Valproic acid up-regulates melatonin MT₁ and MT₂ receptors and neurotrophic factors CDNF and MANF in the rat brain

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Abstract
We have reported that clinically relevant concentrations of valproic acid (VPA) up-regulate the G-protein-coupled melatonin MT₁ receptor in rat C6 glioma cells. To determine whether this effect occurs in vivo, the effects of chronic VPA treatment on the expression of both melatonin receptor subtypes, MT₁ and MT₂, were examined in the rat brain. Reverse transcription–polymerase chain reaction (RT–PCR) and real-time PCR analyses revealed significant increases in MT₁ and MT₂ mRNA expression in the hippocampus, following VPA (4 mg/ml drinking water) treatment for 17 d. Increases in the mRNA and protein expression of the novel neurotrophic factors, conserved dopamine neurotrophic factor and mesencephalic astrocyte-derived neurotrophic factor, were detected in the hippocampus and/or striatum. In addition, significant changes in persephin, glial cell line-derived neurotrophic factor and brain-derived neurotrophic factor mRNA expression were observed. The robust multi-fold induction of MT₁ and MT₂ receptors in the hippocampus suggests a role for the melatonergic system in the psychotropic effects of VPA.

Materials and method

Animals and VPA treatment
Adult male Sprague–Dawley rats (450–500 g) were housed under a 12-h light/dark cycle (lights on 07:00...
hours). Animals had free access to a standard diet and water. All experiments were carried out according to the guidelines set by the McMaster University Animal Research Ethics Board. Animals were randomly divided into two groups (n = 3), with the treatment group receiving VPA via drinking water (4 mg/ml drinking water) and the control group receiving vehicle (water) for 17 d. The dose of VPA selected for chronic treatment produces serum concentrations that are well within the therapeutic range for this drug (Frisch et al. 2009). All animals were decapitated following 17 d of treatment with VPA. Hippocampi and striata were dissected rapidly on ice and stored in RNA later at 4 °C until used for RNA isolation, cDNA synthesis and polymerase chain reaction (PCR) amplification.

Reverse transcription (RT)–PCR
Total RNA was isolated from homogenized tissue with TRIzol, as described by the supplier (Invitrogen Canada Inc., USA). After DNase treatment, cDNA was synthesized from 2 μg total RNA using the Omniscript reverse transcriptase kit (Qiagen Inc., USA) and oligo(dT) primers. All PCR amplifications began with heat activation of the HotStarTaq DNA Polymerase (Qiagen Inc.) at 95 °C for 15 min and ended with a final incubation at 72 °C for 10 min. The MT1 and MT2 receptors were detected by amplifying 10 μl RT product with appropriate primers using the following parameters: 94 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min for 40 cycles. Neurotrophic factors were detected by amplifying 2 μl RT product with the appropriate primers for 33 cycles (CDNF, MANF, PSPN) or 30 cycles (BDNF, GDNF) as follows: 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min. GAPDH controls were amplified using 2 μl RT product and the above parameters for 28 cycles. Negative controls containing DNase-treated RNA without RT were processed to confirm PCR specificity and the absence of DNA contamination. GAPDH forward (F) and reverse (R) primers (5′→3′) were as follows:

MT1 (F) tcagcctcagctctgccag; (R) taatgtagctgttggcaggtctgc (472 bp);
MT2 (F) taacactccctcactgcttg; (R) cacaacactgcaacat cgg (297 bp);
CDNF (F) aacacaaccgcctgttgcta; (R) tcaattttccacaggtca cca (199 bp);
MANF (F) gcaagggccaaagaaactc; (R) agagatggcagaag gcacat (204 bp);
PSPN (F) atggctcaggagacactgg; (R) ggtcttgctctcagga gaagg (418 bp);
BDNF (F) gagatgaggcaccagaggtgc; (R) ttgctatgccccttg cagct (390 bp);
GDNF (F) atgggtgtcgtgcttcgtcg; (R) tctctggagcagggt cagat (643 bp);
GAPDH (F) ttacacatcggagaagcc; (R) ggctggactggtctg catag (237 bp).

