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Elucidation of the Signaling Pathways Regulating *Naip* Expression

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Elucidation of the Signaling Pathways Regulating *Naip* Expression

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ABSTRACT

The Neuronal Apoptosis Inhibitory Protein (NAIP) is neuroprotective in several disease and trauma models. Therapy based on NAIP's upregulation might be possible with a detailed understanding of its expression in neurons. Sodium butyrate (NaB), a *Naip* upregulating compound, was used to investigate its transcriptional regulation. Preliminary studies suggested that *Naip* upregulation was a result of NaB's G protein coupled receptor (GPCR) mediated signaling pathway activation. Kinase and receptor inhibition experiments in the present study revealed no specific pathway responsible and confirmed that NaB mediated *Naip* induction is GPCR-independent. This is consistent with NaB's histone deacetylase inhibitor (HDACi) capacity underlying *Naip* induction, and supported by induction of *Naip* using another potent HDACi, Trichostatin A. We demonstrate that *Naip* induction following HDACi treatment is concomitant with G_1/G_0 cell cycle arrest, p21 induction and E2f1 downregulation, suggesting a cell cycle associated regulation. Further examination revealed that p21 expression and cell cycle arrest are not required for HDACi mediated *NAIP* induction. Direct histone acetylation following HDACi treatment appears to be responsible for *Naip* induction.

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LIST OF ABBREVIATIONS

4-VO – 4 vessel occlusion

6-OHDA – 6-hydroxydopamine

ALS – amylotrophic lateral sclerosis

ANOVA – analysis of variance

BDNF – brain derived neurotrophic factor

BMP – bone morphogenic protein

BIR – baculoviral IAP repeat

Brn-2 – brain-2

CDK – cyclin dependent kinase

CDKI – cyclin dependent kinase inhibitor

ChIP – chromatin IP

CHO – chinese hamster ovary

cIAP – cellular IAP

CNS – central nervous system

CNTF – ciliary neurotrophic factor

CpGV – *Cydia pomonella* virus

DAG - diacylglycerol

DC – dendritic cells

DN – dominant negative

Fr-luc – firefly luciferase

GDNF – glial-cell derived neurotrophic factor

GPCR – G protein coupled receptor

G_qA - [D-Arg^{1},D-$Trp^{5,7,9}$,Leu^{11}] SubstanceP

hCG – human chorionic gonadotropin

HDAC – histone deacetylase

HDACi – histone deacetylase inhibitor

IAP – inhibitor of apoptosis

IKK – inhibitor of kappaB kinase

Inr – initiator element

IP – immunoprecepitation

KA – kainic acid

LPS – lipopolysaccharide

LRR – leucine rich repeat

MAPK – mitogen activated protein kinase

MOI – multiplicity of infection

mTOR – mammalian target of rapamycin

N2A – neuro-2a cells

NaB – sodium butyrate

NAIP – neuronal apoptosis inhibitory protein

NF-κB – nuclear factor kappaB

NIK – NF-κB inducing kinase

NGF – nerve growth factor

NOD – nucleotide-binding oligomerization domain

NT – neurotrophin

Oct – octamer binding transcription factor

OpMNPV – *Orgyia pseudotsugata* nuclear polyhedrosis virus

ORF – open reading frame

PAMP – pathogen associated molecular patterns

PARP – poly ADP-ribose polymerase

PAX – paired box factor

PCD – programmed cell death

PD – Parkinson's disease

PI – propidium iodide

PI3K – phoshpatidylinositol 3 – kinase

PKC – protein kinase C

PKG – protein kinase G

PLC – phospholipase C

PMSG – pregnant mare serum gonadotropin

POU – Pit/Oct/UNC

PTX – pertussis toxin

qRT-PCR – quantitative RT-PCR

RA – retinoic acid

Re-luc – renilla luciferase

RSK – ribosomal S6 kinase

RT-PCR – reverse transcription polymerase chain reaction

SCFA - short chain fatty acid

SD – standard deviation

SE – standard error

SEM – standard error of the mean

SMA – spinal muscular atrophy

SMN – survival of motor neurons

Sp1 – specificity protein 1

TBI – traumatic brain injury

TF – transcription factor

TFI – transient forebrain ischemia

TGF – transforming growth factor

TNF – tumor necrosis factor

TNFR – TNF receptor

Trk – tyrosine kinase receptor

TSA – trichostatin A

XIAP – X-linked IAP

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CHAPTER 1 - INTRODUCTION

Apoptosis

Apoptosis or programmed cell death (PCD) is a well characterized sequence of events in which a cell uses its own energy and machinery to die. In classical apoptosis the characteristic morphological and biochemical events occur due to the activation of cystein-dependent, aspartate-directed proteases called caspases [1]. This differs from necrosis, which is generally considered an uncontrolled process often resulting from physical trauma to the cells leading to rupture of the cell membrane and ultimately an inflammatory response [2].

Apoptosis is a tightly regulated process playing an important role in both the development and homeostatic processes of an organism. Furthermore, its dysregulation has been implicated in numerous disease pathologies. While this is true of most tissues it is acutely obvious in the case of neurons, where during development up to 50% of neurons fail to make functional connections and die by apoptosis [3]. Similarly, the cell death observed in Alzheimer's disease, Parkinson's disease (PD), Spinal Muscular Atrophy (SMA), Amyotrophic Lateral Sclerosis (ALS) and Central Nervous System (CNS) injury exhibit the hallmarks of apoptosis [1, 4, 5]. It is, therefore, critical to understand the proteins responsible for the process of apoptosis and its regulation as they represent attractive therapeutic targets for a multitude of conditions.

Inhibitors of Apoptosis (IAPs)

The first member of the IAP family was identified by researchers studying the baculoviral gene *p35* which is capable of preventing premature host cell death following infection. In an effort to identify genes homologous to *p35,* a genetic complementation assay

was developed whereby SF-21 cells were infected with a *p35* mutant virus along with DNA from other baculoviruses. The researchers identified an open reading frame (ORF) encoding a 31kDa protein from the *Cydia pomonella* virus (CpGV) capable of inhibiting apoptosis following infection with the *p35* mutant virus. This gene was named *iap* for inhibitor of apoptosis. Interestingly, this gene showed no significant homology to the *p35* gene. They also demonstrated that SF-21 cells expressing the CpGV *iap* gene were capable of inhibiting apoptosis induced by treatment with the RNA synthesis inhibitor actinomycin D [6].

The same laboratory later discovered an ORF in the *Orgyia pseudotsugata* nuclear polyhedrosis virus (Op*M*NPV) genome encoding a 30kDa protein capable of inhibiting apoptosis using the same genetic complementation assay as the previous study. This protein which they named Op-IAP shares 58% amino acid sequence homology with Cp-IAP. Both genes contain a C3HC4 finger at their carboxy terminus along with two tandem repeats near the N-terminus. These repeats have been given the name BIR for Baculoviral *Iap* Repeat [7].

The neuronal apoptosis inhibitory protein (NAIP) gene was the first member of the mammalian IAP family identified in 1995 as a candidate gene for SMA, an autosomal recessive genetic neurodegenerative disease caused by the loss of motor neurons in the spinal cord and resulting in voluntary muscle weakness and wasting [8, 9]. The human IAP family has since expanded to include cellular IAP1 (c-IAP1), c-IAP2, X-linked IAP (XIAP), Survivin, Apollon, Livin and Ts-IAP [10-15]. BIR containing proteins have been subdivided into two groups; IAPs containing multiple BIR domains which have anti-apoptotic properties and IAPs with a single BIR domain which play a role in cytokinesis and chromatin segregation [16, 17].

NAIP

The *NAIP* gene maps to 5q13.1, spans 50 kb of genomic DNA encoding 17 exons which leads to the production of a 6.1kb transcript and ultimately a 1403 amino acid, 156kDa protein [9]. The NAIP protein consists of 3 BIR domains, a nucleotide-binding oligomerization domain (NOD), and a leucine rich repeat (LRR). $NAIP_S$ a shorter version of NAIP which encodes a 1295 amino acid protein was identified upon the examination of a fetal brain cDNA library [18]. A splice variant lacking exon 10 and 11 (*NAIP-ΔEx10/11*) which encode the end of the third BIR domain and the COOH terminal tail of NAIP has been identified in a number of cell types [19]. There is also a report of a *ΨNAIP* transcript, an internally deleted *NAIP* lacking the first two BIR domains; however, identification of the protein corresponding to this transcript has remained elusive [20].

Murine Naip[1]

Researchers looking to study the SMA locus without the duplication and inversion seen in humans mapped the homologous locus in mice to chromosome 13. This region in mice contains *Lgn1*, a locus involved in the regulation of resistance to *Legionella pneumophila* infection [21, 22]. The examination of this chromosomal locus led to the identification of multiple copies of the *Naip* gene, with the number varying between mouse strains. The 129 mouse lineage has seven intact *Naip* genes and 3 5' truncated *Naip* pseudo-genes, while the C57Bl6 and A/J mouse lineages have five intact *Naip* genes and a single 5' truncated *Naip* pseudo-gene [23-25]. The *Naip1*cDNA has 77% nucleotide and 68% amino acid homology when compared to human *NAIP* [23]. While it remains unclear why multiple functional copies of the gene have been maintained in the mouse genome, an examination of

[1] *Naip* refers to the mouse gene whereas *NAIP* refers to the human gene.

their mRNA expression in a panel of tissues has revealed a differing pattern of expression for each isoform suggesting tissue specific roles *in vivo* (Z. Yaraghi, N.H. Gendron and A.E. MacKenzie, unpublished observation).

NAIP Localization

Subcellular localization studies have revealed that NAIP localizes to the cytoplasm in all of the cultured and primary cell types which have been examined, with staining extending into the dendritic processes in the case of neuronal cell types [26, 27].

With respect to tissue specificity, *in situ* hybridization in the developing mouse embryo (E9.5-E16.5) revealed *Naip* mRNA expression primarily in the brain and spinal cord; however, at latter stages of development it is also strongly expressed in the intestinal villi [28]. In adult mice *in situ* hybridization has identified *Naip* mRNA expression in the granulosa cells of ovarian follicles and northern blotting has identified *Naip* mRNA expression in testis, ovary, kidney, liver, lung, intestine, spleen, brain, and heart [23, 29, 30]. Western blotting with a polyclonal anti-mouse Naip antiserum has confirmed Naip expression in the intestine, colon, spleen and macrophages [30]. NAIP-like immunoreactivity has also been studied in rat CNS using a polyclonal human NAIP antibody, revealing low levels of expression in the cortex and hippocampus and high levels in subcortical regions [31].

In humans *NAIP* mRNA expression has been observed in adult spleen, thymus, liver, lung, leukocytes and placenta along with fetal brain, lung, liver and kidney by Northern blotting and in brain, heart, kidney, lung, liver, trachea, colon, small intestine, spleen, stomach, prostate, testis, uterus, blood, spinal cord, fibroblasts and lymphoblasts by RT-PCR [8, 18, 32]. Western blotting in human tissues with a highly specific polyclonal human

NAIP antibody has detected NAIP in brain, spleen, kidney, testis, lung, ovary, intestine and placenta [27, 33]. Interestingly, immunohistochemical studies using the same antibody indicate that the NAIP seen in lung and spleen is not in the tissue itself but is rather in infiltrating macrophages [33]. Immunohistochemical studies using a different human NAIP antibody have also identified protein expression in motor neurons of the spinal cord, pyramidal cells of the motor cortex and the choroid plexus [26].

NAIP Function and Role

i. *In Vitro*

In vitro studies have played a fundamental role in the investigation and establishment of NAIP's antiapoptotic properties and the mechanism by which this can occur. Studies in transformed cells and primary cell culture have shown NAIP to be cytoprotective against a variety of apoptotic stimuli including: serum deprivation, potassium withdrawal, menadione and tumor necrosis factor (TNF) treatment[11, 34].

To demonstrate the antiapoptotic properties of NAIP our laboratory generated plasmids and adenoviruses expressing *NAIP* and antisense *NAIP*. These constructs correspond to what was originally believed to be full length *NAIP* but are now known to lack exon 14 and 17 and are referred to as *NAIP-ΔEx14/17*. Chinese hamster ovary (CHO) cells expressing *NAIP-ΔEx14/17* following adenoviral infection or stable transfection displayed a significantly increased resistance to cell death induced by serum deprivation or menadione treatment. Similarly, Rat-1 fibroblast and HeLa cells expressing *NAIP-ΔEx14/17* following adenoviral infection or stable transfection displayed a significantly increased resistance to cell death following menadione and TNF-α treatment respectively [11]. These experiments

demonstrated the suppression of cell death by NAIP, supporting its classification as an antiapoptotic protein.

The antiapoptotic effect of NAIP was also confirmed in primary cell culture. *Adeno-NAIP-ΔEx14/17* infection was used by another laboratory to demonstrate a significant delay in cell death following potassium withdrawal in cultured rat cerebellar granule neurons, a similar delay was seen with *adeno- XIAP*, c*IAP1* and *cIAP2* [34]. At the time it was known that the baculoviral IAPs, XIAP, cIAP1 and cIAP2 were able to exert their antiapoptotic effect by directly inhibiting caspases [35-37]. This study was the first to demonstrate the ability of NAIP to prevent DEVD (the substrate of caspases 3 like caspases) cleavage and activation of caspase 3 following apoptotic stimuli [34].

To better understand the mechanism of NAIP's antiapoptotic function, our laboratory first confirmed that NAIP protected against cell death in a caspase dependent manner and that NAIP's BIR domains alone, the critical antiapoptotic domains, were sufficient to inhibit cell death. To test the ability of NAIP to inhibit caspases, purified recombinant GST-NAIP BIR domain fusion proteins were generated. Using these recombinant proteins in fluorogenic caspase activity assays it was shown that BIR123, BIR2 alone and to a lesser extent BIR 3 alone were sufficient to inhibit the group II effector caspases, caspases-3 and -7. The ability of the BIR domain deletion constructs to protect against etoposide induced cell death when transfected into HeLa cells was confirmed, although interestingly BIR3 alone inhibited cell death more effectively than BIR2 alone in this assay [38]. This supports the model that NAIP and particularly BIR2 and 3 play a role in apoptotic regulation by inhibiting effector caspase activity.

Subsequent to this study, our laboratory investigated the ability of NAIP to inhibit caspase-9, an effector caspase which had previously been shown to be inhibited by the BIR3

domain of XIAP, cIAP1 and cIAP2, and the potential effects this interaction may exert on Smac, a protein capable of binding to caspase-9 displacing IAPs and allowing unrestricted caspase activity [39-42]. NAIP is unique among the IAPs, it is the only family member to posess NOD and LRR domains. NOD containing proteins are a diverse group with roles ranging from apoptosis to pathogen recognition; this study also attempted to address the role of NAIP's NOD and LRR domains with respect to the BIR domains. Once again purified epitope tagged recombinant NAIP fusion proteins were used. Using an *in vitro* study where their interaction with purified active caspase 9 was assessed, it was determined that the interaction of full length NAIP requires ATP. It was also determined that the requirement for ATP is likely structural in nature where it displaces the LRR domain which is proposed to sequester the BIR domains. This is suggested by the fact that in a constitutively active NOD domain mutant, the LRR deletion mutants are able to interact with caspase 9 in the absence of ATP. A fluorogenic caspase 9 activity assay confirmed that NAIP-BIR3 inhibited caspase 9; however, this could not be determined for full length NAIP due to degradation of the protein in the expression system of choice. Finally, immunoprecipitation studies of HeLa cells cotransfected with myc-*NAIP* and HA-*Smac* constructs revealed that there is no interaction between NAIP and Smac [43]. These experiments serve to further elucidate the mechanism of NAIP action and particularly BIR3s antiapoptotic function while also suggesting a role for its NOD and LRR domains.

A yeast two hybrid screen in which NAIP's BIR domains were the bait revealed that NAIP's most potent interactor is a member of the neuron specific family of calcium binding proteins, hippocalcin [44]. This interaction was confirmed *in vitro* with both the NAIP-BIR domains and full length NAIP in Co-IP experiments. Ionomycin and thapsigargin induce cell death in a calcium dependent manner. NSC-34 cells, a spinal cord motor neuron like

cell line, stably overexpressing NAIP-BIR123 display an increased resistance to ionomycin and thapsigargin. However, cells stably co-expressing NAIP-BIR123 and hippocalcin were significantly more resistant. Interestingly, studying the intracellular calcium levels before and after ionomycin or thapsigargin treatment reveals no significant changes in the presence of NAIP, which suggests that its protective effects are not directly related to changes in calcium concentration. Using deletion constructs of individual BIR domains in the yeast two-hybrid system and later Co-IP studies it was determined that BIR3 alone is responsible for binding hippocalcin. NAIP-BIR3 alone is insufficient to protect cells against thapsigargin induced cell death but when co-expressed with hippocalcin it is as effective as all 3 BIR domains combined with hippocalcin. To elucidate the apoptotic cascade through which thapsigargin is inducing cell death, caspase 3/7 activity was examined in NSC-34 and Neuro2A (N2A) cells, a mouse neuroblastoma. The NAIP-BIR domains alone inhibited caspase activation in the NSC-34 cell line and the inhibition was more potent when NAIP was combined with hippocalcin. However in the N2A cell line there was no caspase 3/7 activation and increased cell death was observed which could not be rescued using a caspase inhibitor [44]. These results suggest that thapsigargin induces cell death through both caspase dependent and independent mechanisms. This study was the first to demonstrate NAIP's interaction with hippocalcin and its ability to enhance its efficacy to protect against calcium-induced cell death.

Hippocalcin is a member of the visinin-like protein family of neuronal calcium sensor genes which have many functions in nerve cell physiology. Hippocalcin is highly expressed in the CNS, particularly the hippocampus and pyramidal neurons, but has no ascribed function. The Lindholm laboratory went on to further characterize the NAIP-hippocalcin interaction, determining that the interaction is dependent on D283 and V296 in BIR3 which

are unique to NAIP when compared with other IAPs. Subcellular localization studies using NAIP and hippocalcin fused to fluorescent proteins led to the identification of a relocalization of the two proteins; from the cytoplasm when expressed alone, to the plasma membrane when expressed together. In an effort to examine the role of the interaction in a pure neuronal system they assessed cell survival following NGF withdrawal in mouse sympathetic neurons injected with hippocalcin and NAIP expression vectors, there was a small rescue effect observed with hippocalcin alone but not NAIP alone or the two vectors together [45]. While this was unexpected, it suggests the beneficial effects of the NAIP-hippocalcin interaction are specific to calcium-induced cell death.

ii. *In Vivo*

Proof of NAIP's antiapoptotic and cytoprotective properties is not limited to *in vitro* studies, there have been a number of reports of its efficacy *in vivo*. *In vivo* studies have revealed that the loss of expression of a single murine Naip isoform significantly increases the susceptibility of the neurons to excitotoxic cell death. Increased endogenous expression of Naip has been reported in mouse models of ischemia and brain injury. Additionally, exogenous overexpression of NAIP in these models of ischemia and injury along with models of axotomy and Parkinson's disease has been reported to increase neuronal survival.

Transient forebrain ischemia (TFI) is known to cause damage and apoptotic cell death with the neurons in the neocortex, CA1 region of the hippocampus and the reticular thalamus being the most vulnerable [46]. Researchers studying endogenous NAIP levels following four-vessel occlusion (4-VO), a model of ischemia, observed a marked induction of NAIP in neuronal populations resistant to the ischemic injury and a significant decrease in those which are susceptible. The decrease in NAIP levels in the affected populations is

consistent with prior reports of depressed protein synthesis following transient ischemia and suggests that the susceptibility of these neurons to injury may be due, at least in part, to decreased NAIP levels [47]. To determine if increased NAIP levels would reduce the severity of the ischemic insult, the researchers employed two methods to increase *NAIP* expression: intra-hippocampal injection of adenovirus expressing *NAIP-ΔEx14/17*, and treatment with K252a, a neuroprotective compound which elevates NAIP levels through an unknown mechanism. Induction of NAIP using both methods was shown to significantly reduce neuronal cell loss and specifically apoptotic cell death in the CA1 region of the hippocampus [48]. This report confirms that endogenous NAIP expression plays a role in response to cellular stress and is consistent with the observation in *in vitro* studies that it is neuroprotective.

Changes in endogenous NAIP levels have also been observed following traumatic brain injury (TBI). It was reported by Hutchison *et al* that cell death following TBI reach maximal levels at 24 hours and displays classical characteristics of apoptotic cell death in a mouse model of TBI. They also observed increased Naip protein levels 6 hours post-TBI which decreased by 24 hours. These changes in Naip expression mirrored decreased procaspase-3 levels and increased cleavage of poly ADP-ribose polymerase (PARP), a caspase 3 substrate [49]. In a later study, transgenic mice that ubiquitously overexpress human *NAIP* were used to assess the neuroprotective effects of NAIP following TBI. The severity of the TBI in tg-NAIP mice, as measured by the amount of inflammation observed 24 hours post-injury, was no different from that of controls; however, there was significantly less apoptosis seen in both the white matter and the cortex of the transgenic mice. The decreased apoptosis observed in the transgenic mice was also correlated with a decrease in

caspase 3 activity, which is suggestive of a causal explanation of the decrease in apoptosis observed (J.S. Hutchison, unpublished observation).

Caspase 3 dependent apoptotic cell death has also been observed following axotomy [50]. Perrelet *et al* demonstrated that motor neuron loss following sciatic nerve axotomy in rats was significantly reduced when the proximal nerve stump was capped with a capsule containing adenovirus expressing *NAIP- ΔEx14/17*, *cIAP1* or *cIAP2* up until 4 weeks post-axotomy [51]. In this study the neuronal rescue by *IAP* expression was comparable to that of ciliary neurotrophic factor (CNTF) and brain-derived neurotrophic factor (BDNF), two proteins well established as motor neuron survival factors post-axotomy, but adeno-*BDNF* and *CNTF* infection did not induce *IAP* expression [51-53]. A latter study by the same researchers examined the neuroprotective effects of adenoviral *XIAP* expression in the same model of axotomy. They once again compared the protective effects of the IAPs to that of neurotrophic factors: CNTF, BDNF and glial cell-derived neurotrophic factor (GDNF). This study revealed that XIAP is more effective than the other IAPs at preventing cell death in this model, its protective effects still being observed at 5 weeks post-axotomy. More interesting, however, was the observation that in contrast to CNTF and BDNF, GDNF is able to induce *XIAP* and *NAIP* expression. Combined infection of adeno-*GDNF* and adeno-*antisense NAIP* or *XIAP* resulted in loss of GDNF mediated neuronal survival [54]. These studies indicate that XIAP and NAIP play a critical role in the GDNF, but not the CNTF and BDNF mediated intracellular pathway for motor neuron survival; however, the mechanism through which this is mediated remains elusive.

Parkinson's disease (PD) is a chronic neurodegenerative disorder characterized by the loss of the pigmented dopaminergic neurons of the substantia nigra [55]. Evidence points to the aberrant generation of free radicals as the cause of neuronal death in PD. NAIP's

efficacy to reduce cell death in response to free radicals has been demonstrated *in vitro* and leads to the hypothesis that NAIP may be able to rescue cell death in PD [11]. In this study rats received an intrastriatal injection of *adeno-NAIP-ΔEx14/17* and an injection of 6-hydroxydopamine (6-OHDA) to induce a progressive degeneration of nigral dopamine neurons, a commonly used model of PD [56]. Rats who received the *adeno-NAIP-ΔEx14/17* had a 38% increase in neuronal survival and significantly decreased amphetamine-induced rotational behavior when compared with control animals [57]. This study provides *in vivo* demonstration of NAIP's ability to reduce free radical induced cell death and demonstrates that its neuronal rescue translates into functional rescue.

Having shown that increased NAIP levels are neuroprotective a knockout (KO) mouse model was developed to determine the role of endogenous NAIP expression. The *Naip1* gene is expressed predominantly in the brain and as such was selected as the target for knockdown to assess NAIP's neuroprotective properties [23]. A 1.8kb fragment of the *Naip1* gene containing BIR1 and 2, the domains critical for IAP function, was deleted using a replacement targeting vector. The *Naip1-/-* mice showed normal sex ratios, litter sizes, growth, morphology, histology, behavior, and learning. To determine if Naip1 plays a role in neuronal survival under pathological conditions a seizure model was used. Kainic acid (KA) administration in rodents results in seizure activity and widespread excitotoxic neuronal damage [58]. Excitotoxic cell death is distinct from apoptosis; the two do however, share certain features including caspase 3 activation. This suggested that an antiapoptotic protein capable of inhibiting caspase activation such as NAIP may attenuate the neuronal loss following KA treatment. Wildtype and *Naip1 -/-* mice treated who received a single KA injection had seizures of comparable intensity however there was a 2.5 fold increase in the

cell death observed in the CA3 neurons of the *Naip1* KO mice [59]. This study confirmed that endogenous NAIP expression is critical for neuronal survival.

iii. Differentiation

Thus far the majority of the evidence for NAIP's role has been in the CNS; however, as previously discussed NAIP is expressed in a wide variety of tissues. While tissue-specific expression of Naip isoforms has been observed in mice, its role in non-neuronal cells is unknown. One suggestion stems from a body of evidence pertaining to NAIP expression in differentiating cells. Altered NAIP expression has been reported in oocyte, intestinal epithelial cell, adipocyte, neuronal, and dendritic cell maturation.

Mammalian females are born with a finite number of germ cells. A small number of them reach the ovulatory stage while the rest are lost via atresia of the ovarian follicles, a process which is mediated by apoptosis. *Naip* expression in the mouse ovary has been observed with maximal expression levels seen in the granulosa cells of primary follicles of sexually mature mice [29]. Changes seen in the granulosa cell are significant as they are key players in the development of the oocytes, providing a nutritive environment. An examination of sequential histological sections of mouse ovary either by TUNEL assay to identify apoptotic cells, or *in situ* hybridization for *Naip* mRNA expression, revealed weak or no expression in atretic follicles and high *Naip* expression in the granulosa cells of the majority of non-apoptotic follicles. Gonadotropic hormones can regulate the incidence of atretic follicles; *Naip* mRNA expression in mice who received human chorionic gonadotropin (hCG) increased 1.6 fold and whereas mice that received pregnant mare serum gonadotropin (PMSG) in addition to the hCG showed a 2.4 fold increase. Finally the effect of decreased *Naip* expression was studied by administering intrabursal injections of *Naip*

antisense oligonucleotides or the appropriate controls. Decreased *Naip* expression did not affect the total number of ovulated oocytes but decreased the number which were morphologically normal by 33% [29]. This study suggest that Naip's antiapoptotic role is critical for the survival of ovarian follicles during folliculogenesis and that it is hormonally regulated although no mechanism has been demonstrated.

