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Review article

An Afterwards Analysis of Neural Culture States Using Neurofilament Immunocytochemistry

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Abstract

Various culturing and neurophysiological techniques are used to study neural or neuronal networks, often expressed by aggregates. The neural aggregates in chemically defined medium cultures tend to a stabilized amount of neurofilament positive cells and fibres after day 12 in vitro (12 DIV), with a minor difference of 1 DIV related to cell density plating. The total of positive cells and fibres is related to the diameter of the aggregates. The larger the aggregate diameter the higher the neurofilament positivity. This result opens the possibility to check afterwards the quality of cultures.

Keyword: Nervous system; mitochondria; multi electrode arrays; neurofilaments; neurodegenerative diseases

Definitions

Neural networks differ from neuronal networks: neural networks will contain neurons but also other nervous system cell types. Neuronal networks exist of only neurons. Most networks are made by dissociation of (a part of) the nervous system, thus including other types, among which glia (Figure 1). Bursts: "A burst is a train of closely timed action potentials (spikes). It is convenient to treat doublets (two spikes) and triplets (three spikes) as short bursts. The functional significance of generating a burst of spikes is: i) bursts are a prerequisite to enhance the reliability of the communication between neurons, and ii) burst can be an effective mechanism for selective communication between neurons" [1]. First reliable bursts were noted at 7 days in vitro (DIV) [2] (Figure 2). Multi electrode arrays are 20-80 titanium nitride electrodes on silicon substrate with a Si3N4 insulating glass cover layer. Placed in a square grid with inter-electrode distance of 100µm and electrode diameter of 10µm. Electrodes can be made of Pt, Au, TiN, IrOx or conductive polymers [3]. A glass or plastic ring changed it into a culture chamber (see MEA ring options, multi-channel systems, 2021) (Figure 3).

Neurofilament proteins are constructed from five neuronal intermediate filament proteins. Central base is a group of 310 amino acids with amino-and carboxyl ends. Heavy (200 kD), medium (150 kD) and light (70 kD) chain neurofilaments are discerned to which also belong alpha internexin and peripherin. The high and medium neurofilaments are characterized by a strong phosphorylation of the end groups. Neurofilaments, containing a diameter of 80-100 Å, supports neuronal growth of axons, axon and mitochondrial stability, and microtubule quantity. Assembly occurs intracellular and breakdown by the ubiquitin-proteasomal pathway [4]. Neurofilaments are related to various neurodegenerative diseases e.g.: Alzheimer's disease, ALS and Huntington's disease [5] (Figure 4). Impedance sensing. The study of the first 6DIV (144 hrs) has been

done with impedance sensing on electrodes for several substrate coatings (Figure 5). PEI overcomes the possibility to group into aggregates and electrodes stay occupied. Fibronectine and laminin supports the aggregation or do not inhibit aggregate formation. PLL inhibits aggregate formation till 100-120 hrs. Coating substances do manipulate the aggregate formation within the first 6DIV.

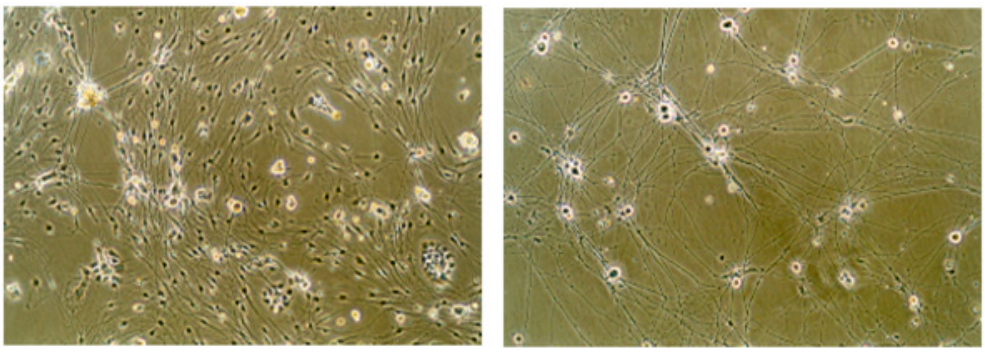

Figure 1: Cortex cultures not treated (neural, left) and treated for 8 DIV with Arabinose C (neuronal, right; Courtesy J. Buitenweg).

