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NMDA receptor-dependent glutamate excitotoxicity in human embryonic stem cell-derived neurons

Kunal Gupta[a,b,c,*], Giles E. Hardingham[b], Siddharthan Chandran[c]

[a] Cambridge Centre for Brain Repair, University of Cambridge, Cambridge CB2 0SZ, United Kingdom
[b] Centre for Integrative Physiology, University of Edinburgh, Edinburgh EH8 9XD, United Kingdom
[c] Centre for Neuroregeneration, University of Edinburgh, Edinburgh EH16 4SB, United Kingdom

HIGHLIGHTS

- Human embryonic stem cell derived neurons develop NMDA and AMPA receptors in culture.
- Enriched HESC-derived neurons develop functional glutamate responses *in vitro*.
- MK 801 confers a protective effect on glutamate-induced excitotoxic neuronal death.
- Studies using human pluripotent stem cells may aid the discovery of novel therapeutic targets.

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ABSTRACT

Thanks to the development of efficient differentiation strategies, human pluripotent stem cells (HPSC) offer the opportunity for modelling neuronal injury and dysfunction in human neurons *in vitro*. Critically, the effective use of HPSC-derived neural cells in disease-modelling and potentially cell replacement therapies hinges on an understanding of the biology of these cells, specifically their development, subtype specification and responses to neurotoxic signalling mediators. Here, we generated neurons from human embryonic stem cells and characterised the development of vulnerability to glutamate excitotoxicity, a key contributor to neuronal injury in several acute and chronic neurodegenerative disorders. Over two months of differentiation we observed a gradual increase in responsiveness of neurons to glutamate-induced Ca^{2+} influx, attributable to NMDA receptor activity. This increase was concomitant with an increase in expression of mRNA encoding NMDA and AMPA receptor subunits. Differentiated neurons were vulnerable to glutamate excitotoxicity in a dose-dependent manner, which was reduced by NMDA receptor antagonists.

1. Introduction

Glutamate excitotoxicity, both acute and chronic, has been implicated widely in the pathogenesis of a range of neurological diseases, including Alzheimer's disease, Huntington's disease and traumatic brain injury [25,28]. Knowledge of the role and mechanisms of glutamate excitotoxicity can potentially enhance our understanding of key downstream signalling processes that mediate neuronal death and identify novel therapeutic targets. A major cause of glutamate excitotoxicity is activation of the NMDA subtype of glutamate receptor (NMDAR), which mediates Ca^{2+}-dependent cell death [26]. Most NMDARs contain two obligate GluN1 subunits plus two GluN2 subunits [11], of which there are four subtypes, GluN2A-D, with GluN2A and GluN2B predominant in the forebrain [30,40]. While physiological patterns of synaptic NMDAR activity have the potential to be neuroprotective [15,19,39], excessive or inappropriate activity is harmful. The work of many laboratories has advanced our understanding of how loss of glutamate homeostasis, ionic imbalance and inappropriate NMDAR activity can contribute to degenerative disorders [8,16,22,25,27].

Antagonism of pathological glutamate-signalling in rodent models of neurological diseases has yielded promising results [6,10], though unsuccessful clinical trials highlight the need for further studies [1,31]. Currently, excitotoxic neuronal cell death has primarily been characterised in rodent systems. Advances in human pluripotent stem cell (HPSC) technology permit the

Abbreviations: aCSF, artificial cerebrospinal fluid; AMPA, 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid; AMPAR, 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid receptor; D-APV, aminophosphonopentanoic acid; HESC, human embryonic stem cell; HPSC, human pluripotent stem cell; iPSC, induced pluripotent stem cell; NPC, neural precursor cell; NMDA, N-methyl-D-aspartic acid; NMDAR, N-methyl-D-aspartic acid receptor.

* Corresponding author at: Department of Neurological Surgery, Oregon Health and Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97239, USA.
E-mail address: kg244@cantab.net (K. Gupta).

