RESTORING MOTOR FUNCTION USING OPTOGENETICS AND NEURAL ENGRAFTMENT

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Abstract:

Controlling muscle function is essential for human behaviour and survival, thus, impairment of motor function and muscle paralysis can severely impact quality of life and may be immediately life-threatening, as occurs in many cases of traumatic spinal cord injury (SCI) and in patients with amyotrophic lateral sclerosis (ALS). Repairing damaged spinal motor circuits, in either SCI or ALS, currently remains an elusive goal. Therefore alternative strategies are needed to artificially control muscle function and thereby enable essential motor tasks. This review focuses on recent advances towards restoring motor function, with a particular focus on stem cell-derived neuronal engraftment strategies, optogenetic control of motor function and the potential future translational application of these approaches.

INTRODUCTION

Virtually all human behavioural output is governed by motor functions, ranging from locomotion and articulated hand movement, to speech and emotional expression. Thus, even minor impairment of motor function can have serious implications for the quality of life of affected individuals, whilst severe loss of motor function can be immediately life-threatening – as in the case of traumatic spinal cord injury (SCI) [1] or neurodegenerative conditions affecting the motor system, such as amyotrophic lateral sclerosis (ALS) [2]. To date, efforts to induce spontaneous regeneration of trauma-induced lesions within the central nervous system (CNS) that affect motor function have not been successful, and there are no existing therapies that can delay or reverse the progressive motor neuron degeneration that occurs in ALS, which ultimately results in death. Therefore alternative strategies are being sought to repair neural circuits that mediate motor control and to artificially restore function to specific muscle groups to enable essential motor tasks. This review will focus on recent advances towards the application of these approaches to restore motor function, with a particular focus on the use of stem cell-derived neuronal replacement strategies [3], optogenetic control of motor function and the potential future translational application of these approaches.

Stem cell-based therapeutic strategies for spinal motor neurons: the challenges

In the absence of conventional therapies to restore lost motor function in ALS and SCI, stem cell based strategies have provided a promising avenue of research to overcome paralysis [4]. Early evidence of lifespan extension in transgenic rodent models of ALS following intraspinal transplantation of human neuronal precursor cells (hNPCs) [5] has recently progressed to Phase 1 clinical trials in ALS patients, and has proven to be safe and well tolerated [6]. However, it is now widely accepted that transplanted hNPCs cannot restore the anatomical connectivity of spinal motor circuits or replace lost motor neurons but, rather, they provide trophic support that delays the loss of endogenous motor neurons – an effect that is

restricted to the motor neuron cell body, whilst motor axon integrity and muscle innervation are not preserved [7].

Since the initial method to differentiate murine embryonic stem cells (ESCs) into spinal motor neurons was first described [8], a variety of protocols have been developed that enable the generation of specific subtypes of motor neurons from stem cells, including human pluripotent stem cells [9-11]. Although, this does raise the prospect of more targeted neuronal replacement strategies to repair damaged spinal motor circuits using CNS stem cell-derived neural grafts [12] to restore motor function, this approach is beset with major difficulties. Briefly, these include: i) the absence of developmentally-restricted molecular and genetic programs responsible for formation of extremely complex spinal motor circuits [13] [14] , which makes it unlikely that grafted motor neurons would functionally integrate into existing, damaged motor circuits; ii) the isolation of intraspinally grafted motor neurons from supraspinal inputs; iii) in ALS, the exposure of the grafted neurons to the same toxic environment that contributes to the degeneration of the endogenous motor neurons; iv) the inhibitory CNS-PNS boundary across which grafted motor neurons would have to extend axons out from the spinal cord [15]; v) the great distance along peripheral nerves which any grafted neurons would have to grow to innervate specific target muscles, again in the absence of developmental axon guidance factors, which would take greater than two years in the longest human nerves [16]. In addition, denervated peripheral nerves can only support axonal regeneration for a finite period [17], so target innervation by grafted motor axons would have to occur within this time. Moreover, this timeframe is longer than the lifespan of many patients diagnosed with ALS; finally, vi) during the intervening period between loss of functional innervation and growth of the grafted motor axons, target muscles may become irreversibly atrophied and cease producing reinnervation cues [18]. It is therefore clear, that strategies that depend on transplantation of grafted stem-cell derived motor neurons are not straightforward.

