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DIPLOMARBEIT

Die Rolle von Histondeazetylase 1 in der neuronalen Differenzierung von PC12 Zellen

The Role of Histone Deacetylase 1 in Neuronal Development of PC12 Cells

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1. Zusammenfassung – Summary

1.1 Zusammenfassung

Histondeazetylasen entfernen Acetylgruppen von Histonproteinen und haben damit eine wichtige Funktion in der Repression von Genen. Epigentische Mechanismen steuern biologisch bedeutsame Prozesse, unter anderem die Differenzierung von pluripotente Stammzellen zu neuronalen Zellen. Dieser Vorgang wird durch den Erwerb von definierten Genexpressionsmustern charakterisiert.

Das Hauptaugenmerk der arbeit liegt in der Untersuchung der Histondeazetylase 1 (HDAC1) in der neuronaler Differenzierung. HDAC1 ist ein Mitglied der Proteinfamilie der Histondeazetylasen und wird in verschiedenen nukleären Regulationskomplexen gefunden. PC12 Zellen sind ein oft verwendetes Modelsystem zur Untersuchung von Differenzierungsprozessen. Behandlung von PC12 Zellen mit neuronalem Wachstumsfaktor (Neuronal Growth Factor (NGF)) induziert neuronale Differenzierung und führt zur Ausbildung von Axon-ähnlichen neuronalen Strukturen. Dieses System erlaubt zusätzlich die Untersuchung unterschiedlicher chemischer Verbindungen zur Inhibierung von Histondeazetylasen. Eine seit kurzem bekannte Verbindung mit dem Namen MS-275 zeigt grosse Spezifität in der Inhibierung von HDAC1 und wird in klinischen Versuchen bereits in der Behandlung von Tumoren des Nervensystems mit viel versprechenden Ergebnissen eingesetzt.

In dieser Arbeit wurde eine Methode zur Quantifizierung der neuronalen Differenzierung in PC12 Zellen entwickelt. Es konnte gezeigt werden, dass Behandlung mit MS-275 die NGF induzierte neuronale Differenzierung in PC12 Zellen signifikant verstärkt. Zusätzlich konnte eine erhöhte Expression des neuronalen Markerproteins Neurofilament Medium festgestellt werden. Im Gegenzug dazu führte die ektopische Überexpression von HDAC1 zu einer Blockade der von NGF induzierten neuronalen Differenzierung in PC12 Zellen. Die Ergebnisse dieser Arbeit könnten zu einem besseren Verständnis der Wirkungsweise von Inhibitoren der Histondeazetylasen im Zuge von klinischen Behandlungen beitragen.

1.2 Summary

Histone deacetylases mediate gene repression by the removal of acetyl groups from core histones. Neuronal differentiation relies on a complex signaling network and constitutes a prime example on how epigenetic modifications regulate cell-fate specification. Progression from non-neuronal pluripotent stem cells to a restricted neural lineage is characterized by acquisition and maintenance of distinct patterns of gene expression. The interest of this work was the role of histone deacetylase 1 (HDAC1), a vital member of the protein family of histone deacetylases, in neuronal differentiation, utilizing the rat cell line PC12. HDAC1 constitutes a component of numerous nuclear multi-protein complexes involved in neuronal development and is thereby a potential regulator of neuron-specific gene expression.

PC12 cells represent a well established and feasible model system to study various mechanisms involved in neurogenesis. Neuronal growth factor (NGF) induces reversible neuronal differentiation accompanied by the outgrowth of axon-like neuronal structures, a process that can be conveniently assessed and manipulated in this *in vitro* cell system. A wide collection of histone deacetylase inhibitors (HDIs), varying in specificity for different HDACs, are available to clarify the role of histone deacetylation in the establishment of the neuronal phenotype. The novel HDI MS-275 shows high specificity for HDAC1 and, according to recent clinical trials, represents a promising candidate in the treatment of cancers of the nervous system.

A neuronal outgrowth assay was developed to allow standardized quantification of neuronal development and it could thereby be demonstrated that treatment with the HDI MS-275 significantly enhanced NGF-induced differentiation in PC12 cells. In addition, our studies showed induction of the neuronal marker gene neurofilament medium protein upon treatment with MS-275. Conversely, ectopic overexpression of HDAC1 protein in PC12 cells led to a block neuronal differentiation. These results could provide an explanation for the clinical applicability of histone deacetylase inhibitors in the treatment of cancers of the nervous system and demonstrate the significance of epigenetic mechanisms in the process of neurogenesis.

2. General Introduction

2.1 The Structure and Function of Eukaryotic Chromatin

Eukaryotic DNA is packaged into the nucleus with the help of a number of histone and nonhistone proteins that collectively make up the chromatin fiber. Organising DNA into a higher order structure helps reducing the overall length of DNA in eukaryotic genomes by a factor of more than 10.000, allowing the roughly two meters of DNA of a human cell to be packed into the cellular nucleus. In addition to a reduction in length, chromatin compaction is a mechanism to ensure protection of the cellular genome. The highly dynamic chromatin fiber can exist in several states of compaction that regulate access to DNA for essential cellular processes, such as transcription, replication, repair, and recombination. Highly condensed chromatin adopts a structure called heterochromatin, a state which is characterized by less accessible and frequently transcriptionally silent genes [1]. The nuclear metaphase chromosome constitutes the highest packing unit of eukaryotic DNA [2]. On the other hand, decondensed chromatin, termed euchromatin, is more accessible than heterochromatin, and contains the majority of actively expressed genes.

The fundamental repeating unit of chromatin is the nucleosome, a disc-shaped protein octamer that contains a histone H3/H4 heterotetramer and two H2A/H2B heterodimers. Figure 1 (C) illustrates the 147 bp of left-handed super-helical DNA wrapped around the globular domain of the histone octamer. Nucleosomes are connected by linker DNA, varying from 10 to 60 base pairs. This results in a chromatin fibre of approximately 10 nm in diameter, a state termed “beads-on-a-string” conformation. Chromatin is furthermore organised into 30 nm fibres stabilised by histone H1 protein.

Each of the highly conserved histones (H2A, H2B, H3 and H4) contains a globular domain, known to interact with other histones and the DNA helix within a nucleosome. In addition, each histone has a flexible N-terminal tail, which protrudes from the surface of the histone octamer. Figure 1C depicts the flexible histone tail domains extending radially from the lateral surface of the nucleosome core particle. The flexible tails are highly basic and are the substrates for numerous enzymes that introduce a diverse array of post-translational modifications (see chapter 2.2). As stated above, the chromatin fiber is not a rigid DNA-protein complex, but instead is believed to act more like a dynamic system with confined decondensation and

recondensation events that alter the accessibility of certain stretches of the packed DNA [3].

2.2 Chromatin Modifications

It is known for more than 40 years that histone modifications such as acetylation have an important role in the cell by modulating transcription efficiency [4]. N-terminal histone modifications were known to regulate the interaction between the highly basic histone tails and nucleosomal DNA. Post-translational modifications comprise phosphorylation of serines and threonines, methylation of lysines and arginines, acetylation of lysines and ubiquitylation of lysines [5]. Those modifications highly correlate with changes in chromosomal accessibility. Acetylation of lysines 9 and 14 at histone H3, methylation of histone H3 K4 (histone H3, position 4) and phosphorylation of histone H3 S10 are, for example, often linked to transcriptionally active regions on the genome whereas tri-methylation of histone H3 K9 is characterized as a modification correlating with transcriptional repression.

Several amino acids constitute targets for various modifications, leading to a further increase in complexity of histone modifications. For example, the amino acid lysine can be acetylated as well as mono-, di- or tri-methylated. In addition, modifying the amino acid next to an already established modification can lead to an altered overall effect. It is known that phosphorylation of serine 10 on histone H3 facilitates acetylation of lysine 9 on histone H3. These two modifications in combination are termed phospho-acetylation and are strongly associated with the promoter regions of active genes [6-8].

As stated above, distinct histone post-translational modifications correlate with specific transcriptional states, leading to the proposal of the histone-code hypothesis [3]. This hypothesis suggests that specific patterns of modifications can be read like a molecular “bar code” to recruit the cellular machinery and to determine the biological read-out. The histone code presumes that histone modifications regulate the interaction between the highly basic histone tails and nucleosomal DNA. For example, acetylation of lysines would remove a positive charge and would lead to a decrease in the affinity between histone tails and DNA. On a greater scale, acetylation and deacetylation events would go along with a dynamic transition between transcriptionally active (open) or inactive (closed) chromatin.

In addition, certain modified amino acids are known to present binding motives for effector proteins recognising the modification via chromo-, bromo- or SET-domains. Chromodomains can be found in transcriptional factors like HP1 (Heterochromatin Protein 1) and preferentially interact with methylated histone marks, whereas bromodomain containing proteins are targeted to acetylated lysine marks [3]. The SET domain methyltransferase family is responsible for the transfer of a methyl group to the amino group of a lysine residue on a substrate [9].

Although there is plenty of molecular evidence supporting the histone code theory, the molecular details by which nonhistone proteins are recruited to the chromatin remain open for discussion. For example, most *in vitro* results concerning chromatin dynamics are performed using histone preparations with undefined posttranslational modification states. In a recent study, Dorigo and co-workers used recombinant histone protein in order to study the physical properties of the N-terminal histone tails [10]. Interestingly, deletion of large parts of the histone H4 N-terminus did not impede formation of the 30 nm fiber. Laser cross-linking studies revealed that no disruption of DNA-histone binding could be identified upon hyperacetylation of histones, hydrodynamical properties of unmodified as well as hyperacetylated histones were identical [2]. Post-translational modifications at the globular histone domain could be responsible for the properties of DNA-histone interactions. Dorigo et al. could demonstrate that nucleosome arrays, consisting of core histone octamer-DNA complexes connected by linker DNA, are stabilized by introducing disulfide crosslinks between the base of the histone H4 tail and the core domain of histone H2A and that deletion of the base of the histone H4 tail prevented complete compaction of nucleosomes [10, 11]. These results suggest an important role of the C-terminal histone tails in chromatin fiber compaction, adding complexity to the histone code hypothesis. Furthermore, the histone code correlates certain histone modification always with the same biological read-out. However, in some cases histone acetylation is found to inhibit and not activate gene expression [2].

The N-terminal histone tails do not constitute a unique substrate for histone modification enzymes. Affinity of histones for the nucleosomal DNA could be influenced by C-terminal histone modifications that result in changes of the balance between stationary and mobile nucleosome octamers [12]. Cosgrove and co-workers proposed the “regulated nucleosome mobility” model, which describes nucleosome

mobility as the missing link between histone code and chromatin dynamics and therefore adding more possible regulation of DNA accessibility [5].

Presumably, clarification of the mechanism of chromatin dynamics could answer fundamental questions in nature sciences in the future. Initial sequencing of the human genome revealed that approximately 35.000 protein coding genes are encoded in our DNA [13]. More recent publications reduced the number of expressed protein-coding genes to 25.000 to 20.000 [14]. Despite the difference in complexity between fruit flies, mice and humans, the total number of genes expressed in those organisms is comparable. Efficient chromatin organisation and networking may have led to an increase in complexity and more diversity throughout the evolution of species that goes beyond the basic information found in the genetic code [3].

2.3 Histone Acetylation

Reversible histone acetylation is one of the best-studied histone modifications. Acetylation takes place at the amino groups of lysine residues present within the N-terminal extensions of the core histones. The acetylation state of target lysines (five lysines in histone H3, four in histone H4, four in histone H2B and five in histone H2A) is tightly controlled by the balance of two counteracting enzyme families, histone acetyl transferases (HATs) and histone deacetylases (HDACs) [15]. The complexity of histone acetylation is furthermore increased by the fact that lysines can be mono-, di-, tri- and tetra-acetylated [16]. Acetylation of the core histone tails disrupts higher-order chromatin folding and thereby leads to better accessibility of the DNA for various nuclear factors. In addition, interactions with non-histone chromosomal proteins, containing bromodomains, can be modulated by acetylation and deacetylation of histones [17]. Deacetylation of a lysine residue can further lead to methylation of the respective histone protein and thereby allow recognition by proteins harbouring Tudor and chromodomains or WD40 repeats [18, 19]. For example, the chromodomain protein HP1 specifically binds to trimethylated lysine 9 of histone H3 and subsequently leads to heterochromatin formation [20, 21]. Condensed heterochromatin is generally poorly acetylated and transcriptionally silent, while decondensed euchromatin regions containing transcriptionally active genes are most often found associated with acetylated histones [22, 23].

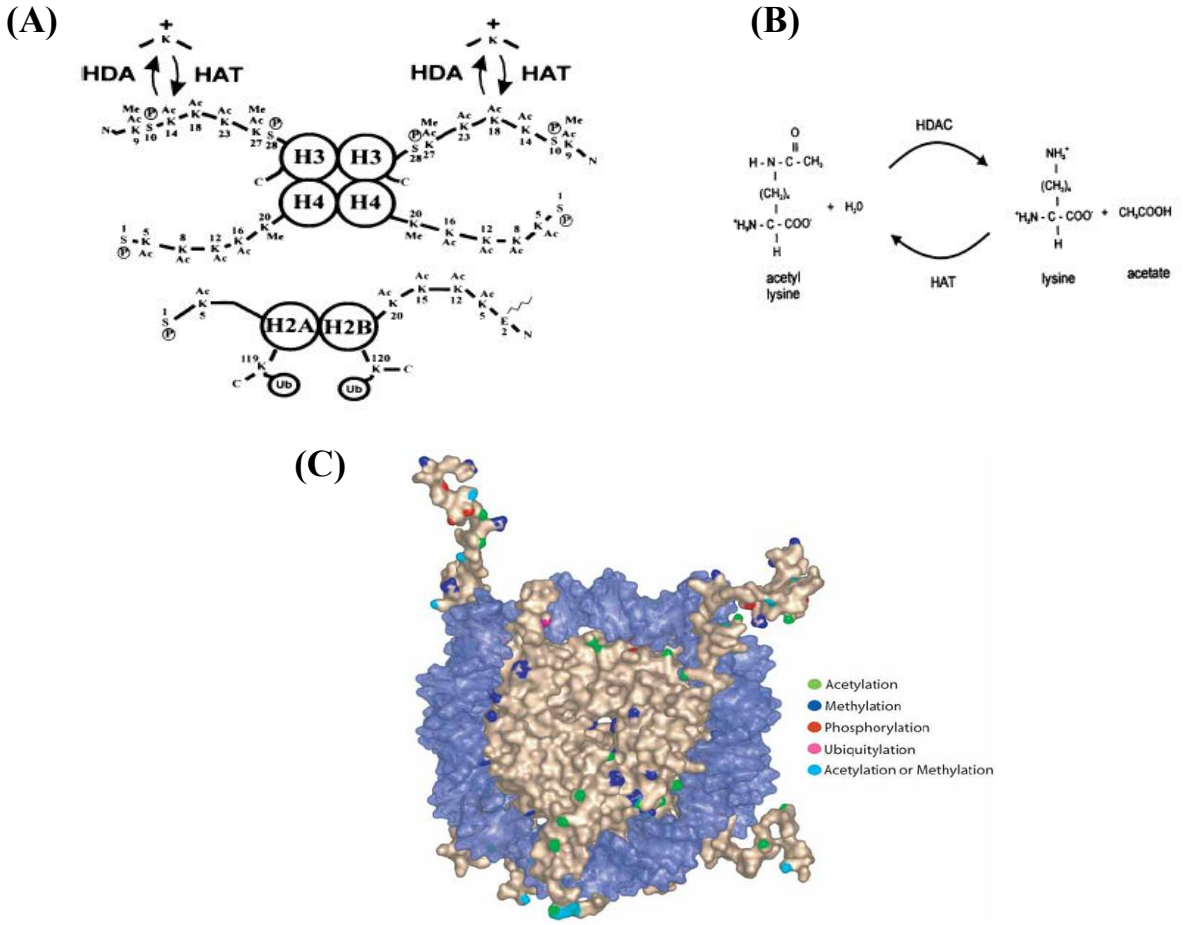


Figure 1. N-terminal tails of core histones are modified by several posttranslational modifications. (A) Acetylation (Ac), methylation (Me), and phosphorylation (P). (B) Dynamic histone acetylation occurs on lysine residues and is catalyzed by histone deacetylases (HDACs) and histone acetyltransferases (HATs). (C) Surface representation of the nucleosome core particle, viewed down the DNA superhelix axis. DNA is coloured light blue, the histone octamer is displayed in wheat. The positions of known histone modifications are shown. Pictures taken from [22], [24].

2.4 Histone Acetyltransferases and Histone Deacetylases

As described above, histone acetyltransferases (HATs) and histone deacetylases (HDACs) perform a central function in the cell by mutual regulation of the acetylation state of histone and non-histone proteins. The discovery of the nuclear acetyltransferase GCN5 [25, 26] and the first mammalian histone deacetylase HDAC1 [27] indicated crucial functions of these enzymes in controlling gene expression. In addition, enzymes which were identified to reversibly acetylate histones were subsequently known to modify non-histone proteins like transcription factors (p53 and E2F-1), nuclear import factors, α-tubulin and the chaperone protein

Hsp90 [28]. Acetylation can influence metabolic stability and the biological function of a modified protein and is a widely used posttranslational mechanism of controlling protein function [15, 29].

HATs as well as HDACs are mainly present within multi-protein chromatin-remodelling complexes. Transcriptional activator complexes containing histone acetyltransferases are known to increase histone acetylation and recruit general transcription factors as well as RNA polymerase II. Conversely, recruitment of repressor complexes with HDAC activity leads to deacetylation of histones, stabilisation of the nucleosome structure and the formation of a chromatin state impeding transcription and elongation processes [3].

Higher organisms have evolved substantial complexity in the HDAC family that was divided into four classes according to phylogenetic analyses and sequence homologies with the yeast proteins homologues (Figure 2). Class I deacetylases are related to the yeast transcriptional repressor RPD3, include HDAC1, HDAC2, HDAC3, HDAC8, can generally be detected in the nucleus and show ubiquitous expression in various human cell lines and tissues. Class II deacetylases are related to yeast Hda1-like proteins, consist of HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10 and can shuttle between the nucleus and the cytoplasm. The class IIb HDACs, HDAC6 and HDAC10, contain two deacetylase domains and are found in the cytoplasm. As can be seen in Figure 2, one of the catalytic domains of HDAC10 is inactive. Currently, HDAC11 is the only known class IV deacetylase. The protein shares sequence similarities with the catalytic core regions of both class I and II enzymes but does not have strong enough identity to be placed in either class. In addition to the enzymes shown in Figure 2, we know one more HDAC class, the class III HDACs (SIRT1, 2, 3, 4, 5, 6 and 7). Class III proteins are evolutionarily unrelated to class I, II or IV and requires NAD^+ for the regulation of gene expression in response to changes in the cellular redox status. For example, SIRT1 has been shown to interact with and deacetylate p53, resulting in repression of p53-mediated transcriptional activity [30].

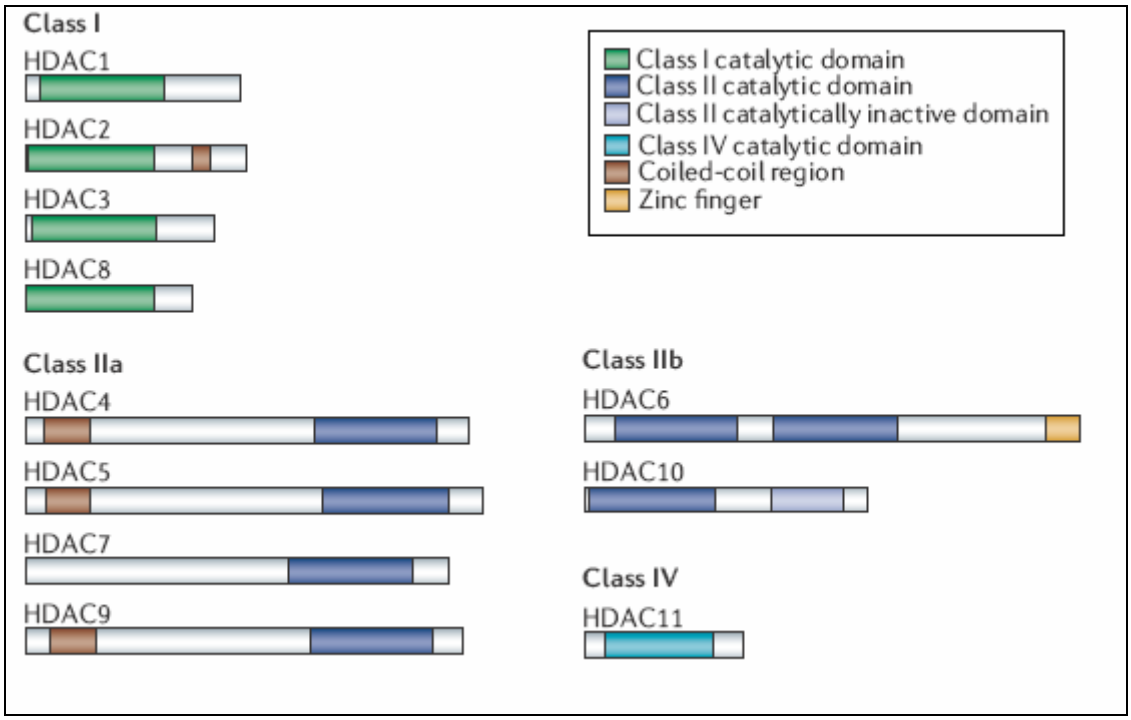


Figure 2. Class I (HDAC1, 2, 3 and 8), class IIa (HDAC4, 5, 7 and 9), class IIb(HDAC6 and 10) and class IV (HDAC11) HDACs with structural/functional domains listed [31].

2.5 Histone Deacetylase 1

Transcriptional repression via HDAC1 exerts crucial functions in various biological processes, such as proliferation, differentiation and cell cycle progression. The generation and study of HDAC1 knockout mice revealed an essential role for HDAC1 in embryonic development [32]. Disruption of the HDAC1 gene causes severe growth retardation and proliferation defects in the mouse embryo and embryonic lethality before embryonic day E9.5 can be observed. Loss of HDAC1 leads to reduced overall deacetylase activity, hyperacetylation of a subset of histones H3 and H4 and changes in other histone modifications. In 2006, Zupkovitz and co-workers could demonstrate that compensatory upregulation of the expression of other class I HDACs in HDAC1 knock-out cells is insufficient to counterbalance the loss of HDAC1, suggesting a unique and crucial function of the enzyme and the existence of regulatory cross-talk between class I HDACs [33]. Interestingly, a small set of genes (Gja1, Irf1, and Gbp2) was found to require HDAC activity and recruitment of HDAC1 for their transcriptional activation, thus describing a novel function for HDAC1 as a transcriptional coactivator.

