Studies on neural differentiation and monitoring with novel biosensor tools

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1 ABSTRACT

Neuronal differentiation is a very complex and sophisticated cellular process that encompasses the development of mature neurons and their specialization. The present work will focus on novel and less frequently cited aspects of neuronal differentiation. After extensive use of classical techniques to elucidate the signal transduction pathways leading to differentiation of N2a neuroblastoma cells, the development and application of a three dimensional tissue-like culture combined with novel biosensoring techniques was addressed. Hence, the first part of the results presented in this thesis will focus on the elucidation of basic aspects of N2a neuroblastoma differentiation, specifically its relation to mitochondrial biogenesis as a downstream consequence of a hormetic response triggered by a moderate superoxide production by the mitochondria and the related signalling cascades. Reactive oxygen species (ROS) have been widely considered as harmful for cell development and as promoters of cell aging by increasing oxidative stress. However, ROS have an important role in cell signalling and they have been demonstrated to be beneficial by triggering hormetic signals, which could protect the organism from later insults. In the present study, we demonstrated that mitochondrial biogenesis and differentiation are mediated by superoxide and MAPK cues, which is accompanied by an increased expression of the peroxisome proliferator-activated receptor gamma (PPARγ) coactivator 1-alpha (PGC-1α). Our data suggest that differentiation and mitochondrial biogenesis in N2a cells are part of a hormetic response which is triggered by a modest increase of superoxide anion concentration within the mitochondria.

The second part of the results will focus on the development of a three dimensional model for neural culture and its combination with novel applications for potentially useful biosensors on the study of neural differentiation. Firstly, a basic bactoagar gel matrix combined with a first pre-prototype impedance biosensor were used as a proof of principle of the ability of this combination to measure electric signals from 3D cultures. Secondly, the impact of gel composition, cell density and selected differentiation factors on differentiation and viability were analysed in order to optimize cell culture conditions for differentiation in three-dimensional matrices. Experimental results revealed that home-made gel matrices based on collagen-laminin mixtures in contact with serum free medium enable increased neural differentiation over other commercial gel matrices. Therefore, collagen-laminin gels appear as a suitable three dimensional model for drug screening in developmental neurobiology. Following optimization of the immobilization process, a second optimized preprototype of impedimetric biosensor and electrical impedance spectroscopy technique were applied to monitor the differentiation process of cells embedded in collagen-laminin gels by means of changes in the dielectric and conductive properties.
Zusammenfassung


Der zweite Teil der Ergebnisse präsentiert die Entwicklung eines dreidimensionalen Modells für neuronale Zellkulturen und seine Kombination mit neuartigen Anwendungen für potenziell nützliche Biosensoren für die Untersuchung von neuronaler Differenzierung. Zuerst wurde eine einfache Bactoagar-Gelmatrix, die mit einem ersten Prototypen eines Impedanzbiosensors verbunden wurde, benutzt, um einen Machbarkeitsbeweis für die Fähigkeit dieser Kombination zu liefern, elektrische Signale von 3D Kulturen zu messen. Im zweiten Schritt wurden die Folgen unterschiedlicher Gelzusammensetzung, Zelldichte und ausgewählten Unterscheidungsfaktoren auf Differenzierung und Lebensfähigkeit analysiert, um die Bedingungen der Zellkulturen für Differenzierung in dreidimensionalen Matrizen zu optimieren. Diese Versuchsergebnisse zeigten auf, dass selbsthergestellte Gelmatrizen basierend auf Kollagen-Laminin Mischungen, die in Kontakt mit einem serum-freien Medium standen, eine neuronale Differenzierung aktivierten.
Folglich stellen sich Kollagen-Laminin Gelmatrizen als ein geeignetes dreidimensionales Modell für Medikamentenscreening in der Entwicklungsneurobiologie heraus. Nachdem der Immobilisierungsprozess verbessert wurde, wurde ein zweiter und optimierter Prototyp eines impedimetrischen Biosensors und eine elektrische Impedanzspektroskopietechnik angewandt, um den Differenzierungsprozess der in Kollagen-Laminin Gel eingeschlossenen Zellen mittels Veränderungen der Impedanz, online zu überwachen.
2  INTRODUCTION

During the development of the central nervous system (CNS), hundreds of different specific neuronal subtypes are generated in an organized and coordinated manner. One of the main goals in developmental neuroscience is the understanding of the mechanisms that underlie these processes, not only for the discovery and control of particular pathways but also as a tool for the development of active treatments to treat neurodegenerative diseases (Briscoe and Novitch 2008).

2.1  Cellular models in developmental neurobiology

Models are necessary for every research activity, in which the effect of natural variability on experimental observations must be reduced; so that there are as few degrees of freedom as possible and the only variable is the one that the researcher wants to induce in the system. In vitro models, essentially cell lines, have been widely used to elucidate the basic mechanisms that underlie neural development, including neurite outgrowth, since these models can recapitulate cellular events of neural development (Radio and Mundy 2008).

Among the available cell models for neuronal differentiation, a distinct group of cell lines has been adopted by the scientific community, probably because they are an endless source of a considerably large number of cells and because each cell line comes from the same clone, which means a high reproducibility of the cell line-specific traits (Radio and Mundy 2008). Cultured neuroblastoma cells are commonly used for the screening of drugs which potentially alter neuronal growth, they are simple and easy to culture, cheap and available, and they are genetically modifiable. Moreover, neuroblastoma is the most common cancer of the children’s nervous system, so that any progress in understanding the physiology of these cells has a direct medical relevance (Connolly 2001).

Primary cultures have also been used as models of neuronal differentiation (Radio and Mundy 2008). Although their growth patterns in vitro resemble closely a physiological developmental process, the complexity of obtaining the cells from an in vivo source, the difficulty of obtaining pure neuronal cultures, the intercultural variability and the ethical issues concerning the use of animals for research places primary cell cultures below the golden standard in developmental neurophysiology.
Another possibility is the use of stem cells that will differentiate to mature neurons. For example, stem cells have shown their ability to differentiate in response to different stimuli such as hypoxia (Pacary, Tixier et al. 2007). Stem cells can be split and induced to differentiate in the appropriate stage of their growth cycle. Furthermore, neuronal networks developed from these stem cells have been used as sensing elements in cell-based biosensors (O'Shaughnessy, Liu et al. 2009). The use of stem cell proliferation as a source of cells allows the performance of a high number of experiments, without the need to sacrifice animals for every experiment, whereas their differentiation into neurons allows the performance of experiments on neurons, which have proved to be similar to ones obtained from primary cultures. Although such experiments are very promising, the ethical concerns around stem cell research, the possible variability between passages and between individuals are facts to take in account. Depending on the subject of study, the benefits of using cell lines can outweigh their potential disadvantages (Connolly 2001).

Some experiments on neural differentiation have demonstrated that it is possible to differentiate cell lines like PC12 when appropriate stimuli are applied to them. PC12 are known to differentiate to neurons after exposure to NGF. PC12 stimulated with NGF is a very extended model of neural differentiation (Read, Herbert et al. 2008). The reason for this is the ease of the visualization of the changes produced in these spherical cells when they differentiate to neurons and produce axons and dendrites (Kathleen Baxter, Uittenbogaard et al. 2009). Other stimuli have demonstrated to induce differentiation in PC12 cells like db-cAMP (De Girolamo, Hargreaves et al. 2001; Ng, Wu et al. 2009), PACAP (Pisegna and Oh; Wang, Qi et al. 2005; McIlvain, Baudy et al. 2006; Ravni, Bourgault et al. 2006), forskolin (Bogacka, Ukropcova et al. 2005; Slaughter and Hobson 2009), estrogens (Merot, Ferriere et al. 2005), hypoxia (Pacary, Tixier et al. 2007; Gutsaeva, Carraway et al. 2008), heat-shock (Read, Herbert et al. 2008) and even microwave irradiation (Inoue, Motod et al. 2008).

The P19 embryonic carcinoma cell type has been used as a differentiation model due to the fact that cells are pluripotent, being able to differentiate in vitro into a wide range of cellular types including neurons after the addition of all-trans retinoic acid. They resemble the behaviour of stem cells (Watkins, Basu et al. 2008).

However, other cell lines i.e. neuroblastoma cell lines have been used for the same purpose, with murine Neuro2a (N2a), human SK-N-SH and SH-SY5Y being the most common among them (Radio and Mundy 2008). N2a cells are known to develop a neuronal differentiated phenotype if exposed to db-cAMP or retinoic acid (Wu, Vaswani et al. 1990). The SH-SY5Y neuroblastoma cell line differentiates to a cholinergic phenotype in the presence of retinoic acid (Miloso, Villa et al. 2004), or a dopaminergic phenotype in response to phorbol esters (Xie, Hu et al. 2010), etc.
Some of the cell lines differ in their responses to some stimuli. For example, N2a cells differentiate in response to db-cAMP and serum deprivation (Flaskos, McLean et al. 1998; De Girolamo, Hargreaves et al. 2001; De Girolamo and Billett 2006). Human neuroblastoma and P19 cells do differentiate in response to retinoic acid while serum deprivation is an apoptotic stimulus for them (Watkins, Basu et al. 2008). Different agents can promote differential gene expression in the same cell line and exhibit synergistic effects on neurite outgrowth (Ng, Wu et al. 2009). It should be also pointed out that the same agents can cause the opposite effect in different types of neurons (Mattson, Taylorhunter et al. 1988), causing either differentiation or death depending on the cell line (Kuenzi, Kiefer et al. 2008).

Therefore, it is important to consider cell lines just as models, which will have limitations when it comes to predicting what the physiological response to a compound will be. However, cell lines preserve the basic cellular physiological mechanisms, allowing researchers to create an endless source of material for pharmacological screening. A very detailed and extensive description of the different models used for the detection of differentiation can be found in the review by Radio et al (Radio and Mundy 2008), as well as references for stimuli depending on the cell line and substances that modify their differentiation. Researchers in the field of neurobiology should take into account the advantages and disadvantages of each cellular model.

2.2 N2a neuroblastoma cell line

For this study the N2a neuroblastoma cell line was selected for its ease of use as an endless source of reproducible material for experimentation without using living animals and as a model that recapitulates the basic features of differentiation when proper stimuli are applied (Figure 1). In addition neuroblastoma is the most common childhood cancer. Hence, the results obtained could be applied to research on pharmacological targets to treat this kind of cancer. The N2a neuroblastoma clone was established from a spontaneous tumor of a strain A albino mouse by R.J. Klebe and F.H. Ruddle. They have neuronal and amoeboid stem cell morphology and grow continuously as a monolayer (ATCC information sheet). Although N2a neuroblastoma are in a rather undifferentiated state, they present some neuronal features: they present a cholinergic phenotype (Gomez, Boutou et al. 1998), they express acetylcholinesterase enzyme (ATCC information sheet) and voltage gated calcium channels (Gaasch, Geldenhuys et al. 2007). N2a murine neuroblastoma cells are a well known model of neuronal differentiation in response to retinoic acid, gangliosides (Wu, Vaswani et al. 1990), db-cAMP and serum starvation (Flaskos, McLean et al. 1998; Radio and Mundy 2008). In the presence of db-cAMP N2a cells differentiate to dopamine neurons (Tremblay, Sikorska et al. 2010). db-cAMP is an analog of cAMP and it is able to subsequently activate the transcription of genes related to cell differentiation and survival through CREB phosphorylation (Tremblay, Sikorska et al. 2010). Forskolin is an activator of
adenylate cyclase, which increases cAMP levels, which in turn induces the differentiation of neural cells in a CREB-dependent pathway (Fernandes, Sun et al. 2007). Forskolin has also been shown to induce C3G, a nucleotide exchange factor involved in actin reorganization during differentiation (Radha, Rajanna et al. 2008) and to induce mitochondrial biogenesis in other tissues like adipocytes (Bogacka, Ukropcova et al. 2005).

Figure 1. Microscopy pictures of N2a cells in undifferentiated (a) and differentiated (b) state.

2.3 Mitochondrial biogenesis

Mitochondrial biogenesis is the process via which cells increase their individual mitochondrial mass. It requires the interaction of the nuclear (nDNA) and the mitochondrial (mitDNA) genome. MitDNA encodes 13 respiratory chain protein subunits and 24 RNA components. The majority of the mitochondrial proteins are encoded by nuclear DNA and are subsequently translocated to the mitochondria (Onyango, Lu et al. 2010) (Figure 2). This cross talk is still poorly understood but there are some nuclear proteins that have been studied in this context in other tissue models. Among them, the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is considered the master regulator of mitochondrial biogenesis (Scarpulla 2002). An increase in the expression of this coactivator has been related to increased mitochondrial mass in various tissues under differentiation such as skeletal muscle (Wu, Puigserver et al. 1999), in adipocytes (Bogacka, Ukropcova et al. 2005), and in pheochromocytoma (Uittenbogaard, Baxter et al. 2010). Mitochondria are essential organelles for cell survival. In fact, some degenerative diseases are related to a loss of function in mitochondria (Onyango, Lu et al. 2010; Pacelli, De Rasmo et al. 2011), and thus triggering of mitochondrial biogenesis has been suggested as a new strategy to treat neurodegenerative diseases (McGill and Beal 2006; Thomas and Beal 2010).
2.4 Differentiation, mitochondrial biogenesis and free radicals

There are some very well known pathways of neuronal differentiation such as the pathway followed by retinoic acid through retinoic acid nuclear receptors (RARs) (Lovat, Lowis et al. 1994), NGF through TrkA receptors or PACAP through PAC1 receptors (Vaudry, Stork et al. 2002). However, other mechanisms seem to be involved in the differentiation process, although they are not so often referred in the literature. Among them, the link between mitochondrial biogenesis, free radicals and differentiation is a main focus of this study.

Nitric oxide (NO) is a volatile free radical with two contradictory roles in developmental neurobiology. On the one hand, a dysregulation of NO homeostasis by an aberrant increase of this diffusible small molecule promotes neural cell apoptosis. On the other hand, small increases in the NO levels (through the cGMP pathway) have been reported in cells under differentiation (Contestabile and Ciani 2004). The regulation of transcription factors is a key mechanism in the effect of NO on neuronal functions. Expression of large number of genes is altered upon neuron exposure to NO, mainly through the activation or inhibition of transcription factors such as CREB, n-Myc, c-fos, c-jun, or zinc-finger transcription factors in a cGMP-dependent way or through nitrosative conformational modification of proteins (Contestabile 2008). A very elegant study on eNOS- and nNOS-deficient mice demonstrated the correlation between the nNOS activity, which is responsible for NO production, and mitochondrial biogenesis through the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1alpha (PGC-1α), the nuclear respiratory factor 1 (NRF-1) and Tfam under hypoxia conditions (Gutsaeva, Carraway et al. 2008). Actually, PGC-1α seems to be the master regulator of mitochondrial biogenesis, acting as a co-activator with NRF-1, NRF-2 and Tfam. It can be activated by NO, HIF1α/β or AMPK.
(Onyango, Lu et al. 2010). Activation of PGC-1α has been found in cells from other tissues under differentiation. Concretely, pharmacological activation of the cAMP or PPAR pathway has been observed to induce differentiation and promote PGC-1α dependent mitochondrial biogenesis in adipocytes (Bogacka, Ukropcova et al. 2005).

PGC-1α is a transcriptional coactivator, which transduces several physiological stimuli into specific metabolic programs, most frequently by stimulation of mitochondrial activity. The activation of mitochondrial biogenesis by PGC-1α depends on the coactivation of ERRα, NRF-1, and NRF-2. For an extensive review on nuclear activators of mitochondrial biogenesis it is recommended to refer to Scarpulla (Scarpulla 2002). PGC-1α promoter contains some highly conservative consensus cis-elements that are critical for its transcription. MEF2 and CRE binding sites are better studied. MEF2 is responsible for the autofeedback regulation of the PGC-1α transcription while CRE can increase transcription in response to cAMP and p38 MAPK pathways (Liang, Yang et al. 2010). PGC-1α and PGC-1β can be induced by ROS and regulate a complex ROS defence system, being part of the ROS homeostatic cycle. In particular they induce SOD1, SOD2 and catalase among others. PGC-1α acts in a dual manner both stimulating mitochondrial electron transport and suppressing ROS levels, thus serving as an adaptive set-point regulator, which provides a precise balance between metabolic requirements and cytotoxic protection from ROS (St-Pierre, Drori et al. 2006).

ROS can also act as second messengers in the differentiation process. ROS have been found to mediate another differentiation mechanism promoted by semaphorin A. The suppression of mitochondrial free radicals by a non-apoptotic rotenone concentration or N-acetyl-L cysteine abolished its differentiating effect (Schwamborn, Fiore et al. 2004). Mitochondria are the major cellular producer of reactive oxygen species (ROS) (St-Pierre, Drori et al. 2006). ROS have been considered as deleterious to cell survival. However, in recent years the role of ROS as signalling molecules has taken weight within the scientific community. Overproduction of ROS results in oxidative stress, which is related to damage of structures and cell death, whereas moderate concentration of ROS is related to beneficial effects, such as defence against later oxidative insults or the maintenance of redox homeostasis by activating a number of signalling pathways (Valko, Leibfritz et al. 2007). Moderate concentrations of ROS have been found to increase life span whereas antioxidants can prevent these ROS from activating life extending-signalling cues (Yang and Hekimi 2010). Interestingly, stimuli that promote life extension have also been related to a promotion of mitochondrial biogenesis (Ristow and Schmeisser 2011).

Some natural substances have been demonstrated to affect mitochondrial biogenesis. Quercetin, a natural flavonoid, known for its antioxidant and anti-inflammatory effect, has been recently demonstrated to use PGC-1α to increase the mitochondrial mass in muscle and brain cells and
increase exercise tolerance in mice (Davis, Murphy et al. 2009). Resveratrol, another natural flavonoid, has been demonstrated to promote mitochondrial biogenesis, differentiation of N2a cells and to be neuroprotective, through the activation of AMPK (Dasgupta and Milbrandt 2007). Forskolin, which is also a natural compound from the tropical plant *Plectranthus barbatus*, also known as *Coleus forskohlii* (Alasbahi and Melzig 2010), has also demonstrated its ability to induce mitochondrial biogenesis in other cell models such as adipocytes (Bogacka, Ukropcova et al. 2005).

However, there is evidence that mitochondrial biogenesis is not only activated by PGC-1α. It was found that ischemia can induce rapid mitochondrial biogenesis in a PGC-1α-independent mechanism, in which the number of mtDNA copies was increased (Yin, Signore et al. 2008). Mitochondrial biogenesis, paradoxically, has also been found in cells under apoptosis. Actually, some of the mechanisms related to differentiation and apoptosis seem to be shared. Aberrant mitochondrial biogenesis and enhanced oxidative stress have been observed in a neuroblastoma cell line after the induction of apoptosis by methamphetamine. The use of vitamin E as antioxidant was able to reverse those effects (Wu, Ping et al. 2007).

Nevertheless, some questions remain open: is mitochondrial biogenesis necessarily concomitant to differentiation? Is it promoted by the same agents through the same signalling pathways? Which are these pathways? Do free radicals play a role in mitochondrial biogenesis and differentiation? If they do, are they cause or effect? (Figure 3)

Figure 3. Interconnection between free radicals, mitochondrial, biogenesis and differentiation. The study of the signaling pathways that interconnect them is a main focus of this study.
2.5 Tissue-like model. Why design a three dimensional model?

Classically, neuronal differentiation has been studied based on cellular models, neuroblastoma or pheochromocytoma cell lines. Those models have the advantage of being an endless source of biological material and resemble neuronal differentiation in response to certain stimuli. The N2a neuroblastoma cell line is a well known model of neural differentiation in response to dibutyryl cyclic AMP (db-cAMP). However, classical studies on cell lines have a limited prediction power due to the fact that cells are cultured in vitro in a two dimensional (2D) environment, which differs drastically from the physiological reality. Three dimensional (3D) models can better resemble 3D physiological structures, where cells can better express their features. It is known that cells in 2D cultures express different genes compared to their 3D counterparts and tissue (Birgersdotter et al. 2005; Li et al. 2007; Wang et al. 2009). This could be the reason of the low efficiency index of drugs that can reach the bed side. Before a new drug is released to market a huge research period is required for preclinical and clinical studies. During preclinical studies, drugs are first tested in cellular models, then in ex vivo tissues, and finally in vivo. Usually cellular models are useful for a first screening in which cytotoxicity and potential of the drug is addressed. However, after this first screening, almost none of the drugs that look very promising are effective when in vivo experiments are performed. If we specifically consider new drugs developed for the treatment of neurodegenerative diseases, the number that finally make it to market, and really are able to ameliorate symptoms is extremely low. That means that the models currently in use are limited when it comes to predicting what actual responses in real organisms will be. Usually cellular models consist of cell lines which are seeded into flasks and maintained in two dimensional layers with nutrient medium. Those cell lines are assumed to reflect the essential physiology of real tissues but actually the environment to which they are subjected is totally different. Tissue architecture, mechanical cues and cell communication are impaired because of the two dimensional structure. It is known that cell to cell and cell to environment interactions are essential for tissue morphogenesis in developing embryos (Pampaloni, Reynaud et al. 2007), which is a three dimensional environment. In this respect new more accurate models are needed, which better resemble physiological reality. Models in which the phenotype of the target tissue is reproduced could give rise to more reliable results. Thus three dimensional models of cultured cells could potentially better mimic physiological conditions. These models used as an intermediate screening step between conventional cell cultures and animal research would result in the reduction of the number of animals used for experimental purposes. Thus, an alternative method to animal research as considered by Russel and Burch in the late fifties, and accomplishing one of the three R’s described by the authors, replacement (L.F.M. Van Zupten 2001). Replacement refers to the substitution of living animals by in vitro techniques that yield the same result without the use of living animals. 3D models, as an intermediate step between
classical 2D cell cultures and animal experimentation can bridge this gap (Pampaloni et al. 2007), thus leading not only to a higher probability of successful drug development but also to a reduction of the number of animals sacrificed for experimental purposes and to a reduction of the related costs. Efforts in this direction have been made in the search for cell culture analogs (CCAs), which resemble the physiological response of the whole body. In these models, different compartments with different tissue-like structures are perfused with a specific substance and the response of the different tissues after the circulation of the drug through other compartments is monitored. Although their development phase is still preliminary, CCAs hold big potential in the fields of toxicology, pharmacokinetics and drug screening (Park and Shuler 2003).

Although 3D models appear as physiologically more relevant, their use is limited due to the fact that not many techniques are adapted to the third dimension. A three dimensional tissue-like model should also resemble the architecture found in physiological tissue (Cullen, Lessing et al. 2007), where neurons are differentiated, emit axons and dendrites in the three space directions and connect between each other, transmitting signals through neurotransmitters. Choosing the right cellular model and the right differentiation stimuli is essential for the development of a tissue-like model from which physiologically relevant data can be obtained. In the present study, the development of a 3D matrix for cell immobilization able to sustain N2a differentiation was addressed (Figure 4), with the aim of obtaining a 3D cellular model able to better resemble physiological reality.

Figure 4. Microscope pictures of differentiated N2a cells immobilized in a collagen-laminin gel matrix (developed in this thesis)
2.6 Cell immobilization

The physical confinement of intact cells to a defined region of space whose activity is preserved is referred to as cell immobilization (Willaert Ronnie and Baron Gino 1996). The immobilized cell system can be divided into three components: Cells, supporting material and interstitial solution that fills the remaining space. Cell immobilization can be done by attaching cells to a porous surface or by entrapping cells within a porous matrix. The ideal immobilization system should accomplish the following properties: have a high cell mass capacity, allow access to nutrients, be biocompatible (both the matrix and the procedure), present a high surface to volume ratio, allow diffusion, be chemically and mechanically stable, reusable, economical, easy to separate and suitable for the desired applications. Although finding an ideal system is not feasible, a compromise can be found between these parameters (Willaert Ronnie and Baron Gino 1996).

Immobilised cell systems can be classified into four categories: self aggregation, barrier containment, surface attachment and porous matrix entrapment. Self aggregation can be inherent to the cell type or facilitated by linking agents such as poly-L-lysine. One example is the use of multicellular spheroids (Kloss, Fischer et al. 2008). Barrier containment includes synthetic membranes, micro capsules and two phase systems. In the next sections surface attachment and porous matrix entrapment will be described in detail, since the selection of one suitable immobilization substrate is a main (crucial) step for the development of a 3D cellular model.

2.6.1 Surface attachment

Surface attachment is the most common way to immobilize cells in a culture. Adherent cells grow on a flat surface in the form of a monolayer either spontaneously or with the help of a coating material (Willaert and Baron 1996).

However, it is possible to control the specific spatial distribution of the cells. Micropatterning of substrates allows us to choose the exact position where cells attach. For example, by patterning neuronal cells within a poly(dimethylsiloxane) (PDMS) hexagonal array of adhesion nodes it is possible to standardize the distance between neighbouring cellular nodes. Neurite outgrowths connect the cellular nodes and develop an axonal network in which the length of the neurite interconnections is standardized. This simple approach can reduce the effort for high throughput screening (Frimat, Sisnaiske et al.). Other micropatterning techniques have been used for neuroblastoma cell attachment like inkjet printing i.e. printing a polyethyleneimine (PEI) solution onto an albumin substrate using a modified commercial inkjet printer (Yamazoe and Tanabe 2009). E-beam lithography on biocompatible hydrogels allowed the generation of micropatterns in which PC12 cells could differentiate and extend their neurites throughout the channels. This approach makes it possible to control the number, shape and length of the neurites (Dos Reis,
Migration, outgrowth and differentiation of human stem cells could be observed thanks to the creation of a micropatterned poly-L-lysine squared surface. Microstructured PDMS surfaces were used as stamps for the printing of Petri dishes with poly-L-lysine (Ruiz, Buzanska et al. 2008). Advances in the field of micropatterning give us the advantage of seeding the cells only on the desired sites, and also give us a chance to explore the differentiation of single neurons and synapse formation depending on their spatial distribution (Liu, Coulombe et al. 2000; Angela, Lars et al. 2003). By micropatterning it could be observed that there are important parameters in stem cell differentiation like colony size and embryoid body size that are usually neglected. Those parameters are easily modifiable by this technique (Bauwens, Peerani et al. 2008).

Depending on the substrate used, micropatterning can directly influence the degree of differentiation. For example, micropatterned polystyrene was observed to positively influence the differentiation of adult hippocampal progenitor cells (Oh, Recknor et al. 2009). Thus micropatterning is a very useful tool in developmental neuroscience, in that it makes possible to guide neuronal differentiation by directing cell protrusion outgrowth, to monitor developmental processes and to explore the influence of different cell arrangements and culture conditions on cell interactions and developmental processes (Buzanska, Ruiz et al. 2009). Unfortunately, these techniques do not currently allow the design of a 3D model. Nevertheless, efforts made in this direction point out micropatterning as a very promising technique for the creation of three dimensional scaffolds for cell entrapment, with respect to nutrient diffusion and control of tissue orientation (Papenburg, Vogelaar et al. 2007).

2.6.2 Immobilization in gels.

Mammalian cells can be immobilized in polymer matrices to form a three-dimensional (3D) culture, which is much more similar to a physiological tissue (Kintzios, Pistola et al. 2001). It consists of a porous support that is formed around cells and maintains cell viability and activity. Different shapes and sizes of the cell-matrix mixture can be achieved, thus adapting to any container shape (Willaert and Baron 1996).

For the immobilization of mammalian cells in three-dimensional matrices a huge variety of carriers have been used as supporting material. Both the polymer and the entrapment procedure are required to be non toxic. These materials can be divided depending on their origin in natural and synthetic polymers (Willaert and Baron 1996). Synthetic polymers have been successfully employed for example on the maintenance of murine embryonic stem cells in three dimensional polyethylene terephthalate in an undifferentiated state (Ouyang, Ng et al. 2007). However, natural polymers present some advantages like their biodegradability and biocompatibility as they are
natural products. The immobilization of living cells within natural polymers is considered a mild technique, with which the damage to living cells can be minimized. Between them, some of the most commonly used are carrageenans, alginites, agar and agarose, cellulose and derivates, pectins, gelatine, polyacrylamide, as well as extracellular matrix (ECM) components such as laminin, fibronectin, collagen or basement membrane extracts obtained from animals (Willaert and Baron 1996). Most of them have been already used in the laboratory of Prof. Kintzios for the entrapment of mammalian cells. A brief description of the natural polymers and other additives used in this study follows.

