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Characterisation of a Cell Culture System for Investigating Nerve Agent Neurotoxicology (Part I)

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Human Protection and Performance Division
Defence Science and Technology Organisation

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ABSTRACT

Neuroblastoma cell lines NB41A3 and SH-SY5Y were evaluated as an in vitro model system for studying organophosphorus (OP) chemical toxicity in central nervous system (CNS) cell lineages. Optimal culturing conditions, neuronal differentiation protocols and appropriate cholinergic gene expression were confirmed. The presence of muscarinic receptors and acetylcholinesterase activity was determined. Importantly, differential acetylcholinesterase inhibition by OP chemicals was demonstrated in live cells. This work has developed expertise in neuronal cell culture, confocal microscopy and enzyme activity assays that will provide the basis for an ongoing research programme. The neuroblastoma cell lines chosen can potentially be used as a model for investigating the toxicity of a range of CNS-acting chemicals of interest to the Department of Defence.

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Executive Summary

The Human Protection and Performance Division (HPPD) of the Defence Science and Technology Organisation (DSTO) was tasked with developing a new research capability for investigating medical countermeasures to toxic organophosphorus (OP) chemicals, including chemical warfare nerve agents. HPPD does not have the facilities, personnel or funding for animal-based toxicology and pharmacology experimental programmes like those of our international Defence partners. It was considered, therefore, that the best avenue for novel research was to utilise cell culture models of the central nervous system (CNS). Such research effort will be complementary to the animal models utilised by our international Defence partners and will provide the opportunity to perform a basic scientific investigation of the precise biochemical mechanisms of nerve agent toxicity in the CNS where there remain many knowledge gaps. Such experiments can provide valuable information for subsequent animal-based medical countermeasure experiments.

The intention is to create an experimental platform and suite of techniques that can be applied to whatever chemical agents are of interest to the client. This platform will consist of cultured mammalian neurons and assays to investigate the cellular receptors and biochemical pathways that are targeted by chemical weapons. The utility of a given neuronal cell line for a given class of chemical warfare agent will need to be validated on a case-by-case basis. Cultured cells will need to express the required receptors and pathways that are applicable for the class of agent.

In order to initiate a nerve agent research programme, neuroblastoma cell lines were chosen and validated as a useful model system. Optimal culturing conditions for experimentation, neuronal differentiation protocols and appropriate gene expression were confirmed. The presence of muscarinic receptors and functional acetylcholinesterase was determined. Importantly, reliable assays for analysis of differential acetylcholinesterase inhibition by OP chemicals were generated using live neurons. This work has developed expertise in neuronal cell culture, confocal microscopy and enzyme activity assays that will provide the basis for an ongoing research programme. The neuroblastoma cell lines chosen can potentially be used as a model for investigating the toxicity of a range of CNS-acting chemicals of interest to the Department of Defence.

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David Mawdsley obtained a Bachelor of Science Degree with Honours (1998) and a Doctor of Philosophy Degree (2002) from the University of Melbourne. His doctoral research focused on developmental biology of the central nervous system. From 2002 to 2006 he continued in the area of nervous system development as a postdoctoral research fellow at Vanderbilt University, Nashville, Tennessee, USA. In late 2006 he moved back to Australia to join DSTO and build their chemical medical countermeasures programme.

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Abbreviations

A	adenine
ACh	acetylcholine
AChE	acetylcholinesterase
BDNF	brain derived neurotrophic factor
bp	base pairs
C	cytosine
cAMP	adenosine 3',5'-cyclic monophosphate
cDNA	complementary deoxyribonucleic acid
ChAT	choline acetyl transferase
CHT1	high-affinity choline transporter 1
CNS	central nervous system
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
FCS	foetal calf serum
G	guanine
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
gDNA	genomic deoxyribonucleic acid
HBS	HEPES-buffered saline
HS	horse serum
IC_{50}	concentration required for 50% inhibition of enzyme activity
IHC	immunohistochemistry
M	molar
mAChR	muscarinic acetylcholine receptor
mins	minutes
mRNA	messenger ribonucleic acid
nAChR	nicotinic acetylcholine receptor
OP	organophosphorus
PBS	phosphate buffered saline
PBST	phosphate buffered saline with 0.1% Tween20
PCR	polymerase chain reaction
RA	*all-trans*-retinoic acid
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SNP	single nucleotide polymorphism
T	thymine
UV	ultraviolet
VAChT	vesicular acetylcholine transporter
VX	O-ethyl-S-2-diisopropylaminoethyl methylphosphonothiolate

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1. Introduction

1.1 Research platforms

Organophosphorus (OP) compounds are commonly used in agriculture as pesticides. Since OPs can be potent neurotoxins, some (e.g. sarin, tabun, soman, cyclosarin and VX) have been developed as chemical warfare nerve agents, making them potential threats in both military and civilian situations (Ohbu et al., 1997; Gosden and Gardener, 2005). Acute exposure to nerve agents causes a characteristic syndrome with symptoms including salivation, lacrimation, respiratory failure, tremor, and seizures (McDonough, Jr. and Shih, 1997; Wiener and Hoffman, 2004). If left unattended, these symptoms can lead to death.

The Human Protection and Performance Division (HPPD) of the Defence Science and Technology Organisation (DSTO) was tasked with developing a new research capability for investigating medical countermeasures to toxic OP chemicals, including nerve agents. Our international Defence partners have long-standing sophisticated animal toxicology infrastructure that is used to research the *in vivo* toxic effects of acute and chronic exposures to nerve agents. The DSTO, however, does not have this animal research capability and, therefore, must rely on *in vitro* models of OP toxicity. Whilst animal models are required for the testing of new drugs and medical countermeasure regimens, an *in vitro* cell biology-based approach utilising cultured mammalian cells would complement animal-based research. Studies of the properties of neurotoxins and development of new therapeutic compounds for disease management often utilise *in vitro* cell culture models (Stacey and Viviani, 2001; Segura-Aguilar and Kostrzewa, 2006). Simple cytotoxic models reveal the gross effects of a drug or compound and, subsequently, more complex and subtle assays are used to elucidate the mode of action of the compound of interest (Frazier, 1992). A basic science investigation of nerve agent toxicity at the cellular level may reveal novel interactions between OP molecules and previously unknown pathways. This will help to elucidate the nature of the cellular damage that occurs, indicate innate cellular repair mechanisms that can be targeted by therapeutics, and help inform *in vivo* pharmacological experiments. Additionally, building experimental platforms that revolve around basic science analyses, such as cell biology, mechanisms of toxicity, cellular repair pathways, receptor activation etc., will enable HPPD to shift and adapt its research capability to whatever chemical agents are of interest to its Defence clients.

1.2 Nerve agent toxicity

The toxicity of nerve agents, pharmaceutical prophylaxis and medical countermeasures for acute exposures have been extensively studied and reported in the open scientific literature (for reviews see Brown and Brix, 1998; Bajgar, 2004; Marrs, 2007; Watson et al., 2009). Briefly, the enzyme acetylcholinesterase (AChE) plays a crucial physiological role in termination of impulse transmission at cholinergic synapses through rapid hydrolysis of the neurotransmitter acetylcholine. Nerve agents exert their effects through binding and irreversible inactivation of AChE, leading to a toxic accumulation of acetylcholine at nicotinic (skeletal muscle and pre-ganglionic autonomic) receptors, muscarinic (mainly postganglionic

parasympathetic) receptors, and central nervous system (CNS) synapses. The CNS toxicity of OP pesticides and nerve agents has been extensively studied. Exposure to symptomatic doses of these agents leads to seizures and characteristic neuropathologic effects that have been examined in a variety of animal models (Myhrer, 2007; Woltjer, 2009).

While the mechanisms underlying OP actions within the CNS are still under investigation, most symptoms are believed to be initially the result of over-activation of the cholinergic system as a result of AChE inhibition (Eyer, 2003). Continued cholinergic firing can also result in massive release of excitatory neurotransmitters, such as glutamate, which over-stimulate their receptors resulting in excitotoxicity and brain damage (McDonough, Jr. and Shih, 1997). Several neurotransmitter systems are hypothesised to become involved sequentially in the initiation and maintenance of seizures induced by nerve agents: an early cholinergic phase, a transitional phase of progressively mixed cholinergic/non-cholinergic modulation, and finally a predominantly non-cholinergic phase (McDonough, Jr. and Shih, 1997; Solberg and Belkin, 1997) (Figure 1).

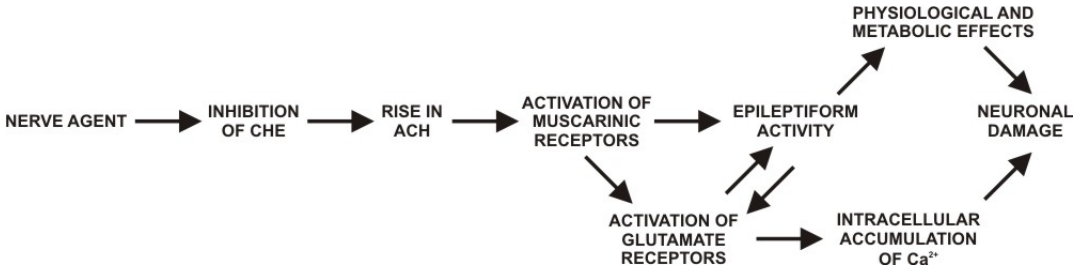

Figure 1 Mechanisms of nerve agent-induced neuronal damage

The proposed sequence of biochemical, neurochemical and electrophysiological events that occur after exposure to nerve agent that initiate and maintain seizure activity and subsequently lead to neuronal damage. CHE, cholinesterase; ACH, acetylcholine. (Taken from McDonough and Shih, 1997.)

