REVIEW

Using induced pluripotent stem cells (iPSC) to model human neuromuscular connectivity: promise or reality?

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Abstract

Motor neuron diseases (MND) such as amyotrophic lateral sclerosis and spinal muscular atrophy are devastating, progressive and ultimately fatal diseases for which there are no effective treatments. Recent evidence from systematic studies of animal models and human patients suggests that the neuromuscular junction (NMJ) is an important early target in MND, demonstrating functional and structural abnormalities in advance of pathological changes occurring in the motor neuron cell body. The ability to study pathological changes occurring at the NMJ in humans is therefore likely to be important for furthering our understanding of disease pathogenesis, and also for designing and testing new therapeutics. However, there are many practical and technical reasons why it is not possible to visualise or record from NMJs in pre- and early-symptomatic MND patients in vivo. Other approaches are therefore required. The development of stem cell technologies has opened up the possibility of creating human NMJs in vitro, using pluripotent cells generated from healthy individuals and patients with MND. This review covers historical attempts to develop mature and functional NMJs in vitro, using cocultures of muscle and nerve from animals, and discusses how recent developments in the generation and specification of human induced pluripotent stem cells provides an opportunity to build on these previous successes to recapitulate human neuromuscular connectivity in vitro.

Key words: Amyotrophic lateral sclerosis, spinal muscular atrophy, neuromuscular junction, stem cells, in vitro model.

Motor neuron diseases (MND) are a group of relatively common, and ultimately fatal, neurodegenerative conditions for which there is presently no cure and few ineffective treatment options. Amyotrophic lateral sclerosis (ALS) is the most common form of adult-onset MND, with an incidence of approximately two per 100 000 (Logroscino et al. 2010), where degeneration of both upper and lower motor neurons causes progressive paralysis and death within 2–5 years of diagnosis (Rothstein, 2009). The vast majority of cases are sporadic, with < 10–15% of cases familial (Schymick et al. 2007). An increasing number of genes have been linked to both familial and sporadic ALS, including SOD1 (Rosen et al. 1993), FUS (Vance et al. 2009) and TDP-43 (Sreedharan et al. 2008). In contrast to ALS, spinal muscular atrophy (SMA) is a predominantly childhood form of MND, and is the most common genetic cause of infant mortality (Lunn & Wang, 2008). Mutations in the survival motor neuron 1 (SMN1) gene cause progressive paralysis and muscle atrophy, resulting from degeneration and loss of lower motor neurons in the brainstem and spinal cord (Lunn & Wang, 2008). In its most severe form (type I SMA), disease symptoms are apparent by 6 months old, and the child dies before the age of 2 years (Russo, 2007).

Despite having distinct aetiologies and clinical symptoms, recent evidence from studies of both ALS and SMA suggests that early pathological changes occurring in the most distal part of the lower motor neuron, at the neuromuscular junction (NMJ), are a significant common feature.
of ALS and SMA (Frey et al. 2000; Fischer et al. 2004; Gould et al. 2006; Murray et al. 2008; Kong et al. 2009). Importantly, NMJ disruption can be readily identified in MND mouse models ‘before’ the onset of symptoms (for review, see Murray et al. 2010a). Thus, the development of experimental systems and technical approaches that allow the examination of functional and physical synaptic pathology at the human NMJ are likely to be required in order to obtain a fuller understanding of the pathophysiology of MND, and for the development and testing of new therapeutics.

Unfortunately, many factors contribute to the fact that it is almost impossible to examine the NMJs of patients with MND, particularly at early stages of disease. These include the absence of reliable biomarkers capable of revealing disease onset as well as late presentation and diagnosis of patients. In addition, current methods allowing the visualisation of human NMJs (e.g. using light or electron microscopy) require an invasive biopsy procedure and only allow the examination of pathology at a fixed point in time. Consequently, there are no techniques that will allow repeated visualisation and/or experimental manipulation of human NMJs in vivo. The next best solution would be the recreation of human neuromuscular connectivity ex vivo.

Recent developments have demonstrated that human induced pluripotent stem cells (iPSCs) have the potential to be converted into a range of different cell types belonging to the neuromuscular system, including lower motor neurons (Inoue, 2010). When coupled with the ability to obtain iPSCs from patients with MND (e.g. Dimos et al. 2008; Ebert et al. 2009), it raises the possibility that iPSC technologies, coupled with traditional co-culture techniques, may make it possible to recreate healthy and diseased human neuromuscular connectivity in vitro.