Furthermore, numerous publications suggest an involvement of HDAC1 in tumour formation [31]. HDAC1 represses gene transcription by interacting with DNA binding proteins such as SP1/SP3 , PCNA, YY1, the pocket proteins pRB, p107, p130 and the tumour suppressors p53 and BRCA1 or, as mentioned above, via its presence in multi-protein complexes. HDAC2 shows closest relationship to HDAC1 with approximately 82 percent identity on the amino acid level which is most probably caused by a past gene duplication event. Both HDAC1 and HDAC2 (in addition to HDAC3) seem to be involved in the regulation of key cell cycle genes such as p21 [34, 35]. In 2002, Lagger and colleagues have reported that HDAC1-deficient embryonic stem cells show reduced proliferation rates, decreased cyclin-associated kinase activities and elevated levels of the cyclin-dependent kinase inhibitors p21 and p27 [36]. Compatible to this hypothesis, elevated p21 and p27 CDK inhibitor levels were also observed *in vivo* in HDAC1 deficient mice. Upon loss of HDAC1 protein, the p21 promoter displays hyperacetylation of histones. This indicates that HDAC1 is a negative regulator of the p21 gene and suggests that the up-regulation of p21 could contribute to the proliferation defect detected in the HDAC1 knock out [32]. However, double knock-out of HDAC1 and p21 did not rescue the lethal phenotype of the HDAC1 knock-out (Meunier, Seiser unpublished). HDAC1 has been shown to be recruited to the p21 promoter by binding to the transcription factor SP1. The p21 gene can be activated by the tumour suppressor p53 upon DNA damage, therefore Lagger and colleagues proposed antagonistic regulation of the p21 gene by competition of HDAC1 and p53 via interaction with SP1 [36].

2.6 Involvement of HDAC1 in Multiprotein Complexes

As can be expected for this vital class of enzymes, the activities of HDACs are tightly controlled. Recruitment of HDAC1 to different co-repressor complexes constitutes an important mechanism for its functional regulation. HDAC1 can be found in three key chromatin-remodelling complexes, the Sin3, CoREST and NuRD complexes. The transcriptional co-repressor complex Sin3 consists of the proteins RbAp46/48, Sin3A, Sin3B, SAP18, SAP30, and Sds3 and is not only involved in the deacetylation of chromatin but also mediates other enzymatic functions including histone methylation, nucleosome remodelling and DNA methylation [37]. The Sin3/HDAC complex lacks any DNA-binding activity and has therefore to be recruited to gene promoters by

interaction with additional transcriptional repressors [37]. RbAp46/48 is involved in the targeting of the histone deacetylase to the N-terminal histone tails whereas Sin3A and Sin3B molecules could be identified as mediators of gene-specific transcriptional repression via their ability to bind sequence-specific transcription factors. A key component of the Sin3 complex is Sds3, a protein essential for maintaining the histone deacetylase activity of the Sin3 complex. Furthermore, Sds3 has a crucial role in the establishment of pericentric heterochromatin [38]. Interestingly, functional overlap between HDAC1 homologues and Sin3 could be detected in yeast and D*rosophila*. For example, transcriptional profiles of Rpd3 mutants, the homologue of HDAC1 in the fruit fly, were found to be similar to those of Sin3 mutants in yeast [39]. These results indicate that the Sin3 complex mainly represses transcription via the histone deacetylase activity of HDAC1. Recent results suggest that Sin3 can also positively regulate transcription besides its role as a corepressor [37].

The second protein complex containing HDAC1 is the NuRD complex. NuRD is unique in that it couples histone deacetylation and chromatin remodeling activities in the same complex. MBD2 and MBD3 have been described as subunits of the Mi-2/NuRD complex but evidently the subunit composition of the enzyme complex appears to vary with cell type and in response to physiologic signals within a tissue [40]. NuRD was shown to lack direct binding ability to methylated DNA. It has been proposed that MBD2, which can bind to methyl CpG, recruits the MBD3-containing Mi-2/NuRD complex to methylated promoters [41]. Additionally, RbAp46/48 and MTA-2 are members of the NuRD complex. It has been proposed that the MI-2/NuRD chromatin-remodeling activity is necessary to initiate and maintain repression of the homeotic (HOX) gene cluster [42]. Remarkably, MBD3 and HDAC1 have been shown to co-localize with the kinase Aurora-A at the centrosomes in the late G2 and early M phase [43].

The third multi-protein complex with involvement of HDAC1 is the CoREST complex. REST (RE1-silencing transcription factor) protein restricts expression of neuronal genes by interaction with the co-repressors Sin3A and CoREST, which in turn recruit HDAC1 and HDAC2 to the gene promoters via a SANT domain [44, 45]. In current understanding, binding of transcriptional activators to REST regulated promoters releases the complex and thereby allows expression of neuronal specific genes [46]. Components of a CoREST-like complex have been recently identified in multiple organisms. In *Drosophila*, the complex is required to regulate neuronal gene

expression whereas *C. elegans* homologues of CoREST regulate the expression of the presenilin gene, proposing a conserved function of the CoREST complex in regulating neuronal gene expression [47].

2.7 The Nervous System

The nervous system (NS) of an animal coordinates the activity of muscles, monitors the organs, constructs and also stops input from the senses and initiates actions. It is divided into the central (CNS), vegetative (VNS) and the peripheral nervous systems (PNS). The PNS is, contrary to the central nervous system, not protected by bones or the blood-brain barrier, leaving it more exposed to chemical agents and mechanical injuries. The PNS consists of the somatic nervous system and the autonomic or visceral nervous system.

The neuronal cell (or neuron) is the building block of the NS. Neurons are typically composed of a soma, or cell body, a dendritic tree and an axon. The majority of vertebrate neurons receive input on the cell body and dendritic tree, and transmit their output via the axon to the terminal buttons, where neurotransmitters are released to stimulate adjacent neurons. However, there is great heterogeneity throughout the NS and the animal kingdom, in the size, shape and function of neurons.

Neurons communicate via electrical or chemical synapses, a process known as synaptic transmission. The process that triggers synaptic transmission is the propagation of the action potential, an electrical signal that is generated by taking advantage of the electrically excitable membrane of the neuronal cell.

2.8 Epigenetic Control of Neuronal Stem Cell Faith

Neural stem cells (NSCs) in the mammalian CNS provide an excellent model system for understanding the basic molecular principles that control cell-fate specification. NSCs are defined as cells that possess the ability to self-renew and they maintain the ability to generate three major CNS cell types: neurons, astrocytes and oligodendrocytes. The connection between neuron-specific gene expression and chromatin regulation was established with the discovery that recruitment of HDACs to the promoter of neuronal genes is crucial for the repression of these genes in non-

neuronal cells [48, 49]. One common feature that is shared by most of these neuron-specific genes is a conserved 21–23-base pair DNA response element, known as RE-1 or NRSE (repressor element 1/neuron restrictive silencer element). The RE-1 is a binding site for the RE-1 silencing transcription factor/neuronal restricted silencing factor (REST/NRSF). The NRSE/RE-1 sequence is found in a large number of neuronal genes, including ion channels, neurotransmitter receptors and guidance/migration molecules [50].

As mentioned above, REST can mediate repression through association with the Sin3 complex, with N-CoR [51] and with the co-repressor complex CoREST. CoREST can recruit other silencing machineries to neuronal target genes, including the methyl DNA binding protein MeCP2, HP1 (heterochromatin protein 1) and a histone lysine methyltransferase (Suvar 39H1) [52].

First studies on the histone deacetylase inhibitor Trichostatin A (TSA) established the link between chromatin state and cell fate [50, 53-55]. More recent publications demonstrate interplay between DNA methylation and histone acetylation. One of the most extensively studied neural activity-dependent genes, brain derived neurotrophic factor (BDNF), is highly expressed in neurons, and its transcription is upregulated by membrane depolarization. Martinowich and colleagues demonstrated that depolarized neurons had decreased methylation within the BDNF promoter, leading to a decreased association with MeCP2 and other silencing proteins, such as HDAC1 and the co-repressor Sin3, and more association with CREB, a transcriptional activator [56]. Furthermore, Bai et al. demonstrated that interaction between DNA methyltransferase DNMT3B and HDAC2 leads to repression of essential genes for neurite outgrowth in PC12 cells [57].

Histone acetylation is proposed to be important for neural stem cell development, including neuronal and oligodendrocyte lineage progression. Adult multipotent neural progenitor cells differentiated predominantly into neurons in the presence of the HDAC inhibitor valproic acid (VPA). VPA treatment also actively suppressed glial differentiation, even in conditions that favoured lineage-specific differentiation. Further analysis revealed that the VPA-mediated neuronal differentiation was correlated with the upregulation of REST/NRSF-regulated genes [55]. HDAC activity was shown to influence progression of oligodendrocyte progenitor cells into mature oligodendrocytes [58].

In addition to effects on neural fate specification, changes in chromatin remodelling have also been associated with changes in neuronal plasticity. Hippocampal neurons induced a rapid and transient phosphorylation at serine 10 and acetylation of lysine 14 of histone H3, as well as an upregulation of c-fos transcription when stimulated with a dopaminergic receptor agonist (SKF82958) or muscarinic acetylcholine receptor agonist (pilocarpine) [59]. As mentioned above, phosphorylation of S10 on histone H3 has been associated with open chromatin and transcriptional activation of immediate-early genes [6, 60]. Furthermore, a connection between long-term memory-related synaptic plasticity and chromatin regulation was observed in cultured Aplysia neurons [61].

Recent studies showed an emerging role of the Swi/Snf protein remodelling complex in the expression of key transcription factors involved in maintaining the proliferation of neural stem-like cells, for example the HMG-box containing protein Sox2. Acetylation of histone H3 K9 at the Sox2 promoter is consistent with the upregulation of Sox2 in neural stem-like cells. It has also been suggested that cell cycle genes including the cyclin-dependent-kinase inhibitors p16 and p19 are targets of the Swi/Snf complex in neural cells [50].

2.9 Histone Deacetylase Inhibitors

In recent years it has been widely recognized that HDACs could present promising targets for therapeutic treatments, for example to reverse atypical epigenetic states associated with cancer. Consequently, there has been considerable effort to develop novel HDAC inhibitors (HDIs). Until now, a large number of structurally diverse HDIs have been purified from natural sources or have been synthetically generated and numerous compounds have progressed to clinical development. In 1975, sodium butyrate was proposed to have anticancer activities based on its capacity to induce cellular differentiation [62]. It was subsequently discovered that butyrate induced histone hyperacetylation, although at that time it was not recognized that HDACs were the target [63].

HDIs can be divided into six classes based on their chemical structure (short-chain fatty acids, hydroxamates, benzamides, cyclic tetrapeptides, electrophilic ketones and others) and these agents inhibit the enzymatic activity of HDACs with varying efficiency. The difficulty of producing recombinant purified HDACs that are

functionally active has prevented comprehensive specificity profiling of those compounds. However, some studies using partially purified and catalytically active recombinant HDACs have been completed, showing that the majority of HDIs do inhibit more than just one particular histone deacetylase. Structurally related HDACs, for example HDAC1 and HDAC2, tend to be inhibited by the same compounds, an outcome that can be explained by the conserved structure of their catalytic sites. For example, MS-275 preferentially inhibits HDAC1 and HDAC2, is less active against HDAC3 and only marginally inhibits HDAC8, whereas Trichostatin A (TSA) was found to be a potent inhibitor for all class I and class II HDACs [31, 64].

HDIs can turn out to be a valuable asset in the fight against cancer. *In vitro* studies showing that transformed cells may be at least tenfold more sensitive to HDIs compared with normal cells provide evidence for HDAC immediate, tumour-cell-selective killing via induction of apoptosis pathways [31]. MS-275 in particular seems to be a promising candidate in the treatment of cancers of the nervous system [65, 66].

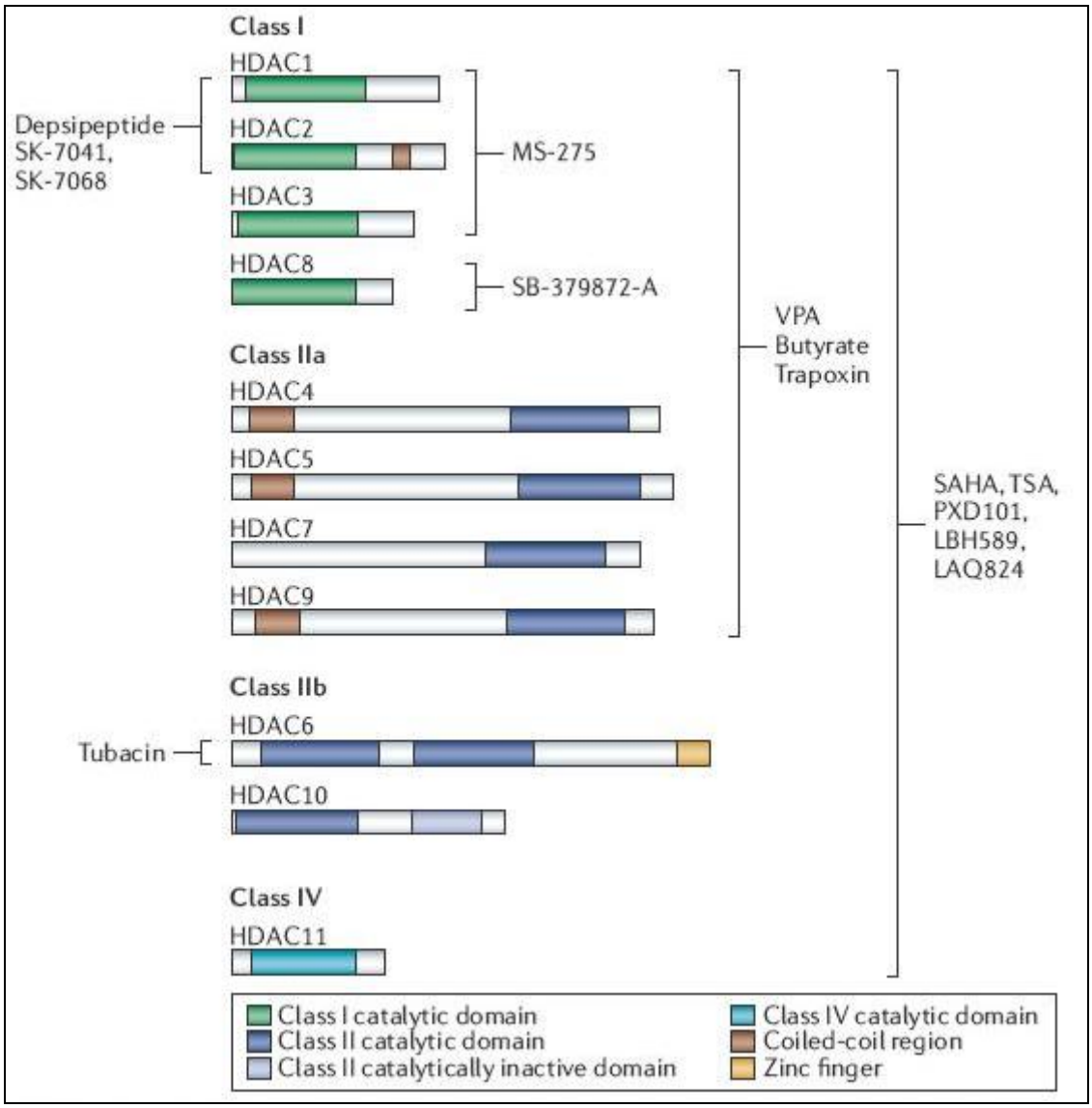


Figure 3. Specificity of various histone deacetylase inhibitors for HDACs [31]. Abbreviations: SAHA: suberoylanilide hydroxamic acid, SIRT: sirtuin, TSA: trichostatin A, VPA: valproic acid.

2.10 The PC12 Cell System

The PC12 cell line was established in 1976 by Lloyd Greene and Arthur Tischler at the Harvard Medical School and is derived from a male rat pheochromocytoma, a tumor arising from chromaffin cells of the adrenal medulla [67]. This line has a homogeneous and near-diploid chromosome number of 40 and, after exposure to neuronal growth factor (NGF), PC12 cells cease to proliferate and begin to extend branching varicose processes, designated neurites. Neurites are similar to processes produced by sympathetic neurons in primary cell culture. After 5 to 6 days of NGF induced neurite outgrowth, PC12 cells are fully differentiated. Removal of NGF is

followed by degeneration of processes within 24 hours and by resumption of cell multiplication within 72 hours [67, 68]. PC12 cells have been widely used in studying different aspects of growth factor-mediated differentiation. For example, Marek et al. discovered that an integrated ERK/JNK pathway is one of perhaps three or more signal conduits required for global gene regulation associated with the differentiation program in PC12 cells [69]. Furthermore, this system has been instrumental for defining the functional NGF receptor, TrkA, as well as the proximal effector pathways, Ras, PLCγ, and c-Src which are required for signal transduction of neural differentiation.

The first steps of NGF induced neuronal differentiation in PC12 cells are well characterised. Binding of NGF to TrkA leads to dimerisation and autophosphorylation of the receptor, which in succession activates downstream kinase pathways [70]. Those signal cascades do not only direct neuronal differentiation of the cell but also have a crucial function in ensuring cell survival [71]. The accumulated data concerning the signal pathways involved in the neuronal differentiation of PC12 cells has led to the assembling of a “connection map” showing the respective signal cascades [68, 72].

3. MATERIALS AND METHODS

3.1 Work with DNA and RNA

3.1.1 Agarose Gel Electrophoresis

Agarose gels with 0.8-2 % agarose in 1 x TAE were prepared depending on the size of the DNA fragments of interest. The agarose was melted using a microwave, cooled down to 60°C, mixed with 0.5 mg/l ethidium bromide and poured into a gel tray. The applied DNA solution was mixed with 6 x DNA loading buffer before loading into the appropriate slots of the gel and subsequently run with 2-8 V/cm.

10 x TAE pH 8.5

400 mM Tris-Acetate

20 mM EDTA

6 x DNA Loading Buffer

4 M Urea

50 % (w/v) saccharose

1 mM EDTA

0.1 % (w/v) bromphenol blue

3.1.2 Excision and Purification of DNA from Agarose Gels

The DNA band of interest was excised with a clean scalpel under low intensity UV light in order to avoid DNA damage. For DNA purification the QIAquick Gel extraction kit (*Qiagen*) was used.

DNA Purification using the QIAquick Gel Extraction Kit (*Qiagen*)

First buffer QG was mixed with the agarose gel slice in a volume ratio of 3:1 and then incubated at 50°C in a heating block until the gel was completely dissolved. One gel volume of isopropanol was added to fragments between 500 bp and 4 kb and the mixture applied to a QIAquick spin column in a collection tube. After centrifugation for 1 min at 12.000 rpm the flow-through was discarded and 750 µl buffer PE were added to wash the column. This was followed by an additional centrifugation step for

1 min at 12.000 rpm and the flow-through was again discarded. To dry the QIAquick membrane, the column was again centrifuged for 1 min at 12.000 rpm and afterwards transferred to a new, clean, labelled 1.5 ml centrifugation tube. To elute the DNA, 30-50 µl dH_2O was applied directly onto the membrane and incubated for 1 to 2 minutes at room temperature. Finally the column was centrifuged for 2 minutes at 12.000 rpm. The purified DNA was either used directly or stored at -20°C.

3.1.3 Enzymatic Reactions

Ligation of DNA Fragments with T4 Ligase

For ligation of DNA fragments, T4 ligase (*Invitroge*n) and the supplied 5 x buffer were used. In general, a molar vector to insert ratio between 1:3 and 1:6, estimated from agarose gel electrophoresis, was applied. The total DNA amount was kept between 10 – 50 ng for sticky end ligation. DNA was mixed with T4 5 x buffer and 1 µl (5 U) of T4 ligase enzyme, in a total volume of 20 µl dH_2O and the reaction was incubated 1 hour at room temperature.

Ligation of DNA Fragments with T4 Ligase in pGEM-T Vector

20 ng plasmid DNA were ligated with 25 ng pGEM-T vector (*Promega*), 5 µl 2 x ligation buffer and 1 µl T4 DNA ligase in a total volume of 10 µl for 1 hour at RT or alternatively at 4°C overnight.

PCR-Reaction using the High Fidelity *Pfu* DNA Polymerase

Pfu DNA polymerase possesses 3' to 5' exonuclease proofreading activity and fragments generated by the *Pfu* DNA polymerase contain fewer errors (1 in 1.3 million base pairs) than PCR inserts generated by the *Taq* DNA polymerase. It has to be considered that the elongation with *Pfu* Polymerase is slow and typically requires 2 minutes to amplify 1kb of DNA at 72° C. In addition, *Pfu* DNA polymerase generates blunt-ended PCR products.

PCR Reaction Mix

Pfu DNA Polymerase 10X Buffer with $MgSO_4$	2.5 µl
dNTP mix, 10mM each	0.5 µl
Upstream primer, 10 pmol/µl	2.5 µl
Downstream primer, 10 pmol/µl	2.5 µl
DNA template (purified vector / cDNA)	0.5 µl / 5 µl
Pfu DNA Polymerase variable	0.25 µl

Nuclease-Free water to final volume of	25 µl

PCR Reaction Program

Initial Denaturation	95°C, 3 minutes	1 cycle

Denaturation	95°C, 1 minute	25–35 cycles
Annealing	60°C, 1 minute	
Extension	72°C, 3 minutes	

Final Extension	72°C, 5 minutes	1 cycle
Pause	4°C, Indefinite	1 cycle

3.1.4 Russian Plasmid Quick-Mini Preparation

This protocol was applied for quick and inexpensive analysis of a large number of bacterial clones. Solutions I and II were made beforehand and stored at 4°C. The desired E. coli colonies were suspended from an LB plate in 2-5 ml LB medium plus ampicillin and incubated at 37°C overnight on a rotor. 1.5 ml of this culture were transferred to an *Eppendorf* tube and centrifuged (12.000 rpm, 30 seconds). The pellet was resuspended in 100 µl cold solution I by vortexing or using a pipette. Next, 200 µl freshly made solution II were added and the tube was inverted four times. After final addition of 150 µl cold solution III and four times inversion, the tube was incubated on ice for 10 minutes and centrifuged (14.000 rpm, 10 minutes). 1 ml 96 % ethanol and 40 µl Na-Acetate were added followed by incubation for 10 minutes at -20°C to precipitate plasmid DNA. The precipitates were collected by centrifugation

(12.000 rpm, 10 minutes) and washed with 70 % ethanol. After drying of the DNA, the pellet was dissolved in 50 µl H_2O and incubated with 1 µl RNase (10 mg/ml) at RT for 30 minutes.

Solution I
50 mM glucose
25 mM Tris/HCl pH 8.0
10 mM EDTA pH 8.0
Autoclaved and stored at 4°C

Solution II (prepare fresh)
0.5 ml 1 M NaOH
1 ml 10 % SDS
3.5 ml H_2O

Solution III
60 ml 5 M KAc
11.5 ml glacial acetic acid
28.5 ml H_2O
Stored at 4°C

3.1.5 Total RNA Extraction

Total RNA was extracted, using Trizol reagent supplied by *Invitrogen*. Cells were harvested at 50-80 % confluence and total RNA was equalised on a 1 % 1 x MOPS/agarose gel. Approximately 0.5 µg total RNA was used for reverse transcription.

