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VALPROIC ACID REDUCES SPATIAL WORKING MEMORY AND CELL PROLIFERATION IN THE HIPPOCAMPUS

J. UMKA,[a]* S. MUSTAFA,[a] M. ELBELTAGY,[b] A. THORPE,[a] L. LATIF,[a] G. BENNETT[a] AND P. M. WIGMORE[a]

[a]*School of Biomedical Sciences, University of Nottingham Medical School, Queen's Medical Center, Nottingham NG7 2UH, UK*

[b]*Department of Anatomy, Menoufiya University, Egypt*

Abstract—Valproic acid (VPA) is widely used clinically, as an anticonvulsant and mood stabilizer but is, however, also known to block cell proliferation through its ability to inhibit histone deacetylase enzymes. There have been a number of reports of cognitive impairments in patients taking VPA. In this investigation we examined the relationship between cognition and changes in cell proliferation within the hippocampus, a brain region where continued formation of new neurons is associated with learning and memory. Treatment of rats by i.p. injection of VPA, reduced cell proliferation in the sub granular zone of the dentate gyrus within the hippocampus. This was linked to a significant impairment in their ability to perform a hippocampus-dependent spatial memory test (novel object location). In addition, drug treatment caused a significant reduction in brain-derived neurotrophic factor (BDNF) and Notch 1 but not doublecortin levels within the hippocampus. These results support the idea that VPA may cause cognitive impairment and provide a possible mechanism for this by reducing neurogenesis within the hippocampus.

Key words: hippocampus, neural progenitor proliferation, cognition.

The widely used anticonvulsant, Valproic acid (VPA) is also used as a mood stabilizing drug in both adults and children (Henry, 2003; Buckley, 2008). Its mechanism of action is not fully understood but it appears to reduce neuronal activity by blocking sodium and calcium channels while elevating GABA and reducing aspartate levels within the brain, for review see (Kwan et al., 2001). Despite its wide use, VPA is a known teratogen causing neural tube defects and has been associated with behavioural problems in children exposed to the drug during gestation (Meador et al., 2008; Nicolai et al., 2008). These effects are likely to be caused by its activity as a histone deacetylase inhibitor (Gurvich et al., 2005; Phiel et al., 2001). Exposure to VPA causes hyperacetylation of DNA leading to increased expression of growth arrest and pro-differentiation genes, for review see (Kostrouchova et al., 2007). Both *in vitro* and *in vivo*, exposure to VPA leads to an inhibition of cell proliferation by up regulation of p21/waf1, a cyclin-dependent kinase inhibitor, together with an increase in apoptosis (Das et al., 2007; Li et al., 2005).

A number of reports have documented a range of mild to moderate cognitive impairments, including memory deficits, in adult patients taking VPA (Senturk et al., 2007; Cysique et al., 2006; Gualtieri and Johnson, 2006; Carpay et al., 2005). This effect of the drug is supported by reports of improvements in cognition when VPA is discontinued (Lossius et al., 2008; Hommet et al., 2007; Ristic et al., 2006; Masmoudi et al., 2006). The causes of these cognitive changes could be the generalized neuro-suppressant effect of this compound, but may also be due to more specific effects on those brain regions in which adult neurogenesis continues.

There are two regions, the hippocampus and the olfactory system, in the adult mammalian brain which continue to generate new neurons throughout life (Ehninger and Kempermann, 2008; Eriksson et al., 1998; Abrous et al., 2005). Proliferation of neural stem cells within the subgranular zone (SGZ) of the dentate gyrus, within the hippocampus, continuously produces new granule cell neurons which are incorporated into the dentate gyrus (Ehninger and Kempermann, 2008; Abrous et al., 2005). It has been postulated that the newly generated neurons in this region contribute to hippocampal function and the rate of adult neurogenesis in the hippocampus can be correlated with the degree of hippocampus-dependent learning and memory (Kitabatake et al., 2007). Specifically factors which affect neurogenesis are also found to affect performance in hippocampal-dependent memory tasks (Bruel-Jungerman et al., 2005; Snyder et al., 2005; Mustafa et al., 2008; Duman et al., 2001; Green et al., 2006). A recent study has shown that chronic VPA treatment reduces cell proliferation and induces cell differentiation within the SGZ of adult animals (Hsieh et al., 2004).

