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Electrophysiological properties of neurons derived from human stem cells and iNeurons *in vitro*

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ABSTRACT

Functional studies of neurons have traditionally used nervous system tissues from a variety of non-human vertebrate and invertebrate species, even when the focus of much of this research has been directed at understanding human brain function. Over the last decade, the identification and isolation of human stem cells from embryonic, tissue (or adult) and induced pluripotent stem cells (iPSCs) has revolutionized the availability of human neurons for experimental studies *in vitro*. In addition, the direct conversion of terminally differentiated fibroblasts into *Induced* neurons (iN) has generated great excitement because of the likely value of such human stem cell derived neurons (hSCNs) and iN cells in drug discovery, neuropharmacology, neurotoxicology and regenerative medicine. This review addresses the current state of our knowledge of functional receptors and ion channels expressed in neurons derived from human stem cells and *iNeurons* and identifies gaps and questions that might be investigated in future studies; it focusses almost exclusively on what is known about the electrophysiological properties of neurons derived from human stem cells and iN cells *in vitro* with an emphasis on voltage and ligand gated ion channels, since these mediate synaptic signalling in the nervous system and they are at the heart of neuropharmacology.

Contents

1. Introduction

Functional studies of neurons have traditionally used nervous system tissues from a variety of non-human vertebrate and invertebrate species, even when the focus of much of this research has been directed at understanding human brain function. Over the last decade, the identification and isolation of human stem cells from embryonic, tissue (or adult) and induced pluripotent stem cells (iPSCs) has revolutionized the availability of human neurons for experimental studies *in vitro*. In addition, the **direct** conversion of terminally differentiated fibroblasts into *Induced* neurons by ectopic expression of defined transcription factors or microRNAs (thus avoiding re-differentiation processes and circumventing the pluripotent stage) has generated enormous excitement because of the likely value of such human stem cell derived neurons (hSCNs)

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and induced neurons (iNs) in drug discovery, drug safety testing, neuropharmacology, neurotoxicology, the study of neural development and neuropsychiatric disorders as well as in neuroregenerative medicine. Several publications have addressed these important areas, including two recent reviews considering new developments in the use of human stem cells for modelling neuropsychiatric diseases *in vitro* (Haggarty et al., 2016) and to advance drug discovery for the treatment of neuropsychiatric illnesses (Wen et al., 2016) and they are briefly considered in the conclusions section of this review.

The characterization of neurons (or more strictly, *neural cells*) derived from an increasing variety of available human cell sources is still, however, in its infancy; this review will address the current state of our knowledge of functional receptors and ion channels expressed in *neurons* derived from human stem cells and iNeurons and identify gaps and questions that might be investigated in future studies. The field of stem cell-derived neurons has grown exponentially over the last decade; this review will therefore focus almost exclusively on what is known about the electrophysiological properties of *neurons* derived from human stem cells and iN cells *in vitro* with an emphasis on voltage and ligand gated ion channels, since these are the mediators of synaptic signalling in the nervous system and the epicentre of neuropharmacology.

2. Tissue-derived stem cells

The original and still an important source of human pluripotent stem cells are embryonal carcinoma (EC) cells, which were first isolated from germ cell tumours (teratocarinomas) more than half a century ago (Andrews, 2002). These EC stem cells are considered the adult counterparts of Embryonic Stem (ES) cells and their study gave rise to many of the protocols to derive and culture human ES cells (in the 1990s) and provide a well-established model to study cell differentiation throughout embryogenesis and neurogenesis (Przyborski et al., 2000). Andrews and colleagues were the first to characterize a clonal cell line (the NTERA2cl.D1 or NT2 cells) derived from human TERA2 EC stem cells. Using retinoic acid, NT2 cells differentiated into cells with a neural morphology, expressed neurofilament proteins and voltage-activated sodium currents that were blocked by tetrodotoxin (TTX); moreover, under current clamp conditions, these cells were able to generate immature action potential-like responses (Andrews, 1984; Rendt et al., 1989). Using microfluorimetry, subsequent experiments also showed that these neural cells responded to acetylcholine, NMDA and kainate with increases in intracellular calcium ion concentrations, consistent with the expression of functional muscarinic acetylcholine (mACh) and ionotropic glutamate receptors (Squires et al., 1996). More convincingly, an elegant study by Hartley et al. (1999) showed that NT2 neurons, differentiated with retinoic acid and co-cultured with rodent glial cells, formed intricate synaptic contacts, fired high-amplitude, regenerative action potentials in response to depolarizing current and survived more than a year *in vitro*. Moreover, spontaneous miniature excitatory and inhibitory postsynaptic currents (mEPSCs and mIPSCs) were recorded by voltage-clamping cells at −40 mV enabling the investigators to detect excitatory inward and inhibitory outward currents, respectively. Synaptic currents were also evoked by stimulating one neuron and recording responses from a second neuron, confirming functional synaptic neurotransmission in these human neurons. In the majority of such paired recordings, the inward synaptic components reversed at 0 mV (the equilibrium potential for cations in these experiments) and were blocked by CNQX and APV, indicating that the mEPSCs were mediated through AMPA and NMDA ionotropic glutamate receptors; in a smaller proportion of paired recordings, the synaptic currents reversed around −70 mV (the approximate equilibrium potential for chloride ions) and were blocked by bicuculline, indicating that the mIPSCs were mediated through $GABA_A$ receptors (Hartley et al., 1999).