Stem cell-based therapeutic strategies targeting peripheral motor nerves

Strategies targeting the peripheral rather than central nervous system [19] may circumvent the challenges described above and this approach has been used to restore specific motor functions in animal models [3].

Transplantation of motor neurons into peripheral nerves has several important advantages; there is no requirement of the grafted motor neurons to integrate into complex spinal motor circuits, the neurons are isolated from the toxic/inhibitory environment of the spinal cord, which is particularly important in ALS, and the transplanted cells can be placed close to the target muscle, near the motor nerve entry point, greatly accelerating the time taken to reinnervate the muscle, thereby avoiding diminished schwann cell support of axon growth and muscle atrophy and enabling specific targeting of individual muscles. However, since peripherally engrafted motor neurons lack input from the CNS, the activity of these grafted neurons has to be elicited by a means of artificial stimulation.

Artificial control of motor neuron function: Electrical Stimulation

Functional electrical stimulation (FES) is one approach that has traditionally been employed to artificially stimulate motor nerves to induce muscle contraction [20]. FES technology has made significant advances over recent years, with the development of implantable electrodes capable of differentially controlling multiple muscles to drive coordinated, complex movements, such as hand grasping [20]. However, this approach has some fundamental drawbacks, particularly in the context of ALS. For example, FES relies on the integrity of the motor nerve to induce muscle contraction; although direct electrical stimulation of muscle can induce contraction, the voltage required is prohibitively high for sustained use. In ALS, as well as cases of contusion-induced loss of motor neurons in SCI, motor axons degenerate resulting in loss of and muscle innervation, rendering FES of peripheral nerves redundant. Additionally, even when some motor axons remain, electrical stimulation cannot discriminate between motor efferent fibres and sensory afferents present in peripheral nerves, so that FES not only

triggers muscle activity, but also simultaneously activates sensory fibres, including nociceptors, which can cause pain, depending on the stimulus intensity. In ALS patients, the sensory system remains largely intact and in cases of SCI, although transmission of pain signals may be completely blocked, local activation of pain circuits may have unforeseen consequences. Most importantly, it is known that FES results in a reversed or random recruitment of motor units [21], such that the largest (strongest) most fatigable motor axons and the muscles that they innervate are recruited at the lowest stimulus intensity, whilst small motor units, which are weaker but more fatigue resistant, are activated by higher intensity stimuli. This non-physiological reversal in the graded recruitment of motor unit by FES has very significant consequences, most critically, that muscles become rapidly fatigued following sustained FES. Thus, for long-term stimulation of muscles, in particular of critical muscles such as the diaphragm, the use of FES may be inappropriate, as it is unlikely to support long-term rhythmic contractions that are essential to maintain breathing.

Indeed, a recent Phase 1 clinical trial assessing the safety and tolerability of electrical pacing of the diaphragm muscle in ALS patients was terminated early due to evidence of decreased survival of patients fitted with the device; patient life-expectancy was decreased by an average of 11months, compared to patients on non-invasive ventilation alone [22]. Although the reasons behind this negative effect of electrical pacing of the diaphragm is not clear from the study, it did not appear to be associated with the surgery itself. It is therefore possible that the FES resulted in enhanced muscle fatigue, possibly forcing the surviving phrenic motor axons to work harder to drive normal respiration, potentially accelerating their degeneration and diaphragm muscle denervation.

Artificial control of motor neuron function: Optical Stimulation

A solution to the significant drawbacks of FES, in terms of specific and physiological control of muscle function, is provided by the now-established technique of optogenetics. Optogenetics relies on the biological activity elicited by photosensitive proteins in response to

light (for a comprehensive review see [23]). Channelrhodpsin-2 (ChR2) is a light-gated ion channel originally isolated from algae that, when expressed as a transgene in neurons and activated, can depolarize these neurons and trigger action potentials [24].

