Neuronal Cell Culture from Human Embryonic Stem Cells as *in vitro* Model for Neuroprotection

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Summary
In the context of efficacy testing of pharmacological compounds in animal models, replacement of some of these models with a relevant human in vitro system appears attractive, in particular with regard to large scale screening. Here, we show results from initial phases of a project, which attempts to explore the outstanding potential of human embryonic stem cell (hESC)-based in vitro models with special regard to neuronal stress as a potential replacement of animal models for human neurodegenerative diseases.
We show the functionality of neurons derived from hESC precursors by calcium imaging, mitochondrial potential measurements and Western blots and moreover demonstrate that this model reproduces crucial mechanistic aspects observed during ischemia and excitotoxicity that are thought to be at the core of some neurodegenerative diseases. Also, the broader possibilities for refining surrogate molecular information emerging from the detailed analysis of this model are discussed.

Zusammenfassung: Stammzellbasierte menschliche *in vitro* Modelle zum Ersatz der Wirksamkeitsprüfung bei Tieren
Im Kontext von Wirksamkeitsstudien von pharmakologischen Formulierungen und Medikamentenkandidaten in Tiermodellen besteht das starke Bedürfnis Ersatzmodelle auf der Basis menschlicher in vitro Systeme zu entwickeln. Wir zeigen hier Ergebnisse aus den frühen Phasen eines Projekts, welches das besondere Potential humaner embryonaler Stammzell- (hESC)-Modelle als entsprechende in vitro Systeme erforschen möchte, mit einem speziellem Fokus auf neuronalen Stress und mit dem Ziel Tiermodelle für humane neurodegenerative Krankheiten zu ersetzen.
Wir konnten die Funktionalität von Neuronen, die aus hESC Vorläufern differenziert wurden, durch Calcium Imaging, Potentialmessungen der mitochondrialen Membran und Western Blots zeigen. Wir konnten auch nachweisen, dass das Modell wesentliche mechanistische Aspekte, wie sie während der Ischämie oder unter erregungstoxischen Bedingungen auftreten, gut widerspiegelt. Es wird vermutet, dass diese Prozesse einer Reihe von menschlichen neurodegenerativen Krankheiten zugrunde liegen. Auch weitergehende Möglichkeiten zur Entwicklung besserer und genauerer, auch molekularer Endpunkte, die sich aus der detaillierten Analyse dieser Modelle ergeben könnten, werden diskutiert.

Keywords: human embryonic stem cells, excitotoxicity, in vitro models, neurodegeneration

1 Introduction

Examples for efficacy models include xenograft models for various human cancers; models for stroke, in which severe cerebral ischemia is induced by permanent occlusion of cerebral arteries in test animals (MCAO); various transgenic rodent models for neurodegenerative diseases, or extremely tough experimental models for autoimmune encephalomyelitis induced by vaccination of experimental animals with autoantigens as preclinical models of multiple sclerosis (MOG-EAE) (Bähr, 2004; Schrattenholz and Klemm, 2006). Despite a high degree of sophistication, there is a general sense of caution towards these models, because their read-outs are often insufficient or misleading.

Generally, in the area of efficacy testing, there is an urgent need for the development of novel *in vitro* methods, which can balance disadvantages in terms of organ specific barriers and metabolism with advantages regarding the higher relevance of humanised systems, more precise functional and molecular read-outs and the potential of higher throughput. In the given situation of regulatory frameworks in the EC and elsewhere, the purely diagnostic use of human embryonic stem cell models promises to potentially provide highly attractive alternatives (Schrattenholz, Klemm and Cahill, 2004). The cells can be differentiated to organotypic cell cultures and serve as a flexible substrate for a variety of functional molecular endpoints. Moreover, they are ideal for validation purposes using modern silencing technologies in a framework of genetically homogeneous differentiations to various

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"tissue"-like cell culture substrates like neural cells, cardiomyocytes, various types of muscle cells and adipocytes (Schrattenholz and Klemm, 2006).

Related results from corresponding murine ESC-screening systems, in combination with quantitative differential proteomic display techniques, have already provided biomarkers for the potential replacement of some related animal models (Buesen et al., 2004; Genschow et al., 2004; Schillo et al., 2005; Schrattenholz et al., 2005; Sommer et al., 2004). Here we show the first results of the adaptation of mESC models to corresponding hESC systems.