Algal polysaccharides are extracted from algae and are widely used as immobilization agents (Willaert and Baron 1996). The Agar Bacteriological (Bactoagar) is a solidifying agent prepared by mixing different agar from several sources and is recommended for gelling culture media where great transparency and brightness is required (Gross, Kartalov et al. 2007). The gelation point of bactoagar depends on the concentration, being around 37 ºC for concentrations between 0.8% and 3%. This makes bactoagar a suitable natural polymer for three dimensional entrapment of living mammalian cells. Agarose, as a purified preparation of agar, is responsible for the gelation of agar when the temperature decreases below its gelling point. The mechanism of gelation involves the shift from a coil solution to the formation of double helixes, that confer a rigid structure and pores large enough for the penetration of proteins (Willaert and Baron 1996).

Alginate gels have been extensively studied in Prof. Kintzios’ laboratory for the entrapment of mammalian cells for biosensoring techniques (Kintzios, Yiakoumetis et al. 2007). Alginate gels have been demonstrated to preserve the membrane properties of neuroblastoma cells as far as voltage dependent ionic channels and calcium dynamics are concerned (Willaert and Baron 1996; Kintzios, Yiakoumetis et al. 2007). The gelation process consists of mixing the cell suspension with a sodium alginate solution and dripping this solution in a multivalent cation solution to form gel spheres. Other approaches include the entrapment of cells in alginate microcapsules, which in turn are encapsulated in a macrocapsule, which allows better diffusion of nutrients (Zhang, Xie et al. 2009). However, preliminary experiments form the laboratory of Prof. Kintzios showed that they do not allow neural differentiation.

Extracellular matrix (ECM) is a three dimensional structure that surrounds the cells and provides the necessary substrate to support intercellular communication via integrin receptors (Desai, Kisaalita et al. 2006). ECM molecules are differentially expressed in the developing embryo and the adult. These differentially expressed components play a role in the signalling pathways that lead to differentiation, synaptogenesis and axonal guidance, whereas the complex three dimensional structure of ECM components provides the appropriate physical cues to support cell growth and integration. For example, laminin was suggested to regulate neuronal migration.
Collagen fibers provide the cells with structural support for the development of neurites (Krewson, Chung et al. 1994; Desai, Kisaalita et al. 2006), whereas differential expression of VGCC was found in differentiating neuroblastoma cells in 3D collagen gels in comparison with 2D cultures (Desai, Kisaalita et al. 2006). Collagen is a fibrous hydrophilic protein with a triple-stranded helical structure that swells in the presence of water. Cell immobilization in collagen gels is performed at low temperatures, gelation is achieved by raising the pH and temperature, whereas cells and collagen form multiple ionic interactions such as hydrogen bonds and van der Waals forces (Willaert and Baron 1996). Laminin is a multi-adhesive matrix protein, which is intercalated and interacts with collagen, other extracellular matrix components (ECM) and cell-surface integrins. Sometimes it is found as a protein network of variable heterodimers (Manaster 2008). Cell immobilization in laminin gels shares the same gelation protocol with collagen gels, so it is possible to combine laminin and collagen to form a 3D matrix. Basement membrane extracts (BME) is an extract of the specialized extracellular matrix that form an interface between cells. In the case of the trade mark used in this study, Engelbreth-Holm-Swarm (EHS) tumor cells are used as a source of BME, which consists of laminin I, type IV collagen, entactin, and heparan sulfate proteoglycan. The differential combination of two or more ECM components and their concentration has proved to modify gel matrix properties and thus facilitate or hinder the progression of differentiation features (Krewson, Chung et al. 1994). Furthermore, the combination of agarose with ECM components has also been demonstrated to support differentiation (Krewson, Chung et al. 1994).

HEPES can be used in 3D matrices as a pH buffer to control the cell culture conditions when the maintenance of pH is not assured, resulting in an improvement of cell viability. HEPES or 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid is a zwitterion buffer, originally designed to avoid the use of CO$_2$ for buffering culture media, because CO$_2$ presents inherent pH fluctuation during in vitro cell culture. In contrast, HEPES presents a stronger buffering capacity at physiological pH, and its use is justified when it is not possible to maintain pH through the bicarbonate/carbon dioxide buffer, for example in very acidic environments or in experiments outside an incubator (Brown, MacLellan et al. 2008), which will be required for some of the experiments in this study.

### 2.7 Techniques to detect differentiation

After an overview of the classical techniques generally used to measure differentiation, this work will focus in novel biosensing techniques that could revolutionize the field of developmental neurobiology. Among them electrical impedance spectroscopy (EIS) will be discussed more deeply because of its usefulness in the study of three dimensional cell cultures.
2.7.1 Conventional techniques to measure differentiation

Conventional techniques to measure differentiation can be classified as semi-quantitative (or non-calibrated), quantitative (or calibrated) and biochemical. Quantitative and semi-quantitative techniques are based on cell morphology i.e. if the cells are presenting neurites (Radio and Mundy 2008). Multiple parameters can be measured such as the number of cells and neurites, the number of neurites per cell, the identification of axons and neurites, the number of cells measured per optical field, the number of fields measured per culture well, number of secondary tertiary neurites, neurite length, soma area, etc (Connolly 2001).

To increase contrast for clearer imaging, one option is cell fixation and staining, for instance with Coomassie brilliant blue (Sachana, Flaskos et al. 2001) (Figure 5), crystal violet, Ramón y Cajal's silver-nitrate staining technique or antibodies for later immunohistochemistry or flow cytometry analysis. Although fixation has some disadvantages such as the possible cell shrinkage, it allows the staining with antibodies for the detection of biochemical markers, allows the storage of samples, the imaging of fine neurites and their tips and allows the collection of data from a huge amount of samples at the same time point (Connolly 2001).

![Figure 5. Microscope pictures of N2a cells: (a) living unstained cells and (b) fixed and Coomassie brilliant blue-stained cells.](image)

Biochemical techniques are based on the detection of some proteins that are overexpressed or some genes that undergo a higher transcription only when the cell enters the differentiation process. For example, neurofilaments or Tau can be detected by immunoblotting or immunocytochemistry with the help of a fluorescence microscope (Radio and Mundy 2008) or mRNAs encoding proteins of interest detected by RT-PCR. Other very useful technique for fluorescence detection is flow cytometry, which not only allows detection of any parameter that is stained with a fluorescent dye or fluorescent antibodies at a single cell level (Watkins, Basu et al. 2008), but also makes cell cycle analysis possible (Georgopoulou, Hurel et al. 2006). During differentiation, the cell cycle must change as far as cells stop growing and enter the differentiation process. Usually, cancer cells as neuroblastoma or PC12 are continuously dividing, thus their cell
cycle would be that of dividing cells. When differentiation starts, division gradually stops and cells differentiate to a concrete cell type. Cell cycle arrest and differentiation have a fateful relationship (Tabata, Yamazaki et al. 2009). They are highly coordinated and interactive processes, governed by cell cycle genes and transcription factors, which will decide the neural cell's fate (Cremisi, Philpott et al. 2003; Pacary, Tixier et al. 2007). Withdrawal from the cell cycle accompanied by a reorganization of the cytoskeleton and an up-regulation of mitochondrial proteins was found in a quantitative proteomic analysis (Watkins, Basu et al. 2008). The complex interplay between cyclins, CDKs, cyclin kinase inhibitors, transcription factors, etc. are responsible for the expression of lineage-specific genes and thus will control the neural cell's fate (Galderisi, Jori et al. 2003). Detection of cell cycle proteins has also been used for the description of neuronal differentiation (Watkins, Basu et al. 2008). The increase or decrease of some cyclins indicates in which phase of the cell cycle the cells are, i.e. if they exit the cell cycle and differentiate. Those proteins can be detected by immunoblotting, by immunocytochemistry, or even their mRNAs detected by RT-PCR (Georgopoulou, Hurel et al. 2006). Moreover, new technical approaches have been developed to detect the biogenesis of mtDNA material in vitro (Lentz, Edwards et al.)

Morphological studies can be automated with a camera and software that detects and counts neurites (Ramm, Alexandrov et al. 2003) or can be done manually by the researcher. Without automation high throughput screening is not a possibility. Therefore, a big step in developmental neuroscience has been the development of cameras and software adapted to the necessities of this discipline. With the aid of these tools, the same work can be done in much less time while the researcher can program the software, interpret the results and continue her/his research in a time-efficient manner. A practical review of the techniques that may be used for cell imaging, including tips to choose the appropriate software and charge-coupled device cameras at an affordable price for two- and three-dimensional imaging, as well as tips to get successful images and choose the appropriate model of study, was made by Connolly et al. (Connolly 2001).

In the field of three-dimensional imaging, confocal microscopy rises as the most extended technique able to deal with problems associated with 3D structures. Other, less common techniques are revised by Pampaloni et al. (Pampaloni, Reynaud et al. 2007). Among them two-photon and multiphoton microscopy, optical coherence tomography, optical projection tomography, confocal theta fluorescence microscopy, stimulated emission depletion fluorescence microscopy or single plane illumination microscopy and light-sheet-based microscopes (Keller, Pampaloni et al. 2006) seem to be the most promising for 3D culture analysis.

Although considerable improvement has been done in terms of automation and resolution, classical techniques are still the researchers' favourite choice, even though they depend on their
subjective appreciation, require bulky and expensive equipment and are not suitable for high throughput screening.

2.7.2 Novel biosensors and advanced techniques

It is possible to improve the cost- and time-efficiency of neuronal differentiation studies. Novel biosensors and advanced techniques offer a wide spectrum of possibilities. Microelectrode arrays (MEAs), the Bioelectric Recognition Assay (BERA) and impedance analysis offer the possibility of non-invasive on-line monitoring of the electrical behavior of electrogenical cells, while cell immobilization and microfluidics permit the manipulation of the microenvironment around the cell, thus allowing a deeper study of physiological processes and allowing the spatio-temporal modulation of the application of stimuli. The use of those advanced techniques as complementary tools for the study of neural differentiation could revolutionize this field. A description of the most suitable candidate techniques follows.

2.7.2.1 Microelectrode arrays (MEAs)

MEAs are planar substrates in which an array of microelectrodes is embedded and are able to measure extracellular action potentials from electrogenic cells and tissues (Kang, Lee et al. 2009). In contrast to the classical patch-clamp technique, which allows only single cell measurements and is quite expensive and time-consuming, MEAs offer the possibility of performing electrophysiological recordings in whole neuronal networks, thus making it possible to monitor intercellular signalling. Another advantage is the possibility to perform on-line and non-invasive electrical measurements for long time periods, for example during the differentiation process. It even offers the chance to monitor electrical activity of subcellular domains such as axons (Dworak and Wheeler 2009). MEAs appear as a non-invasive technique that has been successfully used in the study of neuronal synaptic plasticity, long term potentiation on acute slices and the development and regeneration of organotypic co-cultures (Hofmann and Bading 2006).

MEAs are suitable for tissue studies such as detection of spontaneous excitatory spike firing on slices (Ma, Weston et al. 2008). The creation of a CMOS-based microelectrode system with more than 11,011 electrodes opens the possibility of performing extracellular electrophysiological recordings in brain slices and to simultaneously detect 126 selected recording sites on one slice (Frey, Egert et al. 2009). The addition of three-dimensional tip-shaped electrodes (Dimaki, Vazquez et al.) on a MEA instead of the planar ones provides the advantage of allowing deeper insertion of electrodes into tissue slices or 3D structures and the detection of larger signals (Kopanitsa, O Afinowi et al. 2006)
The electrical response of a whole neural network can be detected by single cell extracellular recordings with MEAs. The temporal network activity progression from stem cells to a mature neuronal network has been studied with the help of MEAs over the course of several weeks, due to the non-invasive nature of the technique. Synchronization of spikes during natural development, the effect of neuroactive drugs on developed networks (Illes, Fleischer et al. 2007), as well as the effect of different trophic factors on stem cell differentiation were studied (O’Shaughnessy, Liu et al. 2009). A micropatterning strategy with poly-D-lysine on the surface of an MEA showed the electrical activity of neurons forming a reproducible triangular axonic network, which was maintained for several weeks. The main advantage of this strategy is the appropriate adhesion of neurons to the electrodes, giving rise to a better signal to noise ratio (Jungblut, Knoll et al. 2009). By developing an array of coated microwells single neurons can attach to single wells and form networks in an ordered manner, thus facilitating statistical analysis and increasing throughput of the MEA (Kang, Lee et al. 2009). The selection of the appropriate coating substrate is of huge importance, since different coatings can give rise to different degrees of fasciculation and thus different electrophysiological activity (Dworak and Wheeler 2009). Moreover, it was possible to detect neuron to electrode contacts (hybrid synapses) on the surface of a MEA, the morphological and functional characteristics of a growth cone, and measure the impedance of the contact area (Bieberich and Guiseppi-Elie 2004). The avoidance of coating material was achieved by using carbon nanotube-based electrode arrays. Carbon nanotubes were assembled into islands with extremely rough surfaces, which play the double role of selectively anchoring the neurons and facilitating a precise stimulation and recording of extracellular membrane potential (Shein, Greenbaum et al. 2009). Carbon nanotubes can easily be used as coating material for metal electrodes. They have been demonstrated to reduce noise, to have a high biocompatibility and to improve the electrode-electrolyte interface (Gabriel, Gomez et al. 2009).

Electrical stimulation and directed local electroporation of concrete axons have been achieved by using a MEA in combination with thickened microelectrodes with vertical sidewalls separated by the appropriate distance to host an axon (Chang and Sretavan 2009). By combining patch clamp detection of intracellular membrane potential and the application of voltage pulses through the electrodes of a MEA, it was observed that it is possible to controllably electroporate neurons with the underlying electrodes of the MEA, which could be very useful for transfection purposes. Moreover, local stimulation of a subcellular fraction and the subsequent cellular signal spreading was observed by combining MEA with fluorescence monitoring of intracellular calcium (Braeken, Huys et al.).

Of course the possibility of acquiring simultaneous recordings makes MEAs a time- and cost-efficient solution for neuronal studies (Braeken, Huys et al.). Moreover, fabrication costs can be reduced to a minimum by simplifying fabrication procedures (Xiang, Pan et al. 2007), materials
(Morales, Riss et al. 2008) and increasing the quantity of devices produced. All these facts make MEAs a very promising technique for the study of neural differentiation. However, the study of three dimensional cellular networks as a whole is still not achievable with this technique, since MEAs are usually based on planar substrates.

2.7.2.2 Bioelectric recognition assay (BERA)

Most of the techniques currently used for the optical and electrophysiological analysis of experiments are designed for two dimensional cultures, which adds extra difficulty to the design of three dimensional models. For example, mere visual observation of cells on a light microscope becomes tedious as the cells are situated in different planes of focus. Regarding electrophysiological recordings, single cell methods such as patch clamp can address the three dimensional challenge by clamping one cell inside a defined tissue, but measuring the response of a whole cluster of cells or a tissue is something that this technique cannot achieve. Most of the current biosensor approaches are based on the contact of a layer of cells to the electrode surface (Opp, Wafula et al. 2009), which makes the use of a three dimensional model impossible. The laboratory of Prof. Kintzios is a pioneer in the adaptation of electrophysiological methods to the study of three dimensional cellular structures:

The Bioelectric Recognition Assay (BERA), developed at the Agricultural University of Athens by Kintzios et al. is a biosensor technique based on gel embedded cells(Kintzios, Pistola et al. 2001). The immobilization within gel matrices preserves cell physiological functions. Responses of stimulated cells are measured with micro electrodes by means of changes in their surface/membrane potential. A typical BERA sensor consists of a measurement and a reference micro electrode, one in contact with immobilized cells (Moschopoulou and Kintzios 2006). The analyte in contact with the cell/gel matrix induces to a characteristic, signature-like change in electrical potential. BERA is a passive recording method which consists on the detection of those signals without the application of any external current. The overall impedance between the measuring electrode and the reference electrode influences the voltage that is recorded by the BERA system (Kintzios, Pistola et al. 2001).

The BERA technique has been improved since its origin in terms of miniaturization, characterization of cell clusters (Moschopoulou and Kintzios 2006) and spread of applications (Moschopoulou, Vitsa et al. 2008; Perdikaris, Vassilakos et al. 2011), already giving rise to six generations of biosensors. The BERA technique has been used for the detection of a wide range of substances and organisms: It was able to detect human viruses like Hepatitis B and C and herpes viruses and plant viruses in tobacco and cucumber (Kintzios, Bem et al. 2004); it was applied for the detection of pesticides and herbicides (Flampouri, Mavrikou et al. 2010) and for the
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Detection of superoxide free radicals (Moschopoulou and Kintzios 2006). Furthermore, artificial neural networks have been designed for data analysis with BERA, demonstrating that these networks can be trained to recognize plant viruses according to biosensors’ responses (Frossyniotis, Anthopoulos et al. 2006). This technique has been and is currently being used in the laboratory of Prof. Kintzios to detect cellular responses of several cell lines, including N2a cells (Flampouri, Mavrikou et al. 2010) and membrane-engineered Vero cells (Moschopoulou and Kintzios 2006) by other students, being the subject of their PhD studies. Therefore, this technique was not employed in the present study.

2.7.2.3 Electrical impedance spectroscopy

As discussed before, most of the techniques currently used for the analysis of electrophysiological cell responses are designed for 2D cultures. The field of biosensing in three dimensional matrices is still poorly developed. Another electrophysiological technique with proved efficacy in adapting to the third dimension is Electrical impedance spectroscopy (EIS). EIS is just a specific branch of the tree of electrical measurements. EIS is a well established technique for the analysis of liquid or solid ionic and dielectric materials by means of a conventional lab-scale or miniaturized impedance analyzer (Barsoukov and Macdonald 2005; Schroeder et al. 2004). It is a powerful method of characterizing many of the electrical properties of materials and their interfaces with electrically conductive electrodes. It involves a relatively simple electrical measurement that can be readily automated and whose results may often be correlated with many complex material variables (mass transport, rates of chemical reactions, corrosion, etc).

Impedance spectroscopy has been used extensively to investigate membrane behaviour in living cells (Barsoukov and Macdonald 2005). Furthermore, it represents an established analytical method to monitor cell biological processes in 2D cultures, e.g. the spreading of adherent cells to artificial surfaces (Wegener et al. 2000) and virally induced cell death (Campbell et al. 2007). Electrical impedance spectroscopy of two dimension cell cultures represents the current state of the art for monitoring adherent two dimensional cell cultures by means of planar electrodes (Lisdat and Schafer 2008). For example, this technique is used to analyse the kinetics of wound healing (Keese et al. 2004). In basic neurodevelopmental research impedance biosensors have emerged as a very powerful tool for the on-line detection of cellular properties such as shape, attachment, differentiation and cell death (Kloss, Fischer et al. 2008). EIS has already been proved to be able to detect differentiation of PC12 cells in response to Nerve Growth Factor (NGF), dexamethasone and forskolin in 2D (Slaughter and Hobson 2009). EIS has also been demonstrated to be a useful tool for the study of differentiation since it is able to distinguish between different electrical characteristics depending on the differentiation degree of the cells (Dalmay, Cheray et al.). In this context, EIS is better known as electric cell substrate impedance sensing (ECIS). Impedance
analysis has also been proved to be a powerful tool for 3D applications. EIS has already been applied to the determination of cell toxicity, growth and membrane integrity in 3D (Kyle et al. 1999), cell growth in 3D matrices (Lin et al. 2009) and the effect of drugs on spheroids (Kloss, Fischer et al. 2008). Implantable impedance sensors record single neuron spikes and refractory times (Mercanzini, Cheung et al. 2008) and they can detect the reactivity of the brain tissue where they are inserted. The more cells attach to the electrode the bigger the magnitude of the impedance (Williams, Hippensteel et al. 2007). An alginate 3D cellular model developed for the simulation of this tissue reactivity in vitro was demonstrated to resemble in vivo conditions in terms of mitochondrial function and cell division, which combined with impedance spectrometry is a good model for better understanding the mechanisms by which the reactive response occurs (Frampton, Hynd et al. 2007).

Impedance spectroscopy has a wide range of applications since it does not require the presence of electrogenic cells to perform electric measurements. On the contrary, every cell is susceptible to impedimetric analysis. Of special interest is the potential use of this technique on the study of stem cell viability (Ehlers, Stempin et al.), development and differentiation (Hildebrandt, Büth et al.), drug effectiveness and toxicity (Wolf, Hartl et al. 2011) and detection of toxins in water (Curtis, Tabb et al. 2009; Brennan, Widder et al. 2012). EIS can be combined with other optochemical oxygen sensors (Wolf, Hartl et al. 2011) or combined with oxygen and pH sensors (Bohrn, Stütz et al. 2011) in order to monitorize different parameters in a time-resolved way. Moreover, EIS experiments have been commonly conducted at frequencies lower than 1 MHz (Kloss, Fischer et al. 2008). Little is known about changes of alternating electric fields at 1 MHz going through differentiating neuronal cells embedded in a gel matrix. All these facts make impedance spectroscopy a very promising candidate for the detection of differentiation in 3D matrices.

2.7.2.4 Microfluidics

Microfluidic devices are usually defined as miniaturized versions of their macro-scale counterparts, systems in which a laminar flow perfuses into tiny channels where the actual experiment is performed. Microfluidic devices present a lot of advantages in sample handling, reagent mixing, separation and detection. They are ideal for the analysis of hard-to obtain or high-cost substances and for parallelization. They have been widely used in methods requiring sophisticated equipment like flow cytometry, but also less sophisticated ones, like electrophoresis, dielectrophoresis, proteomics, immunoassays, sample preparation for mass spectrometry, RT-PCR improvement, etc (Weigl, Bardell et al. 2003)
The potential applications of microfluidic devices in neurobiology are vast. Cells can be seeded within microfluidic channels or perfused into the system. A wide spectrum of applications has already been developed in the field of neuronal differentiation and many challenges remain for the future. For example, it is possible to control the axon outgrowth and its direction. By using a microfluidic device with a microchannel, a microvalve, and a nano-hole array, one can control the spatio-temporal administration of NGF to a PC12 cell line and thus control neurite outgrowth in a very precise manner (Nakashima and Yasuda 2007).

A microfluidic device composed of two separated chambers has been demonstrated to effectively separate neurons from their axons for further proteomic and RNA analysis. This chip has other potential applications such as facilitating research into nerve injury and axon regeneration (Wu, Cheng et al. 2010). Another compartmentalized microfluidic platform has also been demonstrated to effectively separate cell soma from axons, thus making possible to study axon to glia interactions (Hosmane, Yang et al. 2010).

A microfluidic device designed for the generation of gradient concentrations without the generation of significant shear stress on the cells is a powerful tool for the investigation of the mechanisms behind growth cone guidance, since cells can live in the microfluidic channel and respond to the gradients in ways resembling those under physiological conditions (Wang, Li et al. 2008). Microfluidic devices can be combined with MEA or impedance technology to get the best of both. The cellular nutrient requirements or stimulation protocols can be satisfied with the help of a microfluidic system while maintaining the small sample volume of the biosensor and avoiding the modification of the cell extracellular environment. Constant medium supply allows the performance of long term experiments while parallelization of channels facilitates the comparison between samples and controls. MEAs can get extracellular simultaneous recordings of a large number of cells in the network while a microfluidic device supplements drugs or nutrients to a certain point of the network (Kraus, Verpoorte et al. 2006). In the same manner, by combining microfluidic devices with impedance sensing the electrical response of a whole network can be monitored. As another example, the design of narrow and long microtunnels with integrated electrodes allows the recording of single axon spikes and their propagation (Dworak and Wheeler 2009). The appearance of nanostructures will revolutionize the research and treatment of neurodegenerative diseases, since many applications, such as the use of microrobots for axonal guidance, present huge advantages compared to conventional methods (Modi, Pillay et al. 2009).
2.8 Outline

The central subject of the first part of this study revolves around the hypothesis that mitochondrial biogenesis is induced during cAMP-mediated differentiation of N2a neuroblastoma cells. The interaction between pathways related to differentiation and mitochondrial biogenesis will be described. We hereby provide evidence that N2a differentiation and mitochondrial biogenesis are closely associated, with superoxide preceding both processes as a trigger. The concomitant triggering and coordination of these two phenomena leads to the complete development of a neural phenotype in N2a neuroblastoma cells, by guaranteeing both the expression of the differentiation pattern and provision of sufficient energy to the emerging neuronal clusters.

The purpose of the second part of this study was the development of three dimensional tissue-like neural cultures, in which cells can be preserved and differentiate, and to demonstrate that high frequency electrical impedance sensing can be a promising technique for the on-line monitoring of neural electrical responses to neurotransmitters and neural differentiation in 3D matrices. The combination of impedance analysis with a microfluidic system allows on-line monitoring of the responses in real time. The first objective was achieved by triggering of the acetylcholine receptor (AChR) with acetylcholine in bactoagar-embedded N2a cells and studying the outgoing signals by means of electrical impedance analysis. The second objective was achieved by immobilizing N2a cells in a collagen laminin matrix and observing differentiation by means of electrical impedance analysis.
3  THEORY

This section includes basic general knowledge regarding the cellular biology of neurons and will more deeply discuss mitochondrial biology, as well as differentiation and intracellular signalling pathways leading to differentiation and mitochondrial biogenesis. Finally, the theory underlying the technique used to measure differentiation in 3D matrices (electrical impedance spectroscopy) will be described.

3.1  Biology of neurons

3.1.1  Neuronal structure

Neurons are one of the most specialized cell types. Their structure is not similar to any other tissue. Neurons present a series of specialized processes (neurites) that either receive (dendrites) or transmit (axons) electrical signals to other cells/neurons (Figure 6). They exhibit an extended shape, with a long axon and numerous branching dendrites connecting them to other neurons and other cells (Alberts, Johnson et al. 2002). The stereotypical image of a neuron depicts a stellate cell body or soma with several dendrites emerging from one pole and a long axon emerging from the other pole. However this picture does not reflect many other varieties of neurons. For example the Golgi cells of the cerebellum exert several branched axons. As the neuron is the most polymorphic cell in the body, its classification depends on different parameters such as shape, location, function or neurotransmitter.

Figure 6. Neuronal structure.
Neurons present similar cellular compartments as do other cell types: a large nucleus, endoplasmic reticulum, Golgi apparatus, lysosomes, Nissl substance, subsurface cisternae, multivesicular bodies, neurotubules, neurofilaments and mitochondria are present in the neuronal soma. Organelles are differentially distributed along the neuronal axon and dendrites. For example, dendrites usually lack neurofilaments and the axon is structurally divisible in regions that differ in membrane morphology and organelle content. ATP-dependent molecular motors (dinesin and dynein) allow the transport of organelles bidirectionally along the axon up to the axonal termination. The synapse is a specialized junctional complex by which axons and dendrites from different neurons intercommunicate. Usually a higher accumulation of organelles is observed in presynaptic than in postsynaptic terminals. Synapses can be morphologically classified as axodendritic, axosomatic, axoaxonic, dendrodendritic, somatosomatic and somatodendritic. Synapses can also be physiologically classified as excitatory, inhibitory and modulatory, or according to the neurotransmitter released into the synaptic cleft (Siegel, Agranoff et al. 2006).