The first demonstration of the use of optogenetics to control motor activity was shown in transgenic mice expressing ChR2 under the neuronal *Thy1* promoter (*Thy1::ChR2* mice). In this study, the authors used optical stimulation applied to the primary motor cortex via a tethered optical fiber, to induce motor activity [25]. More recently, in *Thy1::ChR2* transgenic mice, in which ChR2 is expressed in motor neurons, it was shown that motor axons could be optically stimulated, using a nerve cuff coupled to a laser light source, to induce highly controlled muscle contractions [26]. Importantly, this study also demonstrated that motor units activated by optical stimulation are recruited in normal physiological order, with smaller, fatigue-resistant motor units being recruited at lower optical stimulus intensities and larger fatigable motor units only being activated at higher intensities [26]. The same physiological recruitment of ChR2 expressing motor units and prevention of muscle fatigue was also verified in our recent study [3], discussed below. Theoretical modelling of the orderly recruitment of motor units in peripheral optogenetic neural stimulation (PONS) suggests that the reduced internodal distance in small diameter myelinated motor axons is an essential parameter underlying this phenomenon [27]. In addition to the major advantage of physiological, graded recruitment of motor units, optical stimulation also has the significant advantage that it only induces activity in neurons that express the light-responsive opsin. This makes it possible to specifically activate motor axons using PONS, and avoids the indiscriminate activation of non-targeted motor axons as well as nociceptive afferent axons [28].

Translational optical control of motor function

To date, most optogenetic studies investigating motor function *in vivo* have utilized either transgenic mouse models or viral transduction of neurons in rodents and non-human primates to express opsins. Although important experimental information has been learned from the

use of optogenetics in transgenic mice, this is not a viable translational strategy for humans. Adeno-associated viruses (AAVs) and other viruses have shown promise as potential gene-therapy delivery vectors and are capable of targeting opsin expression to specific neuronal populations [29], for example to sensory versus motor axons in peripheral nerves in rodents [28]. However, whilst viral transduction to express opsins, to enable optogenetic control of epileptiform activity for example [30], appears a promising approach, it is not without certain disadvantages and risks. For example, it has recently been shown that high-level expression of ChR2 in rats, following *in utero* electroporation, and to a lesser extent viral expression, can result in axonal pathology [31], indicating that the level of opsin expression in neurons must be carefully controlled. Moreover, in the case of ALS, even if viral transduction could safely and effectively deliver appropriate opsins to surviving endogenous motor axons as a therapeutic strategy to restore motor function, the ongoing degeneration of these axons and resulting muscle denervation would rapidly render the approach ineffective. A third and perhaps most translationally viable option would be to take advantage of recent advances in gene targeting technology, such as the highly specific CRISPr/Cas9 method [32] [33], along with advances in iPSC technology [12], to generate optogenetically-modified neural grafts, targeted to peripheral nerves, in order to restore control over paralyzed muscles. Human embryonic stem cell-derived neurons that stably express ChR2 have recently been shown to survive for >6 months following transplant into SCID mice [34]. This is in agreement with our observations of long-term murine ESC-derived motor neuron survival in peripheral nerves of wild-type mice (unpublished data).

Indeed, in a recent study, we developed a combinatorial strategy that utilizes the advantages of both stem cell-derived neural engraftment into denervated peripheral motor nerves and optogenetic control of motor neuron function, as a translationally relevant approach to restoring lost muscle function in mice following nerve injury [3]. In this study, we transplanted mouse ESC-derived motor neurons, modified to express ChR2 as well as the neurotrophic factor GDNF, into a denervated peripheral nerve of adult mice, and showed that not only were

these grafted motor neurons able to survive in this peripheral environment, but to also extend axons to reinnervate specific target muscles in the hindlimb. Moreover, optical stimulation of these ChR2-expressing grafted motor neurons resulted in controlled contraction of the target muscles. Importantly, this optically-controlled muscle function avoided the rapid muscle fatigue associated with electrical neuromuscular stimulation [3], since optical stimulation of the grafted neurons resulted in the normal, physiological recruitment of motor unit, thus confirming the findings of other groups [26]. This approach may be ideally suited as a translational strategy to enable optical control of the diaphragm muscle in ALS patients, using an optical pace-maker to maintain respiratory function. This would avoid the need for mechanical ventilation and the problems associated with electrical pacing of the diaphragm muscle [22]. Indeed, the ability to experimentally control diaphragm function in rodents, using optogenetics, has already been demonstrated [35] and existing pacemaker technology could readily be adapted to enable implementation of this approach in the immediate future.

The combinatorial approach described above demonstrates the advantages of optogenetic control of motor function, along with the ability to functionally reinnervate muscles that have been paralyzed. However, the next major hurdle to overcome is to develop suitable optical stimulation devices to enable chronic control of muscle activity, rather than in our acute proof-of-principle study [3]. This is essential, since the structure and function of neuromuscular junctions (NMJs) are highly dependent on synaptic activity [36], without which, the quiescent motor nerve terminal begins to detach from the NMJ and the muscle fiber begins to undergo atrophy. Thus optical stimulation devices that can deliver chronic, patterned neuromuscular activity are required to maximize the reinnervation and muscle strength achievable using this approach.