1.3 Knowledge gaps

Despite the considerable research into OP toxicity, many knowledge gaps remain in our understanding of the biochemical mechanisms that underlie CNS symptoms of exposure. One area of contention is the relative importance of AChE inhibition as the cause of CNS toxicity. For instance, mathematical analyses have indicated that AChE inhibition is the primary mechanism of acute OP toxicity (Maxwell et al., 2006). However, a growing number of reports have indicated that OP compounds have direct toxic effects on other enzymes, acetylcholine receptors and receptor/channel complexes that are independent of AChE inhibition (Sultatos, 1994; Pope, 1999; Schuh et al., 2002; Casida and Quistad, 2005). Due to their reactive nature, it is conceivable that OP molecules might alter the function of other enzymes and/or structural proteins (Casida and Quistad, 2005; Lopachin and Decaprio, 2005). Additionally, AChE knockout mice were used to investigate inhibition by OPs of targets other than AChE. It was

concluded that the toxicity of VX must be attributed to inhibition of non-AChE targets in the AChE –/– mouse, and that OP toxicity in wild-type mice is probably due to inhibition or binding to several proteins, only one of which is AChE (Duysen et al., 2001). These potential non-AChE targets for the initiation of CNS toxicity have not been definitively demonstrated, however nuclear (Nishio and Uyeki, 1981; Mentzschel et al., 1993), enzymatic (Ehrich et al., 1997), cytoskeletal (Tuler and Bowen, 1989), and plasma membrane (Antunes-Madeira et al., 1994) alterations have been described.

Another area of uncertainty is the extent to which low-level sub-clinical OP exposures can produce long-term CNS damage and/or deficits. Although acute OP poisoning is a well-established clinical entity, the existence of chronic poisoning due to exposure to low levels of OPs (below the threshold required for cholinergic clinical symptoms) is an issue of debate (Pope, 1999; Petroianu et al., 2001). Studies to determine the potential long-term psychological and neurological sequelae are confounded by factors such as low response rates, possible selection and follow-up biases, compensatory psychological responses and possible co-exposures (Romano, Jr. et al., 2001).

Studies in guinea pigs (Hulet et al., 2002; Shih et al., 2006) and rats (Kassa et al., 2001) have assessed the effects of single and repeated low-dose exposure to the nerve agent sarin and found that non-convulsive symptomatic and clinically asymptomatic concentrations of sarin caused only few and subtle long-term signs of neurotoxicity and immunotoxicity with no evidence of brain or heart pathology. However, exposure conditions may be an important factor for whether threshold OP doses for clinical symptoms cause long-term CNS damage. Rats repeatedly exposed under heat-stress conditions to sub-clinical levels of sarin demonstrated delayed development of brain alterations in cholinergic receptor subtypes that may be associated with memory loss and cognitive dysfunction (Henderson et al., 2002).

An analysis of human OP exposure data concluded that threshold exposure levels for known long-term effects are at, or above, intermediate-level exposure (i.e. exposures producing clinical cholinergic symptoms). Long-term health effects seen at intermediate-level exposures, or in many survivors of high-level exposure, are subtle, detectable in exposed populations but not individuals, and not reported in individuals experiencing low-level exposure alone (Brown and Brix, 1998). In contrast, chronic cognitive and neurological symptoms have been described after long-term low-dose exposure to OPs in agricultural settings (Stallones and Beseler, 2002), and many long-term consequences of nerve agent exposure arising from the Tokyo subway attack and exposed Gulf War US military personnel have been reported (Yanagisawa et al., 2006; Heaton et al., 2007; Yamasue et al., 2007). Interactions of OP chemicals with non-cholinesterase targets may contribute to the more delayed and persistent effects observed following chronic exposure to subthreshold doses (Lotti and Moretto, 2005; Costa, 2006).

These knowledge gaps, and others, can be addressed by cell culture models of the CNS. Cultured neurons are ideally suited to screening for non-AChE OP interactions, subtle cellular alterations, changes in gene expression, mechanisms of cell death, dose-response toxicity experiments, and more.

1.4 Choice of cell lines

Primary cell cultures continue to be important in neurotoxicology as they are considered to closely mimic the *in vivo* state and generate more physiologically relevant data (Ferro and Doyle, 2001). However, where appropriate continuous cell lines are available they offer major advantages including reproducibility, ease of propagation, the availability of quality controlled cell banks and the ability to routinely use cells of human origin. Numerous types of immortalised cells now provide key tools for *in vitro* neurotoxicology studies (Frazier, 1992; Stacey and Viviani, 2001).

Therefore, the decision was made to use mammalian adherent neuroblastoma cell cultures to initiate the nerve agent medical countermeasures research programme. OP toxicity on the cellular level has been demonstrated in a diverse array of immortal cell lines *in vitro* (Mochida et al., 1988; Veronesi and Ehrich, 1993; Ehrich et al., 1997; Greenman et al., 1997). The option to utilise primary neuronal cultures or other tissue culture models, such as brain slice cultures and neurospheres, can be explored at a later date, if required.

Two neuroblastoma cell lines were chosen for initial study, a mouse line (NB41A3) and a human line (SH-SY5Y). The open literature reports both lines as having been used for OP chemical toxicity studies, particularly the SH-SY5Y line. NB41A3 cells have been used to study the metabolism and baseline activities of the major target esterases of OP chemicals (Veronesi and Ehrich, 1993), the differential inhibition of AChE and neuropathy target esterase by OP compounds to differentiate between neuropathic OPs and acutely neurotoxic OPs (Ehrich et al., 1997; Li and Casida, 1997), and the concentration-related OP-induced inhibition of carboxylesterase (Ehrich and Correll, 1998). The SH-SY5Y cell line has commonly been chosen to study the pathogenesis of neurodegeneration (Chang et al., 2002; Xue et al., 2006; Zheng et al., 2006) and for drug screening (Levites et al., 2002). Of particular relevance to our work programme, it has been used to study nicotinic acetylcholine receptors (Friederich et al., 2000; Ridley et al., 2002), muscarinic acetylcholine receptors (Wojcikiewicz and Nahorski, 1991; Ehrich et al., 1994), OP toxicity (Carlson et al., 2000; Hong et al., 2003), and nerve agent toxicity (Pachiappan et al., 2009).

Having chosen some initial cell lines, our intention was to systematically determine their optimal handling and culture conditions, and to validate their suitability as a model for our purposes. Much of the experimental data we aimed to generate has already been published in the open literature. However, as this was a new capability area for HPPD, the intention was to build expertise in cell culture and the required biochemical assays. Developing a familiarity with the appearance, morphology and behaviour of the cells in response to routine handling and varying experimental conditions will provide a sound basis for elucidating novel cellular responses to toxic OP chemicals.

Our experimental plan was as follows:

Cell culture conditions:

- Determine routine culturing conditions (medium, serum, flasks, differentiation)
- Assay pre- and post-differentiation cell morphology

Genetics:

- Determine cholinergic gene expression
- Assay for single nucleotide polymorphisms in AChE

Biochemistry:

- Assay AChE enzymic activity
- Assay parameters of toxicity (e.g. apoptosis)

Cell biology:

- Determine protein and organelle localisation
- Perform confocal microscope imaging
- Perform transfection of plasmid constructs

2. Materials and Methods

2.1 Cell lines

Neuroblastoma cell lines were purchased from American Tissue Culture Collection (ATCC) via their Australian distributor Cryosite who processed all the required AQIS import permits as part of their service. Cell lines purchased are listed in Table 1.

Table 1 Neuroblastoma cell lines evaluated for in vitro models of organophosphorus chemical toxicity

Name	ATCC number	Organism	Cell type
NB41A3	CCL-147	Mouse	Adherent neuroblast-like
SH-SY5Y	CRL-2266	Human	Adherent neuroblast-like

2.2 Culturing of mammalian neuroblastoma cells

2.2.1 Routine culturing

NB41A3 and SH-SY5Y neuroblastoma cells were cultured in a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12 nutrient mix with 10% foetal calf serum (FCS) in a humidified incubator at 37°C with 5% CO_2. Culture vessels used were Nunclon™Δ surface flasks and plates or Lab-Tek™ II chambered coverslips (NUNC, Denmark). NB41A3 cells were seeded at 1.2 x 10^3 cells/cm^2 in flasks, 3 x 10^3 cells/cm^2 in plates and 6 x 10^3 cells/cm^2 in chambered coverslips. SH-SY5Y cells were seeded at 3 x 10^3 cells/cm^2 in flasks, 10^4 cells/cm^2 in plates and 1.5 x 10^4 cells/cm^2 in chambered coverslips.

2.2.2 Preparing frozen stocks

For long-term storage, cell aliquots were frozen and stored in liquid nitrogen. Cells were stored in cryovials (Nunc) in full growth medium with 10% DMSO at a cell density of 10^6 cells/ml in aliquots of 0.5 ml for NB41A3 cells, and 1 ml for SH-SY5Y cells. Cryovials were cooled at 1^o/min in a controlled cooler box in a -80^{o}C freezer then placed in liquid nitrogen.

2.2.3 Thawing frozen stocks

Cryovials were thawed rapidly in a 37^{o}C water bath. Once thawed the cells were immediately transferred to a 25cm^2 flask containing 5ml pre-warmed growth medium with FCS and incubated overnight at 37^{o}C and 5% CO_2 in a humidified incubator to allow the cells to attach. A medium change was performed early next day to remove dead cells and residual DMSO.

2.2.4 Subculturing

Cells were rinsed twice with pre-warmed HEPES-buffered saline (HBS) and detached from the culture vessel with 0.25% trypsin-EDTA for 5-10 minutes at 37^{o}C. The trypsin was inactivated with 25% horse serum (HS) and the detached cells pelleted by centrifugation (125x g, SH-SY5Y 7 mins, NB41A3 10 mins). Cell pellets were resuspended in full growth medium (NB41A3 cell pellets were disassociated by gentle pipetting with a P100 Gilsen pipette), counted in 0.1% trypan blue solution using a haemocytometer and seeded into new culture vessels as above.