NAIP expression at both the transcript and protein level have been observed in the intestine [18, 30, 33]. The intestinal epithelium undergoes continuous, rapid proliferation of cells with immature precursor cells differentiating as they travel from the crypts to the tips of the intestinal villi, complete turnover of these cells occurs every 3 to 5 days. Immunohistochemical studies of NAIP expression in intestine reveal selective expression in the upper crypt and the surface layers of epithelial cells connecting the crypts. The pattern of NAIP expression co-localizes with that of p21, an important cell cycle regulator required for growth inhibition, cell cycle withdrawal and terminal differentiation [33, 60]. The NAIP - p21 link suggests that NAIP may play a role in differentiation and/or be induced directly or indirectly by p21 mediated differentiation.

The 3T3-L1 cell line can be induced to differentiate from preadipocytes to adipocytes with growth factor withdrawal. Interestingly, these cells have been shown to develop increased apoptotic resistance during differentiation [61]. In an effort to identify the mechanism responsible for the increased apoptotic resistance, antiapoptotic proteins were considered. In 3T3-L1, 3T3-F442A, and 3T3-C2 preadipocytes NAIP protein is barely detectable however after 8 days of serum starvation, which results in differentiation of approximately 90% of the cells, a greater than 20 fold increase in NAIP level was observed in the 3T3–L1 and –F442A cells, but not the –C2's which are resistant to differentiation. These changes in NAIP level were in contrast to cIAP-1 levels which did not change. Over

the 8 day course of differentiation, an increase in NAIP is seen as early as day 2 with maximal levels attained by day 4 and is maintained for the rest of the time course. This induction was blocked in the presence of rapamycin. Rapamycin is a compound known to inhibit adipocyte differentiation by blocking the induction of C/EBPα, a critical adipogenic transcription factor, suggesting that NAIP may also be under its control [62]. Western blotting for NAIP in rat white adipocyte lysates confirmed that NAIP expression is not unique to immortalized cell lines [63]. These results suggest that NAIP induction may play a critical anti-apoptotic role over the course of adipocyte differentiation although knockdown studies are required for confirmation. The same laboratory later examined preadipoctye differentiation in human primary cells; however, they failed to observe the characteristic increase in apoptotic resistance and saw a decrease in NAIP level [64]. These results call into question the link between NAIP and differentiation seen in the immortalized 3T3-L1 cell line and serves as an important reminder of the potential limitations of using such models.

PC12 cells are a well established neuron-like rat cell line used for the investigation of neurotrophic factor mediated signaling pathways in which neurite extension is observed within 24 hours of nerve growth factor (NGF) treatment [65, 66]. PC12 cell lines overexpressing both full length *Naip2* and *BIR-deleted-Naip2* were established to assess Naip's role in NGF mediated signaling. NGF treated cells expressing *Naip2* or *BIR-Δ-Naip2* showed severe impairment of neurite extension which could be rescued using other differentiation triggers or the expression of the activated Cot kinase, a MEK activator [67]. The Ras/Raf/MAPK pathways are required for the action of NGF with ERK, a member of the MAPK family, phosphorylation being a well characterized downstream event of NGF treatment. *Naip2* expression did not alter the ERK phosphorylation levels, indicating that its inhibitory effects are independent of these pathways [68]. In normal PC12 cells, apoptosis is

suppressed when serum is withdrawn with NGF treatment [69]. An increase in cell survival and decreased caspase 3 activation following NGF-withdrawal and TNF-α treatment was observed in the *Naip2* expressing cells but not in those expressing *BIR-Δ-Naip2* [67]. These experiments suggest that Naip plays an inhibitory role in NGF mediated neurite outgrowth and differentiation, a role that is distinct from its antiapoptotic role as it occurs independently of its BIR domains.

Dendritic cells (DCs) are antigen presenting cells important for infection and immunity. Primary immature DCs (iDCs) harvested from peripheral blood monocytes were induced to differentiate into mature DCs (mDCs) with lipopolysaccharide (LPS) and TNF-α [70]. Researchers examining DC gene expression over a time course of LPS treatment using RT-PCR differential display identified 7 genes with differential time dependent expression, one of which was *NAIP*. RT-PCR with sequence specific primers confirmed that *NAIP* was the only member of the IAP family highly expressed in iDCs and that it is downregulated over the time course of LPS or TNF-α treatment, suggesting a control mechanism distinct from other members of the family. However, a similar decrease in expression was seen following transforming growth factor–β (TGF-β) treatment, which induces differentiation into Langerhan cells and inhibits DC maturation as well as in control untreated DCs. This indicates that the changes in *NAIP* expression observed are likely independent of the differentiation process and is rather an artifact related to the harvesting procedure [71]. There is, however, evidence in other immune cell types of IAP downregulation with differentiation. Retinoic acid (RA) is commonly used to differentiate the human promyelocytic leukemia cell line HL-60 into neutrophil-like cells. Once differentiated these cells die by apoptosis [72]. Northern and western blots from HL-60 cells over the course of RA treatment reveal a time dependent decrease in XIAP, cIAP-1, cIAP-2, and NAIP levels

which is not caspase mediated although their cleavage is [73]. It must, however be considered that the differentiation and apoptotic processes are separate mechanisms; therefore, the change in IAP levels may be related to either process.

Clearly there is evidence for both increases and decreases in NAIP expression over the course of differentiation. These differing reports may be related to cell type specific properties of NAIP or may be artifacts of the model systems used in these studies. However, both increased and decreased NAIP expression upon differentiation is plausible. Increased NAIP expression has been postulated to occur for the purpose of preventing apoptosis during differentiation as these cells are more susceptible to cell death as a result of high levels of metabolic stress [63]. There is, however a growing body of evidence supporting the involvement of activated caspases in differentiation, considering the caspase inhibitory action of NAIP and other IAPs BIR domains, it is proposed that they are downregulated during differentiation to allow the necessary caspase activation to occur [74]. While continued study is required to fully elucidate its role in differentiation and the regulation of its expression under these conditions a link between NAIP and differentiation has clearly been demonstrated.

iv. Innate Immunity

Studies in mice have also revealed a role for Naip in the innate immune system. *Lgn1* is a mouse locus involved in the resistance to *Legionella pneumophila.* This locus was contained in the region encoding the murine SMA locus which encodes multiple copies of the *Naip* gene [21]. *L.pneumophilla* infection requires phagocytic uptake by macrophages. To prevent contact with the early endosomal compartment *L.pneumophilla* replicates within a vacuole eventually resulting in the lysis of the cell and the initiation of a new infectious

cycle (reviewed in [75]). *Legionella* can replicate in macrophages from the majority of mammalian sources [76]. It was noted that inbred mouse strains, with the exception of the A/J strain, are permissive to infection but resistant to its replication [77, 78]. The A/J strain has only the *Naip1,2,3,5,6,* and pseudo-*Naip3* genes, refinement of the *Lgn1* locus lead to the discovery that *Naip5* is associated with *L.pneumophilla* resistance [79, 80].

While NAIP's NOD and LRR domains are unique amongst IAPs, they are common to a family of proteins known as NOD-LRR proteins (also known as NACTH and CATERPILLAR). These proteins are characterized by the presence of LRRs, which recognize various pathogen associated molecular patterns (PAMPs), NOD domains and often possess additional effector domains such as BIR, CARD or PYRN/PAAD domains. At present it is believed that the NATCH proteins induce inflammation through two pathways: activation of NF-κB and regulation of caspase-1 activation (reviewed in [81]). These observations have led to the hypothesis that Naip and its LRR domain in particular acts as an intracellular sensor for PAMPs and plays a role in the regulation of caspase 1 activation and therefore inflammation. Subsequent studies have confirmed this hypothesis using *Naip 5* deletion constructs to demonstrate that the LRR domain is required for *L.pneumophilla* infection recognition and caspase 1 dependent cell death, while the NOD, BIR1 and 2 domains are responsible for the regulation of caspase 1 activation [82]. At present, the possibility that Naip plays a role in apoptosis in the latter stages of infection has not been precluded and should be considered. However, a clear role for Naip in *Legionella* recognition has been demonstrated and further study should be undertaken to determine if it plays a role in the recognition of other pathogens and the innate immune system as a whole.

Regulation of NAIP Expression

Despite a solid body of work on the function of NAIP there is relatively little known about the regulation of its expression. Xu *et al.* studied the *NAIP* promoter to identify transcriptional regulatory elements. It is however, important to note that their findings are relative to a novel transcriptional initiation site they identified using RT-PCR screening which we and other laboratories have been unable to replicate. Using *NAIP* 5' deletion constructs cloned into a luciferase reporter vector, a 350bp region critical for *NAIP* transcription between -338 and +12 was identified with a core element located between -44 and +12. This critical region contains a GC box and within the core element there are putative binding sites for Brain-2 (Brn-2) and TATA box binding proteins, as well as initiator elements (Inr) and octamer-binding transcription factor 2 (Oct-2). The proximity of the Inr binding site to the TATA box suggests that an initiator binding protein may be the rate limiting step in transcription of *NAIP*, and that in addition to the basic transcriptional factors (TFs) which associate with the TATA box, *NAIP* may be regulated by CNS-specific POU-family TFs (**P**it/**O**ct/**U**nc transcription factors) such as Brn-2 and Oct-1 which are involved in the differentiation and development of neurons and the expression of neuronal specific genes [20, 83-85]. Two silencers for *NAIP* expression were also identified in this study in the -1791/-339 and +8/+226 regions, although the particular elements which are responsible for the silencing and if they are specific for *NAIP* remains to be determined [20].

Paired box factor 2 (PAX2) is a member of the paired-box family of transcription factors. It plays a critical role in determining nephron number in the kidney and is anti-apoptotic, suppressing cell death during branching morphogenesis in the kidney [86-88]. Researchers examining the mechanism by which PAX2 suppresses cell death identified that a human embryonic kidney cell line, HEK293, stably expressing PAX2 selectively

upregulated *NAIP* mRNA levels and that Pax2 heterozygous mice showed a downregulation of *Naip1* mRNA in the kidney. A PAX2 recognition motif was identified 140bp upstream of the transcription initiation site in the human *NAIP* promoter. To confirm *NAIP*'s PAX2 responsiveness a luciferase reporter construct under the control of a 780bp fragment of the 5' *NAIP* sequence was transfected into NIH3T3 cells, a 7 fold increase in luciferase activity was observed in the presence of a PAX2 expression vector; direct PAX2 binding to the *NAIP* promoter was also confirmed using EMSA. This is an interesting observation as PAX2 regulation is specific to *NAIP* amongst IAPs and PAX2 is not only expressed in the kidney but also the midbrain-hindbrain, the eye and the ear. This suggests that *NAIP* may also be under its selective regulation in those tissues [86].

Apart from these two studies, the majority of the information we have regarding the regulation of *NAIP* expression are suggestions stemming from NAIP function and drug studies. Neurotrophins (NTs) are proteins which antagonize cell death and attenuate neuronal death *in vitro* by various triggers, including the Aβ-induced apoptotic death characteristic of Alzheimer's disease [66]. NTs act on tyrosine kinase receptors (Trk), resulting in the auto-phosphorylation of tyrosine residues in their cytoplasmic tail which becomes a docking site for kinases such as PI3K [89, 90]. Researchers examining the mechanism by which NT-3 protects against Aβ-induced cell death observed a 2-2.5 fold increase in NAIP protein with NT-3 treatment which was inhibited in the presence of LY294002, a PI3K inhibitor. This study was also the first to demonstrate an interaction between NAIP and SMAC using immunoprecipitation studies. The interaction was observed following Aβ treatment but not in control or Aβ and NT-3 co-treated cells; the interaction was; however, restored upon co-incubation with LY294002 [91]. This suggests that NT-3 is able to protect against Aβ-induced cell death by both upregulating NAIP levels and

preventing its interaction with its inhibitor SMAC in a PI3K dependent manner. There are however, some technical concerns calling into question the validity of the results in this paper. The authors refer to the NAIP protein being examined in the mouse primary cortical neurons as being NAIP1, there is however very high homology between the murine Naip isoforms making it highly unlikely that this antibody is detecting a single isoform. Additionally the western blotting band which they indicate is full length NAIP1 is labeled as being approximately 120kD, full length NAIP protein is however, 156kD. The majority of NAIP antibodies detect a number of NAIP cleavage products, the major species of which is approximately 100kD, suggesting that they are not actually looking at changes in full length NAIP protein but rather a cleavage product.

TNFα is a potent inflammatory cytokine which induced two types of signals within a cell: one signal induces cell death while the other activates NF-κB and mediates an inflammatory response [92, 93]. TNF mediated neuroprotection appears to occur through the p55/TNF receptor type 1 (TNFRI) by activating NF-κB [94, 95]. A rapid increase in TNF production is characteristic of a number of cell death models in which NAIP has been shown to be neuroprotective, including: TBI, excitotoxins and ischemia [96, 97]. XIAP, cIAP1 and cIAP2 have all been shown to be induced in an NF-κB dependent manner following TNFα treatment [98]. This suggests that NAIP induction may also be NF-κB mediated, a hypothesis which is supported by the elimination of Naip induction following KA treatment in TNFRI/II knockout mice [99].

Evidence from research in cancer therapeutics also suggests the involvement of NF-κB in NAIP's regulation. Currently, many anti-cancer therapies are focused on the induction of apoptosis as a means of eliminating their target cells. One of the major obstacles with this approach is multidrug resistance, which is conferred by the overexpression of multidrug

efflux pumps such as P-glycoprotein [100, 101]. Adenosine and its analogs can act through their receptors to inhibit cell proliferation and trigger death [102]. Researchers comparing the effect of an adenosine A_3 receptor antagonist, IB-MECA (1-deoxy-1-[1[[(3-iodophenyl)methyl]amino]-9H-purine-9-yl]-N-methyl-β-D-ribofuranuronamide) on the acute myeloid leukemia cell line HL-60 and its multidrug resistant form, HL-60R, observed a weaker cytotoxic effect of IB-MECA on the multidrug resistant cells. HL-60R cells are known to express higher levels of IAPs, including NAIP. This suggests that the increased IAP levels may contribute to their IB-MECA resistance; this was confirmed by the observation that IB-MECA down regulates *NAIP*, *cIAP2* and *survivin* mRNA expression in HL-60 but not HL-60R cells. HL-60 cells are known to constitutively express activated NF-κB, a transcription factor which can induce *XIAP*, *cIAP1* and *cIAP2* transcription [103, 104]. Since IB-MECA has been shown to downregulate NF-κB in a prostate carcinoma cell line the researchers speculate that a downregulation of NF-κB may be responsible for the downregulation of *NAIP*, *cIAP2* and *survivin* in the HL-60 cells although this hypothesis remains untested [105]. The same laboratory later examined the antitumor effects of a novel NF-κB inhibitor dehydroxymethylepoxyquinomicin (DHMEQ), which inhibits the nuclear translocation of NF-κB in hepatocellular carcinoma (HCC), a common and aggressive tumour type. They demonstrated a significant decrease in *cIAP1* and *NAIP* mRNA levels following DHMEQ or cisplatin treatments an effect which was additive when the two treatments were combined. These results are consistent with their hypothesis from the IB-MECA work. They do however see an increase in *XIAP* mRNA under the same condition which is counterintuitive given the clear role for NF-κB in its regulation [106].

It has been previously shown using selective antagonists that JNK1, a member of the mitogen activated protein kinase (MAPK) family, is required for the antiapoptotic activity of

XIAP but not cIAP1 or cIAP2, which led researchers to examine its requirement for other IAPs [107]. *In vitro* studies revealed that the expression of a NAIP-BIR123 construct leads to a significant activation of JNK1 and to a lesser extent JNK2; but not activation of JNK3, p38 or ERK2. They also showed that JNK1 activation was required for NAIP mediated protection against TNFα and interleukin converting enzyme (ICE, caspase1) induced cell death. Activation of JNK1 was independent of its classical activators MKK4 and 7 and rather occurred through TAK1, an upstream MAP3 kinase which is required for XIAP mediated NF-κB activation following TNFα treatment. TAK1 activation in this model required TAB1 its coactivator and adapter molecule to the bone morphogenic protein (BMP) receptor [108-110]. Using co-immunoprecipitation NAIP was shown to interact directly with TAK1 and TAB1, but not JAK1, indicating that TAK1/TAB1 mediate their functional interaction [111, 112]. These results suggest that a JNK1/TAK1 dependent mechanism may be involved in NF-κB mediated NAIP regulation.

A high-throughput screening of compound libraries by our laboratory identified that cAMP, sodium butyrate (NaB), genistein and serum withdrawal could upregulate *Naip* transcript levels approximately 2-3 fold (C. Craig, Z. Lahoua and A. MacKenzie, unpublished observation) in N2A cells, a mouse neuroblastoma cell line, selected for their neuron-like characteristics. Research had previously shown that NaB treatment led to significant phenotypic improvements in SMA-like mice and that NAIP acts as a modulator of SMA severity, making NaB a logical choice for further study of the signaling pathways involved in *Naip*'s transcriptional induction [113, 114]. NaB is a naturally occurring four carbon fatty acid which can act through the G protein coupled receptors, GPCR41 and GPCR43 to activate numerous signaling pathways and also acts as a histone deacetylase inhibitor (HDACi) [115-117]. A Kinexus screen, large scale western blotting to compare the

expression levels of protein kinases in water and NaB treated N2A cell lysates, was conducted to obtain an indication of the signaling pathways involved in the upregulation of *Naip* by NaB. RSK1 and IKKα showed the greatest upregulation following NaB treatment while PKCμ, ZIP and JAK2 were downregulated to the greatest extent. The roles of individual kinases were assessed using specific chemical inhibitors for JAK2, ERK, p38, PKA and a general serine-threonine kinase inhibitor. N2A cells were treated with the kinase inhibitor alone or in combination with NaB following which *Naip* mRNA levels were assessed by quantitative RT-PCR (qRT-PCR). Through these studies it was determined that the NaB mediated *Naip* induction is independent of JAK2, ERK, p38 and PKA. They did however, suggest that JAK2 inactivation may be required for a non-NaB mediated *Naip* regulatory pathway. Treatment with the broad spectrum serine-threonine kinase inhibitor, H-7, which inhibits protein kinase A, C and G, lowered *Naip*'s expression to half of the vehicle treated control and when combined with NaB it was able to completely inhibit *Naip* induction suggesting that PKC or PKG is required for NaB mediated *Naip* induction as PKA had already been ruled out using Rp-8-cAMP and H-89 [118]. Extant scientific literature demonstrates that NaB acts through its GPCR leading to the activation of many kinases including PKC, Akt and NF-κB independently of its HDACi properties. Intriguingly, as previously outlined, PKC dependent NF-κB activation has been shown to induce IAPs which in turn act as part of a positive feedback loop to activate NF-κB transcription, a role for NF-κB in the regulation of *NAIP* expression has also been suggested by many researchers [104, 119]. Further research was required to confirm if NaB mediated *Naip* induction is dependent upon PKC and/or PKG and their downstream targets such as NF-κB or if alternatively its HDACi capacity is responsible.

Project Outline

The literature which has been reviewed reveals the enormous potential for altered NAIP expression as a therapeutic approach in a multitude of conditions including neurodegenerative disease and CNS trauma. In order to develop potential *NAIP* inducing strategies a thorough understanding of the mechanisms regulating its endogenous expression are required. Identified in a high-throughput screen for *Naip* inducers, NaB, a short chain fatty acid (SCFA) capable of activating numerous signaling pathways through its GPCR and acting as a HDACi, has been used to further elucidate the mechanisms regulating *Naip*'s expression [115-117]. Preliminary research in our laboratory suggests that it is NaB's GPCR signaling pathway modulatory activity and more specifically its effects on PKC or PKG which are responsible for *Naip* induction at the transcriptional level [118]. The goal of the current study is to further investigate the mechanism by which NaB mediates *Naip* induction.

NaB's known receptors, GPCR 41 and 43, signal through some common pathways [115-117]. Focusing on these pathways and specifically those involving PKC, PKG or those which lead to NF-κB activation, the role of NaB's receptor mediated signaling pathway modulatory capacity in *Naip* induction was further examined. Specific kinase inhibitors for key members of these pathways: PI3K, PKC, PKG, mTOR, Akt, and IKK, along with a constitutively active NF-κB inducer and its dominant negative were tested but failed to identify a specific kinase or pathway required for NaB mediated *Naip* regulation.

These results suggested that the HDACi property of NaB may play a greater role in the regulation of *Naip* expression than originally thought. We were able to confirm that NaB mediated *Naip* induction is receptor independent using antagonists for the G_{α} subunits of its GPCRs and observed that TSA, a potent HDACi, is also able to induce *Naip* expression

supporting the hypothesis that it is NaBs HDACi property which is responsible for *Naip* induction[120].

HDAC inhibitors are known to induce cell cycle arrest and differentiation, an effect we were able to observe in N2As treated with NaB or TSA by neurite extension and flow cytometry [121, 122]. Similarly following treatment with the non-HDACi differentiating stimuli, retinoic acid, increased *Naip* expression was observed. Differentiation in response to both stimuli is dependent on p21, a cyclin dependent kinase inhibitor, and the downregulation of E2F1, a transcription factor responsible for the expression of genes required for transition into S phase and the induction of apoptosis in response to high dose HDACi treatment [123-127]. This, in addition to the established links between IAPs and p21, as well as altered Naip expression and differentiation resulted in the hypothesis that HDACi mediated *Naip* induction is p21 dependent [128-132]. Quantitative RT-PCR and western blotting confirmed that the p21 induction and E2f1 downregulation required for differentiation is occurring in this model. Interestingly, no significant changes in the expression or activation of Sp1, a key regulator of p21, were observed. The inability to significantly knockdown p21 protein levels in N2A cells using siRNA led to studies in the human colon cancer line HCT116 and its p21 null daughter cell line [133, 134]. Treatment with NaB or TSA resulted in the characteristic *NAIP* induction in both the wildtype parental line and in the p21 null line indicating that HDACi mediated *NAIP* induction is p21 independent. Ultimately these results suggest that NaB and TSA mediated *Naip* induction occurs as a direct result by their histone deacetylase inhibitory capacity at the promoter level.

CHAPTER 2 - MATERIALS AND METHODS

Media and Reagents

All cell culture supplies including plastic wear, medium, and supplements were obtained from Fisher Scientific (Ottawa, On). Histone deacetylase inhibitors (NaB and TSA), kinase inhibitors (AKTI, KT5823, LY294002, rapamycin, rottlerin and wedelolactone), and 8.8'[Carbonylbis(imino-3,1-phenylene)]bis-(1,3,5-naphthalenetrisulfonic Acid)6Na (NF023) were obtained from Calbiochem (SanDiego, CA). [D-Arg^{1},D-Trp^{5,7,9},Leu^{11}]Substance P was obtained from BACHEM Biosciences Inc. (King of Prussia, PA). All other chemicals were obtained from Sigma-Aldrich Inc. (Oakville, On) unless otherwise specified.

Cell Culture

Neuro2A (N2A), a murine neuroblastoma cell line, was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in α-minimum essential medium (α-MEM) with 10% fetal bovine serum (FBS), 1% l-glutamine and 1% penicillin/streptomycin (pen/strep). PANC-1, a human pancreatic cancer cell line, was obtained from the ATCC (Manassas, VA) and maintained in Dubelcco's Modified Eagle's Medium (DMEM) with 10% FBS and 1% pen/strep. The human colon cancer cell line HCT116 and its p21 null daughter cell line were obtained from Dr. Bruce McKay (OHRI, Ottawa, On). p21 null cells were generated by Dr. Bert Vogelstein (Johns Hopkins Oncology Center and the Program in Human Genetics and Molecular Biology, Baltimore, MD) [135]. Both HCT116 cell lines were maintained in McCoy's 5A medium with 10%FBS and 1% pen/strep. All cells were cultured at 37°C and 5% CO_2.

i. HDACi Experiments

N2A or HCT116 cells were seeded in the appropriate culture medium at $5x10^4$ cells/ml 24 hours prior to treatment. Medium was removed and replaced with medium supplemented with HDAC inhibitors. Except when noted, cells were treated with 2mM NaB or 100nM TSA and were harvested for analysis 48 hours after treatment. If treatment extended beyond 48 hours medium and HDAC inhibitors were renewed every 48 hours.

ii. Inhibition Experiments

N2A cells were seeded at $5x10^4$ cells/ml 24 hours prior to treatment. Inhibitors and HDAC inhibitors were diluted separately in complete medium and cells were treated with inhibitors 1 hour prior to HDACi treatment. HDACi treated cells were supplemented with the same volume of DMSO as inhibitor treated cells. Cells were harvested for analysis 48 hours after treatment.

iii. Differentiation Experiments

N2A cells were seeded at $2.5x10^4$ cells/ml 24 hours prior to treatment. Cells were treated with 60uM all-trans retinoic acid (RA) diluted in normal medium which was renewed every 48 hours.

iv. Adenoviral Infection and NF-κB Reporter Assay

For adenoviral infection N2A and PANC-1 cells were seeded at $3.5x10^5$ cells/ml 24 hours prior to infection. Medium was removed from the cells and replaced with medium containing the *Lac Z*, *NIK* or *DN-NIK* expressing adenovirus (generated in house) at a multiplicity of infection (MOI) of 100 for 1 hour. The adenovirus containing medium was then removed and replaced with medium without antibiotics. Cells were allowed to recover for 1 hour prior to transfection with 0.1ug empty pCDNA3 vector, 0.15ug NF-κB inducible

firefly luciferase (Fr-luc) reporter construct and 0.03ug SV40 renilla luciferase (Re-luc) plasmid (Stratagene, La Jolla CA) with lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturers protocol. Five hours post transfection medium was replaced with culture medium without antibiotic, with or without 2mM NaB as indicated. Cells were analyzed 48 hours after treatment. NF-κB activity was expressed as a ratio of Fr-luc and Re-luc measured using a dual luciferase assay (Promega, Madison, WI) and a luminometer (BMG POLARstar Galaxy, Durham, NC) according to the manufacturer's protocol.

v. Sp1 Activity Assay

N2A cells were seeded at $3.5x10^5$ cells/ml in culture medium without antibiotics 24 hours prior to transfection and treatment. Cells were transfected with 0.1ug pCDNA3 empty vector, 0.03ug SV40 Re-luc plasmid and 0.09ug Sp1 inducible Fr-luc reporter construct (Panomics, Freemont, CA) with lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturers protocol. Six hours post transfection, medium was replaced with medium without antibiotics and treated with 2mM NaB or 100nM TSA as indicated. Cells were analyzed 48 hours after treatment. Sp1 activity was expressed as ratio of Fr-luc and Re-luc measured using a dual luciferase assay (Promega, Madison, WI and a luminometer (BMG POLARstar Galaxy, Durham, NC)) according to the manufacturer's protocol.

