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Extra-pineal melatonin in perisynaptic Schwann cell–muscle fiber cross talk at the regenerating neuromuscular junction

Samuele Negro*, Cesare Montecucco*, Michela Rigoni*

The neuromuscular junction and its pro-regenerative niche: The mammalian peripheral nervous system, unlike the central nervous system, has preserved throughout evolution the ability to regenerate and fully restore function. Key factors for effective nerve regeneration include a supportive neuronal environment and a coordinated tissue response (Brosius Lutz and Barres, 2014).

The neuromuscular junction (NMJ) is the synapse formed between motor neurons and muscle fibers (MF) responsible for triggering muscle contraction. As a prototypical synapse, the NMJ has been central to advancing our understanding of synaptic physiology over the years (Sanes and Lichtman, 1999). The NMJ demonstrates a remarkable capacity for spontaneous regeneration, often achieving complete recovery following damage and degeneration of the motor axon terminal (MAT) caused by various insults, including mechanical trauma, neurotoxins, autoimmune attacks, and genetic diseases. This regenerative capability is essential for maintaining functions critical to survival, such as respiration and locomotion.

The NMJ is a four-component system comprising the MAT, perisynaptic Schwann cells (PSCs), MF and a unique basal lamina lying between MAT and MF and providing the structural support to this unique synapse (Sanes and Lichtman, 1999). The close anatomical association of these components facilitates the rapid exchange of signals and messengers, including acetylcholine and adenosine triphosphate, which are released from neurons through the exocytosis of synaptic vesicles and granules, ensuring proper system function (Gould et al., 2024). Disturbances in this homeostatic signaling, triggered by neuropathogens and damage-induced alarm signals within the NMJ - such as hydrogen peroxide and mitochondrial DNA (Duregotti et al., 2015) - initiate an orchestrated tissue response that promotes repair and regeneration (Brosius Lutz and Barres, 2014; Negro et al., 2017; Rigoni and Negro, 2020).

While PSCs are known to play a pivotal role in NMJ functional recovery (Gould et al., 2024), the contributions of MF and the basal lamina to this process are less well understood. Impairments in the NMJ's supportive environment may contribute to synaptic instability and denervation, which are observed in aging and in chronic, progressive neurodegenerative disorders where the NMJ is an early target, such as amyotrophic lateral sclerosis (Gould et al., 2024).

Neurotoxins as tools to dissect NMJ plasticity: A reliable and reproducible experimental approach for identifying novel components of intercellular signaling at the NMJ, as well as potential therapeutic agents for treating MAT degeneration, involves the use of neurotoxins derived from spiders or snakes, such as α-Latrotoxin (α-LTx) and presynaptic snake PLA2 neurotoxins (nPLA2), respectively (Rigoni and Negro, 2020). These neurotoxins trigger a rapid influx of Ca^{2+} into the MAT from the extracellular medium, leading to the activation of several Ca^{2+}-dependent hydrolases and subsequent MAT fragmentation. Notably, neurodegeneration caused by spider and snake neurotoxins is reversible, with full anatomical and functional regeneration occurring within days in mice and within weeks in humans. Using this *in vivo* model, several promoters of NMJ functional regeneration have recently been identified, most of which are involved in MAT-PSC communication (Duregotti et al., 2015; Negro et al., 2017; Gould et al., 2024), with the neurohormone melatonin playing as a key mediator in the PSC-MF cross talk at the injured NMJ (Stazi et al., 2021).

Melatonin: an ancient molecule with multiple functions: Melatonin is an ancient and evolutionarily conserved molecule with diverse cellular functions that may occur independently of, or be mediated by, specific receptors (abbreviated as MT1 and MT2). Melatonin first evolved in photosynthetic bacteria, where it likely acted as an antioxidant neutralizing reactive oxygen species generated during photosynthesis, and later in plants, where it serves as a hormone (Manchester et al., 2015). Numerous studies have shown that melatonin provides essential protection against free radicals in all cells and organelles. This primary and ancient action is widely accepted by the scientific community (Manchester et al., 2015). However, these antioxidant properties alone cannot account for melatonin's functional diversity. Its broad range of functions is likely linked to its early evolutionary origin and to the cell-context-dependent nature of signaling downstream of the conserved G-protein-coupled receptors MT1 and MT2. Melatonin receptors (MTRs) can form homo- or heteromeric complexes with altered functional properties, activating distinct intracellular pathways. For instance, MT1/MT1 dimers preferentially activate Gi signaling, whereas MT1/MT2 heterodimers are more closely associated with Gq signaling, leading to activation of the ERK1/2 pathway. Furthermore, MTR activity can be modulated by dimerization with other G-protein-coupled receptors (Okamoto et al., 2024).

