The potential of metabolomic analysis techniques for the characterisation of α_1 -adrenergic receptors in cultured N1E-115 mouse neuroblastoma cells

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Abstract Several studies of neuropathic pain have linked abnormal adrenergic signalling to the development and maintenance of pain, although the mechanisms underlying this are not yet fully understood. Metabolomic analysis is a technique that can be used to give a snapshot of biochemical status, and can aid in the identification of the mechanisms behind pathological changes identified in cells, tissues and biological fluids. This study aimed to use gas chromatography-mass spectrometry-based metabolomic profiling in combination with reverse transcriptase-polymerase chain reaction and immunocytochemistry to identify

functional α_1 -adrenergic receptors on cultured N1E-115 mouse neuroblastoma cells. The study was able to confirm the presence of mRNA for the α_{1D} subtype, as well as protein expression of the α_1 -adrenergic receptor. Furthermore, metabolomic data revealed changes to the metabolite profile of cells when exposed to adrenergic pharmacological intervention. Agonist treatment with phenylephrine hydrochloride (10 μ M) resulted in altered levels of several metabolites including *myo*-inositol, glucose, fructose, alanine, leucine, phenylalanine, valine, and n-acetylglutamic acid. Many of the changes observed in N1E-115 cells by agonist treatment were modulated by additional antagonist treatment (prazosin hydrochloride, 100 μ M). A number of these changes reflected what is known about the biochemistry of α_1 -adrenergic receptor activation. This preliminary study therefore demonstrates the potential of metabolomic profiling to confirm the presence of functional receptors on cultured cells.

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Introduction

The alpha adrenergic signalling system has important effects in the adult peripheral and central nervous systems (PNS and CNS, respectively), influencing

pain perception and modulation, reducing stress-induced neuronal cell death, and affecting behavioural and motor activity in response to environmental change (Luttinger et al. 1985; Millan et al. 1994; Hong and Abbott 1996; Stone et al. 1999; Hedo and Lopez-Garcia 2001; Kingery et al. 2002; Millan 2002; Ohashi et al. 2007). Abnormal adrenergic signalling may give rise to a range of pathological conditions such as cancer (Cole and Sood 2012), depression (Wakabayashi et al. 2011) and various pain syndromes (Choi and Rowbotham 1997; Ali et al. 2000; Drummond et al. 2014). In order to study abnormal adrenergic signalling, appropriate robust techniques are needed to dissect the biology of underlying changes in receptor expression and function. Thus, emerging technologies need to be assessed in order to study receptor activity and function.

The adrenergic receptor (AR) is a G protein-coupled receptor, mediating its action via endogenous catecholamines such as adrenaline or noradrenaline (NA). Three different isoforms of the α_1 -adrenergic receptor (α_1 -AR) have been identified using molecular cloning techniques (α_{1A} -AR, α_{1B} -AR, and α_{1D} -AR) (Michel et al. 1989; Schwinn et al. 1990; Pieribone et al. 1994; Chang et al. 1998; Xiao et al. 1998). The distribution of subtypes has been described both in humans and a variety of experimental animals, with subtype-specific differences in various brain regions (Pieribone et al. 1994; Day et al. 1997; Suzuki et al. 1997; Schambra et al. 2005; Khan et al. 2007; Hertz et al. 2010). Neurons and microglia of the CNS have both been shown to express α_1 -AR (Feldstein et al. 1986; Day et al. 1997; Papay et al. 2006; Hertz et al. 2010).

It is well established that agonist-stimulation of α_1 -AR results in activation of protein kinase C, increased intracellular levels of calcium and smooth muscle contraction (Wu et al. 1992). In addition, ARs regulate blood pressure, are involved in the release of neurotransmitters in the CNS and modulate central responses to nociception (Luttinger et al. 1985; Millan et al. 1994; Dunbar 2000; Kamibayashi and Maze 2000; Tanoue et al. 2002). Previous studies have shown that stimulation of α_1 -AR by the α_1 -AR agonist phenylephrine results in increased calcium transients, an effect that can be blocked by the use of the α_1 -AR antagonist prazosin (Thorlin et al. 1998). In addition, glial α_1 -AR activation by NA promotes intracellular calcium release with downstream effects on

membrane depolarisation, neurotransmitter release and cell proliferation (Legendre et al. 1988; Stone et al. 1999; Alpini et al. 2011). It has also been demonstrated that α_1 -AR stimulation enhances glutamate uptake into cultured astrocytes (Hansson and Ronnback 1989; Fahrig 1993; Hertz et al. 2010), and Ohashi et al. (2007) showed that stimulation of α_1 -AR by phenylephrine suppressed stress-induced death and promoted cell survival in embryonic neural progenitor cells. In experiments using cultured keratinocytes Li et al. (2013) showed that exposure to NA led to increased expression of multiple adrenergic receptor subtypes on these cells as shown by real-time PCR.

Noradrenaline is a natural ligand for the α_1 -AR and does not induce pain in human or animal subjects under normal physiological conditions (Torebjork et al. 1995; Moon et al. 1999). However, in neuropathic pain states such as complex regional pain syndrome, a subset of patients appear to have a pathological catecholamine sensitivity with hyper-responsiveness of α_1 -ARs to NA (Jorum et al. 2007). Findings to date support a link between abnormal α_1 -AR signalling and pain development in various models of neuropathic pain (Raja et al. 1991; Moon et al. 1999; Li et al. 2013; Drummond et al. 2014). Thus, experimental methods should be assessed for their prospective use in the study of dysfunctional α_1 -AR signalling in neurons. This study aimed to determine whether metabolomic profiling, a technique that provides a snapshot of the biochemical status of cells, could be used to confirm the presence of functional α_1 -ARs in cultured immortalised N1E-115 mouse neuroblastoma cells. This was compared with data obtained from immunocytochemistry and reverse transcriptase (RT)-PCR.