RNA preparation and qRT-PCR

After treatment cells were lysed and total RNA isolated using the RNeasy plus mini kit as per the manufacturer's protocol (Qiagen, Mississauga, On). RNA concentration was determined using spectrophotometry at 260nM. 100ng of RNA were used for human *NAIP* and mouse *Naip1*, 50ng for *E2f1* and *p21* and 10ng for *Sp1* RT-PCR reactions. The Qiagen (Mississauga, On) QuantiTect primer assay was used for the *Sp1* RT-PCR, all other

primer/probe sequences and reaction conditions are outlined in table 2-1. As a control, RT-PCR for *GAPDH* was carried out for each sample using ABI *GAPDH* control reagents (Applied Biosystems, Foster City, CA) and the reactions were multiplexed with human *NAIP* and mouse *Naip1*. For all other genes the reactions for *GAPDH* and the target gene were run in separate wells on the same plate. Human *NAIP* and mouse *Naip1* RT-PCR were done using the ABI TaqMan EZ RT-PCR kit (ABI, Foster City, CA), all other reactions were done using the QuantiTect SYBR Green RT-PCR kit (Qiagen, Mississauga, On), see table 2-1 for individual master mix details. Reactions were run on the ABI 7700 real-time cycler (ABI, Foster City, CA) and analyzed with ABI 7000 SDS software (ABI, Foster City, CA). Individual samples were run in duplicate or triplicate with a no template control and displayed single melting point peaks indicative of a single amplified product. The ABI 7000 SDS software calculates the threshold cycle (Ct) for each well and the delta Ct (ΔCt) is calculated by taking the difference in the Ct value for the gene of interest and that of GAPDH. The delta delta Ct (ΔΔCt) value is calculated by subtracting the average ΔCt value of the vehicle or untreated control cells and the relative expression level is then calculated as $2^{-\Delta\Delta Ct}$ [136].

Preparation and Western Blot Analysis of Protein

Cells were washed three times with phosphate buffered saline (PBS), cells in 10cm dishes were recovered by scraping into 10 ml of PBS followed by centrifugation and resuspended for lysis in 100ul of RIPA buffer (7.2mM PBS, 1%IGEPAL, 1% taurocholic acid and 0.1% sodium dodecyl sulfate) with 10ug/ml pepstatin A and leupeptin, 1ug/ml aprotinin (Roche Molecular Biochemicals, Mississauga, On) and 1mM polymethylsulfonyl fluoride (PMSF) and EDTA. Cells in 6 well dishes were lysed directly in the plate with 100ul of RIPA buffer

Table 2-1 - Primer/Probe Sequences, Master Mix Components and Reaction Conditions Used for qRT-PCR

GENE OF INTEREST	PRIMER AND PROBE SEQUENCE 5'-3'	
Human *NAIP*	Forward	GCCATTTTATGTCCAAGGGATATC
	Reverse	CTTCCCAATTTCCTAAACATCCA
	Probe	FAM-CTGTACCGTGTCCTGTTTACCTGTAAAGACAAAGC-TAMRA
Murine *Naip1*	Forward	TTCCTGTGGCGGAAGCTT
	Reverse	TGGGCAATTTCCTCTGAAGATT
	Probe	FAM-AGCATGCCAAGTGGTTCCCCAAATG-TAMRA
Murine *E2f11*	Forward	AGAAACGGCGCATCTATGAC
	Reverse	CCATCTGTTCTGCAGGGTCT
Murine *p21*	Forward	AGATCCACAGCGATATCCAG
	Reverse	ACACACAGAGTGAGGGCTAA

GENE OF INTEREST	MASTER MIX (PER REACTION)
Human *NAIP* multiplexed with *GAPDH*	5ul 5x EZ-buffer, 3ul 25mM $Mn(OAc)_2$, 3ul 10mM dNTPs, 1ul rTth polymerase, 0.25ul AmpErase UNG, 0.25ul each of 10uM *GAPDH* forward and reverse primers, 0.5ul 5uM probe, 0.15ul each of 100uM *NAIP* forward and reverse primers, 0.5ul 10uM *NAIP* probe, 5.95ul RNase free water
Murine *Naip1* multiplexed with *GAPDH*	5ul 5x EZ-buffer, 3ul 25mM $Mn(OAc)_2$, 3ul 10mM dNTPs, 1ul rTth polymerase, 0.25ul AmpErase UNG, 0.25ul each of 10uM *GAPDH* forward and reverse primers, 0.125ul 20uM *GAPDH* probe, 0.15ul each of 100uM *Naip1* forward and reverse primers, 0.5ul 10uM *Naip1* probe
Murine *E2f1* and *p21*	12.5ul 2x QuantiTect SYBR Green, 0.25ul QuantiTect RT Mix, 1.25ul each of 10uM forward and revere primers, 4.75ul RNase free water
Murine *Sp1*	12.5ul 2x QuantiTect SYBR Green, 0.25ul QuantiTect RT Mix, 2.5ul *Sp1* QuantiTect Primer Mix, 4.75ul RNase free water
Murine *GAPDH*	12.5ul 2x QuantiTect SYBR Green, 0.25ul QuantiTect RT Mix, 0.25ul (*Sp1*) or 0.63ul (*E2F-1* or *p21*) each 10uM forward and reverse *GAPDH* primers, RNase free water to a final volume of 20ul

GENE OF INTEREST	REACTION CONDITIONS
Human *NAIP*	2min@50°C,30min@60°C,5min@95°C, 45x[20sec@94°C,1min@60°C]
Murine *Naip1*	2min@50°C,30min@60°C,5min@95°C, 40x[20sec@94°C,1min@60°C]
Murine *E2f1* and *p21*	30min@50°C,15min@95°C, 40x[20sec@94°C,1min@60°C]
Murine *Sp1*	30min@60°C,15min@95°C, 40x[20sec@94°C,1min@60°C]

with protease inhibitors and collected. Samples were kept on ice for 1 hour and centrifuged at 13000 rpm for 15min at 4°C after which the supernatant was collected. Protein concentration was determined by spectrophotometry at 595nm of 5ul of diluted protein samples or bovine serum albumin standards (New England BioLabs, Pickering, On) combined with 250ul of Bradford Reagent (Sigma-Aldrich Inc., Oakville, On). Standardized amounts of protein (5ug to 30ug) were denatured at 70°C for 10 min in NuPAGE reducing agent (Invitrogen, Carlsbad, CA) and NuPAGE lithium dodecyl sulfate buffer (Invitrogen, Carlsbad, CA) before separation on pre-cast NuPAGE Novex 4-12% BIS-Tris mini-gels (Invitrogen, Carlsbad, CA). Proteins were transferred to a polyvinylidene fluoride membrane (Bio-Rad, Mississauga, ON) by wet transfer using the XCell II blot module (Invitrogen, Carlsbad, CA). Membranes were blocked overnight with 5% skim milk in PBST (1xPBS pH7, 0.1%Tween-20) at 4° C. The blots were washed four times in PBST (15min each) before incubation with primary antibodies (see table 2-2 for details). The membranes were washed four times (15min each) with PBST and incubated for 2 hours with the appropriate anti-mouse or anti-rabbit IgG horseradish peroxidase conjugated secondary antibody (Amersham, Baie d'Urfe, Qc) diluted 1:2000. Following four washes (20min each) the antibodies were detected using an enhanced chemiluminescence (ECL) plus detection kit and ECL Hyperfilm (Amersham, Baie d'Urfe, Qc). To ensure equal loading all membranes were re-probed for GAPDH.

Cell Cycle Analysis

Cells were detached from the culture dish with trypsin, once lifted the reaction was stopped by adding αMEM supplemented with 10%FBS, cells were recovered by centrifugation (1000rpm, 10min) and fixed with cold 100% methanol (Sigma-Aldrich Inc., Oakville, On).

Table 2-2 - Primary Antibody Specifics

Primary antibodies used for western blotting, dilutions, manufacturers and incubation times.

ANTIBODY	MANUFACTURER	DILUTION	INCUBATION TIME
Anti-NAIP	Gift from Aegera, manufactured by Upstate Cell Signalling Solutions	1:1000	overnight
Human Anti-p21	abcam	1ug/ml	overnight
Murine Anti-p21	Delta Biolabs	1:500	overnight
Anti-Sp1	abcam	1:1000	1 hour
Anti-E2F1	Cell Signalling Technology	1:500	overnight
Anti-GAPDH	Advanced ImmunoChemical Inc.	1:500	1 hour

Cells were pelleted by centrifugation (3000rpm, 5min) and washed twice with cold PBS, incubated with 10mg/ml RNaseA (Roche Molecular Biochemicals, Laval, Qc) for 30min at 37°C and stained with 10mg/ml Propidium Iodide (Sigma-Aldrich Inc., Oakville, On) before analysis with BD FACSAria flow cytometer (BD Biosciences, Mississauga, On). Analysis was completed using DeNovo FCS Express software (DeNovo, Thornhill, On).

Statistical Analysis

Data has been analyzed using GraphPad Prism software. Student T-tests or one-way analysis of variance (ANOVA) followed by a Tukey's *post hoc* test was used to identify conditions that are statistically different. In each analysis p values less than 0.05 were considered significant. Values are presented as mean ± standard error of the mean (SEM).

CHAPTER 3 - RESULTS

The potential for NAIP in therapeutic interventions was realized soon after its discovery. Exogenous overexpression studies in both *in vitro* and *in vivo* systems have allowed for a better understanding of its role at the cellular level and proof of its therapeutic potential. However, in order to better promote the development of therapeutic interventions, an understanding of its endogenous regulation at the transcriptional and translational levels is of critical importance. To investigate the intracellular signals controlling *Naip* expression at the transcriptional level, previous work in our laboratory identified an inducing compound, sodium butyrate (NaB). NaB is a naturally occurring four-carbon fatty acid which is known to activate a number of signaling pathways through GPCR mediated signaling and acts as a histone deacetylase inhibitor (HDACi) [117]. Following a Kinexus screen to identify changes in protein kinase expression with NaB treatment, inhibitors for selected kinases were used in combination with NaB. *Naip* RNA level was then assessed to determine the role of the affected kinase in its induction. The results from this preliminary study suggested that it is NaB's GPCR mediated signaling pathway modulatory activity and more specifically its effects on PKC or PKG which is responsible for *Naip* induction at the transcriptional level, though its HDACi capacity has not been ruled out [118]. The current study aims to further investigate the mechanism by which NaB mediates *Naip* induction.

The effect of NaB on Naip expression

To remain consistent with the preliminary work, Neuro-2A (N2A) cells, a neuron-like mouse neuroblastoma cell line, were used in this project as the model system and

quantitative RT-PCR (qRT-PCR) for *Naip1*[2], the *Naip* isoform expressed at the highest level in CNS, was used to assess transcript level. As was previously observed, NaB upregulates *Naip* RNA level in a dose responsive manner. The basal level of *Naip* transcript in untreated cells was set to a value of one. A maximum induction of 2 fold can be seen 48 hours following 2mM NaB treatment (figure 3.1). No significant change in viability, as determined by trypan blue exclusion and WST assays, was observed in N2A cells following 2mM NaB treatment. 2mM was therefore selected as the concentration to be used for further study.

Naip RNA induction following 2mM NaB treatment is also time responsive (figure 3-2, panel A). When the basal *Naip* level in untreated cells was set to one, a significant and increasing induction is seen at all time points following treatment with 2mM NaB. The maximum induction observed was 6.1 fold following 120 hours of treatment. The increase in *Naip* RNA expression over time correlates with increased protein level (figure 3.2 - panel B). It is, however, important to note that the change observed in Naip protein level is general and not specific to the Naip1 isoform.

Investigation of the role of NaB mediated signaling pathways in the regulation of Naip expression

i. Kinase Inhibition

Using the same approach as our preliminary studies, the roles of individual kinases were assessed using inhibitory compounds in order to determine the key players in *Naip*'s regulation. All the inhibitors have been previously characterized and described in the

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[2]from here on it will be referred to as *Naip*

Figure 3.1 - *Naip* RNA level in N2A cells treated with increasing doses of NaB

48 hours following treatment of N2A cells with 0.1, 0.5, 1, 1.5 or 2mM NaB *Naip* RNA level was determined by qRT-PCR. Each value represents the mean ± SE (N=3) and the data presented is representative of three independent experiments. Statistical significance with respect to untreated cells: * $p<0.05$, ** $p<0.01$ as determined by ANOVA and post-hoc Tukey tests.

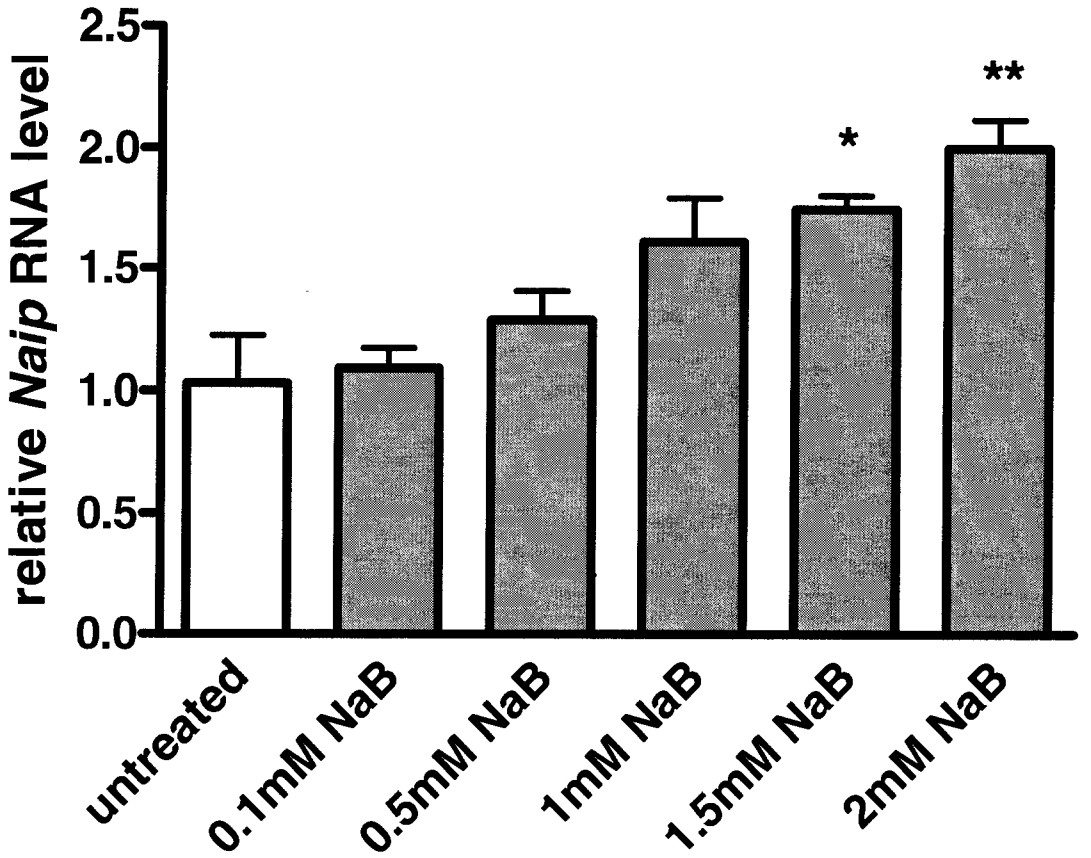
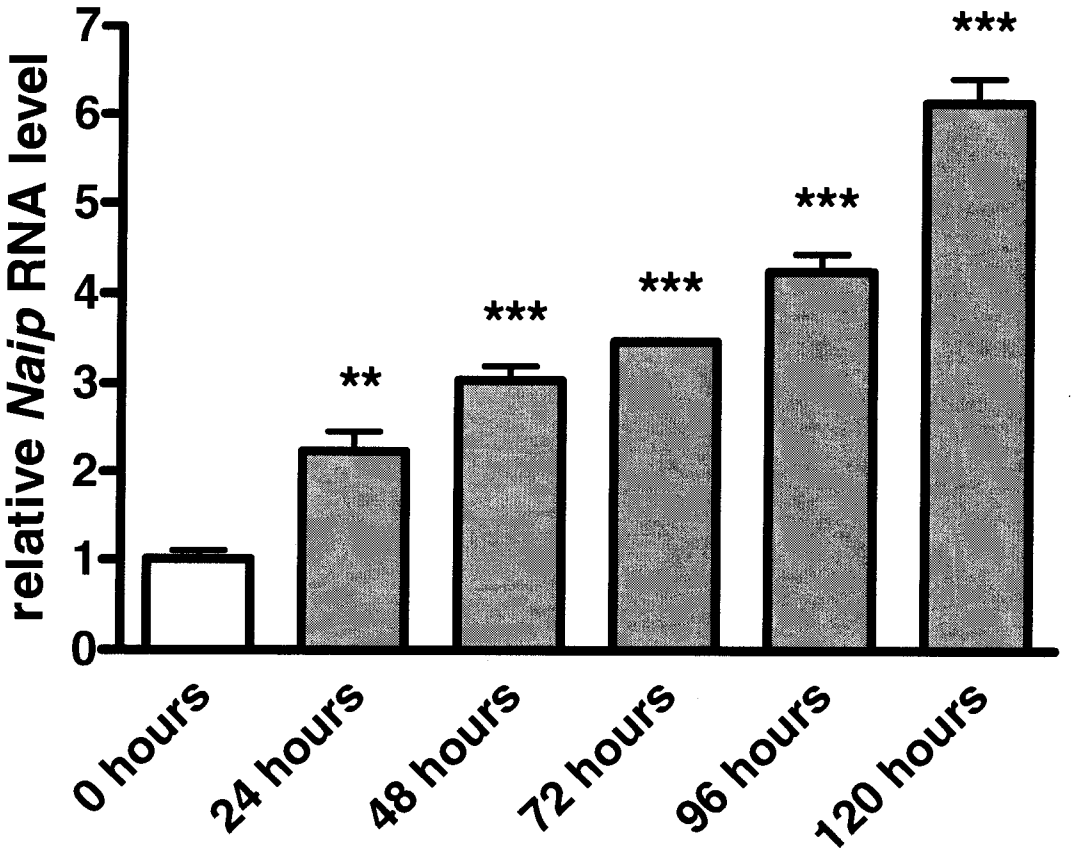

relative Naip RNA level
2.5
2.0
1.5
1.0
0.5
0.0
*
**
untreated
0.1mM NaB
0.5mM NaB
1mM NaB
1.5mM NaB
2mM NaB

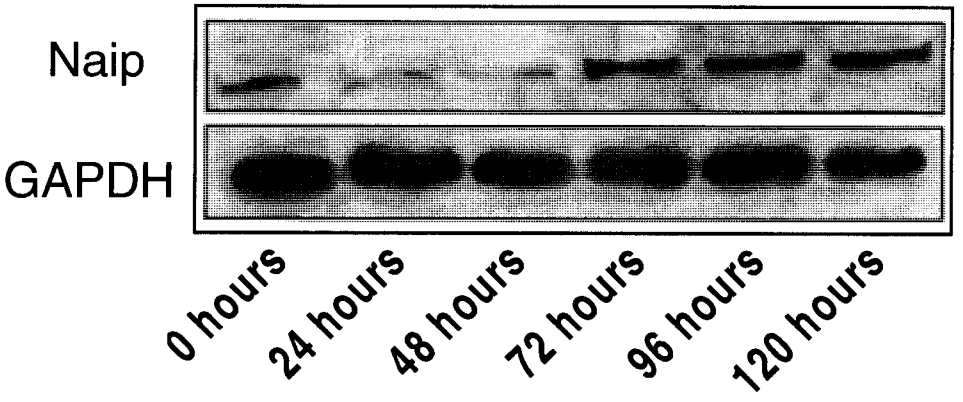
Figure 3.2 - Naip levels in N2A cells over a time course of 2mM NaB treatment

Following treatment with 2mM NaB N2A cells were harvested at 0, 24, 48, 72, 96 and 120 hours and Naip level was determined by (A) qRT-PCR and (B) western blotting. Each value represents the mean ± SE (N=3) and the data presented is representative of three independent experiments. Statistical significance with respect to untreated cells: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ as determined by ANOVA and post-hoc Tukey tests.

A


relative Naip RNA level
0
1
2
3
4
5
6
7
**
***
***
***
***
0 hours
24 hours
48 hours
72 hours
96 hours
120 hours

B


Naip
GAPDH
0 hours
24 hours
48 hours
72 hours
96 hours
120 hours

litterature. The dosages employed were significantly higher than their respective K_i or IC_{50} values to ensure inhibition, and were assessed over a range of dosages to limit toxicity to the N2A cells (data not shown). Confirmation of the inhibition of each individual kinase was not carried out as this was intended to be a preliminary screen from which the findings would be confirmed using the appropriate siRNA, dominant negative, or overexpression studies.

The preliminary results were consistent with the hypothesis that NaB mediated *Naip* induction was a receptor mediated event, therefore in the current study receptor associated kinases were selected first for further investigation. Within the GPCR family 1, which consists of approximately 90 receptors, there are at least 50 members for which the ligands are unknown; this group is known as the orphan GPCRs. Researchers examining a subset of the human orphan GPCRs using a ligand fishing strategy discovered that GPCR41 and GPCR43 are receptors for short chain fatty acids (SCFAs) including NaB [115, 116, 137]. This class of receptors consists of a seven transmembrane domain receptor which is coupled to the interior of the cell by the heteromeric G protein made up of α, β and γ subunits. A conformational change in the receptor is induced by the ligand binding and leads to an exchange of GDP for GTP at the G_α subunit which then dissociates from the $G_{\beta\gamma}$ dimer. Following the release of G_α-GTP, the $G_{\beta\gamma}$ dimer can regulate a number of downstream effectors [138]. Of the 17 possible G_α subunits, $G_{i/o}$, G_q and G_{12} have been identified as being involved in SCFA signaling. Various studies have also shown that they are capable of signaling through phosphoinositide-specific phospholipase C (PLC) and/or phosphatidylinositol 3- kinase (PI3K) [115, 116].

PI3K is a ubiquitously expressed lipid kinase that is involved in receptor mediated signal transduction. It regulates cell growth, vesicular uptake and trafficking, apoptosis and differentiation [139]. The activation and recruitment of PI3K to the plasma membrane

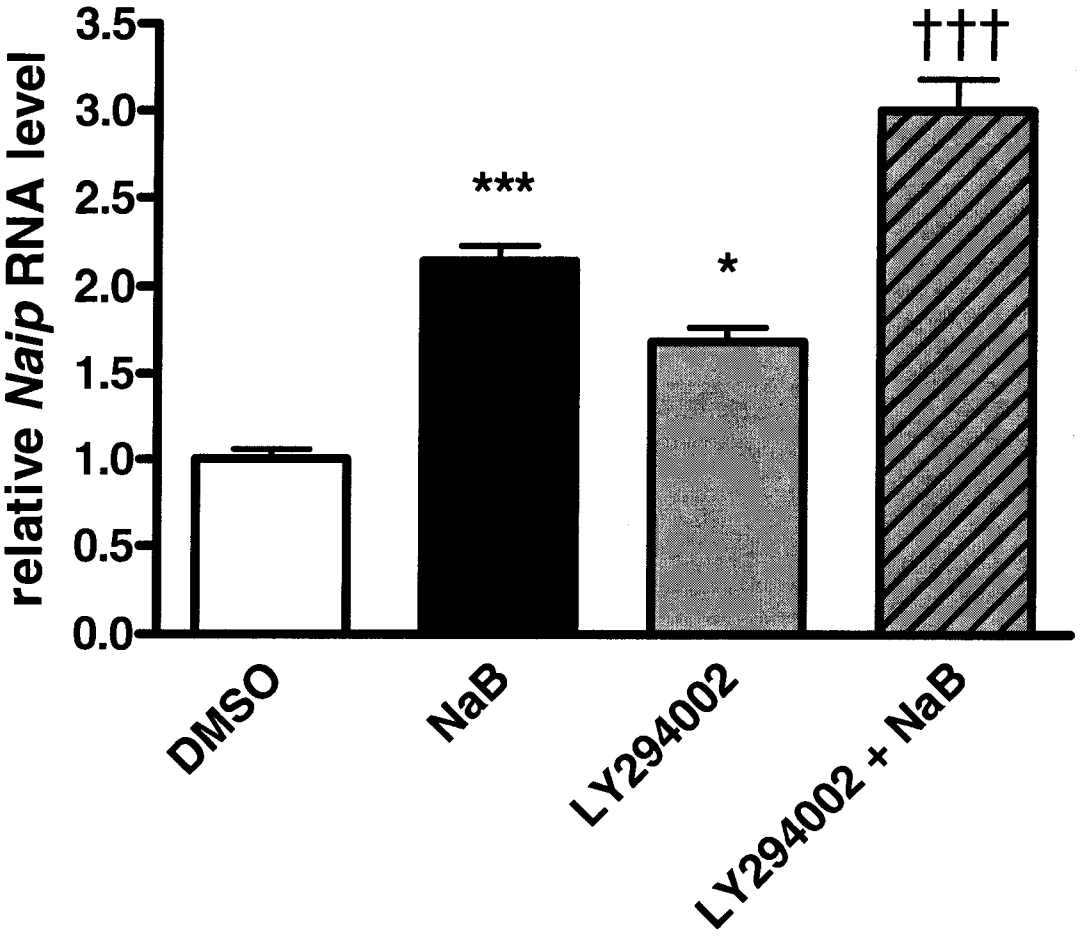
results predominantly in the phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP_2) at the D3 position of the inositol ring, producing phosphatidylinositol-3,4,5-trisphosphate (PIP_3). This goes on to bind the pleckstrin homology domain of proteins which directly or indirectly results in their activation [140]. PI3K has been implicated in HDACi mediated effects by researchers examining the ability of apicidin and TSA, two strong HDAC inhibitors, to activate NF-κB's transcriptional activity. They determined that NF-κB activation is dependent on PI3K and can be completely blocked using the PI3K inhibitor LY294002 [141].

To determine if PI3K is involved in NaB mediated *Naip* induction, LY294002 was used in combination with NaB (Figure 3.3). LY294002 is reconstituted in DMSO. The *Naip* RNA level in cells treated only with DMSO was set to one. Cells treated with NaB alone were the positive control and gave the expected 2.1 fold increase in *Naip*. Cells treated with LY294002 displayed a 1.7 fold increase, while cells pretreated with LY294002 and then NaB showed a 3 fold increase in *Naip* RNA levels. The increase in relative *Naip* RNA level seen when LY294002 and NaB treatment are combined is additive in nature, suggesting that there are two or more pathways which can induce *Naip* expression but that NaB mediated *Naip* induction does not require PI3K.

