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Short Communication

Development and pharmacological modulation of embryonic stem cell-derived neuronal network activity

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Abstract

Embryonic stem cells can be differentiated into neurons of diverse neurotransmitter-specific phenotypes. While the time course of functional progression of ES cell-derived neural precursors towards mature neurons has been described in detail on single-cell level, the temporal development and pharmacological modulation of ES cell-derived neuronal network activity have not been explored yet. Neuronal network activity can be assessed by the microelectrode array (MEA) technology that allows simultaneous monitoring of the electrical activity exhibited by entire populations of neurons over several weeks or months *in vitro*.

We demonstrate here that ES cell-derived neural precursors cultured on MEAs for 5 to 6 weeks develop neuronal networks with oscillating and synchronous spike patterns via distinct states of activity and change electrophysiological characteristics even after 5 to 6 weeks in culture pointing towards late maturational processes. These processes were accompanied by an increasing density of presynaptic vesicles. Furthermore, we demonstrated that ES cell-derived network activity was sensitive to synaptically acting drugs indicating that pharmacologically susceptible neuronal networks were generated. Thus, the MEA technology represents a powerful tool to describe the temporal progression of stem cell-derived neural populations towards mature, functioning neuronal networks that can be applied to investigate pharmacologically active compounds.

Keywords: Embryonic stem cell; Neuron; Neuronal network; Multielectrode array; Embryonic stem cell derived neurons; Embryonic stem cell derived neuronal network

Embryonic stem (ES) cell-derived neurons open up new ways for the treatment of neurodegenerative diseases, e.g., by the substitution of lost neurons via cell therapy. For this purpose, ES cell-derived progeny needs to develop and to integrate into functional neuronal networks. Neuronal networks can be described as populations of synaptically interconnected neurons capable of generating electrophysiological activity that can spread spatially and temporally. Thus, neuronal networks represent the basic principle for brain activity.

In a multitude of *in vitro* and *in vivo* studies ES cell-derived neurons were characterized in detail concerning their specific genetic phenotype (Brüstle and McKay, 1996; Kim et al., 2002; Lee et al., 2000; Okabe et al., 1996). In addition, detailed electrophysiological investigations *in vitro* and *in vivo* after transplantation using the patch clamp method were performed to demonstrate on a single-cell level the basic electrophysiological properties of ES cell-derived neurons, e.g., the capacity to generate action potentials and form functional synapses (Bain et al., 1995; Bühnemann et al., in press; Finley et al., 1996; Jüngling et al., 2003; Strübing et al., 1995). Transplanted single ES cell-derived neurons were also documented to integrate into cortical and hippocampal neuronal networks and receive synaptic input (Benninger et al., 2003; Wernig et al., 2004). Very recently, it was reported that populations of ES cell-derived neurons can form an intrinsic functional neuronal network *in vitro* as assessed by the microelectrode array (MEA) technology (Ban et al., 2006). MEAs render possible a close contact between cultured cells and multiple electrodes, which detect action potentials via extracellular recordings. Combinations of MEAs and neurons are then referred to as bio-electronic hybrids or neurochips (Fromherz et al., 1991). In contrast to the patch clamp technique, MEA recordings are non-invasive and are capable of visualizing the spatial and temporal distribution of action potentials generated by entire neuronal populations. In the present study we use the MEA technology to describe the gradual immunocytochemical and functional development of ES cell-derived neuronal populations

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over several weeks *in vitro* towards synchronously oscillating neuronal networks and to determine their reactions to synaptically active substances.

An ES cell line constitutively expressing enhanced green fluorescent protein (EGFP) under the influence of the chicken β-actin promoter and the R1 cell line was used for this study (Dihné et al., 2006; Nagy et al., 1993). Maintenance of undifferentiated ES cells, embryoid body formation and selection of neural precursor cells were carried out as described (Lee et al., 2000; Okabe et al., 1996) with minor modifications. Briefly, undifferentiated (stage 1) ES cells were expanded in the presence of 1000 U/ml leukemia inhibitory factor (LIF; Chemicon, Temecula, CA). After formation of embryoid bodies (stage 2) in the absence of LIF, selection of neural precursors was initiated with Dulbecco's minimal essential medium (DMEM)/F12 supplemented with 5 μg/ml insulin, 50 μg/ml transferrin, 30 nM selenium chloride and 5 μg/ml fibronectin for 8 days (stage 3). Selected neural precursors were expanded (stage 4) in DMEM/F12 medium supplemented with 2% B-27, 2 mM L-glutamine, penicillin 5000 U/ml, streptomycin 5000 μg/ml (stage 4–medium) and FGF-2 (20 ng/ml, ProSpec.). Neural precursor cells in stage 4 were seeded either onto poly-L-ornithine-coated cell culture dishes for propagation or glass coverslips for immunocytochemical investigations at a density of 100,000 cells/ml medium. For the neurochip experiments stage 4 neural precursor cells were seeded onto poly-L-ornithine (15 μg/ml, Sigma)- and laminin (5 μg/ml, Sigma)-coated MEAs at a density of 15,000 cells/mm^2.