3.1.5.1 RNA Isolation using TRIZOL Reagent (*Invitrogen*)

Cells were directly lysed in a culture dish by adding 1 ml of TRIZOL Reagent and passing the cell lysate several times through a pipette. At this point, the lysate can be stored at -20°C for up to 1 month. After addition of 200 µl chloroform per 1 ml of TRIZOL, the tubes were shaken vigorously by hand for 15 seconds and incubated at room temperature for 2 to 3 minutes. The samples were centrifuged at 12,000 × *g* for 15 minutes 4°C. Following centrifugation, the mixture separates into a lower red,

phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase, which was transferred to a fresh tube. The RNA was precipitated by mixing with 0.5 ml isopropyl alcohol. The samples were incubated at room temperature for 10 minutes and centrifuged at 12,000 × *g* for 10 minutes at 4°C. The RNA precipitate forms a gel-like pellet on the side and bottom of the tube and was subsequently washed once with 75% ethanol and centrifuged at 7,500 × *g* for 5 minutes at 4°C. At the end of the procedure, the RNA pellet was briefly dried. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. The RNA was dissolved in 30 µl RNase-free water containing 0,5 µl Turbo DNAse by passing the solution a few times through a pipette tip and incubating it for 15 minutes at 37°C and 5 minutes on 85°C to inactivate the DNAse.

10 x MOPS pH 7.0

0.2 M MOPS

50 mM Na-Acetate pH 5.0

10 mM EDTA

RNA Loading Dye

15 ml formamide (deionised)

3 ml 10 x MOPS

4.8 ml 37 % formaldehyde

2 ml H_2O

2 ml 87 % glycerol

1.6 ml 10 % bromphenol blue

100 µl ethidium bromide

3.1.5.2 Reverse Transcription

0.5 µg total RNA was reversely transcribed using the iScript cDNA Synthesis Kit (*BioRad)* and the manufacturer's instructions were followed.

<u>i-Script Program:</u>

25°C 5 minutes

42°C 30 minutes
85°C 5 minutes
4°C pause
The reaction was diluted 1:10 for use in RT-PCR.

3.1.5.3 Real Time PCR

Quantitative Real Time PCR was performed using a *BioRad* iCycler. 5 µl template cDNA was applied per reaction and amplified to double-stranded (ds) cDNA. Direct detection of PCR products can be monitored by detecting the increase in fluorescence caused by the binding of the intercalating fluorescence dye Syber Green™ to double-stranded (ds) DNA. The fluorescence was measured by a detector and increased via a photomultiplier. FITC (Fluorescein isothiocyanate) was used as an internal standard. Each reaction was carried out in duplets and a standard cDNA was diluted in order to determine cDNA concentrations. Analysis of the Real Time data was performed using software provided by *BioRad*.

Mastermix for Real Time PCR

1 x Taq buffer (without $MgCl_2$)
25 mM $MgCl_2$
10 mM dNTP´s each
10 pmol/µl upstream primer
10 pmol/µl downstream primer
0.4 µM FITC
0.4 x Syber Green™
5 Units/µl Taq DNA polymerase (*Fermentas*)

The reaction mixture was filled up with H_2O to 20 µl total volume. Enzymes and solutions were bought from *Fermentas*, polymerase and fluorescence dyes were added last.

Real Time PCR Program:

95°C 1 min

95°C 30 sec
60°C 30 sec
72°C 30 sec
} repeat 40 x

95°C 1 min

20°C pause

3.2 Work with Proteins

3.2.1 Bradford Protein Assay

To measure protein concentration, 1 ml Bradford solution from *BioRad* diluted 1:5 in H_2O was set up in a plastic cuvette and 1 µl protein extract was added. The extinction at 595 nm was measured against a blank just containing diluted Bradford solution. Protein concentration can be calculated using the formula:

$$\underline{OD_{595}\text{ nm } 0.1 = 1\ \mu g/\mu l\ \text{protein}}$$

3.2.2 Total Protein Extract (Hunt Extract)

This protocol, also known as Hunt protein extraction, was used for isolating total protein extracts from cultured cells. Basically, it takes advantage of ripping of cells by multiple freezing and thawing steps. Cell culture medium was sucked off and cells in culture dish were washed one time with 10 ml cold 1 x PBS. When needed, cells were scratched from the dish using a rubber policeman. In the case of the PC12 cell line, cells could be simply washed off using 10 ml cold 1 x PBS. Cells were centrifuged 5 minutes at 1.200 rpm at 4°C, PBS was decanted and the cell pellet again resuspended in 1 ml cold 1 x PBS. The cell suspension was transferred to an *Eppendorf* tube and a centrifugation step at full speed for 15 seconds was performed to pellet the cells. Cells were resuspended in 60-200 µl Hunt buffer according to the size of the pellet. The lysate was then frozen in liquid nitrogen and thawed at RT. After resuspension, the lysate was frozen a second time in liquid nitrogen and thawed

again at 37°C. A final freezing step in liquid nitrogen was followed by thawing the lysate slowly on ice. Finally, the lysate was centrifuged at 14.000 rpm for 10 minutes at 4°C and the supernatant, containing the total protein extract, was transferred to a new *Eppendorf* tube.

Hunt Buffer

20 mM Tris/HCl pH 8.0
100 mM NaCl
1 mM EDTA
0.5 % NP-40
1 Proteinase inhibitor tablet per 50 ml total volume (*Roche*)
10mM Na-butyrate

The Hunt buffer can be made in advance and stored at 4°C, except the volatile Na-butyrate and the proteinase inhibitor tablet, which have to be added right before usage.

3.2.3 Histone Isolation

Cells were washed with cold 1 x PBS and harvested. If acetylation states were analysed, 1 x PBS was supplemented with 10 mM Na-butyrate. After a short centrifugation step, 14.000 rpm for 15 seconds, the cell pellet was resuspended in 1 ml ice-cold histone lysis buffer by vortexing and centrifuged at 14.000 rpm for 15 seconds. Three washes with histone lysis buffer and one wash with histone wash buffer were followed by adding 100 µl ice-cold H_2O and vortexing. To elute basic histone proteins, concentrated acetic acid (H_2SO_4) was added to a final concentration of 0.4 N. For proper acidic elution, the histones were incubated with the diluted acidic acid for at least one hour on ice. The solution was centrifuged at 15.000 rpm for 5 minutes. This was followed by precipitation of the histones from the supernatant with 10 x volumes acetone at -20°C for one hour or alternatively overnight at RT. The precipitates were collected by centrifugation (15.000 rpm, 10 minutes, 4°C), the pellet was air-dried and histones were resuspended in 30 µl H_2O.

Histone Lysis Buffer pH 6.5

10 mM Tris base

50 mM Na-Bisulfite

10 mM $MgCl_2$

1 % Triton X-100

8.6 % sucrose

10 mM Na-butyrat

Histone Wash Buffer pH 7.4

10 mM Tris base

13 mM Na_3EDTA

3.3 Western Blot Analysis

3.3.1 SDS Gel Electrophoresis

The following protocol was used to separate protein extracts on acrylamide gels and visualise through antibody-antigen reaction with chemiluminescent reagent.

3.3.1.1 SDS Gel Preparation

Generally, a SDS polyacrylamide gel consists of two parts. The lower part, the resolving gel, separates the proteins and the upper part, termed stacking gel, where the samples are loaded.

The stacking gel has a lower concentration of acrylamide (larger pore size), lower pH and a different ionic content. This allows the proteins in a lane to be concentrated into a tight band before entering the resolving gel and produces a gel with tighter and better separated protein bands.

The percentage of a resolving gel is usually dependent on the respective protein size. For resolving proteins between 40 kDa and 100 kDa, a 10 % gel was used. For smaller proteins, such as histone proteins, the percentage was increased to 16 %. For larger proteins, the percentage was decreased to 8 % to achieve optimal separation. The stacking gel was always made containing 5% polyacrylamide.

For loading, 15-60 µg protein extract were diluted in SDS sample buffer and denatured at 95°C for 5 minutes. 5 to 7 µl protein prestained ladder from *BioRad* was used for size determination. Gel was separated in 1 x running buffer at 25 to 35 mA.

Acrylamide

30 % acrylamide

0.8 % N, N´ methylene-bisacrylamide

The stock was stored at 4°C.

SDS Sample Buffer

100 mM Tris/HCl pH 6.8

20 % glycerol

5 % SDS

10 % ß-mercaptoethanol

0.01 % bromphenolblue

1 x Running Buffer

25 mM Tris base

192 mM glycine

0.1 % SDS

3.3.1.2 Western Transfer

After protein separation on a SDS gel, the stacking gel was removed. The filter paper (*Whatman 3MM*) and the nitrocellulose membrane (*Schleicher & Schuell* BA 83) were cut slightly larger then the gel and soaked in the appropriate buffers. The components were than assembled as following: two 3MM papers soaked in anode I buffer, one 3MM paper soaked in anode II buffer, the nitrocellulose membrane soaked in anode II buffer, the gel and finally two 3MM papers soaked in cathode buffer. Blotting was performed at 4°C for 30 minutes at 15 V and 200 mA in a semi-dry blotter (*BioRad)*. After blotting, the membrane was stained in 1 x Ponceau S solution and destained using dest. H_2O. The visible protein ladder was marked with a ball pen.

Cathode Buffer pH 9.4

25 mM Tris base

40 mM 6-aminohexanoic acid

20 % methanol

Anode I Buffer pH 10.4

100 mM Tris base

20 % methanol

Anode II Buffer 10.4

25 mM Tris base

20 % methanol

10 x Ponceau S

2 % Ponceau S

30 % sulfosalicylic acid

30 % trichloroacetic acid

3.3.1.3 Antibody Incubation

The nitrocellulose membrane was incubated with blocking solution for 30 minutes at RT on a shaker to block unspecific antibody binding to the membrane. The first antibody was diluted to the appropriate concentration in blocking solution and the membrane was incubated shaking overnight at 4°C. After three washes with 1 x PBS, containing 0.1 % Tween-20, for 5 minutes respectively, the membrane was incubated with the secondary, horseradish peroxidase (HRP)-coupled, antibody diluted 1:10.000 in 1 x PBS, 0.1 % Tween-20, for one hour at RT. This was followed by another three washing steps with1 x PBS, containing 0.1 % Tween-20.

Blocking Solution

1 x PBS

1 % PVP

1 % non-fat dried milk powder

0.1 % Tween-20

0.01 % NaN_3

Blocking solution was stored at 4°C.

3.3.1.4 Immunodetection by Enhanced Chemo Luminescence

The membrane was incubated for 1 minute in equal amounts of enhanced chemiluminescence (ECL) solution 1 and 2 (*PerkinElmer*). This mixture was used to rinse the membrane for 1 to 2 min at room temperature. Subsequently the membrane was transferred to a transparent plastic foil and air bubbles were removed. The membrane was then exposed to a light sensitive X-ray film *(Kodak)* in the dark room for appropriate time periods.

3.3.1.5 Protein Quantification using the *Odyssey Imaging System*

Direct infrared fluorescence detection via the Odyssey Imaging System facilitates quantification of low-abundance proteins. Infrared detection effectively eliminates background fluorescence, resulting in clear, sharp bands. In addition, strong and weak bands on the same blot can be accurately imaged without multiple exposures which would be needed with chemiluminescence.

For protein quantification, standard western transfer and primary antibody incubation was performed. A pencil has to be used for marking the blot. The fluorescence-labelled secondary antibody (IRDye™ 700 or IRDye™ 800) was diluted 1:10.000 in 1x PBS, containing 0.1 % Tween-20 and incubation time was 30 minutes at RT followed by another three washes with1 x PBS, containing 0.1 % Tween-20. Exposure of the secondary antibody to light has to be avoided. The membrane is now ready to scan on the *Odyssey Infrared Imaging System* and respective protein levels can be determined using the provided software.

3.3.2 Indirect Immunofluorescence Analysis

The protocol for indirect immunofluorescence experiments was applied for fibroblast cells and an adapted protocol for the PC12 cell line.

3.3.2.1 Indirect Immunofluorescence Protocol for Fibroblast Cells and the PC12 Cell-line

Cells were seeded on sterile glass cover slips in tissue culture six well dishes 24 to 36 hours before use. Culture medium was decanted and cells were gently washed with 1 x PBS. Cells were fixed onto the cover slips with 1 % paraformaldehyde for 5 minutes and with 2 % paraformaldehyde/0.1 % Triton X-100 for 10 minutes. This was followed by 3 washing steps with 1 x PBS for 5 minutes each. The fourth wash was carried out with 1 x PBS /Tween-20. In order to block any unspecific antibody reactions, 10 % goat serum (GS) in 1 x PBS was added to the first antibody mix overnight at 4°C in a humid chamber or alternatively 90 minutes at 37°C. The first antibody was removed and another three washing steps with 1 x PBS for 5 minutes each were performed. The following steps were performed in the dark to ensure signal intensity of the fluorescent antibody. The secondary *Alexa* fluorescence antibodies were diluted 1:1000 in 1 x PBS containing 10 % GS and incubated for one hour at RT in a humid chamber. Following, slides were washed 3 times with 1 x PBS. Before sealing of cover slips with nail polish, vectashield (*Vector Laboratories*) containing 0.1 % DAPI was applied for optimal signal preservation and detection of the nucleus. The slides were stored at 4°C in a dark box.

PC12 cells generally lack strong surface adhesion, even to laminin or collagen coated cell culture dishes and cover slips. Therefore, the described protocol was followed but the first washing step with 1 x PBS was skipped and all washing steps were performed very gently and without shaking

3.3.3 Cytoskeleton Preparation

Fully differentiated PC12 cells were washed very gently with 1 x PBS and 1ml of Lysis low buffer was added and incubated for 2 min on ice. The supernatant was removed and 1 ml Lysis high buffer was added. Cells were incubated for 3 min on ice, while carefully pipetting the Lysis high buffer on the cells every 30 sec to allow complete lysis. Thereafter, 200 µl 5M NaCl were added and incubated again for 3

min on ice. Analogue to the previous step, the supernatant was pipetted gently over the remaining cells every 30 sec. Subsequently, the supernatant was centrifuged for 15 min, 1400 rpm at 4°C. The resulting supernatant was discharged and the pellet, containing the insoluble components of the cytoskeleton, was resuspended in premixed 25µl 8M urea and 25 µl SDS sample buffer. The sample was denatured at 95°C for 15 minutes and centrifuged for 15 min, 13000 rpm at room temperature. Before loading onto an 8% SDS polyacrylamide gel, 10 µl SDS sample buffer were added to 10 µl of the sample and vortexed thoroughly.

Lysis Low Buffer (10 ml)

0.5 ml 10 x PBS
0.5 ml 1M MOPS ph 6.8
0.1 ml 0.1M EGTA
0.1 ml 1M $MgCl_2$
0.1 ml 10% NP-40
75µl PMSF (sat.)
1 Proteinase Inhibitor tablet diluted in 100 µl H_2O
10 mM Na-butyrate
8.425 ml dH_2O

Lysis High Buffer (10 ml)

0.5 ml 10 x PBS
0.5 ml 1M MOPS ph 6.8
0.1 ml 1M $MgCl_2$
1 ml 10% NP-40
75µl PMSF (sat.)
1 Proteinase Inhibitor tablet diluted in 100 µl H_2O
350 µl DNAse (10mg/ml)
10 mM Na-butyrate
7.275 ml dH_2O

3.3.4 Spinal Chord and Brain Tissue Preparation

Spinal chord or brain tissue was obtained from euthanized mice and cleaned once with 1 x PBS. To avoid protein degradation, every step of the protocol was performed on ice and all solutions used were precooled to 4°C. Depending on its size, the mouse spinal chord or brain tissue was homogenized thoroughly with 200 to 400µl of E1A lysis buffer (high salt) using a homogenizer, The homogenate was transferred to an *Eppendorf* tube and centrifuged for 15 min, 10 000 rpm at 4°C. The pellet, containing insoluble components of the cellular cytoskeleton, was stored and the supernatant was transferred to a fresh *Eppendorf* tube and the volume was estimated. Finally the supernatant was diluted with an equal volume of E1A lysis buffer (no salt). Protein concentration was measured and 20-40 µg protein were diluted in SDS sample buffer, denatured at 95°C for 5 minutes and loaded on an 8% SDS polyacrylamide gel.

Alternatively, about 10 µg of the pellet were transferred to an *Eppendorf* tube using a spatula and mixed with 20 µl SDS sample buffer by vortexing vigorously. The sample was then denatured at 95°C for 10 minutes and centrifuged at 14 000 rpm for 10 minutes at room temperature. 10µl of the supernatant were then loaded on an 8% SDS polyacrylamide gel.

E1A Lysis Buffer (with salt)

50 mM Tris-HCl pH 7.5
250 mM NaCl
5 mM EDTA pH 8.0
0.1% NP-40
1 Proteinase Inhibitor tablet (*Roche*)

E1A Lysis Buffer (no salt)

50 mM Tris-HCl pH 7.5
5 mM EDTA pH 8.0
0.1% NP-40
1 Proteinase Inhibitor tablet (*Roche*)

3.4 Tissue Culture

3.4.1 Work with PC12 Rat Pheochromocytoma Cells

3.4.1.1 Coating Procedure for Tissue Culture Dishes for PC12 Cells

PC12 cells lack strong surface adhesion, therefore culture dishes had to be coated with collagen prior to use. 10-cm tissue culture dishes and six well plates were covered with 10 % rat tail collagen I (*BD Biosciences)* in 0.1 % glacial acetic acid and incubated at least for 1 hour. After removal of the coating solution, the plates were dried and washed three times with tissue culture grade H_2O (tcH_2O). This was followed by final sterilisation of the plates under UV-light for not less than 10 hours. Coated plates could be stored at 4°C in the dark for up to 8 weeks

3.4.1.2 Preparation of Cover Slips for PC12 Cell Immunofluorescence

Glass cover slips had to be incubated in nitric acid for at least 24 hours, followed by sterilization in boiling water for 2 hours. From this point on, work was done in a sterile cell culture hood. Cover slips were transferred to tissue culture six well plates and three times washed with tcH_2O. After drying, 1 ml of 10 % poly-L-lysine solution (*Sigma*) in H_2O was applied on the cover slips and incubated for 1 hour. After removal of the poly-L-lysine solution, cover slips were dried and washed three times with tcH_2O. This was followed by sterilization overnight under UV-light. The secondary coating solution, consisting of 10 µg/ml CultrexR Mouse Laminin I (*R&D Systems;* [*10 µg/ml*]*)* diluted in RPMI medium, was applied onto the cover slips with a pipette and incubated in the in the cell culture incubator for 24 to 36 hours. Thereafter, the solution was removed and the glass slides were washed once with tissue culture grade 1 x PBS. From this point on, it is important that the cover slips do not get dry. For this reason, 3 ml RPMI medium were added immediately after the washing step and the plates were used on the same day.

3.4.1.3 Culture and Splitting of Cells

In contrast to many other cell lines, PC12 cells can be directly resuspended and split to new Petri dishes without prior washing or trypsinisation. Medium was sucked off and cells were detached from the Petri dish by 5 to 10 gentle up-and-down pipetting steps. Subsequently, the cells were transferred to a new plate in the desired dilution.

In adequate culture conditions, PC12 cells need approximately 40 hours to double their cell number. A cell culture density between 20 % and 70 % is optimal for cell growth.

3.4.1.4 Freezing and Thawing of PC12 Cells

Freezing of PC12 Cells

Logarithmically growing cells were resuspended in 10 ml complete RPMI-Medium and collected by centrifugation at 1.200 rpm for 5 minutes. The cell pellet was dissolved gently in newly prepared 90 % FCS + 10 % DMSO and transferred to sterile cryotubes (*Nunc*). After 30 minutes incubation on ice, cryotubes were cooled to -80°C for at least three days and long-time stored in liquid nitrogen.

Thawing of PC12 Cells

Cells were removed from liquid nitrogen and quickly thawed in a 37°C water bath for 2 to 3 minutes. The cells were transferred to a fresh Petri dish containing 10 ml complete, CO_2 saturated RPMI-medium.

3.4.1.5 Stable Transfection of PC12 Cells with Lipofectamine

24 hours before transfection, PC12 cells were seeded on six well plates to reach 60 to 80 percent confluency the next day, using medium without antibiotics. For determination of the transfection efficiency after one day, a vector expressing orange fluorescent protein (OFP) was used as control. 10 µl Lipofectamine (*Invitrogen*) were mixed with 250 µl Optimem medium (*Invitrogen*) and incubated for 5 min at room temperature. For each transfection, 4 µg of the desired construct or 2 µg of control plasmid were mixed gently with 250 µl Optimem medium and added to the Lipofectamine mix. After 20 min incubation at room temperature, the DNA – Lipofectamine mix was added to the plate by dropwise pipetting, followed by gentle rocking of the plate and incubation for 6 hours. The transfection medium was removed, exchanged by cell culture medium containing antibiotics and incubated for 24 hours. The following day, the cells were seeded on 10 cm cell culture plates at dilutions between 1:100 and 1:50, using medium containing antibiotics and

puromycin (1 µg/ml). After one week of puromycin selection, small, discrete colonies form, each representing a stable single clone cell line, which can be transferred to a six well plates and be propagated.

3.4.1.6 Neuronal Differentiation of PC12 Cells

PC12 cells were seeded on cell culture plates and incubated for 2 hours to allow surface attachment. The medium was subsequently supplied with 50 ng/ml Neuronal Growth Factor (NGF, *R&D Systems*). Medium was replaced every 2 days by fresh medium containing 50 ng/ml NGF. Neurite outgrowth can be observed after 24 hours and cells are fully differentiated after 6 days.

3.4.2 Media and Solutions

RPMI Medium (*Invitrogen)*

RMPI medium had to be replenished with 5 % fetal calf serum (FCS), 10 % horse serum (HS) and antibiotics (AB). The complete medium was stored at 4°C not longer than 20 days and warmed to 37°C in a water bath before use.

Dulbecco's Modified Eagle Medium (DMEM)

Prior to usage, 450 ml DMEM were supplemented with 50 ml fetal calf serum (FCS) and antibiotics (streptomycin, penicillin). The medium was stored at 4°C and prewarmed to 37°C before use.

10 x Phosphate Buffered Saline (PBS)

80 g/l NaCl
2 g/l KCl
16.5 g/l Na_2HPO_4
2 g/l KH_2PO_4

Trypsin/EDTA Solution (TE)

25 ml 10 x PBS

700 mg (2.2 Units/mg) trypsin

5 ml 1 % Na-EDTA, pH 7.4

H_2O was added to a final volume of 250 ml

100 x Antibiotic Stock Solution (AB)

10 g/l streptomycin sulfate

6 g/l penicillin G potassium salt

Dissolved in 1 x PBS

3.5 Work with Bacteria

3.5.1 Growth of Bacteria

Bacteria were grown in LB medium on a rotor system or on LB agar plates at 37°C overnight.

2.5.2 Freezing of Bacteria

200 to 500 µl of logarithmically growing bacterial culture were mixed with the same amount of 2 x freezing medium and long time stored at -80°C. Alternatively, pure glycerol could be used instead of 2 x freezing medium.