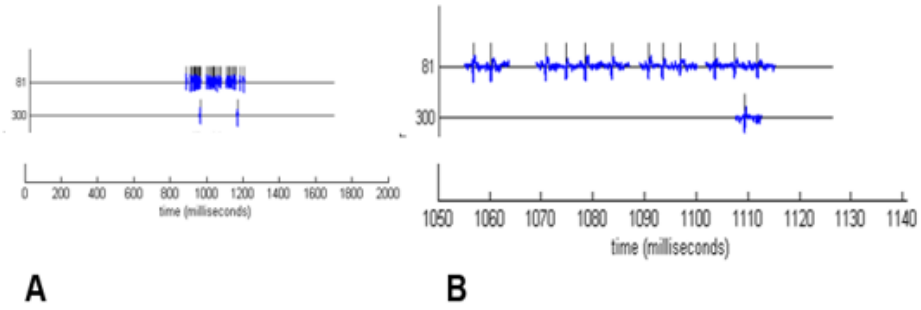

Figure 2: A: Trains of action potentials in a cortical culture from a multi electrode array. The recorded action potentials at electrodes 81 and 300 are shown. B: At electrode 81 four bursts, including a double and triplets can be detected. The 300 electrodes in both shows a single spike (Courtesy J. Stegenga).

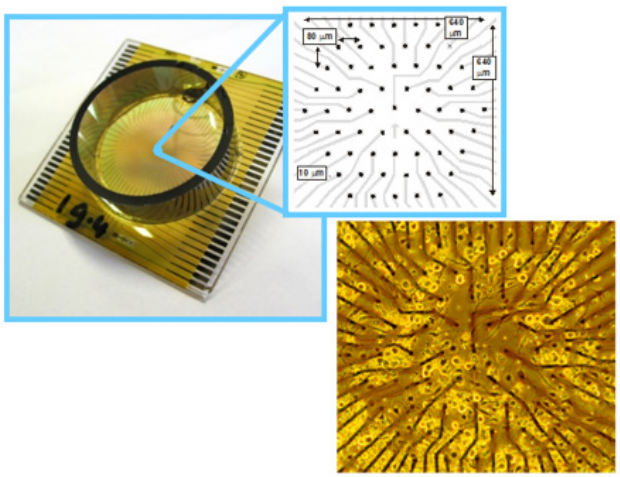

Figure 3: Multi electrode array (with dimensions and 61 hexagonally ordered electrodes) from the department Biomedical Signals and Systems, University Twente and covered with a culture of dorsal root ganglion cells.

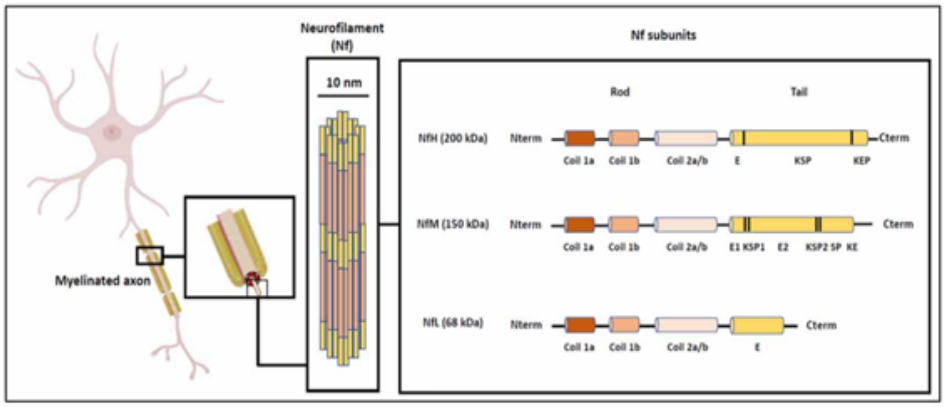

Figure 4: Neurofilament elements and structure assembly. N term amino end part, C term carboxyl end part (Delaby et al., 2022).