2 Materials and methods

Culture and differentiation of hES cells

Undifferentiated hES cells (human embryonic stem cell line (hES) H9 from WiCell Research Institute, NIH Code WA09) were cultured on a feeder layer of irradiated CF1 mouse embryonic fibroblasts (MEF) in hES-medium [DMEM/F12 supplemented with 20% (v/v) Knockout™ Serum Replacement (defined, serum-free formulation), 0.1 mM β-mercaptoethanol, 200 mM L-glutamine, 0.1 mM non-essential amino acids, penicillin (100 IU/ml), streptomycin (100 µg/ml) from Gibco and 4 ng/ml basic fibroblast growth factor (bFGF, Invitrogen)] with a daily change of medium and passage once a week at a ratio between1:3 and 1:6.

For differentiation procedures see Figure 1. As differentiation medium DMEM/F12 was supplemented with N2 (Gibco), penicillin (100 IU/ml, Gibco), streptomycin (100 µg/ml, Gibco) and heparin (2 µg/ml) in the presence of bFGF (20 ng/ml) or BDNF (10 ng/ml) and finally replaced by neurobasal medium.

Routinely APase staining was performed to characterise differentiation (Fig. 2A) by fixing hES cells in 4% paraformaldehyde at 4°C for 20 min, washing several times in Tris-maleate buffer and staining with naphthol AS MX phosphate/Fast Red TR salt mix.