Phosphoinositide-specific phospholipase C (PLC) is also involved in G protein coupled receptor mediated signaling. PLC's activation and recruitment to the plasma membrane results in it catalyzing the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) into two second messengers: inositol-1,4,5-trisphosphate (IP_3), which leads to the release of Ca^{2+} from intracellular stores; and diacylglycerol (DAG), which recruits protein kinase C (PKC) to the membrane and activates it [142]. In the preliminary studies H-7, a

Figure 3.3 - Relative *Naip* RNA levels in N2A cells following treatment with a PI3K inhibitor LY294002, and NaB

Following a one hour pretreatment with 14uM LY294002 and treatment 2mM NaB for 48 hours; the level of *Naip* RNA was determined by qRT-PCR. Each value represents the mean ± SE (N=3) and the data presented is representative of three independent experiments. Statistical significance with respect to the vehicle (*) or NaB (†) treated cells: */† $p<0.05$, **/†† $p<0.01$, ***/††† $p<0.001$ as determined by ANOVA and post-hoc Tukey tests


relative Naip RNA level
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
*
†††
DMSO
NaB
LY294002
LY294002 + NaB

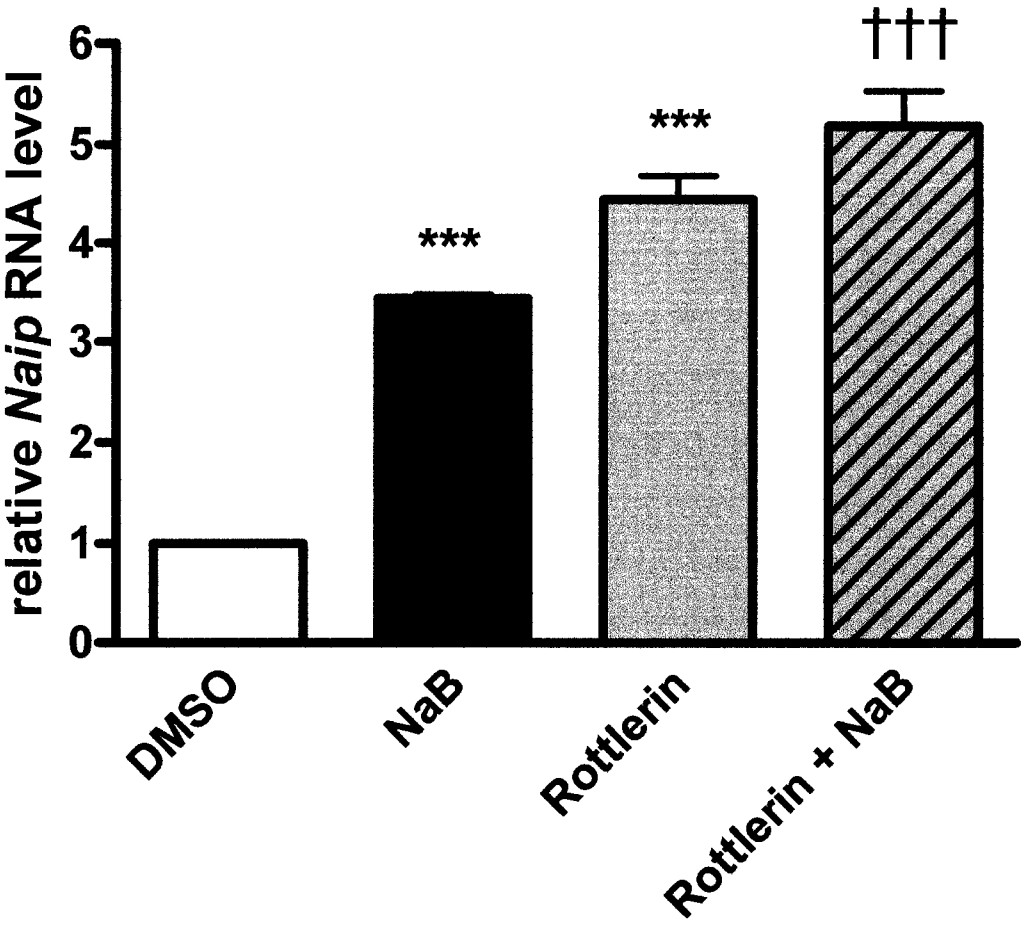
PKA, PKC and PKG inhibitor, was found to inhibit NaB mediated *Naip* induction suggesting that further research into the specific kinases involved would be beneficial [118].

PKCs are a family of serine/threonine-specific protein kinases that regulate a wide variety of cellular functions that include proliferation, differentiation, and apoptosis [143]. The PKC family has three major categories: the classical PKCs (α,β,γ), which require both an increase in intracellular calcium levels and are responsive to phorbol esters, the novel PKCs (δ,ε,θ,η,μ), which are activated by phorbol esters but not an increase in intracellular calcium levels; and finally, the atypical PKCs (ξ, λ) which are sensitive to neither intracellular calcium levels nor phorbol esters [144]. H-7 does not inhibit the classical PKCs, and N2A cells express only the α, β, δ, ε, λ and ξ isoforms of PKC. This leaves the role of the δ, ε, λ, and ξ isoforms to be investigated further [145, 146]. All four PKC isoforms of interest are abundantly expressed in the nervous system, associated with cell survival and NF-κB activation. PKCδ is the isoform responsible for the NF-κB mediated induction of XIAP and cIAP1/2. Researchers examining kainic acid induced cell death in cortical neurons, a model in which *Naip* expression is critical for neuronal survival, have shown that inhibition of PKCδ with rottlerin significantly increases cell death [59, 98, 147]. Therefore, PKC and its δ isoform in particular were selected for further investigation.

To determine if PKC is involved in NaB mediated *Naip* induction, rottlerin was used (figure 3.4). Rottlerin was dissolved in DMSO and the *Naip* RNA level in cells treated with DMSO alone were set to one. NaB treatment resulted in an expected 3.4 fold increase in *Naip* transcript. Rottlerin treated cells showed a 4.4 fold increase, while cells pretreated with rottlerin which were then exposed to NaB showed a 5.1 fold induction of *Naip* RNA. The increase in Naip level in the presence of rottlerin alone suggests that PKC is playing a role in the basal expression of Naip. When rottlerin and NaB are combined; the increase in *Naip*

Figure 3.4 - Relative *Naip* RNA levels in N2A cells following treatment with a PKC inhibitor rottlerin, and NaB

Following a one hour pretreatment with 6uM rottlerin and treatment with 2mM NaB for 48 hours; the level of *Naip* RNA was determined by qRT-PCR. Each value represents the average ± SE (N=3) and the data presented is representative of four independent experiments. Statistical significance with respect to the vehicle (*) or NaB (†) treated cells: */† $p<0.05$, **/†† $p<0.01$, ***/††† $p<0.001$ as determined by ANOVA and post-hoc Tukey tests.


relative Naip RNA level
0
1
2
3
4
5
6
***
***
†††
DMSO
NaB
Rottlerin
Rottlerin + NaB

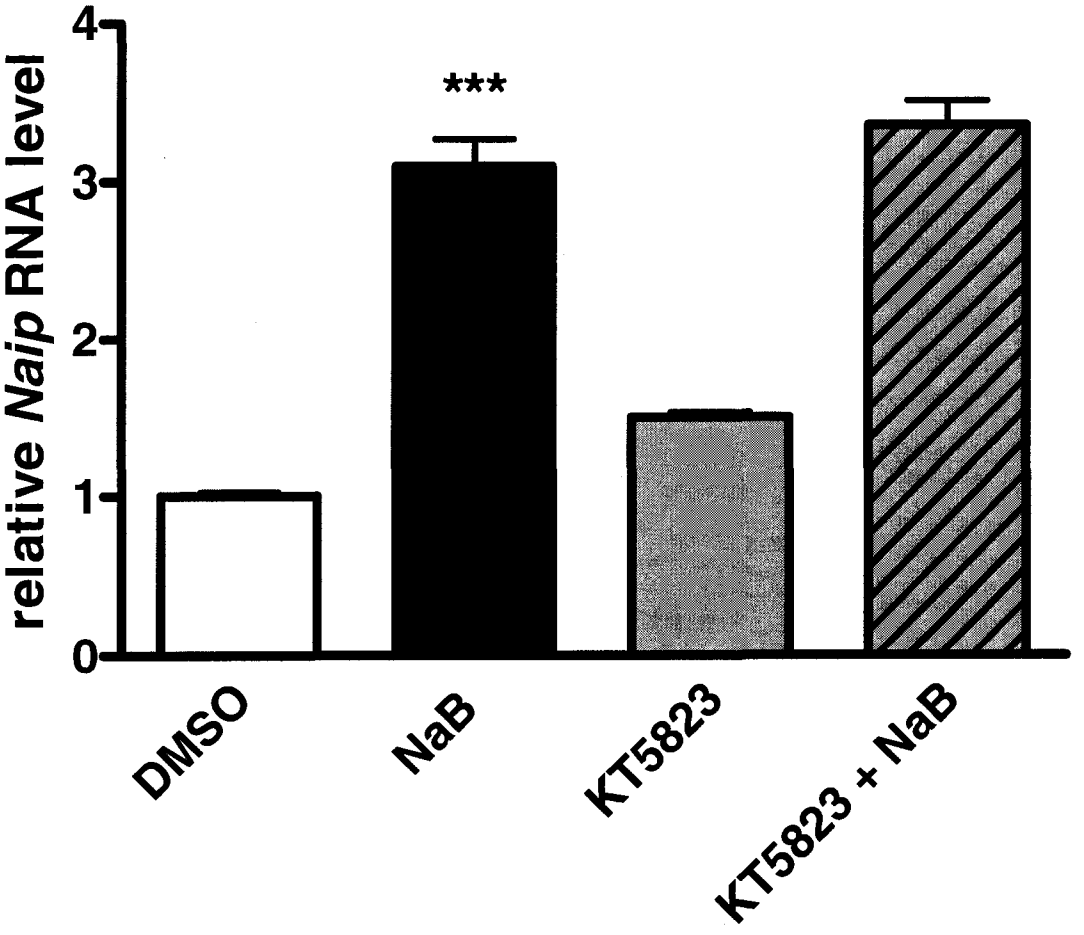
transcript level is neither additive nor synergistic; PKC's role is therefore less clear. It does however appear that PKC is not required for NaB induced *Naip* expression and likely plays a role in another regulatory pathway. It should also be noted that rottlerin does not inhibit all PKC isoforms equally. At the concentration used, PKC δ is the isoform expected to be most effectively inhibited. Further clarification and confirmation of these results could be obtained using a panel of PKC inhibitors.

Of the kinases which H-7 is known to inhibit, PKA was ruled out in the preliminary studies and PKC gave results opposite to those expected, leaving only PKG to be examined further. Researchers studying the receptor meditated anti-apoptotic effect of progesterone on ovarian granulosa cell survival, an effect which has been linked to increased *NAIP* expression, demonstrated that this effect is PKG dependent and can be blocked when these cells were pretreated with the PKG inhibitor KT5832 [29, 148]. To determine the role of PKG in NaB mediated *Naip* induction, the PKG inhibitor KT5823 was used (Figure 3.5). The *Naip* level in cells treated with DMSO alone was set to one. NaB treatment yielded a 3 fold increase in *Naip* transcript. However, KT5823 alone had no significant effect on *Naip* levels, nor did it alter the induction of *Naip* when combined with NaB treatment. These results indicate that PKG is not required for the NaB mediated *Naip* induction.

Akt is the predominant serine/threonine kinase downstream of PI3K signaling [140]. Signaling through Akt can result in the activation of numerous kinases including the p70 ribosomal S6 kinase (RSK), serum and glucocorticoid-induced protein kinases (SGK), and the atypical isoforms of PKC [149]. Researchers investigating the potential of HDAC inhibitors as cancer therapeutics revealed their inability to induce apoptosis is due to their activation of NF-κB. This study demonstrated that the transcriptional activation of NF-κB

Figure 3.5 - Relative *Naip* RNA levels in N2A cells following treatment with a PKG inhibitor KT5823, and NaB

Following a one hour pretreatment with 1uM KT5823 and treatment with 2mM NaB for 48 hours; the level of *Naip* RNA was determined by qRT-PCR. Each value represents the average ± SD (N=3) and the data presented is representative of three independent experiments. Statistical significance with respect to the vehicle (*) or NaB (†) treated cells: */† $p<0.05$, **/†† $p<0.01$, ***/††† $p<0.001$ as determined by ANOVA and post-hoc Tukey tests.


relative Naip RNA level
4
3
2
1
0
***
DMSO
NaB
KT5823
KT5823 + NaB

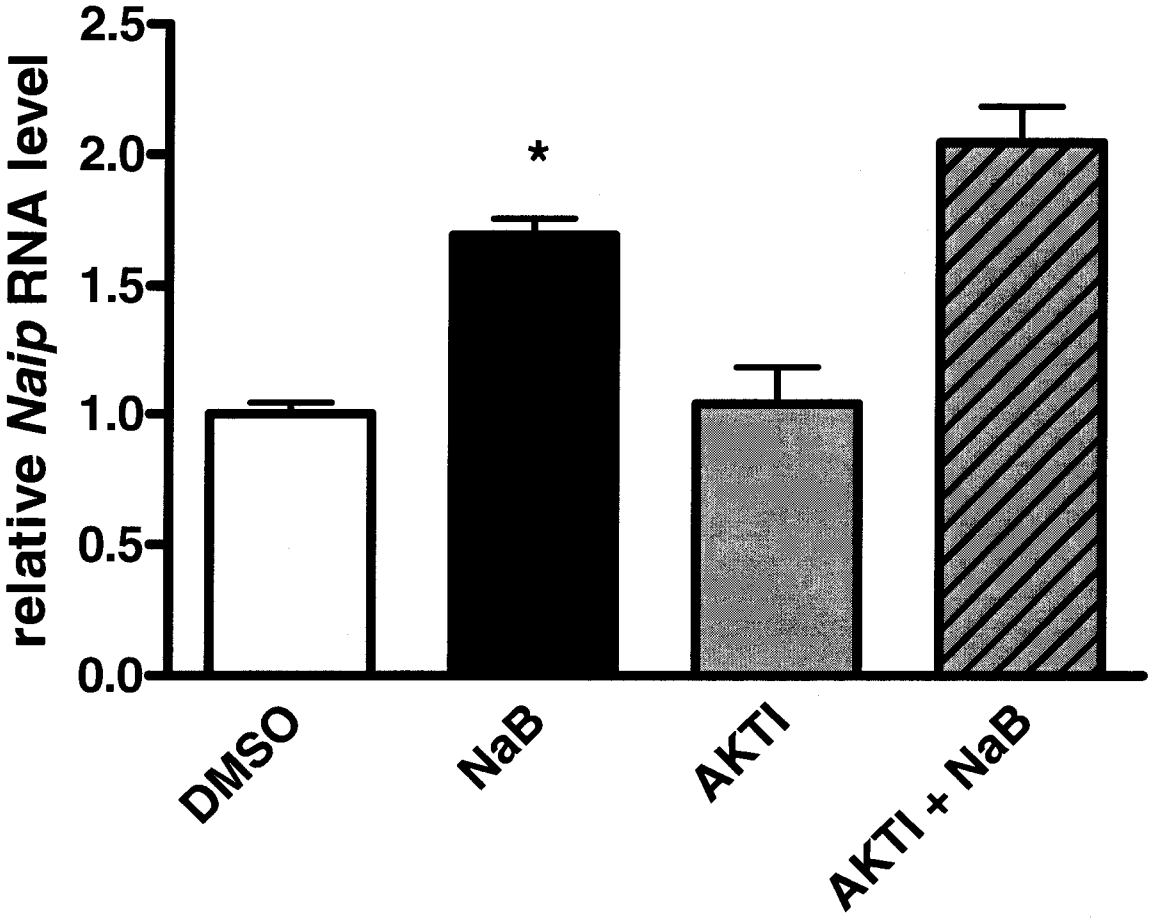
occurred through a PI3K/Akt dependent pathway and that inhibition of this pathway sensitized the cells to apoptosis [150]. Considering that Akt is downstream of PI3K, similar results to those seen when using LY294002, a PI3K inhibitor, were expected. AKTI, an Akt inhibitor,

is prepared in DMSO; therefore, the *Naip* RNA level in cells treated with DMSO alone was set to one (Figure 3.6). An expected 1.7 fold induction with NaB treatment was observed. AKTI treatment alone or in combination with NaB resulted in no significant change in *Naip* expression. This indicates that NaB mediated *Naip* induction does not require Akt, which in turn suggests that the alternate pathway which mediated *Naip* induction following PI3K inhibition is Akt independent.

The mammalian target of rapamycin, mTOR, is a serine/threonine protein kinase conserved amongst all eukaryotes and plays a role in cell growth and metabolism [151]. mTOR can be activated directly by PI3K or its downstream mediator Akt, and can regulate the activation of downstream kinases including the ribosomal S6 kinases (RSK) [152]. Regulation of the RSKs is of interest as an increase in this kinase in the Kinexus screen was observed. Adipocyte differentiation in the 3T3-L1 cell line has been shown to require RSK p70 activation and leads to increases in NAIP levels, both of which are inhibited in the presence of rapamycin, an mTOR inhibitor [63]. While the results obtained with the PI3K and Akt inhibitors do not suggest that mTOR will be required for the regulation of NaB mediated *Naip* induction it may play a role in the secondary pathway which leads to *Naip* induction when PI3K is inhibited. This is also an interesting avenue to examine as mTOR mediated activation of RSK has been implicated in a negative feedback loop which inhibits PI3K and could therefore potentially promote the induction of *Naip* expression observed

Figure 3.6 - Relative *Naip* RNA levels in N2A cells following treatment with an Akt inhibitor AKTI, and NaB

Following one hour pretreatment with 10uM AKTI and treatment with 2mM NaB for 48 hours; the level of *Naip* RNA was determined by qRT-PCR. Each value represents the average ± SE (N=3) and the data presented is representative of four independent experiments. Statistical significance with respect to the vehicle (*) or NaB (†) treated cells: */† $p<0.05$, **/†† $p<0.01$, ***/††† $p<0.001$ as determined by ANOVA and post-hoc Tukey tests.


relative Naip RNA level
2.5
2.0
1.5
1.0
0.5
0.0
*
DMSO
NaB
AKTI
AKTI + NaB

with LY294002 [152]. The vehicle in which rapamycin is prepared is DMSO. DMSO treated cells have their *Naip* level set to one. NaB treatment alone resulted in an expected 2.5 fold increase in *Naip* transcript (figure 3.7). Rapamycin treated cells showed no change in their *Naip* level. Similarly, when pretreatment with rapamycin was combined with NaB, no significant change in *Naip* level was seen relative to NaB treatment alone. This indicates that mTOR is not involved in NaB mediated *Naip* induction.

The IKK family of kinases is responsible for the phosphorylation of IκB which are the inhibitors of NF-κB. Phosphorylation of IκB leads to its ubiquitination and degradation by the proteasome pathway, releasing NF-κB so that it can translocate to the nucleus and stimulate the expression of genes suppressing apoptosis, promoting cell growth and stimulating the immune system [153]. NF-κB has been shown to induce IAPs (XIAP and cIAP1/2) and as part of a positive feedback loop they activate NF-κB transcription [104, 119]. The kinexus screen also revealed that NaB treatment led to an upregulation of IKKα, supporting further examination of its involvement in NaB mediated *Naip* upregulation. Wedelolactone, an IKK inhibitor, was used for this purpose (figure 3.8). The *Naip* level in cells treated with the vehicle, DMSO, alone was set to one. As expected, a 2.4 fold *Naip* induction was seen in cells treated with NaB. Wedelolactone treatment resulted in no significant change in *Naip* level; however, wedelolactone pretreatment combined with NaB resulted in a 7 fold *Naip* induction. The *Naip* induction seen when NaB and wedelolactone treatment are combined is synergistic in nature, which suggests that the inhibition of IKK and therefore the sequestration of NF-κB promotes NaB mediated *Naip* induction.

The results of these experiments conflict with the previous hypothesis that NaB mediated *Naip* induction requires the activation of NF-κB, suggesting instead that NF-κB regulates a repressive element to *Naip*'s transcription in N2A cells. This is unexpected as

Figure 3.7 - Relative *Naip* RNA levels in N2A cells following treatment with an mTOR inhibitor rapamycin, and NaB

Following a one hour pretreatment with 5nM rapamycin and treatment with 2mM NaB for 48 hours; the level of *Naip* RNA was determined by qRT-PCR. Each value represents the average ± SE (N=3) and the data presented is representative of three independent experiments. Statistical significance with respect to the vehicle (*) or NaB (†) treated cells: */† $p<0.05$, **/†† $p<0.01$, ***/††† $p<0.001$ as determined by ANOVA and post-hoc Tukey tests.

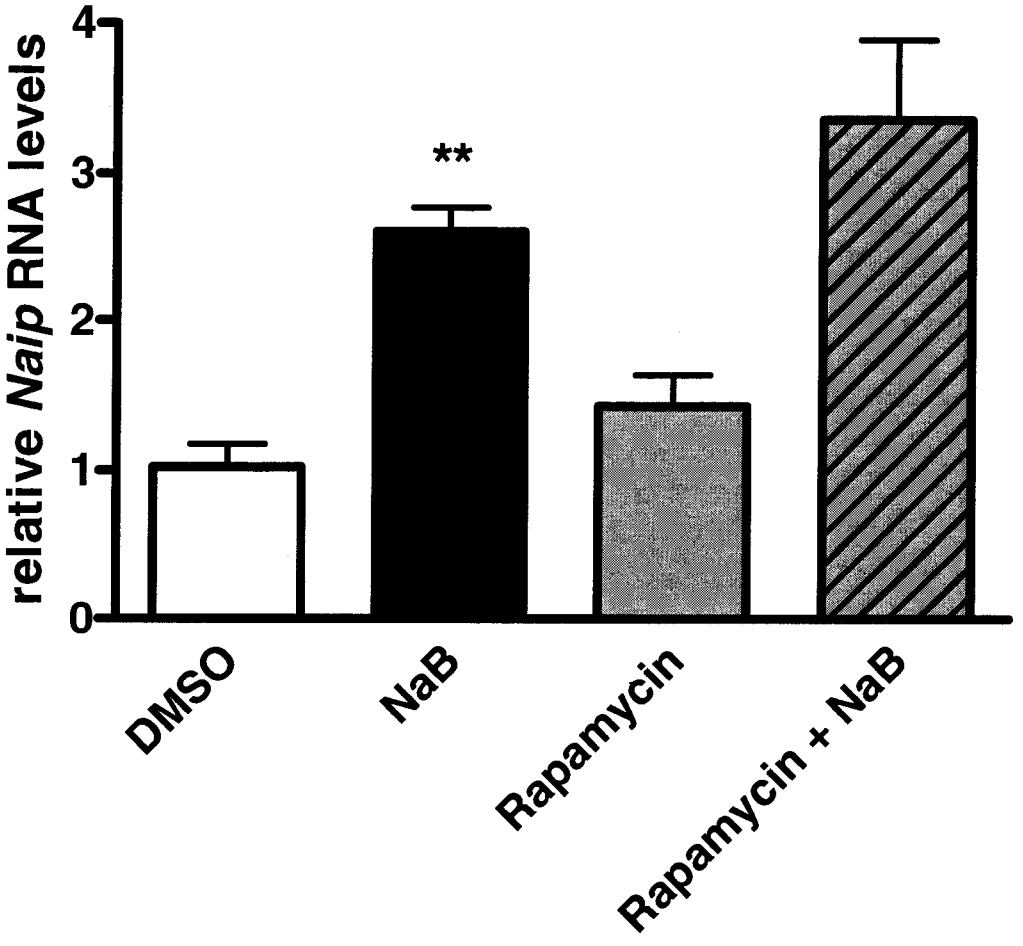
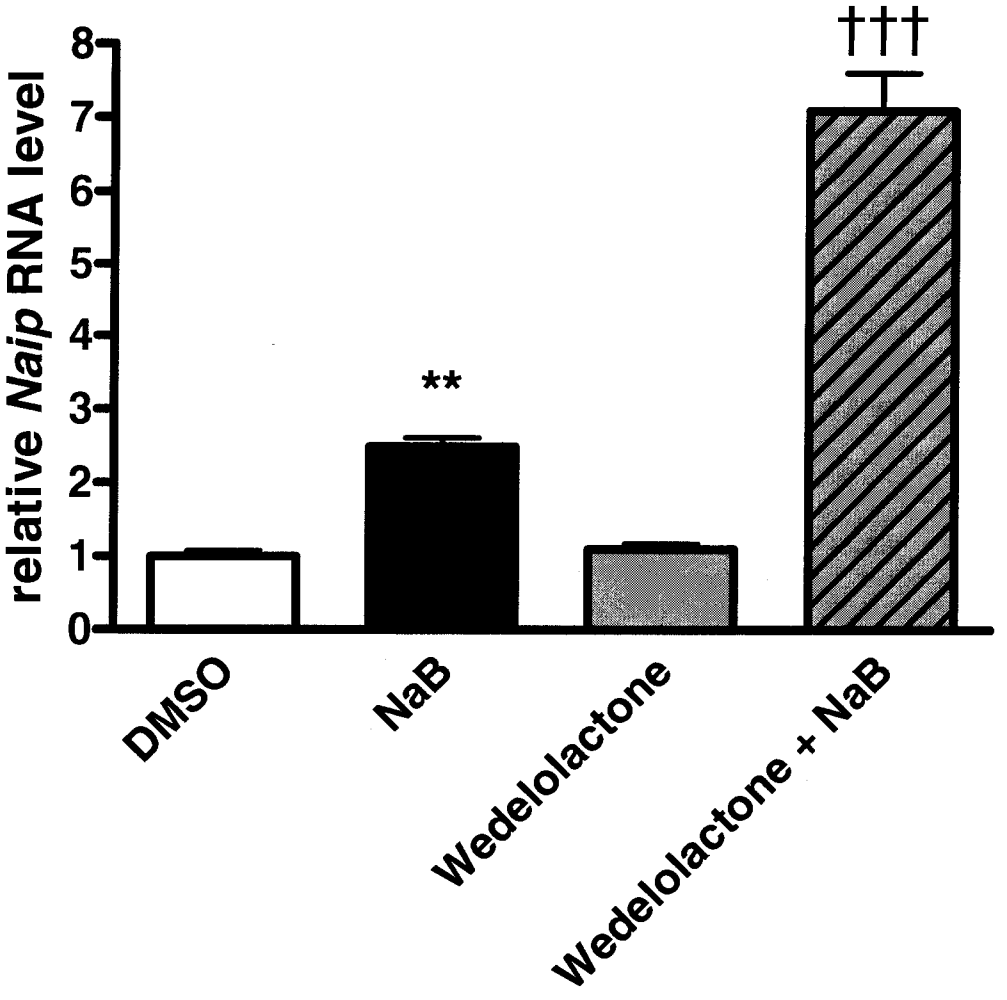

relative Naip RNA levels
4
3
2
1
0
**
DMSO
NaB
Rapamycin
Rapamycin + NaB

Figure 3.8 - Relative *Naip* RNA levels in N2A cells following treatment with an IKK inhibitor wedelolactone, and NaB

Following a one hour pretreatment with 50uM wedelolactone and treatment with 2mM NaB for 48 hours; the level of *Naip* RNA was determined by qRT-PCR. Each value represents the average ± SE (N=3) and the data presented is representative of three independent experiments. Statistical significance with respect to the vehicle (*) or NaB (†) treated cells: */† $p<0.05$, **/†† $p<0.01$, ***/††† $p<0.001$ as determined by ANOVA and post-hoc Tukey tests.


relative Naip RNA level
0
1
2
3
4
5
6
7
8
**
†††
DMSO
NaB
Wedelolactone
Wedelolactone + NaB

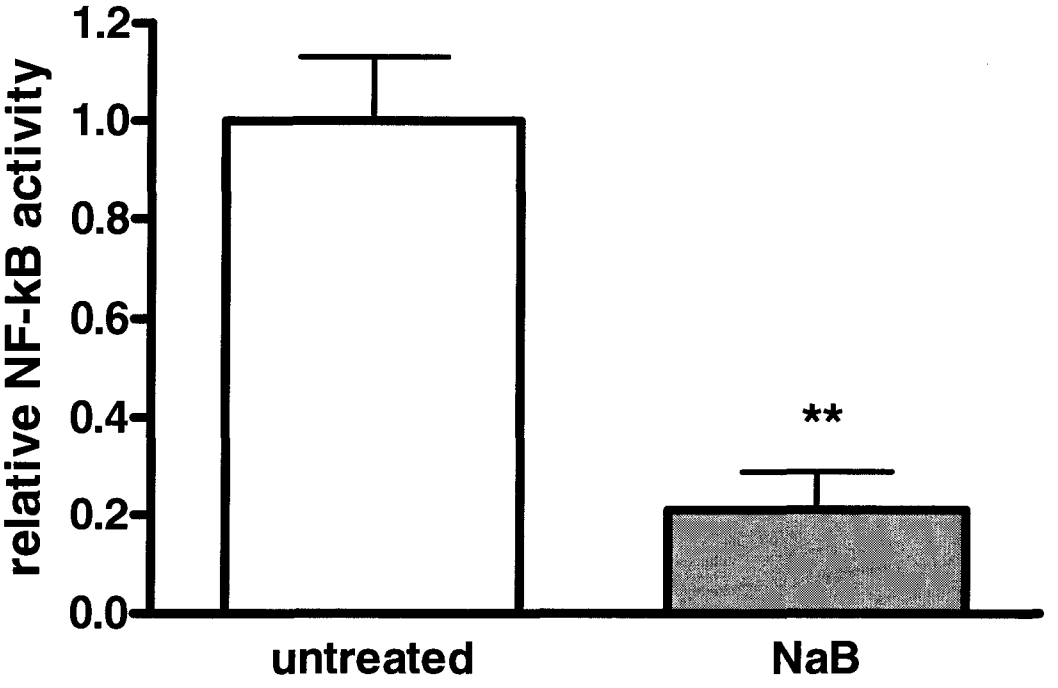
NaB has previously been shown to induce NF-κB mediated transcription through a PI3K/Akt dependent pathway, and induction of other members of the IAPs have also been shown to occur via the activation of NF-κB [104, 119, 150]. Cell line specificity may be responsible for the differences observed. Additionally, the involvement of Akt has already been excluded suggesting that a different pathway is at play in N2A cells. Therefore, a closer look at the role of NF-κB in NaB mediated *Naip* induction is necessary to elucidate the events occurring in this particular system.