3.1.2 Membrane potential

The function of neurons is mainly the communication of information between neurons and muscles or peripheral organs, which requires that signals travel over considerable distances (Byrne and Roberts 2004). How this functionality is achieved is mainly determined by the properties of the membrane. The cytoplasmic membrane is a lipidic bilayer that allows the accumulation of ions and charges on both of its sides, whereas specific protein channels allow the selective diffusion of certain ions. Thanks to the operation of ionic pumps and buffer mechanisms the concentration of important ions such as Na⁺, K⁺, Cl⁻ or Ca²⁺ is precisely maintained. Active mechanisms maintain K⁺ concentrated inside the cell and Na⁺, Cl⁻ and Ca²⁺ in the extracellular space. However, inside the cells other anions, to which the membrane is also impermeable, compensate for the high concentration of K⁺. The differential distribution of ions and charges generates an electrochemical ion force, which is the combination of an electrical force (due to the difference in voltage) and a chemical force (due to an unequal ion concentration). Ions tend to move down their concentration gradients. For example, when a K⁺ channel opens, K⁺ ions diffuse from the high concentration compartment (usually cytoplasm) to the less concentrated one (extracellular space). As K⁺ ions are positively charged this diffusion promotes an increased negativity inside the cell and generates an electrical force that drives diffusion of the ion to the cytoplasm. The potential stops increasing when the equilibrium potential for this ion is reached, i.e. when the electrical force and the chemical force are balanced. At equilibrium it is equally likely that K⁺ ions exit the membrane due to the concentration gradient as it is that they enter the cell due to electrostatic attraction. The equilibrium potential for each ion is therefore determined by the concentration of the ion at both sides of the membrane and is described by the Nernst equation:
where $R$ is the universal gas constant, $T$ is the absolute temperature, $F$ is the Faraday constant and $z_s$ is the ion charge, whereas $[S]_o$ and $[S]_i$ are the extracellular and the intracellular ion concentrations, respectively.

However, the membrane is permeable to more than one type of ion. Different ion channels open and close during excitation and even at rest several ion channels are opened simultaneously. Actually, real cells are never at equilibrium. Steady-state membrane potential of actual membranes presents values between those of the equilibrium potential of the permeant ions. The membrane potential is therefore controlled by the sum of contributions of the permeant ions following the Goldman-Hodgkin-Katz equation:

$$
V_m = \frac{RT}{F} \ln \left( \frac{P_{Na}[Na^+]_o + P_{K}[K^+]_o + P_{Cl}[Cl^-]_i}{P_{Na}[Na^+]_i + P_{K}[K^+]_i + P_{Cl}[Cl^-]_o} \right)
$$

Where $P$ is the relative permeability of each ion and $V_m$ is the membrane potential. The membrane potential is defined as the difference between the intracellular potential and the extracellular potential ($V_m = V_i - V_o$). In modern electrophysiology the extracellular potential is defined as ground $V_o = 0$, so all membrane potentials are equal to the intracellular potential. Briefly, membrane potentials appear as a result of the ion diffusion across a permselective membrane in a concentration gradient-dependent manner.

Usually rest membrane potential is between -30 and -100 mV. Different types of neurons exhibit different potential values and some types do not even have a true resting membrane potential. Making this membrane potential more positive is considered depolarization while making it more negative is considered hyperpolarization. (Byrne and Roberts 2004; Siegel, Agranoff et al. 2006; Jue 2009).

3.1.3 Neuronal activity. Action potentials

Action potentials are the basic elements that permit the transmission of an electric signal within the neuron. At resting potential (-60 to -70 mV for neurons), voltage dependent channels are usually closed. However, incoming signals continuously promote changes in the membrane
potential. During the generation of an action potential the membrane polarization is removed and reaches values approaching and even beyond 0 mV. It is generally promoted by the rapid influx of Na\(^+\) ions, which make the intracellular environment less negative. Depolarizing inputs trigger the opening of voltage gated channels and therefore elicit the propagation of an action potential. Na\(^+\) channels are rapidly inactivated and therefore Na\(^+\) influx decreases. This depolarization is followed by a swing of the membrane potential towards more negative values due to a slightly slower efflux of K\(^-\) ions through K\(^+\) channels that allow positive charge to exit the cell. This process is referred to as hyperpolarization. During hyperpolarization termination of the impulse propagation is achieved. After termination of the action potential, the membrane potential remains at a value even more negative than the original resting potential for a short period, which is referred to as afterhyperpolarization or undershoot. Afterhyperpolarization is generated by the persistence of the K\(^+\) current for a few milliseconds. During this time Na\(^+\) channels return to their original state, which prepares the membrane for the next action potential. For a period of time after the generation of an action potential, no other action potential can be generated; this period is referred to as the refractory period, which prevents the reverberation of signals.

Thus, the action potential is an electrical event generated by the change in the distribution of ions across the membrane. However, it is not associated with substantial changes to the extracellular or intracellular concentrations of the ions. These small deviations of the ion concentrations are corrected by the use of ionic pumps that transport ions against their concentration gradients. For example, the Na\(^+\) - K\(^+\) pump is activated when the concentration of Na\(^+\) is increased in the cytoplasm and actively interexchanges three Na\(^+\) ions for two K\(^+\) ions by hydrolyzing ATP.

The variety of ionic channels presented in neurons is very wide, and hence so is the variety of action potential patterns occurring within single neurons. The propagation of action potentials allows the communication of information from the soma of the neuron to the synapses, and thus communication with other neurons and muscles through the release of neurotransmitters (Byrne and Roberts 2004; Davies and Morris 2004).

### 3.1.4 Synapse

The synapse is the primary place of functional contact at which information is transmitted from neuron to neuron or from neuron to gland or muscle. This contact can be achieved by direct electrical interaction, for example through gap junctions, or by a chemical interaction, through the release of neurotransmitters into the synaptic cleft. Most interneuronal communication relies on chemical transmission, in which a single presynaptic action potential leads to the release of a vast amount of neurotransmitters, which bind in turn to specific receptors and can generate a large
postsynaptic potential. The effect can be excitatory or inhibitory depending on the identity of the neurotransmitter released and the postsynaptic receptors. There is a large diversity of neurotransmitters and receptors. This fact guarantees the multiplicity of postsynaptic responses. In fact, few if any neurons contain a single type of transmitter, indicating that different transmitters can be used by a neuron to transmit different kinds of information to the postsynaptic neuron (Byrne and Roberts 2004).

3.1.5 Neurotransmitters. The example of acetylcholine (ACh)

Classic neurotransmitters are defined as endogenous substances that are synthesized by and released from neurons; they act on postsynaptic receptor sites, producing functional changes in target cells. These include ACh, serotonin, GABA, catecholamines and glutamate.

ACh was the first neurotransmitter identified. It was discovered by Loewi and Navratil as being responsible for neuromuscular junction transmission. ACh is an ester of acetic acid, which is synthesized from acetyl-coenzyme A and choline by the enzyme choline acetyltransferase (ChAT). Then it is stored in vesicles, which release the ACh to the synaptic cleft upon the appearance of an action potential. ACh can then activate nicotinic (nAChR) and muscarinic acetylcholine receptors (mAChR) and is rapidly inactivated by the enzyme acetylcholinesterase (AChE) (Figure 7). Choline reuptake is facilitated by a high affinity transporter. nAChR receive their name from the plant alkaloid nicotine which is an agonist of this receptor. nAChR is a heteromeric protein complex with five transmembrane subunits that enclose a central pore permeable to most cations (Na⁺, Ca²⁺ and K⁺). When two molecules of ACh bind the nAChR, it suffers a conformational change that is translated into an increase of the pore diameter, so that Na⁺ and Ca²⁺ can enter whereas K⁺ can exit the cell, according to their concentration gradients. The opening of ACh receptors causes a membrane depolarization that can be transmitted as an action potential throughout the membrane of the neuron up to the next synapse. ACh has been widely used as a control for nAChR triggering since it is its natural ligand. ACh can also activate muscarinic acetylcholine receptors (mAChR), which also receive their name from the naturally occurring alkaloid muscarine. The activation of mAChR, which is a metabotropic receptor, does not produce a direct membrane depolarization but the activation of modulatory signalling pathways by second messengers. Furthermore, it is known that Alzheimer’s disease is mediated by a shortage of this neurotransmitter among other symptoms.

Nonclassical neurotransmitters such as peptide neurotransmitters differ from the above in several ways: they are not always locally synthesized nor stored. “Unconventional neurotransmitter” is a wider term that refers to a heterogeneous group of substances that mediate the communication
between neurons. This category includes nitric oxide (NO), carbon monoxide (CO), growth factors (BDNF, NGF) or steroids (Byrne and Roberts 2004).

![Figure 7. Schematic representation of a cholinergic synapse.](image)

### 3.1.6 Neuroplasticity

The structural and functional adaptive response of neural cells to physiological or pathological perturbations is referred to as neuroplasticity. Some examples of neuroplasticity include the production of neurons from progenitors (neural differentiation), the growth of axons and dendrites (neurite sprouting and outgrowth), synapse formation and strengthening, and the formation and recognition of dendrites (dendritic remodelling) (Cheng, Hou et al. 2010)

### 3.1.7 Neuronal development

During animal development, dramatic cell movements take place. Embryo gastrulation allows the formation of three germ layers: endoderm, mesoderm and ectoderm. Part of the ectoderm thickens, rolls up and pinches off to form the neural tube and neural crest. Neurons and glial cells of the central nervous system derive from the neural tube, whereas peripheral nervous system neurons derive mainly from the neural crest. The neural tube consists of a single layer of epithelial cells, which are the progenitors of neurons and glia. Progenitors and glial cells multiply to increase the thickness of the epithelium and to maintain its cohesiveness. Nerve cells are generated by cell
division and new-born neurons migrate along this substrate to find an appropriate location to maturate and extend axons and dendrites. Neurons born at different times and places possess a different gene expression pattern which will determine the connections they will form and the neuronal subtype they will become. The development of axons and dendrites is dependent on the presence of chemotactic factors, cell-cell-adhesion molecules, extracellular matrix components, neurotrophic factors, etc., which guide the enlargement of the growth cones and their direction. However, after having reached the targets, many of the innervating neurons die by apoptosis as a result of the competition for neurotrophic factors such as NGF. Some connections are also discarded in order to find a precisely ordered connection pattern, which allows the constant modification of the connection network to be adapted to the external environment (Alberts, Johnson et al. 2002).

The regenerative capacity of the adult mammalian brain is vastly reduced, so that for many years it was believed that regeneration was nonexistent. Neuronal population is known to be completed shortly after birth, while mature neurons do not divide and there is a high rate of daily dropout of neurons. Nevertheless, nowadays it is widely known that regeneration takes place in the adult brain, i.e. proliferation and neurogenesis occur. New born neurons and glia derive from cells with stem cell-like properties that are present in certain regions of the nervous system. The process of neurogenesis is dependent on a wide range of modulators such as growth factors, hormones and neurotransmitters. (Lanza, Gearhart et al. 2006; Siegel, Agranoff et al. 2006).

During neurogenesis stem cells can divide to generate one daughter cell that is a stem cell and another daughter cell that can produce differentiated progenitor cells. This second cell looses its regenerative potential, and start to differentiate into a neuron or a glial cell. The study of the different factors that can regulate stem cell differentiation into neural cells provides us with the tools necessary to suggest new potential therapeutic mechanisms for repair and regeneration of the central nervous system (Weiner 2008).

### 3.1.8 Neural differentiation and cell cycle arrest

The term “cell cycle” defines the process via which a cell reproduces by performing a series of ordered events, in which it duplicates its content and divides in two. A typical cell cycle stems of several phases: $G_1$, $S$, $G_2$, and $M$. During the $S$ phase the duplication of the chromosomes occurs, so that the two daughter cells are genetically identical. The chromosomes are segregated into individual nuclei during the $M$ (mitosis) phase. These two phases are usually separated by two extra gap phases: a $G_1$ phase between $M$ and $S$ phase and a $G_2$ phase between $S$ and $M$ phase. These gap phases provide enough time to the cell to check if the environment is propitious for its
commitment to division. An independent cell-cycle control system, composed by a complex network of regulatory proteins, regulates that those events occur in the correct order and only once per cycle. This control system is, in turn, susceptible to regulation by different intracellular and extracellular signals, mainly during the gap phases. There are several points in the cell cycle in which the progression can be arrested if previous events have not been completed, the so-called check points (Alberts, Johnson et al. 2002).

In order to start the differentiation process, progenitor cells must undergo cell cycle arrest, i.e. cell proliferation must cease before the differentiation process can take place. As a result, differentiation and cell cycle arrest are closely related during the final decision of the fate of a single specific progenitor cell. The co-ordination of cell cycle regulation and the differentiation/determination of a particular cell fate is a key step in neuronal development (Galderisi, Jori et al. 2003).

3.2 Biology of mitochondria

3.2.1 Cellular compartmentalization

Eucaryotic cells are subdivided into different membrane-enclosed compartments. Each compartment (organelle) is specialized in a different function. Membranes provide not only for the division of the cell into different compartments but also a surface where many biochemical processes take place. For example, oxidative phosphorylation requires a membrane to couple $H^+$ transport to ATP synthesis. As membranes are lipid bilayers, they are impermeable to most hydrophilic compounds. That makes the existence of transport proteins necessary for the import and export of metabolites. In the case of mitochondria, which need to import most of the proteins, unfolded proteins are translocated into the mitochondrial matrix through the TOM (translocase of the outer membrane) and TIM (translocase of the inner membrane) complexes. Translocation is dependent on ATP, electrochemical gradient and hsp70 chaperone protein family, which maintain the protein in an unfolded state in the cytoplasm and pull them into the mitochondrial matrix (Alberts, Johnson et al. 2002).

3.2.2 The endosymbiotic origin of mitochondria

Eucaryotic cells have a separate compartment to keep their DNA isolated from the rest of the cell, a cytoskeleton to allow movement, elaboration of different compartments for digestion and secretion, and a metabolism that is dependent on the oxidation of organic compounds by
mitochondria. All these properties suggest that eucaryotic cells originated as predators of other cells. Mitochondria are similar to bacteria in size, in DNA organization as a circular molecule and they have their own ribosomes and transfer RNAs. Therefore, mitochondria most probably originated from aerobic bacteria that were engulfed by a predator anaerobic ancestral cell, which was not able to make use of the oxygen that appeared in big quantities 1.5 billion years ago. So the aerobic bacteria and the predator cell evolved in symbiosis giving rise to modern eucaryotic cells.

The genetic information in eucaryotic cells therefore has a dual origin, the ancestral eucaryote and the bacteria adopted as symbionts. This genetic information is mostly stored in the nucleus but a small amount remains in the mitochondria, which is individually analyzed and sequenced. Mitochondrial DNA codes for 13 proteins, two ribosomal RNA components and 22 transfer RNAs. However, mitochondrial DNA lacks genes for many essential functions in comparison to bacterial DNA. The command of these functions has been assumed by nuclear DNA. Hence, it is the subtle interplay between mitochondrial and nuclear DNA which controls mitochondrial protein synthesis (Alberts, Johnson et al. 2002).

### 3.2.3 Mitochondrial structure

Mitochondria consist of two specialized membranes with different functions, which create two compartments: the matrix and the intermembrane space. The outer membrane contains a high number of large hydrophilic channels called porins, which allow the entrance of big molecules (up to 5000 daltons in size) to the intermembrane space. Thus intermembrane space is chemically equivalent to the cytosol. In contrast, the inner membrane is highly impermeable to big molecules and to anions, which keep the mitochondrial matrix isolated from the cytoplasm. Some specialized transport proteins allow the transport of required molecules. The inner membrane is highly convoluted, forming projections into the mitochondrial matrix, the cristae, which increase the inner membrane area, and thus the energy production capacity (Figure 8). The inner membrane contains the electron transport chain proteins, the ATP synthase, and the transport proteins. The electrochemical gradient necessary to drive ATP production is produced across this membrane. The mitochondrial matrix contains several copies of the mitochondrial DNA, mitochondrial ribosomes, tRNAs, the enzymes required for the citric acid cycle, for pyruvate oxidation and for mtDNA expression (Alberts, Johnson et al. 2002).
3.2.4 Mitochondrial plasticity

Mitochondria are usually depicted as elongated cylinders that resemble bacteria. However, mitochondria are plastic organelles which constantly change their size and shape, fuse with other mitochondria and separate again. They move around the cytoplasm, usually associated to microtubules. Each type of cell presents a unique distribution pattern of mitochondria. In some cells they form long moving filaments whereas in other types they remain packed next to sites with high energy necessities as in the flagellum of a sperm. (Alberts, Johnson et al. 2002). In vivo, mitochondria form a highly plastic and mobile tubular network extended throughout the whole cell. Network mobility and plasticity are necessary to meet unsteady cellular energy requirements. The shape of this network is generated by a series of fusion and fission events (Figure 9), while the movements of mitochondria throughout the cell are facilitated by the cytoskeleton. In neurons, mitochondria are transported to growth cones and synapses thanks to specific kinesin proteins (Mullins 2005).

Figure 8. Mitochondrial structure (by Fotini Tikkou, with permission).

Figure 9. Schematic representation of a fission event. Rapid changes in mitochondrial shape, along with fusion and fission processes can be observed in mitochondria in living cells.
3.2.5 Mitochondrial biochemistry

Mitochondria have been essential in the evolution of eucaryotic cells for the evolution of complex multicellular animals. Without mitochondria the energy supplies would be restricted to those coming from anaerobic glycolysis. Thanks to mitochondria, the pyruvate produced during glycolysis is oxidized to CO$_2$ and H$_2$O, allowing the production of 15 times more ATP than glycolysis. ATP is considered the monetary system of the cell.

Glycolysis is the process via which one molecule of glucose is metabolized to two molecules of pyruvate, with the concomitant production of two molecules of ATP. Pyruvate can be further processed through different mechanisms. It can be anaerobically processed by fermentation to lactate or ethanol, whereas under aerobic conditions pyruvate is completely oxidized to CO$_2$ by mitochondria, generating a much bigger amount of ATP. This oxidation driven by the citric acid cycle or Krebs cycle takes place in the mitochondria. The Krebs cycle is also an important source of precursors for the synthesis of other molecules. High energy electrons from the carbon fuels are harvested during the Krebs cycle and used to form NADH and FADH2, which are subsequently used by oxidative phosphorylation to produce energy. These electrons then flow through a series of membrane proteins (the electron transport chain) until the final acceptor, the oxygen, to generate a proton gradient across the mitochondrial membrane. Electron flow through these transmembrane complexes leads to the transport of protons from the matrix to the intermembrane space to create a transmembrane electrochemical potential, which includes contributions of both a pH difference and a membrane potential. The protons finally flow through ATP synthase that generates ATP, based on the high free energy released when protons flow back to the matrix (Figure 10). The whole process is called oxidative phosphorylation (OXPHOS). The regulation of OXPHOS is mainly driven by ADP levels. Electrons usually do not flow through the chain unless this process is coupled to ADP phosphorylation to ATP. This regulation is called respiratory control or acceptor control. In the same manner, the citric acid cycle rate is controlled by the levels of ADP, since NAD$^+$ and FAD coming from the electron transport chain are needed. From a physiological point of view, the production of energy in the form of ATP should be dependent on the necessity of the cell. Electrons do not flow from donors to oxygen unless the balance ATP/ADP is unfavourable to the cell, unless ATP needs to be synthesized. OXPHOS is susceptible to inhibition at different stages. For example rotenone inhibits the complex I, preventing the utilization of NADH as a substrate. Cyanide or carbon monoxide inhibit the cytochrome C, inhibiting also the ATP synthesis. The coupling between electron transport and phosphorylation can also be disrupted by the presence of certain uncouplers, which carry protons across the inner membrane without producing ATP. A regulated uncoupling of the OXPHOS by uncoupling proteins is very important in physiological processes such as heat production, which is
essential for mammals adapted to the cold and hibernating animals (Alberts, Johnson et al. 2002; Berg, Tymoczko et al. 2002).

Figure 10. Summary of energy-generating metabolism in mitochondria. Schematic representation of the interaction between the citric acid cycle and the electron transport chain to produce ATP. C, Complex; Cyt C, Cytochrome C; Q, coenzyme Q.

3.2.6 Mitochondrial free radical production and buffering.

The high affinity of oxygen for electrons allows the release of a large amount of free energy when it is reduced to water. However, once an oxygen molecule has accepted one electron it forms the superoxide radical, which is highly reactive and dangerous for the cells. Usually cytochrome oxidase (C IV) retains the superoxide anion at its special bimetallic centre. Only when it has picked up a total of four electrons is released in the form of two molecules of water. Although cytochrome C oxidase is remarkably successful in avoiding the release of intermediates, the efficiency of this process is not 100%. Small amounts of superoxide and hydrogen peroxide can escape and are unavoidably released to the mitochondrial matrix as a by-product. These by-products are commonly referred to as reactive oxygen species or ROS. Released ROS can then damage membranes, proteins and DNA, but mitochondria possess a special free radical buffering system which reduces the quantity of free radicals released. Superoxide dismutase (SOD) scavenges superoxide by catalyzing its conversion to hydrogen peroxide and molecular oxygen. The hydrogen peroxide produced in this reaction is in turn scavenged by the catalase enzyme to produce water and molecular oxygen (Figure 11). In addition, cells possess other antioxidant
defences such as vitamin C, vitamin E and peroxidases, which scavenge hydrogen peroxide. The existence of all these mechanisms demonstrates the importance of the redox balance maintenance. Several genetic diseases are associated with an increased production of ROS by the mitochondria such as Friederich’s ataxia or Wilson disease. The role of mitochondrial ROS formation has also been proposed to play a role in diabetes. Moreover, it is known that inhibition of complex I by rotenone or MPTP causes the destruction of the substantia nigra and thus Parkinson’s disease through the formation of ROS in the mitochondria. Finally, mitochondrial ROS have been implicated as one of the main factors responsible for aging. (Alberts, Johnson et al. 2002; Berg, Tymoczko et al. 2002; Schaffer and Suleiman 2007).

![Figure 11. Mitochondrial superoxide production and buffering](image)

**Figure 11. Mitochondrial superoxide production and buffering.** Schematic representation of superoxide leakage from complex IV at the mitochondrial electron transport chain and buffering by mitochondrial specialized enzymes. Cyt C, cytochrome C; C IV, Complex IV; SOD, superoxide dismutase; O$_2^-$, superoxide anion.

### 3.2.7 Mitochondrial biogenesis

Organelle biogenesis is concomitant to organelle inheritance during cell division. It is necessary that organelles double their size and divide to give rise to two identical daughter cells. Mitochondrial biogenesis occurs by growth and division of pre-existing organelles and is temporally coordinated with cell cycle events (Mullins 2005). However, mitochondrial biogenesis is not only produced in association with cell division. It can be produced in response to oxidative stimuli, increases in the energy requirements of the cells, in response to exercise training, electrical stimulation, hormones, during development, or in certain mitochondrial diseases.
Mitochondrial biogenesis is therefore defined as the process via which cells increase their individual mitochondrial mass (Onyango, Lu et al. 2010).

As a result of the endosymbiotic evolutionary history of mitochondria, the study of mitochondrial biogenesis is especially complex because of the coexistence of both eukaryotic and prokaryotic mechanisms. The presence of inner and outer membranes, their own small genome and the continuous fusion and fission events make mitochondria the most complex and unique organelles. Moreover, along with endosymbiosis, novel mitochondrial biogenesis pathways have evolved. Mitochondrial biogenesis is of special importance in modern neurochemistry because of the broad spectrum of human diseases arising from defects in mitochondrial ion and ROS homeostasis, energy production and morphology (Mullins 2005).

Mitochondrial biogenesis requires the interaction of the nuclear (nDNA) and the mitochondrial (mitDNA) genome. MitDNA encodes 13 respiratory chain protein subunits and 24 RNA components (Figure 2). The majority of the mitochondrial proteins are encoded by nuclear DNA and are subsequently translocated to the mitochondria (Onyango, Lu et al. 2010). This process is regulated by several transcriptional activators including NRF-1, NRF-2 and PPARα. The members of the PGC-1 family of transcriptional coactivators act as intermediaries between signalling molecules and transcription factors. Among them, PGC-1α is considered the master regulator of mitochondrial biogenesis (Scarpulla 2002). After nuclear-encoded proteins are synthesized, mitochondria have an elaborated apparatus that allows the import of nuclear-coded proteins as well as the export of mitochondrial-coded proteins (Mullins 2005).

3.2.8 Mitochondria distribution and function in neurons

Mitochondria are distributed not only throughout the cytoplasm of the cell but also throughout the length of the axons up to the presynaptic terminals. Similarly in dendrites mitochondria are localized in the dendritic shafts and occasionally associated with spines. It is known that during neuroplasticity mitochondria are actively transported to different cellular structures; they undergo fusion and fission processes as well as calcium and membrane potential changes in response to neurotransmitters and growth factors, and participate in signalling cascades. The major function of mitochondria is the production of energy. However, they are also known to participate in the regulation of calcium dynamics, redox homeostasis and apoptosis. Mitochondria dynamics are very important to neural functions since they regulate the morphology, number, location and function of mitochondria, which in turn regulate a plethora of functions within the cell (Cheng, Hou et al. 2010).
3.2.9 Role of mitochondria in neuroplasticity.

Mitochondria have been discovered to play important roles during neuroplasticity processes. For example during neurogenesis, it is known that oxygen concentrations influence both mitochondrial function and the self renewal capacity of stem cells. During differentiation, various mechanisms enable neurite outgrowth: mitochondria generate the necessary energy to support the development of fundamental structures; By uncoupling the oxidative phosphorylation UCPs are able not only to reduce ATP production but also to reduce ROS production and modify calcium dynamics; furthermore, mitochondria have been found to congregate around the base of developing axons and in the growth cone (Cheng, Hou et al. 2010)

3.3 Biology of tumors. Neuroblastoma.

Multicellular organisms operate as an ecosystem or society, in which cells represent single individual members. Cells divide, collaborate with other cells and even self sacrifice for the sake of the organism’s well-being and its reproduction. Somatic cell lineages support germ cells during all their existence while they are committed to die without leaving any progeny. Competition and natural selection between cells are not part of the strategy of a multicellular organism to survive. Instead, cooperation and collaboration assure that germ cells will have a chance to transmit copies of their own genome to the progeny. Cells therefore act in a coordinated and responsible manner by receiving, interpreting and elaborating signals that act as social controls for the good of the whole organism.

Cancer cells break all the basic rules by which multicellular organisms are built and maintained. Millions of cells suffer punctual mutations everyday. In case that one of these mutations gives a competitive advantage to this cell with respect to its neighbours and especially if this mutation causes an increase in its division rate, this cell can form a mutant clone that grows into the physiological tissue. Several rounds of mutation, competition and selection can make things worse by allowing mutant clones to live at the expense of other cells and invade their neighbours’ territories. This kind of selfish behaviour can lead to the destruction of the whole cellular society.

Punctual mutations in one single cell are not dangerous unless these mutations promote the disregulation of proliferation. If this is the case, this cell can give rise to a growing mass of abnormal cells, a so called tumor or neoplasm. This tumor is refered to as benign as long as abnormal cells are restricted to a clustered single mass. A tumor is only considered a cancer if it is malignant, i.e. if it is able to invade the surrounding tissue, migrate and produce metastases in other parts of the body. Several properties make a cell successful as a cancer cell: Disregarding
signals that control cell proliferation, avoiding apoptosis, replicative senescence and
differentiation, invading other tissues and proliferating as metastases.

Cancers are classified according to the tissue from which the initial tumor arises. Thus,
carcinoma, sarcoma, leukemia and neuroblastoma designate the cancers derived from epithelial
cells, connective tissue cells, hemopoietic cells and neural progenitors respectively (Alberts,
Johnson et al. 2002).

Neuroblastoma is the most common type of solid cancer in childhood. Its prognosis is extremely
variable depending on the age of the patient. Most infants experience a regression with minimal
therapy, whereas the increasing the age of the patient increases the probability of tumor
progression (Brodeur 2003). Neuroblastoma tumors derive from primitive embryonic cells of the
sympathetic nervous system, which remain in an undifferentiated and proliferative state.

In the search of an effective treatment for this type of cancer several animal and cellular models
have been developed. Neuroblastoma cell lines have provided the basic knowledge necessary to
understand the mechanisms underlying signal transduction and have been extremely useful for
drug screening, whereas animal models (to which neuroblastoma cells are usually
subcutaneously injected) have provided a preclinical testing source for the discovery of related
genes and the application of new drug administration systems. Clinical data have suggested the
induction of differentiation as a novel therapeutic strategy to treat this kind of cancer. In fact,
retinoids have been demonstrated to significantly improve event-free survival in high-risk
neuroblastoma in clinical trials. These findings, in turn, have provided the basis to study the
mechanisms that underlie neuroblastoma differentiation and the pharmacology of the drugs that
can alter these mechanisms. The most commonly used drugs to induce differentiation are:
phorbol esters (that activate PKC), cAMP analogs (that activate PKA and/or Epac), retinoids (that
modulate hormone receptors) and growth factors. Some of these pathways seem to converge on
common signalling proteins such as MAPKs (Leondaritis, Koliou et al. 2012). In the next section
the basic aspects of the signalling pathways addressed in this study will be described.