Development of implantable optical stimulators

The significant benefits of optical versus electrical stimulation warrant the rapid development of more sophisticated technological and bioengineering solutions to expedite the clinical

application of this approach. Indeed, major advances have been made towards the development of optical stimulation methods in rodent models in the past few years. Initial optical stimulation experiments employed tethered optical fibers connected to a laser light source [25]. However, whilst this approach has been elegantly used to control muscle function in awake freely moving rats, following viral transduction to express ChR2 in peripheral nerves [37], it remains technically challenging and impractical for large stimulation experiments requiring long-term stimulation; this approach also prohibits normal social behaviour [38,39].

A recent elegant study has described the development of fully implantable, wirelessly-powered mini-LED devices that are small enough for use in mice, weighing as little as 20mg [40], which are likely to greatly facilitate the investigation of optogenetic techniques to control motor function in translationally relevant model systems. This is particularly important for investigation of motor neuron activation of muscle function, since the formation and maintenance of neuromuscular junctions is dependent on chronic, long term stimulation of the transplanted neurons and subsequent muscle activity, which is not provided by intermittent activation paradigms afforded by tethered systems. A remaining engineering requirement for these devices, in terms of enabling normal control of muscle function, is to enable gradual ramping of light intensity to recruit motor units in a normal, graded physiological order and thereby avoid muscle fatigue. Nonetheless, this technology represents a significant advance towards the ability to reliably control motor function using optogenetics. Indeed, we believe that the ability to optically control more complex motor functions, is largely restricted at present by the sophistication of optical stimulation devices, since either neural replacement or viral transduction strategies can readily confer optogenetic control of spatially discrete, opposable muscle groups, at least experimentally [37].

Further advances towards optogenetic control of motor function

An additional requirement for the translational application of optogenetics to control motor function is the development and refinement of optimized opsins. As noted above, too high an

expression level of ChR2 has been shown to induce axonopathy, therefore opsins that enable greater (more selective) cation flow and respond to weaker optical stimuli, such as the channelrhodopsin Cheriff [41], and the red-shifted channelrhodopsins, ReaChR [42] and Chrimson [43]. These red-shifted opsins have the advantage of requiring less energetic activation wavelengths in the orange-red spectrum, that have greater tissue penetration, thereby enabling more flexibility in terms of optical stimulator development and avoiding potential cellular damage from comparatively high-energy blue light [44]. Moreover, further characterization of existing optogenetic actuators remains to be undertaken. For example, it was recently shown that the well-established neuronal activation by ChR2 is actually reversed at lower temperatures and causes inhibition of motor activity in mice under such conditions [45]. Indeed, the use of halorhodopsins to block motor activity represents an additional means to therapeutically inhibit motor function, which may be of use, for example, in cases of spasticity.

Finally, a recent development in terms of using optogenetics to control muscle function is the use of direct optogenetic control of muscle contraction, using both transgenic ChR2 expressing mice and viral transduction of muscle in vivo, to optically control the muscles of the larynx [46]. This approach may provide a complementary therapy to prevent muscle wasting in the intervening period between muscle denervation and reinnervation by regenerating axons [47].

Conclusions

Recent advances in a range of different fields have resulted in the development of novel methods to restore motor function that circumvent the need to repair damage to the CNS, which has so far proven to be an elusive goal. By combining the potential of stem cell differentiation and the ability to optogenetically control motor neurons, together with the development of more sophisticated optical stimulation devices, it may eventually be possible to couple this approach with advanced methods that can interpret intended motor output.

Indeed, a synthesis of brain-machine interface (BMI) technology with FES to control muscle function [20] has recently been shown to be a feasible approach to enable overground walking in SCI [48], proving that the technolgy to relay motor commands from the brain to target muscles already exists. Thus, in the long-term, a combination of intraneural stem cell-derived motor neuron engraftment and optogenetics together with BMI may enable paralyzed patients to exert control over their own musculature. Recent developments in BMI, from the neurotechnology company BrainGate, have produced a device that can wirelessly transmit intended motor commands collected from a brain implant to steer a wheelchair or robotic arm [49]. Moreover, paralysed patients, some with ALS, are currently taking part in trials of similar technology [50].