First, it was important to determine what effect NaB treatment of N2A cells was having on NF-κB activity. Cells were transfected with a reporter plasmid expressing firefly luciferase under the control of a basic TATA promoter with NF-κB repeats to assess NF-κB activity, and a plasmid expressing renilla luciferase under the control of the SV40 promoter to control for transfection efficiency. Luciferase activity was measured 48 hours following NaB treatment using a dual luciferase assay and NF-κB activity is expressed as a fold change in the ratio of firefly to renilla luciferase. NF-κB activity in untreated cells was set to one. NaB treatment resulted in a significant reduction in NF-κB activity to 0.2 fold (figure 3.9) consistent with the possibility that inhibition of NF-κB activity plays a role in the NaB mediated transcriptional induction of *Naip* in N2A cells.

Adenoviral constructs expressing a constitutively active form of the NF-κB inducing kinase (*NIK*), its dominant negative (*DN-NIK*) or *LacZ* as a control were selected to further study the role of NF-κB in NaB mediated *Naip* induction. NIK is capable of directly phosphorylating and activating IKKα, leading to the activation of NF-κB [154]. The *NIK* and *DN-NIK* expressed in these adenoviruses are the human genes and had not been previously tested in murine cells. It was therefore necessary to confirm their ability to induce and inhibit NF-κB activation respectively in the N2A cell line. N2A cells and PANC-

Figure 3.9 - NF-κB activity following NaB treatment

Following transfection with a NF-κB inducible firefly luciferase reporter construct and a renilla luciferase reporter construct N2A cells were treated with NaB for 48 hours and NF-κB activitiy was measured by dual luciferase assay. Each value represents the mean ± SE (N=5) and is representative of 3 independent experiments. Statistical significance with respect to the untreated N2A cells: * $p<0.05$, ** $p<0.01$ as determined by ANOVA and post-hoc Tukey tests.


relative NF-kB activity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
untreated
NaB

1 cells, a human pancreatic cell line in which the adenoviruses had previously been tested, were infected with the *LacZ*, *NIK* and *DN-NIK* expressing adenoviruses. The cells were then transfected with the same reporter constructs used in the previous experiment and NF-κB activity was once again measured by dual luciferase assay (figure 3.10). In both PANC-1 and N2A cells infected with *adeno-LacZ,* NF-κB activity was set to 1. *Adeno-DN-NIK* led to a downregulation of NF-κB activity to 0.15 fold in PANC-1 cells and 0.25 fold in N2A cells, both of which are statistically significant. *Adeno-NIK* led to a statistically significant increase in NF-κB activity of 2.9 fold in PANC-1 cells and 2.1 fold in N2A cells. It was therefore concluded that these adenoviruses could effectively activate and inhibit NF-κB activity in N2A cells and could therefore be used for further investigation into the role of NF-κB in NaB mediated *Naip* induction.

In order to confirm the role of NF-κB in NaB dependent *Naip* induction, the adenoviruses were combined with NaB treatment (figure 3.11). 48 hours following treatment, NF-κB activity and *Naip* transcript level were assessed in parallel. In both cases, *adeno-LacZ* infected cells are used as the control, with the *Naip* and NF-κB activity levels being set to one. As shown in the previous experiment, *adeno-NIK* and *DN-NIK* were able to induce and repress NF-κB activity respectively relative to *Lac Z* alone. In the presence of NaB, the same trend was seen; though overall, the NF-κB activity was lower as expected due to the inhibitory action of NaB (figure 3.11-panel A). Relative to the *adeno-LacZ* infected cells, neither *adeno-NIK* nor *adeno-DN-NIK* infection caused a significant change in *Naip* expression. NaB treatment of each of the adeno infected cultures resulted in a significant induction of *Naip* transcript relative to their adeno infection alone. However, relative to *adeno-LacZ* infected cells treated with NaB, only, *adeno-DN-NIK* infection combined with

Figure 3.10 - NF-κB activity in PANC-1 and N2A cells infected with adenoviruses expressing *Lac Z*, *NIK* or *DN-NIK*

Following infection at an MOI of 100 with *Adeno-LacZ*, *NIK* or *DN-NIK*, N2A and PANC-1 cells were transfected with a NF-κB inducible firefly luciferase reporter construct and a renilla luciferase reporter construct, NF-κB activation was measured by dual luciferase assay 48 hours later. Each value represents the mean ± SE (N=3). Statistical significance with respect to the *LacZ* N2A(*) or PANC-1 (†) cells: */† $p<0.05$, **/†† $p<0.01$, ***/††† $p<0.001$ as determined by ANOVA and post-hoc Tukey tests.

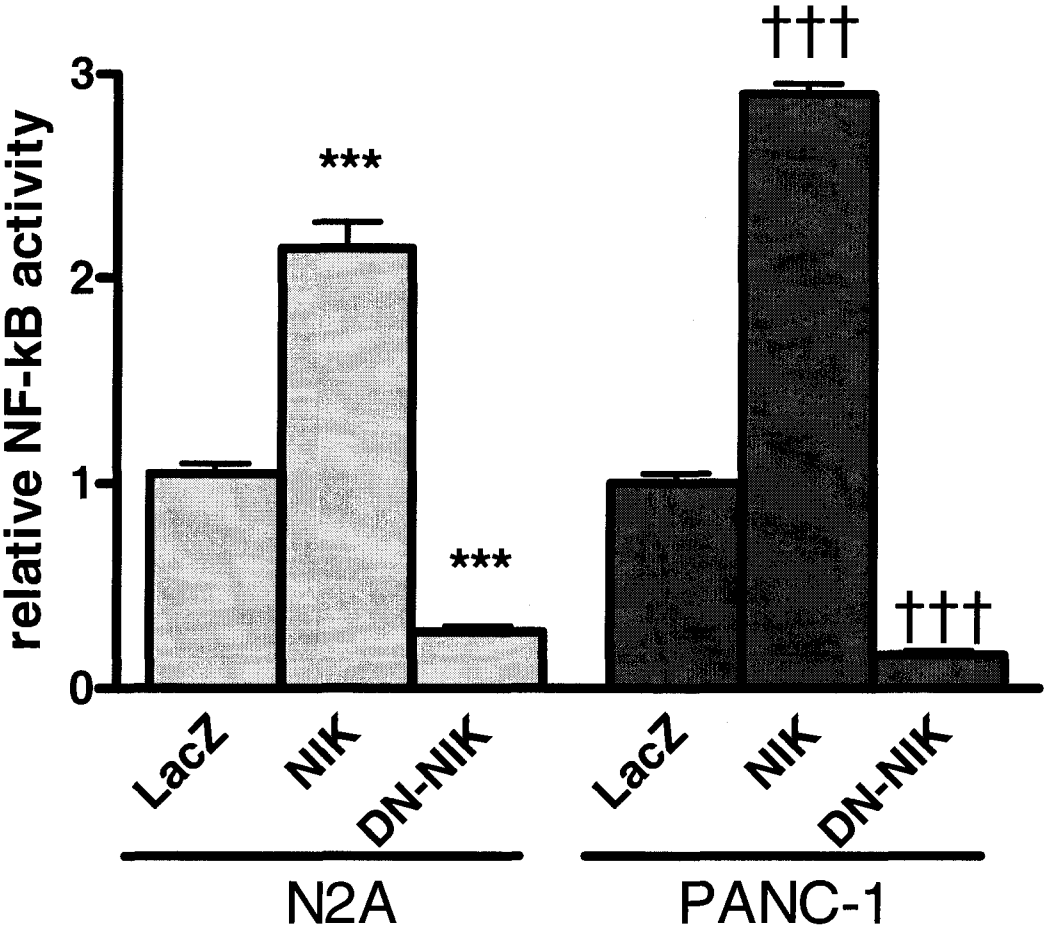
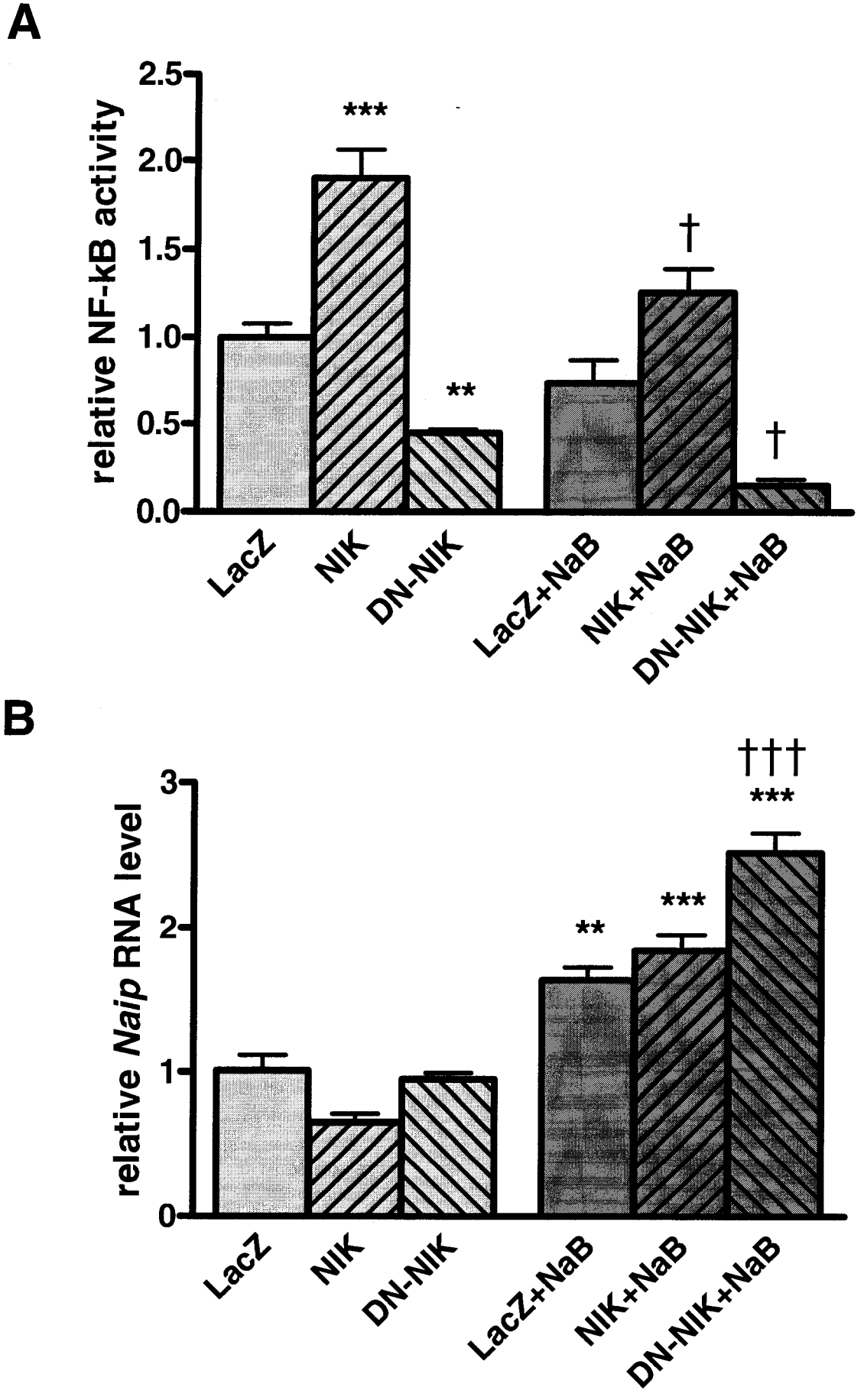

relative NF-kB activity
3
2
1
0
***
***
†††
†††
LacZ
NIK
DN-NIK
LacZ
NIK
DN-NIK
N2A
PANC-1

Figure 3.11 - Relative NF-κB activity and *Naip* RNA levels in N2A cells following infection with adenovirus expressing *Lac Z*, *NIK* or *DN-NIK* and NaB treatment

Following infection at an MOI of 100 with adenovirus expressing a constitutively active *NIK, DN-NIK or LacZ* control, transfected with an NF-κB inducible firefly luciferase reporter construct and a renilla luciferase construct and 48 hour 2mM NaB treatment NF-κB activity was determined measured by dual luciferase assay (panel A) and the *Naip* RNA level was determined by qRT-PCR (panel B). Each value represents the mean ± SE (N=3) and the data presented is representative of three independent experiments. Statistical significance with respect to their respective infection alone control (*) or LacZ+NaB treated (†) cells: */† $p<0.05$, **/†† $p<0.01$, ***/††† $p<0.001$ as determined by ANOVA and post-hoc Tukey tests.


A
relative NF-kB activity
2.5
2.0
1.5
1.0
0.5
0.0
***
**
†
†
LacZ
NIK
DN-NIK
LacZ+NaB
NIK+NaB
DN-NIK+NaB
B
relative Naip RNA level
3
2
1
0
**
***
***
†††
LacZ
NIK
DN-NIK
LacZ+NaB
NIK+NaB
DN-NIK+NaB

NaB treatment resulted in a significantly higher induction of *Naip* (figure 3.11-panel B). This is in agreement with those results obtained using the IKK inhibitor wedelolactone, suggesting that the inhibition of NF-κB activity promotes NaB mediated *Naip* expression; however, given that an induction of NF-κB activity with *adeno- NIK* infection did not inhibit NaB mediated *Naip* induction, it does not appear that it is required for the control of *Naip* expression.

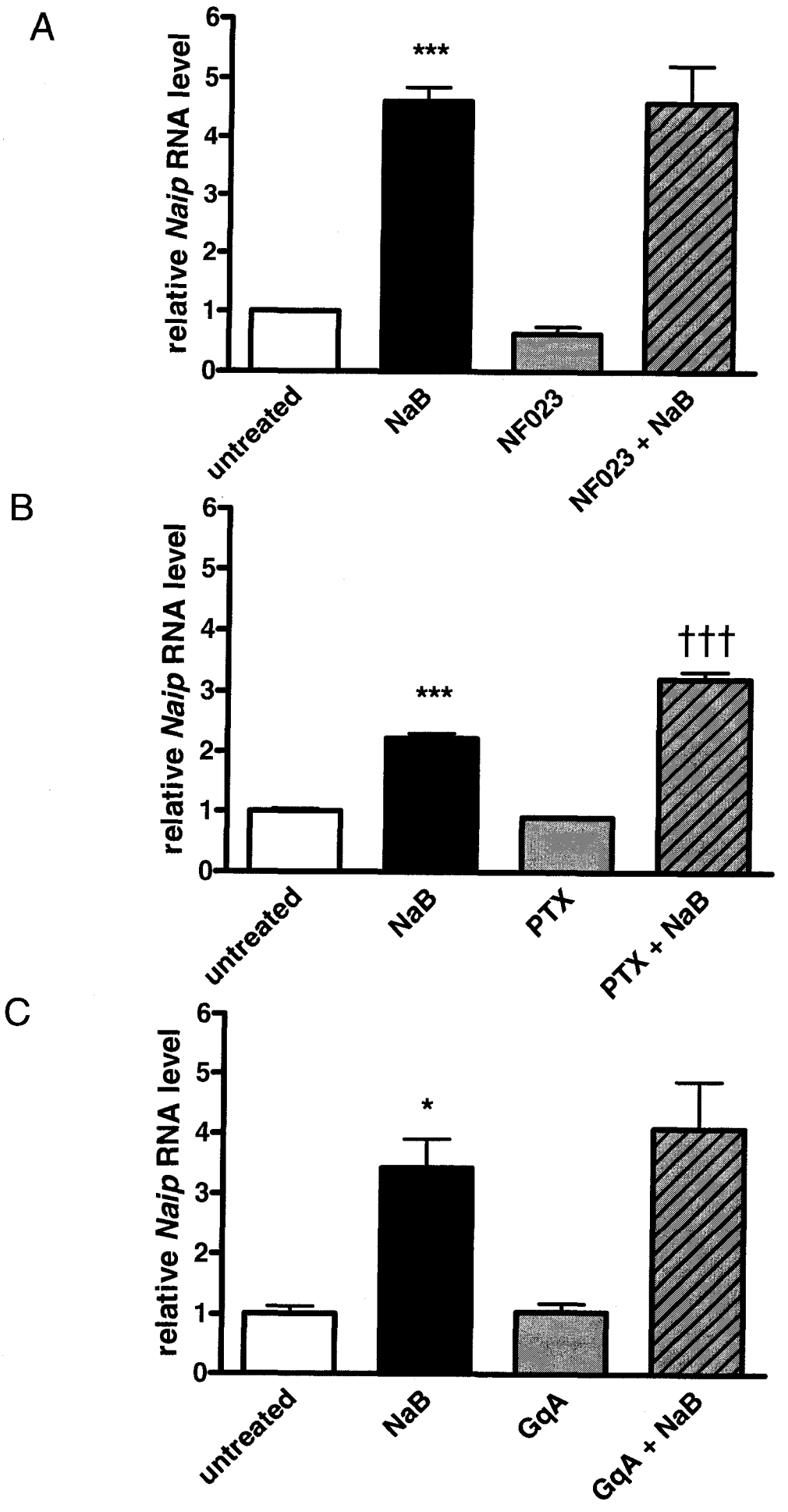
ii. Receptor Inhibition

Strong evidence from preliminary studies in our laboratory suggested that NaB mediated *Naip* induction is receptor mediated, and reports from other investigators indicated that changes in promoter specific histone acetylation level alone following NaB treatment were insufficient to induce the observed modulation in gene expression. Despite this, my results to date have not revealed a particular kinase or signaling pathways being necessary for NaB mediated *Naip* induction, suggesting that it is a receptor independent event [155]. As previously discussed, NaB and other SCFAs signal through the GPCR 41 and the GPCR 43 [115, 116, 137]. While these receptors have been identified in the nervous system where signaling through them in a rat neuronal model system leads to alterations in neurotransmitter-related gene expression it remains to be seen if the NaB mediated *Naip* induction observed in N2A cells is receptor mediated [116, 156].

To confirm that the induction of *Naip* expression by NaB is independent of GPCR mediated signaling antagonists for the specific G_α subunits were used (figure 3.12). 8.8'[Carbonylbis(imino-3,1-phenylene)]bis-(1,3,5-naphthalenetrisulfonic Acid)6Na (NF023) and pertussis toxin (PTX), are antagonists of the $G_{i/o}$ family and [D-Arg^1,D-$Trp^{5,7,9}$,Leu^{11}]Substance P (GqA), is an antagonist of the G_q and G_{12} families [157-159]. The

Figure 3.12 - Relative *Naip* RNA levels in N2A cells following receptor inhibition and NaB treatment

Following a one hour pretreatment with (A) 3uM NF023 (B) 100ng/ml PTX or (C) 10uM GqA and 48 hour 2mM NaB treatment *Naip* RNA level was determined by qRT-PCR. Values represent the mean ± SE (N=3) and the data presented is representative of three independent experiments. Statistical significance with respect to the untreated (*) or NaB treated (†) cells: */† $p<0.05$, **/†† $p<0.01$, ***/††† $p<0.001$ as determined by ANOVA and post-hoc Tukey tests.


A
relative Naip RNA level
0
1
2
3
4
5
6
***
untreated
NaB
NF023
NF023 + NaB
B
relative Naip RNA level
0
1
2
3
4
5
6
***
†††
untreated
NaB
PTX
PTX + NaB
C
relative Naip RNA level
0
1
2
3
4
5
6
*
untreated
NaB
GqA
GqA + NaB

Naip level in untreated cells was set to one. NaB treatment yielded the expected induction of *Naip* transcript and neither NF023, PTX nor G_qA alone altered *Naip* expression levels. Pretreatment with either NF023 or G_qA individually followed by NaB treatment resulted in a *Naip* induction normally seen with NaB alone. PTX combined with NaB resulted in a significantly higher induction of Naip than NaB alone. ERK phosphorylation is a receptor dependant NaB mediated event and was therefore assessed to confirm receptor inhibition [160]. NaB treatment in the presence of any of the three receptor antagonists abrogated ERK phoshporylation thereby confirming effective receptor inhibited (data not shown) This indicates that NaB mediated *Naip* induction does not occur through the $G_{i/o}$, G_q or G_{12} subfamilies of the GPCRs. In the larger picture, this confirms that NaB mediated *Naip* induction is receptor independent and is therefore likely occurring as a result of its HDACi properties. The possibility remains, however, that NaB is signaling though another family of the G_α subunits of the GPCRs or another as of yet unidentified receptor.

The effect of TSA on *Naip* expression

While the discovery that NaB can bind GPCRs, activating signaling cascades within the cell is relatively recent, many of the compounds other properties were established long ago. NaB's ability to arrest DNA synthesis and proliferation, induce differentiation, alter cell morphology and modulate gene expression was first highlighted in a series of papers in the mid 1970s [121, 122]. It was discovered in the late seventies that NaB was able to increase histone acetylation by inhibiting HDACs [161, 162]. HDACi treatment has been reported to be non-specific, altering the expression of many gene families and has thus generally been considered to be a poor choice of potential therapeutic target due to the likelihood of off-target effects. More recently, HDAC inhibitors have been shown to be

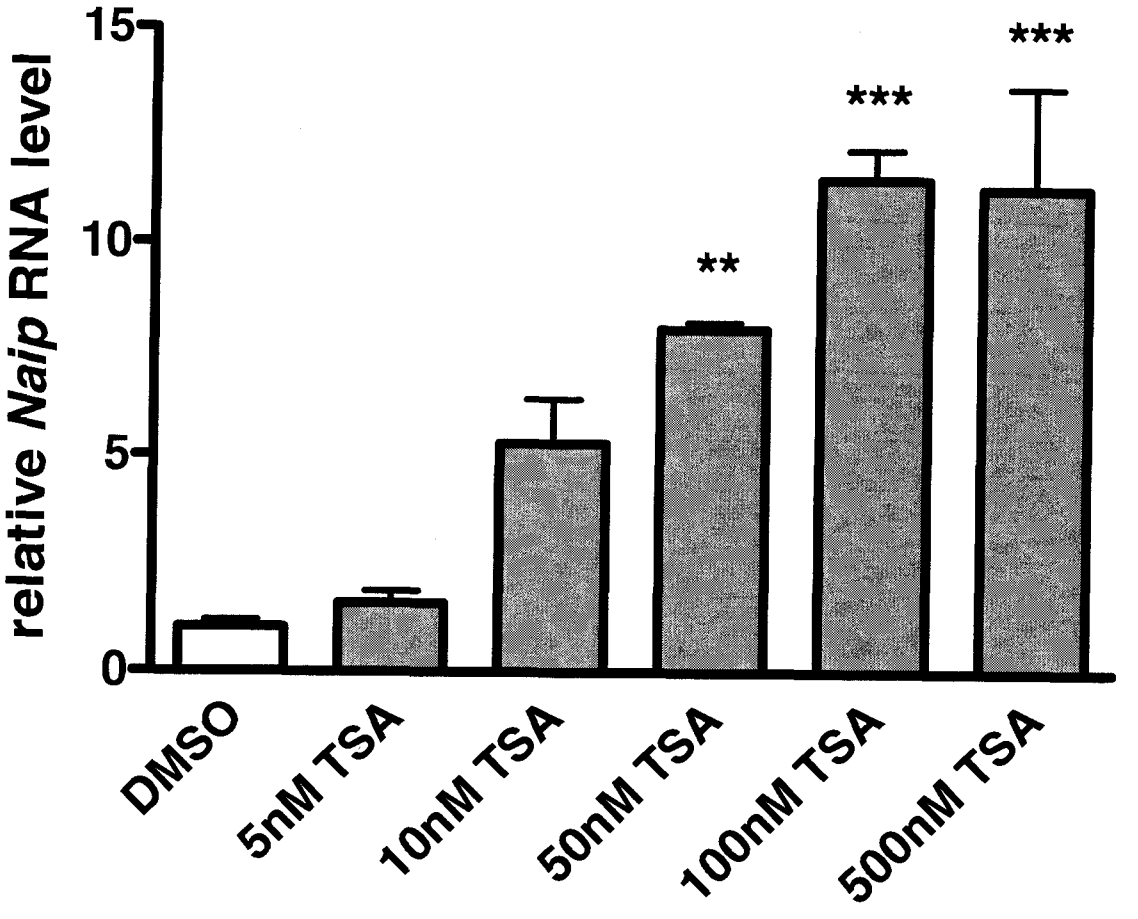
more selective than originally thought affecting the expression of as little as 2-5% of the genome [163]. Their potential therapeutic relevance has thus been revisited for many conditions.

