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Therapeutic concentrations of valproate but not amitriptyline increase neuropeptide Y (NPY) expression in the human SH-SY5Y neuroblastoma cell line

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ABSTRACT

Neuropeptide Y (NPY) is a peptide found in the brain and autonomic nervous system, which is associated with anxiety, depression, epilepsy, learning and memory, sleep, obesity and circadian rhythms. NPY has recently gained much attention as an endogenous antiepileptic and antidepressant agent, as drugs with antiepileptic and/or mood-stabilizing properties may exert their action by increasing NPY concentrations, which in turn can reduce anxiety and depression levels, dampen seizures or increase seizure threshold.

We have used human neuroblastoma SH-SY5Y cells to investigate the effect of valproate (VPA) and amitriptyline (AMI) on NPY expression at therapeutic plasma concentrations of 0.6 mM and 630 nM, respectively. In addition, 12-O-tetradecanoylphorbol-13-acetate (TPA) known to differentiate SH-SY5Y cells into a neuronal phenotype and to increase NPY expression through activation of protein kinase C (PKC) was applied as a positive control (16 nM). Cell viability after drug treatment was tested with a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. NPY expression was measured using immunofluorescence and quantitative RT-PCR (qRT-PCR). Results from immunocytochemistry have shown NPY levels to be significantly increased following a 72 h but not 24 h VPA treatment. A further increase in expression was observed with simultaneous VPA and TPA treatment, suggesting that the two agents may increase NPY expression through different mechanisms. The increase in NPY mRNA by VPA and TPA was confirmed with qRT-PCR after 72 h. In contrast, AMI had no effect on NPY expression in SH-SY5Y cells.

Together, the data point to an elevation of human NPY mRNA and peptide levels by therapeutic concentrations of VPA following chronic treatment. Thus, upregulation of NPY may have an impact in anti-cancer treatment of neuroblastomas with VPA, and antagonizing hypothalamic NPY effects may help to ameliorate VPA-induced weight gain and obesity without interfering with the desired central effects of VPA.

1. Introduction

Neuropeptide Y (NPY) is a 36 amino acid peptide that belongs, together with polypeptide YY (PYY) and pancreatic polypeptide (PP), to the pancreatic polypeptide family [1,2]. Within the brain, NPY is involved in physiological processes like feeding, energy balance, circadian rhythms, learning, memory and hippocampal neurogenesis, and is important in obesity, anxiety disorders, depression, nociception, addiction and epilepsy [3–9]. It is localized in GABAergic interneurons often coexpressed with somatostatin, nitric oxide synthase (NOS) and NADPH diaphorase (NADPHd) and in monoaminergic neurons [10–15]. In the periphery, NPY has long-lasting vasoconstrictor activity upon release from sympathetic terminals and the adrenal gland, where it is co-stored with noradrenalin (NA) in secretory granules, but some parasympathetic neurons also contain NPY. Moreover, NPY is evident in the gastrointestinal tract, liver, pancreas, spleen, heart, endothelial cells of blood vessels and megakaryocytes [8,10]. Peripheral NPY can be coexpressed with NOS and peptides like bombesin, cholecystokinin, vasoactive

Abbreviations: AMI, amitriptyline; AP-1, activator protein 1; Atoh1, atonal homolog-1; BDNF, brain-derived neurotrophic factor; bp, base pair; CREB, cAMP response element-binding protein; Cp, crossing point; ERK, extracellular signal-regulated kinase; DMEM/F12 Ham, Dulbecco's Modified Eagle's Medium F12 Ham; FCS, fetal calf serum; GSK3, glycogen synthase kinase-3; HBSS, Hanks balanced salt solution; HDAC, histone deacetylase; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NA, noradrenalin; NADPHd, NADPH diaphorase; Ngn1, neurogenin-1; NOS, nitric oxide synthase; NPY, neuropeptide Y; PI3K, phosphatidylinositide 3-kinase; PBS, phosphate buffered saline; PIMT, protein L-isoaspartyl methyltransferase; PKC, protein kinase C; PP, pancreatic polypeptide; PYY, polypeptide YY; RT, reverse transcription; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SDHA, succinate dehydrogenase complex, subunit A, flavoprotein; SSRI, selective serotonin reuptake inhibitor; TPA, 12-O-tetradecanoylphorbol-13-acetate; trk, tropomyosin-receptor kinase; VPA, sodium valproate.