Over the past few years, great progress has been made in the development of the biological and technological components that would enable a "body-machine interface" to be constructed that would enable optical control of less complex, but essential motor functions, such as respiration, swallowing and bowel/bladder function, through the use of optical pacemaker-like devices in the near future. The continued development of more sophisticated optical control devices, which has already rapidly evolved during the short history of optogenetics, could see the longer-term goal of restoring more complex motor functions, such as dexterous hand movements and locomotion, come to fruition in the foreseeable future.

Graphical Abstract Legend:

Schematic representation of a combinatorial closed-loop system to restore functionality to paralyzed muscles, enabling control of specific motor functions. Briefly, intraneural grafts of optogenetically-engineered stem cell-derived motor neurons are placed closed to the motor-entry point of the target muscle, leading to its reinnervation (1) or, where intact, viral vectors are used to express opsins in endogenous motor axons. Next, a brain machine interface embedded in the primary motor cortex (2), relays intended motor commands via an external neural decoding and processing unit (3), which then wirelessly transmits execution signals to an implanted optoelectronic stimulator (4) that activates the engrafted motor neurons (5) to induce muscle contraction (6). The implanted optoelectronic device can then relay feedback information from the muscle or nerve to the processing unit (not shown).

References:

1. Nicaise C, Putatunda R, Hala TJ, Regan KA, Frank DM, Brion JP, Leroy K, Pochet R, Wright MC, Lepore AC: Degeneration of phrenic motor neurons induces long-term diaphragm deficits following mid-cervical spinal contusion in mice. *J Neurotrauma* 2012, 29:2748-2760.

2. Peters OM, Ghasemi M, Brown RH, Jr.: Emerging mechanisms of molecular pathology in ALS. *J Clin Invest* 2015, 125:1767-1779.

3. Bryson JB, Machado CB, Crossley M, Stevenson D, Bros-Facer V, Burrone J, Greensmith L, Lieberam I: Optical control of muscle function by transplantation of stem cell-derived motor neurons in mice. *Science* 2014, 344:94-97.

●● **This study describes a novel strategy to restore lost muscle function using optogenetically-engineered ESC-derived motor neurons engrafted into denverated peripheral nerves. The engrafted motor neurons functionally reinnervated distal muscle targets. Importantly, optical stimulation was then shown to induce finely-controlled muscle contraction in a manner that avoided rapid fatigue, normally associated with electrical stimulation, thereby demonstrating a potential strategy to restore lost motor function following paralysis.**

4. Thomsen GM, Gowing G, Svendsen S, Svendsen CN: The past, present and future of stem cell clinical trials for ALS. *Exp Neurol* 2014, 262 Pt B:127-137.

5. Xu L, Yan J, Chen D, Welsh AM, Hazel T, Johe K, Hatfield G, Koliatsos VE: Human neural stem cell grafts ameliorate motor neuron disease in SOD-1 transgenic rats. *Transplantation* 2006, 82:865-875.

6. Feldman EL, Boulis NM, Hur J, Johe K, Rutkove SB, Federici T, Polak M, Bordeau J, Sakowski SA, Glass JD: Intraspinal neural stem cell transplantation in amyotrophic lateral sclerosis: phase 1 trial outcomes. *Ann Neurol* 2014, 75:363-373.

7. Suzuki M, McHugh J, Tork C, Shelley B, Klein SM, Aebischer P, Svendsen CN: GDNF secreting human neural progenitor cells protect dying motor neurons, but not their projection to muscle, in a rat model of familial ALS. *PLoS One* 2007, 2:e689.

8. Wichterle H, Lieberam I, Porter JA, Jessell TM: Directed differentiation of embryonic stem cells into motor neurons. *Cell* 2002, 110:385-397.

9. Chambers SM, Fasano CA, Papapetrou EP, Tomishima M, Sadelain M, Studer L: Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nat Biotechnol* 2009, 27:275-280.

10. Maury Y, Come J, Piskorowski RA, Salah-Mohellibi N, Chevaleyre V, Peschanski M, Martinat C, Nedelec S: Combinatorial analysis of developmental cues efficiently converts human pluripotent stem cells into multiple neuronal subtypes. *Nat Biotechnol* 2015, 33:89-96.

