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Viral Vectors as Tools to Investigate the Role of Dysregulated Proteins in Nervous System Pathologies: The Case of Acquired Motor Neuropathies

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1. Introduction

Gene therapy involves the insertion, removal or alteration of genes within cells and tissues of a living organism. Although it emerged as a tool to correct hereditary diseases due to a non-functional or missing protein, gene therapy also constitutes an experimental tool to investigate gene function. Since the 1960s, most gene therapy studies are aimed at cancer and hereditary diseases. As a research tool, the most traditional form of gene therapy involves the insertion of a functional gene at a specific location in the host genome in order to replace a mutated or missing gene. This is accomplished by isolating and amplifying the gene of interest, generating a construct containing the necessary elements for the right expression, and inserting this construct into the host organism. Another current approach to the study of gene function involves the genetic engineering of knock-out animals (generally mice), in which a specific gene is turned off through a targeted mutation. The resulting phenotype of a knock-out mouse may provide valuable information regarding the function of the missing gene. However, in some cases the targeted gene is necessary for the embryonic development, in which case the knock-out of that gene is not feasible. Alternatively, individual development may be directed towards compensating for that knock-down gene, in which case the phenotype would not provide much relevant information. Another research tool to study gene function is to create a transgenic organism, which can be a microbe, a plant, a fruit fly, a mouse, a zebrafish, a worm ... in which foreign, recombinant DNA (i.e., a transgene) is transferred directly into embryos to result in modified or novel genes. The resulting phenotype can provide valuable information about the role of genes in development, physiology, and disease. Although a transgene integrates in the host cell in a chromosomal location different from the endogenous site, the pattern of expression usually mimics that of the endogenous gene. Transgenic organisms are broadly used for agriculture, production of pharmaceutical drugs and proteins such as insulin, biomedical research, and gene therapy in experimental medicine.

Recent developments have provided us with different kinds of vectors to deliver, remove or modify genes within individual cells and tissues, bypassing undesirable effects of broad

knock-down or transgene expression. Basically, vectors used in gene therapy for release of specific genes can be viral or non-viral. Non-viral vectors include naked DNA, oligonucleotides, dendrimers, lipoplexes, and polyplexes, as well as nanoengineered, organically modified silicates. However, whereas artificial DNA transfer methods have very low efficiency and are not useful for some types of cells, natural selection and evolution have led viruses to naturally develop specialized molecular mechanisms to efficiently transport their genomes into the cells that they infect. Delivery of genetic material by a virus is called transduction, and the infected cells are termed as transduced. Actually, the earliest DNA transfer method for bacteria was bacteriophages, which are still commonly used in experimentation. For mammalian cells, the first viral vectors were based on the monkey tumor virus SV40, in which some viral genes can be replaced by foreign genes. Nonetheless, the advantages of viruses like SV40 are limited because only a few genes can be inserted into their genome before the length of the DNA molecule becomes too large to be packed into the viral coat.

Additionally, the genetic material of SV40 is usually degraded after it infects the cells. Therefore, viral vectors experimentally designed for gene therapy are usually created from pathogenic viruses, whose infectivity is much more effective, but they are modified to minimize the biohazard. Modifications usually involve the elimination of part of the viral machinery necessary for their replication, so that the virus can efficiently infect the cells but lacks the necessary proteins for the production of new virions. Experimentally modified viral vectors also have low toxicity, which means minimal unwanted effects on the physiology and viability of the transduced cells. Additionally, lytic viruses, which kill the cells after replicating into them to propagate the infection, cannot be used as vectors. Thus, a limited number of nonlytic viruses modified from the *retrovirus*, *adenovirus* (AVV), and *adeno-associated virus* groups can be used in gene therapy to stably introduce a gene into a cell. Retroviruses insert the genetic material in the form of a RNA molecule, which will produce a DNA copy by the action of the enzyme reverse transcriptase. This viral DNA is efficiently inserted into the host genome as a provirus, where it permanently replicates together with host DNA at each cell division. However, most retroviruses can only infect dividing cells. *Lentiviruses* (LVVs, like HIV) are a subtype of retroviruses. AVVs and adeno-associated viruses insert their genome in the form of a DNA molecule. Adeno-associated viruses, so named because they need the help of an adenovirus for replication, are small viruses whose genome consists of a single stranded DNA molecule. Once the adeno-associated virus enters the nucleus, their single-stranded DNA becomes double-stranded and integrates into the host genome, like retroviruses. However, AVVs enter the nucleus but do not replicate, remaining as extrachromosomal double-stranded DNA molecules. As many differentiated cells in the brain and other tissues lack the capability to divide, LVVs, AVVs, and adeno-associated viruses are acquiring increasing popularity in gene therapy applications because they can transduce non-dividing cells.

Currently, gene therapy is mainly being focused on the study of genetic disorders such as cancer, diabetes mellitus, cardiovascular diseases and nervous system pathologies, among others. More specifically, regarding the applications in the nervous system, this chapter will highlight the potential of viral vectors as tools for the investigation of the role of altered proteins in neuropathological processes and review our recently published findings (Sunico et al., 2008; 2010; Sunico & Moreno-López, 2010; Montero et al., 2010) in the context of the existing literature. Briefly, we have used AVVs and LVVs to study the function of 2 dysregulated proteins in pathological events occurring at the peripheral

(nerve) and central (motoneuron) levels after the severe crushing of a motor nerve in adult rats. The use of AVVs is proposed as a feasible gene therapy strategy for the enhancement of peripheral nerve regeneration in acquired peripheral neuropathies. Additionally, our outcomes support the promising benefits of gene therapy for the delivery of diverse genetic elements into specific populations of brain cells to treat several neurodegenerative disorders.

