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Increasing pro-survival factors within whole brain tissue of Sprague Dawley rats via intracerebral administration of modified valproic acid

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ABSTRACT

Neural tissue exposure to valproic acid (VPA) increases several pro-survival phospho-proteins that can be used as biomarkers for indicating a beneficial drug response (pAkt^{Ser473}, pGSK3β^{Ser9}, pErk1/2$^{Thr202/Tyr204}$). Unfortunately, targeting VPA to neural tissue is a problem due to severe asymmetrical distribution, wherein the drug tends to remain in peripheral blood rather than localizing within the brain. Intracerebral delivery of an amide-linked VPA–PEG conjugate could address these issues by enhancing retention and promoting cerebro-global increases in pro-survival phospho-proteins. It is necessary to assay for the retained bioactivity of a PEGylated valproic acid molecule, along with locating an intracranial cannula placement that optimizes the increase of a known downstream biomarker for chronic VPA exposure. Here we show an acute injection of VPA–PEG conjugate within brain tissue increased virtually all of the assayed phospho-proteins, including well-known pro-survival factors. In contrast, an acute injection of VPA expectedly decreased signaling throughout the hour. Needle penetration into whole brain tissue is the intentional cause of trauma in this procedure. The trauma to brain tissue was observed to overcome known phospho-protein increases for unmodified VPA in the injected solution, while VPA–PEG conjugate appeared to induce significant increases in pro-survival phospho-proteins, despite the procedural trauma.

1. Introduction

Valproic acid (VPA) is a simple 8-carbon carboxylic acid that has been shown to increase pro-survival biomarkers in cell culture, peripheral administration and chronic intracerebral delivery (1). Several of the increased biomarkers are the phospho-proteins, pAkt^{Ser473}, pGSK3β^{Ser9} and pErk1/2$^{Thr202/Tyr204}$; with total protein increases seen in factors such as glial-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) (2). While neurologically beneficial, peripheral administration of VPA usually carries unwanted side effects on non-targeted organs, which can lead to life-threatening complications (1). Intracerebral delivery of VPA could circumvent endangering peripheral organs by releasing drug directly into target tissue at a significantly lower concentration, while still enhancing the protection of neurological function (1,3,4). Agents capable of increasing survival signaling events involving Akt and Erk, like VPA, have been shown to confer protection during various insults, such as chemical exposure, traumatic brain injury (TBI) and

Abbreviations: Akt, v-Akt Murine Thymoma Viral Oncogene Homolog; APP, amyloid precursor protein; BAD, Bcl-2-associated death promoter; BBB, blood-brain barrier; BCSFB, blood-CSF barrier; BDNF, brain-derived neurotrophic factor; CREB, cAMP response element-binding; CSF, cerebrospinal fluid; ER, endoplasmic reticulum; EP, elevated plus maze; Erk, extracellular signal-regulated kinase; FOXO1, Forkhead box other 1; GAP-43, growth cone associated protein 43; GDNF, glial-derived neurotrophic factor; GSK3β, glycogen synthase kinase 3 beta; ICV, intracerebroventricle; MAPKAPK-2, MAP kinase-activated protein kinase 2; MDM2, Mouse double minute 2 homolog; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MRP, multidrug resistance proteins; mTOR, mammalian/mechanistic target of rapamycin; OF, open field maze; PDK1, phosphoinositide-dependent protein kinase 1; PEG, poly-(ethylene glycol); Pgp, P-glycoprotein transporter; PI3K, phosphoinositide 3-kinase; SD, Sprague Dawley; VPA, valproic acid; VPA–PEG, valproic acid–poly ethylene glycol conjugate.

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nutrient depravation (5–10). VPA also functions as a histone deacetylase inhibitor (HDI), which is a drug class that has been identified to promote survival in neurodegenerative disease models (11–13). Conversely, while VPA has many examples of preventing cell death, it has been reported to increase apoptosis and immunogenicity in tumor cells (14).

In a previous paper (1) we postulated the use of intracerebral VPA administration, but the application is limited due to the drug's poor blood-to-brain ratio (15–19). Cornford et al (15) reported the rat brain-to-blood efflux of radiolabeled VPA from CSF to be faster than that of water and such a change was observable within 4 min. Furthermore, Kakee et al (17) intracerebrally injected Sprague Dawley (SD) rats with 25 mM VPA and found an 89.1% removal after 5 min, which was not significantly different from their 100% clearance control. This perhaps explains how patients taking peripheral VPA have been reported to show wide variations in concentration ranging from 39 μM to 185 μM in brain tissue and 18 μM–262 μM in cerebrospinal fluid (CSF; (20)). The main cause for this asymmetrical distribution is still unknown, but VPA appears not to be transported by Pgp, MRP1 or MRP2 at the blood–brain barrier (BBB)/blood-CSF barrier (BCSFB; (21)). Such evidence for carrier mediation is provided by Kakee et al (17), who reported VPA clearance to significantly decrease to 40.2% after 5 min upon intracerebrally injecting 50 mM VPA in an attempt to overload the unknown mechanism.

Combining intracerebral infusion with a simple amide-linked VPA–PEG conjugate molecule could address this rapid 'leak' issue by increasing intracerebral drug retention, rather than it exiting into peripheral circulation shortly after entering the brain. Borrowing from a prodrug design concept, to 'lock-in' the therapeutic agent, we would enhance the size and water solubility with a biocompatible poly-ethylene glycol (PEG) molecule to promote brain compartmentalization (22,23). We would also exploit the rapid clearance of unmodified VPA from the cerebral compartment to test for successful enhancement of tissue retention and preserved bioactivity by assaying for the known downstream biomarkers of VPA exposure within whole brain tissue.