Western Blot Analysis

The protein concentration was determined by BCA assay according to Smith

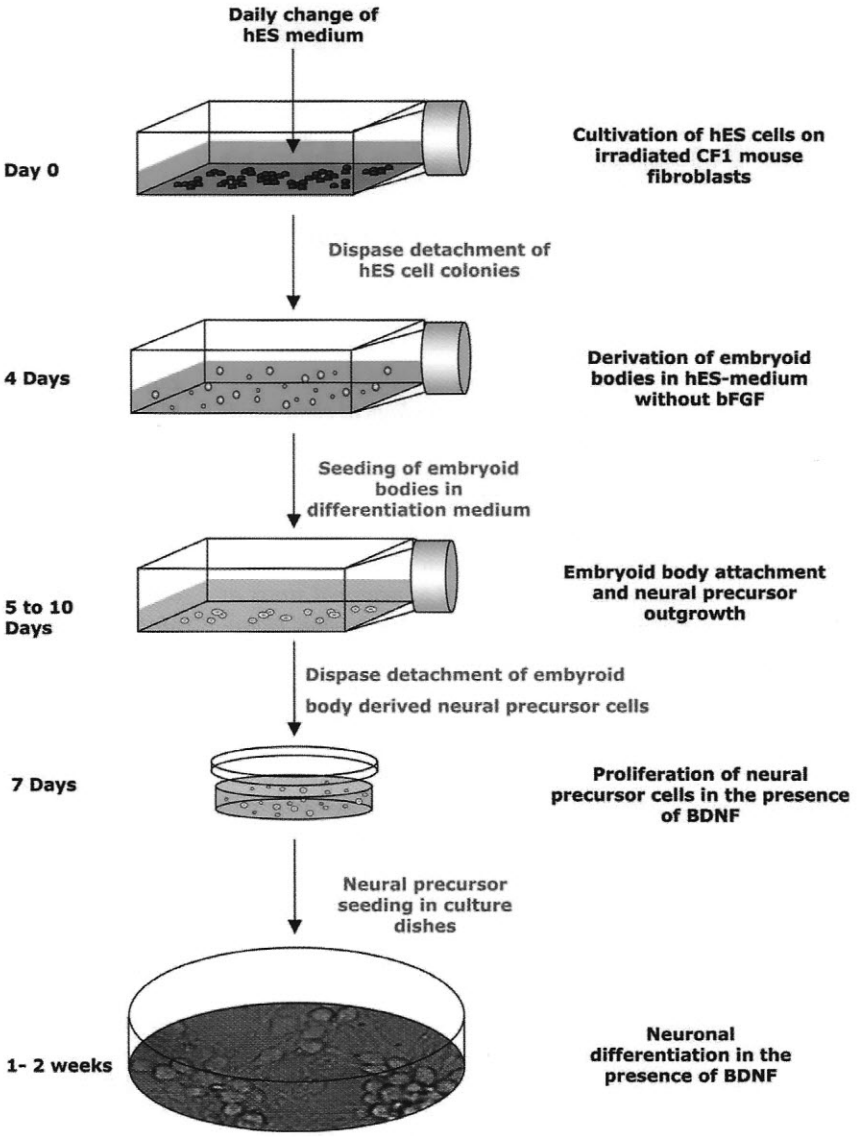

Fig. 1: Differentiation of hESC from totipotent precursor cells to mixed neural cell cultures including functional neurons; bFGF, basic fibroblast growth factor, BDNF, brain derived neurotrophic factor.

(Smith et al., 1985) with adaptation for microplates. Samples were mixed with sodium dodecyl sulphate polyacrylamide gel electrophoresis- (SDS–PAGE) or XT-sample buffer and heated (95°C) for 5 min. Individual samples were run on 12% Criterion-XT Bis–Tris gels (Biorad, Cat # 345-0119) and transferred to PVDF membranes (Biorad). After blocking with 30 mM Tris-HCl (pH 7.5), 100 mM NaCl and 0.1% Tween 20 (TBS-T) containing 5% fat-free milk powder for 1 h at room temperature, the membranes were incubated with the primary antibody: anti synaptophysin (SYP, D-4, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C, followed by a peroxidase-labelled goat anti-mouse secondary antibody for 1 h at room temperature. Visualisation was performed using the ECL method and a CCD camera (Diana system, raytest, Straubenhardt). Immunoreactive bands were quantified using the AIDA Software