Methods

Chemicals

Unless otherwise stated, chemicals were purchased from Sigma-Aldrich (Sydney, Australia) in the highest purity available. Solvents were purchased from LabScan (Seacliff, Australia), in the highest purity available. Primers were purchased from Integrated DNA Technologies Inc. (Coralville, IA, USA). Foetal bovine serum (FBS) and Trypsin–EDTA were obtained from Serana (Bunbury, Australia). RNAzol[®] RT reagent was

purchased from Molecular Research Center Inc. (Cincinnati, OH, USA). SYBR Safe Green and Pro-Long Gold Antifade solution were purchased from Life Technologies (Mulgrave, Australia).

Cell culture

N1E-115 mouse neuroblastoma cells (obtained via Sigma-Aldrich from the European Collection of Cell Cultures) were maintained in Dulbecco's modified Eagle's medium supplemented with 5 % (v/v) heat inactivated FBS, 1 % L-glutamine (2 mM), and 1 % penicillin/streptomycin solution (penicillin 100 units/mL; streptomycin 100 µg/mL). Cells were cultivated in a humidified incubator at 37 °C in a 5 % CO₂ atmosphere. The medium was changed every 4 days and cells were subcultured at a density of 2×10^4 cells/cm² once they reached 75–85 % confluence. Confluent cells between passage 5 and 20 were used in all experiments.

Screening for mycoplasma contamination

After subculturing cells, the culture medium was replaced with antibiotic-free medium. Cells were harvested by trypsinisation using 0.25 % Trypsin-EDTA in PBS, once they had reached 70 % confluence (approximately 5 days). The cell suspension was transferred into a 10 mL tube and cell counts were obtained using an improved Neubauer haemocytometer. An aliquot containing 4×10^6 cells were transferred into a sterile 10 mL tube and centrifuged for 10 min at $150 \times g$ at room temperature. The supernatant was removed and the cell pellet was washed with 2.5 mL PBS and centrifuged at $150 \times g$ for 10 min at room temperature. The majority of the PBS was removed and discarded and the pellet was transferred into a cryovial and stored at −20 °C until extraction and PCR detection. Genomic DNA extraction was performed using the QIAamp DNA mini kit (QIAGEN, Victoria, Australia) as per the manufacturer's instructions. PCR amplification of the 16 S RNA region for species *M. arginini*, *M. hominis*, *M. hyorhinitis*, *M. fermentans*, and *M. pharyngis*, were done in 20 µL reactions using 80 ng of DNA, 0.25 mM dNTPs (dATP, dTTP, dCTP, dGTP), 0.25 µM each of forward and reverse primer, 3 mM MgCl₂, and 0.025 units Platinum Taq DNA polymerase. Thermocycler conditions were initial denaturation

for 5 min at 95 °C, followed by 40 cycles of 95, 55, and 72 °C, each held for 15 s. The dNTPs, MgCl₂, and Platinum Taq DNA polymerase were all sourced from Invitrogen (Victoria, Australia). Cultured N1E-115 cells were confirmed free from mycoplasma contamination by PCR, no amplification product was found for any of the mycoplasma species screened (Supplementary Fig. 1).

RNA extraction and RT-PCR

Mouse brain from 8- to 12-week old female C57BL/6 mice, stored in RNAlater, obtained from the Animal Resource Centre (Murdoch, WA) was kindly provided by Dr. Philip Stumbles (Murdoch University, Animal Ethics permit number R2503/12). Total RNA was extracted from N1E-115 cells and whole mouse brain (for positive control) using RNeasy[®] RT reagent according to the manufacturer's instructions. One-step RT-PCR reactions (Access RT-PCR System, Promega, Sydney, Australia) were carried out using 1 µg RNA in 50 µL reactions. Total RNA was reverse transcribed into cDNA by the avian myeloblastosis virus reverse transcriptase (AMV RT) in the presence of 0.1 units/µL AMV RT, 0.1 units/µL Taq DNA polymerase, 0.2 mM dNTPs (dATP, dTTP, dCTP, dGTP), 2 mM MgSO₄, and 1 µM of each upstream and downstream primer, for 45 min at 45 °C. An initial denaturation and AMV RT inactivation step was performed at 94 °C for 5 min. Thermocycler conditions for PCR amplification were 94 °C for 1 min, 55 °C (for α_1 -adrenergic receptor subtype A and B) or 60 °C (for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the α_1 -adrenergic receptor subtype D) for 30 s, and 72 °C for 1.5 min for 40 cycles. A final extension step at 72 °C was carried out for 7 min. 15 µL of PCR product was analysed using gel electrophoresis on a 2 % agarose gel stained with SYBR Safe Green. A 100 bp ladder was used as a marker to determine the size of the PCR amplicons. The four previously published receptor-specific primer pairs used in the PCR reactions are listed in Table 1. The online Basic Local Alignment Search Tool (BLAST) database was used to verify the specificity of the individual primers and the amplicon sizes. In addition, RNA extracted from mouse brain was used in reactions as a positive control for the subtype specific primers. Experiments were performed in duplicate.