The receptor inhibition experiments confirmed that NaB mediated *Naip* induction is receptor independent, leaving its HDAC inhibitory property to be investigated. While there is no method available to isolate NaB's HDACi property and thereby confirm that it is responsible for the induction of *Naip,* the use of other non-SCFA HDAC inhibitors gives an indication of the ability of HDAC inhibitors as a family to modulate *Naip* expression. Trichostatin A (TSA) is an HDACi isolated from *Streptomyces plantesis* and is generally accepted as the most potent of all HDAC inhibitors; it was therefore selected for further study [120]. *Naip* levels in cells treated with DMSO, the vehicle in which TSA is prepared, were set to one (figure 3.13 - panel A). Cells treated with TSA revealed an induction of *Naip* in a dose responsive manner on a scale which had not previously been observed. *Naip* was induced up to 30 fold although in repeated experiments it was more commonly observed in the 5 to 10 fold range. Western blotting for Naip also revealed increased expression at the protein level (figure 13.3 - panel B). It should be noted that with increasing TSA concentration there is an associated increase in cell death, 100nM was selected as the concentration to be used for further study as it induces *Naip* significantly with no significant change in N2A viability as determined by trypan blue exclusion and WST assays. As was seen in the case of NaB, *Naip* induction by TSA is also time responsive at both the RNA and protein levels (figure 3.14). The observation that *Naip* is induced following TSA treatment supports the hypothesis that NaB mediated *Naip* induction is occurring due to HDACi.

Figure 3.13 - Naip level following treatment with increasing concentrations of TSA

Following a 48 hour treatment with TSA (5-500nM), Naip level was determined by (A) qRT-PCR and (B) western blotting. Each value represents the mean ± SE (N=3) and the data presented is representative of six independent experiments. Statistical significance with respect to the untreated control: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ as determined by ANOVA and post-hoc Tukey tests.

A


relative Naip RNA level
15
10
5
0
**
***
***
DMSO
5nM TSA
10nM TSA
50nM TSA
100nM TSA
500nM TSA

B

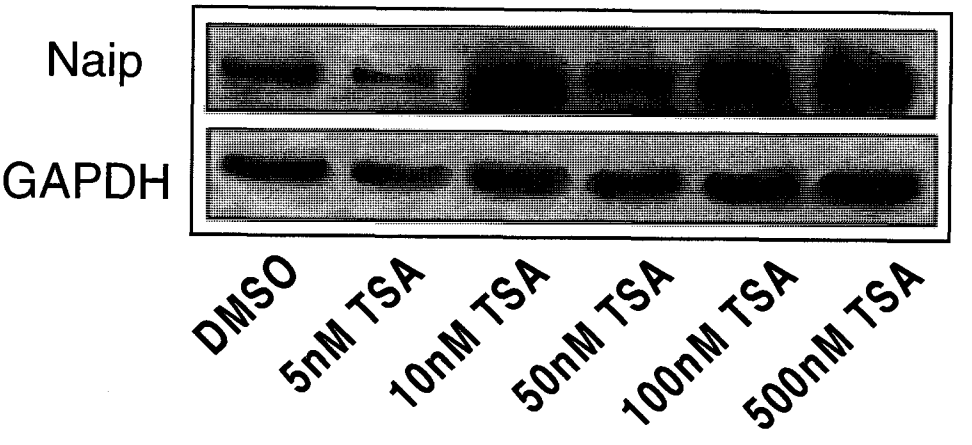
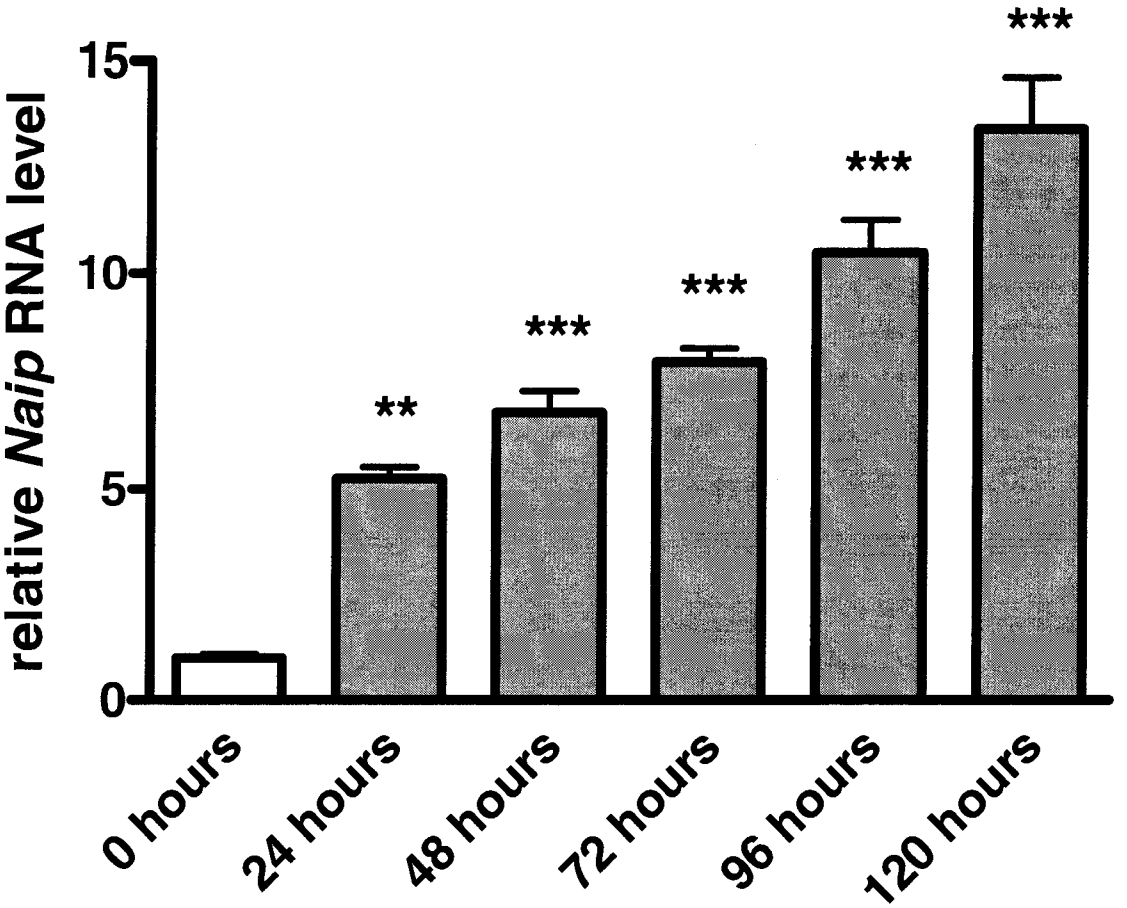

Naip
GAPDH
DMSO
5nM TSA
10nM TSA
50nM TSA
100nM TSA
500nM TSA

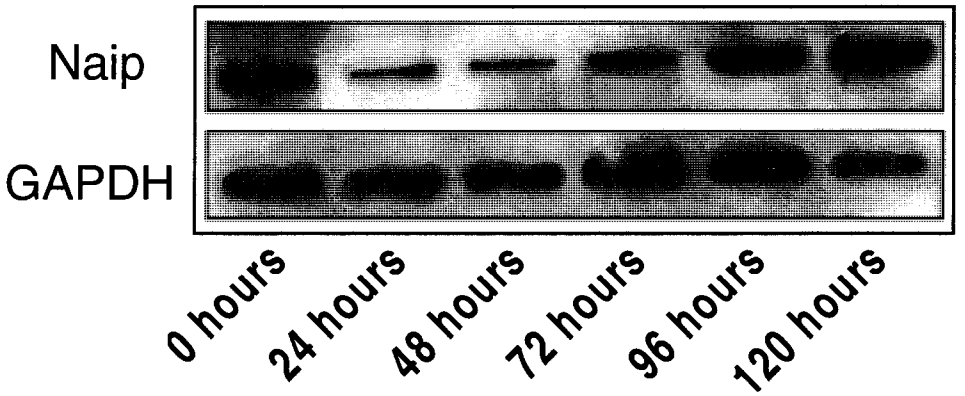
Figure 3.14 – Naip level over a time course of 100nM TSA treatment

Following treatment of N2A cells with 100nM TSA cells were harvested at 0, 24, 48, 72, 96 and 120 hours and Naip level was determined by (A) qRT-PCR and (B) western blotting. Each value represents the mean ± SE (N=3) and the data presented is representative of four independent experiments. Statistical significance with respect to untreated cells: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ as determined by ANOVA and post-hoc Tukey tests.

A


relative Naip RNA level
15
10
5
0
**
***
***
***
***
0 hours
24 hours
48 hours
72 hours
96 hours
120 hours

B


Naip
GAPDH
0 hours
24 hours
48 hours
72 hours
96 hours
120 hours

Differentiation and *Naip* expression

HDAC inhibitors including NaB and TSA induce differentiation in a number of cell types, including neurons [123, 164]. Interestingly, many of the kinase inhibitors that were used in the earlier experiments have also been associated with differentiation, as were a number of the other *Naip* upregulating compounds identified in our original high-throughput screen (A. MacKenzie, personal communication). This raises the possibility that the induction of *Naip* by NaB and TSA as well as the additivity and synergism seen with some of the kinase inhibitors may be occurring as a result of differentiation rather than their direct effects.

This would not be the first demonstrated link between the IAPs and the cell cycle. The yeast and *C. elegans*'s BIR-containing proteins play a critical role in cytokenesis [165, 166]. Similarly survivin, the mammalian IAP most closely related to yeast and *C.elegans,* having only one BIR domain, associates with the mitotic spindle and plays a key role in the G_2/M cell cycle checkpoint [167]. Finally, XIAP, a traditional mammalian IAP, is associated with cell cycle arrest. XIAP has been shown to interact with a number of cell cycle regulators, including p21, MAGE-D1 and NRAGE and its overexpression results in G_0/G_1 arrest in 32D cells [129, 168, 169]. Although increases in NAIP expression have been associated with adipocyte, ovarian granulosa and intestinal epithelial cell maturation, no change in NAIP level has been reported following PC12 cell differentiation, a model of neuronal differentiation [29, 33, 63, 170].

i. The effect of retinoic acid on *Naip* expression

In order to determine if differentiation due to HDACi treatment is potentially inducing *Naip*, transcript and protein levels following differentiation with a non-HDACi

stimulus were examined. Retinoic acid (RA) treatment of N2A cell was used, which is a well established model for the study of differentiation along with its associated morphological and biochemical changes [171-174]. N2A cells display a rounded, flattened morphology with less than 10% of the cells exhibiting neurites. Following treatment with RA, an increase in neutrite extension was observed as early as 6-12 hours with the majority of the cells displaying significant neurite outgrowth by 48 hours. *Naip* RNA levels in N2A cells treated with RA for increasing lengths of time were compared with untreated cell where the level of *Naip* was set to one (figure 3.15-panel A). A time responsive increase in *Naip* RNA expression was seen following treatment, reaching a statistically significant level at 48 hours. Western blotting revealed a concurrent increase in Naip protein level (figure 3.15 – panel B). While *Naip* induction is not necessarily occurring though the same pathway following RA and HDACi treatment, these results indicate that the differentiation associated with the kinase and HDAC inhibitors used in previous experiments may be at least partially responsible for their *Naip* inducing effects.

Cell cycle effects of HDAC inhibitors

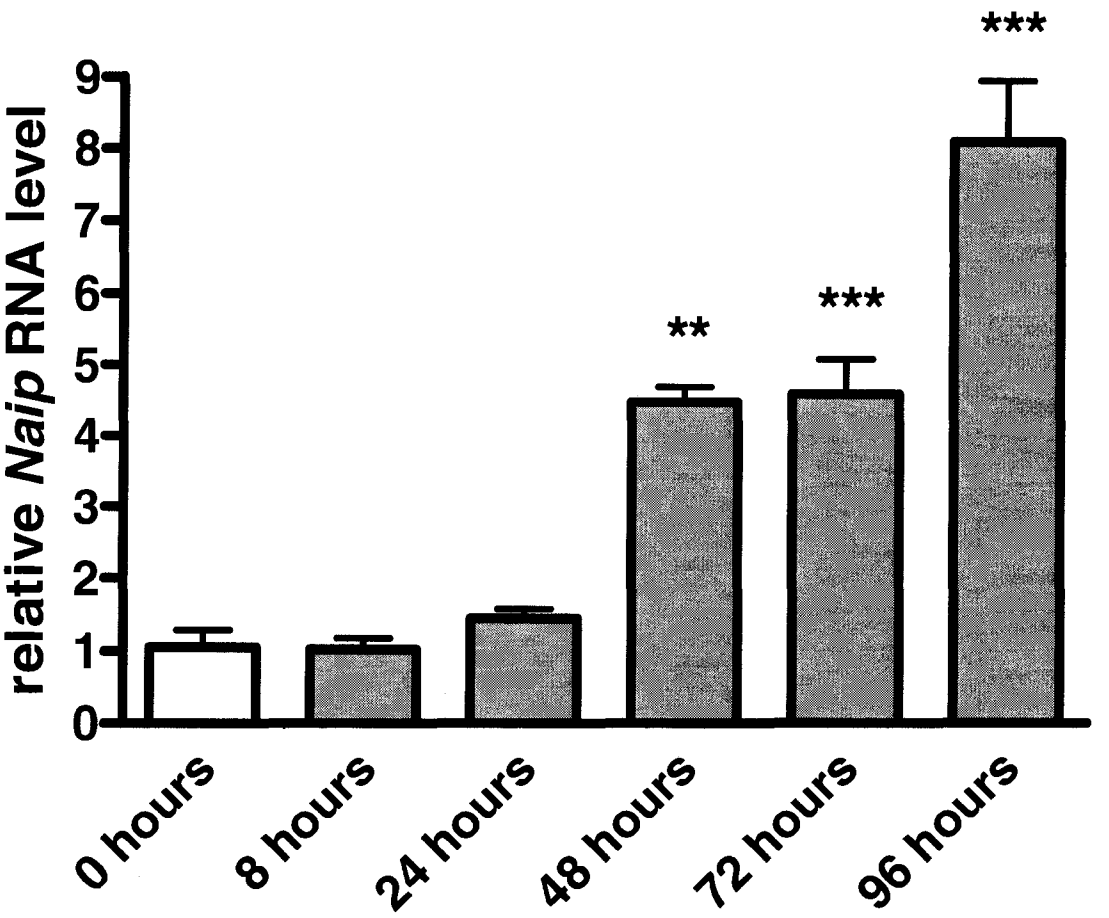
i. Cell cycle arrest following HDACi treatment

Cell cycle arrest following NaB and TSA treatment most commonly occurs in G_1/G_0 but, there have also been reports of arrest in G_2 with both HDAC inhibitors, though not in neuronal models [123, 175-177]. DNA content of propidium iodide (PI) stain N2A cells was analyzed using flow cytometry to determine if and at which stage in the cell cycle arrest is occurring following 2mM NaB and 100nM TSA treatment (figure 3.16). Approximately 55% of untreated N2A cells are found in G_1/G_0, while 29% and 17% are found in S and G_2/M respectively. 24 hours following treatment, a significant increase was observed in the

Figure 3.15 - Naip level in N2A cells over a time course of retinoic acid treatment

Following treatment of N2A cells with 60uM retinoic acid for 0, 8, 24, 48, 72 and 96 hours *Naip* transcript level was determined by (A) qRT-PCR. Following treatment of N2A cells with 60uM retinoic acid for 0, 24, 48, 72, 96 and 120 hours Naip protein level was determined by (B) western blotting. Each value represents the mean ± SE (N=3) and the data presented is representative of three independent experiments. Statistical significance with respect to the untreated control: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ as determined by ANOVA and post-hoc Tukey tests.

A


relative Naip RNA level
0
1
2
3
4
5
6
7
8
9
**
***
***
0 hours
8 hours
24 hours
48 hours
72 hours
96 hours

B

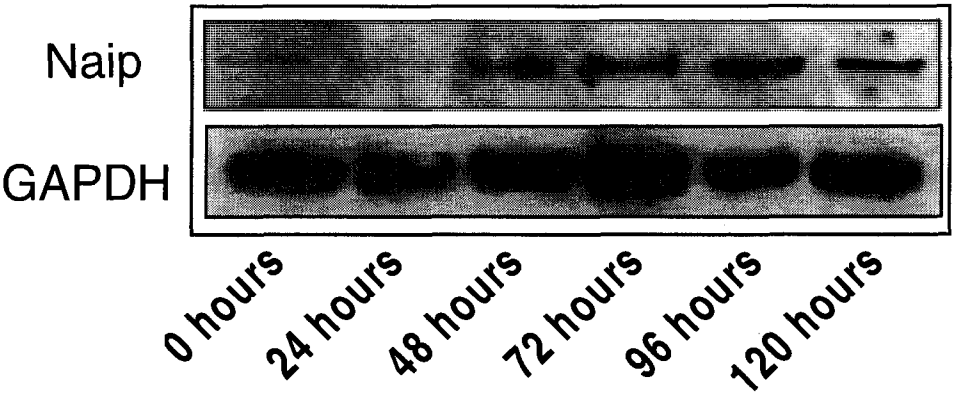
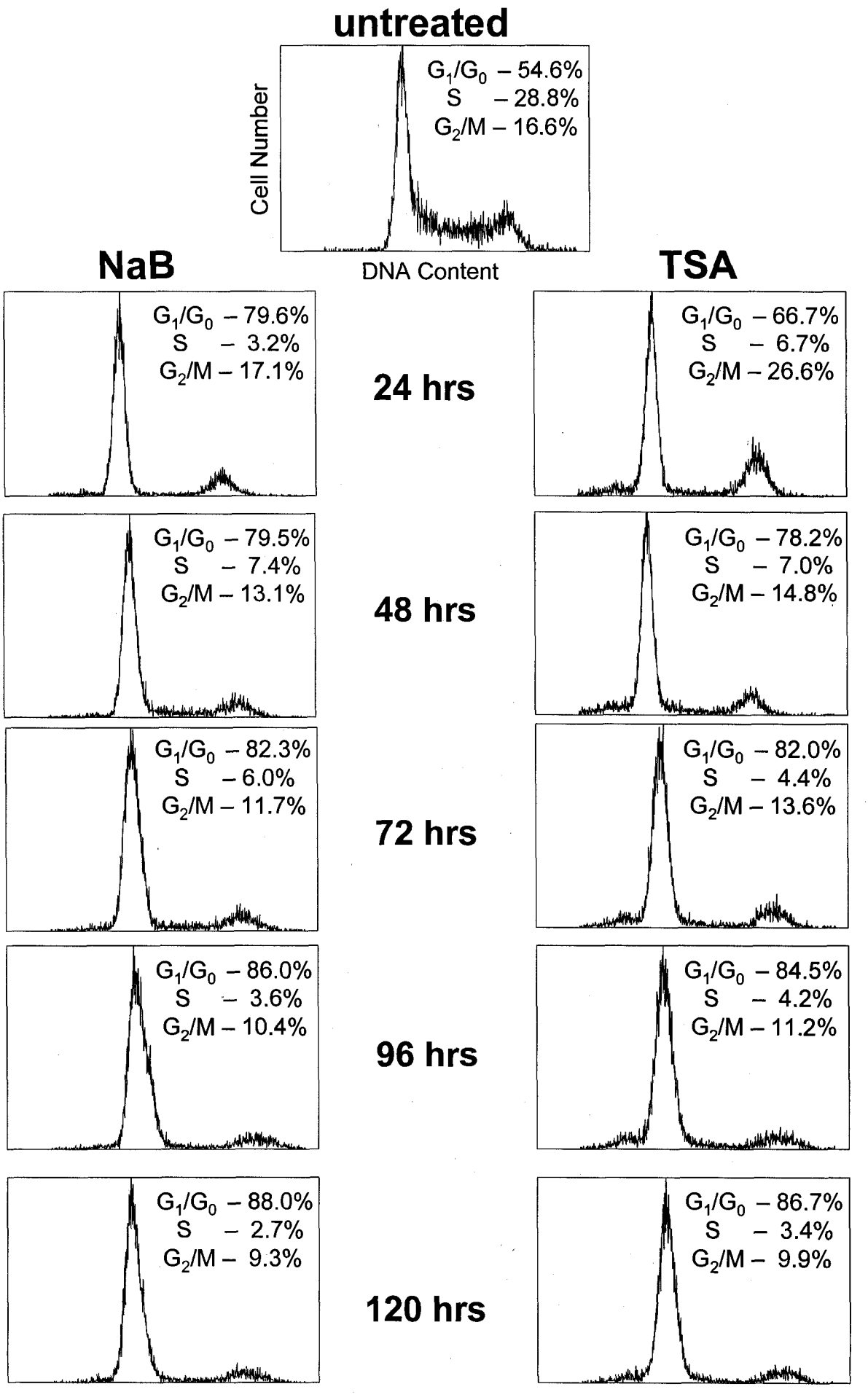

Naip
GAPDH
0 hours
24 hours
48 hours
72 hours
96 hours
120 hours

Figure 3.16 - Flow cytometry analysis of cell cycle stage following a time course of NaB and TSA treatment

Following 0, 24, 48, 72, 96 or 120 hours of treatment with 2mM NaB or 100nM TSA N2A cells were harvested, fixed and stained with PI for flow cytometry analysis. Results are presented as cell number vs. DNA content for 10000 cells and are representative of 3 individual experiments.


untreated
Cell Number
G1/G0 – 54.6%
S – 28.8%
G2/M – 16.6%
DNA Content
NaB
TSA
G1/G0 – 79.6%
S – 3.2%
G2/M – 17.1%
24 hrs
G1/G0 – 66.7%
S – 6.7%
G2/M – 26.6%
G1/G0 – 79.5%
S – 7.4%
G2/M – 13.1%
48 hrs
G1/G0 – 78.2%
S – 7.0%
G2/M – 14.8%
G1/G0 – 82.3%
S – 6.0%
G2/M – 11.7%
72 hrs
G1/G0 – 82.0%
S – 4.4%
G2/M – 13.6%
G1/G0 – 86.0%
S – 3.6%
G2/M – 10.4%
96 hrs
G1/G0 – 84.5%
S – 4.2%
G2/M – 11.2%
G1/G0 – 88.0%
S – 2.7%
G2/M – 9.3%
120 hrs
G1/G0 – 86.7%
S – 3.4%
G2/M – 9.9%

percentage of cells found in G_1/G_0. NaB treatment increased the percentage to 80% and TSA resulted in an increase to 67%. The transition to G_1/G_0 is slightly delayed in TSA treated cells, which display a greater number of cells than NaB treated cells in G_2/M until 96 hours. Following 120 hours of treatment with either HDACi, the ratios are similar, with close to 90% of all cells being found in G_1/G_0.

ii. *p21* and *E2f1* expression following HDACi treatment

Progression through the cell cycle is regulated by the cyclin, cyclin-dependent kinase (CDK), and CDK inhibitor (CDKI) families [178]. Of interest here is the G_1 – S checkpoint: following mitogenic stimuli, the retinoblastoma (Rb) protein becomes phosphorylated by the cyclinD-CDK4/6 complexes, leading to its release from E2F and allowing for the derepression and transactivation of the E2F responsive genes required for the transition to S phase. p21 is a member of the CKDI Kip/Cip family, whose primary responsibility is the regulation of this process at the G_1-S checkpoint [179, 180]. Not surprisingly, p21 is required for the differentiation of many cell types and for cell cycle arrest in response to HDACi or RA treatment [123-125, 173, 181-185]. In addition to its role in cell cycle regulation, p21 can act as a highly specific regulator of transcription. A cDNA microarray analysis of HT1080 human fibrosarcoma cells transfected with an IPTG inducible p21 construct revealed a total of 77 repressed and 54 induced genes. These changes are attributed directly to p21 and not the associated cell cycle arrest [186].

HDAC inhibitors induce p21 in a p53 independent and gene specific manner through the cooperation of two mechanisms. The *p21* promoter has a low acetylation of histones in the steady state, which favors a condensed chromatin structure and therefore an inactive promoter. The inhibition of HDACs allows the histone acetyltransferase p300 to increase

histone acetylation, which results in chromatin opening, a more favorable conformation for transcription [185]. Secondly, the *p21* promoter has a cluster of six specificity protein 1 (Sp1) sites between -112 and -52 which are involved in both the basal and inducible transcription of the gene. HDAC inhibitors induce Sp1 acetylation, increasing its DNA binding activity as well as inducing *Sp1* transcription itself [133, 134].

E2F1 is a transcription factor which acts to induce the expression of genes required for S phase entry and DNA synthesis as its primary role [187]. Along with the p21 induction occurring with HDACi mediated G_1/G_0 arrest, there is a characteristic downregulation of E2F1 at the gene transcription, protein expression and activity levels [127]. Interestingly, in addition to cell cycle arrest and differentiation, HDAC inhibitors have been shown to induce apoptosis in numerous cell lines including N2A cells. This induction of apoptosis is dependent upon the direct activation and induction of E2F1; blocking it leads to significantly reduced cell death [126, 188, 189].

Given the well documented changes in p21 and E2F1 following NaB and TSA treatment, the next logical step was to confirm that they were occurring under the conditions used in the present study. While the concentration at which HDAC inhibitors induce apoptosis is typically higher than those used in the previous experiments, it is nonetheless important to determine if E2f1 is being induced or suppressed following treatment with the 2mM NaB or 100nM TSA. Considering that G_1/G_0 arrest has already been confirmed, an induction of p21 and a downregulation of E2f1 at both the transcript and protein levels were expected.