The NMJ in MND

The mammalian NMJ is a cholinergic synapse formed between a lower motor neuron and a skeletal muscle fibre (Fig. 1; Sanes & Lichtman, 1999). Four different cell types are now considered to contribute to the NMJ; the presynaptic motor nerve terminal, the skeletal muscle fibre, one or more non-myelinating terminal Schwann cells, and one or more NMJ capping cells (known as kranocytes; Court et al. 2008). Importantly, the presence of cell types other than motor neurons and skeletal muscle at the NMJ should not be underestimated for normal synaptic form and function. For example, terminal Schwann cells play a key role in stabilising the NMJ and coordinating regeneration responses after injury (Son et al. 1996). Similarly, kranocytes may also be involved in NMJ maintenance and injury response (Court et al. 2008).

Historically, it was accepted that ALS pathology occurred primarily as a result of disturbances in the cell body of motor neurons, with ubiquitinated accumulations of proteins resulting in death of the cell body. However, recent evidence suggests that early pathological events occurring at the NMJ may ultimately be responsible for the early disruption of neuromuscular function observed in patients with MND, occurring in advance of pathological changes in other regions of the motor neuron (Frey et al. 2000; Fischer et al. 2004; Gould et al. 2006; Murray et al. 2010a). For example, Frey et al. (2000) showed that there was extensive denervation of limb muscles in SOD1<sup>G93A</sup> mice (a commonly used animal model of ALS) about 40 days before any symptoms, such as muscle weakness, were apparent. Fischer et al. (2004) reported similar observations in SOD1<sup>G93A</sup> mice, and also in rare post mortem material from a patient with ALS who died unexpectedly during the early stages of the disease.

Fig. 1 The NMJ. Upper motor neurons synapse onto lower motor neurons in the spinal cord. Lower motor neurons then send their axons out to target skeletal musculature via peripheral nerves. Once a lower motor neuron axon has reached its target muscle (magnified box) it will form neuromuscular synapses with individual muscle fibres. The motor nerve terminal (green) makes up the presynaptic component of the NMJ, and features numerous synaptic vesicles containing the neurotransmitter acetylcholine. The postsynaptic muscle fibre (orange) is also specialised, with accumulations of acetylcholine receptors at the motor endplate (red). The motor nerve terminal is insulated by one or more terminal Schwann cells (blue). A second ‘NMJ capping cell’ – the kranocyte – also overlies the terminal Schwann cell (pink).
disease – NMJ breakdown was present during pre-symptomatic stages of the disease when no cell death of motor neuron cell bodies could be detected in the spinal cord (Fischer et al. 2004). Further experiments in ALS mice demonstrated that deletion of the pro-apoptotic gene Bax from SOD1G85A mice rescued death of motor neuron cell bodies in the spinal cord, but did not prevent NMJ breakdown and only modestly affected disease progression (Gould et al. 2006). Similar evidence demonstrating functional and structural disturbance of the NMJ in ALS and ALS-like conditions has been presented from a range of other animal models, including mice, dogs, zebrafish, Caenorhabditis elegans and Drosophila (Rich et al. 2002; Ferri et al. 2003; Feiguin et al. 2009; Wang et al. 2009; Ramesh et al. 2010).

Early disruption of NMJs has also been reported in mouse models of SMA (Cifuentes-Diaz et al. 2002; Kariya et al. 2008; Murray et al. 2008, 2010b; Kong et al. 2009). For example, Murray et al. (2008) demonstrated that denervation of NMJs was present in particularly vulnerable muscles from a mouse model of severe SMA, even at pre-symptomatic ages. Subsequent functional studies revealed evidence of impaired synaptic transmission at the NMJ in SMA mice, resulting from a reduction in the number of synaptic vesicles present in motor nerve terminals (Kong et al. 2009). This reduction in synaptic vesicle density could also account for the widespread muscle weakness observed in SMA (Kong et al. 2009). Rare post mortem material from patients with SMA has also revealed disruption of NMJs (Kariya et al. 2008).