Table 1 Previously published oligonucleotide primers used in RT-PCR experiment, their gene target and product size

Mouse Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Product size (bp)	References
α_{1A} -AR	TTTTCTTGGCTAACAGACAGGC	GTAACAATGCCTCTTGGAAAAACAC	333	Liu et al. (2009)
α_{1B} -AR	ATACCTGGGTCTGTGGAACG	GGAGCTTGAAAGTGAAGAGTGG	140	Cikos et al. (2007)
α_{1D} -AR	CGCTGTGGTGGGAACCGGCAG	ACAGCTGCACTCAGTAGCAGGTCA	282	Nishiura and Abe (2007)
GAPDH	TGCACCACCAACTGCTTA	GGATGCAGGGATGATGTT	167	Ikeda et al. (2012)

α_1 -AR, α_1 -adrenergic receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Immunocytochemistry

Prior to cell seeding, sterilised poly-L-lysine coated glass microscope slides (76 × 26 mm, StarFrost®, ProSciTech, Townsville City, Australia) were pre-incubated with DMEM for 24 h to aid cell adhesion. Confluent N1E-115 cells were seeded onto the slides at a density of 1.5×10^5 cells/mL and incubated at 37 °C in a humidified atmosphere of 5 % CO₂ and 95 % air for 24 h. After 24 h, the cells were utilised for immunocytochemical staining. Unless otherwise stated, all steps were carried out at room temperature with 3 × 2 min washes with PBS between steps. Cells were washed in PBS for 5 min, fixed with 4 % formaldehyde in PBS (v/v) for 10 min, permeabilised for 2 min using 0.2 % TritonX-100 then incubated for 1 h with blocking solution containing 10 % donkey serum in PBS. Primary antibodies diluted in blocking solution were added to cells and slides incubated for 48 h at 4 °C, after which time cells were incubated with secondary antibodies diluted in 5 % donkey serum in PBS in the dark for 1 h- then coverslipped with ProLong Gold anti-fade solution. Slides were stored in the dark at 4 °C prior to imaging. Cells were dual- or triple-labelled with antibodies directed against the α_1 -AR and the pan-neuronal marker TUJ1, NF200 for myelinated neurons, TRPV1 and CGRP for peptidergic neurons and the TRITC-conjugated lectin *G. simplicifolia* for non-peptidergic neurons as detailed in Table 2. Images were acquired using a Bio-Rad MRC-1024 confocal laser scanning microscope (Carl Zeiss, Sydney, Australia). Images were taken sequentially with DF32 emission filters 522, 605, and 608 for excitation wavelengths 488, 568, and 647 nm using DyLight 488/Cy2, 549, and 649 fluorophores, respectively. The scans were converted

to TIFF files using Confocal Assistant 4.02 (Todd Clark Brelje).

Metabolomic analysis

N1E-115 cells were seeded onto 6-well plates and incubated until confluent. Incubation of cells was carried out under the same conditions as described for cell culturing. Three biological replicates per treatment were used, and each biological replicate included pooling of 5 wells of a 6-well plate. Cells were pre-treated with 20 mM lithium chloride (LiCl) for 10 min followed by 20 min incubation with either LC-MS grade water (control), 10 μ M phenylephrine hydrochloride (PE), 100 μ M prazosin hydrochloride (PRH), or co-administration with both PE and PRH (PRH + PE). Treatments were ceased by placing the plates on ice followed by immediate quenching and harvesting for metabolomic analysis.

Rapid sampling was performed for each biological replicate by transferring 40 μ L of growth medium from five of the six wells and pooling into a pre-chilled 1.5 mL microcentrifuge tube that was frozen at –80 °C. The remainder of the medium was aspirated from the wells and discarded. The cells were gently washed with 1 mL ice cold PBS and quenched and scraped into 1 mL ice cold PBS using a rubber cell scraper. For each biological replicate, cell suspension from five of the six wells was pooled into a pre-chilled 50 mL centrifuge tube and immediately frozen at –80 °C. Frozen samples were lyophilised (Labconco FreeZone® Plus, Labconco, Kansas City, MO, USA) and stored at –80 °C prior to extraction.

The dry matter was re-suspended in a solution of ¹³C-sorbitol in methanol (3.25 μ g/mL), transferred into fresh tubes and lysed with a Precellys 24 tissue

Table 2 Antibodies used in immunofluorescence studies with source and dilution

Lectin, antibody and their host species	Product source	Dilution
Lectin from <i>G. simplicifolia</i> -TRITC	L5264: Sigma-Aldrich, St. Louis, MO, USA	1:250
Primary antibodies		
Anti α_1 -adrenergic receptor: rabbit polyclonal	A270: Sigma-Aldrich, St. Louis, MO, USA	1:250
Anti CGRP: goat polyclonal	1720-9007: AbD Serotec, Raleigh, NC, USA	1:500
Anti NF200: chicken polyclonal	AB5539: Millipore, Billerica, MA, USA	1:5000
Anti TRPV1: goat polyclonal	Sc-12498: Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:250
Anti TUBJ1: mouse monoclonal	MMS-435P: Covance, Sydney, Australia	1:4000
Secondary antibodies		
DyLight-488: donkey anti-goat IgG	Jackson ImmunoResearch, West Grove, PA, USA	1:500
DyLight-488: donkey anti-rabbit IgG	Jackson ImmunoResearch, West Grove, PA, USA	1:600
DyLight-549: donkey anti-goat IgG	Jackson ImmunoResearch, West Grove, PA, USA	1:500
DyLight-549: donkey anti-rabbit IgG	Jackson ImmunoResearch, West Grove, PA, USA	1:1000
DyLight-649: donkey anti-chicken IgG	Jackson ImmunoResearch, West Grove, PA, USA	1:200
DyLight-649: donkey anti-mouse IgG	Jackson ImmunoResearch, West Grove, PA, USA	1:1000
Cy2: donkey anti-chicken IgG	Jackson ImmunoResearch, West Grove, PA, USA	1:500
Cy3: donkey anti-mouse IgG	Jackson ImmunoResearch, West Grove, PA, USA	1:750

CGRP calcitonin gene related peptide, *Cy2* cyanine 2, *Cy3* cyanine 3, *NF200* heavy neurofilament, *TRITC* tetramethylrhodamine isothiocyanate, *TRPV1* transient receptor potential vanilloid receptor 1, *TUBJ1* class III β -tubulin

lyser (Bertin Technologies, Aix-en-Provence, France) for 2×20 s at 6500 rpm. Lysed extracts were centrifuged at $16.1 \times 10^3 g$ for 10 min and the supernatant was transferred into 1.5 mL microcentrifuge tubes and concentrated using an Eppendorf Concentrator Plus rotary vacuum concentrator (Eppendorf South Pacific Pty. Ltd., North Ryde, Australia). Extracts were then lyophilized and stored at -80°C prior to derivatisation.