Introduction

In general, various types of neurons together with their glia are brought in culture depending on the parts of the (central) nervous system used, for instance: cortex (various articles, say) [6], spinal cord ganglia [7,8], arcuate nucleus [9] hippocampus [10] and suprachiasmatic neurons [11]. Culture fluids are either MEM- (Minimum Essential Medium) based, generally supplemented with D-glucose, NaHCO3, glutamine and antibiotics (habitually penicillin and streptomycin) to which is added (fetal calf) serum or DMEM (Dulbecco's Modified Eagle Medium) with analogue additions has been used. Different properties of culture media are out of the scope of this article. Serum-free media (e.g., now regularly produced by industries) [12,13] with or without added NGF (Nerve Growth Factor) are frequently used, surmounting serum impurities and its hormone changes with the advantage to hamper glia over-growth in neural cultures.

The Chemically Defined Media (CDM) without or with NGF let survive 5 to 10 times more neurons per surface unit respectively compared to MEM with or without NGF or chemically defined medium with added fetal calf serum (Figure 6). CDM importance for cultures of embryonic stem cells is widely accepted [14]. Production of neural precursors from cultured embryonic stem cells needs neural induction techniques [15]. Pure neuronal cultures are also made by mitotic suppression after 1DIV, mostly done by 1-b-arabinofuranosylcytosine (Ara C) for 3 to 4 days or longer (Figure 2) [16]. Purification of other cell types, especially of Schwann cells [17,18], is often a prerequisite for experimental research [19]. Dissociated dorsal root ganglion cells (DRG's) can regroup and reconstitute a dorsal root ganglion in neural cultures [20,21]. Unambiguousness for the use of the types of culture media does not exist.

An underground or coating is repellent for neurons if it is hydrophobic. In hydrophilic circumstances neurons, but also other CNS cell types, will adhere to the surface. Coatings are natural ones like fibronectin, laminin or non-naturals like poly-D (Figure 5) and poly-L lysine, poly-L-ornithine, and they normally induce aggregates. Poly-Ethylene-Imine (PEI) is strongly hydrophilic provoking a nearly monolayer of neurons. Such a "random" distributed neural monolayer allows the creation of planar structures. Islands with hydrophilic (PEI) surfaces surrounded by hydrophobic (fluorocarbon) underground show that island-distances of over 100 mu cannot be crossed by neuronal neurites [22]. Monolayer neural cultures that self-organize into spontaneously bursting networks are in contrast with micro patterned cultures, by which the structural component as present in the central nervous system is mirrored. In general, the neuronal axons have the tendency to grow straight on, however the printed pattern on the bottom of the culture disk forces axons to follow these patterns.

Turning behavior can be statistically predicted. In structured neuronal networks neurons synapse with each other, neurotransmitters are produced and spontaneous electrical activity develops as in non-structured ones. Structured neuronal networks demonstrate enhanced recordable networks, enhanced cellular activity, increase in neuronal activity, greater survival of neurons, accelerated synaptogenesis, presumably by the five times higher presence of glia [23-26]. Detecting the state of neural networks during development by bursts has frequently been applied (Figure 2) [27]. The global state of networks has been done using time inter-falls between bursts, duration and number of its action potentials, and its recruitment rate. Seal enhancement effects are unknown.