The neuromuscular junction – a new extra-pineal source of melatonin: In vertebrates, two primary sources of melatonin have been identified: (i) the pineal gland, which accounts for approximately 5% of total melatonin and primarily regulates circadian rhythms and sleep through receptor-mediated actions on central and peripheral clocks, and (ii) extra-pineal tissues distributed throughout the body that synthesizes melatonin within mitochondria, which consequently acts within the cell of origin, or in nearby cells. Since the pineal gland is unique to vertebrates, extra-pineal melatonin is considered the most ancient form (Acuna-Castroviejo et al., 2014).

Noticeably, the NMJ is highly enriched in mitochondria located both pre- and post-synaptically, which are potential sites for melatonin release. It is therefore likely that during MAT injury, the generation of alarm signals (positive cues) and/or a reduction in the physiological release of adenosine triphosphate and acetylcholine (negative cues) (Gould et al., 2024) could trigger the local release of melatonin by the MF into the synaptic cleft. This hypothesis is supported by recent findings showing that local administration of Luzindole, an antagonist of MTR, to the α-LTx-intoxicated NMJ delays the recovery of neurotransmission (Stazi et al., 2021). Following MAT injury, PSCs upregulate melatonin receptor type 1 (MT1), a response also observed in myelinating SCs following mechanical compression of the sciatic nerve, leading to activation of ERK signaling via phosphorylation (Stazi et al., 2021). Thus, melatonin acts as a pro-regenerative factor at the NMJ, with MT1 serving as the primary receptor mediating its effects.

To test the possibility of local melatonin synthesis at the NMJ, we performed immunostaining to detect AANAT (Aralkylamine N-acetyltransferase, also known as serotonin N-acetyltransferase), the rate-limiting enzyme in melatonin biosynthesis (**Figure 1**). In control muscle tissue, AANAT was detected along the nerve and at the neuromuscular synapse (Panel A, first and second rows). Following acute MAT degeneration induced by α-LTx (16 hours), AANAT signal predominantly localized within MF beneath the NMJ (Panel A, third row, and orthogonal projection in Panel B), co-localizing with the mitochondrial marker Tom20 (Panel C). These findings identify the postsynaptic MF as a primary source of melatonin at the injured NMJ.

Melatonin receptors–druggable targets to promote nerve recovery of function: Poisoning and reversible degeneration of the MAT caused by α-LTx, where regeneration is accelerated by melatonin, is rare in humans. In contrast, peripheral neuroparalysis leading to respiratory impairment due to snake venoms containing nPLA2 is highly prevalent and is estimated to cause tens of thousands of human deaths annually. However, the resulting paralysis is fully reversible in both mice and humans if they are mechanically ventilated. Recently, we demonstrated that melatonin significantly accelerates recovery from neuroparalysis and respiratory dysfunction induced by nPLA2 in mice (D'Este et al., 2024); however, the required doses are too high for practical therapeutic use in humans.

Among the wide range of available MTR agonists, we selected Ramelteon and Agomelatine, both of which have been approved for human use: Ramelteon for treating insomnia (FDA, 2005) and Agomelatine for depression and anxiety (EMA, 2009). Both drugs significantly enhance NMJ recovery following intoxication with potent nPLA2-containing snake venoms, as shown through immunofluorescent analysis of NMJ markers, electrophysiological testing, and respiratory function assays in mice (D'Este et al., 2024).