For immunocytochemical investigations, cultured cells were washed in phosphate-buffered saline (PBS), pH 7.3 and fixed for 15 min in 4% paraformaldehyde before incubation with 1% normal goat serum (NGS, Sigma) in PBS for 1 h. Primary antibodies were monoclonal mouse antibodies to β-tubulin III (1:750, R&D Systems), nestin (1:750, Chemicon) and, synaptophysin (1:100, Sigma). They were applied over night at 4 °C. After washing in PBS, appropriate secondary antibodies coupled to Cy2 or Cy3 (1:750, Dianova, Hamburg, Germany) were applied for 1 h at room temperature. Cell cultures were counterstained for 1 min with DAPI (2 μg/ml, Serva) to visualize cell nuclei. More than 95% of stage 4 neural precursor cells expressed nestin and the neural cell adhesion molecule (NCAM) and proliferated in the presence of FGF-2. Removal of FGF-2 5 days after seeding of ES cell-derived precursors led to the differentiation of stage 4 neural precursors into the three principal cell types of the central nervous system. This stage is referred to as stage 5 (Okabe et al., 1996). The composition of stages 4 and 5 neural cell populations has been characterized (Dihné et al., 2006).

MEA recordings and immunocytochemistry were performed weekly, commencing 7 days after initiating the differentiation. Attachment of ES cell-derived neural precursors on MEAs was similar in comparison to the attachment on culture dishes or glass coverslips. Also the temporal development of confluency and the morphology of single cells were similar under all cell culture conditions. To preserve re-usability of MEAs, immunocytochemistry was done only on glass coverslips as paraformaldehyde fixation would possibly cause corrosion of the electrodes. To measure electrophysiological activity of ES cell-derived neurons we used MEAs with a square grid of 60 planar Ti/TiN microelectrodes (30-μm diameter, 200-μm spacing) with an input impedance of <50 kΩ according to the specifications of the manufacturer (Multi Channel Systems, Reutlingen, Germany). Signals from all 60 electrodes were simultaneously sampled at 25 kHz, visualized and stored using the standard software MC_Rack provided by Multi Channel Systems (Reutlingen, Germany). Spike and burst detection was performed off-line by custom-built software (Result, Tönisvorst, Germany). Individually for each channel, the threshold for spike detection was set to eight standard deviations (SDs) of the average noise amplitude during a 10% "learning phase" at the beginning of each measurement. An absolute refractory period of 4 ms and a maximum spike width of 2 ms were imposed on the spike detection algorithm. All spike waveforms were stored separately and visually inspected for artefacts. The burst detection relied on an entropy-based algorithm. Minimum

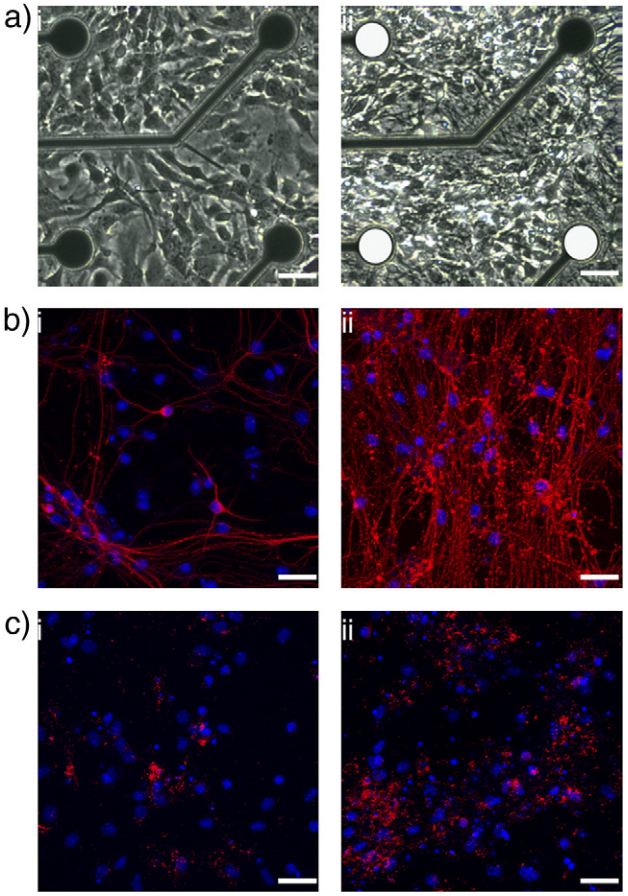

Fig. 1. ES cell-derived neurons at different time points after initiating the differentiation. Phase-contrast images (a) of ES cell-derived cells on MEAs and immunocytochemical stainings for (b) β-tubulin or (c) synaptophysin of ES cell-derived neurons on coverslips at 7 (i) or 28 days (ii) after initiating the differentiation. Note the dense network of neurites at 28 days after initiating the differentiation in comparison to 7 days (a, i and ii) correlating to increased numbers of β-tubulin$^+$ or synaptophysin$^+$ structures (b, c, i and ii). White-labeled electrodes indicate the appearance of spontaneous action potentials while black-labeled electrodes detected no action potentials (a, i and ii). Scale bar=30 μm.