More recently, our lab has investigated the development and pharmacological properties of ion channels expressed in neurons derived from an additional clone of the TERA2 line, TERA2.cl.SP12 EC stem cells (see Fig. 1; Stewart et al., 2004; Coyne et al., 2011; Cao et al., 2015). Our experiments show that neurons derived from these EC cells generate TTX-sensitive voltage-activated sodium currents and TEA-sensitive voltage-gated potassium currents. These neurons show immature action potentials in response to depolarizing current but rarely display spontaneous synaptic or action potential events even with long-term (≥3 months) culture *in vitro*. The majority of neurons however do display large, concentration-dependent whole-cell currents in response to the inhibitory neurotransmitters, glycine and GABA. Like mature native neurons, the glycine currents are blocked by strychnine. Similarly, GABA currents are blocked by picrotoxin and bicuculline and potentiated by the allosteric $GABA_A$ receptor modulators, chlordiazepoxide, diazepam, pentobarbital, allopregnanolone and mefenamic acid. From such electrophysiological recordings we can deduce that the $GABA_A$ receptors expressed in these human EC stem cell-derived neurons are likely composed of $\alpha x\beta 2/3\gamma 2$ subunits (where x is one of several possible α subunits) to enable the rich and complex pharmacological responses consistent with native $GABA_A$ receptors. In addition, around 22% of these neurons respond to glutamate and NMDA (that are inhibited by magnesium ions in a highly voltage-dependent manner) but with much smaller currents, even after 25–50 days *in vitro*. These observations indicate that glutamate receptors are expressed much later in development in human neurons, consistent with development *in vivo* (Maric et al., 2000). One other notable observation from this study is that morphological changes and the neuronal antigen, βIII-tubulin, appear within 5–7 days of neural induction whereas ion channel-mediated currents take 2 or more weeks; these data and, those outlined below, therefore indicate that cell shape, gene expression and immuno-labels do not necessarily equate with functional cells *in vitro*.

Another attractive source of stem cell is human umbilical cord blood cells because they are easy to obtain and are not saddled with major ethical concerns. In a well-conducted study, Sun et al. (2005) induced a non-hematopoietic ($CD34^-/CD45^-$) fraction of human umbilical cord blood stem cells (UCBSCs) to differentiate into neuron-like cells. Microarray analysis indicated these cells expressed genes for voltage-gated Na and K ion channels and 14 neurotransmitter receptors, as well as several neural antigen markers including β-tubulin III and neurofilament, NF-200. Patch-clamp recordings showed these neural cells expressed functional inward rectifying potassium currents (Kir) and outward rectifying potassium currents (I_K). However, further electrophysiological analysis showed that they did not express functional sodium channels and could not fire TTX-sensitive action potentials and only kainic acid was able to induce whole cell currents in some cells indicating they were, at best, highly immature *neurons*. Importantly, these data also emphasize that gene microarrays are also insufficient evidence that a stem cell has successfully differentiated into a functional neuron.

In a later study, Zwart et al. (2008) tested a range of neurogenic stimuli and co-culturing with rat neural cells to induce human umbilical cord blood-derived multipotent mesenchymal stem cells to differentiate into neurons and reported they possessed '*no ionic currents typical of neurons before or after neural induction protocols*'. More recently, however, Zeng et al. (2013) showed that UCBSCs differentiated into neural-like cells when cultured in a serum-free medium conditioned by rat olfactory ensheathing cells (OECs).