In the current study, we assayed for differential phosphoprotein levels in brain tissue after an acute injection of VPA or VPA–PEG conjugate in a whole animal model. This helped us to verify injection site retention and preserved bioactivity of the conjugate versus unmodified drug performance. In addition, three separate groups of rats were chronically infused for 25 days at a different location for each group to help address infusion site importance in biomarker elevation (BDNF) and long-term exposure to the drug solution (sagittal fissure, lateral ventricle, caudate putamen tissue). We also ran behavioral paradigms on the chronically infused rats to determine if there were any overt behavioral perturbations brought on by the conjugate.

2. Material and methods

2.1. Animals

Adult male Sprague Dawley rats (Harlan SD, Indianapolis ID) were housed in pairs in shoe box cages with aspen chip bedding, prior to experiments, under constant temperature (21 °C, ±1 °C) and 12 h lighting (lights on from 6 a.m. to 6 p.m.). Rats were given access to water and rodent chow (Harlan Teklad; Indianapolis, IN) *ad libitum*. Procedures have been approved by the Institutional Animal Care and Use Committee (IACUC, Veterans Affairs Medical Center, Eastern Colorado Healthcare System) and follow American Association for Laboratory Animal Science (AALAS) guidelines.

2.2. Conjugation of valproic acid with tri-branched poly-(ethylene glycol) molecule

Widely used zero-length crosslinking agents were reacted to form a stable amide bond between a tri-branched PEG and VPA molecule (2.3 kDa). Both molecules have single parking areas, creating an optimal conjugation by allowing only one VPA–PEG conjugate species to form. For the reaction mix, amino-$dPEG_4$-$(m\text{-}dPEG_{12})_3$ (Quanta BioDesign Ltd., 10400; Powell, OH) was added to 0.1 M MES buffer (pH 5; 2-(*N*-morpholino)ethanesulfonic acid), rocked at room temperature in a glass vial until completely dissolved, then filter sterilized into a new glass vial (National Scientific, B7800–3; Rockwood, TN). Each component was slowly added dropwise to the reaction vial while being filter sterilized (0.2 μm pore; EMD Millipore, SLGV004SL; Billerica, MA): valproic acid, sulfo-NHS (sulfo-hydroxysuccinimide) and EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide). The final reaction mixture was wrapped in foil and gently rocked at 4 °C for 4 h. The reacted mixture was then glass pipetted into a dialysis cassette with a molecular weight cutoff of 2 kDa (Pierce/Thermo Scientific, 87718; Rockford, IL). Dialysis series occurred in a glass beaker with stirring bar: 12 h in 2.3 L of PBS (phosphate buffered saline; 4 °C; pH 7.4), then three more exchanges of buffer at room temperature (2 h each in 2.3 L). A glass syringe was then used to remove the solution, wrapped in foil, and stored at 4 °C. All glass and metal utensils were washed four times with Optima LC/MS water (Fisher Chemical, W6-500; Kalamazoo, MI) and Optima LC/MS methanol (Fisher Chemical, A456-500; Kalamazoo, MI) before use.

2.3. Acute intracranial injection surgery

Rats (275–300 g) were placed in a stereotaxic instrument (Model 900, KOPF Instruments; Tujunga, CA) while anesthetized under a vaporized isoflurane and oxygen mix (901807, Mobile Laboratory Animal Anesthesia System, VetEquip Inc.; Pleasanton, CA). The scalp was incised and a burr hole opened at coordinates: [+1 mm anterior to bregma; +2.6 mm lateral to midline]. Reference coordinates and outline image used from Paxinos and Watson (1). A 25 gauge, 5/8″ needle attached to a 1 ml syringe loaded with 100 μl of 3 mM PEG/VPA was slowly inserted into the burr hole and pulled back to create a pocket (syringe attached to stereotax arm). The solution was then injected over the course of 2.5 min to clear contaminating blood/bone and deposit drug within the pocket (allowed to backfill while slowly retracting needle). Approximately 5 μl of solution remained within the pocket after the backfilling procedure was completed and clearing solution was absorbed with sterile surgical spears (18105-01, Fine Science Tools; Foster City, CA). After injection, the rat was kept under anesthesia until sample collection (Fig. 1A).

2.4. Implant surgery

Rats (275–300 g) were anesthetized as described above. The groups were stereotaxically implanted with a cannula (Brain Infusion Kit 1, ALZET, Durect; Cupertino, CA) at one of the following coordinates: [+1 mm anterior to bregma; +2.6 mm lateral to midline and; 4.0 mm ventral to skull; right CPu tissue], [+1 mm anterior to bregma; +1.3 mm lateral to midline and; 4.0 mm ventral to skull; right lateral ventricle], [+1 mm anterior to bregma; 0 mm lateral to midline and; 2.0 mm ventral to skull; mid sagittal fissure; spacers added to position above corpus callosum]. Reference coordinates and outline image used from Paxinos and Watson (1). The cannula was attached by Micro-Renathane tubing (MRE040, Braintree Scientific; Braintree, MA) to an osmotic minipump (Model 2004, 0.25 μl/h, ALZET, Durect; Cupertino, CA) loaded with