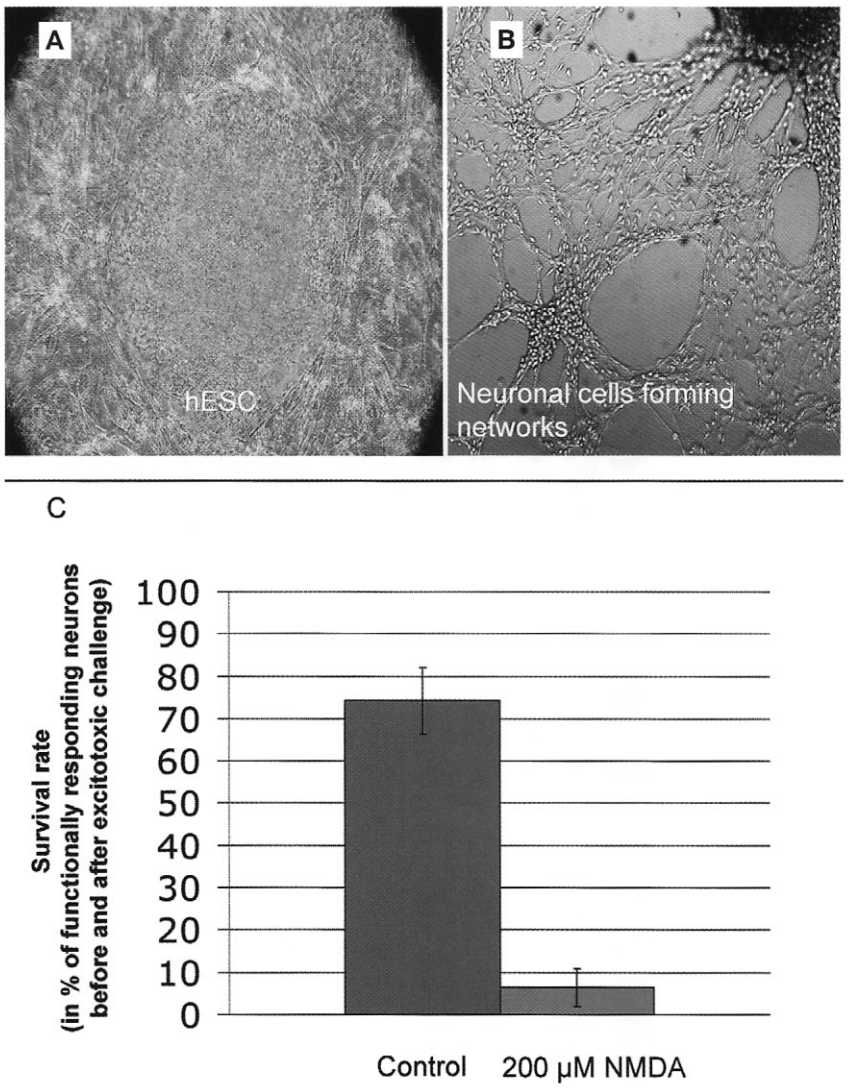

Fig. 2: A, human embryonic stem cells (hESC) stained by alkaline phosphatase. B, embryoid bodies differentiated to cells with neuronal morphology (V41 differentiated cells) in the presence of BDNF. These neural cells had morphological, functional and molecular characteristics typical of neurons. C, cells were tested for neuronal survival of a standard excitotoxic insult by application of 200 µM NMDA. They thus provide a model for testing efficacies of neuroprotective substances.

package (raytest) as described elsewhere (Schillo et al., 2005).

Assessment of in vitro functionality of hES-derived neurons by calcium-imaging

The calcium imaging procedure for quantification of functional hES-derived neuronal responses was described previously (Sommer et al., 2004). Generally, the neurons were kept in washing buffer (130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM HEPES, 10 mM Glucose, pH 7.3) and stimulated by incubation for 30 s in stimulation buffer (130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose and 200 µM NMDA or 10 µM glutamate).

Imaging of mitochondrial membrane potential ($\Delta\psi$) changes

Fluorescent probe rhodamine-123 was used to monitor qualitative changes of mitochondrial membrane potential ($\Delta\psi$). Slices were loaded with rhodamine-123 were excited at 480 nm and emission was monitored at 565 nm. Mitochondrial rhodamine-123 accumulation and quenching results in a fluorescence decrease, whereas depolarisation provokes signal dequenching and an increase of fluorescence after mitochondrial dye release (Metzger et al., 2005).

3 Results

As shown in Figure 2, human embryonic stem cells (hESC, APase staining Fig. 2A, H9 from NIH registry, NIH Code WA09) were differentiated via embryoid bodies to cells with neuronal morphology (V41: neuronal differentiated hES cells, Fig 2B), which were tested for neuronal survival upon a standard excitotoxic insult, i.e. application of 200 µM NMDA (Fig 2C). These conditions were similar to experimental protocols used for description of surrogate protein biomarkers in mESC models and produced a comparable effect on the viability and functional responses of neurons (Sommer et al., 2004), thus providing the prerequisite for investigating neuroprotective compounds and conditions.

On the molecular level, the maturation of neuronal derivatives from hESC precursors was confirmed by staining corresponding cell extracts with an antibody against the human form of the presynaptic marker protein synaptophysin (SYP, D-4, a synaptic vesicle marker). As shown in Figure 3, Western blots from 1-dimensional polyacrylamide gels of proteins from undifferentiated precursor cells (hESC prec) and neuronal differentiated derivatives (V41 diff), stained with a specific antibody against human synaptophysin, showed the strong emergence of this protein at the expected molecular mass of 38 kDa (lanes 9 and 10 in Fig. 3).

Calcium-driven excitotoxicity and mitochondrial apoptosis, which follows the opening of the mitochondrial permeability transition pore, contribute to essential pathomechanisms underlying most human neurodegenerative diseases (Schrattenholz and Soskic, 2006). Therefore, during development of new

lead structures for treatment of e.g. Alzheimer's disease or stroke, the testing of their efficacy with regard to these mechanisms can play a pivotal role. Moreover, as outlined in the scheme shown in Figure 4H, it is of advantage if a variety of converging pathomechanisms can be addressed with similar read-outs. Here, excitotoxic conditions (NMDA insult), which are considered to play a role in Alzheimer's and Parkinson's diseases, certain aspects of stroke, traumatic brain injury and amyotrophic lateral sclerosis (ALS); ischemia, which is a key event in stroke related disorders, and massive calcium-overload of neurons induced by brief applications of neurotoxic amyloidogenic peptides, like Aβ1-40 (relevant for Alzheimer-related mechanisms) (Cappai and White, 1999; Pereira et al., 2004), were all tested and quantified on a single cell level.

