular nerve sprouting during muscle reinnervation promoted a wider multiple-innervation of target muscle cells, thus bolstering the functional recovery after nerve crush [6,7]. In line with this evidence, the electrophysiological data obtained here confirmed that an intermittent mid-intensity running activity increases nerve-terminal sprouting during the reinnervation process in nerve-crushed rats and that this effect is TrkB-dependent.

Moreover, we extended previous findings [6,7], demonstrating that axonal adenosine A2A receptor activation can mimic the effects of training on axonal sprouting in sedentary animals. In this respect, compelling evidence emphasized the relevance of the A2ARs activation during synaptogenesis [18,19] as well as A2ARs and TrkB interplay in modulating the developmental axonal competition [34,35] and adult neurotransmission at NMJ [15,36]. Indeed, by using quantitative immunohistochemistry, Nadal et al. showed that the activation of presynaptic A2A and TrkB receptors are involved in delaying the axonal elimination during the early NMJs developmental stages (postnatal day 7) of *Levator auris longus* muscle in mice. In keeping with this evidence, our data demonstrated that antagonizing either A2ARs (ZM group) or TrkBs (ANA group) in trained nerve-crushed rats completely prevented the activity-related increased axonal sprouting compared to sedentary animals. Conversely, the selective pharmacological activation of A2ARs (CGS group) or TrkBs (DHF group) in sedentary nerve-crushed rats induced a significant enhancement of intramuscular nerve-ending sprouting with similar values to those collected in trained nerve-crushed controls (RUN group). Collectively, our data establish that, as occurs during development [34,35], A2ARs and TrkBs have a pivotal role in modulating synaptic plasticity even in a model of PNI. Intriguingly, from a translational point of view, our demonstration that the A2ARs activation, mirroring the activity-related TrkB-dependent effect, can boost multiple-innervation during the reinnervation process prodromal to a full functional recovery, poses A2ARs as a novel pharmacological target for developing neurotrophin-based-like therapies for treating PNIs. In this reasoning, using A2A as a pharmacological target to transactivate presynaptic TrkBs could represent a novel therapeutic strategy to mirror the

BDNF effect on reinnervation process upon a PNI. At the same time, it could be a helpful strategy to mitigate some common limiting factors associated with the use of exercise-based therapies in the early stages of PNIs, such as pain and immobility, thus broadening the therapeutic window.

In addition, we showed that, in the ZM-DHF sedentary animals, the block of A2ARs abolished utterly the effect of TrkBs agonism on nerve endings sprouting, providing evidence that tonic A2A activation is required to gate the presynaptic TrkB effect at NMJ. In light of this data, we might speculate that, during the reinnervation process after a PNI, A2AR primes the TrkB receptor's functionality in controlling synaptic plasticity at NMJ. In this view, similar to some extent to what occurs for the adenosine control of transmitter release at motor nerve endings [15], in basal condition - i.e. no muscular activity – the activation of presynaptic A2ARs by the resting adenosine tone may be necessary to prime TrkBs into a preactivated state during the reinnervation process, while in depolarizing condition – i.e. during motor activity – A2ARs may sense the rising in the adenosine levels and respond to this increased adenosine tone by facilitating the muscle-derived BDNF effect on axon sprouting TrkB-dependent.

Finally, our immunohistochemical data, on one side, confirmed that A2ARs are mainly localized at the axonal and presynaptic level [30,31] and, on the other side, provided the first evidence of proximity between A2A and TrkB receptors at the muscle plasmalemma of soleus muscle in undenervated controls, thus posing the existence of postsynaptic A2AR-TrkB heteroreceptor complexes. This latter finding aligns with prior evidence indicating that A2A receptors and TrkB receptors interact directly at the dendritic level in cultured rat spinal motor neurons [32], suggesting that adenosine may modulate BDNF activity within the neuromuscular system via distinct signaling platforms. Precisely, at the postsynaptic level, namely at dendrites and muscle plasmalemma, adenosine might modulate