2. The case of acquired motor neuropathies: an useful experimental model to analyze protein dysregulation in neuropathological conditions

Most neurodegenerative disorders and prion diseases have common cellular and molecular mechanisms, including dysregulation of protein expression, function and/or aggregation. Alteration in the expression level of nitric oxide (NO) synthase (NOS) is a hallmark of Alzheimer (AD), Parkinson (PD), and Huntington diseases (HD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), and HIV dementia in humans and/or in animal models, as well as after peripheral and central traumatic lesions of the nervous system (Montero et al., 2010; Moreno-López et al., 2011). Subsequently, dysregulation of protein expression and/or function is a key event in a broad spectrum of neuropathological states. Unmasking the role of imbalanced proteins in anatomo-functional alterations of neurons in neurodegenerative disorders deserves attention. Our research has shown that viral vectors may be useful tools to explore the role of altered proteins in neuropathological processes. Using AVVs and LVVs, we studied the role of 2 NOS isoforms in pathological events occurring at the peripheral nerve and at the motoneuron after XIIth nerve crushing in adult rats.

NO is a short-lived, bioactive free radical which, as a gaseous molecule, freely crosses plasma membrane. NO and L-citrulline are the products of hydroxylation of a guanidine nitrogen of L-arginine and subsequent oxidization of the N^ω-hydroxy-L-arginine intermediate by NOS, a heme-containing enzyme that utilizes tetrahydrobiopterin (H₄B) as a redox cofactor. Electron transfer reactions carried out by NOS are regulated by a Ca²⁺-binding protein (calmodulin). NOS also needs NADPH as an electron donor and requires molecular oxygen to carry out the reaction. In their active form, the known NOS enzymes form dimers in which each NOS monomer is associated with a calmodulin molecule. Three major isoforms of NOS have been identified, coded by different genes and differing in localization, regulation, catalytic properties, and inhibitor sensitivity. The nNOS isoform was the first to be purified and cloned (also known as NOS-I) and is predominantly found in the neuronal tissue. Through their specialized postsynaptic density-95/disk large/zona occludens-1 domains, nNOS can physically associate with postsynaptic density protein-95 (PSD-95). In turn, PSD-95 binds to motifs in the C-terminus of NMDA receptor NR2 subunits. These molecular interactions may provide the mechanistic basis for a functional coupling between Ca²⁺ influx through NMDA receptors and NO production. The iNOS isoform (also known as NOS-II) is inducible in a wide range of cells and tissues, including activated macrophages and, astroglia and microglia in the pathological CNS. This isotype is Ca²⁺-independent and always catalytically active when expressed. However, iNOS is not the only isoform induced in pathological conditions. Finally, eNOS (or NOS-III) is the primary isoform found in vascular endothelial cells (Moreno-López et al., 2011).

Injured nerve fibers in the peripheral nervous system maintain the capacity to regenerate even over long distances in adult mammals. However, after nerve transection, stumps of

damaged nerves must be surgically joined to guide regenerating axons into the distal nerve stump. Even so, severe functional limitations persist after restorative surgery. Since most previous studies have used nerve transection, it is important to signal distinctions between different forms of nerve lesion. Regenerative and survival capacities could be differentially compromised in adult motoneurons following transection or avulsion of the peripheral nerve. However, crushing of a motor nerve causes an immediate and complete suppression of neuromuscular connectivity but, unlike transection, it preserves the endoneural tube, thus providing neurotrophic support and a physical guide for the growing proximal axonal endings (Moreno-López, 2010). A few weeks after nerve crushing, muscle re-innervation takes place without significant neuronal loss. Therefore, traumatic injury by crushing is a plausible model for the identification of molecules that regulate degenerative and regenerative processes after acquired peripheral neuropathies.

2.1 A model of acquired motor neuropathy: the crushing of the XIIth nerve

We have developed a model of acquired peripheral motor neuropathy induced by a traumatic insult that causes a well-characterized range of functional and synaptic impairments in the insulted motoneurons. This experimental model has been useful to test the role and effectiveness of diverse factors in degenerative/regenerative processes after nerve injury by means of functional and anatomical approaches. The hypoglossal system - hypoglossal motoneurons (HMNs), XIIth nerve and their target muscle (the tongue)- has been well characterized by our group in physiological and pathological conditions (González-Forero et al., 2004; Montero et al., 2008; 2010; Sunico et al., 2005; 2008; 2010; 2011). This motor system offers several advantages, since muscle and motoneuron activities can be accurately measured and its peripheral and central partners are easily accessible to carry out damage strategies, local microinjections and electrophysiological recordings.

Crushing the XIIth nerve immediately induces a complete suppression of neuromuscular connectivity, demonstrated by the complete absence in the tongue of the compound muscle action potential (CMAP) evoked by electrical stimulation of the injured nerve at the proximal portion (Fig. 1). CMAP was completely absent for 1, 3 or 7 days after crushing, even when supramaximal nerve stimulation was applied. These results indicate that our method of nerve crushing effectively disconnected most HMNs from their targets for at least 1 week. Evidence of muscle re-innervation was obtained at 15 days after crushing. Subsequently, there was a slow and progressive recovery of CMAP and at 30 days after crushing its amplitude was similar to the control condition (Fig. 1).

In the hypoglossal nucleus (HN), most motoneurons discharge bursts of action potentials synchronized with the inspiratory phase of breathing (Fig. 2). This characteristic activity persists even after animal decerebration and/or under anesthesia. The main alterations observed in the firing pattern of HMNs following XIIth nerve crushing were an overall reduction in firing rates and an almost complete loss of modulation by chemosensory afferents. The inspiratory activity of motoneurons was modulated by chemoreceptor-driven changes in response to alterations in end tidal CO_2 (ET_{CO_2}). The activity bursts of HMNs increased when ET_{CO_2} rose and decreased when ET_{CO_2} declined. One week after XIIth nerve crushing the chemosensory-mediated responsiveness of HMNs to ET_{CO_2} changes decreased (Fig. 2). Synaptic stripping of HMNs after nerve injury is at least partially responsible for reduced response of motoneurons to chemoreceptor-modulated inspiratory drive (Fig. 2).