Another relevant NMJ disorder involving peripheral neuroparalysis that could benefit from pro-regenerative therapies is the Guillain-Barré Syndrome. Guillain-Barré Syndrome arises from the production, during infections, of autoantibodies that cross-react with polysialogangliosides highly enriched on the presynaptic membrane. These autoantibodies bind to the MAT surface, recruit

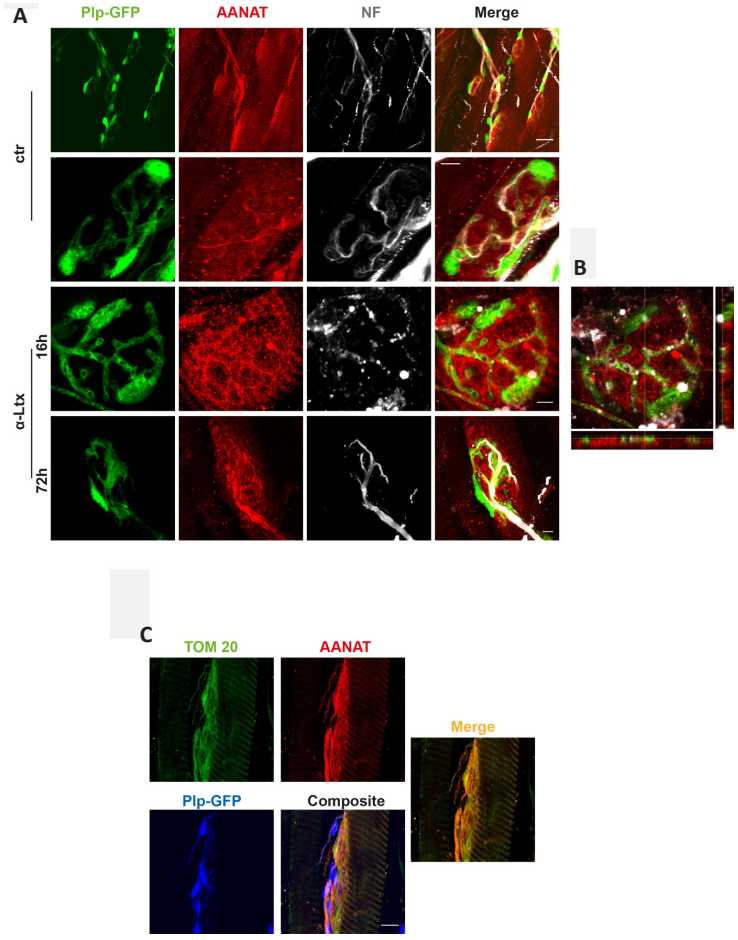

Figure 1 | **AANAT expression at the murine NMJ.**
(A) Immunostaining of control (upper panels) and α-LTx treated (lower panels) NMJs from the *Levatoris auris longus* muscles of *plp*-GFP mice, showing GFP-positive PSCs (green), AANAT expression (red), and the axon terminal identified by anti-neurofilament (NF) antibodies (white). Scale bars: 20 µm (upper rows) and 5 µm (bottom row). (B) Orthogonal projection of one α-LTx-poisoned NMJ (16 hours) showing that the AANAT signal is concentrated underneath the NMJ. (C) The mitochondrial markers Tom20 (green) and AANAT (red) co-localize at the NMJ. PSCs are in blue. Scale bar: 10 µm. Created with Adobe Illustrator. AANAT: Aralkylamine N-acetyltransferase; Ctr: control; GFP: green fluorescent protein; NF: neurofilaments; NMJ: neuromuscular junction; plp: proteolipid protein; PSC: perisynaptic Schwann cell; Tom20: mitochondrial import receptor subunit TOM20 homolog; α-LTx: α-Latrotoxin.

serum complement, and trigger the formation of a transmembrane attack complex. This event causes cytosolic Ca^{2+}-induced neurodegeneration similar to the effects of mechanical trauma or presynaptic membrane damage by spider toxins or snake nPLA2 (Rodella et al., 2016). Given these parallels with venom-induced neuroparalysis, we suggest that melatonin and its agonists should be tested on Guillain-Barré Syndrome models (Rodella et al., 2016) to promote a faster and more effective functional recovery of neurotransmission.

Conclusion: Given the efficacy of melatonin in promoting nerve repair across various types of peripheral nerve injuries, the extensive range of MTR agonists now available with high specificity for either MT1 or MT2 (Okamoto et al., 2024) and recent advancements in drug design that have produced compounds with enhanced properties, we propose expanding the study of diverse MTR agonists to additional models of peripheral neuropathies. Since MTRs can form homomeric or heteromeric complexes with each other and with other receptors, altering intracellular signaling responses (Okamoto et al., 2024), further investigation into the complexity of melatonin signaling in neurons and surrounding cells could uncover novel, effective therapeutic approaches for mitigating the progression of neurodegenerative diseases.

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