2.3 Determination of cell morphology and growth conditions

2.3.1 Media and Serum

Cells were seeded in 6-well cell culture plates and grown in DMEM, Ham's F12 or a 1:1 mixture of DMEM and Ham's F12 medium. Serum concentrations were 0%, 10%, 15% or 20% (v/v) with either HS, FCS or a 1:1 mixture. After one week of incubation cells were detached with trypsin and counted using trypan blue solution and a haemocytometer.

2.3.2 Growth Surfaces

Cells were seeded in standard 6-well cell culture plates coated with laminin, poly-L-lysine or a mixture of both. Poly-L-lysine was diluted to 10µg/ml in HBS, spread over the wells with a cell scraper, incubated for 5 minutes at room temperature, rinsed with sterile H_20 and left to dry in a Class II cabinet under aseptic conditions. Laminin was diluted in HBS (or 10µg/ml poly-L-lysine solution) to 1µg/10µl/cm^2, spread over the wells, incubated at 37^{o}C for 2 hours, rinsed with sterile H_20 and left to dry.

2.3.3 Differentiation and cell morphology

Cells were seeded in 6-well cell culture plates and grown in the presence of 30µM cAMP, 10µM RA or 50ng/ml BDNF. Cells were photographed 48 hours after addition of growth factors using an Olympus CK2 inverted microscope and a PowerShotG6 digital camera.

2.4 Gene expression

2.4.1 RT-PCR

Cells were grown to confluency in 25cm^2 cell culture flasks then differentiated with 30µM cAMP, 10µM RA, 50ng/ml BDNF or control buffer for 24 hours. Cells were then harvested by trypsin and centrifugation and total RNA immediately extracted using the RNeasy Plus kit according to the manufacturer's instructions (Qiagen). RNA was eluted from the spin columns in 50µl RNase-free H_20 and stored at -80^{o}C. RNA concentration was determined by UV spectrometry. First-strand cDNA was synthesised from the purified total RNA using the Omniscript Reverse Transcription kit according to the manufacturer's instructions (Qiagen). A combination of 2.5µM random nonamers (Sigma Aldrich) and 1µM oligo-dT primers (5'-TTTTTTTTTTTTTTTVN-3'; Sigma Aldrich) with 2µg RNA was used for the cDNA synthesis.

To obtain positive control mouse RNA, whole mouse brains were obtained from the Ludwig Institute for Cancer Research Animal House Facility. The brains from euthanized mice were immediately removed, snap frozen in liquid nitrogen and stored at -80^{o}C. To extract RNA, ~70mg of frontal cortex tissue was dissected from a frozen brain, ground to powder with a mortar and pestle on dry ice, dissolved in Qiagen RNA extraction buffer and total RNA isolated using the RNeasy Plus kit.

PCR with cDNA was performed in 50µl using REDTaq DNA polymerase according to the manufacturer's instructions (Sigma Aldrich). The PCR reactions contained 2.5U REDTaq enzyme, 200µM of each dNTP, 20pmol of each primer (see Tables 2 and 3) and 0.5-2µl of the reverse transcription reaction (first-strand cDNA). Oligonucleotide primers were designed using sequences from Ensemble, GenBank and (Nguyen et al., 2000). The thermocycling protocol for all reactions was as follows: 94^{o}C for 2 minutes, 30 cycles of amplification (94^{o}C, 55^{o}C – 65^{o}C and 72^{o}C) for 45 seconds, and finally 5 minutes at 72^{o}C. The annealing temperatures are shown in Tables 2 and 3. Nested PCR was used for amplifying the ChAT fragment from NB41A3 cDNA; PCR was performed using the outer primers, then 5µl of the reaction was used as the template for amplification using the inner primers. PCR products were electrophoresed in 0.8% agarose gels containing 0.5mg/ml ethidium bromide alongside a DNA ladder of known fragment sizes and photographed under UV light.

2.4.2 DNA sequencing

PCR products were cut from the agarose using a razor blade and purified using the QIAquick Gel Extraction kit according to the manufacturer's instructions (Qiagen). Purified PCR products were sub-cloned into the pCR2.1 vector using the TA Cloning Kit according to the manufacturer's instructions (Invitrogen), and the recombinant vectors transformed into

TOP10F *E.coli* according to the manufacturer's instructions (Invitrogen). Plasmids containing the PCR inserts were purified from *E.coli* overnight cultures using the QIAprep Spin Miniprep kit according to the manufacturer's instructions (Qiagen). The identity of the PCR inserts was confirmed by DNA sequencing. Sequencing reactions were performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with 1.6pmol/µl M13F primers and 400ng plasmid DNA. BigDye-labelled DNA fragments were precipitated from the sequencing reactions using ethanol and were sequenced externally at the Institute of Medical and Veterinary Science sequencing centre (Adelaide). Sequences were aligned with Ensemble reference sequences using the DNAStar software (Lasergene).

2.5 Fluorescent immunohistochemistry

The *in situ* fluorescent labelling of proteins for confocal microscopic analysis in cultured neurons was performed using immunohistochemistry (antibody labelling).

Cells were grown to 70-90% confluency in 2-, 4- or 8-well chambered coverslips (Nunc) and differentiated 24 hours prior to experimentation with 30µM cAMP (NB41A3) or 10µM RA (SH-SY5Y). Cells were then:

1. washed 3x with PBST,
2. fixed in 4% paraformaldehyde in PBST at room temperature for 1 hour,
3. washed 3x with PBST,
4. blocked in PBST with 2mg/ml BSA and 2% HS at room temperature for at least 1 hour, or overnight at 4^{o}C.
5. incubated with 1^{o} antibody in block solution at room temperature for 1 hour,
6. washed 3x with PBST,
7. incubated with fluorescent 2^{o} antibody in block solution at room temperature for 1 hour,
8. washed 3x with PBST,
9. stored at 4^{o}C in PBS until imaged.

Antibodies used in immunohistochemistry experiments, their working concentrations and suppliers are listed in Table 4.

Table 2 PCR parameters for gene expression analysis of NB41A3 cells

*Primer sequences are located in regions highly conserved between nAChRα 3 and 4.
#Primer sequences are located in regions highly conserved between nAChRβ 2 and 4.

Gene	Sequence source	Forward primer (5'-3')	Reverse primer (5'-3')	Fragment size (bp)	Annealing temperature
GAPDH	ENSMUSG00000057666	TGGTGAAGCTCGGTGTGAACG	TAGGAACACGGAAGGCCATGC	847	55°C
β-Actin	ENSMUSG00000029580	CGACATGGAGAAGATCTGGCA	CGTACTCCTGCTTGCTGATCC	702	
AChE	ENSMUSG00000023328	GTGGCAATGACACCGAGCTGA	AGTGGTCGAACTGGTTCTTCC	933	
ChAT	ENSMUSG00000021919	Outer primers	Outer primers	975	65°C reduced by 0.5°C/cycle to 50°C
		GGCAGCCTCTCTGTATGAAGC	CAGCCTTGTGGTCAGTCATGG		
		Inner primers	Inner primers	576	
		ATGAACGCCTGCCTCCAATCG	GGATGAAGCCATCAGGACTGC		
CHT1	ENSMUSG00000023945	TCGGTCATTGTCTCTGCACTC	GGCTCTCCTCCAGTAATTCTC	866	
VACHT	NM_021712	TCCTGCAGCTTCTGGTGAACC	CCATTGTGTGCTTCATCCACG	564	
nAChRα*	ENSMUSG00000032303 ENSMUSG00000027577	ACGACAAGGAMAAGATYGAC	TGCASAGCGTSACCTTCTC	~286	60°C
nAChRβ#	ENSMUSG00000027950 ENSMUSG00000035200	CGAGARCAGATCATGACCACCA	GGYGAACGGTGGTGCACAT	~761	

Table 3 PCR parameters for gene expression analysis of SH-SY5Y cells

*Primer sequences are located in regions highly conserved between nAChRα 3 and 4.
#Primer sequences are located in regions highly conserved between nAChRβ 2 and 4.

Gene	Sequence source	Forward primer (5'-3')	Reverse primer (5'-3')	Fragment size (bp)	Annealing temperature
GAPDH	ENSG00000111640	AGGTGAAGGTCGGAGTCAACG	GTGGCAGTGATGGCATGGACT	544	60°C
β-Actin	ENSG00000075624	GATGATGATATCGCCGCGCTC	ACTCGTCATACTCCTGCTTGC	1090	
AChE	ENSG00000087085	CCTGGTGAACCACGAATGGCA	CCGCAGGTCCAGACTAACGTA	648	
ChAT	Nguyen *et al*, 2000 ENSG00000070748	GAAATGCTCCCCGGAAATTC	CTCACAAAAGCCAGTGCCTC	291	
CHT1	ENSG00000115665	CCACCATCAGCGTGATCATCG	CATGCTCTGTCGTGGCTTCAC	1202	
VACHT	ENSG00000187714	TTGCTTCCAAGGCTATCCTGC	AGACTGAGACATGGCGCACGT	867	
nAChRα*	ENSG00000080644 ENSG00000101204	CTCAGCTGGTKRABGTGGATG	CATGGTGAACAGSAGGTACT	731	
nAChRβ#	ENSG00000160716 ENSG00000117971	CAAGAGCGCMTGCAAGATTGA	CGATGGAGAAGGTGACAAGCA	485	

Table 4 Antibodies used for immunohistochemical analysis of protein expression in NB41A3 and SH-SY5Y cells

Antibody	*Supplier Cat#*	*Organism*	*Primary / secondary*	*Suggested dilution*
M1	Sigma M9808	rabbit	Primary	1 in 500
M2	Sigma M9558	rabbit	Primary	1 in 500
AChE	Santa Cruz sc-11409	rabbit	Primary	1 in 200
AlexaFluor 488 goat anti-rabbit IgG	Invitrogen A11018	goat	Secondary	1 in 10000

2.6 Confocal microscopy

Chambered coverslips containing cells for fluorescent immunohistochemical analysis were prepared as above. Fluorescence confocal microscopy was performed using a Leica TCS SP5 spectral confocal system based on a DMI6000 inverted microscope with the following specifications:

Objectives: 10x (air), 40x (oil), 63x (oil), 63x lambda blue (oil) and 100x (oil).
Differential interference contrast (DIC) prisms: available on the 40x and 63x objectives.