3.4 Metabolomics. Intracellular signalling pathways.

Cells are programmed to respond to external stimuli produced by other cells. These external
stimuli can be in the form of neurotransmitters (in the case of synaptic transmission), hormones (if
the substance is transported through the blood) or they can be locally secreted and affect just
neighbouring cells. In any case, a set of protein receptors is ready for receiving external information. Some of the signals, such as steroid and thyroid hormones, nitric oxide or carbon monoxide can diffuse across the membrane to activate intracellular receptors. But there are other hydrophilic substances that can only activate proteins on the surface of the cell. These receptor proteins then act as a transducer that transmits the information to the intracellular environment. Membrane receptor families can be sorted into three main categories: Receptors linked to ion channels, receptors linked to G proteins and receptors linked to enzymes. Enzyme and G protein-linked receptors can then activate the production of second messengers or activate cascades of intracellular signalling proteins that can amplify, spread, transduce or integrate the signals (Alberts, Johnson et al. 2002). In this section some of the signalling pathways, signalling proteins and molecules potentially related to differentiation and mitochondrial biogenesis will be described. Some of those pathways along with the inhibitors that block them are depicted in figure 12.

![Figure 12. Outline of the signalling pathways potentially related to differentiation and mitochondrial biogenesis and some inhibitors of those pathways. AC, adenylate cyclase; cAMP, cyclic AMP; PKA, cAMP dependent protein kinase; NOS, nitric oxide synthase; NO, nitric oxide; cGMP, cyclic GMP; PKG, Protein kinase G; MEK, mitogen activated protein kinase kinase; ERK, extracellular regulated protein kinase; CREB, cAMP reponse element binding protein; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; ROS, reactive oxygen species. Inhibitors of the signalling pathways are depicted in red.](image-url)
3.4.1 cAMP

Cyclic AMP (cAMP) is a small second messenger, which is synthesized from ATP by the trans-membrane-bound protein adenylate cyclase. Adenylate cyclase is usually activated by G proteins in response to physiological stimuli but it can be also activated by chemical substances such as forskolin. The synthesis of cAMP by adenylate cyclase results in an increase in the local concentration of cAMP that can then activate other proteins such as PKA or Epac. cAMP breakdown is achieved by cyclic AMP phosphodiesterases that hydrolyze cAMP to adenosine 5'-monophosphate. cAMP analogs such as dibutyryl cyclic AMP (db-cAMP) mimic the effect of cAMP on target proteins (Carranza, Rousselot et al. 1998; Alberts, Johnson et al. 2002).

3.4.2 PKA

Although cAMP is known to activate a wide variety of proteins, most of the effects of this second messenger are related to the activation of the cyclic-AMP-dependent protein kinase (PKA). This protein kinase consists of two catalytic subunits and two regulatory subunits. When cAMP binds to the regulatory subunits, the catalytic subunits are released and they can then phosphorylate other proteins. PKA catalyzes the transfer of a phosphate group from ATP to the target protein, which regulates the activity of this protein. There is a big variety of substrate proteins that are phosphorylated and activated by PKA. One of the most important proteins activated by PKA is the so called cyclic AMP response element binding protein (CREB). CREB is a transcription factor that recognizes CRE (cAMP response element) sequences in specific genes and, upon recruitment of the CREB binding protein (CBP), it stimulates the transcription of these genes (Alberts, Johnson et al. 2002).

3.4.3 MAPK

cAMP can also activate Epac, that acts as a GTP exchange factor (GEF) to activate Rap1. GEFs promote the dissociation of the nucleotide GTP from the substrate protein and the uptake of GTP from the cytosol, thus activating this protein. Rap1, in turn, is able to activate B-RAF and therefore lead to the activation of the mitogen activated protein kinase (MAPK) cascade (Vossler, Yao et al. 1997), which is related to the induction of differentiation (Wasilewska-Sampaio, Silveira et al. 2005). Briefly, MEK1/2 (or MAP-kinase-kinase) phosphorylates ERK1/2 (or MAP-kinase protein) in two different aminoacids. Once activated, ERK phosphorylates important proteins such as the transcription factor CREB. Thus, PKA and MAPK converge to the same substrates (Lee and Nikoderm 2004), phosphorylating identical or different phosphoacceptor sites and thus modulating
the activity of the substrate (Gerits, Kostenko et al. 2008). The fact that CREB can be phosphorylated on the residue Ser-133 both by PKA and ERK allows the modulation of the kinetics and stoichiometry for CREB phosphorylation and thus the maintenance of the signal beyond the time point when one of the protein kinases becomes refractory (Gerits, Kostenko et al. 2008). Moreover, MAPK can also be inhibited by cAMP or PKA. The existence of these opposite phenomena seems to depend on the cell type, the timing of the signal, the scaffolding of the related kinases in a protein complex and the compartmentalization of the different signalling cascades (Wang, Dillon et al. 2006).

3.4.4 Free radicals.

Molecules or molecular fragments containing one or more unpaired electrons in atomic orbitals are referred to as free radicals. These unpaired electrons confer a high reactivity to free radicals both with other molecules and amongst themselves. Both reactive oxygen species (ROS) and reactive nitrogen species (RNS) are included in this category of signalling molecules. They are known for exert a dual role in cellular physiology, both as deleterious and beneficial to living systems. (Valko, Leibfritz et al. 2007).

3.4.4.1 ROS signalling

Reactive oxygen species (ROS) is the general term that defines the species that are generated from the incomplete reduction of molecular oxygen. The ROS term includes superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroperoxyl radical (HO$_2^-$) and hydroxyl radical (OH) (D’Autreaux and Toledano 2007). ROS arise mainly from leakage from the electron transport chain of the mitochondria and from the overstimulation of NADPH during normal cellular metabolism. ROS play a dual role, being both deleterious and beneficial to cell physiology. Overproduction of ROS results in oxidative stress, which causes damages to cellular structures and has been implicated in several pathological conditions such as cardiovascular disease, cancer, neurological disorders, degenerative diseases and ageing. However, moderate ROS concentrations are known to mediate important physiological processes. The balance between the rate of ROS production and the rate of removal by antioxidants is referred to as redox homeostasis.

The concept of redox signalling describes the regulation of cellular processes by redox reactions. It requires that this balance or homeostasis is disturbed by an increase in the ROS production or a decrease in the antioxidant activity and that the ROS interact as a second messenger with specific signalling molecules. One of the most significant signalling pathways known to be activated by
changes in the redox state is the MAPK pathway. Superoxide and hydrogen peroxide can activate the MAPK cascade at the level of MEK1/2. The activation of MAPK leads to the activation of nuclear transcription factors and thus the expression of important antioxidant genes. ROS signalling has also been related to cell cycle. Generally, a reduced state promotes proliferation, a more oxidized one promotes differentiation, and highly oxidized states can lead to cell death (Maher and Schubert 2000; D’Autreaux and Toledano 2007; Valko, Leibfritz et al. 2007).

3.4.4.2 RNS: NO signalling.

Reactive nitrogen species (RNS) are a family of molecules, whose head is nitric oxide, characterized by the presence of nitrogen in their composition. Overproduction of reactive nitrogen species is referred to as nitrosative stress and it can be highly deleterious to cells. This may occur if the production of RNS exceeds the buffering mechanisms of the cell, and it may lead to nitrosylation and inhibition of proteins. However, RNS and more specifically NO is an important signalling molecule that mediates a wide variety of physiological processes such as blood pressure maintenance and neurotransmission (Valko, Leibfritz et al. 2007).

Nitric oxide (NO) is a volatile free radical that acts as a signalling molecule by regulating the activity of specific target proteins. It is diffusible through cellular membranes, thus serving as a local signalling molecule to adjacent cells. NO production is catalyzed by the enzyme NO synthase by deamination of the aminoacid arginine. It diffuses to neighbour cells and is rapidly converted to nitrates and nitrites by oxygen and water. NO can bind guanylyl cyclase and activate it to produce cyclic GMP (cGMP). cGMP can then diffuse and activate the cyclic GMP-dependent protein kinase (PKG), which phosphorylates specific substrates on serine or threonine residues (Alberts, Johnson et al. 2002). Among these substrates very important transcription factors are found such as CREB, n-Myc, c-fos, c-jun, or zinc-finger transcription factors, which promote the expression of a large number of genes.

NO signalling plays multiple roles in the nervous system, like promoting survival and differentiation of neural cells. Some of its effects are mediated by CREB phosphorylation (Contestabile 2008). Furthermore, cAMP was also found to mediate the activation of NOS and to promote the induction of the master regulator of mitochondrial biogenesis (Bossy-Wetzel and Lipton 2003).
3.4.5 PGC-1α

PGC-1α is a transcriptional coactivator which is considered the master regulator of mitochondrial biogenesis. It is known that the effect of the overexpression of PGC-1α on mitochondrial biogenesis is mediated by the nuclear respiratory factor NRF-1 (nuclear respiratory factor). NRF-1, in turn, is responsible for the transcription of the genes related to mitochondria. Some of these genes encode subunits of the five respiratory complexes, some are involved in the assembly and function of the respiratory machinery, others encode constituents of the mtDNA transcription machinery, and still others encode components of the protein import system. PGC-1α also upregulates important genes of the fatty acid oxidation pathway through PPARα trans-activation. Furthermore, PGC-1α mediates an H$_2$O$_2$-inducible antioxidant program, which includes important mitochondrial antioxidant defences such as superoxide dismutase, catalase and others. It thus appears as an extremely important link between ROS production, and a sophisticated anti-ROS program.

Among the signalling pathways known to induce PGC-1α overexpression, we can find the activation of the transcription factor CREB in a cAMP dependent pathway or in a NO dependent pathway. However, these are not the only pathways known to activate PGC-1α overexpression. A remarkable complexity is found in the mechanisms by which PGC-1α is expressed and activated, and also in its molecular interactions with other coactivators (Scarpulla 2002; Nisoli and Carruba 2006; D'Autreaux and Toledano 2007).
3.5 Techniques to study differentiation: Impedance sensing

Impedance detection is a very useful tool for the measurement of cell electrical properties. Actually, cells can be introduced into an electronic circuit forming an electrochemical cell. When applying an AC voltage perturbation, the current flows through all the components of the circuit, including the cells. Impedance (the cell’s opposition to the current), which will be a sum of the component’s contributions, can be monitored on-line (Lisdat and Schafer 2008).

3.5.1 Concept of impedance

Impedance is an important parameter used to characterize electronic circuits. Impedance (Z) is generally defined as the total opposition a device or circuit offers to the flow of an alternating current at a given frequency. The concept of electrical resistance is general knowledge. It is the ability of a circuit element to resist the flow of electrical current.

Ohm’s law defines resistance in terms of the ratio between voltage $E$ and current $I$.

$$R = \frac{E}{I}$$

The use of this equation is limited to one single circuit element, the ideal resistor.

However, in the real world it is common to find circuit elements that exhibit much more complex behavior and force us to replace the simple concept of resistance for a more general circuit parameter, impedance. Like resistance, impedance is a measure of the ability of a circuit to resist the flow of electrical current (Agilent-Technologies 2009).

Impedance has been widely used to characterize electronic circuits, components, and the materials used to make components. Impedance (Z) is generally defined as “the total opposition a device or circuit offers to the flow of an alternating current (AC) at a given frequency”. It is represented as a complex quantity that can be graphically represented on a vector plane. The impedance vector consists of a real part (resistance, R) and an imaginary part (reactance, X).

Impedance can be expressed using two different equations:

- the rectangular-coordinate form $Z = R + jX$
- the polar form as a magnitude and phase angle: $|Z| \angle \theta$.

The unit of impedance is the ohm (Ω) (Figure 13)
The concept of electrical impedance was first introduced by Oliver Heaviside the 1880s, while the development of vector diagrams and complex representation was done by A. E. Kennelly and C. P. Steinmetz. As impedance takes phase differences into account, it is a more general concept than resistance and it has become a fundamental and essential concept in electrical engineering (Barsoukov and Macdonald 2005).

### 3.5.2 Measuring impedance

In order to calculate impedance, it is necessary to measure at least two parameters due to the fact that impedance is a complex number. Modern impedance measuring instruments are usually programmed to measure the real and the imaginary parts of the impedance vector and then convert them into the desired parameters, making the measurement process as simple as connecting the unknown component to the impedance analyser.

However, in the real world components can exhibit a natural behavior that differs from their idealistic one. The real properties of one component will be defined as the combination of primary elements plus parasitics. At this point it is necessary to define three kinds of values:

- The **true** value is the value that excludes parasitics. It would be the mathematical relationship describing the component’s physical composition.

- The **effective** value takes into account the effects of a component’s parasitics and it is frequency dependent.

- The **indicated** value is the measured value obtained with the help of the measurement instrument. Indicated values contain errors depending on the accuracy of the instrument.
Studies on neural differentiation and monitoring with novel biosensor tools

The effective value is what we would like to measure, so that we would like that the indicated value is as close as possible to the effective value. By comparing the effective value with the indicated value under different conditions it is possible to judge the measurement’s quality. The indicated impedance value of a component is affected by measurement conditions. As a result some component dependency factors must be taken into consideration, such as frequency, test signal level, DC bias, temperature, humidity, magnetic fields, light, vibration and the age of the device (Agilent-Technologies 2009).

3.5.3 Impedance spectroscopy

Electrical Impedance spectroscopy is able to quantify cell environment macroscopic parameters by measuring the inherent electrical properties of individual cells. In theoretical terms, cells and the extracellular environment act as a simple electrical circuit. Cell cytoplasm and extracellular space are conductive (due to the presence of salt ions) and contribute to the resistive components, $R_i$ (intracellular) and $R_e$ (extracellular); while the cell membrane is isolating and contributes the capacitive effect, $C_m$ (Figure 14) (Kyle, Chan et al. 1999).

![Figure 14. Illustration of a simplified cell environment and a model of equivalent electrical circuit. This model takes in account membrane capacitance ($C_m$) intracellular ($R_i$) and extracellular ($R_e$) conduction. (Kyle, Chan et al 1999)](image)

Cell membrane serves to isolate the interior of the cell from the environment in order to maintain the cellular homeostasis. The membrane is impermeable to different substances including ions. The hydrophobic nature of lipid membranes makes them a perfect candidate to exclude charged particles. In the absence of any conductor (artificial membranes) the resistance across the membrane is in the order of hundreds of gigaoohms. A lipid membrane is therefore a very good resistor. In addition, a lipiddic membrane is also a very good capacitor, since it allows the
accumulation of charges on both sides of the membrane. The resistance depends mainly on membrane proteins, which transport charged molecules and ions across the membrane, whereas the lipid bilayer of the membrane acts as a capacitor by allowing the accumulation of charges. The difference of electric potential and the gradient of ions between the two sides of the lipid bilayer will determine the circuit driving force or electrochemical driving force (Jue 2009).

We can use the following model in order to explain the immobilized cell behavior: The matrix-cell sensor system can be considered as a series of n cell/matrix layers acting both as bipolars and as capacitors whereas the potential varies in relation to the total impedance between the measuring electrode and the reference electrode. Each immobilized cell has a conductance $G_{i,j}$ and a capacitance $C_{i,j}$ (where $i=1…n$ and $j=1…m$) depending on its position within the probe. In addition, and in particular at higher gel densities, there is an additional resistance $R$ of the matrix (gel and pores with solution). Thus the sensor resembles an RC circuit consisting of a group of $n$ capacitors serially connected to each other [model developed by Kintzios S and Simonian A. (Valero, Moschopoulou et al. 2010)].

We can calculate the impedance of the system assuming that an electric current in the system travels through the sensor in two ways: (1) through the cell (current entering and exiting the cell), and (2) through the extracellular environment. In this way, we can represent each part of the system as a “unit cell”, which consists of an individual cell and its environment (matrix + solution). We assume that a three-dimensional parallelepiped with dimensions $L_x$, $L_y$, $L_z$ is packed with such “unit cells”. We also assume, that the x dimension contains n such unit cells, and y and z dimensions contain m and k unit cells. Because all impedances through the x axis are connected in series, we may calculate the total impedance $Z_{tot}$ of such a circuit, taking into account that m and k impedances are connected in parallel:

$$Z_{tot} = \rho \frac{L}{S}$$

and $L = L_x$, $S = L_y L_z$. All information about the unit cell is contained in $\rho$, which may be presented as:

$$\rho = Z_1 \frac{\Delta y \Delta z}{\Delta x}$$

where $Z_1$ is the impedance of the unit cell and $\Delta y$, $\Delta z$, $\Delta x$ are the dimensions of the unit cell, where the actual (‘biological’) cell is located [model developed by Kintzios, S. and Simonian, A. (Valero, Moschopoulou et al. 2010)].
It is necessary to make an estimation of the frequencies where dispersion of impedance may occur. In general, there are three characteristic ranges of frequencies related to the polarization of biological objects. In the region of high frequencies (10 GHz) polarization of water is observed, middle frequencies (1 MHz) correspond to properties of cell membranes and low frequencies range (100 Hz) are related to the cell surface (Hölzel and Lamprecht 1994).
4 MATERIALS AND METHODS

4.1 Reagents

Neuro2a cell line was originally provided from LGC Promochem (Teddington, UK). Dulbecco’s Modified Eagle’s Medium (DMEM), L-Alanyl-L-Glutamine, Penicillin/Streptomycin, Pyruvate, Trypsin/EDTA, Hepes were purchased from Biochrom AG (Berlin, Germany). Foetal Bovine Serum (FBS), 2’,7’-dichlorodihydrofluorescein diacetate (H$_2$DCFDA), MitoTracker® Green MitoSOX, MitoTracker® Orange CMTMRos, MitoSOX® and UltraPure LMP Agarose were purchased from Invitrogen (Carlsbad, CA, USA). Mn (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP) was purchased from Cayman (Ann Arbor, MI, USA). Coomassie brilliant blue R, dimethylthiazol-2-2,5 diphenyl tetrazolium bromide (MTT) were provided by MP Biomedicals (Solon, OH, USA). Collagen, laminin, basement membrane extract (BME) were provided from Trevigen (Gaithersburg, MD) and Agar bacteriological from Scharlau (Barcelona, Spain). Finally, Forskolin, db-cAMP, Propidium iodide (PI), N$^G$-Methyl-L-arginine acetate salt (L-NMMA), Protein Kinase A inhibitor fragment 14-22, myristoylated trifluoroacetate salt (PKI), Ribonuclease A and all other reagents were provided by Sigma (St. Louis, MO, USA).

4.2 Biological methods

4.2.1 Cell culture

Murine neuroblastoma Neuro2a cells were grown in Dulbecco’s modified Eagle’s medium supplemented with L-Alanyl-L-Glutamine, penicillin/streptomycin, pyruvate and 10% fetal bovine serum at 37ºC in an standard incubator (95% humidity 5% CO$_2$) and subcultured three times per week. After determining the number of cells they were subcultured at the desired density depending on the experiment to be performed.

For 2D experiments, N2a cells were subcultured at 50% confluency. After waiting 24 hours for attachment and stabilization, cells were treated with serum free medium (SS), 10 µM forskolin (F), 0.3mM db-cAMP (A) in serum-free medium or serum supplemented medium for basal conditions (B) for 48 hours, unless otherwise specified. Inhibitors of the different signalling pathways were all pre-incubated for 30 minutes and co-incubated with the differentiating agents during the entire stimulation time. For 3D experiments cells were subcultured in different matrices as specified in 4.3.2, 4.3.4 and 4.3.5.

4.2.2 Coomassie brilliant blue staining

After two days in the presence or absence of differentiating agents (SS, F, A, as described above) cells were fixed and stained with coomassie brilliant blue as described previously (Flaskos,
McLean et al. 1998). Briefly, cells were fixed with 90% (v/v) methanol in PBS at -20 °C; fixed cells were stained for 1 min at room temperature with coomassie brilliant blue (1.25% coomassie brilliant blue R-250, 40% methanol, 20% acetic acid), washed with PBS and observed in an inverted microscope. Five random fields were taken per well giving a total cell count of at least 200 cells per well. Axon-like processes were defined as extensions greater than two cell body diameters in length. The number of cells bearing axon-like processes was related to the total amount of cells. Neurite length was measured with the help of the imageJ plugin NeuronJ (Meijering, Jacob et al. 2004).

4.2.3 Metabolic activity by MTT assay

Metabolic activity of differentiated cells was measured after 48 hours exposure to the respective treatments as described by (Nisoli, Clementi et al. 1998) with some modifications. After the treatments, cells were counted and 10<sup>5</sup> viable cells from each well were assayed with the MTT test. The constant amount of cells guarantees the avoidance of changes due to cell proliferation, while measuring the metabolic activity of the mitochondria. As described previously (Mosmann 1983) MTT was dissolved in PBS at 5 mg/mL stock solution. MTT (at a dilution 1 to 10) was finally added to all wells of an assay, and plates were incubated at 37°C for 30 min. Cells were centrifuged in order to collect the dark blue formazan crystals, which were subsequently dissolved in DMSO and the absorbance measured at 560 nm using a PowerWave 340 (Biotek, Winooski, VT, USA) spectrophotometer. Moreover, protein content was measured by the Bradford assay as a control of cell counting.

4.2.4 Fluorescence microscopy

N2a cells cultured in the presence or absence of serum and in the presence of 10 uM forskolin, 0.3 mM db-cAMP for 48 hours were stained with 50 nM MitoTracker Orange. Subsequently cells were washed twice with PBS and fluorescence microscopy pictures were taken using a Keyence BZ8000 digital microscope.

4.2.5 Confocal microscopy

N2a cells were seeded on poly-L-lysine-treated 10 mm diameter coverslips and after 48 hours of treatment with the aforementioned drugs, cells were stained with 200nM Mitotracker green, washed twice with PBS, fixed with 4% formaldehyde and mounted with Vectashield hard set mounting medium (Vector laboratories, Inc., Burlingame, CA) on glass slides for fluorescence detection using a confocal Leica TCS SP5 on a DMI 6000 inverted microscope with a 488 nm argon laser and a ×63 oil immersion objective. MTG was recorded through a band pass 507 to 553 nm filter.
4.2.6 Flow cytometry analysis.

N2a cells were cultured in the presence or in the absence of the above mentioned differentiation treatments (B, SS, F, A) and harvested in PBS, pH 7.4, supplemented with 1 mM EDTA. For cell cycle analysis, cells were fixed in 70% cold ethanol for 30 min at 4°C. Ethanol was removed by centrifugation and the cell pellet was washed twice in phosphate-citrate buffer, pH 7.8. Finally, cells were stained by adding 75 μM propidium iodide (PI) supplemented with 0.1 mg/mL Ribonuclease A. Mitochondrial mass, mitochondrial membrane potential, ROS concentration and superoxide concentration were assessed by staining with 70 nM MitoTracker Green (Baxter, Uittenbogaard et al. 2009), 50 nM MitoTracker Orange, 10 μM H$_2$DCFDA (Mackey, Sanvicens et al. 2008) and 5 μM MitoSOX (Mukhopadhyay, Rajesh et al. 2007), respectively. Briefly, cells were incubated with each dye for 30 minutes at 37°C and subsequently washed twice and harvested in PBS. Flow cytometry analysis was performed on a FACSCANTO II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Flow cytometry figures were prepared using the FlowJo software (Tree Star, Ashland, OR, USA). Mean fluorescence was determined from samples of 20,000 cells. Experiments were repeated at least four times.

4.2.7 Western blot

N2a cells treated for 30 minutes, 1 hour or 24 hours were washed twice and harvested in ice cold PBS. Cells were incubated with 100 μL of protein extraction buffer (20 mM Tris –HCl, 137 mM NaCl, 20 mM NaF, 1 mM sodium pyrophosphate dibasic, 1 mM Na$_3$VO$_4$, 1% (v/v) Triton X-100, 10% (v/v) Glycerol, 1 mM Phenyl methyl sulfonyl fluoride, 1 μg/mL Leupeptin hemisulfate salt) for 30 minutes on ice and protein quantification was performed following the Bradford assay (Bradford 1976). Equal amounts of proteins (40 μg) were loaded onto a 10% SDS-PAGE gel. Proteins were transferred to a polyvinylidene fluoride membrane (Perkin Elmer, MA, USA) and blocked for 1 hour in 3% Albumin Bovine Serum. After blocking, the membrane was incubated overnight at 4°C with Rabbit polyclonal PGC-1α antibody (1:1000) (Abcam, Cambridge, MA, USA), Anti-Actin antibody (1:200) (Sigma, St. Louis, MO, USA), primary rabbit polyclonal antibodies P-p44/42 ERK or p44/42 ERK (Cell Signaling Technology, Danvers, MA), respectively. Goat Anti-Rabbit Alkaline Phosphatase-Conjugated (Promega, Madison, WI, USA) or horseradish peroxidase-linked whole anti-rabbit IgG secondary antibody (GE Healthcare Buckinghamshire, UK) were used as secondary antibody (1:5000). Blots were developed with 5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt and nitro blue tetrazolium chloride (NBT/BCIP) stock solution (Roche, Basel, Switzerland) and scanned or detected by Pierce ECL 2 (Thermo Scientific, Rockford, IL, USA) and scanned using a Typhoon 9410 scanner (GE Healthcare Europe GmbH, Freiburg, Germany), respectively. Images were analyzed using the Doc-itLS software (UVP, Upland, CA, USA). β-actin or ERK1/2 were taken as loading control, respectively.
4.2.8  Griess reaction for nitrite detection

Nitrites were detected by the Griess reaction (Griess 1879) after 48 hours exposure to the respective treatments (B, SS, F, A). Briefly, cells were harvested and $10^5$ viable cells were sonicated (Kumar, Jyoti et. al 2009). The resulting supernatant was exposed to the Griess reagent (1% sulfanilamide, 0.1% N-(1-Naphthyl)ethylene-diamine, 5% phosphoric acid) and the absorbance measured at 540 nm using a PowerWave 340 (Biotek, Winooski, VT, USA). A standard curve ranging from 1μM to 30μM was parallelly monitored in order to extrapolate concentration values. Protein content was measured by the Bradford assay as a control of cell counting.

4.3  Novel biosensors and advanced techniques

4.3.1  NO sensor

Extracellular NO concentration changes in differentiating N2a neuroblastoma cells were measured with the help of a novel biosensor designed by Kuppusamy Aravindalochanan (Aravindalochanan, Kieninger et al. 2009; Aravindalochanan, Kieninger et al. 2011). Briefly, the amperometric sensor chip was inserted into a cell culture flask for on line monitoring NO concentrations in cellular cultures. The cell culture flasks were in turn inserted into a heating chamber at 37ºC connected to a potentiostat and the analysis software (Figure 15). Hepes (1 mM) was added to culture medium in order to maintain pH values. Then medium was changed after the overnight stabilization period for serum free medium in the presence of db-cAMP. NO concentration changes were measured during the entire period of differentiation.

Figure 15. NO sensor set up. (a) Amperometric chip inserted in a cell culture flask for live cell monitoring; (b) Culture flask inserted in the heating chamber and connected to a potentiostat and analysis software for on line NO sensing.
4.3.2 Immobilization of N2a cells in 3D matrices for impedimetric biosensor I

Cells were harvested from the flasks, counted with a Neubauer chamber and immobilized in a bactoagar (Scharlau, Barcelona, Spain) gel matrix. Bactoagar was diluted in deionised water at concentrations from 0.96% to 4.2% (w/v) in order to get final concentrations from 0.8% to 3.5% after a 4/5 dilution with DMEM. Gels were autoclaved for 20 minutes at 121°C and 1.2 atm and preserved from solidification at 37°C in a water bath. For immobilization in bactoagar gels, cells were centrifuged and concentrated in a total volume of 20 μL in 500 μL Eppendorfs, which were kept at 37°C in a block heater. 100 μL of bactoagar was then poured onto the cells so that gels were diluted (4/5) when mixed with the cell-containing DMEM. After gently mixing, gels containing cells were poured on the desired device or plate. For the preparation of gel films for impedance analysis, gels were poured on the working electrode and before solidifiction a porous ground electrode was placed on the top, pressed and the excess of gel cut off. Electrodes were then pressed onto a 24-well plate, turned upside-down and stored at 37°C and 5% CO₂ in a standard incubator until the experiments were carried out (Figure 16).