- **This study describes a novel screening platform for determining the effects of small molecules on the differentiation of human pluripotent stem cells, which led to the identification of new protocols to r induce differentiation into spinal and cranial motor neurons, as well as spinal interneurons and sensory neurons, which will be useful for future drug screens.**

11. Amoroso MW, Croft GF, Williams DJ, O'Keeffe S, Carrasco MA, Davis AR, Roybon L, Oakley DH, Maniatis T, Henderson CE, et al.: Accelerated high-yield generation of limb-innervating motor neurons from human stem cells. *J Neurosci* 2013, 33:574-586.

- **This study led to the identification of a combination of small molecules that can efficiently lead to the generation of abundant limb-innervating subtype motor neurons, which are particularly susceptible to ALS pathology and may have be useful for motor neuron engraftment to reinnervate paralyzed limb muscles.**

12. Thompson LH, Bjorklund A: Reconstruction of brain circuitry by neural transplants generated from pluripotent stem cells. *Neurobiol Dis* 2015, 79:28-40.

13. Ladle DR, Pecho-Vrieseling E, Arber S: Assembly of motor circuits in the spinal cord: driven to function by genetic and experience-dependent mechanisms. *Neuron* 2007, 56:270-283.

14. Brownstone RM, Bui TV: Spinal interneurons providing input to the final common path during locomotion. *Prog Brain Res* 2010, 187:81-95.

15. Harper JM, Krishnan C, Darman JS, Deshpande DM, Peck S, Shats I, Backovic S, Rothstein JD, Kerr DA: Axonal growth of embryonic stem cell-derived motoneurons in vitro and in motoneuron-injured adult rats. *Proc Natl Acad Sci U S A* 2004, 101:7123-7128.

16. Kang H, Lichtman JW: Motor axon regeneration and muscle reinnervation in young adult and aged animals. *J Neurosci* 2013, 33:19480-19491.

- **This study utilized in vivo imaging of GFP-labelled regenerating motor axons, following nerve injury in young and aged mice. Surprisingly, the authors show that aged axons grow towards distal muscles at the same inherent rate as young axons but decreased clearance of cellular debris in injured endoneural tubes in aged animals obstructs axon growth, explaining longer denervation time and poorer prognosis in aged humans with nerve injury.**

17. Arthur-Farraj PJ, Latouche M, Wilton DK, Quintes S, Chabrol E, Banerjee A, Woodhoo A, Jenkins B, Rahman M, Turmaine M, et al.: c-Jun reprograms Schwann cells of injured nerves to generate a repair cell essential for regeneration. *Neuron* 2012, 75:633-647.

18. Gordon T, Tyreman N, Raji MA: The basis for diminished functional recovery after delayed peripheral nerve repair. *J Neurosci* 2011, 31:5325-5334.

19. Yohn DC, Miles GB, Rafuse VF, Brownstone RM: Transplanted mouse embryonic stem-cell-derived motoneurons form functional motor units and reduce muscle atrophy. *J Neurosci* 2008, 28:12409-12418.

20. Ethier C, Miller LE: Brain-controlled muscle stimulation for the restoration of motor function. *Neurobiol Dis* 2014.

- **In this review, the authors discuss recent advances in both FES and BMI technology and clearly describe how the two approaches could be combined to restore autonomous control of specific motor functions in paralyzed patients. The authors describe the ability of increasingly sophisticated FES devices to control complex motor functions such as grasping and locomotion, whilst being realistic about the limitations of FES, and highlight optogenetics as a more selective means to control muscle function.**

21. Hamada T, Kimura T, Moritani T: Selective fatigue of fast motor units after electrically elicited muscle contractions. *J Electromyogr Kinesiol* 2004, 14:531-538.

22. Di PWC, Di PSGC: Safety and efficacy of diaphragm pacing in patients with respiratory insufficiency due to amyotrophic lateral sclerosis (DiPALS): a multicentre, open-label, randomised controlled trial. *Lancet Neurol* 2015, 14:883-892.

23. Deisseroth K: Optogenetics: 10 years of microbial opsins in neuroscience. *Nat Neurosci* 2015, 18:1213-1225.

24. Arenkiel BR, Peca J, Davison IG, Feliciano C, Deisseroth K, Augustine GJ, Ehlers MD, Feng G: In vivo light-induced activation of neural circuitry in transgenic mice expressing channelrhodopsin-2. *Neuron* 2007, 54:205-218.

25. Aravanis AM, Wang LP, Zhang F, Meltzer LA, Mogri MZ, Schneider MB, Deisseroth K: An optical neural interface: in vivo control of rodent motor cortex with integrated fiberoptic and optogenetic technology. *J Neural Eng* 2007, 4:S143-156.