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intestinal peptide, galanin, calcitonin gene-related peptide, substance P, Met-enkephalyl-Arg-Gly-Leu and peptide histidine isoleucine [16–22].

NPY has recently come into focus, because it has antidepressant and antiepileptic properties, and is an attractive target for treating obesity [6,9,23–27]. In addition, some therapeutic effects of antidepressants or anticonvulsants like valproate (VPA), which have mood stabilizing properties [28–30], may be mediated via upregulation of NPY expression as it was shown in rodents. In rats displaying depressive-like behavior, treatment with the tricyclic antidepressant amitriptyline (AMI) increased immunohistochemical expression of NPY in the hypothalamus [31]. Likewise Brill et al. [32] reported that chronic VPA administration significantly increases NPY peptide and mRNA expression in the rat nucleus reticularis thalami and hippocampus, and reduces spontaneous synchronous thalamic epileptiform oscillations probably through a NPY Y1 receptor mediated mechanism.

NPY is also a tumoral marker for neuroblastomas (pheochromocytomas) originating from the medulla of the adrenal gland or extra-adrenal chromaffin tissue known to secrete NPY and catecholamines [8,33]. The SH-SY5Y cell line, derived from malignant neuroblastoma, exhibits many properties of mature sympathetic neurons like synthesis of NA and NPY, and depolarization-evoked release of NA [34]. Furthermore, SH-SY5Y cells show enhanced NPY expression upon treatment with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) [35,36]. The aim of the study was therefore to elucidate whether VPA and AMI that enhance NPY levels in rodents also upregulate NPY in the human context by using the human neuronal-like SH-SY5Y cell line as an in vitro model and administering TPA as a control treatment. The results show that therapeutic doses of VPA on its own or in the presence of TPA can directly increase NPY gene expression in SH-SY5Y cells, whereas AMI treatment was not effective.

2. Material and methods

2.1. Cell line, MTT assay and drug administration

SH-SY5Y cells obtained from the ATCC (American Type Culture Collection, http://www.atcc.org) were cultured in Dulbecco's Modified Eagle's Medium F12 Ham (DMEM/F12 Ham) supplemented with 10% v/v fetal calf serum (FCS), 1% L-glutamine, 1% v/v 100 units per ml penicillin and 100 mg/ml streptomycin in a humidified incubator with 5% CO2 at 37 °C (ThermoForma Series II Water Jacketed CO_2 Incubator, HEPA Filter, Fischer Scientific, Dublin, Ireland). Cells were maintained in 60 mm-diameter tissue culture Petri dishes (Corning Incorporated Life Sciences, Dublin, Ireland) and passaged every week by rinsing in 2–3 ml of Hanks balanced salt solution (HBSS) and trypsinizing them in a sterile laminar flow Class II Microflow Biological Safety Cabinet (Astec Microflow, Hampshire, United Kingdom). In the current experiments, cells from passages 25–40 were used. Cells were routinely seeded and grown in 24 well plates for immunocytochemistry, and in six well plates to harvest RNA. Cells were plated at a density of 6.5×10^5 cells per 60 mm dish, and treated with TPA, and the drugs VPA and AMI alone or in combination with TPA. Working solutions of therapeutic drug concentrations were made up in supplemented DMEM/F12 Ham media. Cell viability was tested using a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [37] by incubating cells for 72 h with medium only, 0.6 M VPA (control vs. VPA, n = 12 wells per group), 250 nM, 630 nM and 4.7 μM AMI (control vs. drug concentrations, n = 6 wells per group), and 16 nM TPA (control vs. TPA, n = 12 wells per group; VPA vs. VPA + TPA or AMI vs. AMI + TPA n = 8 wells per group). Cells were treated at 37 °C for 3–4 h with 0.5 mg/ml MTT (Sigma-Aldrich, Wicklow, Ireland) added to the medium. After removal of the medium containing MTT, lysis buffer was added, and absorbance of the purple formazan product was measured at 570 nm in a spectrophotometer (Sunrise™, Tecan Group Limited, Switzerland). Based on the outcome of the MTT assays and effective drug concentrations [38], TPA was used at a concentration of 16 nM in the following experiments. Sodium VPA (Epilim™ Bon Secours Hospital, Cork, Ireland) was applied at the upper therapeutic plasma concentration of 0.6 mM (100 mg/l) [39], which is also effective in vitro [40]. For AMI, the nontoxic concentration of 630 nM (~200 ng/ml) was used. This concentration is at the upper level [41] or slightly above the level of the therapeutic plasma concentration of AMI [42]. Stock solutions of TPA, VPA and AMI (1 mM) were prepared with autoclaved sterile water and stored at −80 °C.