As an example, in Figure 4 A-D, consecutive Fura-2 measurements of intracellular calcium changes were monitored on the single cell level of hESC neurons. All cells responded to a moderate stimulus (pulse application of 10 µM glutamate, Fig. 4A) with clearly quantifiable calcium transients, which returned to steady state levels within seconds (not shown). Based on this viability control, the very same cells were subsequently challenged with an excitotoxic concentration of the glutamatergic agonist N-methyl-D-aspartate (200 µM NMDA for 10 min), a treatment which itself evokes calcium transients (Fig. 4B), but with different kinetic characteristics, leading to neurons that are no longer functional (Fig. 4C). Neurons that were not exposed to excitotoxic conditions or, if so, in the presence of neuroprotective agents, continued to respond to the viability control in a reproducible manner for up to several hours (Fig. 4D).

In Figure 4E, a phase contrast image of typical hESC neurons used for the functional experiments described above is shown (scale: E and G: 100 µM, F: 20 µM). As examples of mechanism-related read-outs, in Figure 4F the imaging of the opening of the mitochondrial transition pore (change of mitochondrial membrane potential) was quantified by a fluorescent assay employing Rhodamin-123 (Metzger et al., 2005) and in Figure 4G, a typical Fura-2 calcium image of neurons from hESC after exposure to 10 µM glutamate is shown as a measure of functional viability. (Fig. 4 A,C,D)