**In vitro NMJ models**

Although the NMJ is a fundamental and early pathological target in MND, the range of approaches and models available to study human NMJ pathology are limited. In general, researchers are restricted to studying human post mortem material invariably from advanced patients or animal models that often fail to fully recapitulate the human MND phenotype. The development of new experimental approaches and models that will allow the examination and manipulation of human NMJs is therefore likely to be of significant benefit for our understanding of disease pathogenesis, discovery and testing of novel treatments. One such approach that is currently being actively pursued is the development of *in vitro* co-culture systems using human iPSCs to generate cell types contributing to neuromuscular connectivity.

Early attempts to co-culture spinal motor neurons and skeletal muscle were basic and focused on understanding NMJ development rather than studying disease. Initially, the favoured species for neuromuscular myotome co-cultures was Xenopus, as the embryos were easy to collect and dissect, and the cultures could be maintained at room temperature. Indeed, it was embryonic Xenopus tissue that was used in the earliest documented co-culture of spinal and skeletal muscle tissue (Harrison, 1907). In this experiment, embryonic Xenopus myotomes were removed and the developing neuromuscular connections were observed in culture over several days, providing the first evidence that NMJs could continue to develop as normal under *in vitro* conditions (Harrison, 1907). Over the next few decades, methods for culturing neuromuscular tissue changed very little. Advances were made in the ability to generate myotome cultures from other species, such as embryonic chick and rodent tissue (Szepsenwol, 1941). Improvements in electrophysiological techniques also meant that the culture preparations could be subjected to functional as well as morphological assays. For example, in 1964 Crain showed that neuromuscular connections, which had developed *in vitro* for several months (Bornstein & Breitbart, 1964), were capable of neuromuscular transmission (Crain, 1964). However, the methodology employed in all of these early experiments was limited by the fact that protocols were centred around removing a section of embryonic spinal cord from chicks, rodents or Xenopus with developing ganglia and muscle still attached and undisturbed – the spinal cord and respective muscle tissue were effectively kept as a myotome unit (Szepsenwol, 1941; Bornstein & Breitbart, 1964; Crain, 1964).

The next major technical breakthrough came in 1968 when Crain (1968) cultured physically separate (by about 1 mm) explants of rodent skeletal muscle and spinal cord, and showed that processes extending from the ventral side of the spinal cord could invade the muscle tissue to form structural and functional synaptic connections. In the same year, James and Tresman applied the same co-culture technique to embryonic chick tissue, and used electron microscopy to analyse the morphological aspects of synaptic connections that were formed. Although the connections were immature, they revealed the presence of synaptic vesicles as well as putative postsynaptic specialisations (James & Tresman, 1968). After the discovery that completely separate explants could still form immature neuromuscular connections, other co-culture systems were rapidly developed, such as the use of dissociated cells from chick skeletal muscle and spinal cord (Fischbach, 1970), and monolayer culture systems also using chick spinal cords and muscle (Shimada et al. 1969).

In 1970 Peterson and Crain demonstrated that neuromuscular connections could be formed not only between embryonic tissue from different species (specifically between rat and mouse tissue), but also using adult muscle tissue and embryonic spinal cord from rodents (Peterson & Crain, 1970), as well as skeletal muscle tissue from adult humans and embryonic spinal cord from rodents (Crain et al. 1970). These cultures were maintained for up to 7 weeks, and electrophysiological data showed that synaptic connections formed between rat neurons and human muscle were functional; stimulation of the rat spinal cord resulted in action potentials in the muscle (Crain et al. 1970). Histological analysis also revealed accumulations of
acetylcholinesterase at sites of synaptic contact (Crain et al. 1970). Later adaptations to experimental protocols allowed researchers to successfully maintain similar co-cultures for up to 40 weeks (Peterson & Crain, 1972).