Lasers: diode (405nm), argon (lines at 458, 476, 488 and 496 nm), diode-pumped solid state (561nm), HeNe (633nm).

Detectors: 3 photon multiplier tubes (PMTs); spectral scanning/detection via acousto-optical beam-splitter (AOBS).

Image manipulations of confocal data were performed using Leica Application Suite or CorelDRAW12. Alterations were limited to brightness, contrast, intensity and tone curve adjustments.

2.7 Acetylcholinesterase activity

SH-SY5Y cells were seeded at $8x10^4$ cells/well in clear-bottom black microplates and incubated under standard conditions for 24 hours to allow attachment. Cells were then differentiated for a further 24 hours in 200µl growth medium containing 10µM RA. Prior to assay cells were washed with Hank's balanced salt solution without Mg^{2+} and Ca^{2+}. AChE activity in live SH-SY5Y cells in the presence of AChE inhibitors was measured using a PolarSTAR plate reader (BMG LabTech) and the Amplex Red Acetylcholine/Acetylcholinesterase Assay Kit according to the manufacturer's instructions (Invitrogen). The relative fluorescence units (RFU) were measured every

2 minutes for 60 minutes. The RFU/minute values from time 30–60 minutes were calculated and normalised using the control (no inhibitor) samples.

Physostigmine (eserine, Sigma catalogue number E8375) and VX (synthesised at DSTO Melbourne Chemical Synthesis facility) were diluted in ethanol as stock concentrations such that no final concentration of ethanol was >1% (v/v).

Data analysis and graphing were performed using Prism5 software (GraphPad).

2.8 Detection of single nucleotide polymorphisms

DNA was extracted from SH-SY5Y cells using a Blood Mini Kit according to the manufacturer's instructions (Qiagen). A 2050bp DNA fragment that contained exon 2 and exon 3 of the AChE gene was amplified by PCR. The PCR fragments were visualised by electrophoresis in an agarose gel and purified using an Illustra GFX PCR DNA Gel Band Purification Kit according to the manufacturer's instructions (GE). To identify SNPs, DNA sequencing reactions were performed on the purified fragments using nested oligonucleotide primers and the BigDye Terminator v3.1 Cycle Sequencing Kit according to the manufacturer's instructions (Applied Biosystems). The sequencing products were purified by ethanol/EDTA/sodium acetate precipitation, resuspended in 10µl HiDi formamide (Applied Biosystems) and analysed with a ABI3130xl sequencer (Applied Biosystems).

DNA oligonucleotide primers used for amplification of 2050bp AChE fragment:

F1:	CGACCGACCCTTCACCCT
R4:	CTATCCTGCCCCTGTCCC

DNA oligonucleotide primers used for DNA sequencing of 2050bp AChE fragment:

24982F:	AGGCGGCACTGGTGGGAAT	(exon 2)
R4:	CTATCCTGCCCCTGTCCC	(exon 3)
25680F:	GCTGTGGTCCTGCATTAC	(exon 3)

Cycle sequencing reaction conditions:

	x1 reaction
ReadyMix	0.50µl
5x Sequence Buffer	3.75µl
Primer (3.2 pmol)	1.00µl
Template DNA in H_2O	14.75µl
Total	20.00µl

Cycling Conditions
1. 94°C for 5 minutes
2. 96°C for 10 seconds
3. 50°C for 5 sec x 30 cycles
4. 60°C for 4 minutes
5. Hold at 4°C

3. Results

3.1 Medium and serum

A literature search revealed that both NB41A3 and SH-SY5Y neuroblastoma cells have been routinely cultured in Dulbecco's Modified Eagle Medium (DMEM), Ham's F12 medium or a 1:1 mixture of both. Additionally, these neuronal cell lines require serum in the medium with the literature indicating a range of potential serum concentrations.

We performed experiments to empirically determine which medium and serum concentration is optimal for growth of these cell lines in our laboratory and experimental conditions. Cells were grown in the presence of varying combinations of medium and serum for one week then counted (Figure 2). NB41A3 cells showed an absolute requirement for serum, without which there was almost complete cell death (Figure 2 A). The proliferation rates were highly variable depending on the particular medium and serum combination used. One clear pattern emerged: foetal calf serum (FCS) produced better proliferation of NB41A3 cells than horse serum (HS) regardless of the medium type (Figure 2 A). SH-SY5Y cells also showed a higher proliferation rate when cultured in FCS compared to HS (Figure 2 B). Additionally, they showed a higher proliferation rate when cultured in a 1:1 mixture of DMEM and Ham's F12. These data also demonstrate that NB41A3 cells are capable of faster proliferation than SH-SY5Y cells (Figure 2 A versus B).

The variable medium/serum experiment determined that FCS was the optimal serum condition for the cell lines. To determine what concentration of FCS was optimal, NB41A3 cells were cultured in a 1:1 mixture DMEM:Ham's F12 with varying concentrations of FCS for one week then counted. The results confirmed that the cells have an absolute requirement for serum and showed that the concentration of FCS (up to 20%) made no significant difference to proliferation rates (Figure 2 C).

Therefore, based on the data from these experiments, we adopted a standard culturing protocol of 1:1 DMEM:Ham's F12 with 10% FCS for both the NB41A3 and SH-SY5Y cell lines for all subsequent experiments.

3.2 Cell culturing surface

As NB41A3 and SH-SY5Y cells are adherent neuronal cells that extend long membrane processes (e.g. axons and dendrites), we tested different standard cell culture coatings and surfaces on which to grow the cells. We coated the surface of 6-well plates with laminin, poly-L-lysine or a mixture of both, cultured NB41A3 or SH-SY5Y cells in the coated wells for one week then qualitatively compared the clumping and spreading of the cells by visual analysis. Results were compared to commercially available Nunclon 'CellBind' cell culture vessels.

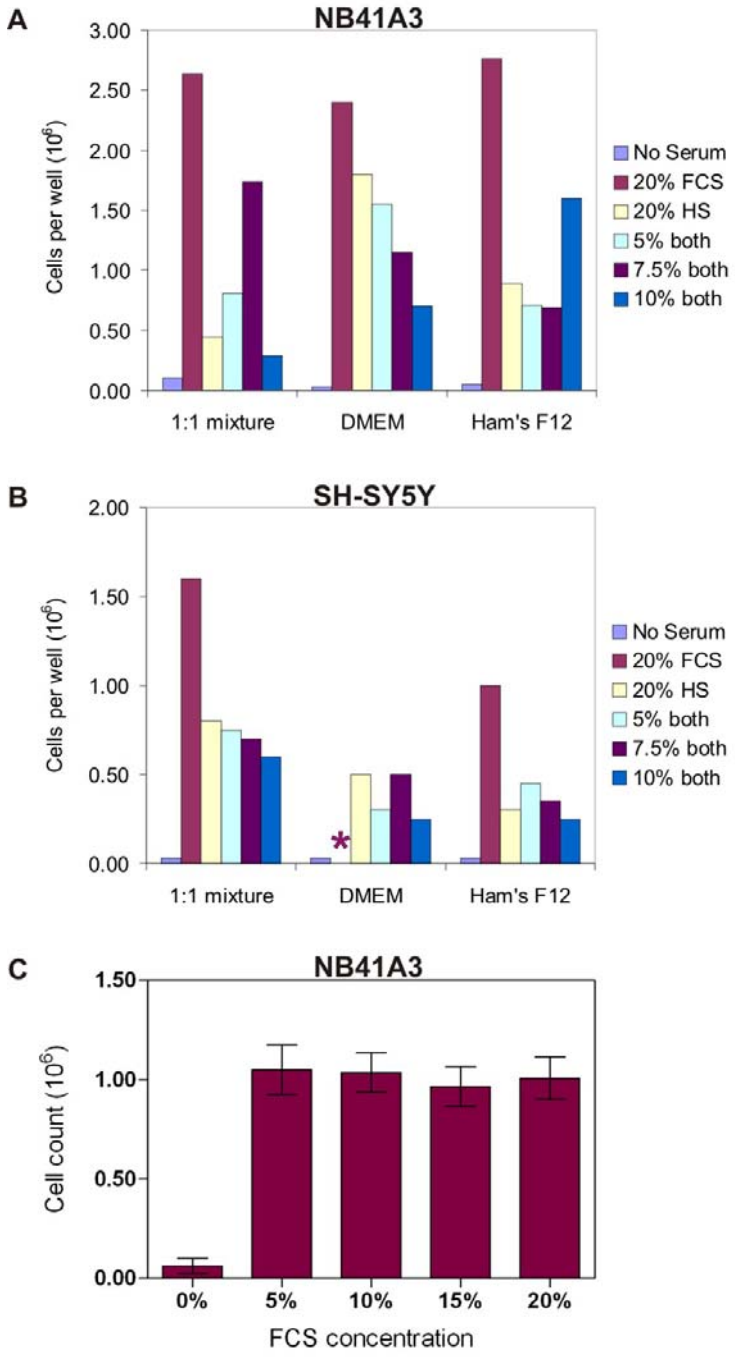

Figure 2 Optimal culture conditions for NB41A3 and SH-SY5Y cells

Cells were cultured in DMEM, Ham's F12 or both with varying concentrations of FCS, HS or both. NB41A3 cells (A) and SH-SY5Y cells (B) showed an absolute requirement for serum in the growth medium and proliferated faster with FCS than HS. There appeared to be no significant difference in proliferation between DMEM and Ham's F12 medium for NB41A3 cells (A), whereas the SH-SY5Y cells proliferated slightly faster in a 1:1 mixture (B). There was no difference in proliferation of NB41A3 cells with varying concentrations of FCS from 5-20% (C). * *Experiment not performed.*

For both NB41A3 and SH-SY5Y cells, the 'CellBind' culture vessels produced cell adherence and uniform spreading, without widespread clumping, at least as well as the laminin and poly-L-lysine manually coated wells (data not shown). Therefore, we adopted the CellBind plasticware for all subsequent experiments.