Derivatisation was carried out as previously described by Abbiss et al. (2012). Briefly, the extracts were agitated at 30°C with 20 μL methoxyamine hydrochloride in pyridine (20 mg/mL) for 90 min at 1200 rpm followed by 30 min agitation at 300 rpm and 37°C with 40 μL of *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) using an Eppendorf Thermomixer Comfort (Eppendorf South Pacific Pty. Ltd., North Ryde, Australia).

Instrumental analysis

Gas chromatography mass spectrometry (GC–MS) analysis was carried out on an Agilent 5973 N series single quadrupole mass spectrometer using an Agilent 6890 series GC system with a 7683 autosampler and

injector (Agilent Technologies, Santa Clara, CA, USA). A Varian Factor Four-fused silica capillary column VF-5 ms (30 m $\times$ ID = 0.25 mm $\times$ DF = 0.25 μm + 10 m EZ-Guard) (Agilent Technologies, Santa Clara, CA, USA) was used. Column temperature was initially increased at 1°C per minute for 5 min and then at 5.63°C per minute to a final temperature of 330°C , and held for 10 min. The inlet was held at 230°C and a volume of 1 μL per sample was injected in splitless mode into the inlet. Helium was used as the carrier gas at a constant flow rate of 1 mL per minute. The ion source and the transfer line were set to 230 and 330°C respectively. A 70 eV electron beam was used to scan masses in the range of m/z 45–600 using a scan rate of 1.56 scans per second.

Data analysis

AnalyzerPro v 2.7.0.0 (SpectralWorks, Runcorn, UK) was used for deconvolution and identification of individual mass spectra for peaks of GC–MS data. The data were compound-matched to an in-house library of metabolite standards (Metabolomics Australia node, Murdoch University) as well as the National Institute of Standards and Technology

Fig. 1 Identification of mRNA expression of the three different α_1 -adrenergic receptor subtypes in cultured N1E-115 mouse neuroblastoma cells. Total RNA (1 μ g) was used in RT-PCR reactions and the PCR product was run on a 2 % agarose gel stained with SYBR Safe. A 100 bp molecular weight marker (lane M) was used for identification of fragment size of the target amplicons, with the brightest band shown at 500 bp. No PCR product was detected for the α_{1A} or α_{1B} subtypes (lanes 2 and 3, respectively). A PCR product was detected for the α_{1D} subtype indicating expression of mRNA for the α_{1D} subtype in cultured N1E-115 mouse neuroblastoma cells (282 bp, lane 4). A single band was also detected for the experimental control GAPDH (167 bp, lane 1)

(NIST) 2005 Mass Spectral library. The data matrix of the integrated peak areas per metabolite per sample, normalised to an internal standard of D-Sorbitol-1- ^{13}C , was exported. Principal component analysis (PCA) was carried out on $\log_{10}$ transformed data using Unscrambler[®] X v 10.1 (CAMO Software, Oslo, Norway) rendering scores plots for identification of separation of metabolite profiles between the different treatment groups. A threshold of 0.05 was chosen, and identified analytes with the highest influence on the

weighted variance, as shown by X-loadings from PCA data, were selected for data interpretation.

Statistical analysis

Changes in abundance levels of specific metabolites as mean $\pm$ standard deviation were analysed using the *F* test for analysis of variance and Student's *t* test for group comparison using IBS SPSS v.21 (SPSS Inc., Chicago, IL, USA) software with a probability level of 95 % for statistical significance.

Results

Cultured N1E-115 mouse neuroblastoma cells express mRNA for α_1 -adrenergic receptors

Expression of mRNA for the different subtypes of α_1 -ARs on cultured N1E-115 mouse neuroblastoma cells was determined using RT-PCR. The experiment demonstrated that cultured N1E-115 mouse neuroblastoma cells expressed mRNA for the α_{1D} -AR subtype but did not express the α_{1A} -AR or α_{1B} -AR subtypes (Fig. 1, lanes 4, 2 and 3, respectively). A single band was also detected for GAPDH, the product of a house keeping gene commonly used as an experimental control (Fig. 1, lane 1). As a positive control, total RNA extracted from whole mouse brain

Fig. 2 Immunoreactivity of α_1 -AR and TUJ1 in cultured N1E-115 mouse neuroblastoma cells obtained by confocal immunofluorescence microscopy. The merged image (a) shows immunoreactivity detected for both α_1 -AR (b) and TUJ1 (c) confirming presence of α_1 -AR and neuronal identity of

N1E-115 mouse neuroblastoma cells. Grey arrows indicates presence of α_1 -AR immunoreactivity in the cytoplasm of neuronal cells. Scale bar is 50 μ m. Abbreviations: α_1 -AR, α_1 -adrenergic receptor; TUJ1, class III β -tubulin

homogenate showed mRNA expression for all three adrenergic subtypes (Supplementary Fig. 2).

