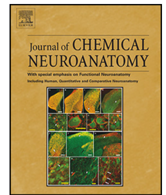
Journal of Chemical Neuroanatomy 81 (2017) 34–41

Contents lists available at ScienceDirect

Journal of Chemical Neuroanatomy

journal homepage: www.elsevier.com/locate/jchemneu



Possible promoting effects of melatonin, leptin and alcar on regeneration of the sciatic nerve

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ARTICLE INFO

Article history:
Received 15 August 2016
Received in revised form 18 January 2017
Accepted 1 February 2017
Available online 3 February 2017

Keywords:
Neuroprotectant
Peripheral nerve regeneration
Stereology

ABSTRACT

Peripheral nerve injury is a widespread and disabling condition that can impair the individual's daily life. Studies involving medications that may positively affect peripheral nerve regeneration are rare. The aim of this study was to investigate new treatments after peripheral nerve injury using various neuroprotectants, melatonin, alcar and leptin, in the regenerative process in an experimental rat model. *Wistar albino* rats were randomly divided into eight groups containing equal number of animals. Intraperitoneal injection of melatonin (50 mg/kg, for 21 days), leptin (1 mg/kg, for 21 days) and acetyl-L-carnitine (50 mg/kg, for six weeks) was performed postoperatively. Histological and electromyographical assessments of the regenerated nerves were performed 12 weeks after surgery. Stereological analysis was performed to estimate myelinated and unmyelinated axon numbers, surface area, myelin thickness and the myelin thickness/axon diameter ratio for each group. The results showed that only alcar has a beneficial effect on the regeneration of unmyelinated axons. Neither melatonin and leptin nor alcar were observed to have any therapeutic effect on the regeneration of myelinated axons. Alcar therapy has a positive effect on the regeneration of unmyelinated fiber in the sciatic nerve. However, the same effect was not observed in myelinated nerve fibers after intraperitoneal application of melatonin and leptin.
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1. Introduction

Varying degrees of nerve damage, including tension, crush or discontinuation, may occur as a result of events such as falls or industrial or motor vehicle accidents. Although nerve injuries do not usually lead to life-threating conditions, they may still cause a social burden in terms of economic costs (Rosberg et al., 2013). Although there has been considerable research into peripheral nerves, the treatment of peripheral nerve injuries is still controversial (Campbell, 2008). Major efforts have therefore been dedicated to resolving this issue. The degree of damage, at the axonal level, is the most important factor determining the speed and duration of recovery following nerve injury. Although, the recovery rate in damaged nerves may vary depending on the pathophysiological situation in the injured area, fully functional recovery generally occurs so long as axonal integrity is preserved (Allodi et al., 2012; Lundborg and Richard, 2003; Robinson, 2000). Studies regarding the effects of neuroprotective agents that potentially increase axonal regeneration following peripheral nerve damage, especially if axonal integrity cannot be preserved, are therefore needed.

A number of studies have investigated the neuroprotective effects of melatonin (Aygun et al., 2012; Kaplan et al., 2011; Leon et al., 2005). This has a potential neuroprotective effect in many conditions affecting the nervous system due to its lipophilic-hydrophilic and free radical scavenger properties. It can therefore protect the nucleus, cell membranes and organelles in particular against free radical damage (Aygun et al., 2012). Melatonin has also been reported to reduce neuronal death after peripheral axotomy. This has been attributed to the preservation of motor neurons and reduction of cell loss in the dorsal horn (Odaci and Kaplan, 2009). In addition, exogenous melatonin administration after nerve injury has a positive effect on myelin sheath thickness and axon numbers by reducing collagen deposition and preventing neuroma (Turgut and Kaplan, 2011). Melatonin thus exhibits neuroprotective effects

Abbreviations: AG, alcar group; AGG, alcar with gap group; CG, control gap group; EMG, electromyography; LG, leptin group; LGG, leptin with gap group; MG, melatonin group; MGG, melatonin with gap group; NGF, nerve growth factor; SOD, superoxide dismutase.

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http://dx.doi.org/10.1016/j.jchemneu.2017.02.003
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by increasing the regeneration process after peripheral nerve injuries and due to its antioxidant properties (Odaci and Kaplan, 2009).

In the literature, numerous studies have investigated the neuroprotective and neurogenetic effects of leptin (Avraham et al., 2011; Folch et al., 2012; Perez-Gonzalez et al., 2011). Exogenous leptin administration reduces neuronal damage following ischemia and stroke. It has also been reported to exhibit neurogenetic and neuroprotective effects by inducing an increase in the number of neurons and glia cells by affecting neural stem cell development (Avraham et al., 2011; Folch et al., 2012; Perez-Gonzalez et al., 2011).

Alcar administration increases axonal regeneration after nerve injury and produces a significant improvement in motor functions. It significantly reduces nerve loss in the dorsal root ganglia in a dose-dependent manner (Flatters et al., 2006). Alcar is thought to exhibit all its neuroprotective effects by increasing intracellular neurotrophic pathways or cholinergic neurotransmission (Flatters et al., 2006; Ori et al., 2002). This study evaluated the possible effects of some neuroprotective agents on sciatic nerve regeneration using stereological and electrophysiological techniques and electron microscopy.