Single neurons show: the thinner the sealing gap between neuron and electrode the higher the potential that is measured. Sealing resistance is studied using impedance spectroscopy. Combination with intracellular recording and stimulation contributes to the further understanding of the neuron-electrode interface [28,29]. Impedance sensing in combination with microscopy has been used to study cell-cell and cell-substrate adhesion adding N-CAM protein and its antibody to the neural cultures [30-32]. Still the uncertainty of culture connectivity development and its final out-grown state

interfere often with coating application, bursting studies and applied drug results, also due to using different methods and various brain areas, also impeding comparison of results. In short, a general measuring method of the state of the neural culture could be helpful. This article proposes a simple histochemical afterwards analysis method to overcome this doubt.

Material and methods

Cell cultures were prepared by anaesthetizing pregnant WAG-rats with aether and the foetuses (E19) were rinsed shortly with 70% ethanol. Under sterile conditions, the cortices were removed from the foetal brains and dissociated by mechanical disruption with a nearly sealed Pasteur pipette. Cell suspensions were centrifugated in chemically defined medium three times (5 min, 1000 rpm) to remove cell debris. One drop (0.07-0.08 ml) of high cell density (10^6 per cm^2) or low cell density (10^5 cells per cm^2) was gently plated on a glass disk coated with poly-D-lysine and placed in a Petri disc. Adherence of cells was allowed for one hour, after which 1-1.5 ml medium per dish was added. Chemical defined medium was used with or without 3.85* 10^{-4} mM nerve growth factor. Cultures were refreshed each two days and stored at 37° C in a 96-100% humidified and 5% CO_2 containing atmosphere.

Immunocytochemistry (examples in Figure 7, cortex peroxidase and Figure 8, SCN fluorescence): cultures were rinsed twice with saline and fixated with acetone, 1 hour, 4° C, and overnight dried. Twice disks were rinsed with PBS and once with PBS 0.1% BSA, each for 5 min. Cultures were incubated with primary antibody NF90 (1:10.000). NF 90 has the ability to recognize also the 70kD subunit, which is the first to appear. Next day cultures were rinsed 3x PBS, 1x PBS0.1%BSA and incubated with HRP-conjugated rabbit-ant-mouse antiserum (1:400) for 1 hour, room temperature. Both antibodies were diluted in PBS 0.1% BSA and 1% normal goat serum. After the second incubation cultures were 3x rinsed in PBS and in 50mM Tris-maliate buffer pH 7.6 and incubated for 20 min with 0.04% 3.3-diaminobenzidine-4HCl and 0.02% H_2O_2 dissolved in the same buffer. For controls of non-specific reactions and endogenous peroxidase the same procedure was performed without first antibody.

Cultures were counterstained with haematoxylin, differentiated in tap water, dehydrated through graded alcohols to xylene and covered with Depex while placed on object glasses. Quantification of neurofilament positive cell bodies and positive fibres was assessed in one culture per day for finally 12 and 31 DIV. Counts were performed in 10 reaggregates per culture with a low or high cell density. Cell bodies were counted positive if staining was as brown as in 12 DIV cultures from a previous normal series. Within one culture both cells and fibres were counted: reaggregates with low cell density (d ±100 µm N=10 per DIV) and with a high cell density (d ±150-200 µm, N=10 per DIV) were screened and the same aggregates were used to count positive nerve connections and the total of connections. Counting was done independently by two persons. A separate series was produced for 21 and 31 DIV for 150-200µm/ high density aggregates.