Confirmation of expression of the α_1 -adrenergic receptor in cultured N1E-115 mouse neuroblastoma cells using immunocytochemistry

Analysis of protein expression of the α_1 -AR on cultured N1E-115 mouse neuroblastoma cells was performed using immunocytochemistry. The results showed that α_1 -AR immunoreactivity was present throughout the cells and the cytoplasm of N1E-115 cells (Fig. 2, grey arrows), and in some instances localised to the nucleus where staining appeared visually more intense in the dense centre of the cells (Fig. 3a, grey arrows). Staining was also present for BS-TRITC, CGRP, NF200, TRPV1, and TUJ1 (Figs. 2, 3). A comparison of staining for the α_1 -AR with the various neuronal markers displayed co-localisation of α_1 -AR with TUJ1 (Fig. 2), as well as with CGRP (Fig. 3a, white arrow), TRPV1 (Fig. 3b, white arrow), and BS-TRITC (Fig. 3c, white arrow) where the staining pattern for these markers overlapped with that of α_1 -AR. Staining pattern for α_1 -AR did overlap to an extent with NF200 (Fig. 3a, b, white arrow), however some NF200 staining did not overlap with that of α_1 -AR (Fig. 3a–c, white arrowhead).

Metabolomic analysis using adrenergic pharmacological intervention confirms functionality of α_1 -AR in N1E-115 cells

To further assess the functionality of the α_1 -AR, metabolomic analysis was carried out on intracellular and extracellular samples derived from cultured N1E-115 cells. N1E-115 cells were exposed to either the α_1 -AR agonist phenylephrine hydrochloride (PE; $n = 3$), the α_1 -AR antagonist prazosin hydrochloride (PRH; $n = 3$), or a combination of both the agonist and antagonist (PRH + PE; $n = 3$). Experimental data show clustering of samples from PE treatment, which does not overlap with any other treatment group in neither intracellular nor extracellular samples, demonstrating that the metabolite profile derived from PE treatment of N1E-115 cells differs from the metabolite profile from all other treatment groups tested (Fig. 4). Conversely, there was no difference in the metabolite

profile of cells exposed to PRH and controls, as shown by overlapping regions of these two treatment groups in the PCA plots of both intracellular and extracellular samples (Fig. 4). Cells exposed to a combination of the antagonist and the agonist (PRH + PE) render clusters of intracellular samples that are separated from the other treatment groups (Fig. 4a), but are appearing in overlapping regions with CONT and PRH in extracellular samples (Fig. 4b).

Across all experiments, a mean of 190 compounds was detected, of which 61 compounds were positively identified. X-loadings data from PCA analysis presenting the analytes that contribute to the variance between the treatment groups render differences between the groups that were best described by between 12 and 28 compounds, primarily amino acids and carbohydrates (Tables 3, 4). Amino acids that show opposing effects in the direction of change in relative abundance level with PE treatment compared to controls and combination treatment compared to PE were leucine, n-acetylglutamic acid, phenylalanine, and valine (Table 3). In addition, the carbohydrates fructose, glucopyranose, glucose, and hexadecanoic acid were all detected at lower levels in intracellular samples with PE treatment as compared to controls, but were detected at higher levels in samples with combination treatment compared to PE (Table 3). Metabolites that influence the difference in extracellular samples with PE treatment when compared to controls and showed a modulated direction of change in relative abundance level by combination treatment were alanine, aspartic acid, glutamic acid, glycine, isoleucine, leucine, methionine, phenylalanine, serine, threonine, valine, glucose, pyroglutamic acid, hexadecanoic acid, mannitol, *myo*-inositol, and urea (Table 4).

Discussion

In this study, the application of GC–MS-based metabolomic profiling to assay the functionality of the α_1 -AR in N1E-115 cell cultures was evaluated. Data from metabolomic analysis were considered in combination with data from RT-PCR and immunocytochemistry, for the identification of functionality of the α_1 -AR in cultured N1E-115 mouse neuroblastoma cells.

Fig. 3 Confocal microscopy images of cultured N1E-115 mouse neuroblastoma cells labelled with anti- α_1 -AR (**d–f**) in combination with various neuronal markers (**g–l**). The figure shows a wide α_1 -AR expression present throughout cells and in the cytoplasm of cultured N1E-115 cells (**e, f**), and sometimes localised to the nucleus (**a** *white arrow*, **d**). N1E-115 cells also show presence of NF200 (**g, h, and i**), CGRP (**j**), TRPV1 (**k**), and BS-TRITC (**l**). The α_1 -AR expression in N1E-115 cells co-localise with expression for CGRP, BS-TRITC, and TRPV1 (*arrow*; **a, b, and c**, respectively). Although expression of NF200 is detected, the pattern does not completely overlap with that of α_1 -AR (*arrowhead*; **a–c**). Scale bar is 50 μ m. Abbreviations: α_1 -AR, α_1 -adrenergic receptor; BS-TRITC, *Griffonia (Bandeiraea) simplicifolia* lectin, tetramethylrhodamine isothiocyanate conjugate; CGRP, calcitonin-gene related peptide; NF200, neurofilament 200; TRPV1, transient receptor potential vanilloid subfamily type 1

PCR analysis confirms expression of mRNA for α_{1D} -AR in cultured N1E-115 cells

To our knowledge, this is the first study to characterise the presence of functional α_1 -ARs in cultured N1E-115 mouse neuroblastoma cells. The present study was able to confirm mRNA expression of the α_{1D} subtype on cultured N1E-115 mouse neuroblastoma cells using RT-PCR (Fig. 1). Alonso-Llamazares et al. (1995) tested seven different regions from mouse brain, and found mRNA expression for the α_{1D} subtype in all brain regions. Similarly, the α_{1A} subtype was found in five out of the seven regions, whereas the α_{1B} subtype

was only found in the striatum, brainstem, and diencephalus (Alonso-Llamazares et al. 1995). Schambra et al. (2005) found mRNA expression for all three subtypes in human cerebellar tissue (Schambra et al. 2005). Thus, the α_{1D} subtype has been shown to be expressed in cerebellar regions in both mouse and human tissue. From the current study we were able to confirm mRNA expression of the α_{1D} subtype in cultured mouse neuroblastoma cells, which is consistent with previous findings of the distribution of the receptor in mouse brain.