Fig. 1. Experimental design and time course of neuromuscular function recovery after XIIth nerve crushing. (a) To evaluate the time course of the neuromuscular function recovery, the compound muscle action potential (CMAP), evoked by electrical stimulation (St.) of the XIIth nerve, was recorded using electrodes implanted in the genioglossus muscle. Inset: Photomicrograph of a sagittal section at the level of the crushing site extracted 3 h after injury, stained by neutral red. Note that axons are fully transected but endoneural tube is preserved. Scale bar: 500 μ m. (b) CMAPs evoked in the genioglossus muscle by single shock stimulation (arrowheads signal stimulus artifacts) of XIIth nerve in control and at 7, 15, 22, 30 and 45 days post-lesion. For comparison, CMAPs evoked by left (L, intact side) and right (R, crushed side) XIIth nerve stimulation are illustrated. Each trace represents an average of 10 individual traces. Dotted lines at 15 and 22 days represent how the Δ latency was calculated. Gray traces at 15 days represent the responses obtained in the same animal after injection of a neuromuscular blocker gallamine triethiodide in the genioglossus muscle. Note complete absence of CMAP on day 7 and the first signs of muscle re-innervation 15 days post-injury. Figure modified from Sunico et al., 2008 and Sunico & Moreno-López, 2010 (inset in (a)). © 2008 Elsevier Ltd and © 2010 Elsevier Ireland Ltd, respectively.

Fig. 2. XIIth nerve crushing induces a reduction of HMNs CO₂-modulated response and of their synaptic coverage. (a) Schematic diagram of the experimental preparation. Unitary discharge activity of HMNs and expired CO₂ were obtained in decerebrated, vagotomized rats, which had been injected with a neuromuscular blocking agent and lightly anesthetized. Right, characterization of firing properties of HMNs. From top to bottom, traces represent the extracellularly recorded spike discharge for a control inspiratory HMN, the instantaneous firing rate (FR, in spikes/s) and the partial pressure of CO₂ as a percentage of the expired air. Mean firing rate (mFR) per burst was measured and analyzed in relation to simultaneous ET_{CO2} measurements (dotted line in the middle trace). End-tidal CO₂ (ET_{CO2}) is indicated on the CO₂ record as a dashed line. (b) Illustrative examples of the time courses of the mean firing rate modulation (mFR, in spikes (sp)/s/burst; right y-axis; gray trace) relative to ET_{CO2} levels (left y-axis; black trace) for a control HMN and a motoneuron recorded 7 days after XIIth nerve crushing. (c) Confocal high-magnification photomicrographs of HMNs identified by the presence of a tongue-injected retrograde tracer (FluoroGold; green) in sections immunostained for the synaptic marker synaptophysin (red) obtained from control animals or 7 days after XIIth nerve crushing. Scale bars: 10 μ m. Figure modified, with permission, from Gonzalez-Forero et al., 2004 (a); Sunico et al., 2005 (c) and Montero et al., 2010 (b). © 2004 The Physiological Society, © 2005 Society for Neuroscience and © 2010 The Physiological Society, respectively.

Up-regulation of NOS also happens as a consequence of different kinds of peripheral neuropathies. More specifically, after traumatic injury of a motor nerve, NOS expression is up-regulated at 3 different levels: in the damaged nerve, in the denervated muscle, and centrally at the injured motoneurons. In the distal stump of the affected nerve, all 3 major isoforms of NOS are up-regulated: iNOS is *de novo* expressed in dedifferentiated Schwann cells and in infiltrated macrophages, whereas nNOS is accumulated in the growing motor axons. However, eNOS, which is constitutively found in blood vessels, is overexpressed in *vasa nervorum* of the distal stump and around the injury site after the damage (Moreno-López, 2010). At the central level, nNOS, but not iNOS, is expressed *de novo* in the soma of motoneurons after traumatic motor nerve injury (Sunico et al., 2005). nNOS is then outwardly transported, accumulating in growing axons at the peripheral level (Moreno-López, 2010). However, eNOS induction has not been reported in injured motoneurons so far (Montero et al., 2010).

2.2 Using an AVV to accelerate and improve muscle re-innervation after acquired motor peripheral neuropathy

Evidences of NO as a harmful molecule for nerve regeneration show that systemic administration of N^o-nitro-L-arginine methyl ester (L-NAME), a nonselective NOS inhibitor, improved motor nerve regeneration in mice after sciatic nerve transection (Zochodne & Levy, 2005). L-NAME also protected motoneurons from death and substantially increased the number that re-innervated the target muscle after facial nerve transection and repair, accompanied by improved motor function recovery (Wang et al., 2009). In the same direction, our studies showed that L-NAME treatment advanced the onset of neuromuscular reconnection after hypoglossal nerve crushing (Sunico et al., 2008). However, the pattern and time course of expression of each NOS isoform (Moreno-López, 2010) suggest that the NO from different origins could regulate diverse degenerative/regenerative processes after peripheral injury in a time-dependent manner. In nNOS or iNOS knockout mice, peripheral recovery was impaired after nerve injury due to delayed Wallerian degeneration, axon breakdown and Schwann cell reaction in the distal stump (Zochodne & Levy, 2005). Nevertheless, axon counts, myelination, and recovery of sensory and motor function in eNOS knockouts and wild-type mice were comparable after transection and reconstruction, although a delay of 2 days in revascularization was observed in eNOS knockout mice (Zochodne & Levy, 2005). The inherent risk of compensatory mechanisms for the disrupted enzyme during brain development is widespread in knockout animals. This may occur though a normally present redundant pathway and may be due to differences in expression of alternatively spliced isoforms of a particular NOS. For example, NOS catalytic activity levels in the brain of nNOS knockout mice are persistently low, which may mask the effect of nNOS deficiency (Moreno-López, 2010). Therefore, results obtained from knockouts, although useful, must be interpreted with caution.