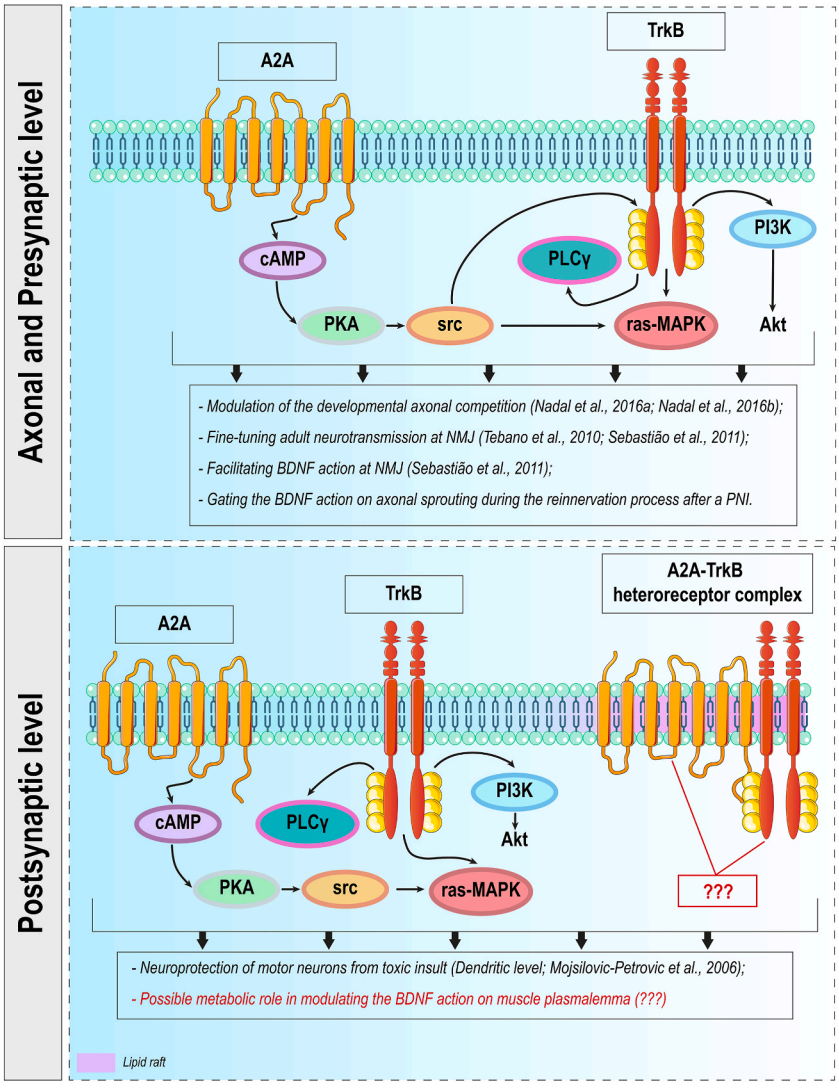

Fig. 4. Putative adenosine/A2A receptor and BDNF/TrkB signalling platforms at neuromuscular level. Abbreviations: cAMP, cyclic adenosine monophosphate; PKA, Protein kinase A; Src, Sarcoma non-receptor tyrosine kinases; MAPK, mitogen-activated protein kinase; Ras, small GTP-binding protein; PI3K, phosphatidylinositol 3-kinase; PLC, Phospholipase C; Akt, Protein kinase B.

TrkBs function on synaptic transmission and muscle metabolism either by a direct physical A2A allosteric modulation or trans-activation process, while at the axonal and presynaptic level, predominantly by transactivation or overlapped downstream pathways (Fig. 4). Therefore, investigating the role of these two potential A2A-mediated pathways in modulating TrkB function could shed light on the molecular underpinnings of critical events shaping NMJ circuitry. Future studies employing genetic models, such as A2ARs conditional knockout strains, alongside imaging techniques like post-embedding immunogold labeling [37], could enable precise mapping of A2ARs distribution and clarify the effects of direct A2A receptor-mediated allosteric modulation or TrkB receptor trans-activation on neuromuscular transmission and muscle metabolism.

5. Conclusion

Achieving complete functional recovery following peripheral nerve injury remains one of the most significant challenges in treatment and rehabilitation. Our findings highlight that presynaptic activation of adenosine A2A receptors is critical for facilitating the activity-dependent, TrkB-mediated enhancement of axonal sprouting during the reinnervation process after nerve crush injury, thus posing A2ARs as a potential pharmacological target for developing neurotrophin-based-like therapies for treating PNIs. However, it is essential to acknowledge that several factors hinder the successful translation of promising findings obtained from preclinical animal experiments. Accordingly, the 10-day post-injury interval used in this study might not fully capture the entire duration of natural recovery in patients and a pharmacological approach may never fully replicate the well-documented pleiotropic effects of activity-associated therapies such as physical exercise. Despite these limitations, the current findings offer promising insights. In future investigations, A2AR agonism treatment could be explored to extend the therapeutic window in the early stages of peripheral nerve injuries, where factors such as pain and immobility may limit the feasibility of physical exercise, thereby potentially enhancing functional recovery in humans.

CRediT authorship contribution statement

Michael Di Palma: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Davide Lattanzi:** Methodology. **Patrizia Ambrogini:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Stefano Sartini:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Data availability statement

Data will be made available on request.

Ethics approval and consent for publication

The animal use was carried out in accordance with the Italian law on animal experiments (D.lgs 26/2014; research project permitted with authorization N° 149/2018-PR by the Italian Ministry of Health).

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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