3.3 Differentiation and cell morphology

We experimented with three differentiation-inducing compounds in our neuroblastoma cell cultures, all of which are well-established methods of differentiating neuronal cultures: adenosine 3',5'-cyclic monophosphate (cAMP), brain-derived neurotrophic factor (BDNF), and *all−trans*−retinoic acid (RA).

NB41A3 and SH-SY5Y cells were incubated in 6-well cell culture plates in the presence of 50ng/ml BDNF, 30µM cAMP, 10µM RA or solvent only (as a control) for 48 hours, then photographed using transmitted white light (Figure 3). In the absence of any differentiation additives in the growth medium, NB41A3 neuroblastoma cells were round to stellate in appearance with multiple projections (Figure 3 A). Many of the projections appeared to connect with nearby cells. When cAMP was added to the medium there was an increase in the number and length of the projections but no change in cell morphology (Figure 3 C). There was no observable increase in apoptosis as judged by morphology and cell density. RA induced a morphological change with the neurons becoming bipolar with single prominent projections from each end (Figure 3 E). This type of differentiation was rejected due to the lack axonal connectivity. BDNF induced an increase in the number and length of the projections similar to cAMP (Figure 3 G).

SH-SY5Y neuroblastoma cells had a more elongated bipolar appearance in their undifferentiated state compared to NB41A3 cells (Figure 3 B). They extended a series of interconnected axonal projections that connected with neighbouring cells. The addition of cAMP to the medium caused a large proportion of the cells to round up and withdraw their extensions, indicating that the cAMP was inducing apoptotic cell death (Figure 3 D). Conversely, RA induced the cells to adopt a more pronounced elongated morphology and intricate axonal connectivity indicative of differentiation (Figure 3 F). BDNF appeared to induce the cells to round up and withdraw their extensions, indicating apoptosis, similar to the effect seen with cAMP (Figure 3 H).

On the basis of these observations, we adopted a standard cell culture protocol of incubating NB41A3 with cAMP and SH-SY5Y cells with RA prior to experimentation.

3.4 Gene expression analysis

An analysis of cholinergic pathway gene expression was performed on NB41A3 and SH-SY5Y cells. Two approaches were utilised: (i) reverse transcription polymerase chain reaction (RT-PCR) using gene-specific oligonucleotide primers, and (ii) immunohistochemistry (IHC) on fixed cells with antigen-specific antibodies.

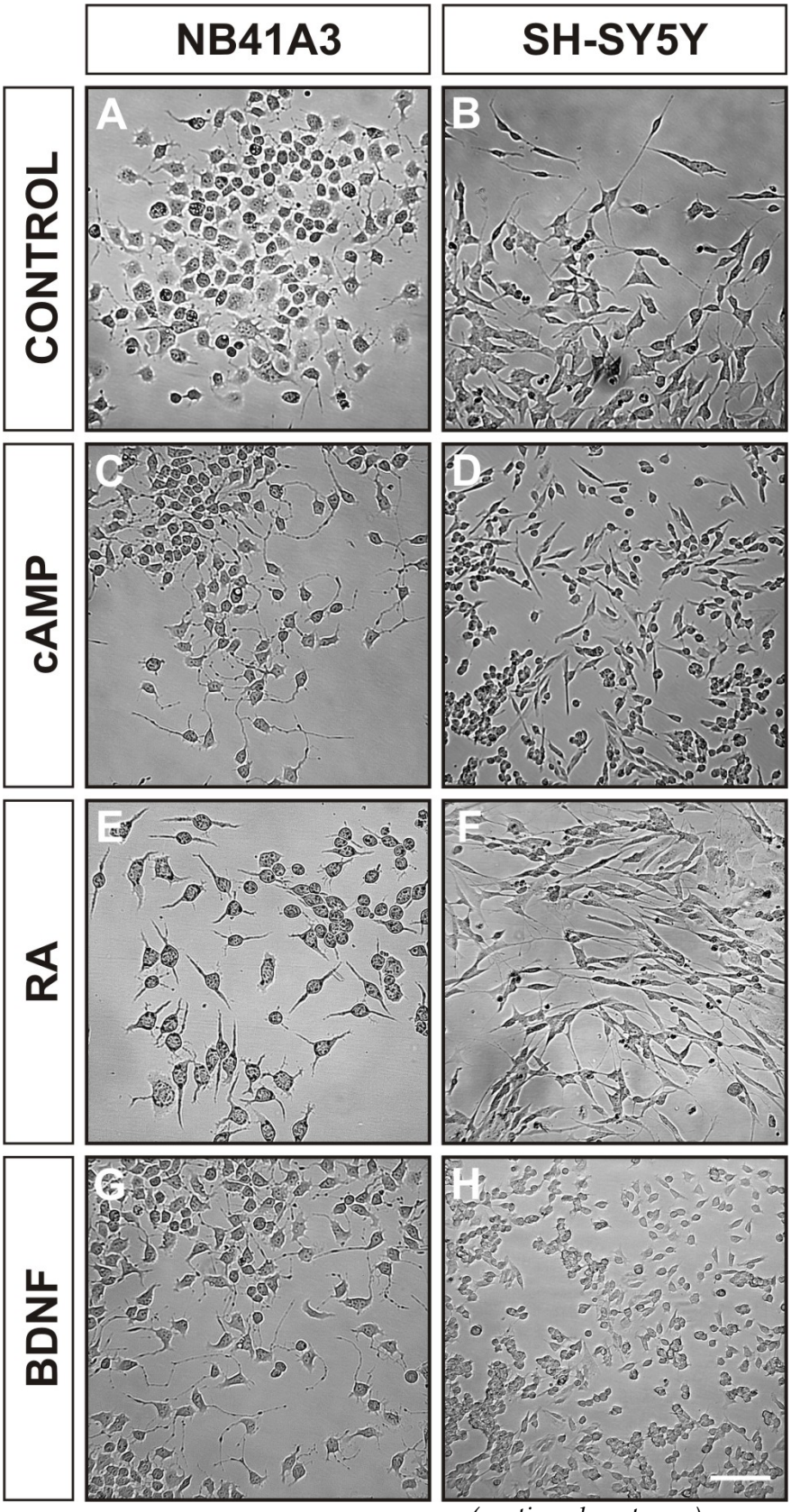

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Figure 3 Morphology of NB41A3 and SH-SY5Y cells with different inducers of differentiation

NB41A3 cells (A,C,E,G) and SH-SY5Y cells (B,D,F,H) were cultured in the presence of different medium additives (cAMP, RA or BDNF), then photographed using transmitted white light. The additives induced morphological changes indicative of neuronal differentiation, such as an increase in axonal number and length. cAMP and BDNF appeared to induce apoptosis in SH-SY5Y cells (D,H). A protocol of incubating NB41A3 with cAMP and SH-SY5Y cells with RA prior to experimentation was adopted. Scale bar: 200 µm.

Determining gene expression by the presence of the encoded protein (IHC) is not as temporally specific as directly assaying the presence of messenger RNA (mRNA) as the translated protein might persist beyond the cessation of gene transcription. However, precise temporal determination of gene expression was not important for the purposes of this study.

Genes chosen for expression analysis by RT-PCR were:

- Acetylcholinesterase (AChE)
- Nicotinic acetylcholine receptor subunits (nAChR)
- Vesicular acetylcholine transporter (VAChT)
- Choline acetyltransferase (ChAT)
- High-affinity choline transporter 1 (CHT1)
- glyceraldehyde 3-phosphate dehydrogenase (GAPDH) *positive control*
- β-actin *positive control*

Genes chosen for expression analysis by IHC were:

- Muscarinic acetylcholine receptor 1 (mAChR1, M1)
- Muscarinic acetylcholine receptor 2 (mAChR2, M2)

3.4.1 Controls for RT-PCR

As a positive control, PCR was performed with primers designed to amplify reference genes known to be expressed in neurons (GAPDH and β-actin; (Thal et al., 2008)). GAPDH and β-actin fragments were successfully amplified from cDNA generated using RNA isolated from both cell lines under all differentiation regimens (Figure 4 A-D). Additional positive controls were performed for the NB41A3 RT-PCR experiments. RNA was extracted from mouse brain cortical tissue and used to generate cDNA. PCR fragments were successfully amplified from the brain cDNA for each NB41A3 primer pair tested (Figure 4 A,C,E,G,I,K,M,O). The positive control results indicated that the RT-PCR assays worked as expected.

As a negative control, primer-only PCR containing no template cDNA was performed for each primer pair tested. These reactions produced no amplification products indicating that the

PCR products in the experimental reactions were specifically generated from the template cDNA and not from environmental DNA contamination (Figure 4).

3.4.2 RT-PCR results

Oligonucleotide primer pairs used in the RT-PCR analysis were designed to span across genomic DNA (gDNA) introns. This allowed for size discrimination between the expected PCR products generated from cDNA (representing expressed mRNA) and false positives from contaminating gDNA. Additionally, each amplification product was purified from the agarose gel and the DNA sequenced to confirm its identity. No false positives were detected from the RT-PCR experiments.

RT-PCR analysis of RNA isolated from NB41A3 and SH-SY5Y neuroblastoma cells indicated that nearly all of the cholinergic pathway genes chosen for analysis were expressed (Figure 4). Human ChAT expression was confirmed by sequencing only with no associated gel (Figure 4 N). The only gene expression not detected was that of CHT1 in NB41A3 cells (Figure 4 O). The presence of the CHT1 genomic locus was confirmed by PCR (data not shown). CHT1 expression was detected in the control brain cDNA reaction (Figure 4 O) suggesting that the lack of amplification from the cell line cDNA represents a specific lack of gene expression in those cells.

The oligonucleotide primer design was such that only a single amplification product was expected, however in two instances, murine ChAT (Figure 4 M) and human AChE (Figure 4 F), there were multiple PCR products. This was likely due to unexpected mRNA splice variants. The different sized amplification products in each instance could be rationalised by the unexpected exclusion or inclusion of exonic DNA (data not shown).