When *p21* transcript levels were compared with those of untreated N2A cells in which the *p21* transcript level is set to one, 2mM NaB or 100nM TSA treated N2A cells exhibited a significant 5-7 fold upregulation at 24 hours and remained elevated at all time

points assessed (figure 3.17 – panel A). Western blotting revealed a concurrent increase in p21 protein level although the levels appear to taper off slightly at 96 hours in TSA treated cells (figure 3.17 – panel B). This increase in p21 following NaB or TSA treatment is consistent with previous reports and supports the hypothesis that the cell cycle arrest associated with HDACi treatment may be responsible for the ensuing *Naip* induction. *E2f1* transcript levels in untreated N2A cells were set to one and compared with N2A cells following 2mM NaB or 100nM TSA treatment (figure 3.18 – panel A). Following treatment with either HDACi, a significant downregulation to *E2f1* RNA to 0.3-0.4 fold of its basal levels was observed at 24 hours and remained low at all time points assessed. Western blotting revealed a concurrent decrease in E2f1 protein level (figure 3.18 – panel B). The downregulation of E2f1 is consistent with previous studies and was expected given the observed G_1/G_0 arrest. The downregulation and absence of significant changes in cell viability also indicates that the HDACi dosages at which *Naip* is induced are not sufficient to cause E2f1 induced apoptosis.

Investigation of the role of HDACi mediated cell cycle effects in the regulation of Naip expression

The presence of multiple murine Naip isoforms and their tissue specific expression patterns suggests that Naip plays roles beyond that of the inhibition of apoptosis. Naip has also been associated with the innate immune response, where its NOD and LRR domains play a part in pathogen recognition [82]. A role for Naip in cell cycle arrest and differentiation is an intriguing one to consider particularly in the case of neurons where cell cycle re-entry following cellular stress has been observed leading to cell death, suggesting that a cell cycle mediated induction of Naip may be a mechanism by which the cell could be rescued.

Figure 3.17 - p21 levels in N2A cells over a time course of NaB or TSA treatment

Following treatment of N2A cells with 2mM NaB or 100nM TSA cells were harvested at 0, 24, 48, 72, 96 and 120 hours and p21 level was determined by (A) qRT-PCR and (B) western blotting. Each value represents the mean ± SE (N=3) and the data presented is representative of three independent experiments. Statistical significance with respect to untreated cells: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ as determined by ANOVA and post-hoc Tukey tests.

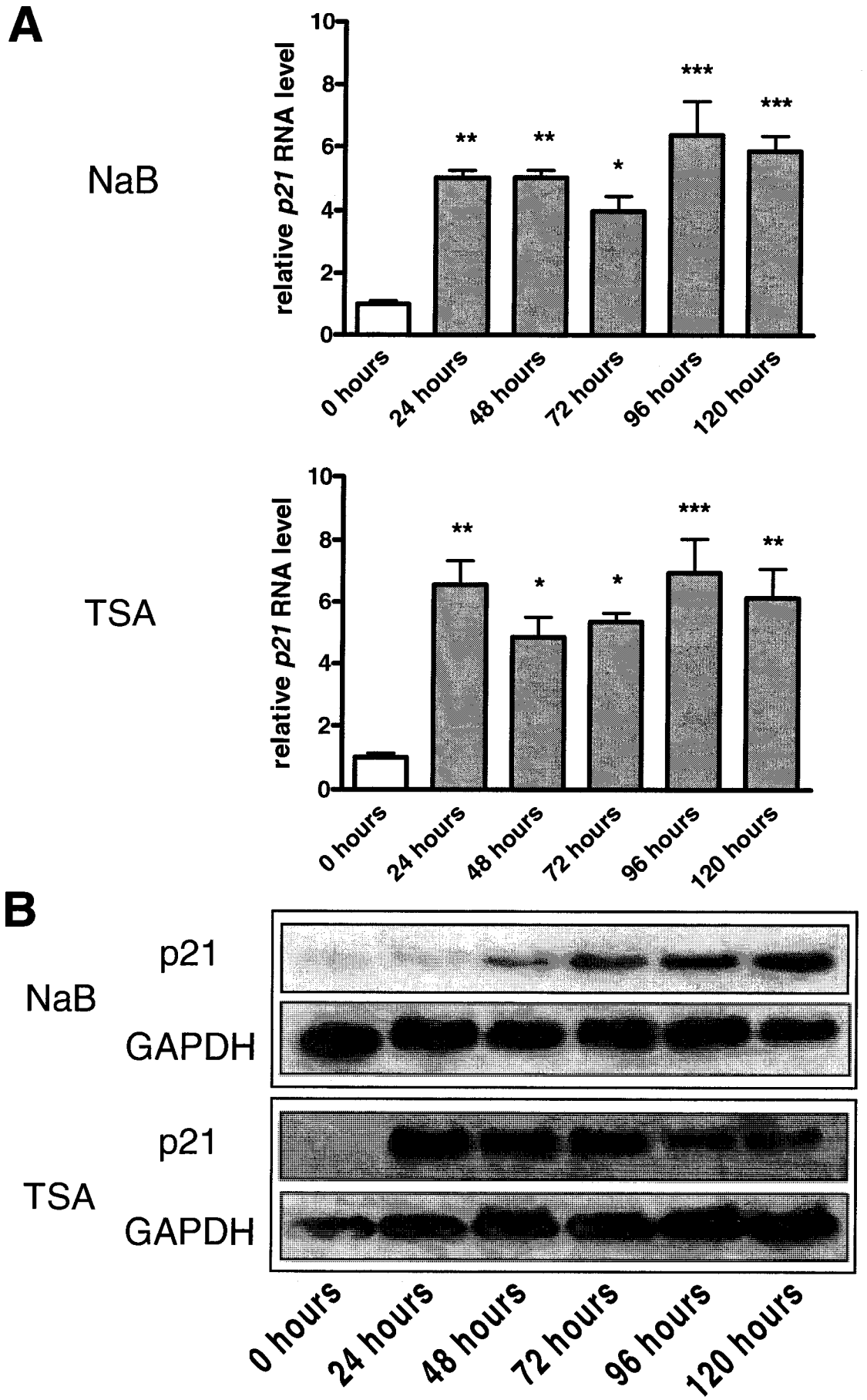
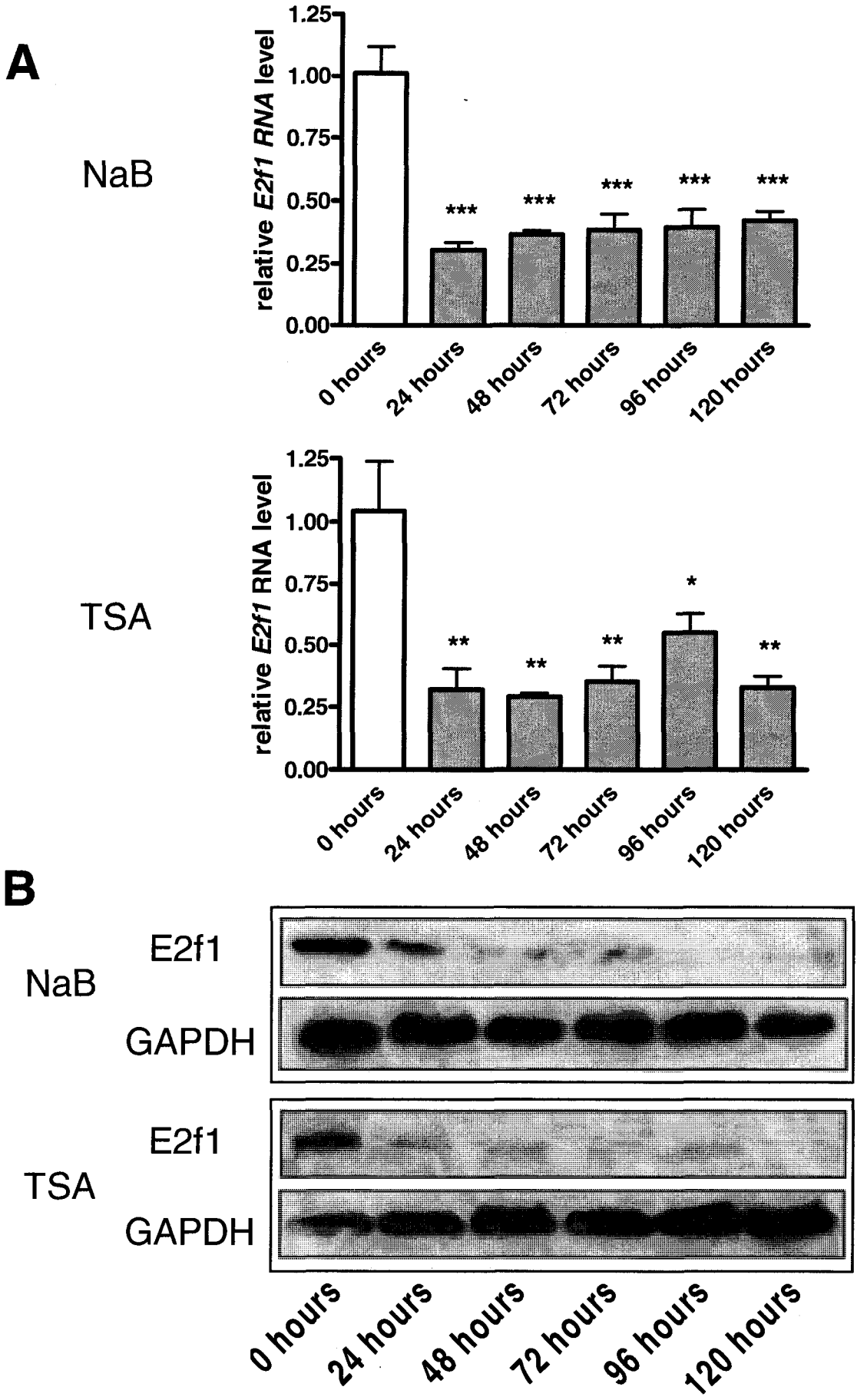

A
NaB
relative p21 RNA level
10
8
6
4
2
0
**
**
*
***
***
0 hours
24 hours
48 hours
72 hours
96 hours
120 hours
TSA
relative p21 RNA level
10
8
6
4
2
0
**
*
*
***
**
0 hours
24 hours
48 hours
72 hours
96 hours
120 hours
B
p21
NaB
GAPDH
p21
TSA
GAPDH
0 hours
24 hours
48 hours
72 hours
96 hours
120 hours

Figure 3.18 - E2f1 levels in N2A cells over a time course of NaB or TSA treatment

Following treatment of N2A cells with 2mM NaB or 100nM TSA cells were harvested at 0, 24, 48, 72, 96 and 120 hours and E2f1 level was determined by (A) qRT-PCR and (B) western blotting. Each value represents the mean ± SE (N=3) and the data presented is representative of three independent experiments. Statistical significance with respect to untreated cells: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ as determined by ANOVA and post-hoc Tukey tests.


A
NaB
relative E2f1 RNA level
1.25
1.00
0.75
0.50
0.25
0.00
***
***
***
***
***
0 hours
24 hours
48 hours
72 hours
96 hours
120 hours
TSA
relative E2f1 RNA level
1.25
1.00
0.75
0.50
0.25
0.00
**
**
**
*
**
0 hours
24 hours
48 hours
72 hours
96 hours
120 hours
B
NaB
E2f1
GAPDH
TSA
E2f1
GAPDH
0 hours
24 hours
48 hours
72 hours
96 hours
120 hours

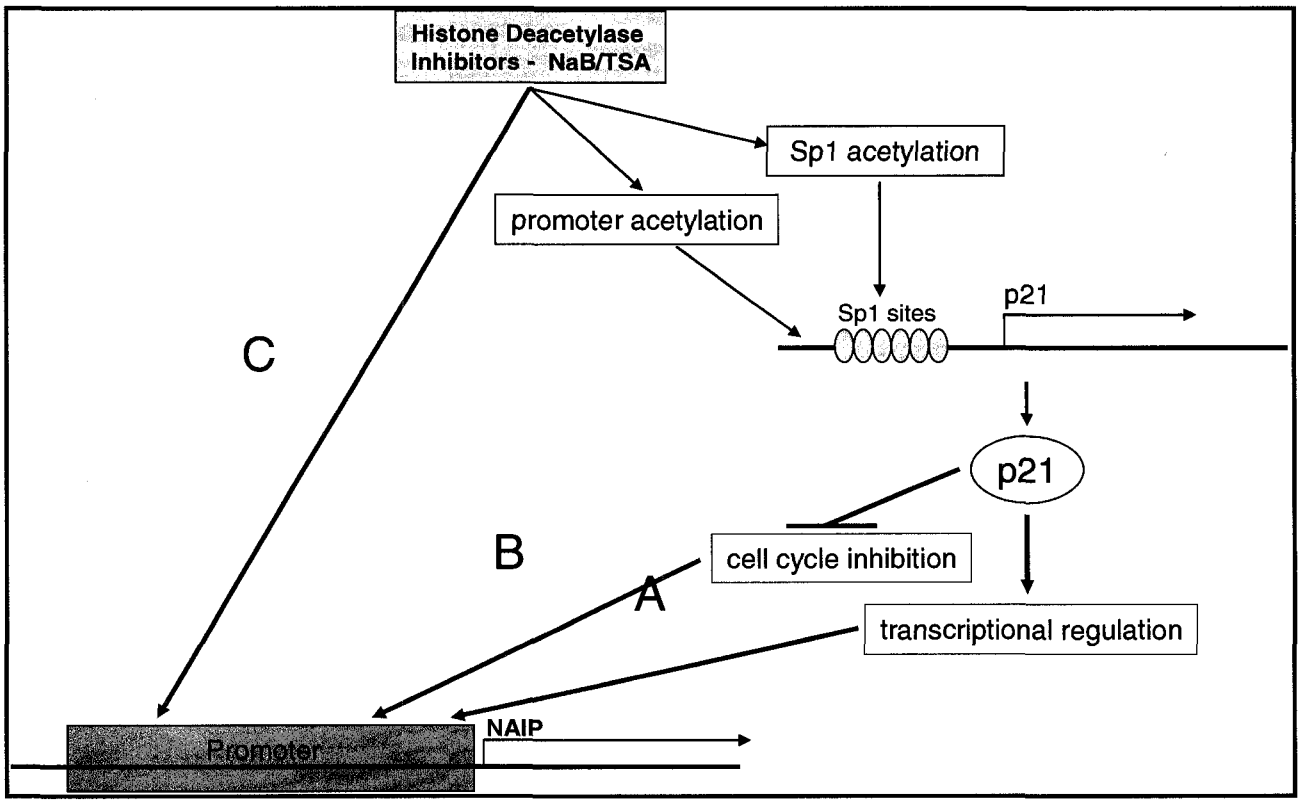
The mechanism by which *Naip* induction is occurring in response to cell cycle arrest and withdrawal is unknown. Interestingly, increased NAIP expression has been linked with the differentiation of many cell types shown to differentiate in a p21 dependent manner. Our laboratory has also shown that NAIP expression matches that of p21 in the intestine where differentiation of cells in the gastrointestinal epithelium occurs progressively from the base of the crypts (immature pluripotent stem cells) to the upper crypt region (terminally differentiated cell) with both p21 and NAIP being restricted to the non proliferative compartments of the intestinal villi [33]. Likewise, experiments in the current study have confirmed G_1/G_0 cell cycle arrest associated with p21 induction, E2f1 downregulation and Naip induction following HDACi treatment. These results suggest that *Naip* induction by HDACi treatment may be regulated by: A) p21 mediated transcription; B) p21's cell cycle effects; or C) the same mechanisms regulating p21 expression. A model outlining this hypothesis can be seen in figure 3.19.

i. p21 regulatory pathways

To assess whether *Naip* is regulated by the same factors as p21, we must first look at how p21 is regulated. As discussed earlier, p21 induction in response to HDAC inhibitors is reported to occur through a combination of histone acetylation and Sp1 activation, with Sp1 also being important in the regulation of its basal expression [133, 185]. Sp1 is a ubiquitously expressed transcription factor which binds to GC rich regions of the DNA through its C-terminal zinc fingers as stacked tetramers. Sp1 tetramers can interact with many proteins to promote or repress transcription. In the neuronal context, Sp1 is generally considered a pro-survival transcription factor [117, 134]. Sp1 is interesting to consider not only with respect to its ability to regulate p21 expression but also from the perspective that it

Figure 3.19 - Proposed model of the regulation of HDACi mediated *Naip* induction by p21

Black arrows represent known events outlined in the literature and red arrows represent proposed regulatory events: A) *Naip* under p21 mediated transcriptional control, B) *Naip* regulated by p21 mediated cell cycle arrest and C) *Naip* under the same HDACi mediated regulatory pathway as p21.


Histone Deacetylase Inhibitors - NaB/TSA
Sp1 acetylation
promoter acetylation
Sp1 sites
p21
C
p21
B
cell cycle inhibition
A
transcriptional regulation
Promoter
NAIP

may directly regulate *Naip's* expression. An examination of the *Naip* promoter reveals several potential Sp1 sites, and previous *Naip* promoter work has revealed the presence of a GC box [20]. Sp1 is also involved in the regulation of *Naip*'s neighboring gene, *SMN*, where its role varies depending on the differentiation state of the cell [190].

Sp1 transcript and protein levels have previously been reported to increase following HDACi treatment and were therefore assessed following NaB or TSA treatment to confirm if this was occurring in N2A cells (figure 3.20 – panel A and B). Unexpectedly, no significant change in transcript or protein level was observed. Sp1 itself is an Sp1 inducible gene and as such an examination of its own transcript and protein levels should allow us to determine if it has been activated. An Sp1 inducible luciferase reporter construct was used to directly confirm the absence of Sp1 activation in this model [191]. A comparison of the relative Sp1 activity level in untreated, NaB or TSA treated cells revealed no significant differences. These results demonstrate that Sp1 is not induced or activated following NaB or TSA treatment in this model system, and indicate that if it plays a role in HDACi mediated *Naip* induction, basal levels of activity are sufficient. Further experiments are required to determine if Sp1 plays a critical role in the transcriptional regulation of *Naip* expression. Promoter studies using reporter constructs with the GC box and/or putative Sp1 sites deleted would be useful in clarifying this matter.

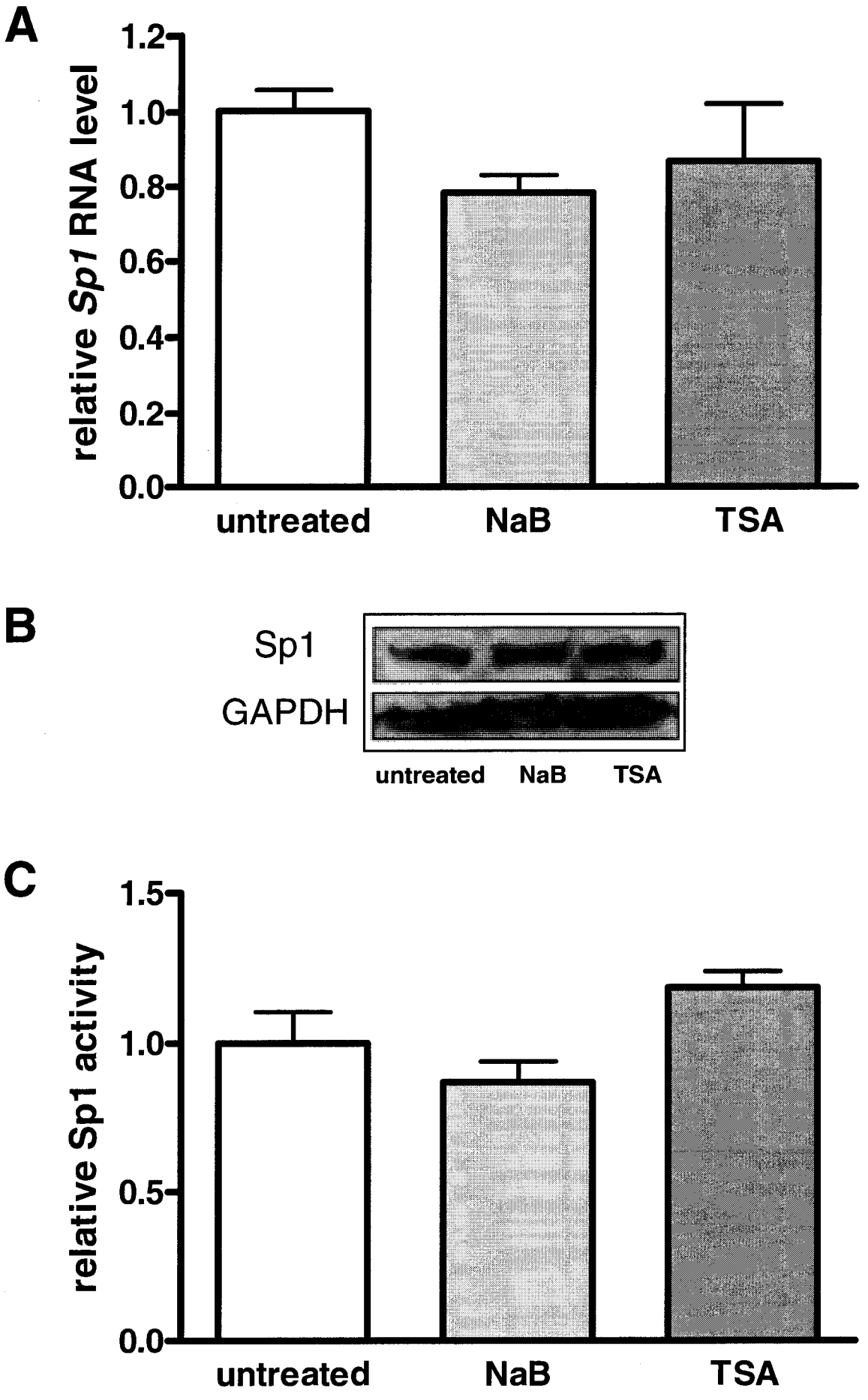
ii. p21 and p21 mediated cell cycle arrest

In order to assess the role p21 itself was playing in the HDACi mediated induction of *Naip,* knockdown of p21 at the protein level was desirable. The initial approach taken was to use siRNA against p21 to achieve knockdown. Afterward HDACi treatment would be administered and *Naip* level determined. Through extensive optimization of the siRNA and

Figure 3.20 - Sp1 expression and activity following NaB or TSA treatment

Panel A and B - Following treatment with 2mM NaB or 100nM TSA for 48 hours the level of Sp1 RNA (A) or protein (B) in N2A cells was determined by qRT-PCR and western blotting respectively. Each value represents the average ± SE (N=3) and the data presented is representative of four independent experiments. Statistical significance with respect to the untreated (*) cells: *$p<0.05$ as determined by ANOVA and post-hoc Tukey tests.

Panel C - Following transfection of N2A cells with Sp1 inducible firefly luciferase and SV40 renilla luciferase reporter constructs, cells were treated with 2mM NaB or 100nM TSA for 48 hours and relative Sp1 activity was determined by dual luciferase assay. Each value represents the mean ± SE (N=5) and is representative of 3 independent experiments. Statistical significance with respect to the untreated N2A (*) cells: * $p<0.05$ as determined by ANOVA and post-hoc Tukey tests.


A
relative Sp1 RNA level
1.2
1.0
0.8
0.6
0.4
0.2
0.0
untreated
NaB
TSA
B
Sp1
GAPDH
untreated
NaB
TSA
C
relative Sp1 activity
1.5
1.0
0.5
0.0
untreated
NaB
TSA

transfection reagents/conditions, excellent knockdown of *p21* at the RNA level was obtained. However, little to no change was obtained at the protein level (data not shown) and as such using this approach yielded no useful clarification of p21's role in HDACi mediated *Naip* induction.

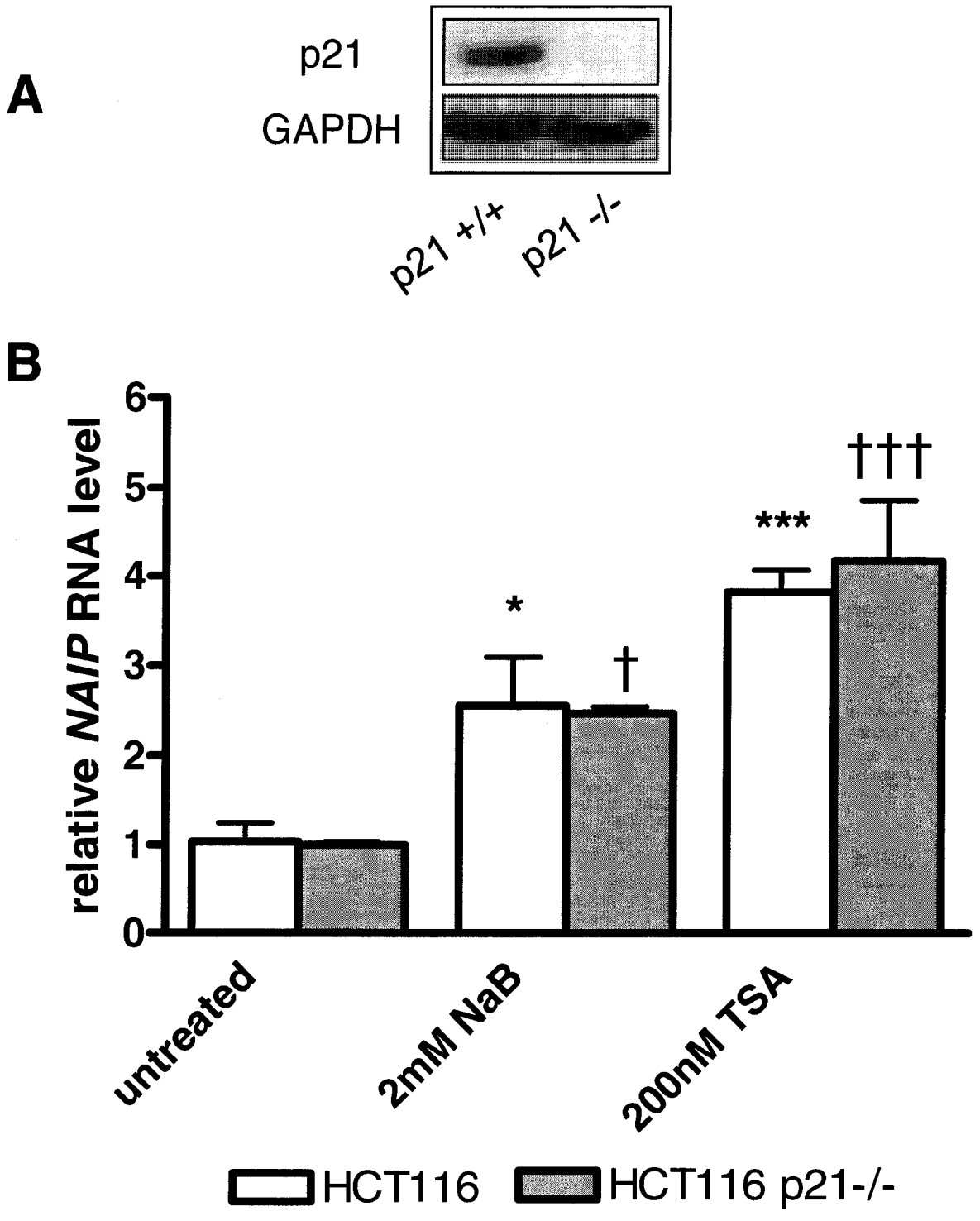
Given the inability to obtain significant knockdown of p21 using siRNA, another approach was required to determine its role in HDACi mediated *Naip* induction. The HCT 116 cell line is a human colon caner cell line in which a p21 null daughter cell line has been created by Dr. Bert Vogelstien's lab using homologous recombination [135]. HDACi treatment of the wildtype HCT116 cell line in previous studies revealed characteristic responses including cell cycle arrest, decreased E2F1 activity and p21 induction [127, 192]. Additionally, studies in the p21 null daughter cell line have shown that they lack a G_1 check point and therefore when cell cycle arrest is induced all cells accumulate in G_2 [135]. This cell line therefore represents a good tool to assess both the role of p21 and also the cell cycle arrest it induces in HDACi mediated *Naip* induction.