Figure 16. Preparation steps of bactoagar gels with N2a-embedded cells. (a) Bactoagar gels containing cells are poured on to the cylindrical measurement electrode. (b) Stainless steel porous ground electrode is pressed on the top; excess of gel comes through the holes. (c) Detail picture of one electrode filled with bactoagar. (d) Electrodes are pressed on a 24-well plate with 1mL DMEM. (e) Plate turned upside down to allow the medium to be in contact with the gel. (f) Detail picture of the gel after removing the top electrode.
4.3.3 Impedimetric biosensor I

In collaboration with the Otto von Guericke Universität Magdeburg (OvG-UNIM) a novel impedimetric biosensor was developed by Thomas Jacobs (Jacobs, Valero et al. 2009), which is able to measure electrical impedance spectroscopy of thin gel films with embedded cells in the range between 10 and 110 MHz. The device mainly consists of a cylindrical measurement electrode and a porous ground electrode, between which the gel films are placed. Both electrodes are made of stainless-steel; the cylindrical measurement electrode is pressed into a PTFE cylinder to form a cavity where the cell-containing gel is placed. The electrodes are inserted into a bioreactor (Figure 17).

Medium flows on the surface of the ground electrode. The holes on this ground electrode allow perfused medium and stimuli to diffuse into the gel, while properties of the liquid phase do not contribute to the measurement signal (Figure 18). An external flow injection system based on standard HPLC equipment combined with a syringe pump was used for the perfusion of media, see, e.g. cell culture medium with and without ACh. The T-valve (V-L-1) allows the selection of the stimulus and avoids the transfer of bubbles and contaminants into the system. After exchanging syringes at V-L-1 a small quantity of the liquid is dispensed to the waste bottle before connecting it to the bioreactor. The experimental protocol involves the following major steps: (0) inserting a sensor that is not used for measurement, (1) filling, cleaning and sterilizing of the system with detergents, (2) flushing with full medium, (3) inserting the sensor used for measurement, (4)
flushing with full medium, (5) placing the chamber in the heating jacket, (6) starting the pump with medium flow and impedance acquisition, (7) waiting ~2 h until a stable base line is present, (8) dispensing of the stimulus in a time range of several hours.

Figure 18. Impedance biosensor I perfusion system. (a) Cross section of the bioreactor configuration (GCC). Medium flows through the inlet, in parallel to the porous ground electrode, which allows diffusion and lately flows out through the outlet. Impedance is measured between ground and measurement electrode. Thus liquid properties do not directly interfere with the measurement. (b) Injection flow system. Flow through configuration for perfusion of the electrode chamber. GCC, Gel Check Chamber; V-L-1, V1, valvs; W1, fresh medium bottle; W2, waste bottle (by Thomas Jacobs).

4.3.4 Immobilization of N2a cells in 3D matrices for the development of a three dimensional tissue-like model.

Cells were collected with trypsin/EDTA, centrifuged 2 min at 1000 rpm and resuspended in 1 % (w/v) agarose or 1 % (w/v) agar bacteriological (bactoagar) at 37 °C using a block heater. BME gels were prepared according to the manufacturer’s instructions. Immobilization in collagen (CGs), and in collagen-laminin gels (CLGs) was performed according to the manufacturer’s instructions with the following modifications. The final composition of the selected CGs and CLGs used was: 10 % cells suspended in DMEM, 50 % Collagen, 2 % NaOH 3N, 1.5 % HEPES (Brown, MacLellan et al. 2008) 1M, 6.2 % DMEM 10x, 2.3 % H2O, 28 % DMEM or 28 % laminin respectively. BME, CGs and CLGs were prepared in an ice bath to avoid solidification. 50 uL of each mixture were poured into 96-well plates and after solidification 200 uL of DMEM were added to each well. The final cell concentration for gel comparison experiments was 1 x 10^6 cells/mL. Gels were stored at 37 °C and 5 % CO2. After 24 hours of culture, determination of differentiation in 3D gels was performed by inverted optical microscopy.
4.3.5 Immobilization of cells in the impedimetric biosensor II

CGs and CLGs were prepared as described in the previous chapter. 30 μL CGs or CLGs were poured inside the electrode-cavities. The final cell concentration for impedance experiments was $2 \times 10^6$ cells/mL. The gel dispensing procedure was optimized in order to obtain uniform and complete filling of the wells: Gels were dispensed between the measurement and ground electrodes through the tiny ground electrode pores with the help of a 100 μL pipette with an extra thin tip. This procedure ensured a homogeneous coverage of all measurement electrodes. The micro-fluidic channel piece was set on the top and all three pieces inserted into a custom made stainless steel bioreactor and kept in a standard incubator at 37 ºC and 5% CO$_2$ until the experiment was performed. After each experiment residues of biological material were removed with a concentrated solution of trypsin and sterilized by EtOH and 20 minutes UV exposure.

4.3.6 Impedimetric biosensor II

For the electrical impedance analysis of differentiating N2a cells in a three dimensional culture an impedimetric biosensor with an electrode array designed by Thomas Jacobs (University of Magdeburg) was used. The device, as shown in Figure 19 consists of 16 gold electrodes on a glass slide, which were deposited by a standard sputtering technique, first a chromium layer followed by a gold layer. An adhesive 1 mm thick silicone foil on top containing 5 mm circular wells defines the shape of the gel films for impedance measurement. The larger diameter of the circular wells compared to the electrode diameter guarantees a constant active measurement area, defined by the uniformity of the gold electrodes. Proper adhesion of the silicon foil to the slide was achieved by applying high temperature and pressure during 24 hours. A thin sheet of stainless steel placed on the top side serves as grounded reference electrode. Thus, gel films poured into each well are squeezed between both electrodes for a proper electrical contact and a well defined gel thickness. Multiple small holes in the stainless steel sheet enable medium diffusion into the gels while four micro-fluidic channels made of a silicon foil on a glass slide allow the perfusion of the cells with culture medium and stimuli. Electrodes are divided by the micro-fluidic channels into four groups, with four electrodes each. For the electrical impedance analysis of neural differentiation, N2a cells were immobilized in a CLG matrix on top of the gold electrodes of the impedimetric sensor. After preparation of the gels in the wells and solidification of the gel in the incubator, the stack as shown in Figure 19 was pressed for proper sealing. The array was inserted into a novel custom-made stainless steel microfluidic bioreactor with four inlets and four outlets which allow the perfusion of the four chambers with different media.
Figure 19. Impedimetric biosensor electrode set-up. Impedimetric biosensor consisting of 16 gold electrodes on a glass slide and a silicone foil on top with circular wells for the gel matrix and a porous reference electrode on top for impedance measurement with four micro-fluidic channels. It is inserted in a stain less steel bioreactor with four inlets and four outlets on top for medium perfusion and 16 holes for pin connectors on the bottom (by Thomas Jacobs).

Subsequently the bioreactor was connected to an external flow injection system consisting of a syringe pump and conventional HPLC equipment and inserted into the heating jacket at 37 °C. DMEM without serum was dispensed at a constant flow rate of 0.5 mL/h into the micro-fluidic channel (Figure 18b). Electrodes were connected by means of a custom made multiplexer to a network analyzer in impedance measurement mode (Agilent 4395A) for impedance measurement of the gel matrix embedded cells in the wells. The output power was fixed to -10dBm. Impedance spectra were acquired in the range of 100 kHz to 50 MHz at a rate of 1 spectrum/min between the signal electrodes and the porous ground electrode. This approach guarantees the penetration of the electrical field through the whole film and the electrical shield of the gel film from the liquid phase. The changes in the dielectric and conductive properties of the bare gel and the cell membrane as well as cellular action potential changes contribute to the sensor response. Multiple electrodes in parallel in a single group enable the detection of enclosed gas bubbles or partly uncovered electrodes due to an improper filling of a well. The overall stability of the measurement system in terms of online impedance analysis is mainly limited by temperature stability. With an appropriate heating jacket and a thermostat we have achieved stability against thermal fluctuations as low as 0.1 K. Experiments were performed in quadruplicates and data were processed and analyzed using MATLAB (The Mathworks, Natick, MA, USA).
4.3.7 Viability assays for 3D cultures

Two different viability assays were used which are not able to differentiate between apoptosis and necrosis, but are suitable for testing viability in 3D matrixes:

4.3.7.1 Trypan blue staining

Gels were exposed to a 0.5% (w/v) trypan blue solution and the number of dark blue and white cells was counted with the help of a phase contrast microscope. Dark blue cells were considered dead, whereas the rest were considered to be alive. In order to be able to focus the microscope and count the cells in a 3D matrix, gels were mounted between slide and coverslip and pressed, so that a 2D focus plane/shot was achieved.

4.3.7.2 MTT viability test. Modifications for 3D gels

MTT viability tests were performed as described previously (Mosmann 1983) with the following modifications. MTT (3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyl tetrazolium) was dissolved in PBS at a concentration of 5mg/ml and filtered. Dilutions 1/10 were applied for 30 minutes in 2D cultures and 2 hours in 3D cultures and absorbance was measured at 560nm with the help of a spectrophotometer (Powerwave 340, Biotek). As in some experiments cells were immobilized in 3D matrices, some problems needed to be addressed such as the possible absorbance given by the gel or the need to dilute in DMSO the formazan salt present inside the gel. The first problem was solved by the use of blanks consisting of bare gels in every experiment. For the second problem we utilized the solubility of bactoagar in DMSO. Both bactoagar gel and cells containing the formazan salt were thus diluted in DMSO, giving rise to a homogeneous solution. In the case of CL gels MTT formazan was extracted by vortexing the samples in DMSO. This made it possible to measure differences in absorbance due to the amount of viable cells living in the gel.

4.4 Statistical analysis

Statistically significant differences between groups were determined by ANOVA and followed by Newman Keuls post hoc test to compare all pairs of columns using GraphPad Prism for Windows, (GraphPad Software, San Diego California USA, www.graphpad.com). The level of statistical significance was taken at p<0.05.
5 RESULTS

5.1 Neural differentiation and mitochondrial biogenesis

5.1.1 Serum starvation, forskolin and db-cAMP promote neuronal differentiation and cell cycle arrest of N2a neuroblastoma cells.

In order to establish a stable differentiation model, murine neuroblastoma N2a cells were subjected to different stimuli and different stimulation periods (data not shown). These included 48 hour-long exposure to serum starvation (SS), 10 µM Forskolin (F) and 0.3 mM db-cAMP (A), both in the absence of serum. They were selected because of their potency in inducing differentiation compared to basal conditions (B) in this particular cell type. As observed in Figure 20a-b, serum starvation for 48 hours promotes an increase in the number of cells bearing neurites of approximately 35%. Differentiation was further stimulated to 50% and 55% if cells were treated with forskolin or db-cAMP in serum starvation conditions for 48 hours, respectively. Serum starvation was found to increase per se differentiation of N2a cells, while the exposure of cells to the same stimuli in the presence of serum did not show any differentiation effect (data not shown). Neurite length measurement was possible by the use of the ImageJ software with the neuronJ plugin, which is able to measure neurite length after calibration with a proper scale. Neurite length means were found to be dramatically elevated in cells treated with forskolin or db-cAMP, whereas serum starvation per se promoted only a moderate growth of the neurites (Figure 20c). In order to check if differentiation was associated with cell cycle arrest of this neuroblastoma cell line, cell cycle analysis was performed by means of propidium iodide staining and flow cytometry. The results, presented in Figure 20d, showed an increase in the G₀G₁ phase of the cell cycle and a reduction of the S and G₂M phases, suggesting that cell cycle is indeed arrested in N2a cells by serum starvation, forskolin and db-cAMP. It is noteworthy that the three differentiating treatments produced a similar cell cycle arrest, in contrast to the differential promotion of neurite growth promoted by the different treatments.
Figure 20. N2a differentiation and cell cycle arrest in response to serum deprivation and its combination with forskolin and db-cAMP. N2a cells treated for 48 hours with: (B) Basal conditions, (SS) serum starved cells and cells incubated with (F) 10 µM forskolin or (A) 0.3 mM db-cAMP under serum starvation conditions. (a) Images of N2a cells stained with coomassie brilliant blue, scale bar, 25 µm; (b) percentage of differentiated cells; (c) average neurite length and (d) representative overlay of N2a cell cycle analyzed by PI staining followed by flow cytometry after 48 hours treatment. Data correspond to the mean ± S.E.M. of eight different batches of cells. ***p<0.001 as compared to basal conditions (B); ###p<0.001 as compared to SS.
5.1.2 Mitochondrial mass increases in the early stages of neural differentiation

Based on previous studies on different tissues where pharmacological activation of cAMP-related signalling pathways produced mitochondriogenesis (Bogacka, Ukropcova et al. 2005) we formulated the hypothesis that cAMP-mediated differentiation of N2a neuroblastoma cells is related to mitochondrial biogenesis. In order to prove this hypothesis, N2a neuroblastoma cells subjected to the above mentioned differentiating treatments were stained with MitoTracker Green (MTG) and analyzed by flow cytometry. We used the fluorescence intensity emitted by MTG as an estimate of the overall mitochondrial mass, since it accumulates in the mitochondria in a membrane potential-independent manner (Baxter, Uittenbogaard et al. 2009). The results revealed an increase in mitochondrial mass following 48 hours of exposure to serum starvation, forskolin and db-cAMP (Figure 21) to at least twice the basal values for all the treatments whereas small differences were found among the three differentiating treatments (SS, F and A).

Figure 21. Mitochondrial mass increases during neural differentiation. (a) MTG fluorescence overlay of representative populations as measured by flow cytometry of 48 hour-treated N2a cells; (b) Relative median MTG fluorescence intensity from 20000 cells treated for 48 hours; (B) Basal conditions, (SS) serum starved cells and cells incubated with (F) 10 µM forskolin or (A) 0.3 mM db-cAMP under serum starvation conditions. Data correspond to the mean ± S.E.M of at least four different batches of cells. **p<0.001 as compared to undifferentiated cells (B); #p<0.05, ###p<0.001 as compared to SS.
5.1.3 Mitochondria are redistributed towards the neurites

In order to further explore the phenomenon of mitochondrial biogenesis in differentiating N2a cells in terms of mitochondrial morphology and distribution, confocal microscopy pictures of MTG-stained N2a cells subjected for 48 hours to the different treatments (B, SS, F, A) were taken. Representative pictures of one single Z-stack layer are showed in (Figure 22). Pictures of four independent experiments did not allow the observation of changes in mitochondrial morphology among the treatments. However, a redistribution of mitochondria towards the neurites can be clearly observed.

![Figure 22. Mitochondria are redistributed towards the neurites during N2a differentiation.](image)

5.1.4 Newborn mitochondria do not loose membrane potential. Mitochondrial oxidative activity seems to increase parallel to mitochondrial mass.

The fact that one cell suffers an increase in its mitochondrial mass does not mean that mitochondrial activity is elevated in this cell. Different phenomena can influence mitochondrial performance such as membrane depolarization. In order to find out if this phenomenon was taking place, we performed experiments in the presence of Mitotracker orange (MTO), similar to those for MTG. Unlike MTG, MTO is sensitive to variations in the mitochondrial membrane potential. Furthermore, MitoTracker Orange CM-H2TMRos, the non-fluorescent reduced form of tetramethylrosamine, is oxidized by molecular oxygen in actively respiring cells. This phenomenon is considered to allow measuring the oxidative activity of the mitochondria (Agnello, Morici et al. 2008). We used MTO fluorescence as an estimate of the healthiness of the mitochondria. Cells treated for 48 hour with the differentiating treatments (serum starvation, forskolin and db-cAMP) and control cells (basal) were stained for MTO and measured by flow cytometry. The results, as depicted in Figure 23, denoted a similar staining to that obtained with MTG, being the values from the three differentiating treatments significantly above the basal values. In our understanding, these results suggest that newly synthesized mitochondria are healthy and present a regular
membrane potential. However, the results obtained with MTO must be interpreted with caution, since it has been reported that MTO is highly sensitive not only to changes in membrane potential or oxidative activity but also to the presence of free radicals (Buckman, Hernandez et al. 2001). Therefore, the values obtained with MTO were not considered as a quantitative measure of mitochondrial membrane potential but as an estimate of the healthiness of mitochondria. MTO stained-N2a cells were also observed by fluorescence microscopy. Representative pictures were selected and presented in Figure 23. Although it was not possible to reach the resolution of the confocal microscope, the same phenomenon of mitochondrial redistribution was observed by a conventional fluorescence microscope on MTO stained cells.

Figure 23. Mitochondrial membrane potential is maintained in the newly synthesized mitochondria. Cells were stained with MitoTracker Orange and fluorescence was measured by (a) fluorescence microscopy and (b,c) flow cytometry. (a) Representative fluorescence pictures of the different treatments. Arrows point to the mitochondria redistributed on the dendrites. (b) Superposed image of a single representative flow cytometry experiment. (c) Relative MTO fluorescence intensity. (B) Basal conditions, (SS) Serum starvation, (F) serum starvation in the presence of forskolin (10 µM) and (A) serum starvation in the presence of db-cAMP (0.3 mM). Data correspond to the mean ± S.E.M. of 4 experiments of different batches of cells. **p<0.01, compared to undifferentiated cells (B); #p<0.05, compared to SS.
5.1.5 **Metabolic activity increases parallel to mitochondrial biogenesis and differentiation**

In order to further test if new mitochondria were metabolically active, the metabolic activity of differentiated N2a cells was tested by means of a modification of the MTT assay. Equal numbers of cells ($10^5$ cells) were constantly collected from each treatment and MTT was added to the culture medium. This approach allows us to avoid any variation in MTT due to changes in cell proliferation or cell cycle arrest. Moreover, protein content was measured as a control of cell counting. As shown in Figure 24, serum starvation and the addition of forskolin or db-cAMP elicited an increased MTT absorbance, i.e. an increased metabolic activity. This increase is parallel to the increase in MTO staining detected by flow cytometry. Thus, after 48 hours of exposure to serum starvation, forskolin and db-cAMP in the absence of serum we found an increase in the mass of healthy mitochondria whose electron transport chain is metabolically active.

*Figure 24. Newly synthesized mitochondria are metabolically active.* MTT absorbance derived from $10^5$ N2a cells subjected for 48 hours to the following treatments: (B) Basal conditions, (SS) serum starvation, (F) serum starvation in the presence of forskolin (10µM) and (A) serum starvation in the presence of db-cAMP (0.3mM). Data correspond to the mean ± S.E.M of 8 experiments of different batches of cells. **p<0.001 as compared to undifferentiated cells (B); ##p<0.01, ###p<0.001 as compared to SS.*
5.2 Intracellular signalling pathways leading to mitochondriogenesis during N2a differentiation

5.2.1 PGC-1α is induced during differentiation

In order to elucidate the intracellular molecular pathways used by serum starvation and its combination with forskolin or db-cAMP to induce mitochondrial biogenesis, the first focus was on the transcriptional coactivator PGC-1α, which has been characterized as the master regulator of mitochondrial biogenesis due to its ubiquity (Onyango, Lu et al. 2010). It has been described that an overexpression of this transcriptional coactivator is directly related to an increase in mitochondrial mass and to have an integrative role in mitochondriogenesis (Scarpulla 2011). Furthermore, a well known pathway that promotes PGC-1α expression includes cAMP signalling, which is simulated by forskolin and db-cAMP. Therefore, PGC-1α appears as a good candidate for mediating the mitochondriogenesis observed in N2a cells. Consequently, the relative expression of PGC-1α was determined by western blot with a polyclonal antibody against the carboxyl tail of the protein. After determination of the maximum expression time of PGC-1α (data not shown), PGC-1α expression was measured at 24 hours of drug exposure and data were analyzed by densitometry. Two bands were detected to be increased upon the treatments (Figure 25). The expected molecular weight of PGC-1α is 92kDa. However, since this protein can suffer a variety of posttranslational modifications and alternative splicing the observed position in the western blot membrane may vary. According to the literature the upper band corresponds to the full length PGC-1α, whereas the stronger lower band corresponds to one shortened form of PGC-1α, as also observed by other authors in different models (Baar, Wende et al. 2002; Aquilano, Vigilanza et al. 2010; Tadaishi, Miura et al. 2011).

Figure 25. PGC-1α is overexpressed in differentiating N2a cells. Representative PGC-1α immunoblot corresponding to protein extracts from 24 hour-treated cells, β-actin was taken as loading control. (B) Untreated cells in standard DMEM supplemented with 10% FBS, (SS) serum starvation, (F) serum starvation in the presence of forskolin (10µM) and (A) serum starvation in the presence of db-cAMP (0.3mM).
Studies on neural differentiation and monitoring with novel biosensor tools

We also observed other weak bands around the full length PGC-1α, whose relevance remains to be elucidated. PGC-1α expression was significantly increased in differentiating N2a cells treated both with forskolin and db-cAMP in the absence of serum. Serum starvation by itself was able to induce only a moderate increase in PGC-1α, in accordance with all the aforementioned results.

5.2.2 Involvement of PKA in mitochondrial biogenesis and differentiation

In the search of possible upstream mediators of mitochondrial biogenesis, PKA appeared as a good candidate since db-cAMP and forskolin are well known activators of this pathway. In addition to this, the PKA pathway has has been shown to play a role in the expression of the oxidative phosphorylation genes (De Rasmo, Signorile et al. 2010) and CREB-mediated PGC-1α expression (Wallace and Fan 2010). With the aim of discovering if PKA is involved in differentiation and mitochondrial biogenesis, cells were pre-incubated for 30 minutes with 10 μM H89, a reversible inhibitor of PKA, and co-incubated for 48 hours in the presence or absence of differentiating agents (B, SS, F, A). Mitochondrial mass and differentiation percentage of N2a cells subjected to the four different treatments in the presence and in the absence of H89 were detected. Inhibition of PKA by H89 not only did not reverse either mitochondrial mass increase or differentiation (Figure 26), but even increased them in most of the treatments. Especially noteworthy is the ability of H89 to increase differentiation even in the presence of serum. These data can be explained by the documented poor selectivity of H89 and other PKA inhibitors (see discussion) For this reason, in order to have a clear picture about the role of PKA on mitochondrial biogenesis and differentiation in N2a cells we decided to use a different inhibitor. PKI, which is the natural PKA ligand, is considered to be highly specific for PKA (Murray 2008). The effects of pre- and co-incubation with 4 μM PKI on differentiation and mitochondrial mass were assessed by means of coomassie brilliant blue staining and flow cytometry, respectively (Figure 27). A slight decrease of both parameters can be observed. Although this decrease is not statistically significant, the participation of PKA in both differentiation and mitochondrial biogenesis can not be totally discarded. In other words, although we cannot discard a small PKA contribution, this contribution is not one of the main pathways that lead to mitochondriogenesis and differentiation in this particular cell type.
Figure 26. PKA inhibition by H89 does not reverse differentiation or mitochondrial biogenesis but even increases them. (a-d) Representative pictures after coomassie brilliant blue staining, (e) percentage of differentiated cells, (f-i) MTG fluorescence overlay of representative populations as measured by flow cytometry and (j) MTG relative fluorescence intensity of N2a cells subjected to 48 hours treatment with: (B) Basal conditions, (SS) serum starved cells and cells incubated with (F) 10 µM forskolin or (A) 0.3 mM db-cAMP under serum starvation conditions in the absence (CTR) or in the presence of 10 µM H89. Data are means ± SEM of at least four different batches of cells. ns, not significant; *p < 0.05, ***p < 0.001, significantly different in comparison to the controls (CTR).
Figure 27. PKA inhibition by PKI does not reverse differentiation or mitochondrial mass increase. (a-d) Representative pictures after coomassie brilliant blue staining, (e) percentage of differentiated cells, (f-j) MTG fluorescence overlay of representative populations as measured by flow cytometry and (j) Relative MTG median fluorescence intensity from N2a cells subjected to 48 hours treatment with: (B) Basal conditions, (SS) serum starved cells and cells incubated with (F) 10 µM forskolin or (A) 0.3 mM db-cAMP under serum starvation conditions in the absence (CTR) or in the presence of 4 µM PKI. Data are means ± SEM of at least four different batches of cells. ns, not significant differences found in comparison to the controls (CTR).
5.2.3 Involvement of MAPK in mitochondrial biogenesis and differentiation

The second candidate that would be able to phosphorylate CREB and promote PGC-1α overexpression is the MAPK pathway. Following the same protocols as previously described, this pathway was studied by means of the pharmacological inhibition of one member of this cascade. Cells were pre-incubated for 30 minutes with 50 µM PD98059, an irreversible inhibitor of MEK 1/2 and co-incubated for 48 hours with PD98059 and the four different stimulation treatments (B, SS, F, A). Subsequently, mitochondrial mass and differentiation percentages were measured by means of MTG staining followed by flow cytometry and Coomassie brilliant blue staining and counting, respectively.

Figure 28 shows that PD98059 almost completely reversed the effect of serum starvation, forskolin and db-cAMP in serum starvation conditions on neural differentiation, as detected by Coomassie brilliant blue staining. Cell morphology appeared to be very similar to basal conditions. In the same time, PD98059 was able to partially reverse the effect of serum starvation, forskolin and db-cAMP on mitochondrial mass. These results suggest that the MAPK is the key pathway involved in both differentiation and mitochondrial biogenesis in N2a cells but probably not the single pathway involved in the effect of SS, F or A on mitochondriogenesis, since mitochondrial mass increases were not totally reversed by PD98059. It is noteworthy that no effect of PD98059 was found when pre- and co-incubated with basal conditions (in the presence of serum) in comparison to basal, indicating that the inhibition of MEK1/2 by PD98059 did not affect basal ERK activation.
Figure 28. MEK1/2 inhibition by PD98059 reverses both neural differentiation and the increase in mitochondrial biomass. (a-d) Representative pictures after coomassie brilliant blue staining, (e) percentage of differentiated cells, (f-i) MTG fluorescence overlay of representative populations as measured by flow cytometry and (j) Relative MTG median fluorescence intensity from N2a cells subjected to 48 hours treatment with: (B) Basal conditions, (SS) serum starved cells and cells incubated with (F) 10 µM forskolin or (A) 0.3 mM db-cAMP under serum starvation conditions in the absence (CTR) or in the presence of 50 µM PD98059 (PD). Data are means ± SEM of at least four different batches of cells. ns, not significant , ***p < 0.001 significantly different from controls (CTR).
5.2.4 ROS are increased in differentiating cells

As mitochondria are the major cellular producer of reactive oxygen species (ROS) within the cell as well as the major antioxidant producer, the presence of an increased mitochondrial mass should affect free radical production and buffering. This section focuses on the involvement of free radicals on the differentiation and mitochondrial biogenesis promoted by serum starvation, forskolin and db-cAMP in N2a neuroblastoma cells. We examined whether the incubation of N2a cells with the above mentioned differentiating agents (SS, F, A) could induce ROS production compared to control conditions (B). Cells were incubated for 30 minutes, 24 hours or 48 hours with the different treatments, stained with 10 µM H$_2$DCFDA, and subsequently green fluorescence intensity of 20000 cells was measured by means of flow cytometry. As observed in Figure 29, serum starvation, forskolin and db-cAMP induced an increase in reactive oxygen species compared to basal conditions (B). This increase was apparent in as early as 30 minutes after exposure to the differentiating agents and further increased during the next 24 hours, while after that ROS levels were maintained for the rest of the experiment. The fact that ROS appeared during the first 30 minutes of exposure to the drugs suggests that ROS are probably part of a signalling cue. Once again, serum starvation promoted only a moderate increase in comparison to the two other inducers, in accordance with the aforementioned results, further supporting the hypothesis of the existence of an inherent link between mitochondrial biogenesis and differentiation. These two hypotheses will be further investigated in the following sections.