Amplified cDNA bands were separated on a 2% agarose gel stained with ethidium bromide and digitally scanned using an AlphaImager™ 2200 program.

Real-time PCR
The mRNA expression of MT1 and MT2 was further assessed by real-time PCR using a LuminoCt SYBR Green qPCR ReadyMix (Sigma-Aldrich Canada Ltd, Canada), with the following forward (F) and reverse (R) primers (5′→3′): MT1 (F) tttgagcgtgttgctgtg (R) gacacctgcagccattaga (140 bp); MT2 (F) atcctggacacct catcag; (R) cttggcagcctgtcatc (126 bp). GAPDH (primers shown above) was used as an internal control. Control and VPA cDNA (1 μl) were amplified on a MX3000P (Stratagene) cycler for 40 cycles, using annealing and melting temperatures of 53 °C (MT1 and MT2) or 55 °C (GAPDH) for 30 s and 94 °C for 1 min, respectively. PCR specificity was confirmed by melting curve analysis, which produced a single peak for each product. Following normalization of data with GAPDH, group differences in gene expression were determined by the ΔΔCt method.

Western analysis
Hippocampal and striatal proteins were extracted in lysis buffer (20 mM Tris–HCl, 137 mM NaCl, 1% NP40, 10% glycerol, 48 mM NaF, 2 mM Na3VO4, pH 7.5) with Complete Protease Inhibitor Cocktail (Roche Diagnostic, USA). Gels with 80 μg protein per lane were run at 200 V for 1 h at 4 °C. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes overnight at 25 V, 4 °C. Blots were blocked for 1 h at room temperature with 5% skimmed milk in TBST buffer (50 mM Tris–HCl, 150 mM NaCl, 0.1% Tween-20, pH 8.5) and then incubated with rabbit anti-MANF (1:500; ProSci Inc., USA) for 48 h at 4 °C. After protein detection (as described below), blots were stripped and then reprobed for 48 h with rabbit anti-CDNF (1:250; ProSci Inc.) at 4 °C. After a second stripping, blots were incubated with anti-β-actin serum (1:2000; Sigma-Aldrich Ltd, Canada). For protein detection, blots were incubated in 5% skimmed milk-TBST buffer containing a horseradish peroxidase-conjugated second antibody (1:3000 for MANF and CDNF; 1:10 000 for β-actin; Santa Cruz Biotechnology, USA) for 2 h at room temperature. After washing, blots were treated with enhanced chemiluminescence reagents...
Melatonin receptors are differentially distributed within the central nervous system (CNS), with the MT$_1$ receptor subtype being relatively enriched in the hippocampus, whereas the MT$_2$ receptor subtype is highly expressed in areas such as the suprachiasmatic nuclei of the hypothalamus (Ishii et al. 2009). The novel finding, that chronic treatment with VPA induces a significant induction of melatonin MT$_1$ and MT$_2$ receptors in the rat hippocampus, suggests that the melatonergic system is an important therapeutic target for this psychotropic agent. Given the antidepressant effects of melatonin or its receptor agonists, such as agomelatine (Fornaro et al. 2010; Hale et al. 2010; Hickie & Rogers, 2011), this finding raises interesting questions about the potential role of the melatonergic system in the antidepressant effects of VPA, which are thought to involve the hippocampus. Although various brain regions including the prefrontal cortex, amygdala and striatum have been implicated in depression, several studies have shown that the hippocampus is a key target in both the

Statistical analysis

The optical density values for PCR products from semi-quantitative RT-PCR were normalized against those of GAPDH, as we have reported previously (Castro et al. 2005). Following conversion to percentage values, data were assessed by unpaired Student’s t test, with $p \leq 0.05$ taken as the level of significance. Data shown are expressed as means $\pm$ S.E.M. Real-time PCR data from controls or VPA-treated animals were normalized with GAPDH and relative fold-differences between groups were determined by the $2^{-\Delta\Delta C_t}$ method, followed by Student’s t test.