2 x Freezing Medium

88.8 g/l glycerol

12.6 g/l K_2HPO_4

3.6 g/l KH_2PO_4

1.8 g/l $(NH_4)_2SO_4$

0.9 g/l Na-Citrate

0.18 g/l $MgSO_4x7H_2O$

3.5.3 Transformation of Bacteria

3.5.3.1 Generation of Chemically Competent E. coli Cells

Top10 bacteria were grown as overnight 3 ml culture in LB medium at RT. The overnight culture was added to 500 ml SOB++ medium and incubated at 25 to 30 °C until the absorbance at 600 nm was between 0.4 and 0.6. When the right optical density was reached, the culture was chilled on ice for at least 10 minutes. Bacteria were centrifuged in a pre-cooled *Sorvall GSA rotor* (4.000 rpm, 10 minutes, 4°C), the supernatant was decanted and the pellet was gently resuspended in 100 ml ice-cold TB buffer. The suspension was incubated 10 minutes on ice, centrifuged in a precooled *Sorvall* rotor (3.000 rpm, 10 minutes, 4°C) and resuspended in 18.6 ml ice-cold TB buffer. 1.4 ml sterile DMSO was added, bacteria were incubated on ice for at least 10 minutes and aliquoted into sterile, cooled *Eppendorf* tubes at 100 µl per tube. Finally, tubes were shock frozen in liquid nitrogen. Chemically competent bacteria can be long time stored at -80°C or in liquid nitrogen.

SOB++ Medium pH 7.0

20 g/l *bacto* tryptone
5 g/l yeast extract
0,5 g/l NaCl
0.186 g/l KCl
10 mM $MgCl_2$
10 mM $MgSO_4$

All components except $MgCl_2$ and $MgSO_4$ were heat sterilized, filter sterilized $MgCl_2$ and $MgSO_4$ was added.

TB Buffer pH 6.7

10 mM HEPES pH 6.7
15 mM $CaCl_2$
55 mM $MnCl_2$
250 mM KCl

All components except $MnCl_2$ were mixed and the pH adjusted to 6.7 with KOH. $MnCl_2$ was added and the solution was sterilized using a 0.22 µm filter.

3.5.3.2 Transformation of Ca2+ Competent Bacteria (Heat Shock)

50 µl chemically competent bacteria were gently thawed on ice and 5 µl of ligation mixture solution was added and incubated on ice for 30 minutes. The *Eppendorf* tube containing the mix was incubated on a 42°C warm heating block for 45 seconds, followed by chilling on ice for 2 to 3 minutes. 300 µl LB medium were added and the bacteria were incubated shaking for at least 30 minutes at 37°C. The transformed bacteria were plated on LB ampicillin plates and incubated overnight at 37°C.

3.5.4 Media and Plates

Lysogeny broth (LB) Medium

10 g/l NaCl
10 g/l *bacto* tryptone
5 g/l yeast extract

Ampicillin

50 mg/ml (*Serva*)
Antibiotic solutions were sterile filtrated through a membrane filter (pore size 0.2 µm) and stored at -20°C.

LB Plates

1 L standard LB medium was supplemented with 15 g agar, autoclaved and cooled down. When the temperature was not higher than 50°C, ampicillin was added to a final concentration of 50 µg/ml. The medium was poured into 10 cm Petri dish and allowed to harden. If necessary, the agar plates could be stored at 4°C up to 6 weeks.

X-Gal/ IPTG LB Plates

To allow LacZ-colour selection of bacteria, X-Gal (= 5-bromo-4-chloro-3-indyl-ß-D-galactosidase; 80 µg/ml) and IPTG (0.5 mM) were added before pouring the LB ampicillin plates.
X-Gal and IPTG could also be applied onto previously prepared LB ampicillin plates.

3.6 Antibody List

Antibody	Dilution IF	Dilution WB	2nd antibody	Company
HDAC1 10E2 C-term.	1 / 250	1 / 5.000	mouse	*Seiser Laboratory*
HDAC1 Aff. purified	1 / 200	1 / 5.000	rabbit	*Seiser Laboratory*
HDAC1 Sat 208 N-term.	1 / 500	1 / 10.000	rabbit	*Seiser Laboratory*
HDAC2 3F3	1 / 50	1 / 100	mouse	*Seiser Laboratory*
HDAC2 Sat 33	1 / 100	1 / 1.000	rabbit	*Seiser Laboratory*
HDAC 3	-	1 / 10.000	rabbit	*Abcam*
Neurofilament NFM-160	1 / 40	1 / 400	mouse	*Sigma*
Acetylated Histone H3 (Ac-H3)	1 / 500	1 / 5.000	rabbit	*Upstate*
Acetylated Histone H4 (Ac-H4)	-	1 / 5.000	rabbit	*Upstate*
C-terminal Histone H3	-	1 / 50.000	rabbit	*Abcam*
ß-Actin (A5316)	1 / 200	1 / 2.000	mouse	*Sigma*
c-myc monoclonal 4A6	1 / 500	1 / 5.000	mouse	*Upstate*
c-myc polyclonal ab9106	1 / 500	1 / 5.000	rabbit	*Abcam*

4. RESULTS

4.1 Affinity Purification of the polyclonal HDAC1 Antibody Sat 244

In order to detect HDAC1 in our experiments, we use several different antibodies directed against this protein. The rabbit polyclonal HDAC1 antibody, sold by *Abcam (HDAC1 antibody - ChIP Grade (ab7028))* and *Upstate (Anti-HDAC1 (06-720)),* was obtained by using the synthetic peptide CKEEKPEAKGVKEEVKLA conjugated to KLH (keyhole limpet hemocyanin) for immunization. The mouse monoclonal HDAC1 antibody 10E2, sold by *Abcam (HDAC1 antibody [10E2] - ChIP Grade (ab46985))* and *Upstate* (*Anti-HDAC1, clone 2E10 (05-100)),* is directed against a 16 amino acid long synthetic peptide with the sequence EEKPEAKGVKEEVKLA. Both antibodies are targeted against the same epitope, corresponding to the C-terminal amino acids, 465-482 and 466-482 respectively, of human and mouse HDAC1 and react with mouse, rat and human HDAC1.

Incidentally, when using the antibodies for indirect immunofluorescence on mouse brain cryosections or differentiated PC12 cells, we did not only visualize nuclear HDAC1 protein, but we could also detect an additional, cytoplasmatic signal. Furthermore, we could see a strong signal for a 160kDa protein on western blots in addition to the 55kDa HDAC1 protein when loading mouse neuronal tissue extracts or cytoskeleton extracts from differentiated PC12 cells.

In order to identify the 160 kDa protein band we utilized the program BLASTP (version 2.2.17) and LALIGN (Huang and Miller, Adv. Appl. Math. 1991) to search for putative sequence overlaps between the peptide KEEKPEAKGVKEEVKLA and mouse neuronal specific proteins. As expected, results did demonstrate highest scores for histone deacetylase 1 (Score 54.9, E-Value 2e-8). Surprisingly, neurofilament heavy protein (Score 26.5, E-Value 5.5) and neurofilament medium protein (Score 24.8 E-Value 18) ranked high. LALIGN also showed amino acid sequences similarities for neurofilament light protein. Table 1 illustrates protein sequence resemblances between the peptide used for generating the protein antibody and HDAC1, neurofilament heavy, medium and light protein.

Peptide 10E2

KEEKPEAKGVKEEVKLA

HDAC1 protein [Mus musculus], locus NP_032254

MAQTQGTKRKVCYYYDGDVGNYYYGQGHPMKPHRIRMTHNLLLNYGLYRKMEIYR
PHKANAEEMTKYHSDDYIKFLRSIRPDNMSEYSKQMQRFNVGEDCPVFDGLFEFC
QLSTGGSVASAVKLNKQQTDIAVNWAGGLHHAKKSEASGFCYVNDIVLAILELLKYH
QRVLYIDIDIHHGDGVEEAFYTTDRVMTVSFHKYGEYFPGTGDLRDIGAGKGKYYAV
NYPLRDGIDDESYEAIFKPVMSKVMEMFQPSAVVLQCGSDSLSGDRLGCFNLTIKG
HAKCVEFVKSFNLPMLMLGGGGYTIRNVARCWTYETAVALDTEIPNELPYNDYFEY
FGPDFKLHISPSNMTNQNTNEYLEKIKQRLFENLRMLPHAPGVQMQAIPEDAIPEES
GDEDEEDPDKRISICSSDKRIACEEEFSDSDEEGEGGRKNSSNFKKAKRVKTEDEK
EKDPEEKKEVTEEEKTKEEKPEAKGVKEEVKLA

Neurofilament, heavy polypeptide [Mus musculus], locus NP_035034

MMSFGSADALLGAPFAPLHGGGSLHYSLSRKAGPGGTRSAAGSSSGFHSWARTS
VSSVSASPSRFRGAASSTDSLDTLSNGPEGCVVAAVAARSEKEQLQALNDRFAGYI
DKVRQLEAHNRSLEGEAAALRQQQAGRAAMGELYEREVREMRGAVLRLGAARGQ
LRLEQEHLLEDIAHVRQRLDEEARQREEAEAAARALARFAQEAEAARVELQKKAQA
LQEECGYLRRHHQEEVGELLGQIQGCGAAQAQAQAEARDALKCDVTSALREIRAQ
LEGHAVQSTLQSEEWFRVRLDRLSEAAKVNTDAMRSAQEEITEYRRQLQARTTELE
ALKSTKESLERQRSELEDRHQADIASYQDAIQQLDSELRNTKWEMAAQLREYQDLL
NVKMALDIEIAAYRKLLEGEECRIGFGPSPFSLTEGLPKIPSISTHIKVKSEEMIKVVEK
SEKETVIVEGQTEEIRVTEGVTEEEDKEAQGQEGEEAEEGEEKEEEEGAAATSPPA
EEAASPEKETKSRVKEEAKSPGEAKSPGEAKSPAEAKSPGEAKSPGEAKSPGEAK
SPAEPKSPAEPKSPAEAKSPAEPKSPATVKSPGEAKSPSEAKSPAEAKSPAEAKSP
AEAKSPAEAKSPAEAKSPAEAKSPATVKSPGEAKSPSEAKSPAEAKSPAEAKSPAE
AKSPAEVKSPGEAKSPAEPKSPAEAKSPAEVKSPAEAKSPAEVKSPGEAKSPAAVK
SPAEAKSPAAVKSPGEAKSPGEAKSPAEAKSPAEAKSPIEVKSPEKAKTPVKEGAK
SPAEAKSPEKAKSPVKEDIKPPAEAKSPEKAKSPVKEGAKPPEKAKPLDVKSPEAQT
PVQEEAKHPTDIRPPEQVKSPAKEKAKSPEKEEAKTSEKVAPKKEEVKSPVKEEVK
AKEPPKKVEEEKTLPTPKTEAKESKKDEAPKEAPKPKVEEKKETPTEKPKDSTAEAK
KEEAGEKKKAVASEEETPAKLGVKEEAKPKEKTETTKTEAEDTKAKEPSKPTETEK
PKKEEMPAAPEKKDTKEEKTTESRKPEEKPKMEAKVKEDDKSLSKEPSKPKTEKAE
KSSSTDQKESQPPEKTTEDKATKGEK

Neurofilament, medium polypeptide [Mus musculus], locus NP_032717

MSYTLDSLGNPSAYRRVTETRSSFSRVSGSPSSGFRSQSWSRGSPSTVSSSYKRS
ALAPRLAYSSAMLSSAESSLDFSQSSSLLNGGSGGDYKLSRSNEKEQLQGLNDRFA
GYIEKVHYLEQQNKEIEAEIQALRQKQASHAQLGDAYDQEIRELRATLEMVNHEKAQ
VQLDSDHLEEDIHRLKERFEEEARLRDDTEAAIRALRKDIEESSMVKVELDKKVQSL
QDEVAFLRSNHEEEVADLLAQIQASHITVERKDYLKTDISTALKEIRSQLECHSDQNM
HQAEEWFKCRYAKLTEAAEQNKEAIRSAKEEIAEYRRQLQSKSIELESVRGTKESLE
RQLSDIEERHNHDLSSYQDTIQQLENELRGTKWEMARHLREYQDLLNVKMALDIEIA
AYRKLLEGEETRFSTFSGSITGPLYTHRQPSVTISSKIQKTKVEAPKLKVQHKFVEEII
EETKVEDEKSEMEETLTAIAEELAASAKEEKEEAEEKEEE*PEAEKSPVKSPEAKEE
EEEGEKEEEEEGQEEEEEEDEGVKSDQAEEGGSEKEGSSEKDEGEQEEEEGETE
AEGEGEEAEAKEEKKIEGKVEEVAVKEEIKVEKPEKAKSPMPKSPVEEVKPKPEAK
AGKGEHKEEEKVEEEKKEVTKESPKEEKVEKKEEKPKDVADKKKAESPVKEKAVEE
VITISKSVKVSLEKDTKEEKLQPQEKVKEKAEEEGGSEEEGSDRSPQESKKEDIAIN
GEVEGKEEEEQETQEKGSGREEEKGVVTNGLDVSPAEEKKGEDSSDDKVVVTKKV
EKITSEGGDGATKYITKSVTVTQKVEEHEETFEEKLVSTKKVEKVTSHAIVKEVTQGD

Neurofilament, light polypeptide [Mus musculus], locus NP_035040

MSSFGYDPYFSTSYKRRYVETPRVHISSVRSGYSTARSAYSSYSAPVSSSLSVRRS
YSSSSGSLMPSLENLDLSQVAAISNDLKSIRTQEKAQLQDLNDRFASFIERVHELEQ
QNKVLEAELLVLRQKHSEPSRFRALYEQEIRDLRLAAEDATNEKQALQGEREGLEE
TLRNLQARYEEEVLSREDAEGRLMEARKGADEAALARAELEKRIDSLMDEIAFLKKV
HEEEIAELQAQIQYAQISVEMDVSSKPDLSAALKDIRAQYEKLAAKNMQNAEEWFKS
RFTVLTESAAKNTDAVRAAKDEVSESRRLLKAKTLEIEACRGMNEALEKQLQELEDK
QNADISAMQDTINKLENELRSTKSEMARYLKEYQDLLNVKMALDIEIAAYRKLLEGEE
TRLSFTSVGSITSGYSQSSQVFGRSAYSGLQSSSYLMSARSFPAYYTSHVQEEQTE
VEETIEATKAEEAKDEPPSEGEAEEEEKE*KEEGEEEEGAEEEEAAKDESEDTKEE
EEGGEGEEEDTKESEEEEKKEESAGEEQVAKKKD

Table 1 Protein sequence similarities between the synthetic peptide KEEKPEAKGVKEEVKLA and HDAC1, neurofilament heavy, medium and light proteins. Red letters denote perfect amino acid complementary, blue letters refer to conservative amino acid changes. Asterisk (*) indicates overlapping complementary sequences.

To prevent the recognition of neurofilament proteins via the polyclonal HDAC1 antibody Sat 244, we wanted to eliminate cross-reacting antibodies by binding them to the synthetic peptide used to generate the monoclonal HDAC1 antibody 10E2. The *SulfoLink Immobilization Kit for Peptides* (*Pierce*) contains all the necessary components for covalent immobilization of sulfhydryl-containing peptides to a beaded

agarose support. The *SulfoLink Coupling Resin* is derivatized to contain iodoacetyl groups that react specifically with free sulfhydryls. For our experiment we used the 18 amino acid long synthetic peptide CKEEKPEAKGVKEEVKLA and covalently bound it to the resin via its N-terminal cysteine. The manufacturer's protocol for peptide immobilization and antibody purification were followed and antibodies were tested on a western blot loaded with cytoskeleton extract of mouse spinal cord (for protocol, see above). No HDAC1 protein should be present in cytoskeleton extracts.

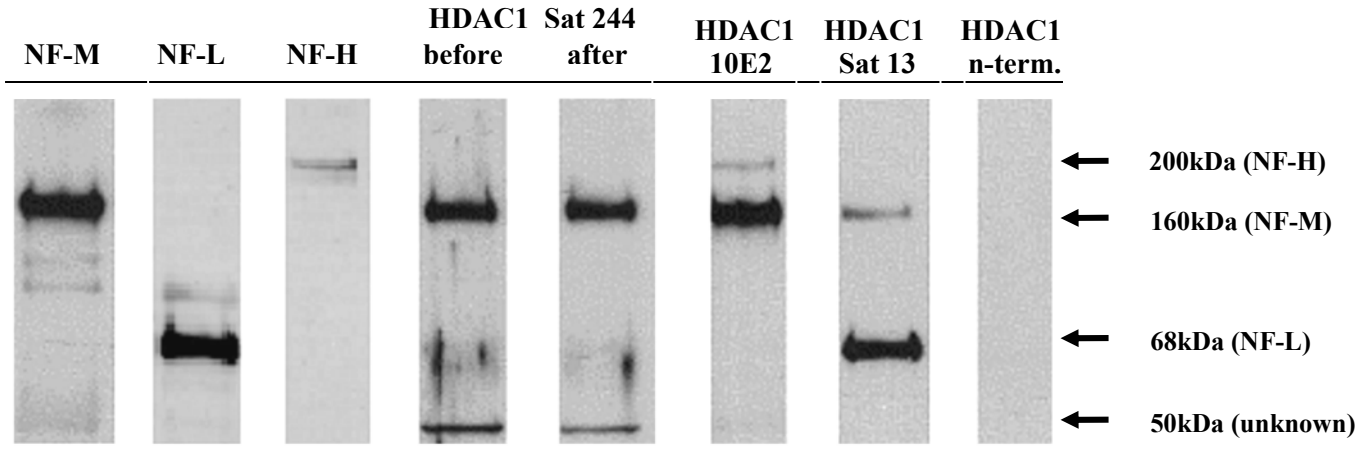


Figure 4. Western Blot Analysis of Different HDAC1 Antibodies. Cytoskeleton extracts of mouse spinal cord tissue were used to determine HDAC1 antibody cross-reaction with different neurofilament proteins. Abbreviations: neurofilament medium (NF-M), neurofilament light (NF-L), neurofilament heavy (NF-H) antibodies, HDAC1 Sat 244 serum before affinity purification (before) and after affinity purification (after). Antibody dilutions are listed in the Antibody List.

Figure 4 shows western blot analysis of mouse cytoskeleton extracts with several HDAC1 antibodies. Affinity purification of the HDAC1 Sat 244 antibody with the 10E2 peptide did not reduce binding of this antibody to neurofilament medium protein. Surprisingly, different HDAC1 antibodies showed varying specificity for neurofilament protein. The polyclonal antibody HDAC1 Sat 244 bound to neurofilament medium protein and additionally to an unknown 50kDa protein, the monoclonal antibody HDAC1 10E2 showed cross reaction with neurofilament heavy and medium protein and the polyclonal antibody HDAC1 Sat 13 recognised neurofilament medium and light protein. As anticipated, the antibody HDAC1 n-term did not cross-react with any neurofilament protein.

4.2 Involvement of HDAC1 in Neuronal Differentiation

4.2.1 NGF-Induced Neuronal Differentiation of PC12 Cells

The rat pheochromocytoma cell line PC12 is a well established cell culture system for investigating neuronal differentiation [57, 67, 69]. PC12 cells are able to differentiate from mitotic neuronal precursors into post-mitotic neuronal cells in a time period of 5 to 6 days via treatment with nerve growth factor (NGF). Binding of NGF to the receptor tyrosine kinase TrkA activates various signalling pathways, leading to the induction of neuronal differentiation programs. Undifferentiated PC12 cells exhibit high cell division rates and are small, round and clonally growing (Figure 5A). Upon treatment with NGF, neurite outgrowth (Figure 5B) up to neuronal network formation (Figure 5C) can be observed.

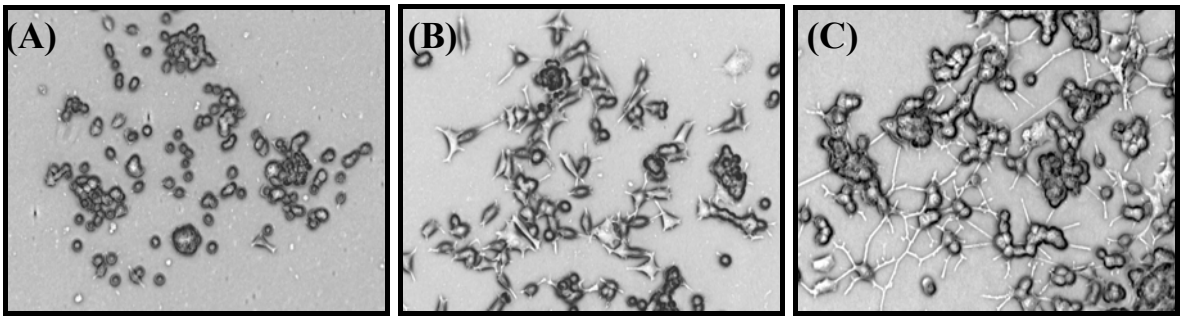


Figure 5. Differentiation experiment of the rat cell line PC12 into neuronal cells upon treatment with nerve growth factor (NGF). Over 6 days culture medium was supplied with NGF and pictures were taken to survey the differentiation process including axonal outgrowth, neuronal network formation and transition into post-mitotic neurons. (A) undifferentiated PC12 cells, (B) differentiation progress on day 3, (C) differentiation progress on day 6.

4.2.2 Histone Deacetylase Inhibitors Induce Histone Acetylation in PC12 Cells

Histone acetylation is known to be induced by histone deacetylase inhibitors [73]. In order to verify the effect of certain HDIs, we treated PC12 cells with comparable concentrations of TSA, VPA and MS-275 for 24 hours. Histone protein isolation was performed according to the protocol (see above) and 1.5 µg histone protein was loaded on a 16 percent SDS polyacrylamide gel.

We could show that all three HDIs induce acetylation of histone H3 and histone H4 after 24 hours (Figure 6). All compounds lead to a robust increase in histone H3 acetylation. Valproic acid (VPA) seems to exhibit a considerably weaker effect on histone H4 acetylation compared to TSA and MS-275.

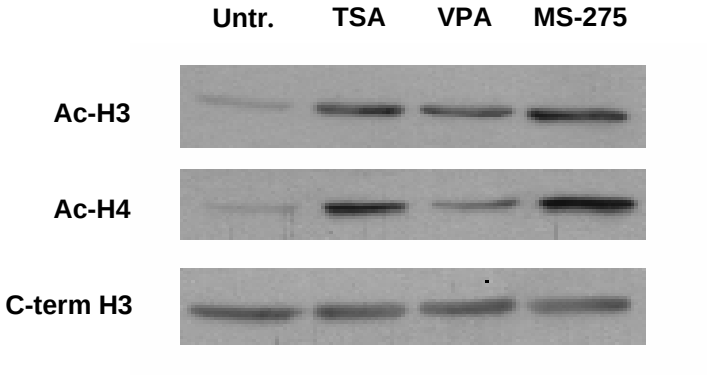


Figure 6. Effect of various HDIs on chromatin acetylation. PC12 cells were treated with 50 ng/µl TSA, 1mM VPA, 10 µM MS-275 or left untreated for 24 hours as a control. After isolation, 1.5 µg histone protein was loaded on a 16% SDS polyacrylamide gel. To analyse chromatin acetylation, primary antibodies recognising acetylated histone H3 (Ac-H3) and acetylated histone H4 (Ac-H4) were used. Antibody recognising the C-terminus of histone H3 (C-term H3) was applied to ensure equal loading. Antibody dilutions are listed in the Antibody List.

4.2.3 Effect of the Histone Deacetylase Inhibitor MS-275 on Neuronal Differentiation

The histone deacetylase inhibitor (HDI) MS-275 preferentially inhibits HDAC1 and, as stated above, was shown to be a potent and promising drug in the therapy of glioblastomas and other cancers of the nervous system [31]. For example, encouraging results have been obtained using a combination of HDIs and retinoids to treat neuroblastomas [74]. The HDI TSA is known to inhibit neuronal differentiation in PC12 cells [53]. Therefore, our group was interested in the effects of MS-275 on NGF-induced neuronal differentiation.