2.2. Immunocytochemistry and image analysis

The medium was carefully removed from the treated cells in the 24 well plates. The wells were washed in pre-warmed HBSS, and then suctioned off with a sterile glass Pasteur pipette. The cells were then fixed with 4% paraformaldehyde in 10 mM phosphate buffered saline (PBS) for 20 min at room temperature. Unspecific binding sites were blocked, and the cells were permeabilized in a single step in the wells for 30 min at room temperature with 20% normal horse serum (Vector Labs, United Kingdom) in 0.02% Triton-X-100 and PBS (10 mM). The blocking/permeabilizing solution was removed after 30 min, and the wells were incubated with the polyclonal anti-human/anti-mouse NPY antibody made in goat (1:500; sc-14728, Santa Cruz Biotechnology Inc., TX, USA) in 10 mM PBS containing 1% normal horse serum for 12 h at 4 °C. The first well of each row of the 24 well plate was left blank, the second well as a negative control, in which cells were incubated with blocking/permeabilizing solution without primary antibody to account for non-specific binding (omitting primary antibody resulted in the lack of NPY immunofluorescence), and cells from the third through sixth wells were stained for NPY immunofluorescence. The next day, the primary antibody was removed and the wells were washed 3 times for 5 min each in 0.02% Triton-X-100 in 10 mM PBS. Incubation with the secondary antibody was carried out using Cy3 coupled anti-goat IgG produced in rabbit (1:50; C-2821, Sigma-Aldrich, Wicklow, Ireland) in 10 mM PBS for 1 h at room temperature. The wells were washed again 3 times for 5 min, and the nuclei counterstained with bisbenzimide (1:2000, 3.33 μg/ml) in 10 mM PBS for 4 min, followed by 2 × 5 minute washes in 0.02% Triton-X-100.

Immunofluorescence was visualized with an inverted microscope (IX70, Olympus, United Kingdom, www.olympus-global.com/en/network/). Images were captured with a 40× objective using a DP50 digital camera (Olympus, United Kingdom) at equal microscope settings. Four images were taken from each of the 4 wells per treatment group stained for NPY immunofluorescence in a plate. In each of these 4 images per well, 11 cells were randomly selected for densitometry analyses (altogether 44 cells per well), and 8–12 wells were analyzed per each treatment group (352–528 cells per treatment group). A background reading was taken from each image, and the fluorescence intensity of cells was measured in four images per 15.6 mm well using densitometry analysis (ImageJ Software; National Institutes of Health (NIH), Bethesda, MD, USA). The relative mean soma fluorescence intensity was calculated according to Gutierrez et al. [43] by subtracting the background reading from the fluorescence intensity of cells. In order to avoid experimental bias between groups, all treatment groups (untreated controls, TPA, drug, TPA and drug) were equally represented in a separate row in each plate. In total, three 96-well plates were analyzed per VPA as well as AMI experiments.

2.3. Standard and quantitative RT-PCR

Standard reverse transcription polymerase chain reaction (RT-PCR) was employed to confirm the presence of NPY expression in our SH-SY5Y cells (primer pair: NPY_Forward 5′-TGCTAGGTAACAAGCGACTG-3′, NPY_Reverse 3′-CTGCATGCATTGGTAGGATG-5′) followed by agarose gel electrophoresis to visualize the 387 bp reaction product. Quantitative RT-PCR (qRT-PCR) was carried out to determine NPY mRNA levels using a gene specific primer/probe combination that was calculated by