Results

Effects of VPA on body weight and water consumption

Every Tuesday and Friday, animals were weighed and water consumption was measured before fresh water with or without VPA was provided. There were no significant differences in body weight or water consumption between controls and animals treated with VPA. Body weight: controls $= 528 \pm 11$ g; VPA $= 568 \pm 37$ g; water consumption: controls $= 55 \pm 3$ ml; VPA $= 54 \pm 2$ ml; $n = 3$ for all groups.

MT$_1$ and MT$_2$ mRNA induction by VPA in rat hippocampus

Chronic treatment with VPA caused a dramatic increase of both MT$_1$ and MT$_2$ mRNA expression in the rat hippocampus. Significant increases of about eight-fold ($p < 0.03$) and 14-fold ($p < 0.01$) were detected in MT$_1$ and MT$_2$ mRNA levels, respectively, by RT-PCR analysis (Fig. 1a, b). These findings were confirmed by real-time PCR, which indicated seven-fold ($p < 0.001$) and 10-fold ($p < 0.03$) increases in MT$_1$ and MT$_2$ mRNA expression, respectively, in the hippocampus of rats treated with VPA.

Effects of VPA on neurotrophic factor mRNA and protein expression in hippocampus and striatum

VPA caused a significant induction of CDNF ($p < 0.03$), MANF ($p < 0.01$), GDNF ($p < 0.01$), PSPN-long transcript ($p < 0.05$), PSPN-short transcript ($p < 0.01$) and BDNF ($p < 0.01$) in the hippocampus, as shown in Fig. 1c–g. Similarly, increases in CDNF ($p < 0.01$), MANF ($p < 0.02$), GDNF ($p < 0.01$) and BDNF ($p < 0.01$) were observed in the striatum (Fig. 2a–d). In addition to the primary GDNF transcript (643 bp), a shorter and less abundant transcript (565 bp), derived from alternate splicing of the GDNF gene, which results in a 78 bp deletion, was consistently detected in the striatum, as reported previously (Schaar et al. 1994). A significant increase in the PSPN-long transcript ($p < 0.05$) was detected in the striatum, but in contrast to the hippocampus, the short PSPN transcript was decreased ($p < 0.05$) following VPA treatment (Fig. 2e). Preliminary Western analysis of VPA-induced changes in CDNF and MANF protein expression in the hippocampus and striatum are shown in Figs 1h and 2f, respectively.

Discussion

In earlier work, we have demonstrated that VPA, at clinically relevant or higher induces melatonin MT$_1$ receptor mRNA and protein expression in rat C6 glioma cells (Castro et al. 2005; Kim et al. 2008). The present study investigated whether this in vitro up-regulation of the MT$_1$ receptor could be replicated in an in vivo model and whether another mammalian G-protein-coupled melatonin receptor, the MT$_2$, would also be influenced by VPA treatment. Our results show that chronic treatment with a therapeutically relevant dose of VPA causes a dramatic increase in melatonin receptor mRNA levels of seven- to eight-fold for the MT$_1$ and 10- to 14-fold for the MT$_2$ in the rat hippocampus, indicating that VPA up-regulates the expression of both melatonin receptor subtypes in this brain region.