Systemic application of relatively isoform-specific NOS inhibitors is another strategy that has been used to study the role of each isoform in degenerative/regenerative processes after nerve injury. nNOS or iNOS inhibition by the relatively specific inhibitors for nNOS 7-nitroindazole (7-NI), S-methyl-L-thiocitrulline (SMTC) and iNOS L-N6-(1-iminoethyl)-lysine hydrochloride (L-NIL), has contributed contradictory results. Whereas systemic administration of 7-NI, SMTC or L-NIL had beneficial effects on axonal regeneration after

facial nerve transection and repair (Wang et al., 2009), positive effects were not obtained after XIIth nerve crushing by treatment with 7-NI or aminoguanidine (AG), another iNOS inhibitor (Sunico et al., 2008). Strikingly, administration of the relatively specific eNOS inhibitor L-N(5)-(1-iminoethyl)ornithine (L-NIO) accelerated the onset of muscle re-innervation after hypoglossal nerve crushing by promoting axonal regrowth (Sunico et al., 2008). Contradictions can be explained by differences in the lesion model and in the dose and relative specificity of NOS inhibitors (Moreno-López, 2010). In any case, these results pointed to eNOS as the major source of detrimental NO in axonal regeneration, at least in the first week after nerve injury.

In general, a strong consensus exists stating that NOS inhibition, at least during the first week after traumatic motor neuropathy, accelerates and improves motor function recovery. However, the administration route must be carefully taken into account. NO has important roles in the immunological, cardiovascular and nervous systems. Key physiological functions, such as learning, platelet aggregation, arterial blood pressure, and immune responses, could be disrupted by chronic and systemic NOS inhibition. NOS inhibition as a plausible treatment to improve functional recovery after nerve injury must bypass unwanted side effects of systemic administration of the inhibitors. The therapeutic strategy should entail specific inhibition of the proper NOS isoform that is detrimental to nerve regeneration and the selected NOS inhibitor should be locally administered (Moreno-López, 2010). Endothelial isoform has been described as having negative effects on functional recovery in the traumatic motor nerve injury model (Sunico et al., 2008), mainly inhibiting axonal regrowth. Due to the apparent involvement of eNOS in vasodilation of blood vessels and platelet aggregation, however, chronic systemic treatment with a specific eNOS inhibitor is not advised. Such a treatment can affect cardiovascular function, inducing hypertension and increasing the risk of thrombosis and atherosclerosis. Finally, systemic administration of NOS inhibitors is not advised because they cause motor deficits in mice and rats (Moreno-López, 2010). Feasible tools to improve motor function recovery after nerve injury without systemic side effects could involve local application of viral vectors. Promising results have been obtained by our group, which has reported that intraneural administration of an AVV expressing the dominant negative of eNOS (AVV-TeNOS) accelerated and improved motor function recovery after XIIth nerve crushing (Sunico et al., 2008).

To elucidate the role of eNOS, recombinant protein expression has been induced by viral transfection. There are different approaches to induce a full or partial “loss of function” using viral transgenesis. In this chapter we illustrate the use of a dominant negative protein, which interferes with the target protein or its function. To suppress eNOS activity, a recombinant AVV was used to express a truncated form of eNOS (TeNOS) under the control of the human cytomegalovirus (hCMV) promoter (Kantor et al., 1996). TeNOS, which lacks catalytic activity, acts as a dominant negative inhibitor of wild-type eNOS by heterodimerization with the native protein (Liu et al., 2011). As a viral control, a recombinant AVV was used to express the enhanced green fluorescent protein (eGFP) directed by the hCMV (AVV-eGFP). This gene therapy strategy successfully revealed the involvement of eNOS from the *nucleus tractus solitarius* in hypertension of spontaneously hypertensive rats (Kasparov et al., 2004; Waki et al., 2003; Waki et al., 2006; Paton et al., 2007). The expression of proteins under control of the hCMV promoter is characterized by a very rapid increase in expression, peaking 10 hr after transfection (Stokes et al., 2003).

Furthermore, when AVV-eGFP and AVV-TeNOS were injected in the nervous system, eGFP and TeNOS were expressed in glia and endothelial cells 7 days after transduction; expression diminished greatly at 14 days after vector injection (Waki et al., 2006). Therefore, a single intranerve injection of AVV-TeNOS could be expected to provide effective chronic inhibition of eNOS for at least 1 week in the transduced cells. It is noteworthy that this AVV did not retrogradely transduce motoneurons when injected into the nerve (Sunico et al., 2008; Moreno-López, 2010).

A single intranerve microinjection (3 μ l) of AVV-TeNOS (2.2×10^{10} infective units/ml) on the day of the XIIth nerve crushing accelerated the recovery of neuromuscular transmission, resulting in a measurable CMAP 1 week after the damage (Fig. 3). Furthermore, the CMAP was fully recovered 22 days after the damage, which is 1 week earlier than in the untreated or eGFP transduced animals (Sunico et al., 2008). Stability of recovery was confirmed by the recording of a control-like CMAP from animals administered with AVV-TeNOS and studied at 62 days post-injury (Fig. 3). These results demonstrated that a single intraneural injection of the AVV-TeNOS efficiently accelerates the functional recovery of the neuromuscular junction after nerve injury (Sunico et al., 2008).