Immunocytochemistry of N1E-115 cells confirms protein expression of α_1 -AR

In order to confirm expression of the α_1 -AR protein in cultured N1E-115 cells, immunocytochemical experiments were conducted using dual- or triple-labelling of N1E-115 cells with antibodies directed against the α_1 -AR, as well as various neuronal markers (Table 2). The results from this study show that cultured N1E-115 mouse neuroblastoma cells express at least one subtype of the α_1 -AR (the α_{1D} -AR), primarily throughout the cytoplasm of cells (Fig. 2, grey arrows), in some instances with additional perinuclear localisation (Fig. 3, grey arrows). Previous studies have also shown intracellular localisation of the α_{1D} -AR subtype in both human aortic smooth muscle and HEK 293 kidney cells (Chalothorn et al. 2002; Khattar

Fig. 4 PCA scores plot from GC-MS analysis of intracellular (**a**) and extracellular (**b**) metabolites derived from N1E-115 cells under various treatment conditions. Samples are normalised to internal standard and to cell number. The figure illustrates the difference in metabolite profile between treatment groups from

cells treated with either phenylephrine hydrochloride (PE, 10 μ M; $n = 3$), prazosin hydrochloride (PRH, 100 μ M; $n = 3$), cells treated with a combination of both PRH and PE (100 and 10 μ M, respectively; $n = 3$), or untreated controls (CONTR; $n = 3$)

Table 3 Fold change (*p* value) of intracellular metabolites contributing to differences between treatment groups in N1E-115 mouse neuroblastoma cells influenced by adrenergic pharmacological intervention

Class	PE/CONTR	PRH + PE/CONTR	PRH + PE/PE
Amino acids			
Alanine ^{b,c}	0.5 (0.094)	1.6 (0.146)	3.4 (0.013)*
Beta-alanine ^c	1.1 (0.882)	1.8 (0.029)*	1.7 (0.097)
Aspartic acid ^c	1.2 (0.490)	1.6 (0.695)	1.4 (0.777)
Glutamic acid ^{a,b,c}	1.2 (0.795)	1.2 (0.890)	1.0 (0.999)
Glycine ^{a,b,c}	1.0 (0.971)	1.5 (0.495)	1.5 (0.443)
Isoleucine ^{b,c}	0.9 (0.810)	1.8 (0.317)	1.9 (0.314)
Leucine ^{a,b,c}	0.9 (0.797)	1.7 (0.352)	1.9 (0.281)
Methionine ^{b,c}	1.2 (0.696)	1.7 (0.607)	1.4 (0.715)
N-Acetylglutamic acid ^{a,b,c}	0.8 (0.359)	1.6 (0.016)*	2.1 (0.028)*
Phenylalanine ^{a,c}	1.0 (0.981)	1.6 (0.540)	1.6 (0.530)
Proline ^c	0.8 (0.653)	1.3 (0.713)	1.5 (0.510)
Serine ^{a,b,c}	1.4 (0.564)	1.5 (0.720)	1.1 (0.898)
Threonine ^{a,b,c}	1.2 (0.635)	2.0 (0.242)	1.6 (0.309)
Tyrosine ^{b,c}	1.1 (0.901)	6.4 (0.320)	5.9 (0.325)
Valine ^{a,b,c}	0.9 (0.847)	1.7 (0.353)	1.8 (0.285)
Carbohydrates			
Fructose ^{a,b,c}	0.6 (0.197)	1.3 (0.323)	2.1 (0.020)*
Glucopyranose ^{a,b,c}	0.1 (0.047)*	1.5 (0.141)	12.6 (0.015)*
Glucose ^{a,b,c}	0.4 (0.062)	1.0 (0.916)	2.4 (0.004)*
Pyroglutamic acid ^{b,c}	0.9 (0.789)	1.6 (0.236)	1.7 (0.238)
Fatty acids			
9-(Z)-Octadecenoic acid ^{b,c}	1.4 (0.537)	1.9 (0.131)	1.4 (0.432)
Hexadecanoic acid ^{a,b,c}	0.6 (0.368)	1.4 (0.372)	2.2 (0.024)*
Others			
Benzoic acid ^b	0.6 (0.581)	0.0 (0.156)	0.0 (0.002)*
Glycerol 3-Phosphate ^c	0.9 (0.838)	1.6 (0.168)	1.7 (0.060)
Malic acid ^{b,c}	0.8 (0.505)	0.0 (0.014)*	0.0 (0.000)*
<i>myo</i> -Inositol ^{b,c}	0.9 (0.837)	2.6 (0.166)	2.8 (0.155)
Sorbitol ^{b,c}	1.0 (0.952)	2.0 (0.059)	2.0 (0.063)
Succinic acid ^c	0.7 (0.481)	1.1 (0.840)	1.6 (0.491)
Tryptamine ^c	1.2 (0.781)	1.5 (0.616)	1.2 (0.743)

* Statistical significance using a threshold of 0.05

^a Contributes to the variance in PE/CONTR, ^b contributes to the variance in PRH + PE/CONTR, ^c contributes to the variance in PRH + PE/PE

et al. 2006; Wang et al. 2007; García-Cazarín 2008). McCune et al. (2000) used Rat1 fibroblasts and found perinuclear expression of the α_{1D} -AR. The current study also found that the immunoreactivity for the α_1 -AR that was detected in N1E-115 cells overlapped with TUJ1, CGRP, TRPV1 and BS-TRITC positive cells (Figs. 2, 3). Positive detection of the neuronal markers supports the neuronal identity of the cultured N1E-115 cells and also indicates that this cell line may be useful in future in vitro studies with requirements for neurons expressing immunocytochemical markers for pain signalling (e.g. TUJ1). This study also investigated the presence of the 200-kD neurofilament

(NF200) which can be used as a marker of myelinated nerves (Trojanowski et al. 1986; Perry et al. 1991). This marker was detected in cultured N1E-115 cells, indicating that this cell line may possess some level of myelination (Fig. 3).