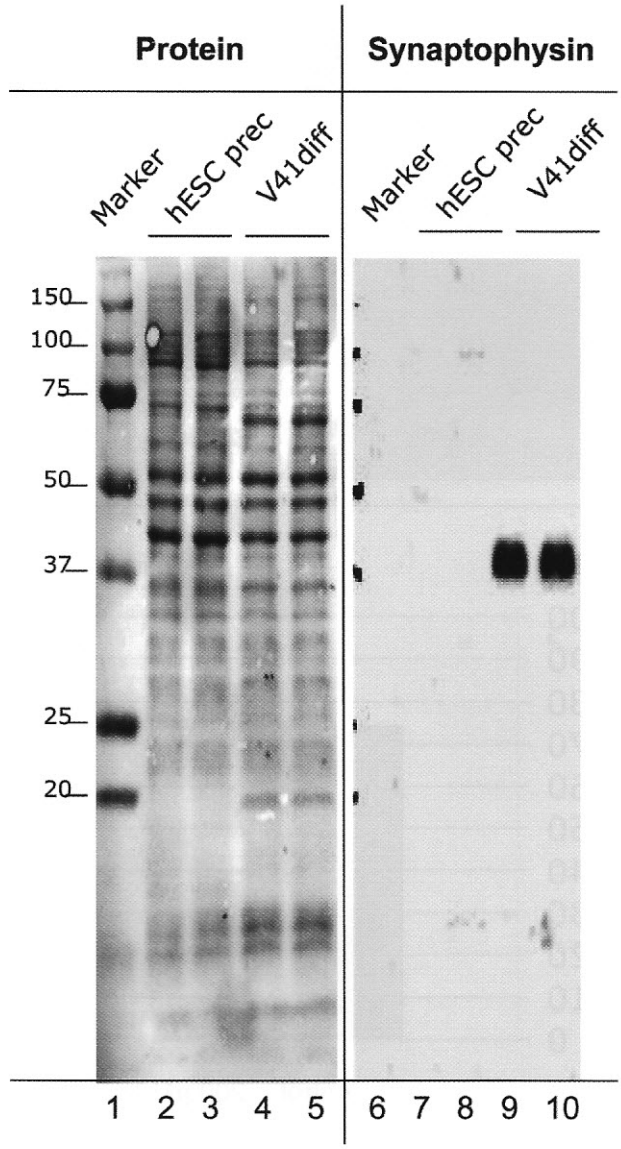

Fig. 3: Neuronal derivatives of hESC precursors expressed protein markers for mature neurons.
In extracts from cultured hESC-derived neural cells (as shown in Fig. 1B), the human form of the presynaptic marker protein synaptophysin (a synaptic vesicle marker) was detected by Western blot, thus demonstrating differentiation to a neuronal endpoint. Left panel, silver-stained 1-dimensional polyacrylamide gels of proteins from undifferentiated precursor cells (hESC prec) and neuronal differentiated derivatives (V41 diff) (lanes 2-5); lane 1, molecular mass standards. Right panel, Western blots of these samples (lanes 7-10) show a strong expression of the protein at the expected molecular mass of 38 kDa (lanes 9 and 10) exclusively in the cultures with cells of neural morphology and NMDA-responses (compare to Fig. 2B,C).

4 Discussion

Taken together, we could show that the human stem cell model represents crucial mechanistic aspects that are thought to play a major role in a variety of neurodegenerative diseases. At the moment,