In addition, axonal regeneration was increased by AVV-TeNOS administration, compared with the AVV-eGFP treated group. Specifically, the number of retrogradely labeled motoneurons at 2 days after nerve crushing and intraneural injection of AVV-eGFP was significantly lower than that observed with AVV-TeNOS (Fig. 3). The number of retrogradely labeled HMNs at 7 days post-lesion in AVV-TeNOS-injected rats was similar to the control condition illustrating that AVV administration did not affect motoneuron viability (Sunico et al., 2008). Therefore, local eNOS inhibition with AVV-TeNOS speeds up neuromuscular functional recovery that is associated with accelerated axonal regeneration, among other possible mechanisms.

2.2.1 Cellular mechanisms that speed up axonal regrowth after nerve injury in AVV-TeNOS-treated animals

Schwann cells (SC) play a very important role in promoting axonal regeneration through the distal stump. After peripheral nerve axotomy, Wallerian degeneration involves axonal degradation as well as myelin breakdown and clearance in the distal stump of the injured nerve. For the subsequent axonal regeneration, SC dedifferentiation is required (Jessen & Mirsky, 2008). Thus, following nerve axotomy, SCs change from a myelin-producing state to a dedifferentiated, proliferating non-myelin-forming state (Stoll & Muller, 1999). In this dedifferentiated state, SCs are characterized by the formation of strands called bands of Bungner, which are the cellular substrate to guide growing axons, together with the up-regulation of several regeneration associated proteins, such as cell adhesion molecules, cytokines, neurotrophins, and growth factors that promote axonal regeneration (Araki et al., 2001; Stoll & Muller, 1999). More specifically, the growth-associated protein 43 kDa (GAP-43) is strongly up-regulated in regenerating axons and in dedifferentiated SCs (Curtis et al., 1992; Plantinga et al., 1993; Scherer et al., 1994). As soon as 1 day after transection or crushing of sciatic nerve, GAP-43 mRNA is significantly increased in the distal stump, remaining at high levels for at least 4 weeks (Plantinga et al., 1993). Thus, GAP-43 protein, which is involved in cell shape plasticity and motility (Curtis et al., 1992; Scherer et al., 1994), contributes to SC reorganization to form bands of Bungner in the degenerative/regenerative processes that take place after nerve injury.

Fig. 3. Chronic intraneural inhibition of a dominant negative for eNOS using an AVV accelerates neuromuscular function recovery and axonal regeneration. (a)-(c) CMAPs evoked in the genioglossus muscle by single shock stimulation (arrowheads point to the stimulus artifact) of XIIth nerve at 7 (a), 22 (b), or 62 (c) days after intraneural injection of AVV-eGFP or AVV-TeNOS. For comparison, recordings obtained by stimulation of the left (intact) and right (crushed) XIIth nerve are illustrated. Each trace represents an average of 10 individual responses. (d), (e) Photomicrographs of coronal sections of the right HN showing FluoroGold-labeled motoneurons in animals injected with AVV-eGFP (d) or AVV-TeNOS (e) on the crushing day. FluoroGold was applied on day 2 post-crushing and the animals were perfused 7 days after retrograde marker application. Scale bar = 100 μ m. Figure modified from Sunico et al., 2008. © 2008 Elsevier Ltd.

Given that AVV-TeNOS intraneural injection speeds up motor function recovery after XIIth nerve injury (Sunico et al., 2008), SCs were targeted to find out a feasible mechanism by which eNOS inhibition could enhance nerve regeneration, with the presumption that endothelial NO could be acting as a negative regulator of SC dedifferentiation after nerve crushing. To approach this issue, GAP-43 protein was analyzed in this study as a marker of regenerative processes, together with the density of SCs and bands of Bungner, in injured nerves after intraneural inhibition of eNOS. Indeed, our results indicate that endothelial NO confers a delaying action on SC dedifferentiation. As soon as 2 days following crushing of XIIth nerve together with AVV-TeNOS microinjection in the distal stump, GAP-43 immunoreactivity increases compared to control treatment with AVV-eGFP (Fig. 4). The reason for this, at least in part, could be an increase in GAP-43-positive cells, presumably SCs, after eNOS inhibition. Formation of bands of Bungner is also favored by TeNOS (Sunico & Moreno-López, 2010). Thereafter, all these data indicate that eNOS inhibition enhances the number of SCs and the subsequent formation of bands of Bungner after peripheral nerve injury (Fig. 4).

To summarize, negative regulators for myelination, which activates the SC dedifferentiation program, involve many extrinsic and intrinsic signals (Jessen & Mirsky, 2008). It is a well established fact that injury-related signals from neighboring SCs and neurons accelerate and influence SC dedifferentiation *in vivo* (Jessen & Mirsky, 2008). However, these recent studies also identify endothelial NO as a negative regulator of SC dedifferentiation, thus pointing to the vascular system as a source of signals that regulate some of the degenerative/regenerative processes that take place after peripheral nerve injuries (Sunico & Moreno-López, 2010). For this reason, the local endothelium is cautiously proposed in this chapter as a gene therapy target in the acute events that take place in peripheral neuropathies, and virally mediated eNOS antagonism is highlighted as a viable novel therapeutic strategy.

Fig. 4. TeNOS transgene increases the number of GAP-43 cells and bands of Bungner after XIIth nerve crushing. (a)-(c) GAP-43-immunoreactive (a) cells co-identified by Hoechst nuclear staining (b); the average number of GAP-43-positive cells (in thousands per cubic millimeter) at 4 and 5 mm distal to the injury site receiving the indicated adenoviruses is plotted (c). (d) Confocal high-magnification photomicrograph showing GAP-43-positive SCs forming two bands of Bungner disposed in parallel. (e)-(g) Confocal micrographs illustrating that after AVV-TeNOS injection the re-organization of SCs in bands of Bungner was more evident than in AVV-eGFP-treated injured nerves. The average number of bands of Bungner (in thousands per cubic millimeter) at 4 and 5 mm distal to the injury site receiving the indicated adenoviruses is plotted (g). Scale bars: (a), (b), (d), 10 μ m; (e), (f), 50 μ m. * $P < 0.05$; non-parametric Mann-Whitney U test. Figure modified from Sunico & Moreno-López, 2010. © 2010 Elsevier Ireland Ltd.