The establishment of a reliable protocol for co-culture led to many groups using in vitro models to study the effects of human disease on neuromuscular connectivity. For example, Liveson et al. (1975) studied the effects of ALS patient sera on co-cultures of embryonic mouse spinal cord and skeletal muscle. Their finding of no resulting abnormalities effectively ended a popular theory that a cytotoxic substance was present in the sera of patients with ALS (Liveson et al. 1975). Witkowski & Dubowitz (1975) were the first to culture embryonic mouse spinal cords with muscle biopsies taken from human patients suffering from a range of neuromuscular conditions, including SMA and Duchenne muscular dystrophy. They found no clear differences between control and diseased muscle groups, and concluded that any pathology observed in sufferers was not primarily caused by the muscle (Witkowski & Dubowitz, 1975). However, muscle regeneration was the focus of this study and so cultures were only kept for a maximum of 11 weeks (Witkowski & Dubowitz, 1975). A later study by Peterson et al. (1986) also used human dystrophic muscle in co-culture and, when maintained for more than 4 months, the regenerated muscle did begin to display pathological features such as myofilament breakdown and cytoplasmic bodies. However, despite these breakthroughs, the only human tissue that could be used in nerve/muscle co-cultures was skeletal muscle. The ability to generate human motor neurons, with or without disease mutations, only became a possibility with the advent of stem cell technology (for review, see Marchetto et al. 2011).

Human stem cell-derived in vitro NMJ models

Human PSCs were first isolated from the inner cell mass of the blastocyst, the pre-implantation embryo, in 1998 (Thomson et al. 1998). These human embryonic stem cells (hESCs) were micro-manipulated and subsequently grown in culture by a variety of methods (Xu et al. 2001; Amit et al. 2003, 2004), generating non-transformed, stable and commercially available cell lines. iPSCs, on the other hand, can be generated from terminally differentiated adult somatic cells (e.g. dermal fibroblasts), which are then ‘reprogrammed’ through transfection with transcription factors, initially four in the pioneering study led by Yamanaka (Takahashi et al. 2007; for review, see Amabile & Meissner, 2009). The resulting cells are embryonic-like, and possess the capacity to both self-renew and differentiate into any cell type. By virtue of the fact that they are non-transformed, hESCs potentially represent a more robust in vitro model for studying human neurodevelopmental processes. However, because iPSCs can be generated from patients with specific inherited diseases, the resulting cells represent an unparalleled opportunity to study human inherited neurodegenerative diseases (e.g. SMN1 mutations in SMA; Ebert et al. 2009). Hence, the advent of disease-specific iPSCs has revolutionised the prospect for creating in vitro human disease model systems to study underlying pathobiological processes at a cellular and molecular level; but also for the consideration of novel therapeutic strategies (Ebert et al. 2009; Lee et al. 2009).

One of the most important pre-requisites in creating an accurate and clinically relevant in vitro model system is to generate the correct, disease-appropriate, cellular subtype. In vivo, motor neurons develop from highly restricted foci in the ventral spinal cord (the pMN domain), while sensory neurons are largely generated more dorsally (Jessell, 2000). Building on such developmental insights, and those gained from studies involving mouse embryonic stem cells, much progress has been made towards generating human motor neurons from both embryonic and induced pluripotent cells (Wichterle et al. 2002; Li et al. 2005; Dimos et al. 2008; Hu & Zhang, 2009; Guo et al. 2010; Marteyn et al. 2011; Patani et al. 2011). A caudal positional identity is initially induced in neural precursor cells, usually by exposure to retinoic acid (Jessell, 2000; Wichterle et al. 2002), although other methods to generate caudalised ESC-derived neural precursors and neurons are recognised (Patani et al. 2009, 2011; Peljto et al. 2010). Next, a ventral positional identity is assigned, by activation of the sonic hedgehog signalling pathway (Wichterle et al. 2002; Li et al. 2005). Once neural precursors have been positionally specified to the ventral spinal cord, they can be plated down for terminal differentiation. In vitro, this usually involves a poly-d-lysine(pdl)/laminin substrate with concomitant withdrawal of mitogens and addition of growth factors, including brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF) and insulin-like growth factor 1. Using this approach in an adherent culture system, OLIG2-expressing motor neuron precursors appear at 3 weeks, and markers of mature motor neurons (such as HB9) appear from 4 to 5 weeks after neural induction from iPSCs (Li et al. 2005). The generation of spinal motor neurons from stem cells can then be validated using a range of morphological and functional techniques to identify features specifically associated with motor neurons, such as the expression of choline acetyltransferase – an enzyme that produces the neurotransmitter acetylcholine.