26. Llewellyn ME, Thompson KR, Deisseroth K, Delp SL: Orderly recruitment of motor units under optical control in vivo. *Nat Med* 2010, 16:1161-1165.

●● **This was the first study showing that ChR2 expressing motor axons could be optically stimulated in the peripheral nerve to induce finely-controlled muscle contractions. Importantly, this revealed that optical stimulation results in physiological recruitment of motor units, with small, fatigue-resistant motor units activated at low stimulus intensities and large, rapidly-fatigable motor units only activated at high optical stimulus intensities. This work paves the way for an optical muscle stimulator that overcomes the problems of rapid muscle fatigue and lack of specificity associated with electrical stimulation.**

27. Arlow RL, Foutz TJ, McIntyre CC: Theoretical principles underlying optical stimulation of myelinated axons expressing channelrhodopsin-2. *Neuroscience* 2013, 248:541-551.

28. Iyer SM, Montgomery KL, Towne C, Lee SY, Ramakrishnan C, Deisseroth K, Delp SL: Virally mediated optogenetic excitation and inhibition of pain in freely moving nontransgenic mice. *Nat Biotechnol* 2014, 32:274-278.

29. Ji ZG, Ishizuka T, Yawo H: Channelrhodopsins-Their potential in gene therapy for neurological disorders. *Neurosci Res* 2013, 75:6-12.

30. Wykes RC, Kullmann DM, Pavlov I, Magloire V: Optogenetic approaches to treat epilepsy. *J Neurosci Methods* 2015.

31. Miyashita T, Shao YR, Chung J, Pourzia O, Feldman DE: Long-term channelrhodopsin-2 (ChR2) expression can induce abnormal axonal morphology and targeting in cerebral cortex. *Front Neural Circuits* 2013, 7:8.

● **The authors of this study reveal possibly the first indication of a deleterious effect of high-level ChR2 expression in the mammalian CNS, which resulted in axonal pathology. This evidence highlights the need to carefully regulate the expression of opsins both for experimental purposes but also for any prospective gene-therapy applications.**

32. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, et al.: Multiplex genome engineering using CRISPR/Cas systems. *Science* 2013, 339:819-823.

33. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM: RNA-guided human genome engineering via Cas9. *Science* 2013, 339:823-826.

34. Weick JP, Johnson MA, Skroch SP, Williams JC, Deisseroth K, Zhang SC: Functional control of transplantable human ESC-derived neurons via optogenetic targeting. *Stem Cells* 2010, 28:2008-2016.

35. Alilain WJ, Li X, Horn KP, Dhingra R, Dick TE, Herlitze S, Silver J: Light-induced rescue of breathing after spinal cord injury. *J Neurosci* 2008, 28:11862-11870.

●● **This groundbreaking study showed possibly the first evidence of a translational application for optogenetics, in the control of repiratory function in a rat model of SCI. Following cervical spinal hemisection, ChR2 was delivered by sindbis virus to the phrenic motor nucleus, which**

enabled recovery of rhythmic respiratory function and diaphragmatic motor activity upon optical stimulation. This study clearly demonstrates the possibility of maintaining respiratory function in paralyzed patients with respiratory compromise using optogenetics.

36. Darabid H, Perez-Gonzalez AP, Robitaille R: Neuromuscular synaptogenesis: coordinating partners with multiple functions. *Nat Rev Neurosci* 2014, 15:703-718.

37. Towne C, Montgomery KL, Iyer SM, Deisseroth K, Delp SL: Optogenetic control of targeted peripheral axons in freely moving animals. *PLoS One* 2013, 8:e72691.

● **Using AAV-expression of ChR2 in lower hindlimb flexor and extensor muscles of rats, the authors succeded implanted a optical stimulator nerve cuff, tethered to an external laser light source, to demonstrate the feasibility of optically controlling muscle function in awake freely-moving animals. This elegant proof-of-concept study paved the way for more sophisticated, fully-implantable optical stimulation devices.**

38. Iwai Y, Honda S, Ozeki H, Hashimoto M, Hirase H: A simple head-mountable LED device for chronic stimulation of optogenetic molecules in freely moving mice. *Neurosci Res* 2011, 70:124-127.

39. Wentz CT, Bernstein JG, Monahan P, Guerra A, Rodriguez A, Boyden ES: A wirelessly powered and controlled device for optical neural control of freely-behaving animals. *J Neural Eng* 2011, 8:046021.