2.3 Combinatory use of LVVs and AVVs to scrutinize the role of nNOS in central events occurring after traumatic nerve injury

Disconnection of motoneurons from their target myocytes interrupts mutual trophic relations, leading to deep alterations in the structural and physiological properties of both motoneurons and muscle fibers. Axotomy provokes changes in axonal, synaptic and

intrinsic membrane properties, including enhanced somato-dendritic excitability, decreased axonal conduction speed, considerable loss of afferent synaptic contacts, and disorders in the firing properties and recruitment order of motor units (González-Forero et al., 2004; 2007). Synapse loss is the major feature underlying cognitive impairment in patients and/or animal models of AD, PD, MS, HD, and HIV-related dementia (Sunico et al., 2010; Moreno-López et al., 2011). Interestingly, synaptic alteration, rather than neuronal death, is the main factor responsible for the age-related downturn in neuronal function. Synapse loss also happens in several motor maladies, including ALS, progressive muscular atrophy, and traumatically-injured motor axons (Sunico et al., 2005; 2010). Interestingly, *de novo* expression of nNOS in motoneurons commonly occurs in response to the physical injury of a motor nerve and in the course of ALS. In both conditions, this event precedes synaptic withdrawal from motoneurons (Moreno-López et al., 2011). Changes in functional properties of injured cells also were prevented using various pharmacological agents targeting nNOS. NO-mediated disturbances involve changes in intrinsic membrane properties and anatomical synaptic deterioration that suggest a major pathological role of nNOS. However, nNOS is only one of the numerous proteins dysregulated after nerve damage. Therefore, the actual role for nNOS is less clear within the complex scenario created by multiple dysregulated proteins (Montero et al., 2010).

In an attempt to scrutinize whether nNOS up-regulation is sufficient to promote alterations after axonal injury on motoneurons, we have virally induced *de novo* expression of nNOS in non-axotomized HMNs together with complementary down-regulation of nNOS expression using virally mediated gene knock-down (Montero et al., 2010; Sunico et al., 2010). Replication-deficient recombinant AVVs (10^{10} - 10^{11} infective units/ml), directing the expression of enhanced green or monomeric red fluorescent proteins (eGFP and mRFP, respectively) or nNOS, were injected into the tongue to retrogradely transduce HMNs (Fig. 5). AVVs expressed eGFP, mRFP, or nNOS downstream the hCMV promoter. The AVV-eGFP and AVV-mRFP vectors were used as controls to test virally induced side effects.

Neonatal HMNs were retrogradely transduced by injecting AVV-nNOS and/or AVV-eGFP into the genioglossus muscle. Ratiometric real-time NO imaging was used to record NO released around transduced HMNs in response to a glutamatergic stimulus (Fig. 5). For that purpose, the NO-sensitive fluorescent probe 1,2-diaminoanthraquinone sulphate (DAA) was perfused together with the reference dye Alexa 633. We found that the slope of DAA fluorescence increased around eGFP/nNOS-transduced motoneurons in response to glutamate, which was prevented by adding the NOS inhibitor L-NAME to the bath. This increase of NO created a gradient of concentration around the transduced HMNs, which in brain parenchyma showed a space constant of 12.3 μm . Thus, HMNs retrogradely transduced with AVV-nNOS express a functional enzyme that synthesizes NO in response to glutamatergic stimulation (Montero et al., 2010).

Retrograde cotransduction of adult HMNs, after injection in the tongue of AVV-eGFP/AVV-nNOS, induced a significant reduction in their synaptic coverage, as shown by immunohistochemistry and electron microscopy. Thus, nNOS transfection mimicked the effect of axonal injury on the synaptic coverage of HMNs. In this way, it seems clear that NO, synthesized by up-regulated nNOS in adult axotomized motoneurons, is not only necessary but also sufficient to trigger the molecular cascade leading to synapse withdrawal from HMN perikarya (Sunico et al., 2010; Moreno-López et al., 2011).

Fig. 5. AVV transfection of HMNs with functional nNOS. (a) Left, AVV injection into the tip of the tongue retrogradely transfected HMNs. Right, an illustrative example of a retrogradely cotransduced HMN after injection in the tip of the tongue of AVV-eGFP+AVV-mRFP. (b) Merged DIC and eGFP channels showing the tip of a pipette (arrow) ejecting dyes close to an eGFP-transfected HMN. The circle indicates the region of interest (ROI) used to construct the plot illustrated in (c). (c), (d) Time courses of the DAA/ Alexa 633 ratio within a 5 μm diameter ROI placed just at the border of transfected HMNs by means of the indicated AVVs before and after addition to the perfusate of L-glutamate (500 μM ; arrows). Slopes of the regression lines adjusted before (S_{bglut}) and after (S_{aglut}) glutamate addition are indicated in (d). The slope increase after Glut application in (d) result from a rise in DAA relative to Alexa fluorescence. This indicates that NO synthesized by transduced HMN interacts with the NO-sensitive dye DAA. (e) Averaged $S_{\text{aglut}} - S_{\text{bglut}}$ was obtained from HMNs transfected with the specified AVVs and incubated with the indicated drugs. Drugs were added to the bath 5 min before glutamate. Inset, illustration signaling the location of ROIs analyzed per motoneuron. The average of $S_{\text{aglut}} - S_{\text{bglut}}$ was taken as the representative HMN value. Prevention of the slope increase after Glut by pre-incubation with the NOS inhibitor L-NAME strongly suggests that the change in the slope is mediated by generation of NO. (f) NO gradient in brain tissue surrounding nNOS expressing HMNs. Average $S_{\text{aglut}} - S_{\text{bglut}}$ was obtained from HMNs transfected with the indicated AVVs relative to the distance from the center of the ROI to the motoneuron border. Measures have been normalized relative to the value obtained in the ROI nearest to the motoneuron. Inset illustrates how ROIs were located for this type of measure. (g) Data presented in (f) were well fitted to the exponential decay equation used to calculate the theoretical space constant of the NO gradient created around AVV-nNOS transfected motoneurons. Scale bars: 50 μm . Figure modified, with permission, from Montero et al., 2010 (a) and Sunico et al., 2010 (b-g). © 2010 The Physiological Society and Society for Neuroscience, respectively.