Interestingly, with one exception, there appeared to be no gross differences in gene expression between differentiated and undifferentiated neurons for either cell line. The one exception was the NB41A3 ChAT gene which was not expressed in undifferentiated cells but expressed in differentiated cells (Figure 4 M). However, RT-PCR performed in this fashion is not quantitative and cannot be used for definitive determination of relative gene expression.

3.4.3 Immunohistochemistry

To confirm mAChR expression, IHC was performed on differentiated NB41A3 and SH-SY5Y cells using anti-M1 and anti-M2 receptor antibodies. IHC demonstrated that NB41A3 and SH-SY5Y neuroblastoma cells expressed M1 and M2 receptors (Figure 5 A-D). At higher magnification, punctate expression of M1 and M2 receptors was observed in the SH-SY5Y neurons (Figure 5 E,F arrows). This is likely to correspond to receptor proteins encapsulated in vesicles as they are trafficked to and from the plasma membrane. Localisation of receptors to axonal projections was also observed consistent with their role in synaptic neurotransmitter reception (Figure 5 E,F arrowheads).

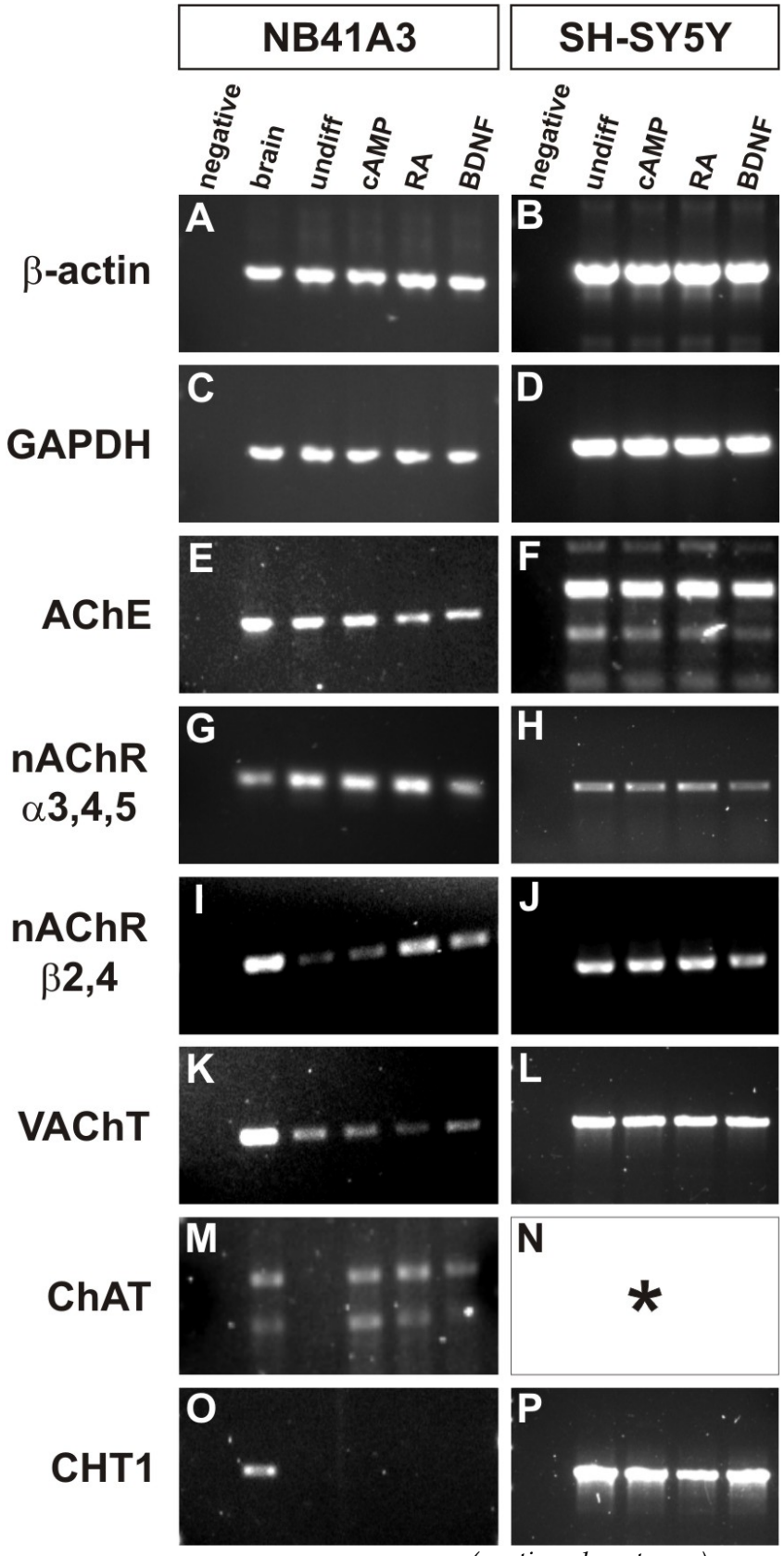

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Figure 4 Expression of cholinergic pathway genes in NB41A3 and SH-SY5Y cells

*Nearly all of the cholinergic pathway genes chosen for analysis were expressed. Human ChAT expression was confirmed by sequencing only with no associated gel (N). The only gene expression not detected was that of CHT1 in NB41A3 cells (O), although it was detected in the control brain cDNA reaction (O) suggesting that the lack of amplification from the cell line cDNA represents a specific lack of gene expression in NB41A3 cells. There were multiple PCR products for murine ChAT (M) and human AChE (F) which were likely due to unexpected mRNA splice variants. With the exception of the NB41A3 ChAT gene (M), there appeared to be no gross differences in gene expression between differentiated and undifferentiated neurons for either cell line. The NB41A3 ChAT gene was expressed in differentiated cells but not in undifferentiated cells. * Expression confirmed by DNA sequencing only.*

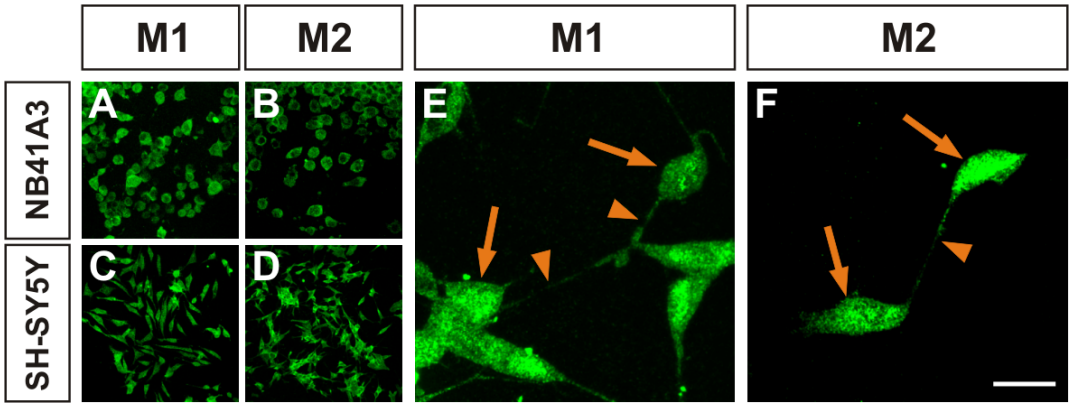

Figure 5 M1 and M2 muscarinic acetylcholine receptor expression in NB41A3 and SH-SY5Y cells

NB41A3 cells (A,B) and SH-SY5Y cells (C-F) expressed M1 and M2 receptors. Punctate expression of M1 and M2 receptors was observed in SH-SY5Y cells (E,F arrows). Localisation of receptors to axonal projections was also observed (E,F arrowheads). Scale bar: A-D = 150 µm; E,F = 25 µm

3.5 Acetylcholinesterase localisation and activity

The expression of AChE mRNA in NB141A3 and SH-SY5Y neuroblastoma cells was confirmed via RT-PCR (Figure 4 E,F). However, the presence of mRNA does not necessarily establish the presence of functional enzyme in the cells. Therefore, experiments were performed to demonstrate the presence of AChE protein (and its cellular localisation) and enzyme activity. IHC with an anti-AChE antibody was performed on fixed SH-SY5Y (Figure 6 A) and NB41A3 (Figure 6 B,C) neurons. The antibody was generated against an epitope corresponding to amino acids 481-614 at the C-terminus of human AChE.

AChE protein was localised widely throughout the cells (Figure 6). This likely represents the protein at various stages of synthesis, post-translational modification and trafficking, as well as varying levels of multimerisation. AChE protein was also localised to synaptic junctions between neurons (Figure 6 B,C arrows) consistent with its role in cholinergic neurotransmitter signalling.

AChE activity in SH-SY5Y cells was measured in the presence of increasing concentrations of the carbamate cholinesterase inhibitor physostigmine or the nerve agent VX. Briefly, SH-SY5Y cells were plated out in a 96-well microplate at 80000 cells per well, incubated overnight, exposed to an inhibitor for 30 minutes then assayed for AChE activity. Baseline AChE activity was inhibited by physostigmine and VX in a dose-dependent fashion with the relative inhibitory potency of the two OP compounds clearly discernable (Figure 6 D).