40. Montgomery KL, Yeh AJ, Ho JS, Tsao V, Mohan Iyer S, Grosenick L, Ferenczi EA, Tanabe Y, Deisseroth K, Delp SL, et al.: Wirelessly powered, fully internal optogenetics for brain, spinal and peripheral circuits in mice. *Nat Methods* 2015, 12:969-974.

●● **This remarkable report details the development of, and provides open access to detailed protocols to contruct, fully-implantable wirelessly powered optical stimulation devices, which are ideally suited for optogenetic studies of motor function in mice. Due to the extremely small size of these optical stimulators, animals can be multiply housed during**

stimulation/training regimens, with minimal effect on innate behaviour. These devices therefore represent a major step forward in the field of optical control of motor function.

41. Hochbaum DR, Zhao Y, Farhi SL, Klapoetke N, Werley CA, Kapoor V, Zou P, Kralj JM, Maclaurin D, Smedemark-Margulies N, et al.: All-optical electrophysiology in mammalian neurons using engineered microbial rhodopsins. *Nat Methods* 2014, 11:825-833.

42. Lin JY, Knutsen PM, Muller A, Kleinfeld D, Tsien RY: ReaChR: a red-shifted variant of channelrhodopsin enables deep transcranial optogenetic excitation. *Nat Neurosci* 2013, 16:1499-1508.

43. Klapoetke NC, Murata Y, Kim SS, Pulver SR, Birdsey-Benson A, Cho YK, Morimoto TK, Chuong AS, Carpenter EJ, Tian Z, et al.: Independent optical excitation of distinct neural populations. *Nat Methods* 2014, 11:338-346.

44. Hockberger PE, Skimina TA, Centonze VE, Lavin C, Chu S, Dadras S, Reddy JK, White JG: Activation of flavin-containing oxidases underlies light-induced production of H2O2 in mammalian cells. *Proc Natl Acad Sci U S A* 1999, 96:6255-6260.

45. Liske H, Qian X, Anikeeva P, Deisseroth K, Delp S: Optical control of neuronal excitation and inhibition using a single opsin protein, ChR2. *Sci Rep* 2013, 3:3110.

46. Bruegmann T, van Bremen T, Vogt CC, Send T, Fleischmann BK, Sasse P: Optogenetic control of contractile function in skeletal muscle. *Nat Commun* 2015, 6:7153.

- **(discussed with Ref [47])**

47. Magown P, Shettar B, Zhang Y, Rafuse VF: Direct optical activation of skeletal muscle fibres efficiently controls muscle contraction and attenuates denervation atrophy. *Nat Commun* 2015, 6:8506.

- **These reports (46 & 47) show that skeletal muscle contraction can be directly control using optical stimulation in either transgenic or virally transduced mouse muscles, expressing ChR2. Suprisingly, optical stimulation efficiently induced tetanic muscle contraction, whereas direct electrical stimulation of muscle is extremely inefficient in comparison to**

nerve-evoked muscle contraction. These findings may provide an additional approach to optically control denervated muscles but the long-term benefits remain to be investigated.

48. King CE, Wang PT, McCrimmon CM, Chou CC, Do AH, Nenadic Z: The feasibility of a brain-computer interface functional electrical stimulation system for the restoration of overground walking after paraplegia. *J Neuroeng Rehabil* 2015, 12:80.

- **This study proves that combining BMI technology, to interpret intended motor functions, with a means of activating skeletal muscles in a coordinated manner (in this case using FES), is already capable of autonomously enbaling complex motor functions in paralyzed patients. Although the study was performed in a single patient and rather than an implanted BMI, a surface, EEG-based device was used, it shows that this approach is readily achievable, albeit with major investment to refine the technology and greatly enhance the scalability.**

49. Homer ML, Nurmikko AV, Donoghue JP, Hochberg LR: Sensors and decoding for intracortical brain computer interfaces. *Annu Rev Biomed Eng* 2013, 15:383-405.

50. Jarosiewicz B, Sarma AA, Bacher D, Masse NY, Simeral JD, Sorice B, Oakley EM, Blabe C, Pandarinath C, Gilja V, et al.: Virtual typing by people with tetraplegia using a self-calibrating intracortical brain-computer interface. *Sci Transl Med* 2015, 7:313ra179.

Graphical abstract