Melatonin receptors are differentially distributed within the central nervous system (CNS), with the MT$_2$ receptor subtype being relatively enriched in the hippocampus, whereas the MT$_1$ receptor subtype is highly expressed in areas such as the suprachiasmatic nuclei of the hypothalamus (Ishii et al. 2009). The novel finding, that chronic treatment with VPA induces a significant induction of melatonin MT$_1$ and MT$_2$ receptors in the rat hippocampus, suggests that the melatonergic system is an important therapeutic target for this psychotropic agent. Given the antidepressant effects of melatonin or its receptor agonists, such as agomelatine (Fornaro et al. 2010; Hale et al. 2010; Hickie & Rogers, 2011), this finding raises interesting questions about the potential role of the melatonergic system in the antidepressant effects of VPA, which are thought to involve the hippocampus. Although various brain regions including the prefrontal cortex, amygdala and striatum have been implicated in depression, several studies have shown that the hippocampus is a key target in both the
Fig. 1. Chronic valproic acid (VPA) treatment induces melatonin receptor and neurotrophic factor mRNA expression in the rat hippocampus. Gel images of (a) MT1, (b) MT2, (c) conserved dopamine neurotrophic factor (CDNF), (d) mesencephalic astrocyte-derived neurotrophic factor (MANF), (e) brain-derived neurotrophic factor (BDNF), (f) glial cell line-derived neurotrophic factor (GDNF) and (g) persephin (PSPN) are shown. Lanes 1 and 2: control (Con) and VPA, respectively. Data shown are means ± S.E.M. (n = 3) for percentage values of optical density (OD) ratios for each gene target/GAPDH, as indicated. *p < 0.05, **p < 0.01 vs. Con. For PSPN: grey bars, long transcript; black bars, short transcript. (h) Immunoblots of CDNF (18 kDa), MANF (20 kDa) and β-actin (42 kDa) from Con (lane 1) and VPA (lane 2).
pathophysiology and the treatment of depression (Samuels & Hen, 2011). Brain imaging has revealed a reduction in hippocampal volume in depressed patients, whereas antidepressants attenuate or reverse this decrement (Duman, 2004), probably by inducing neurogenesis via induction of neurotrophic factors such as BDNF and GDNF (Duman & Monteggia, 2006). Since both BDNF and GDNF are induced by VPA in vitro (Castro et al. 2005) and in the hippocampus (Fukumoto et al. 2001; present study, Fig. 1e,f), it

![Fig. 2. Effects of chronic valproic acid (VPA) treatment on neurotrophic factor mRNA expression in the rat striatum. Gel images of (a) conserved dopamine neurotrophic factor (CDNF), (b) mesencephalic astrocyte-derived neurotrophic factor (MANF), (c) brain-derived neurotrophic factor (BDNF), (d) glial cell line derived neurotrophic factor (GDNF) and (e) persephin (PSPN) are shown. Lanes 1 and 2: control (Con) and VPA, respectively. Data shown are means ± S.E.M. (n = 3) for percentage values of optical density (OD) ratios for each gene target/GAPDH, as indicated. * p < 0.05, * p < 0.02, ** p < 0.01 vs. Con. For PSPN: grey bars, long transcript; black bars, short transcript. (f) Immunoblots of CDNF (18 kDa), MANF (20 kDa) and β-actin (42 kDa) from Con (lane 1) and VPA (lane 2).]
is likely that they are involved in the neurogenic and antidepressive effects of this agent (Samuels & Hen, 2011). Similarly, there is evidence that melatonin can increase the expression of GDNF and BDNF in cultured cells (Armstrong & Niles, 2002; Imbesi et al. 2008; Niles et al. 2004). Moreover, chronic treatment with the melatonin M1/M2 receptor agonist/5-HT2C receptor antagonist, agomelatine, up-regulates BDNF expression in the rat hippocampus (Calabrese et al. 2011). In view of the foregoing, the induction of both the M1 and M2 receptors in the hippocampus by chronic treatment with VPA suggests that additive or synergistic antidepressant and/or neuroprotective effects may be produced by VPA in combination with melatonin or its analogues.