2. Materials and methods

Histological follow-up was performed at the Histology and Embryology Department of Ondokuz Mayıs University, Turkey, and electron microscopic analysis in the laboratory of the Cavalieri Ottolenghi Neuroscience Institute, the University of Turin, Italy. The Animal Experiments Committee and the Ethics Committee of Ondokuz Mayıs University approved the study. All available precautions were taken to minimize pain, suffering and the number of animals used. At the end of the experiment, rats were sacrificed through an overdose of anesthetic.

Forty-eight female *Wistar albino* rats (150–200 g) were divided into eight groups: a pure control group (PG) which didn't undergo any special protocol, a control gap group (CG), a melatonin group (MG) that was given only melatonin, a melatonin with gap group (MGG), a leptin group (LG), a leptin with gap group (LGG), an alcar group (AG), and an alcar with gap group (AGG). Rats received a standard diet over the 12-weeks study period. In the gap groups, following resection of the sciatic nerve, a 5 mm gap was opened between the distal and proximal parts of the nerve and the segment was rotated for 180° was replaced by a fibrin coated tubular scaffold. At this point; gap style is suitable model for examining whether there is a possible regeneration.

Melatonin (Sigma-Aldrich) at 50 mg/kg (Keskin et al., 2015) was daily injected intraperitoneally in the melatonin groups for 21 days. During the same period, 1 mg/kg leptin (Maniscalco and Rinaman, 2014) (Sigma-Aldrich) was administered intraperitoneally to the leptin groups. Additionally, 50 mg/kg alcar (McKay-Hart et al., 2002) (Sigma-Aldrich) was administered intraperitoneally to the alcar groups in the postoperative period. The same protocols of injection have been performed on rats of groups with sciatic nerve not transected. Histological and electromyographical assessments of the regenerated nerves were performed 12 weeks after surgery.

On the 12th week postoperatively, electrophysiological analysis was performed on all rats before sacrifice. Compound muscle action potentials of the gastrocnemius muscle were measured. The time between the beginning of stimulation and the start of deflection (latency) and peak-to-peak amplitude (p-p amplitude) of the existing potential were measured on the action potential curves. Electrophysiological analyses were performed in the Physiology Department laboratory of the Ondokuz Mayıs University Faculty of Medicine. Electromyography (EMG) tests were performed using a 4SP PowerLab (AD Instruments, Sydney, Australia) device and Scope (ver. 3.7.2, AD Instruments) software. After the electrophysiological measurements, nerve samples were processed for resin embedding and the distal part was transversally sectioned using an ultra-microtome (Leica Ultracut UCT, Leica Microsystems GmbH, Germany). Sections of 70-nm were used, for the electron microscopic (JEM-1010, JEOL, Tokyo, Japan) analysis, whereas sections of 2.5 μm thickness were used for light microscopic examination. The number of unmyelinated axons (in the electron microscope images) and the number of myelinated axons, axonal and myelin sheath thickness measurements (in the light microscope images) were evaluated using stereological method which is called 2D fractionator (Stereoinvestigator 9.0. Micro Brield Field, Colchester, USA) (Kaplan et al., 2012).

2.1. Stereological analyses

The basic steps of the fractionator technique that is used in the estimation of the total myelinated axon number in peripheral nerve can be summarized as this: The nerve tissue samples belonging to each subject, following the proper histological tissue processing, should be prepared as blocks in the mounting medium appropriate for the examination method. The important factor in the block preparation is to execute the tissue processing with care in accordance with the properties of the study, size of the tissue samples and characteristics of the tissue. After the preparation of blocks, sectioning can be performed. Because, the peripheral nerve fascicle displays the same features regarding the axonal structure, one section from each block should be enough. Due to the fractionator logic, the systematic sampling will be done on every section optically. For this end, after the sections are properly stained, the number of the parameters of interest (myelinated axon number etc.) can be determined under the microscope. When the counting of the concerning parameters, such as the number of the myelinated axons, are performed, the resulting value depends on their physical existence. Namely, all the myelinated axons in the tissue have an equal chance to be sampled and it is impossible to reach a counting value greater than the number of axons that are present. Thus, the important point in the fractionator method is whether the parameter to be counted is visible. For this reason, it is crucial to render the particles of interest visible by staining processes done on sections, to prevent the result from be affected by the errors (Ross and Pawlina, 2011). When the myelinated axons on the sections are counted under microscope, it requires that the counting areas are as stated above, optically fractionated in accordance with systematic sampling method. If not, the counted particle number would be higher. This type of sampling is called area fractionators (Gundersen, 1986). Firstly, the boundaries of the area of interest are determined and it is decided how the step intervals will be in moving on this area. Area fractioning (in which the ratio of the steps that is to be sampled for counting is learned) is performed on the section by moving on the x and y axes and the myelinated axons are counted by using a counting frame step by step in each area. The size of the counting frame and the step size that limit the movement on x and y axes should be determined optimally during the pilot study by taking the properties like diameter of the nerve tissue, fascicle number etc. into account. The ratio of the counting frame area to step size gives us the area sampling ratio. As a result, by multiplying the total number of myelinated axons counted in a step by inverse of all the used fractionator steps, the total number of myelinated axons in the nerve fascicle is gained (Fig. 1). When it is considered that the estimation is done on only one sample, it is seen that it is very probable that the total myelinated axon number belonging to the peripheral nerve sample of interest is higher than the real value. However, by increasing the number of subjects in each group and consequently repeating the estimation of the total myelinated

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