The wildtype and p21 null HCT116 cell lines were obtained from Dr. Bruce MacKay's lab, and the absence of p21 expression confirmed by western blotting (figure 3.21 – panel A). Both cell lines were treated with 2mM NaB or 200nM TSA and *NAIP* RNA level was assessed using qRT-PCR (figure 3.21 – panel B). The *NAIP* transcript level was set to one in the untreated cells of both the wt and p21 null lines. Treatment of the wt and p21 null cell lines resulted in significant and comparable increases in *NAIP* level of 2-5 fold with NaB and TSA. These results confirm that HDACi mediated *NAIP* induction is not dependent on p21 mediated transcription or the G_1/G_0 cell cycle arrest which it induces.

Figure 3.21 - HDACi treatment of wildtype and p21 null HCT 116 cells

Panel A - Western blotting for p21 and GAPDH in wildtype and p21 null HCT 116 cells.

Panel B - Following treatment of wildtype and p21 null HCT 116 cells with 2mM NaB or 200nM TSA cells were harvested at 48 hours and *NAIP* transcript level was determined by qRT-PCR. Each value represents the mean ± SE (N=3) and the data presented is representative of 4 independent experiments. Statistical significance with respect to untreated cells: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ as determined by ANOVA and post-hoc Tukey tests.


A
p21
GAPDH
p21 +/+
p21 -/-
B
relative NAIP RNA level
0
1
2
3
4
5
6
*
†
***
†††
untreated
2mM NaB
200nM TSA
HCT116
HCT116 p21-/-

Summary

The kinase inhibition experiments demonstrated that PI3K, PKC, Akt, PKG, mTOR and IKK are not required for NaB mediated *Naip* induction. The PI3K and PKC inhibitors, LY294002 and rottlerin, did induce *Naip* transcription on their own and in an additive manner when combined with NaB. NaB was shown to inhibit NF-κB activity. The inhibition of IKK and the direct inhibition of NF-κB activity by *adeno-DN-NIK* infection were able to synergistically enhance the *Naip* induction observed in the presence of NaB, but had no effect on their own. Interestingly, NF-κB activation by *adeno-NIK* did not alter basal or NaB mediated *Naip* levels. The effects of NaB on *Naip* transcription were demonstrated to be receptor independent using specific antagonists for the G_α subunits of their GPCRs. TSA, a potent HDACi, was also shown to induce *Naip* expression, shifting the focus to the elucidation of the mechanism of HDACi mediated *Naip* induction. HDAC inhibitors are known to induce cell cycle arrest and differentiation in a p21 dependent manner, both of which have previously been associated with *Naip* induction [122-124]. G_1/G_0 cell cycle arrest following NaB and TSA treatment was confirmed in N2A cells along with the associated p21 induction and E2f1 downregulation at both the RNA and protein levels. No change in Sp1 expression or activation was observed; however, further research is required to rule out its involvement in the regulation of *Naip* expression. Finally, NaB and TSA mediated *NAIP* induction was shown to be p21 independent using the p21 null HCT 116 cell line. At present it appears that HDACi mediated *Naip* induction is regulated primarily at the level of histone acetylation.

CHAPTER 4 - DISCUSSION

The regulation of NAIP as an anti-apoptotic therapy is an attractive target for a number of neurodegenerative diseases and CNS trauma. Its promise has been shown in multiple *in vitro* and *in vivo* models. However, the development of therapeutic strategies relies on the complete biochemical characterization of the gene and its product, which remains incomplete. Preliminary studies in our laboratory and the present study aims to elucidate the transcriptional regulation of the *Naip* gene in an effort to better understand Naip and promote therapeutic development.

In a preliminary study conducted in our laboratory, an examination of the mechanisms by which *Naip* upregulating agents identified in our high throughput screen act revealed a striking commonality, GPCR mediated signal transduction [118]. Our selected inducing compound, NaB, has been shown to act through GPCR41 and 43 and more specifically the $G_{i/o}$, G_q and G_{12}, G_α subunits [115, 116]. Additionally, the inhibition of NaB mediated *Naip* induction by the broad-spectrum kinase inhibitor H-7 further supported the hypothesis that GPCR mediated signaling through PKC and/or PKG is important in the regulation of *Naip* expression. NaB mediated signaling through GPCRs can induce NF- κB; while the exact signaling pathway remains to be elucidated PI3K and Akt have been implicated [150]. This is significant since we observed an increase in IKKα expression with NaB treatment and IKKα mediates the release of NF-κB from its inhibitor. Also, NF-κB activity is required for the transcription of other IAP family members [104, 118]. Therefore, the present study initially focused on GPCR mediated signaling in response to NaB treatment to determine the mechanisms by which it induced *Naip* transcript levels with the expectation that NF- κB may play a role. A thorough analysis of these pathways of interest using a panel

of specific kinase inhibitors demonstrated that PI3K, PKC, Akt, mTOR, PKG and IKK activity are not required for NaB mediated *Naip* induction.

The tissue specific pattern of expression of NAIP and the apparent specificity of its various murine isoforms suggests that it may be subject to cell type specific regulatory mechanisms. This has also been alluded to in studies of its various cell type specific roles; for example, the receptor meditated anti-apoptotic effect of progesterone on ovarian granulosa cell survival which has been linked to NAIP expression, is PKG dependent [29, 148]. Additionally, adipocyte differentiation requires RSK p70 activation, a process mediated by mTOR, and leads to increases in NAIP expression, both of which are inhibited in the presence of rapamycin, an mTOR inhibitor [63]. While we found that both PKG and mTOR were not required for NaB mediated *Naip* induction in this neuronal model, it would be of interest to further investigate their role in ovarian granulosa cells and adipocytes respectively.

Interestingly, neither an attenuation of NaB mediated *Naip* induction nor a decrease in its basal expression has been observed. The only exception is the H-7 experiment in the preliminary study. This experiment was, however, likely inaccurate as I was unable to replicate it (data not shown) and the inhibition experiments for the individual kinases that H-7 targets were also inconsistent with the initial observation. This lack of decreased or attenuated *Naip* expression suggests the possibility that the gene exists in a repressed state and that the increase in *Naip* transcript level observed following NaB treatment is the result of derepression. This hypothesis is consistent with a prior report that there are two silencer elements located in the -1791/-339 and +8/+226 regions of the human *NAIP* gene [20]. Results observed with a subset of the specific kinase inhibitors in the present study provide further support to this model.

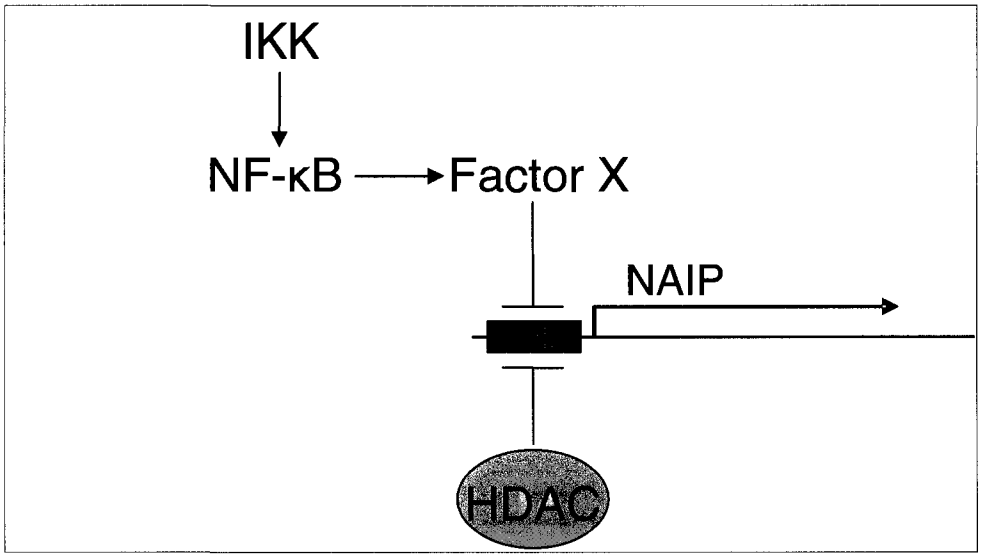
Treatment of N2A cells with either the PI3K or PKC inhibitor alone resulted in an induction of *Naip* RNA. This effect was additive in the presence of NaB (figure 3.3 and 3.4). The additive rather than synergistic effects seen in the presence of NaB indicate that they are part of an alternative *Naip* regulatory pathway which is independent of NaB. Nevertheless, inhibition of these kinases promotes *Naip* induction and therefore suggests that in their active state they mediate the inhibition of the gene. PI3K can directly activate PKC and thus they may be part of the same regulatory pathway; treatment with both inhibitors would serve to further clarify this [140]. Additionally, a panel of PKC inhibitors or specific siRNAs would be useful to narrow down the specific isoforms which are involved in *Naip* regulation.

Treatments which inhibit NF-κB activity in N2A cells do not alter *Naip* transcript levels alone. In the presence of NaB, however, a synergistic increase in *Naip* level is observed (figure 3.8 and 3.11). It is unlikely that *Naip* is directly inhibited by NF-κB as an examination of its promoter does not indicate the presence of κB binding sites. This should be confirmed experimentally. It is more likely that NF-κB regulates the expression or activity of a transcription factor which represses the transcription of *Naip*. Interestingly, the induction of NF-κB following *adeno-NIK* infection does not reduce *Naip* transcript levels and is unable to attenuate the NaB mediated induction, indicating that NaB mediated *Naip* induction is not dependent upon NF-κB inhibition. *Adeno-DN-NIK* infection alone was able to significantly inhibit NF-κB activity but resulted in no significant change in *Naip* expression, indicating that the NaB mediated NF-κB inhibition observed (figure 3.9) is not the primary mechanism by which it regulates *Naip* induction (see figure 4.1 for a model). Therefore, other NaB mediated effects were examined to fully understand its *Naip* inducing properties.

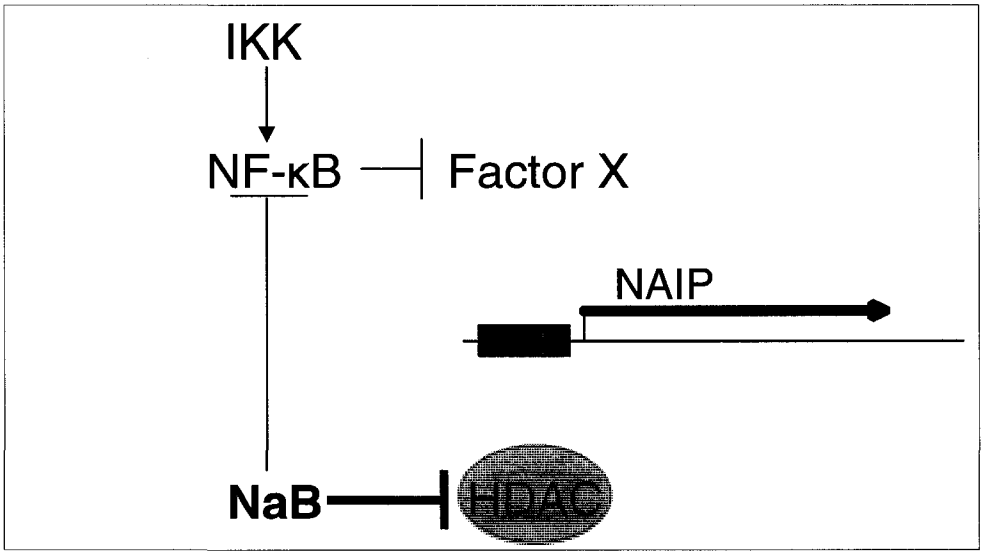
Figure 4.1 - Model of *Naip*'s transcriptional repression and NaB mediated derepression

Model of *Naip* in its hypothesized repressed state where it is under the inhibion of an unknown factor regulated by NF-kB and HDACs resulting in only basal levels of its expression (panel A). When treated with NaB the inhibition with both elements is relieved resulting in derepression of the *Naip* gene (panel B).

A - Transcriptional Repression



B - Transcriptional Derepression



Overall, these results were unexpected for two main reasons. It was expected that NF-κB would be activated in response to NaB treatment as the ineffectiveness of HDAC inhibitors as anticancer agents has been attributed to NF-κB activation and the resultant increase in anti-apoptotic signaling [150]. However, the proteasome plays an important role in the activation of NF-κB by degrading its inhibitor, IκB; and recent studies have demonstrated that HDAC inhibitors are able to suppress proteasome activity thereby preventing NF-κB activation [154, 193]. HDACi regulation of the proteosome is also reported to be affected by the differentiation status and proliferative capacity of the cells, which accounts for the original observation of NF-κB activation [193]. Regulation of the proteasome is not the only mechanism by which NaB regulates NF-κB activity; it has also been shown to impair IKK activity [194]. Therefore, the observation that NaB effectively inhibits NF-κB activity in N2A cells contributing to its ability to induce *Naip* is not inconsistent with the literature. Second, it has been suggested in a number of studies that *Naip* transcription is regulated by NF-κB in a similar manner to XIAP and cIAP1/2. This hypothesis stems from the association between increased NAIP levels and cell survival in response to NF-κB activating compounds or apoptotic stimuli. The role of NF-κB in the induction of other IAP family members makes this a logical hypothesis, but a causal role for NF-κB in the regulation of *NAIP* expression has never been confirmed. While NAIP is a member of the IAP family displaying the expected antiapoptotic function, it is also unique with respect to its role in pathogen recognition and multiple murine isoforms. As such, having a regulatory mechanism distinct from other members of the IAP family is not unreasonable or unexpected.

Interestingly, NF-kB activity is also attenuated in response to other *Naip* inducing compounds identified in our high through-put screen, including genistein and cAMP [195,

196]. This suggests that a downregulation of NF-κB activity may play a more universal role in *Naip* regulation and be important for all *Naip* inducers. It would be of interest to determine what role the inhibition of NF-κB activity is playing in genistein or cAMP mediated *Naip* induction.

While the SCFA GPCRs have been identified in the nervous system, and signaling through them in PC12 cells alters neurotransmitter-related gene expression, it remained to be seen if the NaB mediated *Naip* induction observed in N2A cells is receptor mediated [116, 156]. Antagonists for the specific Gα subunits implicated in SCFA signaling were able to effectively inhibit NaB mediated ERK phosphorylation but not *Naip* induction (figure 3.12) [160]. Having ruled out the involvement of NaB's GPCR mediated signaling a greater role for its HDACi properties was suggested. Consistent with this hypothesis, I have demonstrated that Trichostatin A (TSA), a potent HDACi, is also able to induce *Naip* transcription (figure 3.13 and 3.14). Furthermore, TSA is a more potent HDACi than NaB and the *Naip* induction observed with TSA is of a greater magnitude.

CNS trauma has been shown to induce the accumulation of cell cycle proteins which initiate proliferation in mitotic cells such as astrocytes; however, in post-mitotic neuronal cells they initiate caspase dependent apoptosis [197]. One of the earliest established properties of HDACi was their ability to induce cell cycle arrest and differentiation [123]. It was therefore intriguing to consider the possibility that *Naip* expression could be cell cycle regulated to afford cells some protection against the stresses of differentiation or cell cycle re-entry in response to trauma. Confirmation of the induction of *Naip* in response to the non-HDACi differentiation stimulus, retinoic acid, supports this theory (figure 3.16).

Consistent with prior reports, G_1/G_0 cell cycle arrest, p21 induction and E2f1 downregulation were demonstrated to occur in response to NaB and TSA treatment (figure

3.16 - 3.18). Our laboratory has previously demonstrated a matching pattern of expression of NAIP and p21 in the differentiating cells of the intestinal epithelium [33]. In addition to its role as a cell cycle regulator, p21 is a highly specific regulator of transcription [186]. This suggested that it may be involved in the regulation *Naip* transcription (see figure 3.19 for model).

Upregulation of p21 expression in response to HDACi treatment occurs through a combination of histone acetylation and Sp1 activation [133, 185]. Sp1 is a ubiquitously expressed transcription factor which binds to GC rich regions of DNA and interacts with other proteins to repress or promote transcription [191]. In contrast with published results, no significant changes were observed in *Sp1* transcript, protein and activity levels when examined following NaB or TSA treatment (figure3.20). This suggests that in HDACi mediated N2A cell cycle arrest, histone acetylation is sufficient to promote p21 induction. Also if Sp1 is involved in the regulation of *Naip* transcription, its basal activity level is sufficient. Sp1 involvement in *Naip* regulation appears likely with the *Naip* promoter having several putative Sp1 sites and a previously identified GC box. It may not be involved in the basal expression as these sites are outside of the minimal essential promoter previously identified [20]. Sp1 may instead be important for the derepression or induction of the gene in response to specific stimuli. *Naip* promoter constructs with the GC box and/or putative Sp1 sites deleted would be useful in clarifying the role of Sp1 in its transcription.

While it remains uncertain if Sp1 is playing a role in *Naip's* transcriptional regulation, a recent study examining the TSA mediated Sp1 dependent transcriptional derepression of the luteinizing hormone receptor (LHR) gene revealed a novel Sp1 dependent mechanism of action in the absence of its activation. TSA treatment of JAR cells resulted in no changes in Sp1 DNA-binding activity or expression; however, a significant

increase in phosphorylation at serine 641 was observed. This phosphorylation of Sp1 was dependent on PI3K and PKCζ, although the mechanism stimulating their activity is unknown and not believed to be a direct effect of TSA. The phosphorylation status of Sp1 influences its interaction with cofactors and in this case leads to the release of p107, a transcriptional repressor and close homologue of the retinoblastoma (Rb) protein [198, 199]. Consistent with previous publications examining the HDACi mediated induction of specific genes, these investigators report that histone acetylation alone is playing a minor role in LHR induction as the TSA mediated transcriptional induction is significantly reduced in the absence of Sp1 binding sites in promoter constructs [198]. This is of particular interest given the apparent repressed nature of the *Naip* gene. Should a role for Sp1 be identified in its regulation, an examination of Sp1's phosphorylation status and interacting partners would be useful to gain further insight into its regulation.

p21 has well documented roles in cell cycle arrest and transcriptional regulation, both of which could potentially play a role in the regulation of *Naip* transcription. In an effort to directly assess the role of p21 in N2A cells, siRNA knockdown was first selected. Despite excellent transfection efficiency and 90% knockdown at the mRNA level, minimal and inconsistent knockdown was seen at the protein level. Two of the most common stumbling blocks to achieving significant knockdown of a gene at the protein level are the stability of the protein and the asynchronous nature of cells in culture [200]. We can rule these out in the present study, given the short p21 protein half-life and the rapid doubling time of N2A cells, coupled with the rapid cell cycle arrest following HDACi treatment and the ability to maintain significant p21 mRNA knockdown for 72-96 hours. Therefore no clear explanation for the lack of protein knockdown is apparent.

As an alternative approach, HCT116, a human colon cancer cell line, and its p21 null daughter line were used. While they are a human cell line, many of the experiments outlined in this study were carried out in parallel in a human neuron-like cell line, NT2, showing the same results (data not shown) and indicating that the mechanisms governing human *NAIP* transcriptional regulation are likely the same or very similar to those regulating murine *Naip1*. Additionally, prior studies using HCT116 cells revealed that they respond to HDACi treatment in the expected manner exhibiting; cell cycle arrest, decreased E2F1 activity and p21 induction [127, 192]. As expected, the p21 null line does not arrest in G_1 and therefore when cell cycle arrest is induced all cells accumulate in G_2 [135]. When treated with NaB or TSA both wt and p21 null HCT116 cells showed a comparable and significant induction of *Naip* (figure 3.21). As in N2A cells, the *Naip* induction was greater with TSA. These results indicate that neither p21's transcriptional regulatory or its G_1/G_0 cell cycle arrest inducing properties are involved in *Naip*'s transcriptional regulation.

Despite our preliminary studies suggesting that NaB's effect on *Naip* transcription was occurring through GPCR mediated signaling pathway modulation, and the repeated demonstrations in the literature that histone acetylation alone is insufficient to account for HDACi mediated transcriptional changes, no clear signaling pathway or non-histone protein acetylation event appears to be involved in NaB or TSA mediated *Naip* regulation. Rather, all evidence in this study points to the histone acetylation and the resultant changes in chromatin architecture as being responsible for NaB and TSA's ability to induce *Naip* transcription. Chromatin immunoprecipitation (ChIP) studies could be used to confirm *Naip* promoter specific histone acetylation; however, these studies would not serve to elucidate the mechanisms regulating *Naip* expression. They would instead further highlight the somewhat promiscuous and broad effects of HDAC inhibitors. A panel of HDAC inhibitors whose

specific HDAC targets are known could be used to determine if a specific HDAC family is involved in the regulation of *Naip* transcription. Subsequent to the identification of a particular HDAC family, an examination of its interacting partners could be helpful in identifying regulator of *Naip* transcription.

Conclusions and Future Directions

Kinase inhibition experiments revealed that PI3K and PKC play a key role in a regulatory pathway(s) suppressing *Naip* transcription, as their inhibition results in increased *Naip* transcript levels. Further experiments could be conducted to determine if they are acting through the same or distinct pathways, and identify the specific PKC isoform(s) involved. This would be useful for the identification of the downstream factors directly responsible for the repression of *Naip* transcription.

It was also determined that the inhibition of NF-κB activity synergistically enhances *Naip* transcription in the presence of NaB but is not NaB's principal mechanism of action or sufficient in and of itself to induce *Naip* transcription. This observation suggests that NF-κB regulates an element that represses *Naip*'s transcription. Further investigation is required to identify this repressive element and determine the conditions under which it binds to the *Naip* promoter; constituitively or only in the HDACi mediated hyperacetylated state. While both could explain why the *Naip* inducing effects of NF-κB inhibition are only seen in the presence of NaB, the later would suggest a HDACi specific regulatory mechanism.

Receptor inhibition studies confirmed that NaB mediated *Naip* induction is receptor independent, suggesting that an HDACi mediated mechanism is responsible for the observed induction. Consistent with this hypothesis is the demonstration that TSA, a potent HDACi,

also induces *Naip* expression. Subsequently, G_1/G_0 cell cycle arrest, p21 induction and E2f1 downregulation in response to NaB and TSA treatment were confirmed.

Investigation of the potential cell cycle associated regulation of *Naip* transcription determined that p21 and cell cycle arrest are not required for HDACi mediated *NAIP* induction. No significant change in Sp1 expression or activity levels were observed in response to HDACi treatment; however, promoter studies are required to fully rule out the involvement of this transcription factor in *Naip* regulation.

Overall, the results of this study suggest that the principal mechanism responsible for NaB and TSA mediated *Naip* induction is direct histone acetylation and the resultant changes in chromatin structure which render it more permissive to transcription. ChIP studies could be used to confirm and study the *Naip* specific changes in histone acetylation with these treatments; however, the benefit of these studies in obtaining a general understanding of *Naip's* transcriptional regulation is relatively small.

CHAPTER 5 - REFERENCES

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Education

2005 – Present	M.Sc. Department of Biochemistry, University of Ottawa, Ottawa, ON, Canada *Thesis Topic* – Elucidation of the Signaling Pathways Regulating the Expression of the Neuronal Apoptosis Inhibitory Protein (NAIP)
2000 – 2004	B.Sc. (Hon.) Department of Biochemistry, University of Ottawa, Ottawa, ON, Canada *Honors Thesis* – Phenotypic Assessment of the Spinal Muscular Atrophy Mouse Models
1995 – 2000	High School Diploma with Bilingual Certificate Hillcrest High School, Ottawa, ON, Canada

Research Positions

2004 – Present	M.Sc. Student – University of Ottawa, Dept. of Biochemistry Supervisor – Dr. Alexander E. MacKenzie
2003 – 2004	Honors Student – University of Ottawa, Dept. of Biochemistry Supervisors – Dr. Alexander E. MacKenzie and Dr. Nathalie H. Gendron
Summer 2002/03/04	Summer Student – Apoptosis Research Institute, Children's Hospital of Eastern Ontario (CHEO) Laboratory – Dr. Alexander E. MacKenzie
Winter 2000	Co-Op Student – Hillcrest High School in Affiliation with the Ottawa Regional Cancer Center (ORCC) Laboratory – Dr. Sharon Cassol

Teaching Positions

Fall 2006	Teachers Assistant/Lab Demonstrator for a third year molecular biology lab course (BCH 3356) at the University of Ottawa. Duties included student supervision and lab report correction.
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	Evaluation of grant proposals submitted in partial fulfillment for a third year biochemistry course in cell regulation and control (BCH 4125) at the University of Ottawa.

Conferences Attended and Presentations

Neuroscience 2006 – Society for Neuroscience 36th Annual Meeting
Poster Presentation – Elucidation of the Signaling Pathways Controlling NAIP Expression

Neuroscience 2005 – Society for Neuroscience 35th Annual Meeting
Poster Presentation – Elucidation of the Signaling Pathways Controlling NAIP Expression

Canadian Genetic Disease Network Annual Meeting 2005
Poster Presentation – Elucidation of the Signaling Pathways Controlling NAIP Expression

Neuroscience 2004 – Society for Neuroscience 34th Annual Meeting

Honors and Awards

2005	University of Ottawa, Biochemistry Program Travel Grant
2001 and 2002	Minto Skating Club Scholarship
2000	University of Ottawa Entrance Scholarship
2000	Ontario Scholar Award