To date, a large number of studies using iPSCs have focussed on deriving motor neurons, but have not addressed the issue of which particular subtype is being generated. Two contemporaneous recent studies using mouse (Peljto et al. 2010) and human iPSCs (Patani et al. 2011) have begun to unravel the developmental logic of motor neuronal subtype diversification. Such studies raise the prospect of studying motor neuron subtype selective vulnerability, which is known to exist in MND (Murray et al.
2008; Kanning et al. 2010). Furthermore, the NMJs formed by distinct subtypes of motor neurons may themselves display differential vulnerability – raising important experimental questions around modelling MND pathogenesis using intact NMJs rather than just isolated motor neuronal subtypes.

A number of groups have already attempted to create NMJs in vitro using human stem cells. However, they have had limited success, producing only immature points of contact between neurite tips and myotubes. One of the first studies to attempt to co-culture stem cell-derived motor neurons with skeletal muscle was performed by Li et al. (2005). In this study, motor neuron progenitor cells were cocultured with the murine myoblast cell line C2C12 (Li et al. 2005). The cultures were maintained for up to 3 weeks, during which the motor neuron progenitor cells matured into motor neurons and extended neurites (Li et al. 2005). Co-cultures were stained with the synaptic marker synapsin, and α-bungarotoxin was used to label acetylcholine receptors. This analysis revealed that clusters of acetylcholine receptors were formed on myotubes where they were contacted by neurite processes (Li et al. 2005). Singh Roy et al. (2005) also attempted to generate NMJs in vitro using a modified version of the motor neuron differentiation protocol outlined by Li et al. (2005). Singh Roy et al. (2005) used human stem cells that had been genetically modified to express green fluorescent protein under the motor neuron-specific promoter, HB9. After differentiation, these motor neurons could be isolated from other cell types by fluorescence-activated cell sorting, resulting in a pure motor neuron population. In this study, rather than using a muscle cell line, NMJs were formed with a primary culture of dissociated neonatal rat skeletal muscle (Singh Roy et al. 2005). As in the Li et al. (2005) study, synaptic vesicle-associated protein SV2 clusters were found to co-localise with acetylcholine receptors on myotubes, indicating synapse formation (Singh Roy et al. 2005). No clusters of acetylcholine were observed on skeletal muscle fibres cultured without motor neurons, implying that receptor clustering was induced by the presence of motor neurons (Singh Roy et al. 2005).

In 2010, Guo et al. (2010) reported further developments in methodologies for developing and analysing stem cell-derived motor neurons co-cultured with skeletal muscle. Motor neurons were again derived from human stem cells and were cultured with dissociated skeletal muscle fibres from embryonic rats. However, Guo and colleagues generated a pure muscle culture, devoid of other cell types. Co-cultures were maintained for 4 days in media designed to support motor neuron maturation and survival then, after 4 days, the media was switched to a simpler NbActiv4 media (Guo et al. 2010). NbActiv4 does not contain trophic factors, such as BDNF or GDNF, which support axonal extension and survival but have also been shown to down-regulate production of agrin – a key molecule involved in inducing the clustering of acetylcholine receptors at the developing NMJ (Peng et al. 2003). Co-cultures were maintained for a maximum of 10 days before staining for neuronal-specific cytoskeletal element β-III-tubulin and acetylcholine receptors (Guo et al. 2010). Staining revealed neurites overlying muscle cells expressing clusters of acetylcholine receptors, similar to those reported previously (Guo et al. 2010). A Glut–Curare assay was also used to assess the functional capabilities of their in vitro NMJs. When glutamine was added to their co-cultures (thereby stimulating motor neurons), the number of contracting muscle cells increased, and this was reduced to baseline levels when curare was applied, implying that the points of synaptic contact were capable of synaptic transmission (Guo et al. 2010).

Recently, Patani and colleagues successfully generated medial motor column motor neurons from human stem cells using a retinoid independent protocol. Resulting motor neurons had preserved capacity to establish immature synaptic connections with skeletal muscle: a co-culture with C2C12 myotubes revealed that the stem cell-derived motor neurons were capable of inducing acetylcholine receptor clustering on myotubes after 12 days in culture (Patani et al. 2011).