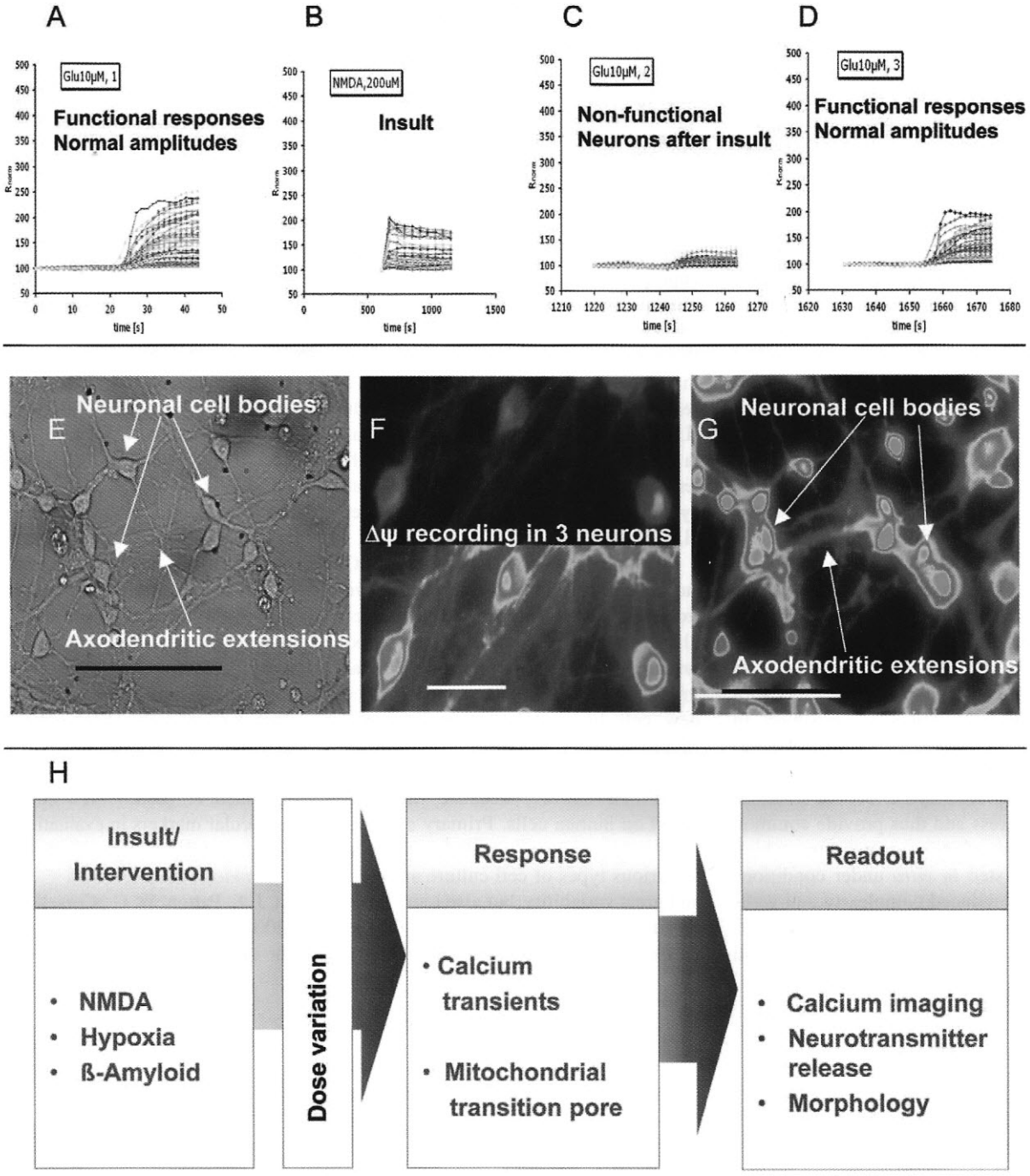

Fig. 4: Calcium-driven excitotoxicity and mitochondrial apoptosis, which follow the opening of the mitochondrial permeability transition pore, are connected to a variety of human pathomechanisms and were tested *in vitro* using neural hESC derivatives.
A, all viable cells responded to pulse applications of 10 µM glutamate. B, subsequent excitotoxic challenge (200 µM NMDA for 10 min). C, non-functional neurons after excitotoxic challenge. D, controls that were not exposed to excitotoxic conditions or, if so, in the presence of neuroprotective agents, still continued to respond to the viability control in a reproducible manner for up to several hours. E, phase contrast image of typical hESC neurons (scale: E and G: 100 µM, F: 20 µM). F, imaging of the opening of the mitochondrial transition pore is quantified by Rhodamin-123 fluorescence. G, typical Fura-2 calcium image of neurons from hESC after exposure to 10 µM glutamate as functional viability.
H, scheme summarising the sequence of functional insults and related read-outs, which can be easily extended to corresponding assays employing β-amyloid-related neurotoxic peptides or hypoxic conditions.

hESC and mESC models are employed in early preclinical phases of drug development and a parallel molecular exploration contributes significantly to understanding the underlying mechanisms, which in the future could help to replace animal tests in efficacy studies (Schrattenholz and Klemm, 2006).

Our work represents only one example among others and a first step en route to further and eventually humanised *in vitro* models (Kim et al., 2005; Huuskonen, 2005; Spielmann, 2005). The development of the full potential of these novel models requires a gradual incorporation of novel concepts concerning *in vitro* pharmacokinetics and bioavailability into related projects (Spielmann, 2005; Whitebread et al., 2005; Li et al., 2005; Hareng et al., 2005). The questions around systemic barriers, endocrine disruptors and metabolism, will certainly be exclusively addressed by *in vivo* models in the foreseeable future. But probably the most important aspect is to convincingly connect the molecular pathologies of various neurodegenerative diseases with corresponding molecular signatures in these *in vitro* models. Here it is noteworthy that various pathological processes converge on the level of calcium-related stress and thus provide common principles of neuroprotection, which can be tested *in vitro* under conditions of highly reduced complexity and well-defined functional read-outs. In particular, calcium-related excitotoxicity and the intrinsic mitochondrial apoptotic pathway, which are targeted by some of the most successful current treatments (Schrattenholz and Soskic, 2006), are directly accessible by fluorescent cell-based imaging technologies, as shown in Figure 4.

In addition, emphasis is on the post-translational protein information emerging from ESC models, which in connection with parallel investigations of appropriate animal models for certain human diseases could help to form the basis of a future replacement of some of these models. Stem cell technologies, maintenance and characterisation have reached certain international standards (Li et al., 2005; Moon et al., 2006; Plaia et al., 2005) suitable for validated alternative methods in toxicity and efficacy testing (Hareng et al., 2005; Seiler et al., 2004; Spielmann, 2005).

The purely diagnostic use of hESC suggested here not only offers attractive experimental advantages and, in particular, the major advantage of being based on human cells, but also avoids ethical implications around the generation of new cell lines and therapeutic risks. Moreover hESC-based *in vitro* models are superior to any type of primary neuronal cell culture, because they are based on human cells. Primary cultures from rodent embryos are not only prone to various types of cell culture artefacts and great variability, but also require the sacrifice of huge numbers of animals, or, more precisely, pregnant animals and embryos. Human neuroblastoma cell lines are not terribly popular, because they are too far off the native physiology, too variable and often too reduced in their repertoire of cellular responses (for a comprehensive overview see Bähr, 2004).

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