For functional studies we injected AVV directly into the HN (Fig. 6). Five to 7 days after AVV administration, numerous neurons were identified as positively transfected at the injection site. Besides, a high number of astrocytes were also transfected, as confirmed by co-immunostaining against the astroglial cell marker glial fibrillary acidic protein (GFAP). Inspection of brainstem slices did not reveal eGFP-positive cell bodies in areas that project to the HN, such as the ventrolateral reticular formation (VLRF; Fig. 6). This argues against the possibility that our results could be affected by retrograde spread of AVV from the injection

Fig. 6. Specificity of viral injections and transfections. (a) Schematic diagram of viral administration. AVV and LVV were administered into the HN. (b) Epifluorescence photomicrograph of a coronal section at the level of the HN obtained 6 days after intranuclear injection of AVV-eGFP. Note that injection was almost fully restricted to the HN; cc, central canal. (c) Photomicrograph of a coronal section obtained 7 days after viral administration at the indicated distance from the injection site showing a broad number of eGFP-expressing hypoglossal neurons. At this level a high frequency of co-transfection was observed after intranuclear injection of AVV-eGFP/AVV-mRFP (right panels). (d) Low magnification photomicrograph of a coronal section at the level of AVV-eGFP injection site, obtained 7 days after viral administration. Note the absence of infected neurons in neighboring regions, such as the ventrolateral reticular formation (VLRF), which project directly to the HN. Asterisk points to the mark that identifies the right side of the brainstem. (e), (f) Photomicrographs from coronal sections at the level of the injection place obtained 7 days after intranuclear injection of LVV-miR-shRNA/nNOS in untreated animals (e) and those receiving doxycycline (Dox) in the drinking water (f). Scale bars: (b), (e), (f) 250 µm; (c), (d) 500 µm; (c) right panels 100 µm. Figure modified from Montero et al., 2010. © 2010 The Physiological Society.

site. A large number of eGFP-positive neurons were identified, strictly within the HN, 200-300 μm rostrocaudal and ipsilateral to the injection site (Fig 6). Furthermore, astroglia was not transduced at these remote locations, as colocalization of eGFP and GFAP was absent there (Montero et al., 2010). Therefore, functional tests were performed in these areas away from the injection site to minimize any contaminating effects of nNOS transduction of astrocytes.

Unilateral AVV-nNOS microinjection in the HN of adult rats induced axotomy-like changes in HMNs such as alterations in axonal conduction properties and reduction in the responsiveness to synaptic drive (Montero et al., 2010). In AVV-eGFP-transfected animals, as in controls, the majority of HMNs display a characteristic respiratory pattern of bursts of action potentials that is synchronized with the inspiratory stage of breathing. In contrast, the mean firing rate in basal conditions ($\text{ET}_{\text{CO}_2}=4.8\text{-}5.2\%$) was considerably reduced after intranuclear microinjection of AVV-eGFP/AVV-nNOS (Fig. 7), likewise 1 week after hypoglossal nerve damage (Gonzalez-Forero et al., 2004). This reduction in the mean firing rate was prevented by chronic treatment with L-NAME or 7-NI, a relatively specific nNOS inhibitor. These results suggest that the reduction in the mean firing rate is evoked by NO synthesized by transduced nNOS in the HN.

To gain specificity in nNOS activity inhibition, we used a neuron-specific LVV to knock-down nNOS. This LVV system has been recently described (Liu et al., 2008; 2011). Briefly, for nNOS knock-down, we used a binary LVV system that requires co-operative action of 2 viral vectors. The first vector expresses tetracycline-sensitive transactivator Tet-off under control of an enhanced synapsin-1 promoter (Liu et al., 2008; 2011). The second LVV harbors an expression cassette for a miRNA30 (miR30)-based short hairpin (shRNA) interference system (Stegmeier et al., 2005) under control of a Tet-sensitive promoter. The system expresses a miRNA30-like hairpin targeting the gene of choice as a fusion with the eGFP, which facilitates targeting of the RNA duplex into the RNA-induced silencing pathway. Tet-off is able to bind tetracycline or similar molecules such as doxycycline (Dox); this renders it unable to bind to the Tet-sensitive promoter and blocks the expression of the hairpin (Fig. 6). This system is then referred to as LVV-miR-shRNA/nNOS.

nNOS-induced effects on mean firing rate were fully prevented by administration of LVV-miR-shRNA/nNOS before AVV-nNOS injection into the HN, indicating that nNOS expression in neurons, but not in glial or endothelial cells, affects mean firing rate (Fig 7).