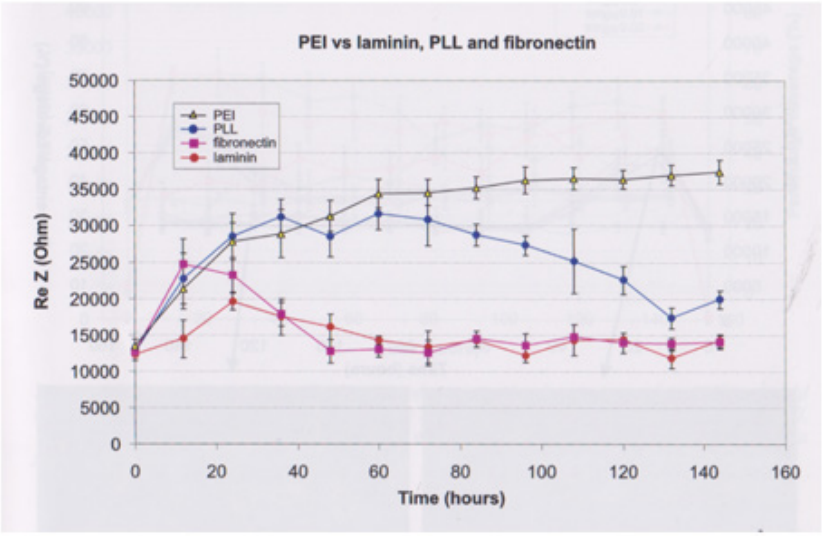

Figure 5: The real impedance during development of neuronal cultures on top of 7850 µm2 gold electrodes measured at a frequency of 10 kHz. Comparison of polyethylene imine (PEI), laminin, poly-L-lysine (PLL) and fibronectin as substrate coatings. (N=6; courtesy Wiertz, 2010 a).

Results

Reaggregates of dissociated fetal (E19) cortical rat neurons showed interconnections by bundles of protrusions (Figure 7). The bundles make synaptic contact within and outside the aggregates, notice the re-looping bundle (Figures 7&8). Within an aggregate interconnection are encountered and dendrites are present. Boutons en-passage and terminal ones are recognized by their

varicosity-like appearance (Figure 8) [33]. Positive bundles can be followed into the centre of the few negative aggregates. Within connecting bundles thick and thin neurofilament positive fibres are noted. After the first 5-DIV in culture aggregates start being formed and the first accumulates are interconnected.

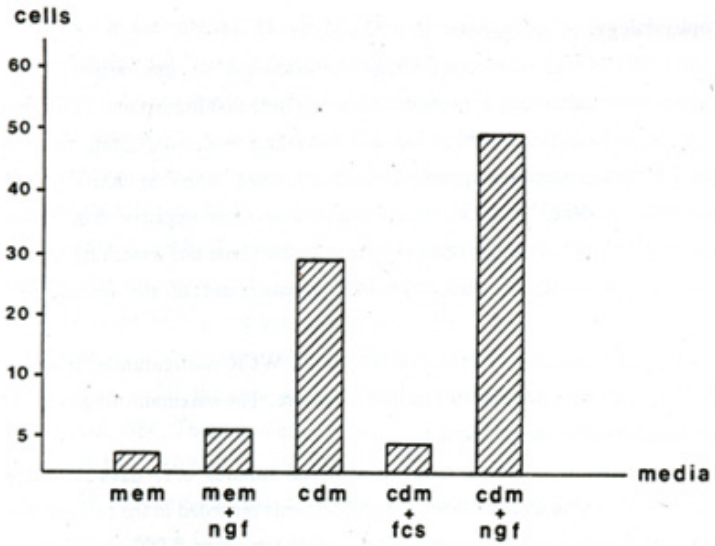

Figure 6: Surviving hypothalamic arcuate neurons per surface unit using different culture media: mem: minimum essential medium; ngf: nerve growth factor; cdm: chemically defined medium; fcs: fetal calf serum (Marani et al., 1988).