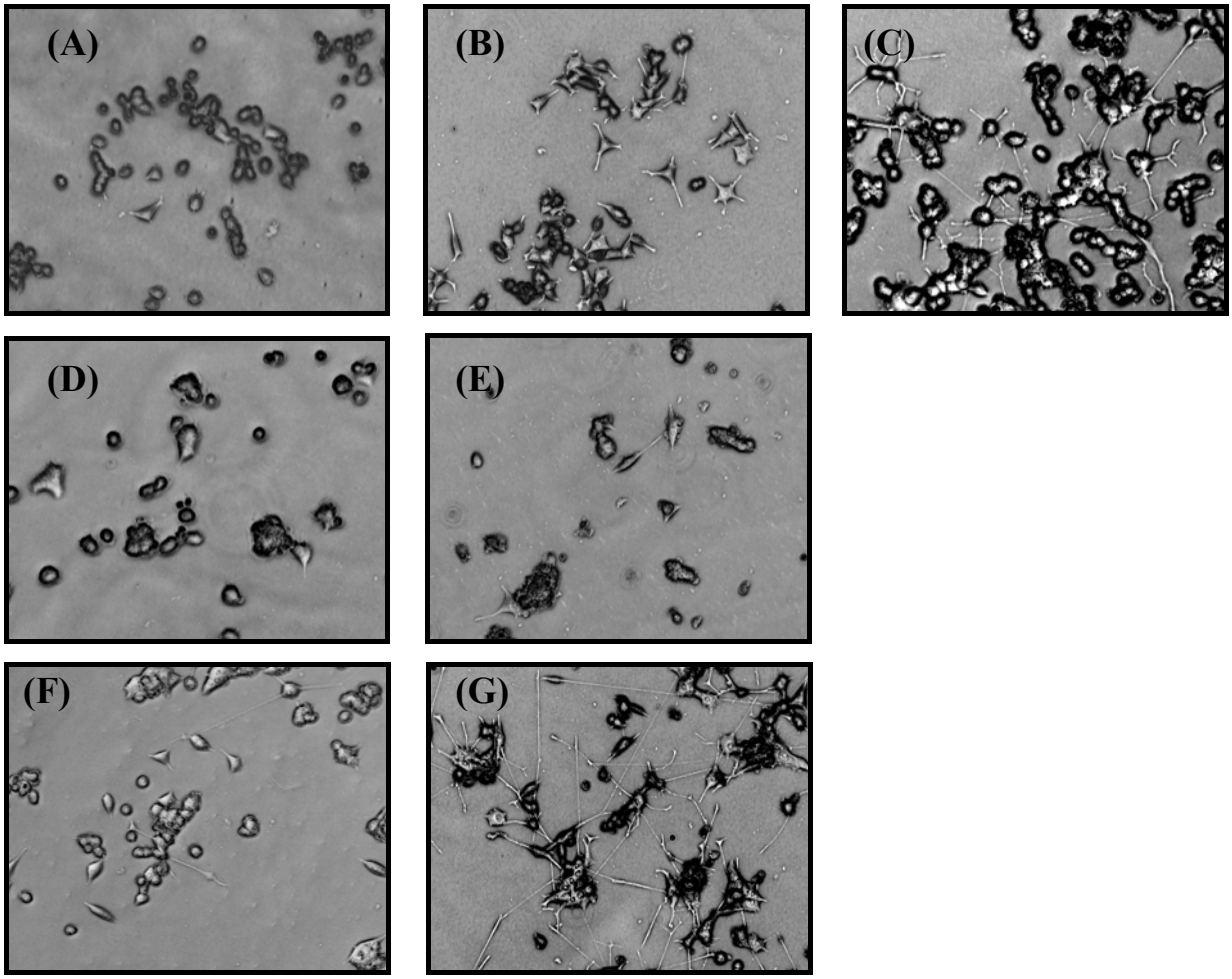


Figure 7. Differentiation experiment of the rat cell line PC12 into neuronal cells upon treatment with nerve growth factor (NGF) and the HDAC Inhibitor MS-275 (5 µM). Over 6 days culture medium was supplied with NGF or MS-275 or both. (A) Undifferentiated PC12 cells, (B) – (C) NGF only, differentiation progress on day 3 and 6, (D) – (E) MS-275 only, differentiation progress on day 3 and 6, (F) – (G) NGF and MS-275, differentiation progress on day 3 and 6.

In this experiment, MS-275 acts as an enhancer of NGF induced neuronal outgrowth of PC12 cells. We could observe an increase in percentage of differentiated cells when treating the cells with a combination of NGF and MS-275 compared to treatment with NGF alone (Figure 7, F-G). In general, the PC12 system is a relatively heterogeneous cell-line. Treatment with NGF induces neuronal differentiation and neurite outgrowth in the majority of cells. Therefore, the observation that MS-275 increased the proportion of cells bearing neurites is an indication for an enhancing effect of this HDI on neuronal development. Furthermore, neurites appeared to be

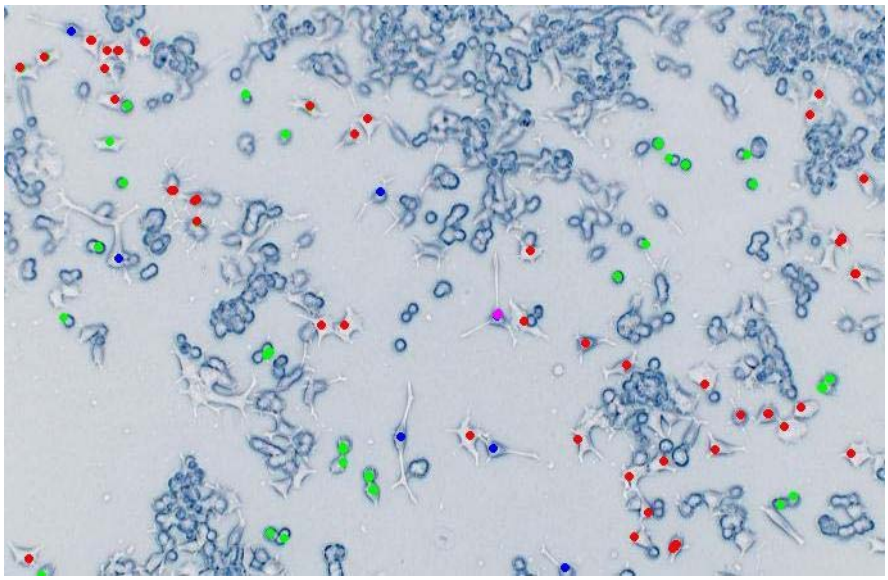
more protracted and less reticulate after 6 days treatment with MS-275 and NGF compared to treatment with NGF alone (Figures 7C and G).

When cells were treated with MS-275, a fraction of cells detached from the plate, pointing towards a possible toxic effect of MS-275. Fewer cells could be found on the cell culture plate after 6 days of treatment with MS-275 compared to the cell number after 3 days (Figure 7, D and E).

4.2.4 Neuronal Outgrowth Assay

We established a neuronal outgrowth assay to quantify the effects of MS-275 on NGF induced neuronal differentiation, using the presence of neurites as a marker for differentiation. In addition, differentiated cells were ranked into 3 different categories by comparing the length neurites to the diameter of the respective cell body (cell body diameter; CBD). Experiments were done in triplicates, approximately 100 cells were classified per plate and cells growing in clusters were not accounted. The purpose of this assay was to quantify the enhancing effect of MS-275 on neuronal differentiation of PC12 cells using a statistic approach.

(A)



Classification

(2 days NGF)

Green: not differentiated

Red: 0 – 2x CBD

Blue: 2 – 4x CBD

Purple: over 4x CBD

(B)

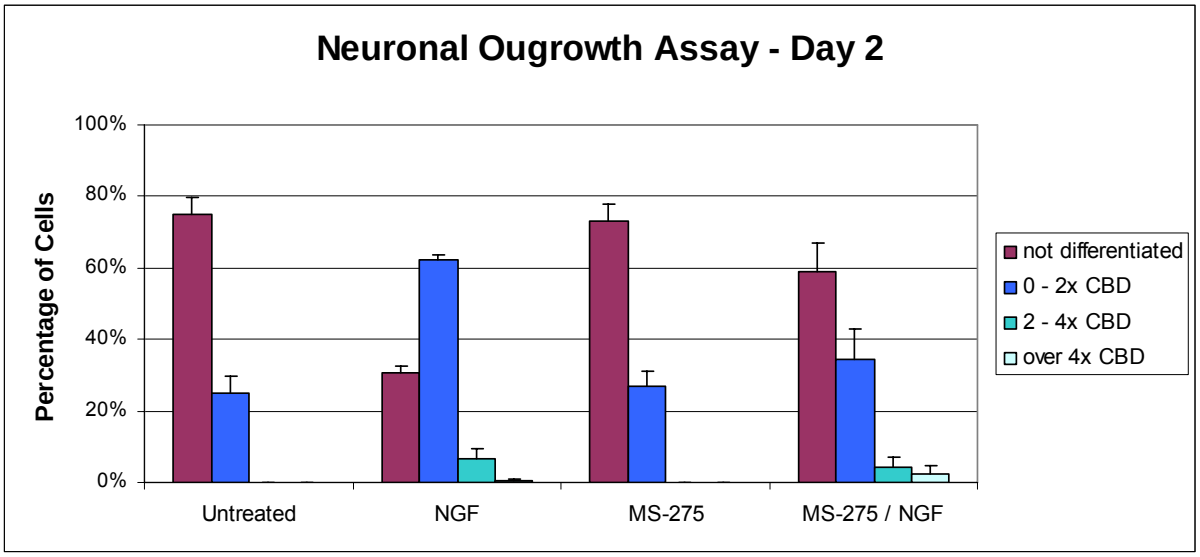


Figure 8. Neuronal Outgrowth Assay of PC12 cells on Day 2. (A) Exemplary classification of NGF treated PC12 cells. Colours were inverted to enhance contrast. (B) Statistical Evaluation: Cells were supplied with cell culture medium (Untreated); medium containing Neuronal Growth Factor (NGF); medium containing 5µM MS-275 (MS-275); medium containing 5µM MS-275 and Neuronal Growth Factor (MS-275 / NGF). Cells were classified according to the presence and length of neurites compared to the respective cell body diameter (CBD).

We could demonstrate that approximately 1 out of 5 cells showed small neuronal extensions when cultured with normal cell culture medium. After 2 days of treatment with NGF, more than 60% of the cells displayed different degrees of neuronal differentiation. On day 2 of the experiment, no effect on neurite outgrowth could be observed for cells cultured in medium containing the HDI MS-275 only (Figure 8B). When supplementing the medium with NGF and MS-275, we could observe a very slight increase in PC12 cells having neurites longer than 4 times their cell body diameter (Figure 8B).

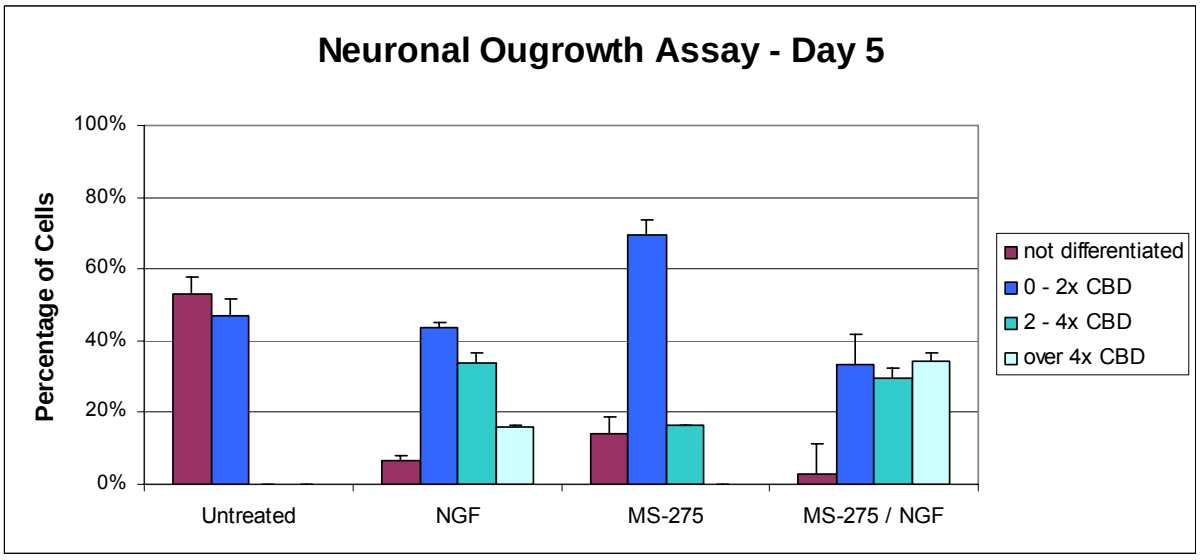


Figure 9. Neuronal Outgrowth Assay of PC12 cells on Day 5. Cells were supplied with cell culture medium (Untreated); medium containing Neuronal Growth Factor (NGF); medium containing 5µM MS-275 (MS-275); medium containing 5µM MS-275 and Neuronal Growth Factor (MS-275 / NGF). Cells were classified according to the presence and length of neurites compared to the respective cell body diameter (CBD).

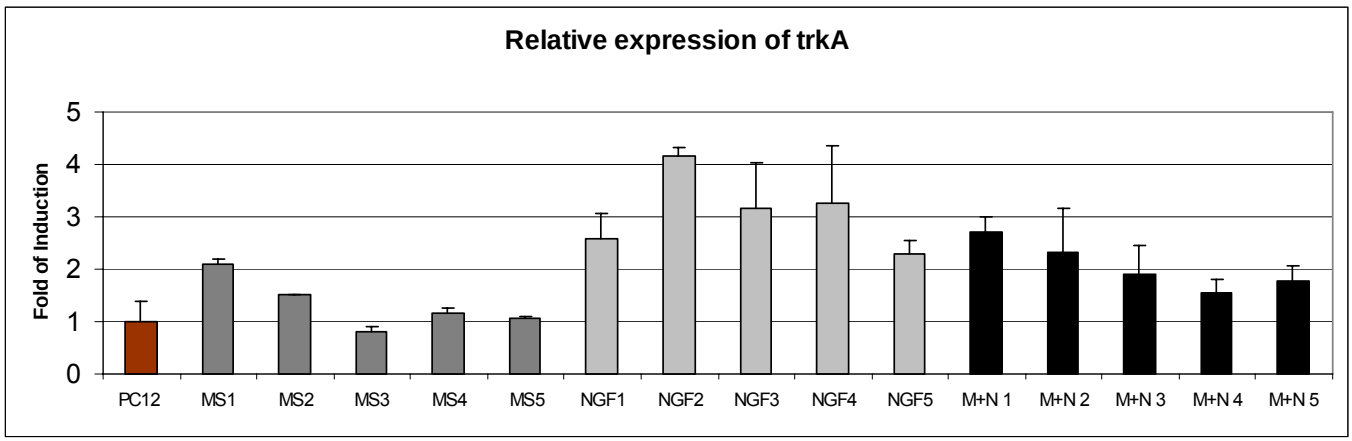
After 5 days of culturing PC12 cells with cell culture medium, approximately half of the cells developed short neuronal extensions but no cells with neurites longer than 2 times their respective cell body diameter were present. Treatment with NGF for 5 days caused neurite outgrowth in over 90% of the total cell number. Approximately half of the cells had neurites longer than 2 times their cell body diameter. In addition, strong neuronal network formation could be observed. Cells supplied with the HDI MS-275 for the same period of time displayed significantly higher levels of neuronal differentiation as untreated cells (Figure 9). Our assay detected an enhancement of

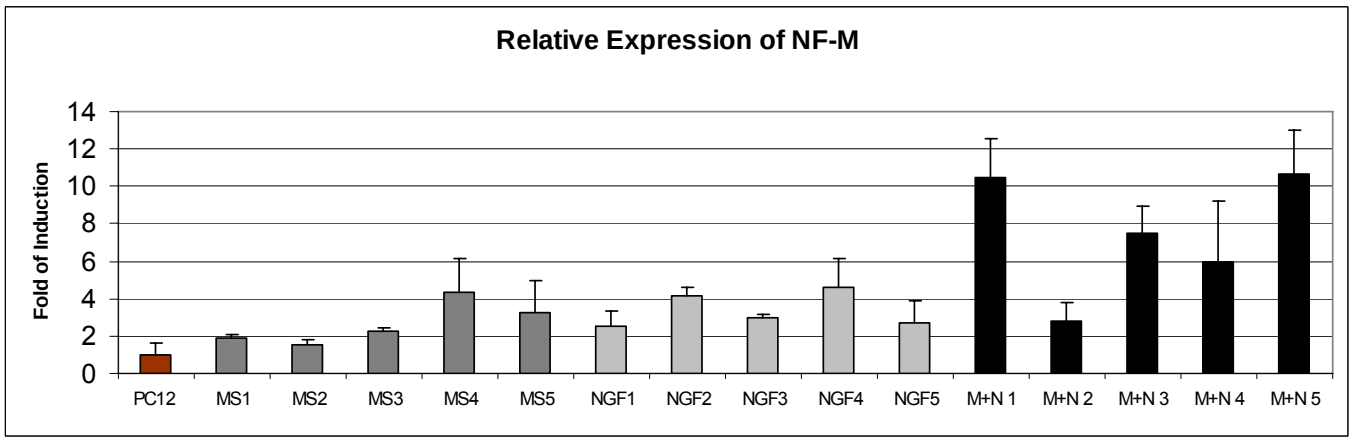
neurite outgrowth and length when treating cells with NGF and MS-275, pointing towards a possible combinatory effect.

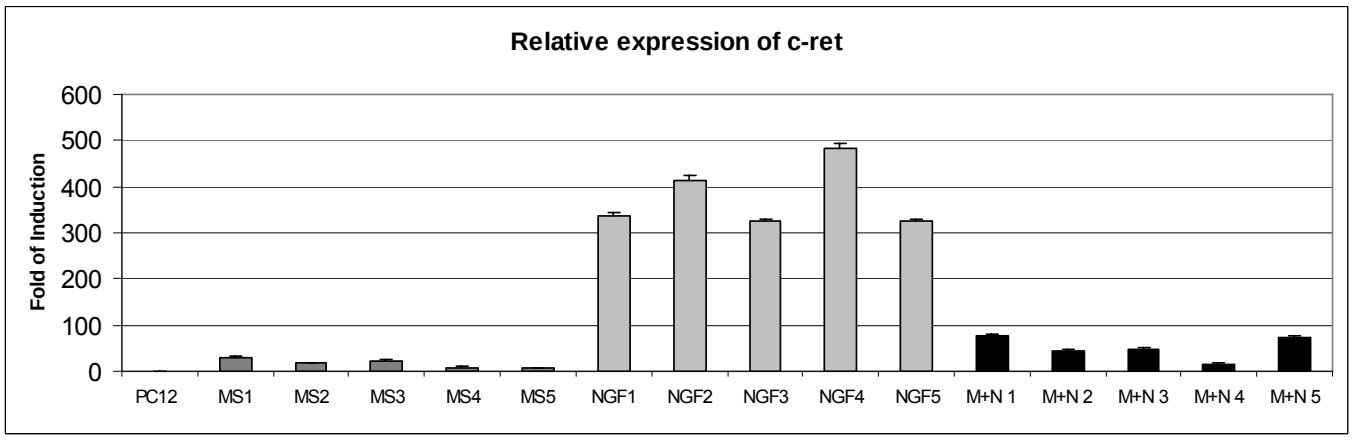
4.2.5 Real Time PCR Analysis of Neuronal Marker Gene Expression

We used Real time PCR (RT-PCR) to investigate putative neuronal target genes in the PC12 cell line. To confirm the results of the Neuronal Outgrowth Assay, we searched for genes induced by NGF as well as by the histone deacetylase inhibitor MS-275. Literature provides a diverse collection of different neuronal target genes, we decided to concentrate on five probable candidate genes; trkA, c-ret, ctrl-b, cortexin and neurofilament medium chain (NF-M). TrkA represents the cellular receptor for NGF [71] and the receptor tyrosine kinase c-Ret is known to promote neurite outgrowth of PC12 cells and to control development of enteric and sensory neurons in different vertebrate species. Cortexin, a peptide bio-regulator of cerebral functions is used in memory and attention recovery. TrkA, c-ret and cortexin are identified neuronal target genes in PC12 cells [69] while clathrin light chain b (ctrl-b) constitutes a known target gene for the REST protein complex [75]. As stated above, neurofilament medium is a structural, filamentous intermediary filament protein induced in differentiated neurons.

PC12 cells were treated with NGF, MS-275 or both compounds in combination for 6 days and cells of each treatment were taken for analysis in intervals of 24 hours. To increase fidelity of the experiment, each treatment was done in triplicates. Previous experiments showed that supplementing the medium with NGF every 2 days can lead to discontinuous induction of target genes caused by degradation or depletion of NGF in the cell culture medium. To achieve a robust and continuous expression of putative target genes, medium was changed every 2 days, including supplying the medium with NGF, and addition of half the amount of NGF on the following day. RNA preparation, synthesis of double-stranded cDNA and RT-PCR were performed according to the protocol. Expression of the neuronal marker genes was determined relative to the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT).