The present study uses an animal model to investigate the cognitive effects of VPA exposure. A hippocampal-dependent memory task, the novel object location test (NOL), was used to assess memory function (Lee et al., 2005; Dix and Aggleton, 1999). The results show that sub-chronic VPA treatment impairs spatial memory and causes a reduction in cell proliferation in the SGZ of the dentate gyrus. Furthermore, drug treatment reduced the levels of brain-derived neurotrophic factor (BDNF) and the receptor Notch 1 but not the early neuronal differentiation marker doublecortin (DCX) in the hippocampus. These results indicate that VPA treatment can induce cognitive deficits which could be due to a reduction in hippocampal neurogenesis.

*Corresponding author. Tel: +44-0-11582-30172; fax: +44-0-11582-30142.
E-mail address: mbxju@nottingham.ac.uk (J. Umka).
Abbreviations: BDNF, brain-derived neurotrophic factor; DCX, doublecortin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NOL, novel object location; SGZ, sub granular zone; VPA, valproic acid.

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EXPERIMENTAL PROCEDURES

Subjects

Adult male Lister Hooded rats (n=20, weight was 220–250 g, Charles River Laboratories) were randomly assigned to control (saline injected) and drug-treated groups. Animals were housed in groups of four under a 12-h light-dark cycle with access to food and water and allowed to habituate in the animal facility for 11 days prior to the start of procedures. Animals were weighed daily and all procedures were carried out in accordance with UK Home Office guidelines and licensing. Animal numbers were the minimum required to obtain statistically valid results and all procedure were passed by the local animal welfare committee.

Treatment

Drug-treated animals received two daily i.p. injections of VPA (300 mg/kg, dissolved in 0.9% saline; Sigma-Aldrich, Inc., St. Louis, USA) at a volume of 1 ml/kg for 10 days, a treatment regime which significantly reduces seizure frequency in spontaneously epileptic rats (Nissinen and Pitkanen, 2007) and has been used in previous studies (Hsieh et al., 2004). Control animals received equal numbers of saline injections.

Behavioural testing

Novel object location (NOL). The NOL test (Ennaceur and Delacour, 1988) used in the present study was modified from Dix and Aggleton (1999) and recorded by video camcorder (EthoVision®, Version 3.1, Noldus, Wageningen, The Netherlands) as described previously by Mustafa et al. (2008). Task apparatus consisted of two open square opaque Perspex boxes (39×23.5×30 cm^3, Whatmore Creative Plastic, UK). The illumination in the behavioural room was 80 lx. Weighted identical commercial plastic water bottles of height 7 cm were used as the objects to be explored. Each animal was habituated to the test boxes for 1 h on the day before behavioural testing.

The NOL test was started 7 days after the end of drug treatment, to avoid acute effects of the drug, and consisted of a familiarization trial (3 min) and a choice trial (3 min) with a 5 min inter-trial interval during which control and treated rats were returned to their home cages. Between trials objects and boxes were cleaned with 20% ethanol to eliminate olfactory cues. The starting location of objects in the familiarization trial was randomized between animals and could be either in adjacent corners of the arena or in opposite corners. In the choice trial, one object was returned to the same (familiar) location whereas the other was moved to a new (novel) location. The exploratory activity measured, was time spent actively exploring the object while directing the nose towards the object at a distance of less than 2 cm (Dix and Aggleton, 1999). The distance moved was measured during the NOL test. The discrimination index is defined as the difference in exploration time between the two objects in the choice trial (Dix and Aggleton, 1999).