The first disease-specific human motor neuron–muscle co-cultures have recently been attempted. For example, Marteyn et al. (2011) generated motor neurons from human embryos with myotonic dystrophy type 1. It was noted that motor neurons generated from affected patients displayed increased neurite outgrowth compared with identically cultured motor neurons from non-affected individuals, so a co-culture approach was used to determine whether the altered neurite outgrowth might also affect synaptogenesis at the NMJ (Marteyn et al. 2011). Here, a human muscle cell line, Mu2bR3, was used in the co-culture, which was maintained for up to 16 days and then stained for the pan-neuronal marker TuJ1 and acetylcholine receptors using α-bungarotoxin (Marteyn et al. 2011). From these co-culture experiments, the authors observed a reduced number of points of contact in the disease-specific motor neurons compared with the wild-type motor neurons, implying that the disease-specific motor neurons have a reduced ability to form NMJs (Marteyn et al. 2011).

**Preactive synapses or mature human NMJs?**

Despite initial successes in developing co-cultures of human motor neurons and muscle to generate NMJs in culture, stem cell-derived NMJs remain immature and unstable in comparison to the NMJs previously established using tissues from other species or NMJs in vivo (Fig. 2). For example, one of the most striking differences between the recent stem cell-derived co-cultures and historical co-cultures using animal tissues is the length of time that the cells could be maintained in vitro. Peterson & Crain (1972) co-cultured
muscle and rodent spinal cord for more than 40 weeks, whereas co-cultures using stem cell-derived motor neurons have not been successful beyond a 3-week period. This issue mainly arises due to differences in cell culture media requirements associated with using stem cell-derived motor neurons. Human motor neurons derived from stem cells are notoriously difficult to culture and require a complex media full of growth and trophic factors, several of which are not ideal for long-term maintenance of skeletal muscle cells. One possible solution to this may be to use compartmentalised co-cultures. Keeping each cell type isolated in its optimal culture media would likely mean increased cell survival and could therefore contribute to increasing the length of time co-cultures can be maintained for, and therefore the maturity of NMJs formed.

One other major factor that is likely to require addressing in order to successfully develop in vitro NMJ models using stem cell-derived motor neurons concerns the presence of other supporting cell types, including terminal Schwann cells and kranocytes (see above). Such cells types are known to play important roles in supporting NMJs in vivo (Koirala et al. 2003; Court et al. 2008; Feng & Ko, 2008), and would have been present in many of the historical co-culture experiments (although they were never specifically examined). In contrast, most recent co-cultures using stem cell-derived motor neurons have used muscle cell lines for the skeletal muscle component of the co-culture (e.g. Li et al. 2005; Marteyn et al. 2011; Patani et al. 2011). This may have had a negative effect on synaptogenesis and/or synaptic maturation. Therefore, the addition of potentially supportive cells, such as Schwann cells, to co-cultures may enhance synaptogenesis and maintain any putative synapses that do form.

Finally, it should be noted that to create a ‘real’ human NMJ in vitro, both co-culture components must be human in origin and, for disease studies, both cell types must carry the disease mutation. And yet, all recent co-cultures using human stem cell-derived motor neurons have either been xeno-cultures or have used primary muscle cell lines, primarily because they are readily obtained and easy to manage. However, it is possible to create human skeletal muscle from stem cells (Kim et al. 2005; Barberi et al. 2007). Attempts to develop NMJs from co-cultures of motor neurons and skeletal muscle both derived from stem cells will therefore be required. The successful development of such an approach would make it possible to address fundamental questions about the roles of nerve and muscle in MND pathogenesis. For example, there is much controversy over whether loss of presynaptic inputs at the NMJ (due to pathological changes in lower motor neurons) indirectly leads to muscle atrophy, or whether skeletal muscle is intrinsically vulnerable to low levels of SMN protein in SMA (Henderson et al. 1987; Braun et al. 1995; Gavrilina et al. 2008; Mutsaers et al. 2011). Similar questions are also being raised with regards to ALS pathogenesis (Miller et al. 2006; Wong & Martin, 2010).
Conclusions

The ability to use human iPSCs to model early MND pathogenesis at the NMJ would be extremely valuable to MND research. Such models would answer fundamental questions about the nature and mechanisms of some of the earliest events occurring across a range of different forms of MND, and would also be useful for rapid evaluation of the therapeutic effectiveness of new and existing treatment strategies. Early attempts to recreate human neuromuscular connectivity using iPSCs have been relatively successful, although there are numerous technical hurdles that will need to be overcome if the approach is going to yield mature NMJs in vitro. The rapid developments occurring in the field of stem cell biology suggest that the major technical hurdles identified will eventually be surmounted, allowing in vitro human NMJ preparations to become routinely available to a large number of research laboratories.

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