Figure 29. ROS are increased during differentiation. (a-c) Overlay of representative flow cytometry curves and (d) relative median fluorescence intensity of 10 µM H$_2$DCFDA-stained N2a cells, subjected to the following treatments during 30 minutes (30 min), 24 hours (24h) and 48 hours (48h): (B) Basal conditions, (SS) serum starved cells and cells incubated with (F) 10 µM forskolin or (A) 0.3 mM db-cAMP under serum starvation conditions. Data are means ± SEM of at least four different batches of cells. *p<0.05, **p < 0.01, ***p<0.001 as compared with time-matched basal conditions (B).
5.2.5 Role of free radicals in differentiation, cause or effect?

In order to find the answer for this question, the degree of differentiation was tested in the presence and in the absence of N-Acetyl-cysteine (NAC), a very well known free radical scavenger. If the effect of the forskolin or db-cAMP would be mediated by free radicals the pre- and co-incubation with NAC should reverse their effect. As shown in Figure 30a-b, NAC could partially but significantly reverse the effect promoted by forskolin and db-cAMP on neural differentiation. In order to confirm that NAC is able to work effectively as a ROS scavenger at the concentration (10mM) and the conditions of the present study (48 hours experiments), control flow cytometry experiments in the presence and in the absence of NAC were performed with the help of the fluorescent dye H$_2$DCFDA. Forskolin was selected for control experiments since it presented the maximal production of ROS at all the tested exposure times. Indeed, as observed in figure 30c, NAC was able to reduce ROS production by forskolin to a very large extent, though not completely. These data suggest that free radicals partially mediate the process of differentiation and they seem to be necessary to complete the differentiation process.

![Figure 30. Free radical scavenging by NAC reverses neural differentiation.](image)

(a) Representative pictures after coomassie brilliant blue staining, (b) percentage of differentiated N2a cells subjected to 48 hours treatment with: (B) Basal conditions, (SS) serum starved cells and cells incubated with (F) 10 µM forskolin or (A) 0.3 mM db-cAMP under serum starvation conditions in the absence (CTR) or in the presence of NAC. (c) Representative curve of a control experiment indicating the ability of NAC to reduce forskolin-induced ROS production in H$_2$DCFDA-stained cells as detected by flow cytometry. Data are means ± SEM of at least four different batches of cells. ns, ###p<0.001 significantly different from controls (CTR).
Based on these results, the question that arises is: Which free radicals are involved in this mediation? Two species were selected because their documented relation to differentiation signalling cues: Nitric oxide (NO) and superoxide anion ($O_2^-$).

5.2.6 Nitric oxide detection in N2a differentiated cells

The effect of pharmacological stimulation of N2a differentiation on nitric oxide production was detected by two different techniques: The Griess reaction for nitrite detection and a novel superoxide sensor for live NO detection.

The Griess reaction (Griess 1879) can detect the concentration of nitrites in the culture medium or, as in this case, in sonicated cells (Kumar, Jyoti et al. 2009). Nitrites are the stable residues after the conversion of NO, which is an unstable free radical. The Griess reaction is an indirect method to acquire endpoint measures of stable nitrite concentrations, as an indicator of the presence of the unstable NO during the course of the experiment. After 48 hours exposure to the different treatments (B, SS, F, A), cells were counted, lysated by sonication and nitrite concentration was measured by the Griess reaction. Although it could hardly be observed a slight tendency to increase in the F treatment, no significant increase in nitrite concentration was detected by this technique for any of the treatments in relation to control conditions (B), whereas the incubation of the cells for the same time with 30 µM sodium nitroprusside (SNP), a nitric oxide donor, was effectively detected by the method (Figure 31a).

![Figure 31. Indirect and direct nitric oxide detection.](image)

(a) Detection of nitrites on cell lysates by the Griess reaction after 48h in the presence of the following treatments: (B) basal conditions, (SS) serum starvation, (F) 10 µM forskolin, (A) 0.3 mM db-cAMP or (SNP) 30 µM sodium nitroprusside in serum starvation conditions. **p < 0.01, significantly different from untreated cells, ns, non significant. (b) Live detection of nitric oxide (NO) by a novel NO sensor (Aravindalochanan, K). After overnight culture stabilization (A) db-cAMP in serum starvation conditions was added to the cells for one or two days, respectively, and compared to control conditions (B). Normalized data of 3 experiments.
The second technique used to detect NO is a novel specific amperometric nitric oxide sensor developed by Kupps Aravinda in the University of Freiburg (Aravindalochanan, Kieninger et al. 2009; Aravindalochanan, Kieninger et al. 2011). This ultra-sensitive sensor can take live measurement of the concentration of NO diffused from live cells. Although a small peak in the concentration of NO seems to appear in one of the experiments (Figure 31b), the addition of db-cAMP in serum starvation conditions did not produce a significant effect in the concentration of NO, at least not bigger than the culture stabilization could produce. These results suggest that NO is not increased in differentiating N2a cells.

5.2.7 Nitric oxide does not appear as a main mediator of mitochondrial biogenesis or differentiation

Although no detectable increase in NO was found, we decided to check its possible role on differentiation and mitochondrial mass increase before discarding it completely as a potential mediator. Nitric oxide production can be suppressed by inhibiting its synthesizing enzyme, nitric oxide synthase (NOS), with a specific inhibitor. In this case, we used L-NMMA, a broad spectrum NOS inhibitor that inhibits all the different isoforms of NOS (Boer, Ulrich et al. 2000). If NO was a mediator in the differentiation process, we would observe a reversion in the differentiation profile. L-NMMA was pre- and co-incubated with the four different treatments and its effect was measured by means of coomassie brilliant blue and counting as well as by means of MTG staining and flow cytometry detection. As it is observed in Figure 32, the differentiation percentage of N2a cells was not altered in most of the treatments, with the exception of db-cAMP that suffered a moderate reduction of differentiated cells. We could attribute this reduction to a side effect of the combination of the two drugs since it is the only treatment that suffered a reduction. We therefore considered the effect of L-NMMA on differentiation in the present conditions as negative, although a deeper study would better clarify this issue. In a similar manner L-NMMA was tested by means of MTG staining and detection by flow cytometry. As showed in Figure 32, no reduction in the fluorescence emitted by MTG was observed under treatment with L-NMMA. As expected, no effect on mitochondrial biogenesis was observed, nor an apparent effect on differentiation. These results are in accordance with the above mentioned findings, in which no increase in nitrite concentration or NO was observed. Thus our data suggest that nitric oxide is not a main mediator in the process of N2a differentiation.
Figure 32. NOS inhibition by L-NMMA does not reverse neural differentiation or the increase in mitochondrial biomass. (a-d) Representative pictures after coomassie brilliant blue staining, (e) percentage of differentiated cells. Data are means ± SEM of at least four different batches of cells. ***p<0.001 significantly different from basal (B); ##p<0.01 significantly different from paired control (CTR); (f-i) MTG fluorescence overlay of representative populations as measured by flow cytometry and (j) Relative MTG median fluorescence intensity from N2a cells subjected to 48 hours treatment with: (B) Basal conditions, (SS) serum starved cells and cells incubated with (F) 10 µM forskolin or (A) 0.3 mM db-cAMP under serum starvation conditions in the absence (CTR) or in the presence of 300 µM L-NMMA.
5.2.8 Superoxide anion is increased in the early stages of differentiation

In order to check if the overall increase in ROS was due to an increased production of superoxide anion by the mitochondria, the fluorescent dye MitoSOX red was employed to selectively detect superoxide at the mitochondrial level (Mukhopadhyay, Rajesh et al. 2007). Subsequently, MitoSOX detection was performed by means of flow cytometry on 20000 cells previously subjected to the four different treatments (B, SS, F or A) for 30 minutes, 24 hours or 48 hours. As observed in (Figure 33), serum starvation, forskolin and db-cAMP promoted an increase in the mitochondrial levels of the superoxide anion, which was correlated with the observed increases in ROS (Figure 29). An early increase of superoxide concentration after 30 minutes of exposure to the differentiating treatments was also observed, which was maintained and further increased mainly during the first 24 hours of experiment. Thus, superoxide appeared as a potential mediator of differentiation and/or mitochondrial biogenesis, worthy of further study.

Figure 33. Mitochondrial superoxide is increased during differentiation. (a-c) Overlay of representative flow cytometry curves and (g) relative fluorescence intensity of 5 µM of MitoSOX-stained N2a cells, subjected to the following treatments during 30 minutes (30 min), 24 hours (24h) and 48 hours (48h): (B) Basal conditions, (SS) serum starved cells and cells incubated with (F) 10 µM forskolin or (A) 0.3 mM db-cAMP under serum starvation conditions. *p<0.05, **p < 0.01, ***p<0.001 as compared with time-matched basal conditions (B).
5.2.9 Superoxide anion mediates mitochondrial biogenesis and differentiation

In order to elucidate whether superoxide anion is a mediator in the process of mitochondrial biogenesis during differentiation we decided to use a well-known SOD mimetic, MnTBAP. First of all, in order to determine if 100 μM MnTBAP could effectively reduce superoxide at the mitochondrial level we performed a control experiment in MitoSOX stained cells by means of flow cytometry. As observed in Figure 34, MnTBAP was able to potently, though not completely, reverse superoxide production promoted by the differentiating treatments at the mitochondrial level. Thus, MnTBAP was considered useful for performing superoxide scavenging prior and during the exposure to the differentiating treatments. Subsequently, we tested the effects of pre- and co-incubation with 100 μM MnTBAP on differentiation and mitochondrial mass. Figure 35 shows a marked reversion of both differentiation and mitochondrial mass with an efficacy similar to PD98059 (Figure 28), as detected by coomassie staining and mitotracker green staining. Thus, the presented data suggest that superoxide is a main character in N2a mitochondriogenesis during N2a differentiation.

![Figure 34](image_url)

**Figure 34.** MnTBAP (100μM) effectively reduces mitochondrial superoxide production. (a) MitoSOX fluorescence overlay of representative populations as measured by flow cytometry and (b) relative MitoSOX fluorescence intensity of N2a cells subjected to 48 hours treatment with: (B) Basal conditions, (SS) serum starved cells and cells incubated with (F) 10 μM forskolin or (A) 0.3 mM db-cAMP under serum starvation conditions in the absence (CTR) or in the presence of 100 μM MnTBAP. Data are means ± SEM of at least four different batches of cells. ns, not significant, **p<0.001 significantly different from controls (CTR).
Figure 35. Superoxide scavenging by MnTBAP reverses both neural differentiation and the increase in mitochondrial biomass. (a-d) Representative pictures after coomassie brilliant blue staining, (e) percentage of differentiated cells, (f-i) MTG fluorescence overlay of representative populations as measured by flow cytometry and (j) relative MTG fluorescence intensity of N2a cells subjected to 48 hours treatment with: (B) Basal conditions, (SS) serum starved cells and cells incubated with (F) 10 µM forskolin or (A) 0.3 mM db-cAMP under serum starvation conditions in the absence (CTR) or in the presence of 100 µM MnTBAP. Data are means ± SEM of at least four different batches of cells. ns, not significant, ***p<0.001 significantly different from controls (CTR).
5.2.10 Superoxide anion is an upstream regulator of the MAPK cue

In order to elucidate if the production of superoxide and the activation of the MAPK were part of the same or different signalling cues, we combined the two inhibitors that were found to reverse differentiation and mitochondrial biogenesis, PD98059 and MnTBAP. If they were part of different signalling cues, they would present additive effects on the final effects of differentiation and/or mitochondrial biogenesis. Otherwise, if they would be part of the same signalling cue the effect of the combination would be similar to that obtained with the two inhibitors separately. The effect of this combination was tested by means of Coomassie brilliant blue staining and counting and MTG staining and flow cytometry, respectively (Figure 36). The effect of the combination was compared to the effect of each inhibitor alone. The combination promoted a reduction in the number of differentiated cells as well as in mitochondrial mass that was similar to that obtained by the individual inhibitors (Figure 28 and Figure 35), the slight differences found not being significant. These findings indicate that superoxide and ERK pathway are indeed part of the same signalling cue that leads to mitochondrial biogenesis and differentiation. However, in order to discard the possibility that superoxide could be a downstream process to ERK activation, we detected superoxide by means of mitoSOX staining and flow cytometry in the presence of PD98059. If superoxide were a downstream process to ERK activation, then PD98059 should reduce superoxide production, otherwise it must be situated upstream. As observed in Figure 37, PD98059 was not able to reduce the formation of superoxide at any of the time frames tested (30 min, 24 h or 48 hours), but it even promoted an increase in superoxide production. This fact indicates that superoxide is not a downstream process to ERK activation, but an upstream one. Furthermore, we performed western blot analysis of ERK phosphorylation in the presence of forskolin and in the presence and absence of the two inhibitors that promoted reversion of its effect. The results show a reduction in ERK phosphorylation in the presence of MnTBAP, which was further reduced in the presence of both inhibitors simultaneously (Figure 37). The fact that MnTBAP reduced ERK phosphorylation promoted by forskolin further supports the hypothesis that moderate superoxide production is an upstream process to ERK phosphorylation. These results completed the elucidation of the signalling cascade that leads to mitochondrial biogenesis and differentiation in N2a cells.
The combination of MnTBAP and PD98059 does not produce additional effects on the reduction of differentiation or mitochondrial mass. (a-d) Representative pictures after coomassie brilliant blue staining, (e) percentage of differentiated cells, (f-i) MTG fluorescence overlay of representative populations as measured by flow cytometry and (j) Relative MTG fluorescence intensity of N2a cells subjected to 48 hours treatment with: (B) Basal conditions, (SS) serum starved cells and cells incubated with (F) 10 µM forskolin or (A) 0.3 mM db-cAMP under serum starvation conditions in the absence (CTR) or in the presence of 100 µM MnTBAP and 50 µM PD98059. Data are means ± SEM of at least four different batches of cells. ns, not significant, ***p<0.001 significantly different from controls (CTR).
Figure 37. PD98059 does not reduce superoxide production but MnTBAP reduces ERK 1/2 phosphorylation. (a-d, f-i, k-n) Overlay of representative flow cytometry curves and (e, j, o) relative median fluorescence intensity of 5 µM MitoSOX-stained N2a cells, subjected to the following treatments during 30 minutes (30 min), 24 hours (24h) and 48 hours (48h): (B) Basal conditions, (SS) serum starved cells and cells incubated with (F) 10 µM forskolin or (A) 0.3 mM db-cAMP under serum starvation conditions in the presence or in the absence of PD98059 (PD). Data are means ± SEM of at least three batches of cells. *p<0.05, **p<0.01, ***p<0.001 as compared with time/treatment-matched control conditions (CTR). (p) Representative p-ERK1/2 immunoblot corresponding to protein extracts from 30 minutes and 1 hour-treated cells (30 min, 1h), total ERK was taken as loading control. (FM) forskolin-treated cells pre- and co-incubated with 100 µM MnTBAP, (FMP) forskolin-treated cells pre- and co-incubated with 100 µM MnTBAP and 50 µM PD98059.
5.3 Detection of differentiation by means of impedance spectrometry in 3D cultures

Although classical techniques could provide us with a broad knowledge about the physiology of N2a cell differentiation and mitochondrial biogenesis, they are still based on two dimensional culture conditions. As discussed in the introduction, the availability of techniques able to deal with three dimensional cultures is very limited. In order to provide an advance in this field, the following study was performed with the aim of developing a three dimensional neuroblastoma culture that would be compatible with a novel impedimetric biosensor able to deal with the third dimension. During our collaboration with the University of Magdeburg in the framework of the Cell Check project, a novel impedimetric biosensor was specially designed by Thomas Jacobs, with the aim of detecting electrical signals from neuronal cells in three dimensional environments (Jacobs 2011). Two versions of this biosensor became prototypes, in order to adapt the sensor capacities to the studies required (see materials and methods). Our part of the work consisted of designing a three dimensional matrix that could sustain neural viability to the maximum possible extent, developing a matrix that would allow neural differentiation, making this matrix compatible with the impedance biosensor and performing experiments to demonstrate its usefulness for neurophysiological studies.
5.3.1 Development of a three dimensional neuronal culture suitable for the impedance biosensor I.

5.3.1.1 Immobilization of N2a cells in bacteriological agar (bactoagar)

Based on the previous knowledge in the laboratory of Professor Kintzios regarding the immobilization of mammalian cells in three dimensional matrices, bactoagar gels with N2a immobilized cells were used with the Impedimetric biosensor I. N2a neuroblastoma cells were immobilized in three dimensional bactoagar gels inside the electrodes and these electrodes containing the cell-gel combination were inserted in a 24 well plate and kept in a standard incubator. After 48 h gels were extracted from the electrodes and observed in an optical microscope. Figure 38 shows neuroblastoma three dimensional cultures at two different magnifications after extracting them from the impedance electrode. At the lowest magnification (4x) the macro structure of the bactoagar gels can be observed. By increasing the gel concentration from 0.8% to 1.6% (w/v), the gel stiffness increases and hence the maintenance of its shape after removing the reference electrode is improved. The observed cylinders correspond to the part of the matrix within the holes of the reference electrode for nutrient diffusion. At a higher magnification (10x) cells can be observed as spheres hanging at a point of the three dimensional matrix. All of the bactoagar concentrations tested were transparent and diffusible, which allow the detection of trypan blue staining and MTT assay as viability tests. One of the problems of working in three dimensions is the difficulty of separating cells from gels in order to get gel-free cells for further analysis. However, Trypan blue and MTT are two simple viability tests, for which no separation step is needed, since bactoagar is permeable to these substances.

![Figure 38. N2a cells embedded in bactoagar gel matrices. Different gel concentrations, ranging from 0.8% to 1.6% (w/v), were observed at 4x (pictures above) and 10x (pictures below) magnification in an inverted light microscope.](image-url)
5.3.1.2 Viability tests in 3D bactoagar gels inside the impedance electrodes. Effect of the presence of the sieve on mass transfer.

Mass transfer is an important parameter to take in account in 3D cultures, especially in this case, due to the presence of a sieve with small pores on the top of the gel, the counter electrode. The presence of the sieve could limit the access of nutrients to the cell environment and, thus affect cell behaviour. In order to discard this possibility, a comparison of cell viability in gel film with and without a counter electrode was carried out. Cells were stained with trypan blue and the number of viable cells was related to the total number of cells. As shown in Figure 39, no differences were found between the two conditions, i.e. in the presence or in the absence of the porous reference electrode. These series of experiments showed that nutrient supply through the small holes of the permeable electrode is sufficient to maintain cell viability through a 48 hour period. No significant diffusion barrier effect could be observed. Thus, the mass transfer of the gel cells inside those cylinder electrodes in the presence of the reference electrode seems to be adequate for electrical impedance measurement.

Figure 39. Viability is not reduced by the presence of the sieve (counter electrode). N2a cells embedded in bactoagar gel matrices, inside the impedance electrodes, either in the presence (w sieve) or in the absence (w/o sieve) of the diffusible reference electrode. After 48h storage cells were stained with trypan blue and results were normalized with the following formula: L x 100 / (L + D); D, dead cells; L, living cells. ns, not significant by using the t test as statistical analysis.
5.3.1.3 Viability tests in 3D bactoagar gels inside the impedance electrodes. Effect of storage conditions.

The next experiments consisted of testing the difference in viability of cells embedded in bactoagar and inserted in the electrodes after 48 hours culture under different storage conditions: in a standard incubator (37°C, 5% CO₂), at room temperature without CO₂ (24°C, atmospheric CO₂) or in the refrigerator (4°C, atmospheric CO₂). The aim of these experiments is to find the optimum conditions for storage and to explore the possibility of distant shipping of the sensors in case of necessity. Figure 40 shows that cells embedded in bactoagar exhibit a high viability both under standard incubation conditions and at room temperature. In contrast, viability is drastically reduced when the sensors were stored at 4°C. Bactoagar seems to preserve the viability of the cells even under suboptimal temperature conditions such as 24°C and even without the pH controller CO₂. However, for the following experiments 37°C and 5% CO₂ were selected as the optimal conditions and used hereafter.

![Graph showing cell survival](image)

**Figure 40.** N2a cells embedded in bactoagar gel matrices, inside the impedance electrodes, stored for 48h in different conditions and temperatures and stained with trypan blue. Results were normalized with the following formula: Dead cells (D) and living cells (L) were counted and the total number related to 100%. Percentage of cell survival corresponds to the formula L x 100 / (L + D). 4 °C, atmospheric CO₂ (4 °C), 24 °C, atmospheric CO₂ (24 °C), 37 °C, 5 % CO₂ (37°C); ns, not significant, ***p<0.001 significantly different to 4 °C.
5.3.1.4 Viability tests in 3D bactoagar gels inside the impedance electrodes. Effect of cell density.

In order to get a maximal response from the neurons embedded in a gel matrix, it is important to consider cell density. The higher the cell density, the bigger the signal should be. However, it is known that very dense cell cultures tend to collapse due to the lack of nutrients and the accumulation of residuals. Consequently, we checked if an increase in cell density could affect cell viability, in order to reach a reasonable maximum cell density. MTT test was used as a measure of cell viability. Pictures at different cell densities show that at high concentrations, intercellular space almost does not exist. Indeed, a first experiment in a wide cell density range showed that viability begins to be impaired when cell density increases above one million cells per 120 μL. A finer experiment in a cell concentration ranging up to one million cells / 120 μL showed a linear dependency between the number of cells per unit of volume and the MTT absorbance detected by photometry (Figure 41). Moreover, an increase in MTT absorbance was observed after 24h of experiment, compared to the 0h point.

![Figure 41. Effect of cell density on viability.](image)

Viability tests of different cell densities were tested by MTT assay. After a first approach (0.1 - 2 million cells / 120 μL) viability was tested between 0.1 - 1 million cells / 120 μL at 0 h and 24 h culture. Maximal cell density for impedance experiments was considered to be 1 million.
5.3.1.5 Viability tests in 3D bactoagar gels inside the impedance electrodes. Effect of gel concentration.

In order to achieve a suitable gel resistivity to the flowing current, different concentrations of bactoagar were tested and the maximum concentration suitable for a healthy cell culture was selected. MTT test was used as a measure of cell viability. A series of experiments covering a wide range of gel concentrations was performed at two different endpoints, which cover the time scale in which impedance experiments are usually carried out. Highly concentrated gels (2.5% - 3.5%) did not support cell survival in the first 24 hours and they were discarded, whereas low concentrations presented excessively low resistance for impedance measurement. Thus, a compromise was reached by using gels with a final concentration of 2% (w/v) for impedance experiments (Figure 42). At this concentration, viability is maintained during 24h, the time range in which the experiments were performed. Thus, based on those experiments, a 2% (w/v) bactoagar gel concentration with 1 million cells per well was considered as the reasonably optimum maximum gel and cell concentration. All the impedance measurements were done following this protocol.

![Figure 42. Effect of gel concentration on viability. Viability test on N2a cells cultured in a three dimensional bactoagar gel matrix at different gel concentrations. 2 % bactoagar gels were considered as the reasonable maximum to maintain cell viability.](image-url)
5.3.2 Impedance spectroscopy measurements. Impedance biosensor I.

Impedance spectra were recorded in the range of 10 to 110 MHz. After a two hour stabilization period, the time needed for temperature to be stable at 37 ºC, stimulation was carried out through perfusion of the system with the desired stimulus or pure medium as a control. Preliminary experiments in the presence or in the absence of gel and in the presence or in the absence of cells were carried out in order to find out the optimal conditions for measurements. Figure 43a shows an impedance spectrum corresponding to control unstimulated N2a cells in a bactoagar gel matrix. The almost linear increase in the magnitude of the impedance denotes a high ionic conductivity of the system, which dominates the spectrum. In comparison to preliminary experiments in the absence of gel (data not shown) the magnitude is ten times higher. This demonstrates that the presence of gel promotes decreased ion mobility, i.e. it increases resistance. Figure 43b shows the stability and noise of the impedance spectrum over time. During the time period of control experiments on unstimulated cells no significant change in the phase or the magnitude of the impedance were found. These results were expected, since prior to the experiments two stabilization phases are carried out: firstly, cells were stabilized in the incubator before the experiment started, so no change due to metabolic processes were expected; secondly, an additional stabilization period of 2h was given for temperature increase and medium diffusion, so no change due to mass exchange were expected. Only small waveform changes were found that are attributed to slight temperature deviations. Furthermore, MTT assay checked after the experiments did not show any significant effect of the current flow on cell viability. Therefore, it was considered that stable basal conditions for performing the experiments were achieved.

Figure 43. Representative cellular response of control unstimulated gel embedded N2a cells by means of electrical impedance spectroscopy: (a) Electrical impedance spectrum; (b) phase and magnitude depending on time. $10^6$ cells / well, 2 % bactoagar.
After reaching a stable base line, cells were stimulated with an excitatory neurotransmitter. Figure 44 shows the change of impedance parameters after perfusion of the system with 10 mM of ACh. Around ten minutes after starting ACh perfusion the magnitude of the impedance started to decrease over several tens of minutes down to a stable plateau. Spectra were acquired until the steady state was reached. Shifts in the impedance spectra (10 to 110 MHz) of gels showed that AChR triggering is accompanied by an increase in the overall conductivity of the gel in a time scale of tens of minutes. These changes in conductivity are attributed to the opening of ionic channels due to the ACh stimulation. The 10 minute delay is attributed to the low flow rate and diffusion within the bactoagar gel. After finishing experiments cell viability was checked, without finding any significant changes, demonstrating that the effect of the current flow on cell viability is negligible.

After this series of experiments during the secondment at the University of Magdeburg it was demonstrated that it is possible to measure the excitatory response of a neuroblastoma cell cluster in a 3D matrix by means of impedance analysis. Although these results were very promising, some questions needed to be addressed: Firstly, bactoagar gels did not support the differentiation of N2a neuroblastoma cells, which is the central aim of this study. So it was considered a priority to design a 3D model that supports differentiation. Secondly, the time-efficiency of the measurement was too low, given the time range in which differentiation is presented and did not allow the parallelization of experiments. So the design of a new miniaturized bioreactor with 8 electrodes in parallel was also considered a priority (by Thomas Jacobs, University of Magdeburg).
5.3.3 Impedimetric biosensor II. Detection of differentiation

Since impedance spectroscopy had been demonstrated to be suitable for 3D applications in neuronal cultures the next step was to adapt the system for the measurement of neural differentiation. From the theoretical point of view, differentiated cells present a bigger membrane surface, which should affect impedance measurements. Furthermore, as mitochondrial biogenesis is concomitant to the differentiation process (previous chapters), the membrane surface of this organelle is also increased, which should affect the impedance recordings. The next sections describe the efforts made in finding a suitable gel that allows differentiation of N2a cells and its optimization. In parallel, new miniaturized parallelized electrodes were constructed by Thomas Jacobs at the University of Magdeburg (Jacobs 2011). Finally, the detection of differentiation in embedded N2a cells by means of impedance analysis was optimized during the secondment at OVG-UNIM (January-March 2010).
5.3.3.1 Effect of gel composition on the differentiation of N2a cells in 3D gels.

For the electrical impedance analysis of cellular differentiation by means of the impedimetric sensor, the composition of the gel matrix and the cell density needed to be optimized. A comparison between five different gel matrices was carried out 24 hours after cell immobilization. Figure 45 shows images of N2a cells immobilized in collagen (CG), collagen-laminin (CLG), basement membrane extract (BME), bactoagar and agarose gels. As described in previous sections, bactoagar gels did not support the differentiation of N2a neuroblastoma cells in a period of 48 hours. In a similar manner, agarose gels and collagen gels also maintained cell viability but did not support differentiation. However, BME supported the formation of short and robust neurites. The addition of laminin to the composition of the collagen gels not only supported the formation of neurites but also allowed a higher growth of these neurites compared to BME, forming long and thin neurites that seemed to be directed to neighbour cells. Preliminary comparative experiments between a range of differentiation stimuli in conventional 2D N2a cultures pointed out db-cAMP as one of the most potent differentiating stimuli for N2a cells. Therefore, all the conditions were tested in the presence and in the absence of db-cAMP. Qualitative data were obtained by optical microscopy. db-cAMP was not able to induce differentiation in those systems that did not have it in its absence. On the contrary, it seems to poorly boost the formation of neurites in those systems which support differentiation i.e. BME and CLGs. Comparing the pictures a, b, c, d and e with f, g, h, i and j, it is interesting to remark that the key factor for differentiation in 3D collagen matrices is laminin and not db-cAMP as expected. A reasonably high differentiation can be obtained without the addition of any differentiating agent when using CLGs.