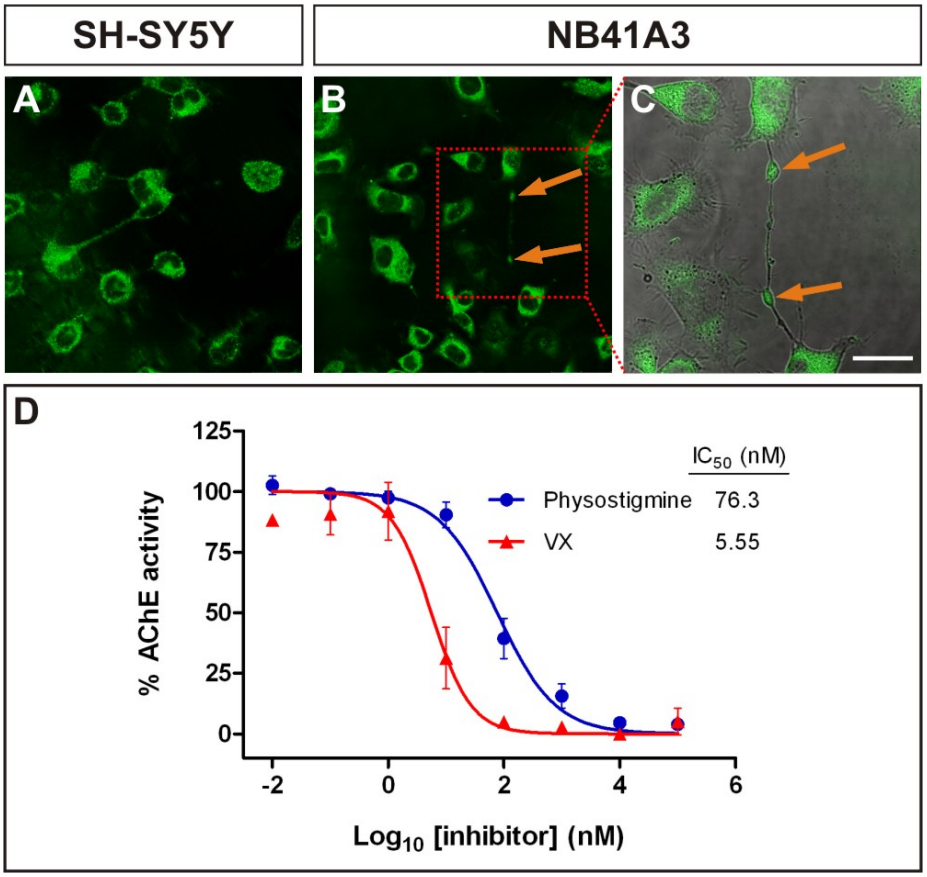

Figure 6 AChE localisation and inhibition in NB41A3 and SH-SY5Y cells

IHC with anti-AChE antibodies was performed on SH-SY5Y (A) and NB41A3 (B,C) neuroblastoma cells. AChE protein was present and localised widely throughout the cells. AChE protein was also observed at synaptic junctions between neurons (B,C arrows). AChE enzymic activity in SH-SY5Y cells was measured against increasing concentrations of physostigmine and VX (D). AChE activity was inhibited in a dose-dependent manner. Scale bar: A = 30 µm; B = 50 µm; C = 25 µm.

Physostigmine achieved 100% AChE inhibition at 10μM whereas VX achieved 100% inhibition at 100nM, a 100-fold lower concentration. The IC_{50} values for physostigmine and VX were 76.3nM and 5.55nM, respectively. A previously reported IC_{50} value for VX was 6.20×10^{-10} M (Sawyer et al., 1992) which is an order of magnitude lower than our value (5.55×10^{-9} M). This difference is probably due to the different cell types used in the experiments. Sawyer et al. utilised mouse embryonic primary neuronal cells which they described as exhibiting extreme sensitivity to nerve agents.

3.6 Acetylcholinesterase gene polymorphisms

The potential presence of AChE single nucleotide polymorphisms (SNPs) in the SH-SY5Y cell line may have consequences with respect to enzyme inhibition studies and the cells' susceptibility to OP chemicals. We screened for the presence of SNPs in the AChE gene by sequencing exon regions of the gene known to contain SNPs (Figure 7). Two SNPs were detected, both of which have been previously reported in the literature. In exon 2, a non-synonymous A/C SNP was detected that produces a His to Asn change (Figure 7 A). This SNP is responsible for the YT-2 blood group phenotype and influences the antigenic properties of AChE (Hasin et al., 2004). In exon 3, a synonymous T/C SNP was detected (Figure 7 B). No novel SNPs were identified.

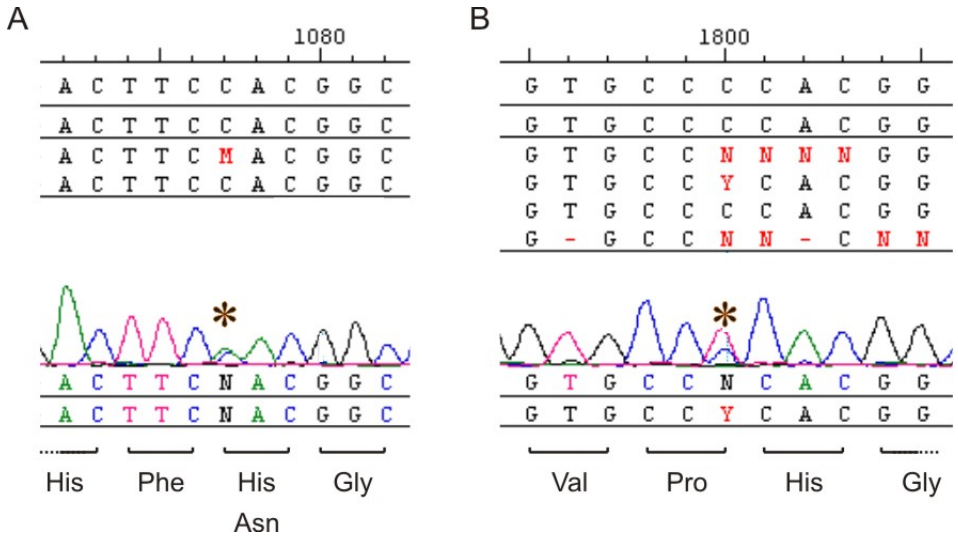

Figure 7 Single nucleotide polymorphisms in the SH-SY5Y AChE gene

Sequencing of exon regions of the AChE gene in SH-SY5Y neuroblastoma cells indicated the presence of a non-synonymous A/C SNP in exon 2 that produces a His to Asn substitution (A), and a synonymous T/C SNP in exon 3 (B). No novel SNPs were identified. A = Adenine, C = Cytosine, G = Guanine, T = Thymine, M = A or C, Y = T or C, His = Histidine, Phe = Phenylalanine, Asn = Asparagine, Gly = Glycine, Val = Valine, Pro = Proline.

4. Discussion

4.1 Cell lines and culturing conditions

Based on the data from the cell culturing experiments, we adopted a standard culturing protocol of 1:1 DMEM:Ham's F12 with 10% FCS for both the NB41A3 and SH-SY5Y cell lines (Figure 2). This is a common medium recipe reported in the literature, so we are confident that the behaviour of the cells and our protocols are consistent with those of other users of these cell lines. The adoption of CellBind culture vessels will greatly simplify the culturing protocols, however successful application of laminin and poly-L-lysine as a substrate coating for culture vessels means this technique can be applied in the future if required.

Neuroblastoma cells frequently resemble relatively undifferentiated neuroblast cells, both in terms of morphology and molecular traits, and are often induced to differentiate by addition of various factors to the growth medium. Differentiation achieves more neuron-like properties, such as neurite outgrowth and morphological changes (Pahlman et al., 1984), activation of survival signalling mechanisms (Lee et al., 2006) and reduction of susceptibility to neurotoxins (Cavanaugh et al., 2006), so as to better mimic the responses of mature *in vivo* neurons in *in vitro* studies. The utilisation of already-differentiated primary neuronal cultures would remove this necessity. However, the ease of propagation, availability and opportunity to use CNS cells of human origin provided the best avenue for rapidly initiating a research capability. A primary brain cell culture capability is a future goal for the HPPD's medical countermeasures research programme.

We evaluated three differentiation-inducing compounds in our neuroblastoma cell cultures, all of which are well-established methods of differentiating neuronal cultures:

1. cAMP
Adenosine 3',5'-cyclic monophosphate (cAMP) is a naturally-occurring activator of cyclic-AMP-dependent protein kinase (PKA). cAMP is an important second messenger that is linked in many systems to neurotransmitter- or hormone-induced receptor stimulation. The cAMP/PKA signaling pathway has been shown to inhibit cell proliferation, induce differentiation and axonal elongation and potentially lead to apoptosis (Chen et al., 1998).

2. BDNF
Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of nerve growth factors (Barbacid, 1994; Patapoutian and Reichardt, 2001). BDNF is important in development and maintenance of neuronal populations within the central nervous system or cells directly associated with it. BDNF has been shown to enhance the survival and differentiation of several classes of neurons *in vitro*, including dopaminergic and cholinergic neurons (Jones et al., 1994; Ghosh et al., 1994; Cohen-Cory and Fraser, 1995; Alcantara et al., 1997).

3. RA
All−trans−retinoic acid (RA) is a retinoid compound that acts through its receptors to regulate the growth and differentiation of neurons (Napoli, 1996) and neuroblastoma cells (Redfern et al., 1995). RA can indirectly modulate differentiation of neurons through the modification of expression of neuronal cell surface receptors to peptide growth factors (Scheibe et al., 1991) and may act as a type of signal molecule working through a nuclear receptor in the regulation of region differentiation of the central nervous system (Durston et al., 1989). Many publications describe RA's mechanisms of action and pharmacokinetics (for example, Orfanos et al., 1987).

On the basis of the morphology results (Figure 3), we adopted a standard cell culture protocol of incubating NB41A3 cells with cAMP and SH-SY5Y cells with RA prior to experimentation in order to induce differentiation. A more thorough analysis of differentiation would involve testing the expression of a variety of molecular markers associated with differentiation, such as withdrawal from the cell cycle (e.g. retinoblastoma hypophosphorylation), neurofilaments, nuclear markers of mature neurons (e.g. NeuN), neuronal polarity markers (e.g. tau, microtubule-associated protein 2), enzymatic functions (e.g. neuron-specific enolase), synaptic proteins (e.g. synaptophysin), and the phosphorylation status of a range of kinases (e.g. JNK, Akt, Erk, PKC).