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generation of enriched neurons of differing positional identities as well as astrocytes from human embryonic stem cells (HESC) and allow investigation of cell injury and rescue paradigms in a human *in vitro* system [12,13,23,24,35,38]. In light of the inter-species differences between rodent and human [34], studies utilising human *in vitro* systems may prove informative in interpreting the relevance of findings in rodent studies to the human pathological condition [3,13,18].

Here, we describe the functional maturation of HESC-derived neurons and the development of glutamate-responsiveness, in order to study neuronal responses to pathologically relevant glutamate concentrations [5].

2. Materials and methods

HESC Culture and neuralisation: The human embryonic stem cell (HESC) line H9 (Harvard University, Cambridge, MA) was cultured and passaged regularly on a feeder layer of irradiated mouse embryonic fibroblasts, and neuralised by established protocols [21]. HESC-derived neurons were generated by methodologies described by Koch et al. [23]. Enriched HESC-derived neural precursor cells (NPCs) were propagated in Advanced DMEM/F12, 1% Glutamax, 1% N2 supplement 1% penicillin-streptomycin, 0.1% B27 supplement, 10 ng/ml FGF and 10 ng/ml EGF. Coverslips were coated with Matrigel (BD Biosciences) for 1hr, diluted 1:30 in DMEM. NPCs were plated in NPC propagation medium without mitogens for 24 h and switched to Neurobasal-A, supplemented with 1% non-essential amino acids, 1% N2 supplement, 1% penicillin-streptomycin, 0.5% Glutamax, 10 ng/ml BDNF, 10 μM forskolin, 0.1 μM retinoic acid for neuronal differentiation. 50 μM DAPT [4] was included in the first medium change. These HESC-neuronal cultures are consistent with those described in Gupta et al. [13] in cellular composition; 95% were neurons and the remainder astrocytes, confirmed by TuJ1 and GFAP immunostaining. Differentiation duration was determined from the day of application of neuronal differentiation medium to NPCs. The day before experiments, HESC-derived neurons were removed from their trophic differentiation medium into a trophically deprived, glutamate-free "minimal medium" [13], which comprises 90% Salt-Glucose-Glycine (SGG) medium [2] and 10% MEM (Minimal Essential Medium, Invitrogen).

Calcium-imaging: The day before imaging, HESC-derived neurons were removed from their trophic differentiation medium into a trophically deprived, glutamate-free minimum medium as described above. HESC-neurons, identified by morphology and confirmed by TuJ1 immunostaining, were imaged at various timepoints in artificial cerebrospinal fluid (aCSF) [39] on a 37 °C-heated stage. Cells were loaded with 11 μM Fluo-3 for 30 min and washed in aCSF. Coverslips were mounted in a perfusion chamber. Fluo-3 fluorescence images (excitation 488 nm/emission 520 nm) were taken at baseline and during saturating glutamate (200 μM) and NMDA (150 μM) stimulation. Glycine (100 μM) was applied as a co-agonist. D-APV (50 μM) was used as an NMDAR antagonist. For calibration, ionomycin was used to saturate the Fluo-3 signal (50 μM), followed by $MnCl_2$ (10 mM) to quench. The quenched Fluo-3 signal corresponds to ~100 nM Ca^{++}. The K_d of Fluo-3 was taken as 315 nM and free Ca^{++} concentration was calculated by the formula $Ca^{++} = K_d\ (F - F_{min})/(F_{max} - F)$, where F is fluorescence. Images were taken every second for the duration of each experiment (~10 min) [17].