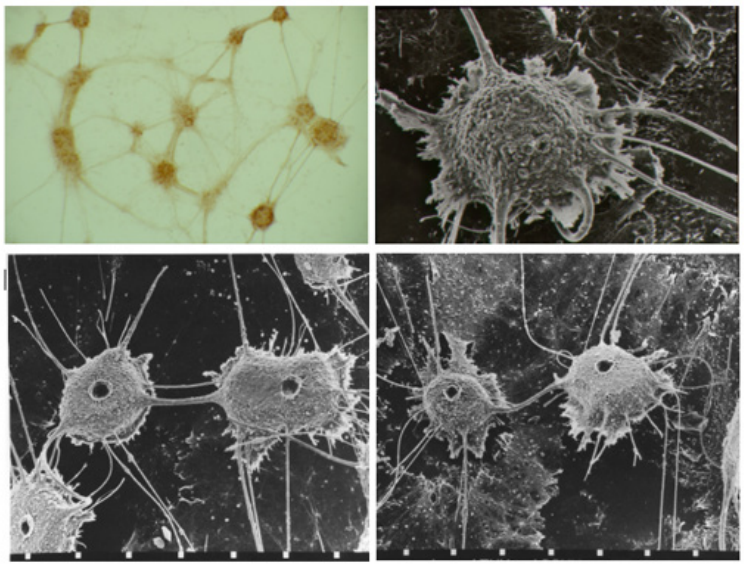

Figure 7: Upper left: Light microscopy of cortical culture with aggregates on poly-D-lysine stained for phosphorylated neurofilament (31 DIV). Upper right: SEM of one aggregate. Lower photographs: SEM overview of aggregates with ground layer of poly-D-lysine. Top of aggregates vacuum imploded due to scanning EM technique. Note aggregates with connective bundles.

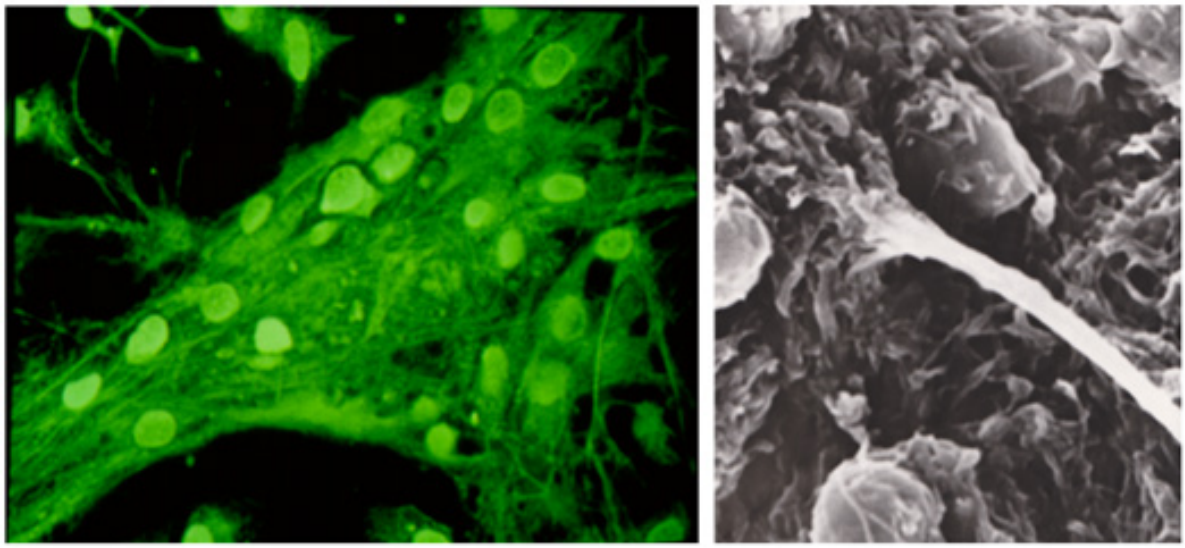

Figure 8: Left: One out of a series of confocal microscopical images of a fluorescent stained aggregate of the Suprachiasmatic Nucleus (SCN) with antibodies recognizing H, M, L kD phosphorylated neurofilament fixed after 14 DIV. Note the positive fibre tangle concentration in the centre of the aggregate and its positive outside neurons (Marani et al., 2003). Right: Connective small bundle (± 1µm) contacting cell protrusions of a cortex aggregate.