However, despite not performing these analyses we are confident we can carefully proceed on to mechanistic work with these cell lines using cAMP and RA to differentiate the cells. This opinion is based on our own morphological analysis and the numerous examples in the open literature of using cAMP (for example, Prasad et al., 1976; Mena et al., 1995) and RA (for example, Encinas et al., 2000; Miloso et al., 2004; Cheung et al., 2009) to differentiate neuroblastoma cells. RA-stimulated differentiation of SH-SY5Y cells is particularly well-studied. Interestingly, there are also reports describing the use of undifferentiated SH-SY5Y cells as a neuronal model (Xue et al., 2006).

4.2 Gene expression

An analysis of cholinergic gene expression was performed on NB41A3 and SH-SY5Y cells. Whilst experiments relating to cholinergic gene expression in these cell types have been reported in the literature (Wojcikiewicz and Nahorski, 1991; Boyano-Adanez et al., 1997; Friederich et al., 2000; Ridley et al., 2002), it was considered important to empirically determine the expression of appropriate cholinergic genes in our cell lines as this is the pathway in which nerve agents exert their well-characterised effect (i.e. inactivation of AChE). Additionally, this would allow the development of gene and protein expression assays that would have an ongoing importance in a cell culture-based research programme.

The results of our analyses of cholinergic gene expression were consistent with the literature and with cholinergic neurons. Interestingly, with the exception of the murine NB41A3 ChAT gene, there were no gross differences in gene expression detected between differentiated and undifferentiated neurons for either cell line (Figure 4). This was probably due to the gene expression experiments having utilised RT-PCR for 30 cycles in order to maximise the chances of detecting the target transcripts. This type of experiment cannot be used to gauge subtle

relative changes in gene expression between samples as the PCRs almost certainly went beyond the log phase of amplification. Thus, the differentiation protocols may have produced alterations in the expression of the assayed cholinergic genes that could only be detected by quantitative PCR. Thus, we cannot draw any firm conclusions regarding differentiation on the basis of the RT-PCR results alone.

The standard culture condition adopted for these experiments was to incubate the cells with the differentiation factor for 1–2 days. However, it was learnt subsequently that a routine differentiation method for the SH-SY5Y line is to incubate the cells in RA for 5–7 days in order to allow the cells enough time to complete the differentiation process (Cheung et al., 2009). The lack of differences in gene expression between differentiated and undifferentiated neurons for both lines may indicate that the cells were not fully differentiated. However, the obvious changes in cell morphology and apparent activation of murine ChAT gene expression after addition of a differentiation factor indicates that at least some level of neuronal differentiation was likely to have occurred.

M1 and M2 acetylcholine receptor expression was confirmed by IHC in both cell lines (Figure 5). The five mAChR subtypes (M1 to M5) can be grouped on the basis of the intracellular signalling mechanisms they initiate upon ligand activation:

> The M1, M3 and M5 receptors are linked to phospholipase C and promote the hydrolysis of phosphatidylinositol 4,5-diphosphate to inositol 1,4,5-triphosphate. This increases intracellular Ca^{2+} and diacylglycerol which in turn activates protein kinase C.
>
> M2 and M4 receptors are linked to adenylate cyclase. Depending on the interacting G protein subtype, adenylate cyclase activity is stimulated leading to conversion of ATP to cAMP which enhances activity of protein kinase A (G_s), or repressed leading to the reverse (G_i).

Therefore, anti-M1 and anti-M2 antibodies were chosen in order to determine that both these signalling pathways are represented in our neuroblastoma cell lines.

4.3 Acetylcholinesterase analyses

4.3.1 Acetylcholinesterase expression and inhibition

The ability of OP nerve agents and insecticides to inhibit AChE activity will not be an area of ongoing interest for the HPPD as this is already a very well-characterised interaction. However, determination of AChE expression, and subsequent enzyme inhibition after OP exposure, in our neuroblastoma cell lines was performed as a validation of our cell culture model. AChE expression was confirmed at the nucleic acid level by RT-PCR (Figure 4 E,F) and the protein level by IHC (Figure 6 A-C). Localisation of AChE to synaptic junctions between neurons increased our confidence that the cultured neurons are forming functional synaptic connections utilising cholinergic signalling (Figure 6 B,C arrows). The enzymic activity of AChE was determined by measurement of specific substrate hydrolysis (Figure 6 D). However, whilst AChE protein and enzymic activity was detectable, the assays indicated that

the NB41A3 and SH-SY5Y cell lines (particularly the latter) had very little AChE protein present in the cells. For instance, the confocal microscopy imaging of the IHC labelled cells required very high gain settings, and the enzyme inhibition assays required seeding densities of 80000 cells instead of the usual 20000 cells for other microplate assays in order to detect endogenous enzyme activity. Western blot assays supported these observations (data not shown).

This is not regarded as a problem with using these lines as an *in vitro* model for OP toxicity as there was enough AChE enzyme present to confirm the well-characterised inhibitory effects of OP chemicals. It is doubtful the cell lines could act as a model for studies of the reactivation of OP-inhibited AChE (e.g. by oximes), but they can serve as a model for ACh receptor binding studies or for non-cholinergic mechanisms of OP toxicity.

Importantly, it was demonstrated that a dose- and compound-dependant inhibition of AChE activity could be detected when the cells were exposed to the known cholinesterase inhibitors physostigmine (a potential human therapeutic carbamate drug for nerve agent prophylaxis) and the nerve agent VX. This enzyme inhibition was quantitatively measured in the context of whole living neuronal cells, not merely purified enzyme.

4.3.2 Acetylcholinesterase SNPs

The pharmacogenomics of OP toxicity is of interest to DSTO as developing a rational means to optimise drug therapy with respect to patients' genotype will help to ensure maximum efficacy with minimal adverse effects. Correlating gene expression or SNPs with a drug's efficacy or toxicity is a primary pharmacogenomic focus. Identification of AChE SNPs allows the study of the possible association of an individual's AChE phenotype with adverse drug responses to carbamate pre-treatments and hypersensitivity to OP chemicals. For instance, SNPs in the AChE promoter have been shown to affect AChE gene expression and are a susceptibility factor for adverse reactions to cholinesterase inhibitors (Shapira et al., 2000). The occurrence of SNPs in the AChE coding region in people with different ethnic backgrounds has been studied with 15 haplotypes and five ethnospecific alleles identified (Hasin et al., 2004). Among the SNPs resulting in an amino acid substitution, three are within the mature protein, mapping on its external surface. They are, thus, unlikely to affect its catalytic properties, yet could have antigenic consequences or affect putative protein-protein interactions.

Our analysis indicated that the SH-SY5Y AChE gene does contain a previously identified non-synonymous SNP (Figure 7), a (A/C) SNP in exon 2 that is responsible for an amino acid change that influences the antigenic properties of AChE (Hasin et al., 2004). The anti-AChE antibody used in our IHC experiment was generated with a peptide corresponding to the C-terminus of the protein; the A/C SNP does not alter any of the amino acids in that peptide. However, it is possible that this SNP alters the affinity of the antibody to the SH-SY5Y AChE protein and is responsible for the relatively low signal in our IHC and Western blot assays.

5. Conclusions

The neuroblastoma cell lines chosen for evaluation as a model system for the nascent HPPD medical countermeasures research programme appear to be suitable as the basis for ongoing research. A basic investigation of aspects of the cholinergic signalling pathway was consistent with the literature and indicates the lines are appropriate for exploratory mechanistic work examining the mechanisms of nerve agent toxicity. Reproducible culturing conditions, storage and handling of cells was confirmed. Sufficient differentiation of cells based on morphological appearance was determined. Further analysis of neuronal differentiation based on molecular data may be undertaken in the future if it becomes necessary. Gene expression was consistent with cholinergic neurons. At the protein level, the presence of muscarinic acetylcholine receptors and functional AChE was determined. Importantly, a reliable assay for analysis of differential AChE inhibition by OP chemicals was developed in live cells. This can be used to compare the relative contribution of inhibited AChE versus non-AChE targets in OP-mediated neuronal toxicity. However, due to the sometimes artefactual nature of physiological responses in neuroblastoma cells, it will be important to confirm the results of novel mechanistic studies in another biological system (e.g. primary cells, brain slice cultures, neurospheres or an animal model).

This work has developed expertise in neuronal cell culture, confocal microscopy, enzyme activity assays and toxicity-related assays. The elements of the proposed experimental plan listed in the Introduction (Section 1.4) not covered in this part of the Technical Report are covered in Part II. Specifically, fluorescent labelling and confocal imaging of proteins and organelles, plasmid transfections of neurons, and assays of OP toxicity are described.

On the basis of our results and the many examples in the literature of the use of these lines for mechanistic investigations, we are confident that we can move forward with the utilisation of these cell lines for ongoing work in HPPD. The ultimate intention is to create a platform for investigating the mechanisms of toxicity of a range of CNS-acting chemicals of interest to the Department of Defence. Whether a cell line can be used as a model for different classes of chemical warfare agents will need to be determined on a case-by-case basis. Different agents exert their toxicity via different mechanisms of action; however there is always some initial indication of the molecular mechanism(s) of toxicity. Thus, whether or not we can use our cell lines as a model for any given agent will depend on whether the cells express the required molecular pathways for that agent. This can be determined before committing significant resources.

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19. ABSTRACT

Neuroblastoma cell lines NB41A3 and SH-SY5Y were evaluated as an in vitro model system for studying organophosphorus (OP) chemical toxicity in central nervous system (CNS) cell lineages. Optimal culturing conditions, neuronal differentiation protocols and appropriate cholinergic gene expression were confirmed. The presence of muscarinic receptors and acetylcholinesterase activity was determined. Importantly, differential acetylcholinesterase inhibition by OP chemicals was demonstrated in live cells. This work has developed expertise in neuronal cell culture, confocal microscopy and enzyme activity assays that will provide the basis for an ongoing research programme. The neuroblastoma cell lines chosen can potentially be used as a model for investigating the toxicity of a range of CNS-acting chemicals of interest to the Department of Defence.