Gene expression analysis: Total RNA was isolated *via* the Absolutely RNA miniprep kit, as described [13]. cDNA synthesis performed *via* the AffinityScript cDNA (Stratagene) synthesis kit using oligo-dT and random hexamer primers, and qRT-PCR performed using Brilliant SYBR Green master mix (Stratagene), following the manufacturer's instructions. Forward and reverse primer sequences: *GRIN1* AGGAACCCCTCGGACAAGTT, CCGCACTCTCGTAGTTGTG; *GRIN2A* TGGACGTGAACGTGGTAGC, CCCCCATGAATGCCCAAGAT; *GRIN2B* TTCCGTAATGCTCAACATCATGG, TGCTGCGGATCTTGTTTACAAA; *GRIN2C* GAGTGGTCAAATTCTCCTACGAC, TGTAGTACACCTCCCCAATCAT; *GRIN2D* CTGGCCTCACTGGATCTGG, GGAAGGAAACCATAATCACGCA; *GRIA1* GGTCTGCCCTGAGAAATCCAG, CTCGCCCTTGTCGTACCAC; *GRIA2* AGTTTTCCACTTCGGAGTTCAG, CCAAATTGTCGATGTGGGGTG; *GRIA3* TCCGGGCGGTCTTCTTTTTAG, TGGGGAATCCTCCGTGAGAAT; *GRIA4* GCCATTGTCCCTGATGGAAAA, GAGGGGCAATAGCAATCTCTG; *GAPDH* AGGCTGGGGCTCATTTG, CAGTTGGTGGTGCAGGAG.

Electrophysiology: Whole-cell NMDA-evoked currents were recorded using an Axopatch 200B amplifier (Molecular Devices) using patch-pipettes made from thick-walled borosilicate glass with a tip resistance of 4–8 MΩ that were filled with an 'internal' solution that contained (in mM): K-gluconate 141, NaCl 2.5, HEPES 10, EGTA 11; pH 7.3 with KOH. Experiments were conducted at room temperature (18–21 °C) in an 'external' solution containing (in mM): NaCl 150, KCl 2.8, HEPES 10, $CaCl_2$ 4, glucose 10, pH to 7.3 with NaOH. Picrotoxin (50 μM), strychnine (20 μM) and tetrodotoxin (300 nM) were also included. NMDAR-mediated currents were induced by the addition of saturating concentrations of NMDA (100 μM) and the co-agonist glycine (100 μM) to the external solution. Access resistances were monitored and recordings where this changed by >20% were discarded. Currents were filtered at 2 kHz and digitised online at 5 kHz *via* a BNC-2090A/PCI-6251 DAQ board interface (National Instruments, Austin, TX, USA).

Neuronal injury and Cell viability assays: The day before application of the excitotoxic insult, HESC-derived neurons were removed from their trophic differentiation medium into a trophically deprived, glutamate-free minimal medium. Neurons were treated with glutamate with and without MK801 (10 μM) [29]. 24 h after excitotoxic challenge, cell viability was quantified using CellTiter-Glo Luminescent Cell Viability Assay (Promega), following manufacturer's instructions. Neuronal injury experiments using high-dose NMDA (150 μM) [29], were performed in magnesium-free SGG medium [2]; cell viability was quantified after 24 h by the same protocol.

Data analysis: All experiments were performed with at least 3 biological repeats; within each, 3 technical repeats were generated. qPCR data were normalised to GAPDH. Data are presented as mean ± standard error of the mean; statistical analyses were performed in Microsoft Excel and GraphPad Prism 5 (GraphPad Software, Inc., San Diego) by univariate and two-way ANOVA with Newman-Keuls post-test and two-tailed Students' *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Temporal profile of in vitro acquisition of HESC-derived neuronal glutamate responses

In order to study excitotoxic responses in a human platform, enriched cultures of HESC-derived neurons were examined for functional glutamate-mediated responses. Neurons were stimulated with saturating glutamate (200 μM) and functional glutamate receptor activity was determined by calcium-imaging over increasing duration in culture. While initially unresponsive to glutamate (including doses of 1 mM glutamate: data not shown), HESC-derived neurons developed calcium currents in response to bath-extracellular glutamate application with increasing duration in culture. 2-week old HESC-derived neurons demonstrated no significant calcium influx, while later time points demonstrated significant calcium influx with glutamate treatment, with peak calcium currents increasing over time to maximal at 6 and

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