In physiological conditions, the activity of HMNs increases when the ET_{CO_2} rises, and decreases when the ET_{CO_2} lowers. Axonal injury of HMNs induces a decrease in their response to chemoreceptor-modulated inspiratory drive (see Fig. 2; González-Forero et al., 2004) which was NO-mediated (Sunico et al., 2005). Strikingly, when HMNs are transduced with transgenic nNOS, their sensitivity to the chemoreceptor-modulated inspiratory drive is dramatically decreased, an alteration that was equivalent to the effects of crushing on motoneuron sensitivity to their afferent drive (Fig. 8). Chronic administration of L-NAME or 7-NI, as well as LVV-miR-shRNA/nNOS injection, protected against the changes in motoneuron sensitivity induced by *de novo* expression of nNOS. Altogether, these findings suggest that nNOS transgene-derived NO is sufficient to activate the molecular processes that lead to a decrease in motoneuron sensitivity to their afferent inputs.

NO-mediated synaptic withdrawal from motoneurons is an underlying factor that is at least partially responsible for functional changes induced by axonal injury of motoneurons

(Gonzalez-Forero et al., 2004; Sunico et al., 2005; 2010). We have recently reported that *de novo* synthesis of NO, induced by retrograde transduction of HMNs through muscle injection of AVV-nNOS, was sufficient to induce a withdrawal of synaptic boutons on HMNs (Sunico et al., 2010). This effect came together with a strong decline in the evoked excitatory postsynaptic potential on motoneurons *in vitro* (Sunico et al., 2010). Additionally, AVV-nNOS microinjection into the HN induced a reduction in the number of synaptic boutons apposed to HMNs, which could be prevented by preceding injection of LVV-miR-shRNA/nNOS (Fig. 8). Taken together, these observations indicate that main functional alterations induced in motoneurons by nNOS transgene expression or axotomy involve NO-directed synaptic re-arrangements.

Fig. 7. AVV-nNOS injection into the HN mimics effects of XIIth nerve crushing on the basal firing activity of the HMNs. Representative examples show the discharge activity of HMNs recorded at basal conditions ($ET_{CO_2} = 4.8\text{--}5.2\%$) at the indicated conditions. For each panel, traces are the raw signals (top) of extracellularly recorded spike activity and the histogram of instantaneous firing rate (in spikes (sp)/s; bottom). Note that AVV-nNOS induced alterations in basal activity of HMNs were prevented by 7-NI or LVV-miR-shRNA/nNOS. Figure modified from Montero et al., 2010. © 2010 The Physiological Society.

In summary, we suggest that *de novo* expression of nNOS creates a repulsive gradient of NO around the motoneurons in an activity-dependent manner that can alter their inherent membrane properties as well as their synaptic coating. These results further point to nNOS as a pivotal target for the development of tools for the treatment of peripheral neuropathies and neurodegenerative disorders characteristically accompanied by central up-regulation of nNOS. Additionally, this opens a line of research for a strategy to elucidate the role of dysregulated proteins in the neuronal impairment taking place in the course of several neuropathological situations.

Fig. 8. AVV-nNOS injection into the HN mimics effects of XIIth nerve crushing on the chemoreceptor-modulated inspiratory activity of HMNs and on their synaptic coverage. (a) Illustrative examples of the time courses of the mean firing rate modulation (mFR, in spikes (sp)/s/burst; right y-axis) relative to ET_{CO2} levels (left y-axis) for HMNs recorded 7 days after the injection of the indicated viruses into the HN. Figure modified from Montero et al., 2010. © 2010 The Physiological Society.

3. Conclusions

Dysregulation of protein expression in specific cell populations of the central nervous system is a common hallmark in most neurodegenerative diseases. Ideally, this problem could be approached by somatic gene delivery targeted to specific cell types within a certain nucleus in order to increase or decrease the expression of a particular gene, with a precise control over the temporal expression of the transgenes. Transfection of cell lines with plasmids is a very efficient process, mainly thanks to the use of specific chemical reagents that increase the permeability of the cells. However, transfer of genes into a brain *in vivo* is still a challenging task. Nonetheless, gene delivery into brain cells using viral vectors has been successfully performed, even for long-term gene expression (Thomas et al., 2003; Papale et al., 2009). Likewise, viral vectors have been successfully used both *in vitro* and *in vivo* for the delivery of new genomic tools such as small interference RNAs (Snove and Rossi, 2006; Paddison, 2008).

As supported by our outcomes using an experimental model of peripheral neuropathy, the re-establishment of a functional gene through the insertion, removal, or modification of genes within cells and tissues is a very promising therapeutic alternative to chemicals for the treatment of human genetic disorders. Unfortunately, although in theory it could be considered as the holy grail, in practice each type of gene therapy still poses many challenges for use as a routine medical practice.

In germ line gene therapy, germ cells can be altered by the insertion of functional genes that integrate into their genomes, thus being inherited by later generations. However, numerous ethical, religious and technical reasons restrict this for application in human beings.

Conversely, in somatic gene therapy, the genes are introduced into somatic cells; therefore the effects will be restricted to the individual and will not be inherited by the offspring. However, we must be especially cautious with the vectors used for the gene delivery, particularly when viral vectors are used. Concerns about the risks of AVVs were raised during a gene therapy clinical trial in 1999, after an 18-year-old participant, Jesse Gelsinger, died because of a massive immune response triggered by the viral vector.

In our view, gene therapy represents a very promising tool that may ameliorate many human genetic diseases and neuropathological conditions accompanied by protein dysregulation at the expression and/or functional levels in the future. However, the nature of the diseases themselves and their genetic links must be more deeply understood before applying gene therapy. In the same way, the biological consequences of gene therapy need further evaluation, including its short-range and long-term side effects. Nonetheless, although gene therapy is still in its infancy, it has already been used *in vivo* with some success. The recent findings related to the treatment of acquired motor neuropathies described in this chapter highlight the therapeutic potential of this novel genetic tool.

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