Relative expression of trkA
Fold of Induction
0
1
2
3
4
5
PC12
MS1
MS2
MS3
MS4
MS5
NGF1
NGF2
NGF3
NGF4
NGF5
M+N 1
M+N 2
M+N 3
M+N 4
M+N 5


Relative Expression of NF-M
Fold of Induction
0
2
4
6
8
10
12
14
PC12
MS1
MS2
MS3
MS4
MS5
NGF1
NGF2
NGF3
NGF4
NGF5
M+N 1
M+N 2
M+N 3
M+N 4
M+N 5


Relative expression of c-ret
Fold of Induction
0
100
200
300
400
500
600
PC12
MS1
MS2
MS3
MS4
MS5
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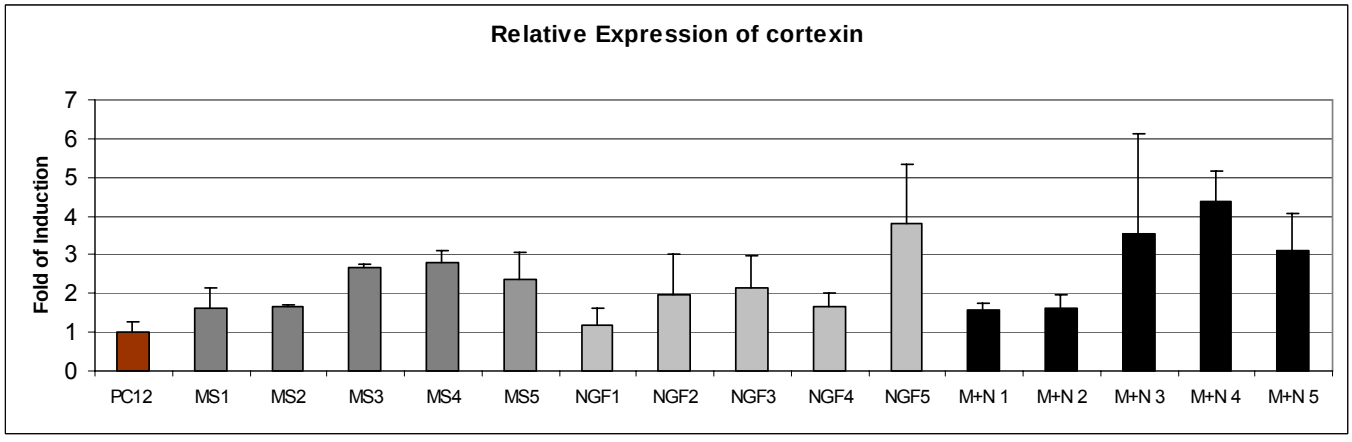
NGF2
NGF3
NGF4
NGF5
M+N 1
M+N 2
M+N 3
M+N 4
M+N 5


Relative Expression of cortexin
Fold of Induction
0
1
2
3
4
5
6
7
PC12
MS1
MS2
MS3
MS4
MS5
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NGF2
NGF3
NGF4
NGF5
M+N 1
M+N 2
M+N 3
M+N 4
M+N 5

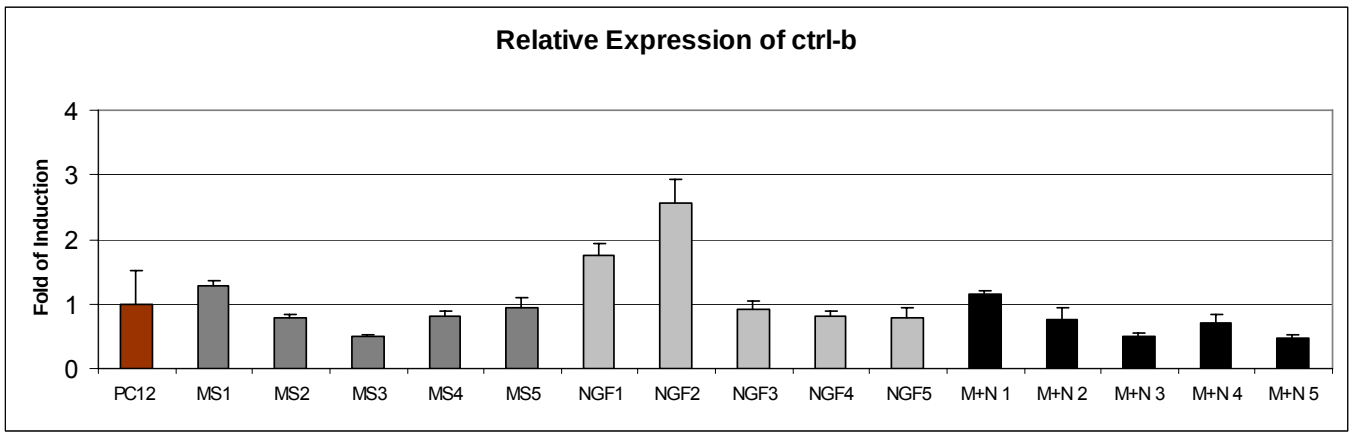


Figure 10. Relative Expression of Marker Genes in NGF-induced Neuronal Differentiation of PC12 cells analysed via Real Time PCR. For 1 to 5 days, cells were treated with 10 µM MS-275 (MS1–MS5), NGF (NGF1–NGF5), MS-275 and NGF in combination (M+N1–M+N5). RNA of untreated PC12 cells was used as a control (PC12) and set to 1.

As can be seen in Figure 10, treatment of PC12 cells with NGF leads to an up to 4 times induction in cellular mRNA levels for the NGF receptor protein, TrkA. MS-275 alone causes just a minor induction after 24 hours whereas treatment with NGF and MS-275 in combination seems to induce TrkA mRNA levels but not as strong as NGF alone. Transcription of c-ret mRNA seems to be induced up to 30 times by treating the cells with the HDI MS-275 and we could see a strong 300 to 600 fold induction when NGF was supplied to the medium. Surprisingly, double treatment of cells with NGF and MS-275 led to a considerable weaker induction of TrkA transcription then NGF alone.

Cortexin mRNA levels seemed to be mildly induced be MS-275 and NGF, the strongest induction could be seen from day 3 to day 5 when treated with a combination of both effectors. Cltr-b mRNA levels were unaffected by treatment with MS-275 and mildly induced by NGF in the first 2 days. Double treatment of cells with MS-275 and NGF did not affect ctrl-b mRNA transcription. Neurofilament medium transcripts were induced up to 4 times by single treatment with NGF or the HDI MS-275. Both substances in combination led to an induction up to 10 times the level of untreated PC12 cells.

4.3 Immunofluorescence - Effects of Different Fixation Methods

Indirect immunofluorescence is a popular tool in the field of cell biology. It facilitates detecting expression of proteins of interest and furthermore helps analyse protein localisation inside a cell. Remarkably, there are many different protocols for immunofluorescence available, using different methods for protein fixation.

In general, two different methods for protein fixation can be implemented. Cross-linking reagents like formaldehyde and glutaraldehyde form covalent bonds between proteins and creating a gel, thus retaining cellular constituents in their *in vivo* association to each other. Soluble proteins are fixed to structural proteins and rendered insoluble, giving mechanical strength to the entire structure which enables it to withstand subsequent processing. This kind of fixation requires additional permeabilisation of cellular membranes by treatment with Triton X-100 or an alternative surfactant.

Secondly, methyl and ethyl alcohols can be used for protein fixation due to their ability to disrupt the hydrophobic bonds which contribute to the maintenance of the tertiary structure of proteins. Hydrogen bonds appear to be more stable in methanol and ethanol than in water, so that while affecting the tertiary structure of proteins, these alcohols may preserve their secondary structure. The dehydrating agents acetone or acetic acid, due to their ability to penetrate cells thoroughly and rapidly, are often used in combination with cross-linking reagents or methyl and ethyl alcohols.

Aim of this experiment was to study the effects of different fixation methods for indirect immunofluorescence on signal strength and localisation of 2 independent antibodies, the monoclonal mouse HDAC1 antibody 10E2 C-term and the polyclonal rabbit HDAC1 antibody Sat 208 N-term. We were especially interested in the effects on the antibody HDAC1 Sat 208 N-term, as we were unable to obtain a specific signal with this antibody when using para-formaldehyde as fixation agent.

For this experiment we used undifferentiated PC12 cells and the indirect immunofluorescence protocol mentioned above, applying standard para-formaldehyde fixation and four additional protein fixation methods.

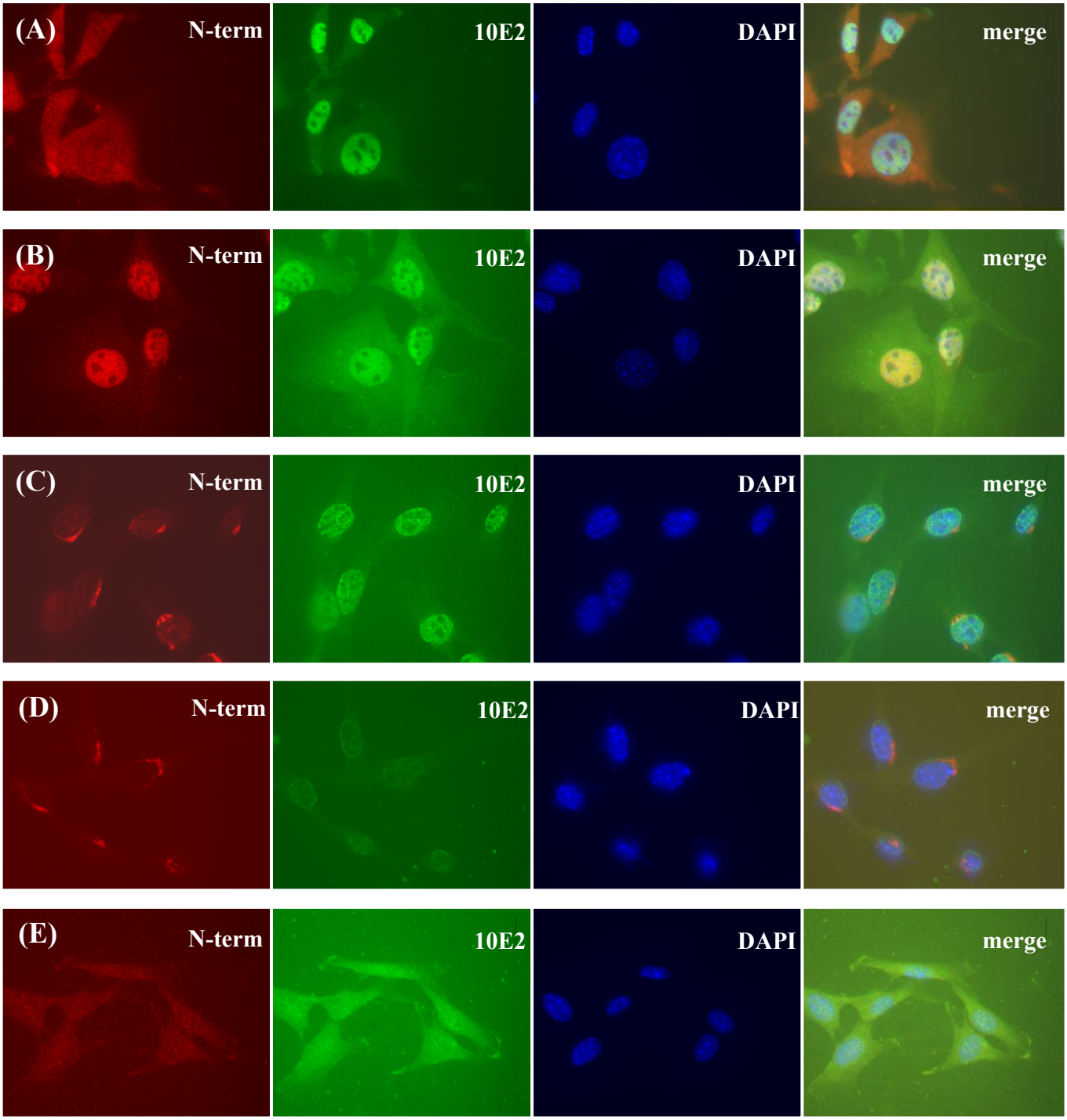


Figure 11. Effects of different fixation methods on the detection of HDAC1 by indirect immunofluorescence in undifferentiated PC12 cells. The methods were compared for the polyclonal rabbit HDAC1 antibody Sat 208 recognising the N-terminus of HDAC1 (N-term) and the monoclonal mouse HDAC1 antibody 10E2 directed against the C-terminus of HDAC1 (10E2). (A) 5 min 1% para-formaldehyde and 15 min 2% para-formaldehyde / 0,1% Triton X-100. (B) 10 min 4% para-formaldehyde and 10 min MeOH (-20°C). (C) 10 min MeOH (-20°C) and 1 min Acetone (-20°C). (D) 10 min PEM buffer (0.1 M PIPES, 1.0 mM EGTA, 0.5 mM $MgCl_2$, pH 6.8) and 10 min EtOH

(-20°C). (E) 10 min EtOH, 5% acetic acid and 5 min 1 x PBS, 0,5% Triton X-100. All antibody dilutions are listed in the Antibody List.

Surprisingly, the selection of the fixation method has profound consequences on signal strength and location. As seen in previous experiments, the Sat 208 N-terminal antibody showed an unspecific, cytoplasmic signal when performing standard para-formaldehyde/Triton X-100 fixation (Figure 11A) but we could obtain specific staining of the cellular nucleus with this particular antibody when using 4% para-formaldehyde followed by MeOH (-20°C) (Figure 11B). Two other fixation methods resulted in a strong, unidentified signal for this antibody at the periphery of the cellular nucleus, possibly caused by antibody-trapping at the contracted nuclear membrane (Figures 11C and D).

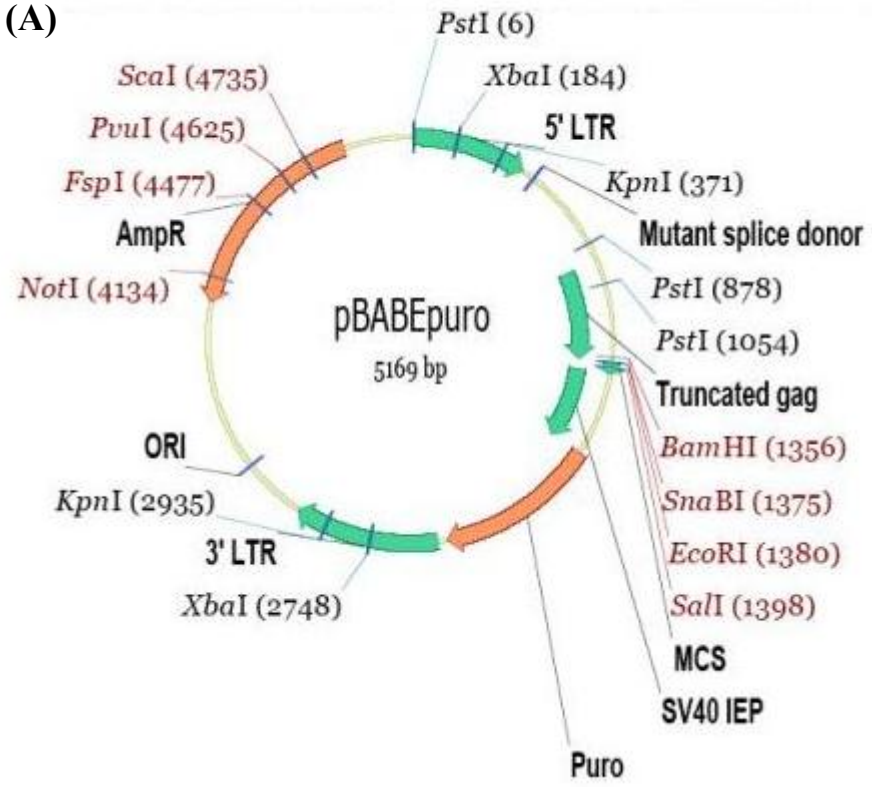
We could also see variations in signal specificity and strength for the 10E2 C-terminal antibody. The best result for this for this antibody could be obtained using standard para-formaldehyde / Triton X-100 fixation (Figure 11B). No specific signal for either antibody could be detected after fixation with EtOH, 5% acetic acid for 10 min and 5 min permeabilisation with 1 x PBS, 0.5% Triton X-100 (Figure 11E).

These results suggest the application of 4% para-formaldehyde/MeOH (-20°C) fixation in indirect immunofluorescence experiments using the polyclonal rabbit HDAC1 antibody Sat 208 N-terminal.

4.4 Cloning of the *HDAC3-myc and HDAC8-myc into the pBABE-puro* Plasmid

The mammalian expression vector *pBABE-puro* constitutes a 5169bp long, high copy plasmid and allows selection for ampicillin (bacteria) and puromycin (mammalian cells) (for details, see Figure 12). We utilized the *pBABE-puro HDAC1-HIS1-myc* plasmid DNA to generate selectable vectors expressing HDAC3 and HDAC8 as *c-myc* fusion proteins. The *pBABE-puro HDAC1-HIS1-myc* vector, coding for a catalytic site mutant of HDAC1, allowed us to cut out the mutated HDAC1 sequence but not the downstream, in-frame *c-myc* epitope tag.

(A)



(B)

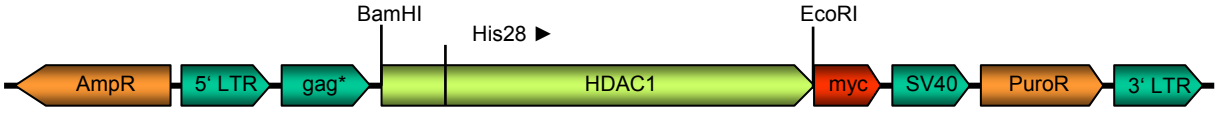
Unique restriction sites

SpeI	616
BamHI	1355
EcoRI	1379
SalI	1397
NcoI	1634
HindIII	1743
SacII	1966
ClaI	2406
NheI	2480
NotI	4133
FspI	4476

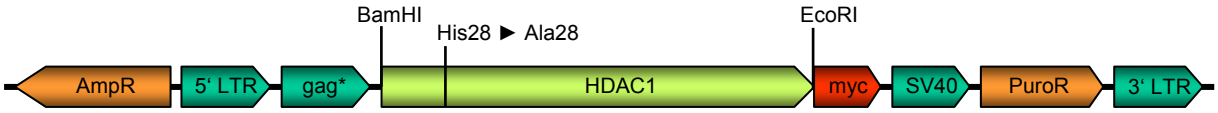
Figure 12. The pBABE-puro Expression Vector. (A) pBABE-puro vector map, (B) unique restriction sites of the pBABE-puro expression vector.

cDNA coding for human HDAC3 was derived from the vector *Flag-HDAC3* used as template for the PCR reaction. Mouse HDAC8 cDNA was obtained by reverse transcription of mouse fibroblast mRNA. PCR-Primers were designed to generate an upstream BamHI restriction site and a downstream EcoRI restriction site. We used high fidelity *Pfu DNA Polymerase* (*Promega*) to ensure accurate DNA synthesis. The *pBABE-puro HDAC1-His1-myc* plasmid was digested using the restriction enzymes EcoRI / BamHI to cut out the HDAC1 coding sequence, resulting in the pBABE-puro vector backbone containing the *c-myc* epitope tag. Following PCR, HDAC3 and HDAC8 inserts were digested with EcoRI and BamHI. Vector and insert DNA were purified using agarose gel electrophoresis and ligation was performed using viral T4 ligase (*Invitrogen*). Finally, transformation of competent bacteria was carried out and vectors were verified by sequencing of the insert. Protocols for digestion, purification, ligation of DNA and transformation of competent bacteria were followed (see above).

(A)

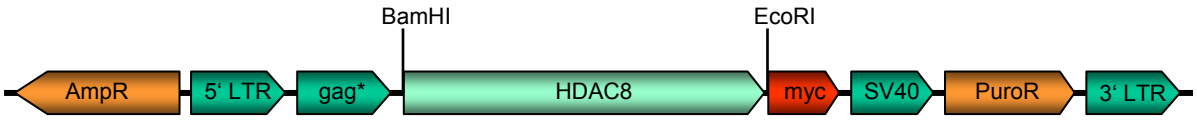


pBABE-puro HDAC1-His1-myc (6645 bp)



pBABE-puro HDAC1-His1-myc (6645 bp)

pBABE-puro HDAC3-myc (6483 bp)



pBABE-puro HDAC8-myc (6330 bp)

(B)

Primers:

human HDAC3:

upstream:	CGGATCCATGGCCAAGACCGTGGCC	Tm: 60°C
downstream:	CGAATTCAATCTCCACATCGCTTTCCTT	Tm: 60°C

mouse HDAC8:

upstream:	CGGGATCCATGGAGATGCCAGAGGAACC	Tm:62°C
downstream:	CGGAATTCGACCACATGCTTCAGATTCC	Tm:60°C

Figure 13. pBABE-puro Vector Maps / Primers for PCR. (A) Abbreviations: AmpR – ampicillin resistance gene (coding for beta-lactamase), 5' LTR – 5' long terminal repeat sequence, gag* – truncated gag, BamHI / EcoRI – Unique restriction sites used for cloning of new vectors, myc – *c-myc* epitope tag (EQKLISEEDL), SV40 – viral simian virus 40 immediate early promoter, PuroR – puromycin resistance gene (coding for puromycin N-acetyl-transferase), 3' LTR – 3' long terminal repeat sequence. (B) PCR primer sequences for human HDAC3 and mouse HDAC8.

4.5 Transfection of PC12 Cells with pBABE-puro HDAC1-myc Plasmid

As described above, we could enhance neuronal differentiation in the PC12 cell system by inhibition of class I histone deacetylases via MS-275. Hence, we were interested in the consequences of stable overexpression of HDAC1 on neuronal differentiation and neurite outgrowth. Following the protocol stated above, PC12 cells were transfected with the *pBABE-puro HDAC1-myc* plasmid, a construct allowing stable expression of *c-myc*-tagged HDAC1 under a strong viral promoter. Simultaneous transfection of PC12 cells with an orange fluorescent protein (OFP) expressing vector confirmed the viability of the cells.

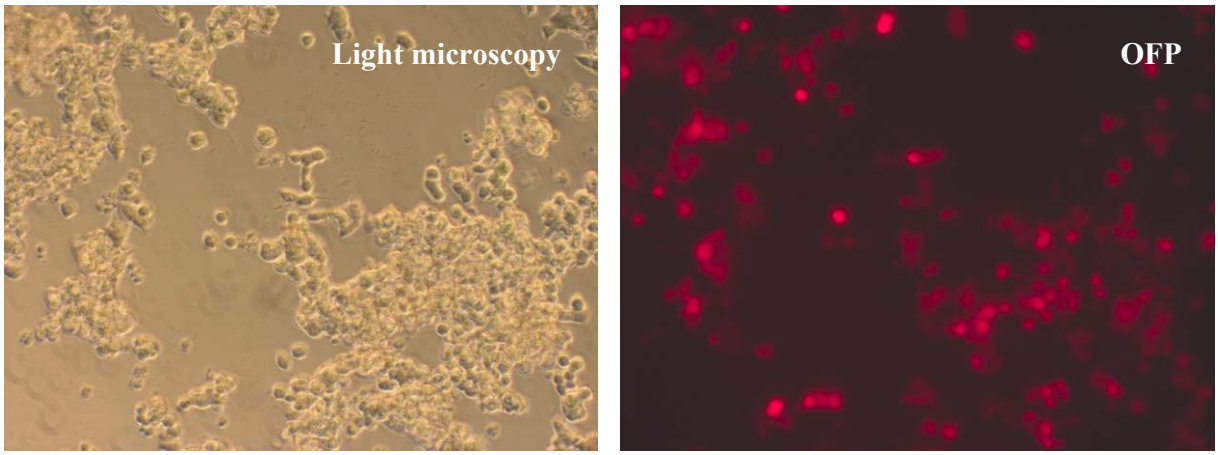


Figure 14. Evaluation of transfection efficiency after 24 hours after transfecting PC12 cells with orange fluorescent protein (OFP) expressing vector.

The protocol for the generation of stable single clone cell-lines was followed for the cells transfected with the *pBABE-puro HDAC1-myc* plasmid, resulting in 12 individual single clone cell lines. Out of the 12 cell-lines, one expressed HDAC1-myc in about 40 percent of the total cell number. As can be seen in figure 15, HDAC1-myc positive cells grew in small, clonal colonies, therefore this cell-line most probably constitutes a

mixed clone and was termed *PC12 HDAC1-myc mixed clone*. Expression of the full length fusion protein HDAC1-myc was furthermore verified by SDS-PAGE (data not shown).