The mechanisms involved in the up-regulation of melatonin receptors by VPA in the hippocampus await clarification but there are several possibilities, given the multiple cellular targets for this drug (Monti et al. 2009). Earlier studies revealed a rapid induction of M1 mRNA expression in primary cultures of ovine pars tuberalis following forskolin treatment, which was linked to increases in cAMP levels (Barrett et al. 1996). The authors suggested that cAMP-dependent gene induction via phosphorylation of the transcription factor, cAMP-response element binding protein (CREB), or MAPK/AP-1 activation could be involved in up-regulating M1 expression in the ovine pars tuberalis (Barrett et al. 1996). It is unlikely that CREB activation is involved in up-regulation of hippocampal melatonin M1 and M2 receptors by VPA, which was found to inhibit forskolin-stimulated cAMP elevation in the rat prefrontal cortex (Montezinho et al. 2007). Chronic treatment with VPA caused a transient increase in CREB phosphorylation in the mouse striatum (Shen et al. 2008) but not in the rat cerebral cortex or hippocampus (Chen et al. 1999). In addition, in vitro studies do not support a role for MAPK/ERK signalling as inhibition of this pathway, by PD98059, failed to block VPA induction of melatonin M1 receptors in C6 glioma cells (Castro et al. 2005). Interestingly, trichostatin A, another HDAC inhibitor, which is structurally distinct from VPA, was found to induce M1 receptor mRNA expression in C6 cells (Kim et al. 2008), suggesting that an epigenetic mechanism, involving HDAC inhibition with associated chromatin remodelling and gene transcription, underlies the up-regulation of melatonin receptor expression by VPA.

Chronic VPA treatment caused a significant increase in hippocampal and striatal mRNA expression of the novel neurotrophic factors, CDNF and MANF, which are members of an evolutionary conserved family of proteins. In keeping with this finding, preliminary Western analysis suggests that VPA increases the protein levels of CDNF in the hippocampus and MANF in the striatum. CDNF has been found to be at least as effective as GDNF in protecting dopaminergic neurons in the substantia nigra in a 6-hydroxydopamine (6-OHDA) model of Parkinson’s disease (Lindholm et al. 2007) with MANF also being neuroprotective in a similar model (Voutilainen et al. 2009). More recently, it was reported that chronic infusion with CDNF is neuroprotective in the above 6-OHDA model, but similar infusions of MANF or GDNF were not effective (Voutilainen et al. 2011), suggesting that CDNF may be particularly beneficial in treating Parkinson’s disease. Relatively high levels of MANF are present in the cerebral cortex, hippocampus and cerebellar Purkinje cells. Status epilepticus or global forebrain ischaemia increases MANF mRNA expression in the dentate granule cell layer of the hippocampus or in the hippocampal formation, respectively. The widespread expression of MANF in both neuronal and non-neuronal tissues, and its sensitivity to brain insults, suggests important physiological roles for this evolutionary conserved protein (Lindholm et al. 2008). Clarification of the roles of both CDNF and MANF, and whether they contribute to the psychotropic and/or neuroprotective effects of VPA, will depend on future studies aimed at identifying the receptors and signalling pathways employed by these neurotrophic factors.

VPA treatment also altered the mRNA expression of PSPN, a member of the GDNF family of neurotrophic factors, which is widely distributed in the CNS and thought to be of both astroglial and neuronal origin (Jaszai et al. 1998). As noted earlier, the expression of both PSPN transcripts was increased in the hippocampus following VPA treatment, whereas, the long variant was increased in the striatum but the short transcript was decreased. This apparent differential regulation of the two PSPN transcripts in the striatum by VPA awaits confirmation by further study, including protein analysis.

In conclusion, chronic treatment with a clinically relevant dose of VPA caused a multi-fold increase in the expression of melatonin M1 and M2 receptor subtypes in the rat hippocampus. This novel finding strongly suggests a role for melatonergic signalling in the neurogenic, antidepressant and other effects of VPA in the hippocampus and other CNS regions. VPA also caused significant increases in the expression of CDNF, MANF and PSPN, indicating that these neurotrophic factors (together with BDNF and GDNF) are potential mediators of the neuroprotective and psychotropic actions of VPA in the CNS.
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Statement of Interest

None.

References