The specificity of the anti- α_1 -AR antibody has been previously shown in immunoblot analysis and has been successfully used for protein identification in western blot analysis (Nakadate et al. 2006; Khan et al. 2007). Thus, the usefulness of this antibody has been established, and the finding from this study is consistent with previous data showing intracellular localisation of the α_{1D} -AR.

Table 4 Fold change (*p* value) of extracellular metabolites contributing to differences between treatment groups in medium samples from N1E-115 mouse neuroblastoma cells influenced by adrenergic pharmacological intervention

Class	PE/CONTR	PRH + PE/CONTR	PRH + PE/PE
Amino acids			
Alanine ^{a,b,c}	0.7 (0.201)	1.3 (0.480)	1.8 (0.101)
Aspartic acid ^{a,b,c}	0.7 (0.381)	0.8 (0.545)	1.2 (0.538)
Glutamic acid ^{a,c}	0.4 (0.265)	0.7 (0.514)	1.8 (0.064)
Glycine ^{a,c}	0.7 (0.316)	1.4 (0.220)	2.0 (0.016)*
Isoleucine ^{a,b,c}	0.7 (0.365)	1.4 (0.268)	1.9 (0.033)*
Leucine ^{a,b,c}	0.8 (0.496)	1.4 (0.271)	1.7 (0.034)*
Methionine ^{a,b,c}	0.7 (0.394)	1.3 (0.338)	1.8 (0.042)*
<i>N</i> -Acetylglutamic acid ^{b,c}	0.5 (0.218)	0.3 (0.039)*	0.5 (0.512)
Ornithine ^c	0.7 (0.251)	1.4 (0.330)	2.1 (0.032)*
Phenylalanine ^{a,b,c}	0.7 (0.227)	1.3 (0.306)	1.9 (0.041)*
Proline ^c	0.7 (0.305)	1.4 (0.298)	2.0 (0.029)*
Putrescine ^c	0.6 (0.276)	1.5 (0.277)	2.4 (0.030)*
Serine ^{a,c}	0.9 (0.832)	1.7 (0.138)	1.8 (0.027)*
Threonine ^{a,c}	0.8 (0.422)	1.4 (0.265)	1.8 (0.034)*
Tyrosine ^c	0.6 (0.287)	1.3 (0.496)	2.0 (0.043)*
Valine ^{a,b,c}	0.7 (0.302)	1.4 (0.271)	1.9 (0.031)*
Carbohydrates			
Dulcitol ^{a,c}	1.2 (0.779)	1.9 (0.144)	1.6 (0.337)
Fructose ^{a,b,c}	1.1 (0.677)	2.0 (0.031)*	1.8 (0.016)*
Galactose ^c	0.8 (0.696)	1.2 (0.801)	1.5 (0.418)
Glucose ^{a,b,c}	0.6 (0.223)	1.6 (0.216)	2.7 (0.019)*
Pyroglutamic acid ^{a,b,c}	0.7 (0.424)	0.8 (0.615)	1.1 (0.796)
Fatty acids			
9-(<i>Z</i>)-Octadecenoic acid ^{a,b,c}	0.4 (0.133)	0.2 (0.075)	0.6 (0.127)
Hexadecanoic acid ^{a,b,c}	0.6 (0.271)	0.9 (0.811)	1.4 (0.235)
Others			
Glycerol ^c	0.6 (0.251)	1.4 (0.330)	2.2 (0.031)
Mannitol ^{a,c}	0.9 (0.767)	1.5 (0.236)	1.7 (0.032)*
<i>myo</i> -Inositol ^{a,b,c}	0.8 (0.467)	1.6 (0.116)	2.0 (0.017)*
Succinic acid ^c	0.7 (0.470)	1.1 (0.847)	1.5 (0.248)
Urea ^{a,c}	0.8 (0.390)	1.4 (0.221)	1.9 (0.021)*

* Statistical significance using a threshold of 0.05

^a Contributes to the variance in PE/CONTR, ^b contributes to the variance in PRH + PE/CONTR, ^c contributes to the variance in PRH + PE/PE

Metabolomic analysis of N1E-115 cells exposed to adrenergic pharmacological intervention confirms functionality of the α_1 -AR

PCR and immunocytochemistry experiments were conducted to confirm the expression of α_1 -AR on N1E-115 cells. In order to determine whether metabolomic analysis could be utilised to assay receptor functionality, cells were cultured in 6-well plates and analysis was performed on cells treated with the α_1 -AR agonist phenylephrine hydrochloride (PE), the α_1 -AR antagonist prazosin hydrochloride (PRH), a combination of both the agonist and the antagonist

(PRH + PE), or on control cells (CONTR). Scores plots derived from analysis of both intracellular and extracellular data show separation of PE-treated cells from all other treatment groups tested (Fig. 4). The results therefore show that treatment of cultured N1E-115 cells with PE induces cellular biochemical changes to render PE-treated cells different from CONTR, PRH, or PRH + PE treated cells. Further investigation of the data identified several changes in metabolite levels that could be directly linked to activation of the α_1 -AR receptor. While additional work is required to confirm that these changes are the direct result of the pathways suggested, they provide

an indication of the ability of the technique to study receptor functionality.