The extensive networks developed by joined axons and dendrites and they supported cell migration, especially near the centres of the cultures. Control procedure for immunoreactivity was negative at 9-DIV and non-specific precipitates were not found. Until 6-DIV aggregates stayed negative with a very light brownish diffuse overall staining. At day 7 in 150-200 µm aggregates and at 8-DIV in 100 µm aggregates the first NF positive perikarya were present in 100 µm ones in a single peripheral islet, containing the low cell density. From 9-DIV on NF positive fibres were observed. Gradually at older DIV's positivity increased in the centres of well-developed aggregates (Tables 1 & 2).

Table 1: Overview of neurofilament positive cells in aggregates of 150-200 µm and 100 µm per DIV.

Neurofilament positive cells within 10 aggregates of 150-200 µm per DIV	
DIV 1-6	0
DIV 7	18
DIV 8	16
DIV 9	13
DIV 10	18
DIV 11	20
DIV 12	24
DIV 21	25
DIV 31	27

Neurofilament positive cells within 10 aggregates of ± 100 µm per DIV		
DIV 1-7	0	106.6± 14.4
DIV 8	1	99.5±13.9
DIV 9	0	95.5±12.7
DIV 10	7,5	93.0±30.8
DIV 11	8	102.0±26.0
DIV 12	13,5	100.3±13.7

Table 2: Number of total bundles larger than 2.5 µm leaving aggregates and neurofilament positive nerve fibres per aggregate and per 100 nerve bundles, included bundles < 2.5 µm, till DIV 12 for aggregates of ± 100µm (diameter and SD).

Total number of Nerve	Bundles > 2.5 µm	Number Positive Nerve Fibers	Per 100 Nerve Bundles	Mean diameter (µm) aggregates n=10 +SD
DIV 9	76.5	0	0	96.9 ± 13.6
DIV 10	69.5	6	8.6	105.7 ± 19.9
DIV 11	48.5	13	26.8	107.9 ± 13.5
DIV 12	80.5	52.5	65.2	115.0 ± 15.0

Discussion

Neurofilaments are cytoskeletal proteins involved in: microtubule organisation, nerve conduction, neurotransmission and organelle dynamics. The neurofilament genetics presented NF importance in neurodegenerative diseases. NF-L is needed for assembly of the higher kD neurofilament proteins and is studied in relation to degenerative diseases. Developmental control in vivo of the NF-L has a yet an unknown biological meaning [34]. Neuronal cultures do play an important role in these neurofilament studies. Cell density in culture starts influencing the structural end situation: high densities, 10^6 per cm^2, compared to low cell density, 10^5 cells per cm^2, produces in general ± 40-50% more bundles and ± 50% more positive cells and a ± 30% higher number of positive fibres. After 12 DIV large aggregates (150-200µm) show a nearly equal number of positive fibres up to the measured 31 DIV.

Smaller aggregates (100 µm) show ± 50 % less positive cells at day 12 DIV and are considered fully outgrown at 12 DIV [35]. Expression of neurofilament in cells is earlier than the expression in fibre bundles. A delay of 1 DIV is noted. Dissociated neurons need a recovery period in the chemically defined medium of around 7 DIV. This delay phase compares to the delay of neurofilament expression in the rat neural tube. As in the neural tube the expression of neurofilament is first in cells (motoneurons) and later in the spinal cord tracts [36,37]. Heterogeneity in vivo for neurofilament is a property of the developmental and mature rat spinal cord and is refound in the aggregates containing positive and negative elements for neurofilaments. The increase of synapses coincides with neurofilament increase in positive areas.

Checks and Balances

Checking the quality of the cultures afterwards supports reliable results:

- Control cultures are produced at 12 DIV in the same medium, coating, type of neural/neuronal cells and kept under the same culturing conditions.
- Perform neurofilament immunocytochemistry.
- Count positive neural/neuronal cells at several aggregates by choice small (100µm) or large (150-200µm) and fibres >2,5 µm.
- Use measured normal results in comparison to the experimental results by its neurofilament immunocytochemistry after experiments.
- Strong differences are or due to the experiments or to critical culture situations.

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