Figure 45. Differentiation of N2a cells depends on gel composition. Pictures of N2a cells embedded during 24 h in CGs (a, f), CLGs (b, g), BME (c, h), bactoagar (d, i) and agarose (e, j) gels in the presence (a, b, c, d, e) and in the absence (f, g, h, i, j) of 0.3 µM db-cAMP. The arrows point to the neurites.
5.3.3.2 Effect of cell density on viability and differentiation

In order to optimize the 3D cell culture conditions to reach the maximum differentiation degree in the novel CLGs, different cell densities were studied: $0.6 \times 10^6$, $1.4 \times 10^6$, $2 \times 10^6$, $4 \times 10^6$, and $6 \times 10^6$ cells / mL. As shown in Figure 46a-e, a high differentiation degree was found in cells cultivated at densities up to $2 \times 10^6$ cells / mL while this differentiation was partially lost when cell density was increased above that level. These results suggest that differentiation degree is strongly dependent on the concentration of cells within the gel matrix. This effect can be attributed to a decrease in diffusion processes, to cell-to-cell contact inhibition, or simply to the lack of nutrients and excess of waste products. In order to understand the effect occurring at higher cell densities, MTT assays were carried out to discover if the lack of differentiation was correlated to a loss in cell viability. As shown in Figure 46f, the increase in cell concentration leads to an increase in MTT absorbance which has a maximum at $4 \times 10^6$ cells / mL, while above this concentration, viability is reduced even though more cells are immobilized in the gel. Thus, two effects are simultaneously taking place in N2a cells embedded in CLGs. On the one hand, differentiation is lost when cell density rises above $2 \times 10^6$ cells / mL. On the other hand, relative viability (viability on per cell basis) is continuously decreasing with increasing cell densities and this loss in viability is especially critical above $4 \times 10^6$ cells / mL. The loss of differentiation at high cell concentrations is attributed to the increase in cell death, probably due to nutrient starvation.

Figure 46. Differentiation and viability of N2a cells is dependent on cell density in 3D CLGs. Pictures (a, b, c, d, e) are representative examples of cells immobilized at different densities. (a) $6 \times 10^5$ cells/mL, (b) $1.4 \times 10^6$ cells/mL, (c) $2 \times 10^6$ cells/mL, (d) $4 \times 10^6$ cells/mL, (e) $6 \times 10^6$ cells/mL. The arrows point to the neurites. (f) Viability as the absorbance of MTT formazan depending on the cell density.
5.3.4 Electrical impedance analysis of 3D gel matrices with embedded differentiating N2a cells

Based on the experimental results using conventional cell culture plates presented in the previous sections, the electrical impedance analysis of cellular differentiation in CLG was focussed. An array of 16 electrodes inserted in 16 wells was designed for the analysis of neural differentiation in 3D matrices (Jacobs 2011). In order to validate the novel electrode array for this application, CLGs and CGs were prepared as described in the materials and methods section. Results of CLGs with differentiating N2a cells were compared with cells immobilized in bare CG as a control experiment for non-differentiating cells. Impedance spectra of the electrodes were acquired for a period of 12 h, while DMEM without serum was continuously dispensed into the micro-fluidic channels. At the end of each experiment the viability, the grade of differentiation and the structure of the deposited gel film were characterized by means of MTT tests and light microscopic pictures. Figure 47 shows CLGs embedded N2a cells in a single well of the impedimetric sensor after the end of the impedance spectrum acquisition. Microscopic pictures revealed that cells further differentiate in the impedimetric biosensor accompanied by the formation of neurites in CLGs but not in CGs. Parallel experiments in an identical bioreactor were performed in an incubator in order to determine the effect of current flow on the cells. When comparing immobilized cells with and without AC-current flow, no differences could be observed based on the viability tests and cell morphology. These results are in accordance to the results observed in conventional cell culture plates. In all the cases cells were found to be differentiated when cultured in CLGs while cells in CGs did not differentiate. Hence, the effect of the AC current flow and the impact of medium flow compared to stop flow regime on the cell behaviour is regarded as negligible.

Figure 47. N2a cells differentiate inside the impedimetric biosensor in CLGs. Differentiated (a) and non-differentiated (b) N2a cells embedded in a CLG and a CG after the end of the experiment in a single well of the impedimetric sensor. $2 \times 10^6$ cells/mL.
Preliminary experiments in the presence and in the absence of gel, showed a significant increase in the magnitude of the impedance due to the presence of gel (data not shown), indicating that the presence of the gel on the electrodes reduces ion mobility and increases the ohmic resistance. (Figure 48a,b) shows the relative impedance shift referred to the initial impedance of two representative electrodes (E1,E2) at 1 MHz of 2 x 10^6 N2a cells/mL in bare collagen (CG). During the first two hours an increase of around 7 Ω in the magnitude of the impedance was observed that is attributed to thermal equilibration. After two hours the temperature was stable at 37 ºC and a stable baseline is present that remains constant in the time frame of impedance measurement. Thus, within 12 h no significant changes in the dielectric or conductive properties of bare collagen and the immobilized N2a cells could be observed. Figure 48c,d shows the corresponding measurement curves of four representative electrodes (E1-E4), when N2a cells were immobilized in CLG. During thermal equilibration a local minimum occurs and subsequently the impedance magnitude starts to increase, while the phase remains almost constant. Kinetics of the sensor signal shift as shown in Figure 48c,d correspond to the time scale of cellular differentiation as observed in 96 well plates. The reduction of up to 2 Ω in the initial impedance might be attributed to cellular migration within the 3D structure. Figure 48e shows the impedance spectrum of 2 x 10^6 N2a cells/mL embedded in a laminin-collagen matrix without compensation of parasitic electric elements. At frequencies higher than 100 kHz and less than 10 MHz the magnitude of the impedance is almost frequency-independent. The phase switches from capacitive to ohmic and inductive behaviour. Hence, the sensor response is mainly mediated by a shift in the specific resistivity of the gel matrix with embedded cells. Variations that appear in between electrodes can be mainly attributed to electrode fabrication tolerances, variations in the number of cells and deviations in the manual gel film preparation. In case one bubble could eventually appear within the gel, it was detected by the impedance measurement as a big increase of the output signal due to the increased resistivity of the whole gel (an entrapped bubble behaves like an insulator) and those signals were discarded from the analysis. The presence of four parallel electrodes for the same treatment allows the exclusion of non-properly filled electrodes. In addition after each experiment gels were visually inspected with a Leica macroscope (MZ16 APO) and with a conventional inverted microscope.
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Figure 48. Impedance recordings of differentiating vs. non-differentiated N2a cells in 3D matrices. Relative impedance shift of two selected electrodes with control non-differentiated N2a cells (E1-E2) at 1 MHz differentiation (2 x 10^6 N2a cells/mL in CGs) referred to the initial impedance at tm = 0: magnitude (a) and phase (b). Relative impedance shift of four selected electrodes (E1-E4) at 1 MHz during cellular differentiation (2 x 10^6 N2a cells/mL in CLGs) referred to the initial impedance at tm = 0: magnitude (c) and phase (d). Impedance spectrum of N2a cells at a concentration of 2 x 10^6 cells/mL embedded in a laminin-collagen matrix in a frequency range between 100 kHz and 50 MHz (e).
6 DISCUSSION

During the development of the CNS a plethora of different processes are taking place. These processes are finely regulated to give rise to hundreds of different cell types. In this study the coupling of the processes of differentiation, cell cycle arrest and mitochondrial biogenesis have been addressed. N2a neuroblastoma cells were used as a model of neural differentiation as they are known to differentiate in response to db-cAMP (a well known cAMP analog) and serum starvation. Indeed, not only db-cAMP promoted a marked differentiation of these cells but forskolin, an activator of adenylate cyclase, also produced a similar effect (Figure 20). By activating adenylate cyclase, forskolin is able to increase cAMP levels, which in turn activates important signalling pathways. Serum starvation by itself was found to increase differentiation of N2a cells, while the exposure of cells to the same stimuli in the presence of serum did not show any differentiation effect. This result is in accordance with other authors which observed the same effect in this (Flaskos, McLean et al. 1998) and other cell types (Radha, Rajanna et al. 2008), although in some cell types like PC12 serum starvation is an apoptotic stimulus (Uittenbogaard, Baxter et al. 2010). Serum starvation was previously found to activate important signalling pathways in N2a cells such as MAPKs that promote differentiation in N2a cells (Evangelopoulos, Weis et al. 2005). However, further stimulation by forskolin or db-cAMP seems to be necessary to induce a neuronal phenotype, both in terms of percentage of differentiated cells and neurite length. In line with our results, (Tremblay, Sikorska et al. 2010) found additional effects of db-cAMP on tyrosine hydroxylase expression and dopamine production on N2a cells over serum reduced conditions.

Differentiation is usually coupled with cell cycle arrest (Galderisi, Jori et al. 2003) during natural neural development. Proliferation has to be stopped in order to start the differentiation program and to give rise to mature neurons. Indeed, serum starvation, forskolin and db-cAMP were found to arrest cell cycle as observed by flow cytometry after PI staining (Figure 20). It was noted that cell cycle arrest was produced by the three differentiating treatments in a similar extent, thus suggesting that the main factor that influences cell cycle progression might be the presence or absence of serum, whereas differentiation might be further influenced by pharmacological treatments. Many authors argue that it is during the last cycle that cell fate is decided (Cremisi, Philpott et al. 2003; Galderisi, Jori et al. 2003). During this last cycle, a unique pattern of gene expression is observed. Some proteins are over-expressed while others are under-expressed (Watkins, Basu et al. 2008). In fact, some cell cycle molecules have been implicated in neuroblastoma differentiation (Munoz, Sanchez et al. 2003) and some proteins have been shown to present a dual role, acting both at the level of differentiation and cell cycle exit during cortical neurogenesis (Nguyen, Besson et al. 2006). However, the implications of cell cycle molecules
were not the focus of this study. Cell cycle analysis results were taken as a corroboration of the fact that cells were committed to differentiation by an arrest of cell division.

Based on previous studies on different tissues where activation of PKA produced mitochondriogenesis (Bogacka, Ukropcova et al. 2005) we formulated the hypothesis that forskolin and db-cAMP-mediated differentiation of N2a neuroblastoma cells could be related to mitochondrial biogenesis. In order to prove this hypothesis, N2a neuroblastoma cells in serum starvation conditions were induced to differentiate by two well-known activators of cAMP signalling, forskolin and db-cAMP. Mitotracker green (MTG) staining detected by flow cytometry was used as an estimate of mitochondrial mass since MTG accumulated in mitochondria in a membrane potential-independent manner (Baxter, Uittenbogaard et al. 2009). The results revealed an increase in mitochondrial mass following 48 hours of exposure to serum starvation, forskolin and db-cAMP (Figure 21). Confocal microscopy pictures of N2a cells treated for 48 hours and stained with MTG (Figure 22) did not allow the observation of changes in mitochondrial morphology among different treatments. However, it can be observed that mitochondria are redistributed to the neurites, suggestive of a functional mitochondrial transport system in the newly formed neurites. According to our results, other agents able to induce differentiation in different models were also found to be responsible for the induction of mitochondrial biogenesis in the early stages of differentiation. For example, NGF is able to induce mitochondrial biogenesis in PC12 pheochromocytoma cells and to cause a redistribution of mitochondria towards the dendrites (Baxter, Uittenbogaard et al. 2009); This study also demonstrated that a PC12 cell model which overexpressed the transcription factor NeuroD6 suffered the same phenomena, together with a rearrangement of actin and microtubules. This increase in mitochondrial mass was dependent on the microtubule network integrity (Baxter, Uittenbogaard et al. 2009). Previous works of the same group already showed that NeuroD6 itself is able to initiate the differentiation of PC12 cells, linking the mitochondrial survival pathway, differentiation, and cell cycle arrest (Uittenbogaard and Chiaramello 2005). Moreover, they found a parallel increase in the expression of differentiation and mitochondrial proteins in a genome-wide microarray study (Uittenbogaard, Baxter et al.). This group’s works provide us a good picture of other models in which a link between differentiation, cell cycle arrest and mitochondrial biogenesis exists. Similarly, in an extensive proteomic analysis of the P19 differentiation cell model, an overall increase in mitochondrial proteins was found to correspond with the differentiation process, accompanied by cell cycle protein down-regulation and cytoskeletal remodeling protein up-regulation (Watkins, Basu et al. 2008).

In our understanding, this generation of new mitochondria could reflect a response to the increased energy demands of the cell under differentiation and the local necessity of energy production in the dendrites due to the appearance of synaptic activity. The induction of mitochondrial biogenesis could also compensate for damages resulting from mitotic arrest and
other types of cellular stress after serum deprivation (Uittenbogaard, Baxter et al. 2010). This mechanism will also be discussed below in association with the role of ROS in neural differentiation.

In order to know if newborn mitochondria were energized and healthy, similar experiments to those performed for MTG were carried out after staining with Mitotracker orange (MTO), which is sensitive to mitochondrial membrane depolarization. Quantification of fluorescence intensity by flow cytometry revealed similar changes in MTO-stained cells (Figure 23) to that obtained with MTG (Figure 21). MTO has been used by many authors to detect changes in mitochondrial membrane potential, since this dye accumulates selectively in the mitochondria and responds to mitochondrial membrane depolarization (Ahmad, White et al. 2001; Buckman, Hernandez et al. 2001). However, results obtained with MTO must be considered with caution, since MTO can respond in a similar manner to different parameters such as changes in oxidative activity (Agnello, Morici et al. 2008) or even to the presence of certain free radicals (Buckman, Hernandez et al. 2001). Other authors have used this dye to measure mitochondrial mass/mitochondrial biogenesis (Pile, Spellman et al. 2003; Aoi, Naito et al. 2010). Therefore, we cannot consider these results as a direct measure of mitochondrial membrane potential, but only as an indicator of the healthy state of the newborn mitochondria, i.e. as a measure of the content of energized mitochondria. The fact that that MTO changes are similar to those obtained with MTG may well indicate that parameters other than mitochondrial mass (membrane potential, free radicals, etc.) do not strongly influence the MTO signal. Apparently, mitochondrial membrane potential is not dramatically altered. However, we cannot rule out that any of the aforementioned factors or some combination of some of them could influence the signal obtained with this dye.

In the same manner, the MTT assay was used as an estimate of the metabolic activity of differentiated cells in comparison to control (Figure 24). The MTT assay performed on equal amounts of viable cells for all the treatments revealed that these newly synthesized mitochondria are metabolically active, i.e. their electron transport chain is functional, since similar increases in MTT activity were found to those obtained with MTG, MTO and Coomassie brilliant blue. This variation of the MTT assay has been previously used as a method to test mitochondrial activity (Nisoli, Clementi et al. 1998), i.e. to detect the increase of activity due to the newly synthesized mitochondria. Although it is widely assumed that MTT is reduced by active mitochondria in living cells, it is also known that MTT is not only reduced by mitochondria but also extramitochondrially by other cellular compartments. In particular, MTT can be reduced by a wide range of enzymes and substances, including the succinate dehydrogenase (complex II) of the mitochondria (77 % of the total succinate reducing capacity), NADPH and NADH (major electron donors for MTT reduction), reducing enzymes throughout the cell, extracellular and intramitochondrial superoxide (around 20% of total MTT reduction), etc. (Berridge, Herst et al. 2005). For this reason, we cannot
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consider MTT measurement as a direct proof of mitochondrial biogenesis but we can consider it as a measurement of an integrated set of enzyme activities that are related in various ways to cell metabolism (Berridge, Herst et al. 2005). In other words, we can consider it an estimate of the integrated metabolic state of the cell. However, further and more specific experiments concerning the energy status of the mitochondria would be necessary to further understand the increases observed by the MTT assay. In our case, taking into account that mitochondrial mass was increased in differentiated N2a cells, newly synthesized mitochondria are most probably strongly influencing the overall signal, by increasing MTT reduction either though succinate dehydrogenase or even through the presence of increased superoxide production. Hence, looking at MTO and MTT results together, they could well indicate the healthiness and metabolic functionality of the newborn mitochondria.

All these findings suggest the important role of mitochondria during differentiation. Mitochondria are the responsible energy-producing organelles. Probably, a higher energy demand during and after differentiation triggers mitochonrdiogenesis and mitochondrial migration to the neurites. This process will involve the activation of transcription factors and gene expression.

Mitochondrial biogenesis is a complex process that requires the coordination of mitochondrial and nuclear genomes. The regulation of the nuclear transcription depends on a network of transcription factors and signalling pathways. Although the coupling of this network varies between tissues, the transcriptional coactivator PGC-1α has been characterized as the master regulator of mitochondrial biogenesis due to its ubiquity (Onyango, Lu et al. 2010). N2a cells induced to differentiate by forskolin or db-cAMP in the absence of serum demonstrated an increased expression of PGC-1α compared to control undifferentiated N2a cells (Figure 25). Two bands appeared to be increased upon the treatments by using a polyclonal antibody raised against the carboxyl terminus of PGC-1α, as also observed by other authors. According to (Miura, Kai et al. 2008), three different isoforms are described for PGC-1α: PGC-1α-a, PGC-1α-b and PGC-1α-c, being PGC-1α-b isoform 4 aminoacids and PGC-1α-c 14 aminoacids shorter than PGC-1α-a. The N-terminal 16 aminoacids in PGC-1α-b and PGC-1α-c differ from that on PGC-1α-a. Recent studies of the same group on transgenic mice with antibodies raised against the carboxyl terminus found three bands to be increased in PGC-1α-b transgenic mice with molecular weights around 110kDa, 85kDa and 45kDa (Tadaishi, Miura et al. 2011), whereas studies on neuroblastoma cells and other models with antibodies directed to the amino terminus detected bands at 113 and/or 90kDa (Aquilano, Vigilanza et al. 2010). Our antibody is detecting the carboxyl terminus of the protein (which is common to all of the isoforms), thus theoretically detecting all of them. Based on the presented data, we cannot determine which specific isoforms are presented in differentiating N2a cells but we can conclude that these two bands correspond to the full length and one shortened isoform of PGC-1α. The appearance of multiple bands is
attributed to posttranslational modifications and/or alternative splicing variants of the PGC-1α (Miura, Kai et al. 2008; Aquilano, Vigilanza et al. 2010), which may alter its apparent molecular weight. It is also noteworthy, that serum starvation per se was able to induce only a moderate increase in PGC-1α expression (compared to the other two inducers). The non-linear association between the expression of PGC-1α and mitochondrial mass (as detected by MTG staining) may also be explained by the complexity of the molecular interactions that influence PGC-1α activity. PGC-1α is known to suffer both post-transcriptional modifications (such as alternative splicing) (Baar, Wende et al. 2002; Miura, Kai et al. 2008) and post-translational modifications (phosphorylation, ubiquitination, methylation or acetylation) (Scarpulla 2002; Wallace and Fan 2010; Fernandez-Marcos and Auwerx 2011), which may regulate its activity. We are possibly dealing with a combination of overexpression, alternative splicing and posttranslational modifications that leads to the final effect of increasing mitochondrial mass. In line with our findings, PGC-1α was observed to be overexpressed in other neural differentiation models such as PC12 in response to the overexpression of NeuroD6 (Uittenbogaard, Baxter et al. 2010). In this study, a recently discovered PGC-1α isoform (Zhang, Huypens et al. 2009), NT-PGC-1α, was found to be responsible for mitochondrial mass maintenance of these cells upon serum deprivation. Unfortunately, due to technical limitations, it was not possible to check for this or other truncated forms. However, the recent discovery of new brain specific truncated forms of PGC-1α and their potential relation to degenerative diseases such as Huntington’s disease, Alzheimer’s disease or Parkinson’s disease (Soyal, Felder et al. 2012), makes PGC-1α and its isoforms a very promising target for treating these neurodegenerative diseases and highlights them as a good candidate for future research in this direction. Indeed, PGC-1α overexpressed in the adult rat nigrostriatal system effectively increased mitochondrial mass (Ciron, Lengacher et al. 2012) but sustained overexpression caused the selective loss of dopaminergic neurons of the striatum, indicating not only the pivotal role of PGC-1α but also the necessity of maintaining physiological PGC-1α levels for dopaminergic neuron survival. In our understanding, the selective pharmacological activation of one or more specific PGC-1α isoforms could probably be the answer to this intricate issue, and remains one of our targets for future research.

In the context of finding and better understanding potential pharmacological targets, the present study continues by following the molecular crosstalk between mitochondrial biogenesis upstream regulators due to the pharmacological induction of neural differentiation. The expression of PGC-1α is controlled, among others, by the transcription factor CREB (Onyango, Lu et al. 2010; Scarpulla 2011), which in turn can be activated by various of protein kinases. PKA and ERK 1/2 are able to phosphorylate CREB at Ser-133 and activate gene transcription (Gerits, Kostenko et al. 2008). The PKA inhibitors H89 and PKI, as well as the MEK 1/2 inhibitor PD98059 served as tools to elucidate the subtle cross-talk between pathways that lead to differentiation and mitochondrial biogenesis.
Although the involvement of the PKA pathway has scarcely been reported in differentiation or mitochondrial biogenesis studies, we found it interesting to study this signalling cue regarding the mitochondrial biogenesis process, since forskolin and db-cAMP are two well known PKA activators and this pathway has been shown to play a role in the expression of the oxidative phosphorylation genes (De Rasmo, Signorile et al. 2010) and CREB-mediated PGC-1α expression (Wallace and Fan 2010). Surprisingly, inhibition of PKA by H89 did not reverse either mitochondrial mass increase or differentiation (Figure 26). On the contrary, H89 promoted an increase in differentiation and mitochondrial mass, with special intensity in those treatments that were less effective in promoting these parameters under control conditions (in the absence of H89). Especially noteworthy is the ability of H89 to increase differentiation even in the presence of serum, which was not observed in the presence of any of the differentiating treatments tested. On the contrary, those treatments that showed a high degree of differentiation and increased mitochondrial mass did not suffer any additional effect in the presence of H89, which may well suggest the presence of an upper limit reachable by this particular cell type, or a convergence of signalling pathways. The ability of H89 to induce differentiation is explained by the documented poor selectivity of H89 for PKA. H89 has been shown to promote differentiation of neuroblastoma cells in an ERK-dependent mechanism (Singleton, Lu et al. 2000) and by blocking Rho kinases (Leemhuis, Boutillier et al. 2002). Although the clarification of the particular mechanism by which H89 induces mitochondriogenesis and differentiation would require further experimentation, these mechanisms are most probably interfering with the signal obtained in the presence of H89 in the conditions of the present study. Consequently, in order to be able to explore the role of PKA in mitochondrial biogenesis and differentiation we selected a highly selective PKA inhibitor. Among the available PKA inhibitors, PKI is reported to exhibit increased specificity, being the natural ligand for PKA (Murray 2008). In the presence of PKI, differentiation and mitochondrial mass suffered a slight reduction in comparison to controls (Figure 27). Although this decrease is not statistically significant, the participation of PKA in both differentiation and mitochondrial biogenesis cannot be totally discarded. Other authors have found that inhibition of PKA by means of KT5720 completely reversed the effect of a combination of forskolin and Isobutylmethylxanthine (IBMX) on N2a differentiation (Leung, Huang et al. 2010). The apparent discordance between the results of this study and ours may be explained by the poor selectivity of KT5720 and other inhibitors for PKA. KT5720 interferes with other protein kinases, some of them at substantially lower concentrations than required for PKA inhibition and some of them at similar concentrations, such as MAPK, which are implicated in cytoskeleton modification and differentiation (Murray 2008). Actually, the lack of selectivity of some PKA inhibitors has been widely reported (Davies, Reddy et al. 2000) and corroborated by the aforementioned experiments in the presence of H89. In this regard, PKI, which is the natural PKA ligand, is considered to be highly specific for PKA (Murray 2008). Consequently, based on the presented results, PKA activation does not seem to be a main
player in the differentiation or mitochondrial biogenesis signalling cues in this particular cell type. Therefore, we subsequently focused on other signalling pathways that could be participating.

The MAPK pathway has been previously associated with neural differentiation. Phosphorylation of ERK 1/2 was associated with differentiation of N2a cells in response to different stimuli such as serum starvation (Evangelopoulos, Weis et al. 2005), forskolin (Lee and Nikoderm 2004), db-cAMP (De Girolamo and Billett 2006), mevastatin (Evangelopoulos, Weis et al. 2009), 2,4-dinitrophenol (Wasilewska-Sampaio, Silveira et al. 2005) or α-lipoic acid (Wang, Wang et al. 2011). In order to elucidate whether the signalling cues that mediate mitochondrial biogenesis in N2a cells are related to those which mediate differentiation, the MAPK pathway was studied by means of the pharmacological inhibition of this cascade with PD98059, an irreversible inhibitor of MEK 1/2. PD98059 almost completely reversed the effect of the treatments on neural differentiation, whereas it reversed strongly but partially the effect of serum starvation, forskolin and db-cAMP on mitochondrial mass (Figure 28). In other words, the inhibition of neural differentiation also reduced novel mitochondrial biogenesis. Our data suggest that serum starvation, forskolin and db-cAMP in the absence of serum activate mitochondrial biogenesis by a MAPK-dependent pathway in differentiating N2a neuroblastoma cells. However, MAPK is most probably not the only pathway involved in the process of mitochondriogenesis, since PD98059 was not able to totally reverse the increases in mitochondrial mass. It is important to point out that PD98059 exerted no effect under basal conditions, which indicates that PD98059 is specific for signal-activated MEK1/2, whereas it is less effective in inhibiting basal MEK1/2 activity. Therefore, the reversion observed is exclusively due to the inhibition by PD98059 of the signal-activated MEK due to the pharmacological activation (SS, F or A) of this pathway. In line with our results, ERK has been found to be required for mitochondrial biogenesis in Schwann cells (Echave, Machado-da-Silva et al. 2009), for acetyl-L-carnitine-mediated neuroprotection and mitochondrial biogenesis under hypoxic conditions (Hota, Hota et al. 2012) and for mitochondriogenesis during osteoblastic differentiation of C3H10T1/2 cells (An, Yang et al. 2010). In the last study, mitochondrial biogenesis was found not only to appear concomitantly to differentiation, but also to be essential for differentiation. The approach of inhibiting mitochondrial biogenesis through the use of zidovudine together with the approach of using cells which are devoid of mtDNA opens a new window for further investigating the possible dependency of differentiation on mitochondrial biogenesis in N2a cells and remains as a challenge for future research. In the same manner, it would be interesting to further research the use of genetic approaches to conditionally silence PKA in order to further clarify the role of PKA in these processes, since a big amount of synergies and interferences are described between PKA and MAPK pathways. (Vossler, Yao et al. 1997; Lee and Nikoderm 2004; Wang, Dillon et al. 2006; Gerits, Kostenko et al. 2008). In this context and in view of the present results, the transcription factor CREB could be a critical, even nodular downstream target for ERK signalling to mediate migration and maturation of neural progenitor.
cells (Shioda, Han et al. 2009). It is then possible that the two distinct PKA and MAPK pathways, far from competing for cAMP and counteracting their effects, are finely coordinated to a common goal, the development of new neurons that are metabolically active. This cross talk would allow an extremely fine tuning of the responses to a joint stimulus, facilitated by the regulation of the timing and the compartmentalization of the different signalling pathways. Further research could be focused on investigating an eventual collaboration of PKA and MAPK and in case of a positive result, determining the time-specific, differentiation-dependent patterns of CREB phosphorylation in N2a and other cell types.