When comparing the various treatment groups, it is apparent that there was a reduction in the levels of *myo*-inositol in both intracellular and extracellular samples of PE treated cells when compared to CONTR (Tables 3, 4). α_1 -AR stimulation results in activation of phospholipase C and the hydrolysis of inositol polyphosphates into inositol trisphosphate and diacylglycerol (Berridge 1984; Wu et al. 1992). Previous in vitro studies have shown that cellular recycling of inositol polyphosphates can be retarded by applying LiCl, which inhibits inositol-1-phosphatase thereby hampering the dephosphorylation of *myo*-inositol-1-phosphate into *myo*-inositol (Naccarato 1974; Hallcher and Sherman 1980). LiCl was used in this study to ensure that any effect induced by adrenergic signalling was captured for metabolomic analysis at the time of sample quenching, essentially functioning to ‘slow down’ the response of the cells. Changes to the levels of *myo*-inositol detected in this study could potentially be explained by α_1 -AR activation by PE inducing the hydrolysis of phosphoinositides, with LiCl preventing the recycling of phosphoinositides, thereby reducing the levels of *myo*-inositol (Berridge 1984; Wu et al. 1992). Conversely, an increase in the levels of *myo*-inositol was found in intracellular and extracellular samples from PRH + PE treated cells as compared to both CONTR and PE (Tables 3, 4). By blocking α_1 -AR using PRH, the activity of the receptor remained unchanged with PE treatment. This would have inhibited both PE induced and baseline hydrolysis of phosphoinositides, thereby sparing inositol, resulting in increased levels of *myo*-inositol (Tables 3, 4).

A comparison of the metabolite profile of PE treatment and CONTR shows a reduction in the levels of carbohydrates as well as specific amino acids in PE treated cells (Table 3). PE-treated cells also had increased levels of glutamic acid and other amino acids when compared to CONTR (Table 3). Based on current knowledge about adrenergic receptor activation (Wu et al. 1992; García-Sáinz 2000; Piascik and Perez 2001), assumptions can be made that stimulation of cultured N1E-115 cells with PE induces energy-dependent cellular processes. It therefore seems plausible that the observed reduction in carbohydrates may be the result of increased utilisation to meet a higher energy demand. Data derived from extracellular samples also demonstrated reduction in the levels

of carbohydrates in the PE-treated group when compared to CONTR (Table 4). Thus, cells may have increased their uptake of carbohydrates from the medium to account for higher energy metabolism. Neurons also have the ability to utilise alanine as an alternative energy source, which could explain the reduction in the level of alanine (Tables 3, 4) (Zwingmann et al. 2000).

Data also showed a decrease in intracellular and extracellular levels of both glucose and alanine, as well as an increase in intracellular levels of glutamic acid, with PE treatment when compared to CONTR (Tables 3, 4). Both neurons and glial cells are capable of metabolising glucose via oxidative phosphorylation into CO₂ and water (Itoh et al. 2003). It is also known that glial cells can utilise glucose for the generation of glutamate via transamination of the TCA cycle intermediate α -ketoglutarate (Sonnewald et al. 1996; Westergaard et al. 1996). Glutamate is the main excitatory neurotransmitter in the mammalian CNS and is therefore strictly regulated to prevent neuronal over-excitability and excitotoxicity (Choi 1988; Greene and Greenamyre 1996; Michaelis 1998). α_1 -AR stimulation has previously been shown to result in increased glutamate uptake into cells (Hertz et al. 2010). Thus, an increase in intracellular glutamic acid may originate from glucose conversion into glutamate, via transamination of α -ketoglutarate with branched-amino acids such as alanine, thereby reducing the levels of glucose and alanine whilst increasing intracellular levels of glutamic acid.

The current study used the α_1 -AR antagonist PRH to assess if agonist-induced changes to the metabolite profile of cultured N1E-115 cells could be modified. Data derived from the study showed overlapping regions of PRH + PE-treated cells with CONTR (Fig. 4). This overlap suggests that treatment of cultured N1E-115 cells with PRH in combination with PE resulted in modification of the effects of PE alone to such an extent that PRH + PE could not be differentiated from control. The same data also identified differences in the metabolite profile of PRH + PE- and PE-treated cells, as shown by separation of these treatment groups on the PCA scores plot (Fig. 4). From the data, it is clear that the levels of carbohydrates, as well as specific amino acids, were increased, and the level of malic acid was reduced in cells exposed to PRH + PE treatment when compared to PE treatment (Table 3). In fact, opposing effects

induced by PE versus PRH + PE treatment were seen for multiple metabolites including many carbohydrates, several amino acids (e.g. alanine, glutamic acid, glycine, leucine, phenylalanine, and valine, see Tables 3 and 4), and *myo*-inositol as described earlier. Thus, whilst PE treatment of cultured N1E-115 cells resulted in a reduction in the levels of carbohydrates, PRH + PE treatment led to an increase (Table 3). As agonist treatment with PE could induce cellular processes which may increase the demand for energy, additional treatment with an antagonist, such as prazosin, is likely to partially or completely block any stimulation of the cells induced by the agonist (Arunlakshana and Schild 1959; Kenny et al. 1995). The differing results found with PRH + PE treatment when compared to PE treatment suggest that treatment of N1E-115 cells with PRH modulated the cellular response to PE, further confirming the presence of a functional α_1 -AR in these cells.

Conclusions

The results obtained from metabolomic analysis show that cultured N1E-115 neuroblastoma cells could be stimulated by the α_1 -AR agonist phenylephrine, which induced biochemical changes within the cells that rendered a metabolite profile that differed from control. Furthermore, these changes were modulated, and for several metabolites even reversed, by additional treatment with the α_1 -AR antagonist prazosin. Changes in many of the metabolites observed in this study were consistent with α_1 -AR stimulation, for example alterations in the levels of carbohydrates and *myo*-inositol. The observed differences in the metabolite profile of cultured N1E-115 mouse neuroblastoma cells by adrenergic pharmacological treatment indicate that the α_1 -AR is likely a functional receptor. This supports the use of metabolomic technologies for studying receptor functionality using pharmacological intervention. The ability to detect changes in the profile of metabolites after drug treatment by using metabolomic analysis also demonstrates the usefulness of this technique in identifying biochemical changes in cells caused by drug treatment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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