Tissue preparation

Animals were killed 15 days after the end of drug treatment, and the brains removed. One half was used for immunohistochemistry while in the remaining half, the hippocampal formation was dissected out and snap-frozen for use in Western blotting. For immunohistochemistry, half brains were cryoprotected in 30% sucrose in phosphate buffer saline (PBS) and then snap-frozen while embedded in OCT-compound (VWR International Ltd, Lutterworth, UK) and stored at −80 °C prior to sectioning.

Immunohistochemistry

Serial 20 μm coronal sections were taken through the entire dentate gyrus (between Bregma −2.3 to −6.3 mm) using a Leica, CM 100 cryostat (Leica Microsystems, Knowlhill, UK). Sections were thaw mounted on APES coated slides and stored at −20 °C. Eight evenly spaced sections for staining were selected from the dentate gyrus using a systemic random sampling method (Mayhew and Burton, 1988) and fixed in 0.5% paraformaldehyde (pH 7.4) for 3 min. All reactions were at room temperature and antibodies were diluted in PBS. Sections were incubated for 1 h in anti-Ki-67 primary antibody (1:150; Novocasta NCL-KI-67-MMI), a marker of proliferating cells (Kee et al., 2002). Following washing, sections were incubated and counter stained with Propidium Iodide (1:3000; Sigma-Aldrich, Inc, St.Louis, USA) for 30 s, and mounted in glycerol. Images were captured with a Hamamatsu (C4742-95) digital camera run by Openlab software on a Nikon EFD-3 fluorescence microscope. The number of Ki-67 positive cells in the SGZ, defined as cells within 3 cell diameters of the dentate gyrus (Kempermaan, 2006) were scored. Previous studies have shown that over 70% of dividing cells in this region differentiate into granule cell neurons in the dentate gyrus, the remainder becoming glia (Zhao et al., 2006). The volume of the dentate gyrus was estimated using the Calvalieri method (Schmitz and Hof, 2005). The number of Ki-67 positive cells in each section were combined to provide the total number of proliferating cells in the SGZ (Huang and Herbert, 2006).

Western immunoblotting

Hippocampal tissue was prepared for Western blotting as previously described (Mustafa et al., 2008). 30 μg proteins per lane were loaded onto 12% SDS-polyacrylamide gels to assess BDNF and DCX while 50 μg proteins per lane were loaded onto 8% SDS-gels to assess Notch 1 levels. Blots were probed with the following primary antibodies, polyclonal anti-BDNF (1:500 Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal anti-doublecortin (DCX) antibody (1:1000 Cell Signaling Technology, Danvers, MA, USA), polyclonal anti-Notch 1 (1:1000 C20 Santa Cruz Biotechnology, Santa Cruz, CA, USA), an antibody which binds to the transmembrane region and produces a 120 kDa band indicating the amount of uncleaved protein, or monoclonal mouse anti-GAPDH antibody (1:20000 Abcam, Cambridge, UK). The blots were then incubated with secondary antibodies at a dilution of 1:10000: IRDye 800CW goat anti-mouse, IRDye 680CW goat anti-rabbit or IRDye 800CW donkey anti-goat (LI-COR Biosciences). Blots were analyzed in accordance with the manufactures' instructions using on an Odyssey scanner (Licor, Application software V 3.0).

Statistical analysis

All statistical parameters were calculated using GraphPad Prism (V 4.0). Student's t-test and repeated measures ANOVA were used to analyze data. A probability level of $P<0.05$ was considered statistically significant.

RESULTS

VPA reduces performance in a spatial working memory task

To test the behavioural effect of VPA in a hippocampal dependent task, the performance of drug and control treated animals were compared using the NOL test. Results were assessed by comparing the total time animals spent on objects in either novel or familiar locations in the choice trial, and by calculating the discrimination index for each group. Neither drug-treated nor control groups showed any preference for either object in the familiarization trial (data not shown). In both the familiarization and