Mitochondria are the major ROS producers within cells (Vieira, Alves et al. 2011). Due to this fact, it was considered appropriate to check if free radicals were altered due to the increase in mitochondriogenesis. ROS have been widely considered as harmful for cell development and as promoters of cell ageing by increasing oxidative stress. Overproduction of ROS results in an increase in oxidative stress which in turn causes damage to lipids, proteins and DNA (Valko, Leibfritz et al. 2007). However, ROS have an important role in cell signalling acting as second messengers after stimulation with cytokines, growth factors and hormones. They can regulate key metabolic pathways such as MAPK (Valko, Leibfritz et al. 2007). ROS have been found to be mediators in neural differentiation in several in vitro models (Vieira, Alves et al. 2011). Furthermore, ROS have been found to mediate the expression of antioxidant enzymes such as hemo oxygenase and catalase in differentiating N2a cells in response to retinoic acid (Shinjyo and Kita 2007). It has been reported that ERK activation requires a moderate concentration of ROS within the cell, whereas antioxidants can prevent this activation (Guyton, Liu et al. 1996; Huang, Chio et al. 2002; Wu, Li et al. 2008; Li, Ji et al. 2011; Tai and Ascoli 2011). Furthermore, they can promote the over-expression of the master regulator of mitochondrial biogenesis, PGC-1α (Irrcher, Ljubicic et al. 2009). Thus ROS exert a dual role in cell signalling, they can be deleterious or beneficial for neural differentiation depending on the redox homeostasis state of the cell.

We therefore examined whether the incubation of N2a cells with the above mentioned differentiating agents (SS, F, A) could modify ROS production compared to control conditions (B). Indeed, the differentiating treatments promoted an increase in ROS production, which was apparent as early as 30 minutes after exposure to the differentiating agents and further increased during the next 24 hours, after which ROS levels were maintained (Figure 29). The very early appearance of a moderate increase in the overall ROS level after exposure to the differentiating agents suggests that the appearance of free radicals apparently is not a consequence of the increased mitochondriogenesis but may represent an early step of a downstream process leading to the induction of both differentiation and mitochondrial biogenesis. It is also noteworthy that serum starvation could only promote a moderate increase in ROS production compared to the other two treatments, being in accordance with the results obtained from the measurement of
other aforementioned parameters such as differentiation percentage, neurite length, mitochondrial mass, MTT activity and PGC-1α expression. This could well indicate the presence of an inherent link between the phenomena of differentiation and mitochondrial biogenesis that involves the production of free radicals.

In order to check if ROS were appearing as messengers of the signalling pathway leading to differentiation, N-acetylcystein (NAC) was used as a free radical scavenger and differentiation percentage was measured. In fact, NAC pre- and co-incubated was able to significantly reduce differentiation (Figure 30), corroborating the hypothesis that this moderate increase in ROS is mediating the signalling cascade that leads to differentiation. In accordance with our results, other authors have found differentiation to be ROS dependent in different models. For example, mitochondrial ROS production is required for neurite induction by Sema3A in PC12 (Schwamborn, Fiore et al. 2004) and also for neurite induction by docosahexaenoic acid (DHA) through MAPK pathway in SH-SY5Y (Wu, Ichikawa et al. 2009).

Subsequently, we investigated whether certain free radical species were involved in the observed mediation of mitochondriogenesis in differentiated neuronal cells. Nitric oxide (NO) and superoxide (O$_{2^-}$) were selected as the most plausible candidates, because of their documented participation in related signalling cascades (Brookes, Levonen et al. 2002; Huang, Chio et al. 2002; Finocchietto, Franco et al. 2009).

It is known that N2a neuroblastoma cells present a detectable basal NO production and express different nitric oxide synthase subtypes (Lopez-Figueroa, Caamano et al. 2001). Therefore, the effect of the differentiation treatments on N2a basal production was checked by means of the Griess reaction for nitrite detection (Griess 1879) and a novel amperometric biosensor for direct live NO detection (Aravindalochanan, Kieninger et al. 2009; Aravindalochanan, Kieninger et al. 2011). Both techniques agreed in showing the changes of NO concentrations as negligible in the conditions of the present study (Figure 31). Nevertheless, before discarding it, we decided to further check if NO was a mediator of differentiation and/or mitochondrial biogenesis, by inhibiting its synthesizing enzyme with L-NMMA, a broad spectrum NOS inhibitor (Boer, Ulrich et al. 2000). Experiments on differentiation percentage and mitochondrial mass also corroborated that NO does not seem to be a main player in these process in the conditions of the present study, at least that we can detect (Figure 32), and suggested that most probably other free radicals may be mediating them. However, we cannot totally discard a contribution of NO, since other authors have found an apparently clear relationship between differentiation and NO production (Evangelopoulos, Wuller et al. 2010; Cerqueira, Cunha et al. 2012; Piantadosi and Suliman 2012). The differences found between our and other author’s results may be due to the different techniques and inhibitors used. However, the lack of a comparative study of different techniques,
inhibitors and cell lines, makes it difficult to assess the real role of this molecule in the differentiation observed in this particular cell model. Thus, NO remains for us as an open question that would require further experimentation in order to discard it completely as a mediator in the differentiation or mitochondrial biogenesis process in N2a cells.

The second free radical selected as a potential mediator was the superoxide anion (O$_2^-$). Superoxide anion, which is mainly generated as a by-product of the oxidative phosphorylation by the electron transport chain of the mitochondria, is released to the mitochondrial matrix, where manganese superoxide dismutase converts it immediately to hydrogen peroxide. Mitochondrial hydrogen peroxide can then diffuse to the cytosol and the nucleus and react with other free radical species, modulate signalling pathways or cause cellular damages (Wallace and Fan 2010). In order to check if the overall increase in ROS was due to an increased production of superoxide anion by the mitochondria, the fluorescent dye MitoSOX red was employed to selectively detect superoxide at the mitochondrial level. Indeed, the differentiating agents promoted an increase in superoxide anion at the mitochondrial level which was correlated to that obtained for ROS. Once again, an early increase of mitochondrial superoxide was found after 30 minutes of exposure, which was maintained and further increased mainly during the first 24 hours of experiment (Figure 33), indicating that superoxide is possibly part of the signalling cascade leading to mitochondriogenesis and/or differentiation. In order to check this hypothesis, we tested the effects of pre- and co-incubation with 100 μM MnTBAP, a well-known SOD mimetic, on these two parameters. A marked reversion of both differentiation and mitochondrial mass with an efficacy similar to PD98059 was found (Figure 35), further supporting our hypothesis. In the same manner, the effect of the combination of PD98059 and MnTBAP on mitochondrial mass and differentiation degree was tested (Figure 36). No significant additive effect was found when comparing the effect of this combination to PD98059 and MnTBAP for any of the treatments tested, suggesting that superoxide production and MAPK activation are part of the same signalling cascade that leads to mitochondrial biogenesis and/or differentiation of N2A cells. In order to discard the possibility that superoxide production could be a downstream phenomenon to MAPK activation, superoxide levels were measured by means of flow cytometry in the presence or in the absence of the MEK1/2 inhibitor PD98059. PD98059 was not shown to reduce the production of superoxide by mitochondria (Figure 37), suggesting that superoxide production does not act downstream of the MAPK cascade, but upstream. It is noteworthy that a significant increase in mitochondrial superoxide levels was found in the presence of PD98059 in combination with the treatments at 24 and 48 hours of treatment but not in basal conditions. Similar results were obtained by measuring H$_2$DCFDA in the same conditions, with free radical increases due to PD98059 being non-significant (data not shown). Increases in free radical production in the presence of PD98059 have been observed by other authors (Caja, Sancho et al. 2009). Although this increase should be further studied, it could be attributed to a compensatory mechanism, attempting to safeguard the...
activation of the MAPK pathway even under PD98059 inhibitory activity, by increasing the concentration of the free radicals (stimulating agents). However, it could also be due to a regulatory feedback loop exerted by ERK1/2 on superoxide production, which would be only unmasked under treatment with PD98059 in differentiating conditions. In other words, it is possible that a second regulatory feedback loop exists, one that allows for ERK1/2 activity to control superoxide production. Furthermore, pre- and co-incubation of MnTBAP with forskolin was able to reduce ERK 1/2 phosphorylation compared to forskolin alone, whereas the combination of MnTBAP and PD98059 totally abolishes ERK 1/2 phosphorylation, reinforcing the hypothesis that superoxide production is an upstream process to the MEK/ERK cascade.

Thus, the presented data suggest that superoxide is a main character in N2a mitochondriogenesis during N2a differentiation. However, the particular mechanism by which differentiating agents, specifically cAMP promoters, can induce an increase in superoxide production is still poorly understood. Recent research conducted in this direction points out Epac activation as a stimulator of free radical production, whereas PKA signalling is reported as a suppressor of free radical production (Hara, Kobayashi et al. 2012). In this study, different cAMP analogs/cAMP promoters, presenting a different selectivity for PKA or Epac, promote a different effect on free radical production. Although it would require further exploration, Epac activation appears as a good candidate to mediate the effects of forskolin, db-cAMP and serum starvation on mitochondrial superoxide production. It is also known that Epac can act as a GTP exchange factor to activate Rap1, which in turn can activate B-Raf and thus can lead to the MEK/ERK phosphorylation cascade (Peace and Shewan 2011). Moreover, since MnTBAP is not able to completely abolish ERK 1/2 phosphorylation, a cooperation of these two signalling pathways that lead to ERK activation cannot be discarded. In the same manner, we cannot discard that other signalling pathways could cooperate with ERK signalling to achieve the final effect of increasing mitochondrial mass, since the combination of PD98059 and MnTBAP does not completely reverse the effect of serum starvation, forskolin or db-cAMP.

In line with our results, ROS-elicited MAPK signalling has been found to mediate neural differentiation in several in vitro models (Schwamborn, Fiore et al. 2004; Wu, Ichikawa et al. 2009; Vieira, Alves et al. 2011) including N2a cells in response to α-lipoic acid (Wang, Wang et al. 2011). Furthermore, hydrogen peroxide directly added to N2a cells has been found to induce the phosphorylation of ERK, which is dependent on the release of calcium from the endoplasmic reticulum (Kemmerling, Munoz et al. 2007), whereas in differentiating N2a neuroblastoma cells heme oxygenase induction was found to be ROS dependent (Shinjyo and Kita 2007). It was also reported that mitochondrial biogenesis is triggered during the first stages of neural differentiation in PC12 promoted by the over-expression of the transcription factor NeuroD6, which integrates the co-ordination of mitochondrial biogenesis and antioxidant response (Uittenbogaard, Baxter et
This response includes the expression of PGC-1α and ROS detoxifying enzymes. However, in PC12 serum starvation promotes apoptosis, whereas NeuroD6 can rescue the cells from this oxidative stress stimulus. On the contrary, in N2a and other cell lines, serum starvation is necessary to trigger differentiation (Flaskos, McLean et al. 1998; Radha, Rajanna et al. 2008). Compared to PC12, N2a seems to both tolerate and require a higher ROS level for differentiation, since the presence of a superoxide scavenger partially prevented both mitochondrial biogenesis and differentiation.

The presented results can be explained by the new concept of mitochondrial hormesis or mitohormesis. Hormesis refers to a beneficial action resulting from the response of an organism to a low-intensity stressor otherwise detrimental when administered at higher concentrations or intensities (Masoro 1998; Ristow and Schmeisser 2011). Thus, small increases in the free radical production by the mitochondria are beneficial for the natural development of neurons (Tsamtali, Walcott et al. 2005; Cheng, Hou et al. 2010; Vieira, Alves et al. 2011). Free radicals have been demonstrated to be beneficial by triggering hormetic signals, which could protect the organism from later insults. In fact, one study on skeletal muscle cells demonstrated that the scavenging of free radicals with N-acetylcysteine was able to reverse the beneficial effect of moderate concentrations of H₂O₂ on the overexpression of PGC-1α and mitochondrial biogenesis (Ircher, Ljubicic et al. 2009). Superoxide was also found to trigger hormetic signals which were related to an increase in longevity in Caenorhabditis elegans (Yang and Hekimi 2010). Thus, regarding the present study and from an evolutionary point of view, a moderate increase in ROS (in particular superoxide) concentration could elicit a cellular defense response (St-Pierre, Drori et al. 2006), expressed as a downstream process leading to novel mitochondriogenesis, mitotic arrest and differentiation. In this way, cells could eventually compensate for any ROS-mediated damages to existing mitochondria (e.g. due to the oxidation of lipid membranes), while providing the sufficient energy supplies to support neural differentiation. In this context, free radicals would act as messengers of this biogenesis process, which involves the activation of signalling pathways, transcription factors and gene expression.

Up to this point all the discoveries presented in this thesis were obtained from cells cultivated in two dimensional/flat culture plates. This was vital, since 2-D cultures represent the regular approach for the initial experimentation and definition of factors affecting neural differentiation in vitro. After the definition of the differentiation mechanism in 2-D culture, the research framework can be adapted with modifications to the more realistic 3-D system. In addition, one of the objectives of this PhD and the whole Cell Check project (which funded the research) was the design of a three dimensional cell culture that could better resemble a physiological tissue, and its combination with novel biosensors. Therefore, a first approach towards the design of a three dimensional tissue-like model that would be compatible with a custom designed impedimetric
biosensor will be described in following. Some examples of signal recordings are given as a proof of the possibilities of this combination to get additional information to that obtained with two dimensional cell cultures.

As previously introduced, the motivation to carry out these studies is based on the knowledge from drug screening that most of the candidate drugs that enter Phase I trials fail to reach the bedside. This is due to the fact that the models commonly used to assess the pharmacological characteristics of one drug are not representative of the physiological reality. The most common cellular models are two dimensional models, in which cells coming from a certain cell line or tumor are grown on flat flask surfaces (Pampaloni, Reynaud et al. 2007). It is known that those cells don’t express part of their natural phenotype (Wang, Wu et al. 2009) i.e. gene expression and protein/enzyme transcription due to this mechanical alteration, the absence of extracellular matrix (ECM) and surrounding cells (Birgersdotter, Sandberg et al. 2005; Li, Livi et al. 2007). That makes 2D models limited in the context of usefulness to predict the cellular responses of real organisms in vivo. However, whole organisms and organs, organ slices, spheroids or simply ECM gels with embedded cells have risen as a new potential model for drug screening, with increased predictive power. For these reasons, designing a three dimensional model whose electric characteristics could be monitored on-line appeared as a main objective of this PhD.

Consequently, we designed a three dimensional neuronal model with the aim of pushing this novel research field a step forward. We successfully cultivated N2a cells in bactoagar three dimensional matrices at different concentrations of bactoagar and different cell densities. Furthermore, we were able to cultivate them inside the electrodes of an impedance analyzer and maintain the cells alive. New protocols for testing viability were developed in order to be able to test cell viability and death within three dimensional environments such as a modification of the MTT assay. With those new protocols it was found that cells were alive inside the matrix. A custom-designed impedance analysis setup was developed by Thomas Jacobs (Jacobs 2009) in order to monitor signals from cells embedded in gel matrices on-line when they are perfused with medium or stimuli (see Materials and Methods). The influence of different parameters such as difusibility (Figure 39), storage conditions (Figure 40), cell density (Figure 41) and gel concentration (Figure 42) were tested in order to find the optimum conditions for cells that would allow impedance measurements by the Impedance biosensor I. It was found that diffusion through the pores of the ground electrode is enough for maintaining cellular survival, whereas the ideal storage conditions for N2a cells embedded in bactoagar gels are 37 °C and 5% CO$_2$, i.e. in a standard incubator. Regarding cell density, it was assumed that the more cells we had, the bigger signals we would get at the time of performing impedance sensing. Therefore, as a reasonable maximum cell density that would not promote cell death we selected 1 million cells per single electrode (120 μL). It is noteworthy that MTT measured 24 h after immobilization appeared to be
increased with respect to 0h. This increase could be due either to an increased metabolic activity of cells after stabilization of the cell culture or even to cell proliferation. In any case, it indicates the healthiness of the cell culture after 24 hours of immobilization. Concerning gel concentration, overly concentrated gels did not allow proper gelation or cell survival and were discarded, while overly watery ones did not support impedance measurement due to their low resistance to the flowing current. By increasing gel concentration, the mobility of charges decreases, the ohmic resistance of the sensor increases and therefore it is adapted to the output impedance (50 Ohm) of the impedance analyzer. Therefore, a gel concentration of 2% was selected as the reasonable maximum concentration for impedance measurements.

As discussed in the introduction, impedance analysis (Doerner, Schneider et al. 2007) appears to be a promising technique for the exploration of electrical properties of 3D cell culture models. During the secondment in OVG-UNIM (February to April, 2009) impedance measurements were set up. Experiments with different concentrations of gel, signals from gels with and without cells, unstimulated cells and responses of cells to stimuli such as the neurotransmitter acetylcholine (ACh) were all recorded (Figure 43 and Figure 44). Special care was taken concerning temperature stability, due to the fact that impedance recordings are extremely sensitive to temperature changes. Impedance spectra were recorded in the range of 10 to 110 MHz and signals were recorded with the help of custom made software. After setting up stable basal conditions in the presence of embedded cells, cells were stimulated with the excitatory neurotransmitter acetylcholine. It was expected that changes in the impedance as a measure of channel opening and depolarization in immobilized N2a cells would be detected. ACh was used as excitatory neurotransmitter, since these cells are known to express a cholinergic phenotype (Gomez, Boutou et al. 1998). Depolarization should promote the opening of voltage gated calcium channels, also present in N2a cells (Gaasch, Geldenhuys et al. 2007), and thus increase the ion conductivity of the system, while the whole cluster should contribute to alterations in the impedance recordings. In this context, the influence of diffusion of ACh within the 3D structure on the kinetics of cellular response was characterized. These series of experiments were performed as a proof of principle of the suitability of this technique to detect electrical changes in the neuronal cluster environment. Impedance analysis results showed the response of these cells to ACh, which induces a decrease in the magnitude of impedance. Thus, changes in cell membrane potential induce the change of the overall conductivity of the gel films. The fact that the response to ACh appears 10 minutes after the perfusion with this neurotransmitter is attributed to the slower diffusibility of the gel compared to pure medium. This work was published as the proof of principle that membrane depolarization in neural cells embedded in three dimensional matrices can be online monitored by means of impedance analysis.
These results were found to be very promising. However, in order to achieve the objective of detecting differentiation in a three dimensional neural culture, some topics needed still to be addressed. Firstly, a different gel matrix that would allow differentiation of neuroblastoma cells needed to be found. Secondly, taking in account the time range in which differentiation happens, a parallelized system that would allow the performance of more than one experiment at a time needed to be developed, in order to make experiments time-efficient.

In the second phase of experiments different gel matrices were studied as candidates for the development of a new 3D model of neural differentiation. Cell density as well as gel composition was optimized mainly in terms of viability and grade of differentiation (Figure 45 and Figure 46). The gel composition and matrix structure are important parameters for cell differentiation similar to the extracellular matrix (ECM) for naturally differentiating cells. Cell survival, organization, growth and differentiation are strongly dependent on cell adhesion to the ECM (Kintzios et al. 2007). Therefore, different gels were compared in terms of their ability to sustain differentiation in 3D: bactoagar, agarose, collagen (CG), basement membrane extract (BME), and a home-made mixture of collagen and laminin (CLG, see materials and methods). In a previous study it was shown that bactoagar gel matrices can be used for cell immobilization and preservation (Kintzios et al. 2004). CGs are extensively used as 3D matrices (Desai et al. 2006; Lin et al. 2005) while BME possess the natural composition of the extracellular environment. In these series of experiments we compared our CLGs with all those known substrates and observed differentiation. Neither bactoagar, nor agarose nor bare collagen were able to support the differentiation of N2a cells in a period of 48 hours. On the contrary, BME and CLGs were able to do it with two main differences: BME promoted the formation of short and robust neurites, while CLGs promoted the appearance of long neurites which tended to touch adjacent neurons. Therefore CLGs were selected for subsequent experiments. The reason why bactoagar and agarose do not support differentiation could be the absence of anchorage proteins in these matrices, which are necessary for a proper dendrite elongation (Cullen et al. 2007). Other authors have used the strategy of mixing different matrices and components of the extracellular matrix in order to favor neurite extension (Cullen et al. 2007; Lin et al. 2005) with similar results on other cell models. Similarly, other authors have also successfully developed 3D cultures for mammalian cell cultures. Some of them were found to preserve cells (Brown, MacLellan et al. 2008), others can maintain ESC in an undifferentiated state, and others can support differentiation (Krewson, Chung et al. 1994), in accordance with our observations. It is also noteworthy that the addition of db-cAMP did not show any further effect on differentiation, probably because laminin by itself was able to reach the maximum differentiation possible to this cell type. Although a deeper study would be needed, it is possible that similar signalling cues could be used by both agents to promote differentiation. Indeed, other authors have found the ERK pathway to be crucial for laminin-induced neural differentiation of mesenchymal stem cells (Mruthyunjaya, Manchanda et al. 2010).
After optimising cell density in order to maintain viability and differentiation, experiments on the detection of differentiating N2a cells were performed during the secondment at the University of Magdeburg OVG-UNIM (January to March, 2010). Differentiation was monitored on-line by means of the improved miniaturized impedimetric biosensor designed by Thomas Jacobs (see materials and methods). After developing protocols for proper gel formation into the electrodes, we performed preliminary experiments to discard any artefacts due to temperature changes, gel instability, etc. in order to set up basal conditions for the subsequent experiments. Viability tests and cell morphology showed no effect of current flow on cell behaviour after experiments compared to control ones, indicating that the effect of current flow through the cells was not affecting the parameters to measure, i.e. the differentiation of the cells (Figure 47). Thus experiments in undifferentiated cells were compared to differentiating cells in the time range of neural differentiation (Figure 48). After thermal stabilization undifferentiated cells presented a stable base line, so no specific change in the dielectric or conductive properties of bare gels with embedded N2a cells were found. In contrast, the electrical impedance analysis of differentiating N2a cells presented a local minimum during thermal equilibration followed by an increase in the impedance magnitude, which is attributed to the differentiation process. However, we cannot totally discard a contribution of the specific properties of the gel films, since both contributions can not be fully distinguished with the actual sensor signal processing. This takes into account that a bare gel and gel matrix embedded cells can not be treated as a linear superposition of individual electrical properties. Similar results were obtained by (Slaughter and Hobson 2009), who detected similar curves during NGF-induced PC12 differentiation to sympathetic neurons. In their study a decrease in normalized impedance followed by a gradual increase and stabilization was observed. However, the subsequent increase in the impedance magnitude can be attributed to the formation of neurites during differentiation. Neurites are thin projections of the neuronal body and thus extensions of the cellular membrane surface. If we consider the cell as a resistor/capacitor (Kyle et al. 1999), an increase in the number of neurites would result in a permanent increase in the ohmic resistance and thus in an increase in the magnitude of the impedance. In the same manner, the increase in the impedance magnitude can also be attributed to the increased membrane content of the cell due to mitochondrial biogenesis. Hence, the two phenomena of neurite elongation and mitochondrial biogenesis are probably collaborating to induce an increase in the impedance magnitude that is detectable by means of impedance analysis.

In any case, impedance sensing appears as a very promising tool for live detection of differentiation in three dimensional cell cultures. Its exploration as well as further miniaturization by the use of microfluidics and the deeper exploration of three dimensional matrices could open new perspectives for future research. 3D models have opened a new window to the future of developmental neuroscience, since they better resemble physiological conditions in vivo. Within normal tissues, cells live in a 3D environment, i.e. they relate to other cells in the three dimensions
of the space and with the extracellular matrix. All these interactions are lost in classical 2D cultures, which confer them with an additional limitation. On the contrary, 3D models can simulate inter- and extra-cellular environments. In line with our discoveries, several authors have applied the strategy of mimicking the extracellular environment with different purposes: for example by mimicking the conditions of nerve regeneration with a collagen sponge populated with Schwann cells and fibroblasts (Gingras, Beaulieu et al. 2008), or developing new matrices which could be transplanted for nerve regeneration (Lin, Wu et al. 2005). The use of 3D models as an intermediate step between classical 2D cell culture and animal research will have a strong impact in the reduction of the number of animals sacrificed for experimental purposes (Pampaloni, Reynaud et al. 2007), due to the fact that more non-efficient compounds will be screened out and will not proceed to the animal experimentation phase. Of course, as 3D culture models are not extensively used within the scientific community, additional comparative studies between already existing 3D matrices (Lin, Wu et al. 2005; Cullen, Lessing et al. 2007), as well as between 2D and 3D environments (O'Connor, Stenger et al. 2001; Mao and Kisaalita 2004; Lin, Wu et al. 2005; Desai, Kisaalita et al. 2006; Li, Livi et al. 2007), are required, along with the development of new techniques for monitoring the activity of neuronal cells in a 3D environment (Mao and Kisaalita 2004) and the development of novel scaffolds with increased biocompatibility and convenience of use (Wu, Zhao et al. 2006), natural, engineered (Wang, Wu et al. 2009) or gel-free (Ong, Zhang et al. 2008). As a rather recent example, three dimensional neural networks have been developed on borosilicate glass spheres. Those beads are packed in layers with a hexagonal symmetry, which makes it possible for neurons to connect with other layers and establish synapses. Moreover, as the beads are transparent, it is possible to stain cells with a fluorescent dye or transfect them with a GFP and visualize the connections between neurons from different layers by fluorescence microscopy or simply by optical imaging. The axonal growth can be directed as desired with the use of chemical attractants or differentiating mediators such as cAMP (Pautot, Wyart et al. 2008). 3D models better resemble the physiological situation and also introduce a new challenge in drug screening, since techniques able to deal with the third dimension are rare. Emerging novel, miniaturized biosensor technologies have the potential to revolutionize the study of neuronal differentiation, since they can overcome these difficulties by detecting neuron-derived electrical signals and differentiation markers, such as shape or attachment in a non-invasive, high-throughput fashion. They offer the possibility of automation, single cell and even subcellular measurements, 3D cell culture and tissue slices testing, they can be manipulated to generate a desired environment, decide the shape, the gradient concentration, use extremely low reactive volumes, etc. and all this at a low price. A new interdisciplinary science is emerging, the future science of neurodevelopmental biosensing.
7 CONCLUSIONS

Differentiation of N2a neuroblastoma cells in response to serum starvation, forskolin or db-cAMP is coupled with cell cycle arrest and mitochondrial biogenesis. In addition, PGC-1α, the master regulator of mitochondrial biogenesis, is overexpressed and there is an increase of mitochondrial metabolic activity in differentiated cells. ROS and specifically superoxide anion are increased in differentiating cells as early as 30 minutes after exposure to differentiating agents. In particular, superoxide scavenging by MnTBAP partially reverses differentiation and mitochondrial biogenesis, as well as the inhibition of the MAPK cue by PD98059. The non-additive effect of the combination of MnTBAP and PD98059, together with the inability of PD98059 to reduce superoxide production and the reduction of ERK 1/2 phosphorylation by MnTBAP, places superoxide as an apparent upstream regulator of MAPK. However, we cannot discard the possibility that other signalling pathways could also be taking place and cooperating with the ones described in the present study, thus opening several windows for further research. These results suggest that differentiation and mitochondrial biogenesis in N2a neuroblastoma cells are coupled and mediated by a hormetic increase in these particular free radical species. The concomitant triggering and coordination of these two phenomena leads to the complete development of a neural phenotype in N2a neuroblastoma cells, by guaranteeing both the expression of the differentiation pattern and sufficient provision of energy to the emerging neuronal clusters.

As a first approach to the design of a three dimensional neural culture, different gel matrices were studied as candidates for the development of a new 3D model of neural differentiation. Cell density as well as gel composition were optimized mainly in terms of viability and grade of differentiation. Depolarization and differentiation were monitored on-line by means of two pre-prototypes of a novel impedimetric biosensor especially designed for 3D cell cultures. Viability tests and cell morphology showed no effect of current flow on cell behavior. The electrical impedance analysis of N2a cells in CLG revealed that the differentiation process is mediated by an increase of the impedance magnitude, which is not observed in non-differentiated cells on CGs. This three dimensional model appears to be promising tool for drug screening in developmental neurobiology, and would be worthy of further research.
8 PUBLICATIONS

Guest editor of the international peer-reviewed journal “Current Pharmaceutical Design” for the special issue “Mitochondrial biogenesis: pharmacological approaches” (under preparation)


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