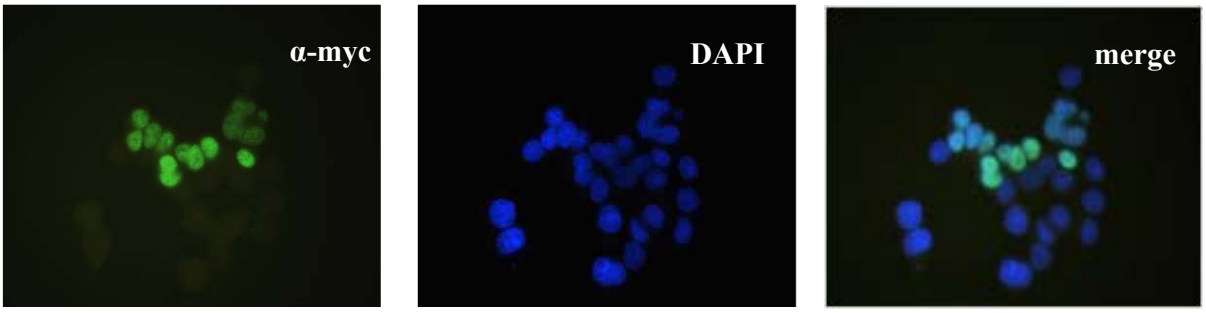


Figure 15. Immunochemical analysis of the HDAC1-myc positive PC12 cell line. Expression of HDAC1-myc was detected via the monoclonal mouse myc-antibody 4A6. Antibody dilutions are listed in the Antibody List.

4.5.1 Effects of HDAC1 Overexpression on NGF-Induced Neuronal Differentiation

We examined the potential of the *PC12 HDAC1-myc mixed clone* cell line to differentiate into neurite-bearing cells after treatment with NGF. In this experiment, we could observe a moderate response of this cell line to NGF compared to wild type PC12 cells. A smaller fraction of cells developed neuronal extensions and many detached from the cell culture dish or formed aggregated clusters.

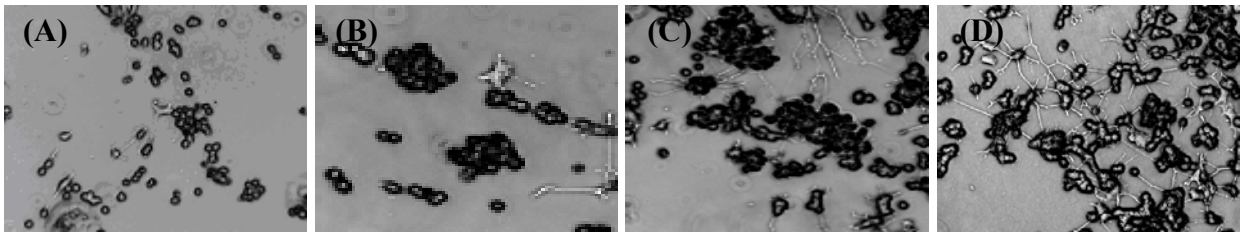


Figure 16. Differentiation experiment of *PC12 HDAC1-myc mixed clone* into neuronal cells upon treatment with nerve growth factor (NGF). Over 6 days culture medium was supplied with NGF. (A) *PC12 HDAC1-myc mixed*, day 1, (B) *PC12 HDAC1-myc mixed*, day 3, (C) *PC12 HDAC1-myc mixed*, day 6, (D) PC12 wild type cell-line on day 6.

We used indirect immunofluorescence to further elucidate the effect of stable HDAC1-myc overexpression on neuronal development, as it was not possible to utilize the Neuronal Outgrowth Assay (described above) on a mixed clone. The polyclonal HDAC1 antibody Sat 13, by not only recognising HDAC1 but also neurofilament protein (described above), could be used for detection of neuronal differentiation in individual cells.

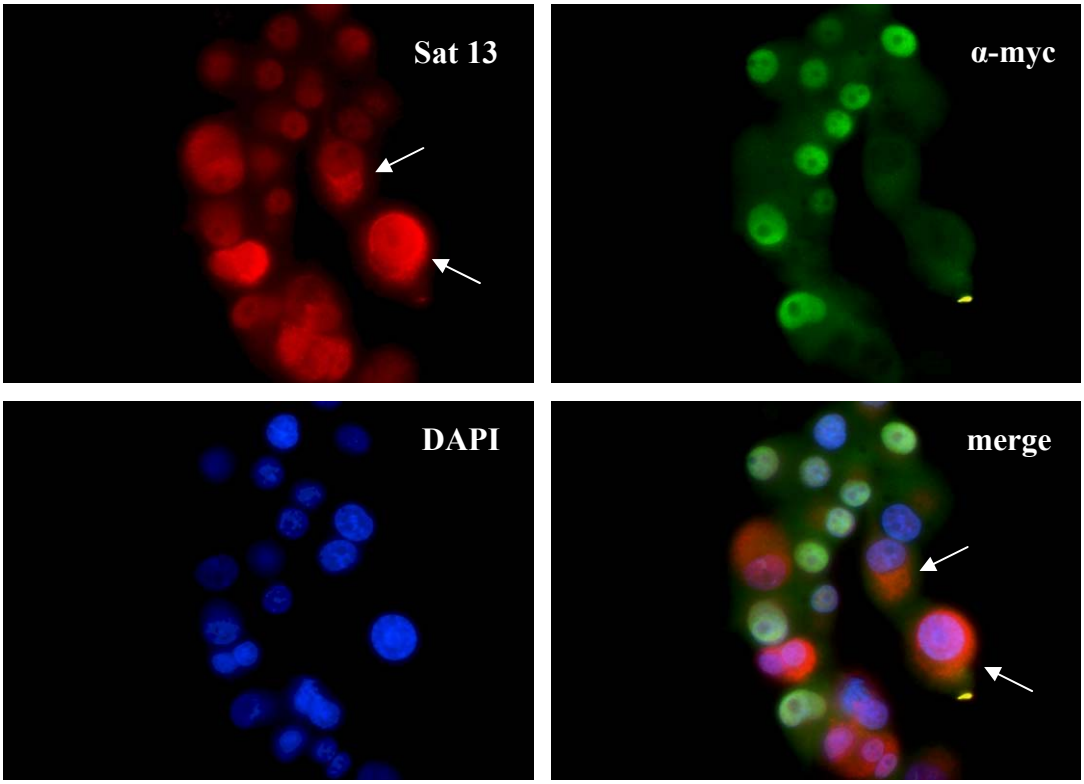


Figure 17. Immunochemical analysis of the *PC12 HDAC1-myc mixed clone* after 6 days treatment with NGF. Expression of HDAC1-myc was identified via the monoclonal mouse myc-antibody 4A6, endogenous HDAC1 and expression of neurofilament protein could be detected using the HDAC1 antibody Sat 13. Arrows depict examples of cytosolic neurofilament protein localisation. Antibody dilutions are listed in the Antibody List.

Cytosolic neurofilament protein can be detected in a fraction of cells after 6 days of treatment with NGF. As can be seen in figure 17, Neurofilament proteins form a basket-like structure in the cytoplasm of the cell, which can be detected by the HDAC1 antibody Sat 13. Interestingly, we did not identify any cells expressing HDAC1-myc and neurofilament protein at the same time.

4.5.2 Statistical Analysis of NGF induced Neuronal Differentiation and MS-275 Treatment on the *PC12 HDAC1-myc mixed clone*

We utilized the *PC12 HDAC1-myc mixed clone* and the HDI MS-275 to get an insight into the role of HDAC1 in neuronal differentiation. We induced neuronal differentiation via 5 days treatment with NGF with or without MS-275 and categorised single cells based on the expression of HDAC1-myc and neurofilament medium protein. The fusion protein and of the neuronal marker were detected using indirect immunofluorescence and approximately 100 cells per treatment were categorised.

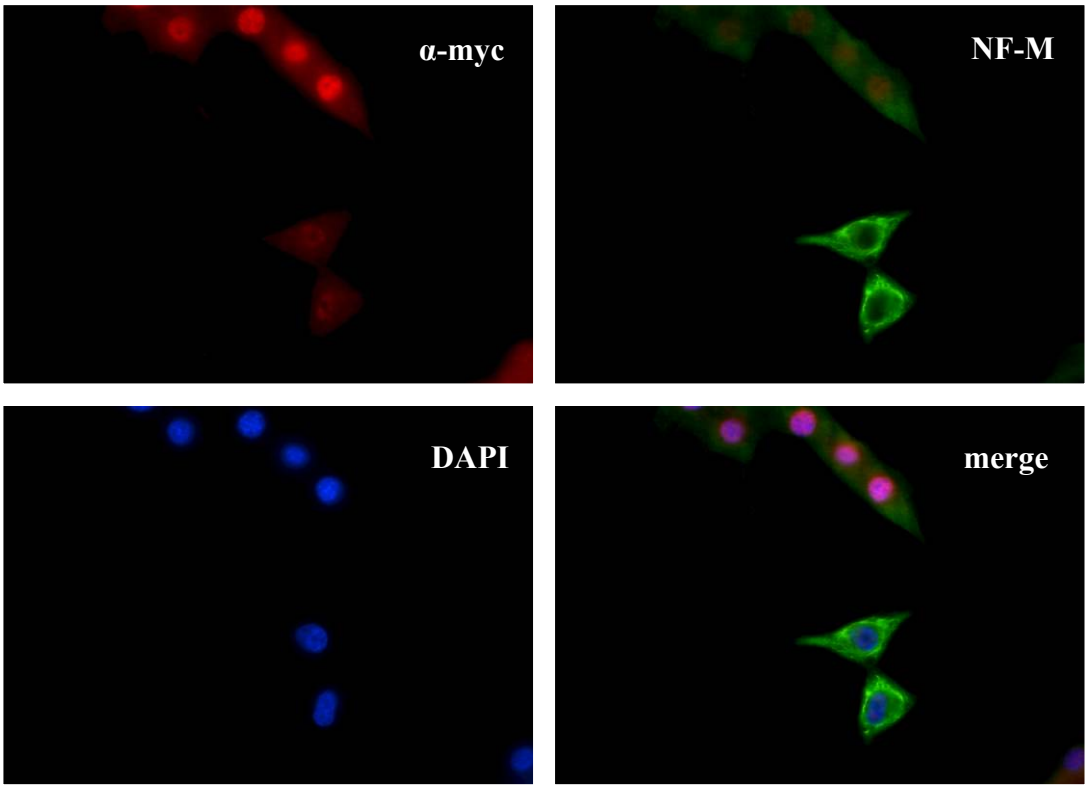


Figure 18. Immunochemical analysis of the *PC12 HDAC1-myc mixed clone* after 6 days treatment with NGF. Expression of HDAC1-myc was identified via a polyclonal myc-antibody, expression of neurofilament protein was detected by the neurofilament medium antibody. Antibody dilutions are listed in the Antibody List.

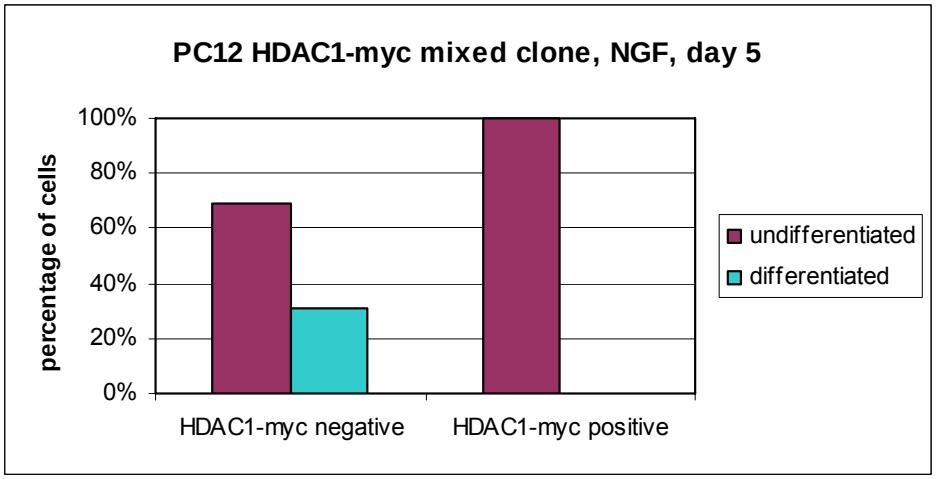


Figure 19. Neuronal Outgrowth of *PC12 HDAC1-myc mixed clone* cells on Day 5. Cells were supplied with cell culture medium containing Neuronal Growth Factor. Cells were classified according to expression of HDAC1-myc and neurofilament medium.

Figure 18 illustrates the analysis of the *PC12 HDAC1-myc* mixed clone after 5 days of NGF induced neuronal differentiation. Four cells express the HDAC1-myc fusion protein but do not express the neuronal marker gene neurofilament medium. Two cells do not express HDAC1-myc but show cytosolic staining for NF-medium protein. Data obtained from multiple microscopy images was combined in Figure 19. PC12 cells tend to form clusters of cells that do not differentiate when treated with NGF. Those clusters are also incorporated into the statistical analysis, accounting for the high percentage of undifferentiated cells.

After 5 days treatment with NGF, 30 percent of HDAC1-myc negative cells showed expression of the neuronal marker protein NF-medium. In contrast, no differentiated cells expressing the fusion protein HDAC1-myc could be identified. This data suggest a possible block in neuronal differentiation induced by overexpression of HDAC1.

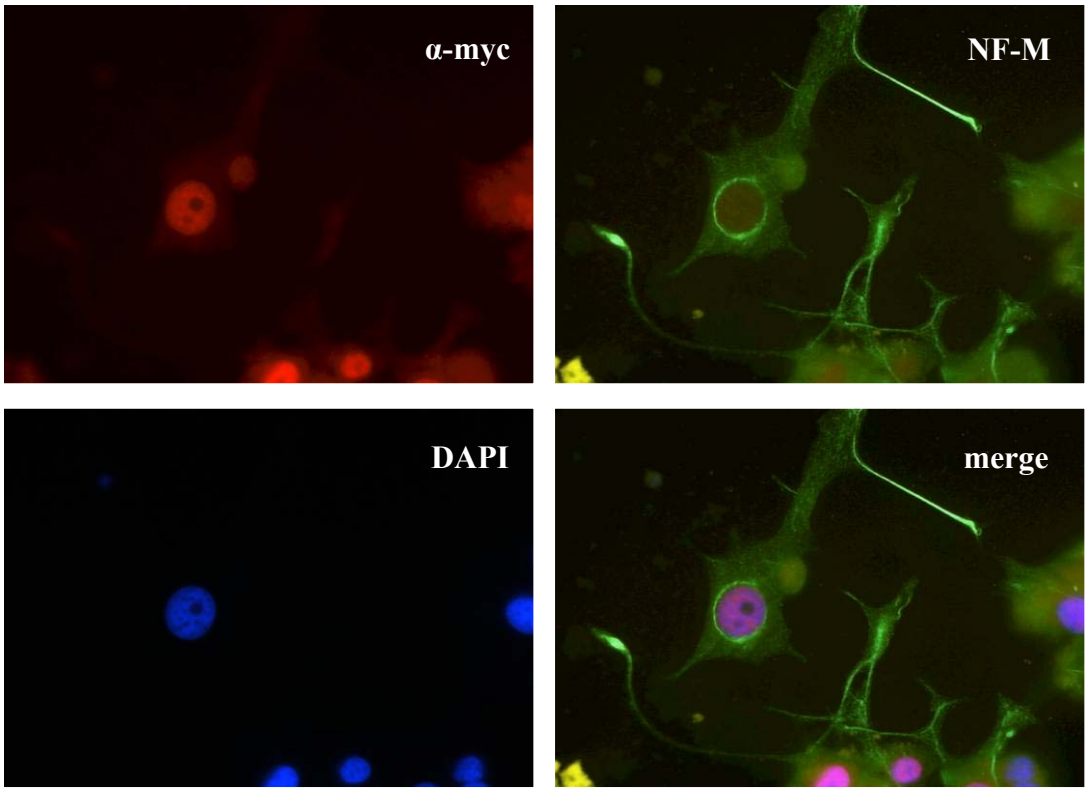


Figure 20. Immunochemical analysis of the *PC12 HDAC1-myc mixed clone* after 5 days treatment with NGF and the HDI MS-275. Expression of HDAC1-myc was identified via a polyclonal myc-antibody, expression of neurofilament protein was detected by the neurofilament medium antibody. Antibody dilutions are listed in the Antibody List.

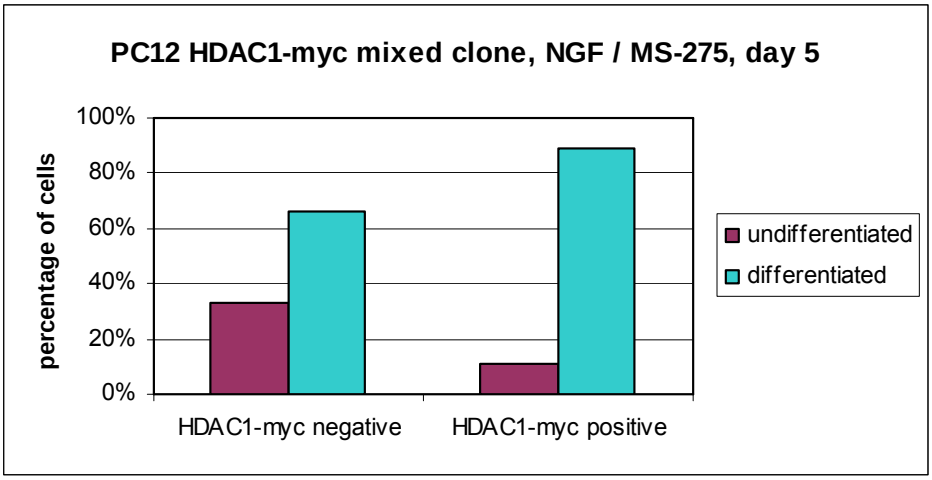


Figure 21. Neuronal Outgrowth of *PC12 HDAC1-myc mixed clone* cells on Day 5. Cells were supplied with cell culture medium containing Neuronal Growth Factor and the histone deacetylase inhibitor MS-275. Cells were classified according to expression of HDAC1-myc and neurofilament medium.

In the presence of the HDI MS-275, NGF induced neuronal differentiation of the *PC12 HDAC1-myc mixed* cell line is enhanced. Over 60 percent of HDAC1-myc negative cells expressed the neuronal marker NF-medium, confirming the results of the neuronal outgrowth assay (see above). Additionally, Figure 20 shows HDAC1-myc positive cells expressing NF-medium protein. This demonstrates that the block in neuronal differentiation induced by overexpression of HDAC1-myc in the *PC12 HDAC1-myc mixed* cell line could be abolished by treatment with the HDI MS-275.

4.5.3 Generation and Analysis of a Pure, HDAC1-myc Expressing PC12 Cell-line

As stated above, the *PC12 HDAC1-myc mixed* cell line represents a mixture of cells expressing the fusion protein HDAC1-myc and presumable wild-type cells. Therefore, mRNA expression profile of this cell-line cannot be studied using RT-PCR. The same applies to analysis of relative HDAC1 protein expression of cells expressing the HDAC1-myc protein and wild type PC12 cells. Therefore, we generated a pure, HDAC1-myc expressing PC12 cell-line. Multiple new transfection round of PC12 cells with the *pBABE-puro HDAC1-myc* plasmid did not result in a single new HDAC1-myc expressing clone. We additionally tried an alternate approach, the isolation of HDAC1-myc expressing cells out of the *PC12 HDAC1-myc mixed* cell-line by limiting dilution.

Out of the *PC12 HDAC1-myc mixed* cell-line, 18 individual single clones were generated. One clone, consisting exclusively of HDAC1-myc expressing cells, could be isolated and was termed *PC12 HDAC1-myc pure clone*. Surprisingly, treatment with NGF induced neuronal differentiation in the *PC12 HDAC1-myc pure* cell-line (Figure 22, C). In addition, Figure 23 demonstrates that transcription of neuronal target genes could be induced in this clone using NGF and MS-275.

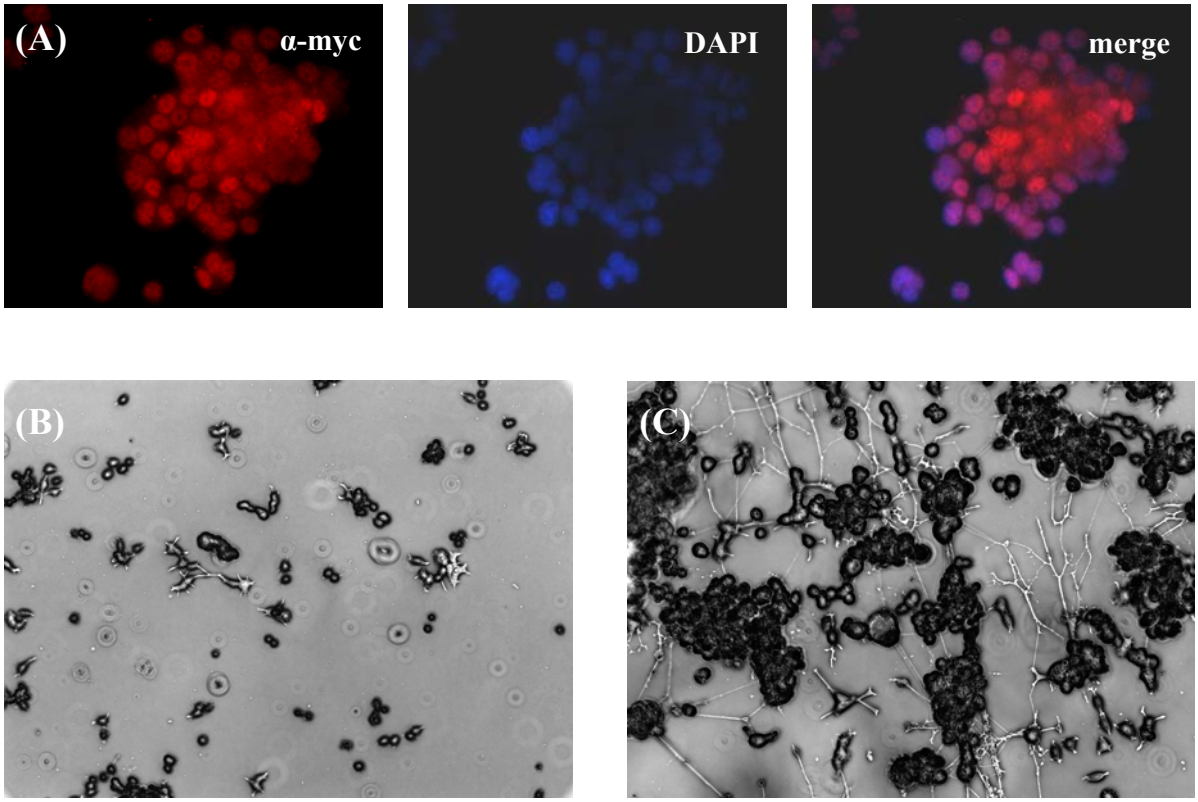
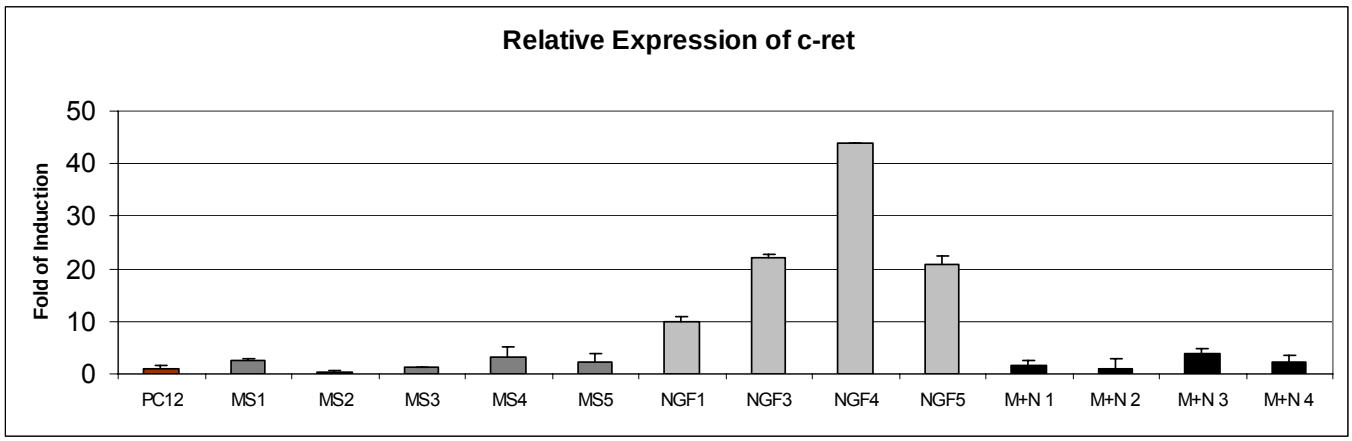
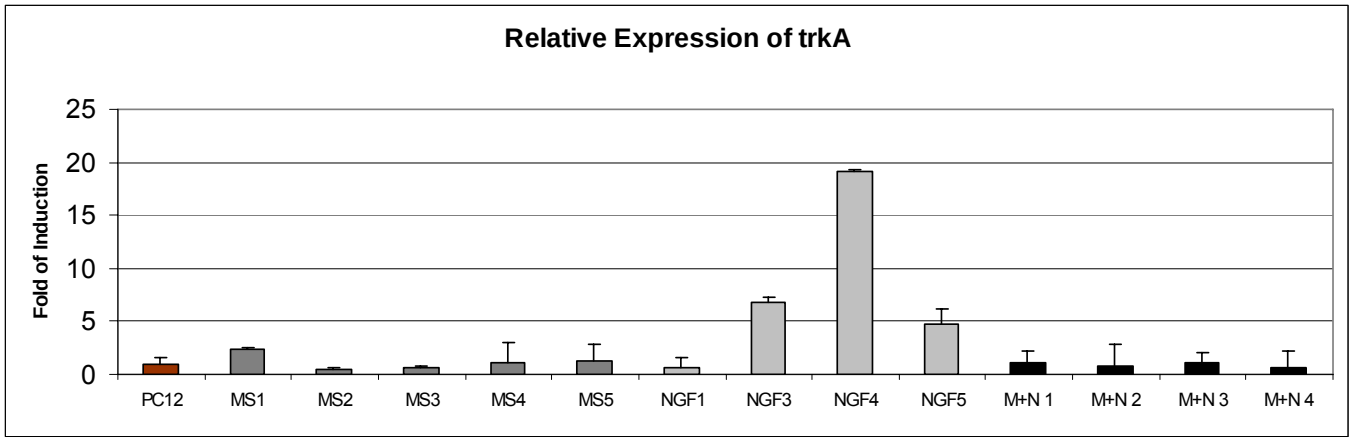
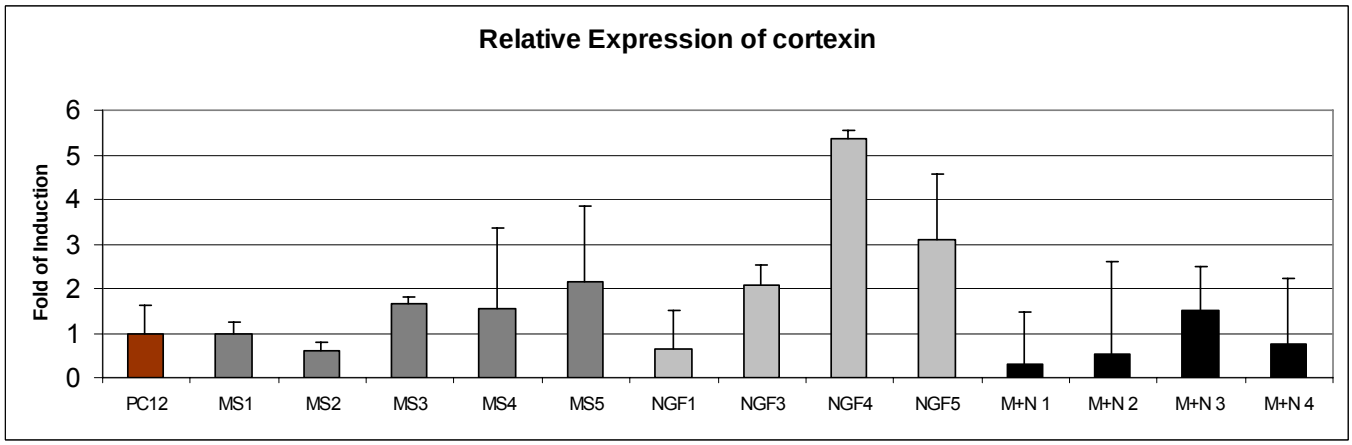


Figure 22. Analysis of the *HDAC1-myc pure* PC12 cell line. (A) Indirect Immunofluorescence, expression of HDAC1-myc was detected via the polyclonal rabbit myc-antibody. NGF induced neuronal differentiation after 2 days (B) and after 6 days (C). Antibody dilutions are listed in the Antibody List.







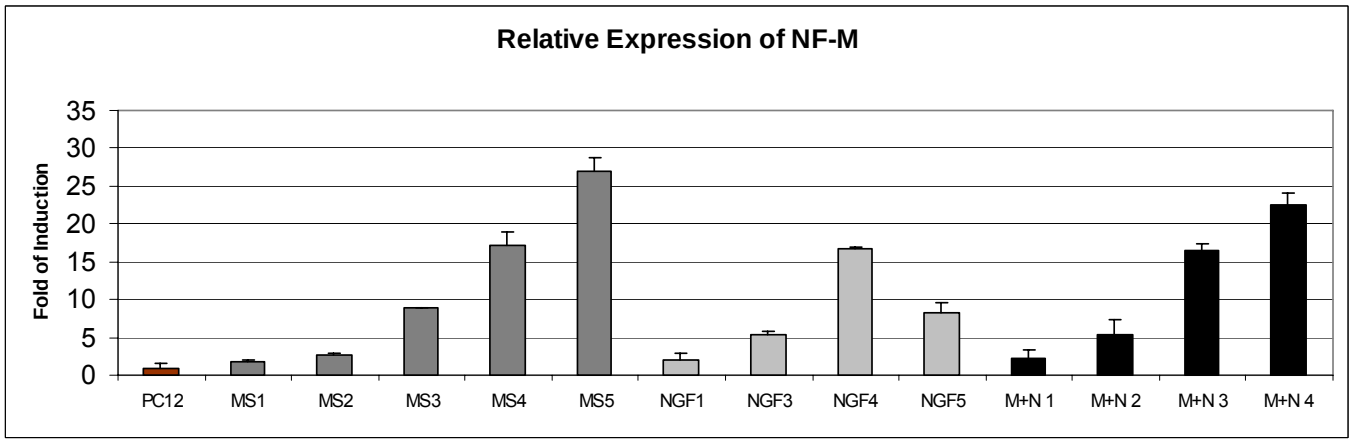


Figure 23. Relative Expression of Marker Genes in NGF-induced Neuronal Differentiation of the *PC12 HDAC1-myc pure* cell-line analysed via Real Time PCR. For 1 to 5 days, cells were treated with 10 µM MS-275 (MS1 – MS5), NGF (NGF1 – NGF5), MS-275 and NGF in combination (M+N 1 – M+N 4, day 5 missing due to lost RNA sample). RNA of untreated PC12 cells was used as a control (PC12).

Robust induction of neuronal target genes could be identified in the *PC12 HDAC1-myc pure* cell-line after treatment with neuronal growth factor (Figure 23). C-ret and trkA were strongly induced by NGF whereas NF-medium expression was induced by NGF, MS-275 and a combination of the two compounds. As seen in wild type PC12 cells (Figure 10), we could observe an attenuating effect of MS-275 in combination with NGF versus NGF alone on the expression of c-ret, trkA and cortexin.

4.5.4 Relative HDAC1 Protein Abundance in the *PC12 HDAC1-myc pure* Cell-line

We determined HDAC1 and HDAC1-myc protein levels using *Odyssey imaging System* following the protocol (see above). Apparently, the *PC12 HDAC1-myc pure* cell-line has reduced HDAC1 protein levels compared to PC12 wild type cells (Figure 24, A). This outcome is quite surprising, but can be interpreted as a substantial downregulation of endogenous HDAC1 protein in the *HDAC1*-myc expressing cell-line (Figure 24, B).

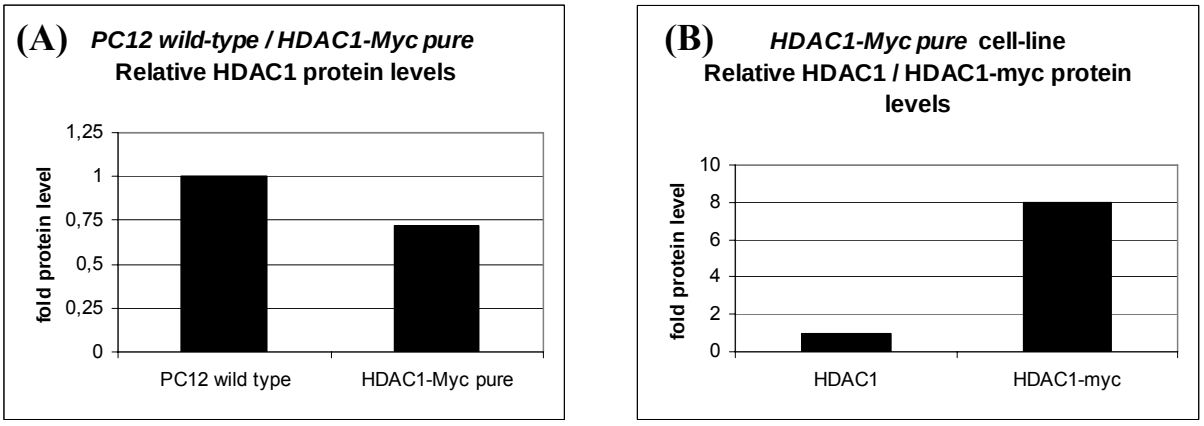


Figure 24. Relative HDAC1 Protein Levels in PC12 wild-type cells and the *PC12 HDAC1-myc pure* cell-line. (A) HDAC1 protein levels in PC12 wild-type cells and HDAC1-myc expressing PC12 cells. (B) HDAC1 – HDAC1-myc protein levels in the *HDAC1-myc pure* cell-line.

5. DISCUSSION

5.1 Cross-Reaction of HDAC1 Antibodies with Neurofilament Proteins and Adaptation of the Indirect Immunofluorescence Protocol

During work with neuronal tissue samples, we did encounter strong cross-reaction of our HDAC1 antibodies Sat 13, Sat 244 and 10E2 with presumably cytosolic, filamentous proteins. BLAST searches and subsequent analysis with the LALIGN program revealed unexpected sequence similarities between the N-terminus of HDAC1 and neuronal-specific neurofilament (NF) proteins. Table 1 depicts sequence resemblances between HDAC1, NF-light, NF-medium and NF-heavy proteins. Consensus between the neurofilament proteins can be observed for large parts of their respective amino acid sequence and can be explained by a shared evolutionary origin of those proteins. However, sequence similarities between neurofilament proteins and the highly immunogenic C-terminus of HDAC1 appear to be a product of chance.

We assumed that the last 17 amino acids of HDAC1, termed the 10E2 peptide, were responsible for the cross-reaction of certain HDAC1 antibodies with neurofilament proteins. Therefore, we coupled the 10E2 peptide to agarose beads and tried to affinity purify the polyclonal HDAC1 Sat 244 antibody serum by isolating cross-reacting antibodies. However, results did show that affinity purification of the HDAC1 Sat 244 antibody serum did not reduce recognition of neurofilament proteins (Figure 4).

Figure 4 demonstrates that different antibodies targeted against the C-terminus of HDAC1 display varying specificity for neurofilament proteins. HDAC1 Sat 244 recognises NF-medium, HDAC1 Sat 13 cross-reacts with NF- medium and NF-light and the monoclonal antibody HDAC1 10E2 binds to NF- heavy and NF-medium.

Fortunately, the HDAC1 Sat 208 N-terminal antibody does not cross-react with proteins other than HDAC1 and works in western blot analysis using standard conditions. Adaptations had to be made for utilizing the HDAC1 N-terminal antibody for indirect immunofluorescence. Protocols with para-formaldehyde and Triton X-100 as fixatives resulted in an unspecific, cytoplasmic signal. We could obtain specific staining of nuclear HDAC1 protein with the HDAC1 Sat 208 N-terminal antibody when using 4% para-formaldehyde followed by MeOH (-20°C). These results provide

a guideline for specific detection of HDAC1 protein via immunofluorescence in neuronal tissues, primary cell cultures and cell lines.

5.2 HDAC1 in Neuronal Differentiation of PC12 Cells

Neuronal differentiation and concurrent cell-cycle arrest can be induced in the rat pheochromocytoma cell-line PC12 by treatment with neuronal growth factor (NGF), a process that is known to be modulated by various chromatin modifications [76]. In order to elucidate the role of histone acetylation in neuronal development, we investigated the effect of the histone deacetylase inhibitor (HDI) MS-275, a promising candidate for the treatment of cancers of the nervous system

We could demonstrate that MS-275 induced acetylation of histone H3 and histone H4 in undifferentiated PC12 cells, comparable to two other well known HDIs, TSA and VPA (Figure 6). Interestingly, we could observe a strong, enhancing effect of MS-275 on neuronal differentiation of this cell-line (Figure 9). PC12 cells treated with NGF and MS-275 in combination produced more and longer neuronal extensions than cells treated with NGF alone. In addition, treatment with the HDI MS-275 alone induced neuronal differentiation. To quantify the impact of MS-275, we developed a neuronal outgrowth assay which allows easy validation of promising candidate compounds and their effect on PC12 cell differentiation in the future.

It could be shown that treatment with MS-275 leads to a significant increase in neurite length. Additionally, MS-275 enhances the total number of differentiated PC12 cells in culture to nearly 100 percent, an interesting observation in this normally relative heterogeneous cell line (Figure 9). This effect, driving proliferating cells into differentiation, could provide a model for the clinical application of this novel HDI. Inducing neuronal development and subsequent cell-cycle arrest could be responsible for the effectiveness of MS-275 treatment in the case of tumors of the nervous system. Interestingly, a recent study demonstrates a novel histone acetylation-independent mechanism by which HDAC inhibitors lower the apoptosis threshold for other therapeutic agents [77]. Chen and co-workers reported that HDAC inhibitors cause Akt (protein kinase B) dephosphorylation in U87MG glioblastoma and PC-3 prostate cancer cells by disrupting HDAC - protein phosphatase 1 (PP1) complexes. Akt dephosphorylation leads to inactivation of the enzyme and abolishes its antiapoptotic features [78]. A novel approach is the use of HDAC inhibitors to

enhance tumor radiosensitivity. Recent preclinical studies indicate that HDAC inhibitors can enhance both the in vitro and in vivo radiosensitivity of human tumor cell lines generated from a spectrum of solid tumors [79].

We could see a much stronger effect of MS-275 on neuronal outgrowth than we could observe with two other well studied HDIs, TSA and VPA (data not shown). Promotion of neurite outgrowth by VPA was reported by van Bergeijk in 2006 [80]. Interestingly, the histone deacetylase inhibitor TSA was reported to inhibit NGF induced neuronal outgrowth of the PC12 cell-line [53]. We could validate the properties of the general HDAC inhibitors, TSA and VPA, but we could observe a much stronger effect of MS-275. Possibly, varying effects of the HDIs TSA, VPA and MS-275 can be explained by the different specificity of those compounds for histone deacetylases (Figure 3). Considering the high specificity of MS-275 to inhibit HDAC1, our results suggest a central role for HDAC1 in neuronal development.

We intended to confirm the effect of the HDI MS-275 on neuronal differentiation on the level of gene transcription. Comparative analysis of genes induced by MS-275 could provide a working hypothesis for the role of HDAC1 in neuronal development. RT-PCR analysis of neuronal target genes demonstrated induction of c-ret, trkA, NF-medium and clathrin light chain b transcripts when cells were treated with NGF. We could observe an up to 500 fold increase in transcript abundance of c-ret mRNA, a receptor tyrosine kinase involved in neuronal development (Figure 10). The HDI MS-275 induced this transcript up to 20 times. Form the data we obtained from the neuronal outgrowth assay, we expected highest induction of this gene by combinatory treatment of the cells with the HDI and NGF than with one of the effector compounds alone. Interestingly, c-ret mRNA was moderately induced up to 80 times when cells were treated with NGF and MS-275, a much lower level as seen after treatment with NGF. Similar induction profile could be observed for trkA mRNA levels. The basis of this attenuating effect of MS-275 on NGF mediated induction of several neuronal target genes remains unclear. It can be speculated that perhaps strong induction of certain unknown regulators of neuronal development results in a negative feedback loop repressing transcription of other regulators. However, double treatment with NGF and the HDI resulted in highest expression of neurofilament medium mRNA, suggesting that NF-medium expression could be modulated by numerous signalling pathways involved in neuronal development [72]. Regulation of NF-medium by more than one pathway is plausible, considering that NF-medium is a

structural protein required for the formation and mechanical stabilization of the axonal and dendritic architecture of various neuronal cell types [81].

HDAC1 is known to be member of the REST complex, involved in repression of neuronal genes in non-neuronal tissues [46]. We did consider the possibility that the promoting effect of the HDI MS-275 was based on induction of REST controlled genes. Conversely, treatment with MS-275 did not induce expression of clathrin light chain b mRNA, a transcript known to be modulated by the REST complex.

Microarray approaches as done by Marek et al. [69] could provide more insight on the effects of MS-275 on gene expression. Furthermore it would allow identification of genes that are promoting neuronal differentiation and are upregulated after treatment with the HDI MS-275. As described above, HDAC1 functions as negative regulator of the CDK inhibitor p21 and elevated p21 levels were also observed *in vivo* in HDAC1 deficient mice. Ectopic expression of p21 in PC12 cells leads to growth arrest and does not directly lead to a differentiated phenotype [82]. Interestingly, Erhardt and co-workers report that expression of p21 greatly enhances the response of PC12 cells to NGF. Therefore, it is possible that augmentation of NGF induced neuronal outgrowth by MS-275 is influenced by a mechanism involving upregulation of p21.

5.3 Stable Overexpression of HDAC1-Myc in PC12 Cells

Results of the neuronal outgrowth assay suggest involvement of HDAC1 in neuronal differentiation of PC12 cells (Figure 8 and 9). Treatment with the HDI MS-275 leads to enhanced neurite outgrowth and induction of neuronal target genes. Therefore, we were interested in the consequences of HDAC1 overexpression on neuronal differentiation. Transfection of PC12 cells with the *pBABE-puro HDAC1-myc* plasmid resulted in the generation of the *PC12 HDAC1-myc mixed* cell-line, expressing the HDAC1-myc fusion protein in about half of the total number of cells.

We could identify a strong effect of HDAC1-myc overexpression on NGF induced neuronal outgrowth. Cell positive for the fusion protein did not express neurofilament medium protein; a structural protein expressed in neuron-like cells, and did not show visible signs for neuronal differentiation (Figure 18). This data suggest that HDAC1 overexpression in PC12 cells leads to a block in NGF induced neuronal differentiation. Simultaneous treatment of the *PC12 HDAC1-myc mixed* cell line with NGF and the HDI MS-275 abolished the block towards neuronal differentiation.

These finding indicate that the enzymatic deacetylase activity of HDAC1 is responsible for the differentiation block observed in HDAC1-myc expressing PC12 cells.

We isolated a pure, HDAC1-myc expressing PC12 cell-line out of the *PC12 HDAC1-myc mixed* clone and were interested in its capability to differentiate and neuronal target gene expression. Interestingly, cells differentiated normally and mRNA profiles (Figure 23) revealed solid induction of c-ret, trkA and neurofilament medium protein upon treatment with NGF. NF-medium was also induced by MS-275 alone. Once more, we could observe an attenuating effect of MS-275 on NGF mediated induction of c-ret and trkA. This date suggest that the block in neuronal differentiation observed in the *PC12 HDAC1-myc mixed* cell-line was lost in the *PC12 HDAC1-myc pure* cell-line.

In order to identify the cause of the differences in neuronal differentiation between the two cell-lines, we compared HDAC1 protein levels between PC12 wild-type cells and the *PC12 HDAC1-myc pure* cell line. The *PC12 HDAC1-myc pure* cell-line showed substantial downregulation of endogenous HDAC1 protein and the overall HDAC1 abundance in the HDAC1-myc expressing cell-line was similar to wild-type cells (Figure 24). Auto-regulation of endogenous HDAC1 expression via HDAC1 protein could provide a model for the comparable levels of total HDAC1 protein in PC12 wild-type cells and the *PC12 HDAC1-myc pure* cell-line [83]. Expression of exogenous HDAC1-myc protein possibly leads to a deacetylation of the promoter region of endogenous HDAC1 and thereby to a block in its expression. In addition, selection against cells expressing high, potential detrimental levels of exogenous HDAC1-myc protein can not be excluded. Transfection of PC12 cells with the pBABE-puro plasmid and subsequent selection for cells carrying puromycin resistance is a time-consuming process, requiring up to 4 weeks before the cells can be analysed. This provides a long enough time window for the selection against cells expressing high levels of HDAC1-myc protein and allows those cells in culture to be overgrown by other cells.

Multiple transfection attempts to generate additional pure HDAC1-myc expressing cell-lines did fail. Tight regulation of HDAC1 protein concentration seems to be crucial for the viability of the cell. Long-term treatment of PC12 cells with HDIs has toxic effects, resulting in cell death and the appearance of individual cells with multiple nuclei. It is known that signal pathways involved in NGF mediated neuronal

differentiation of PC12 cells also contribute to ensure cell survival [84]. Overexpression of HDAC1-myc protein potentially interferes in the signal cascades governing cell survival in addition to neuronal differentiation. In this context, it is also remarkable that the generated cell line constituted a mixed clone. PC12 cells cultured under serum-free, NGF-free medium conditions are known to induce neurite outgrowth by secretion of detectable NGF to the medium [85]. Autocrine regulation of neuronal differentiation by NGF could play an important role in cell survival. The population of HDAC1-myc negative cells in the *PC12 HDAC1-myc mixed* clone may contribute to ensure viability of the HDAC1-myc expressing fraction of cells.

Future investigations of the role of HDAC1 in neuronal development could be facilitated by using an inducible expression system, allowing more accurate analysis of neuronal differentiation and expression of neuronal target genes. Inducible vectors would eliminate effects of long-time overexpression of HDAC1 on the generated cell-lines, such as downregulation of endogenous HDAC1 protein or possible selection against cells expressing high levels of HDAC1-myc.

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7. Appendix

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