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Epigenetic control of GLT-1 gene activity and its modulation by psychoactive drugs in comparison to genome-wide drug effects

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Erklärung

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Eidesstattliche Versicherung

Diese Dissertation wurde eigenständig und ohne unerlaubte Hilfe erarbeitet.

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(Tatjana Perisic)

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The work presented in this cumulative thesis was conducted in the research group of Dr. Theo Rein at the Max Planck Institute of Psychiatry in Munich and comprises the results of two scientific publications:

I

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II

Perisic T, Holsboer F, Rein T, Zschocke J (2012) The CpG island shore of the GLT-1 gene acts as a methylation-sensitive enhancer. *Glia (in press*)

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1 Abbreviations

3MeH3K27	tri-methyl-histone H3 Lys27
AcH4	acetyl-histone H4
AMI	amitriptyline
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AVP	arginine vasopressin
BDNF	brain-derived neurotrophic factor
CBZ	carbamazepine
CER	cerebellum
CIT	citalopram
CREB	cAMP response element-binding
CTX	cortex
DEX	dexamethasone
DNMT	DNA methyltransferase
E1	exon 1
EAAC1	excitatory amino acid carrier 1
EAAT	excitatory amino acid transporter
EGF	epidermal growth factor
ETS	E-twenty six
GADD45α	growth arrest and DNA-damage inducible 45α
GFAP	glial fibrillary acidic protein
GLAST	glutamate-aspartate transporter
Gln	glutamine
GLT-1	glutamate transporter 1
Glu	glutamate
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDAC-I	histone deacetylase inhibitor
HDM	histone demethylase
HMT	histone methyltransferase
HPC	hippocampus
iGluR	ionotropic glutamate receptor
LINE-1	long interspersed nuclear element 1
LTG	lamotrigine
LUMA	luminometric methylation analysis

MBD	methyl-CpG-binding domain
MeCP	methyl-CpG binding protein
mGluR	metabotropic glutamate receptor
NGFI-A	nerve growth factor induced clone A
NMDA	N-methyl-D-aspartate
PAR	paroxetine
RNA pol	RNA polymerase
Sp1	specificity protein 1
SUMO	small ubiquitin-like modifier
SUV39	3–9 suppressor of variegation
TET1	ten-eleven translocation 1
TF	transcription factor
TSA	trichostatin A
VEN	venlafaxine
VPA	valproic acid

2 Summary

Epigenetic mechanisms confer stability of gene expression but are also subject of reprogramming by various stimuli including stress and psychoactive drugs. In this thesis, the epigenetic regulation of the rat glutamate transporter 1 (GLT-1) gene was analysed, focusing on the CpG island and the upstream CpG shore. In particular, I sought to decipher the contribution of chromatin modifications to differential GLT-1 response to the stress hormone dexamethasone (DEX) in cortical (CTX) and cerebellar (CER) astrocytes. In addition to DEX, epigenetic effects of mood stabilizers and antidepressants on GLT-1 including the histone deacetylase inhibitor (HDAC-I) valproic acid (VPA), carbamazepine, lamotrigine, amitriptyline (AMI), venlafaxine and citalopram were assessed. Genome-wide epigenetic effects of these drugs on primary astrocytes were also evaluated. Chromatin analysis demonstrated that repressive marks at the CpG shore region correlated with GLT-1 unresponsiveness to treatment with DEX in CER, whereas positive marks were associated with increased GLT-1 transcription in CTX following treatment with DEX and HDAC-Is. Importantly, the CpG shore acted as a stress hormone- as well as HDAC-I-responsive enhancer element in a methylation-dependent fashion in reporter gene assays. The extent of epigenetic reprogramming at the GLT-1 promoter by DEX and HDAC-Is was more pronounced in CTX, where VPA induced marked DNA demethylation and histone H4 hyperacetylation of the CpG shore. Except for VPA, the GLT-1 gene was not a subject to epigenetic regulation by the other investigated psychoactive drugs and none of them affected GLT-1 transcription. However, VPA as well as AMI promoted genome-wide reversible DNA demethylation at CCpGG sites in CTX astrocytes, as measured by luminometric methylation analysis. In the case of VPA, global DNA demethylation was associated with histon H3/H4 hyperacetylation and 2MeH3K9 hypomethylation, as determined by Western Blot. VPA-associated demethylation at CCpGG sites occurred independently of DNA methyltransferase (DNMT) suppression. In the case of AMI, however, CCpGG demethylation was paralleled by a reduction of DNMT enzymatic activity. The results of this thesis support the notion that the epigenetic landscape of the CpG shore generates different GLT-1 transcriptional outcomes and can be modulated in particular by drugs with HDAC-I properties. Furthermore, the results indicate that VPA and AMI target different genes and interfere with distinct components of the processes leading to DNA demethylation. The data presented here extend our understanding of epigenetic regulation in neural cells and pave the way to identification and characterization of promising novel drug targets.

3 Introduction

3.1. Cellular and molecular features of glutamate transporters

3.1.1. Neuroregulatory functions of astrocytes

Glutamatergic neurotransmission is crucial for brain functions such as cognition, memory and learning. Excitatory potentials are evoked by activation of several transmembrane glutamate receptors: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), N-methyl-D-aspartate (NMDA), kainate and metabotropic (group I-III) glutamate receptors. Glutamatergic firing must be rapidly terminated in order to prevent cytotoxicity and ensure optimal cell-to-cell communication. The key elements of glutamate clearance from the extracellular space are sodium-dependent excitatory amino acid transporters (EAAT), expressed mainly on astrocytes but also on neurons. The glutamate taken up by astrocytes is metabolised to glutamine and transported to neurons. This trafficking of glutamate and glutamine between astrocytes and neurons is commonly referred to as the glutamate-glutamine cycle (Fig. 1). Apart from the role in metabolism and glutamate clearance, astrocytes are integral components of the “tripartite glutamatergic synapse”. Neuron-astrocyte communication within these synapses relies on the expression of neurotransmitter receptors on astrocytes including dopamine and serotonin receptors (Brito et al., 2009; Carson et al., 1996). Active release of neurotransmitters from astrocytes has also been documented (Panatier et al., 2006; Perea and Araque, 2007). A growing body of data indicates that astroglial cells have the capacity to integrate neuronal inputs and modulate synaptic potentials on neighbouring neurons, concomitantly influencing behavioural responses (Halassa and Haydon, 2010).

3.1.2. Types and localisation of glutamate transporters

Glutamate uptake is mediated by a family of membranous transport proteins. High-affinity glutamate transporters were first identified in rodents and designated as glutamate-aspartate transporter (GLAST) (Storck et al., 1992), glutamate transporter 1 (GLT-1) (Pines et al., 1992) and excitatory amino acid carrier 1 (EAAC1) (Kanai and Hediger, 1992). Identification of the human glutamate transporters EAAT1, EAAT2, EAAT3 corresponding to GLAST, GLT-1 and EAAC1 followed up (Arriza et al., 1994). Subsequently, two additional mammalian transporter subtypes were identified, EAAT4 (Fairman et al., 1995) and EAAT5 (Arriza et al., 1997). Localization studies have shown that GLAST and GLT-1 are almost exclusively glial transporters (Fig. 2). They are also present in developing neurons (Plachez

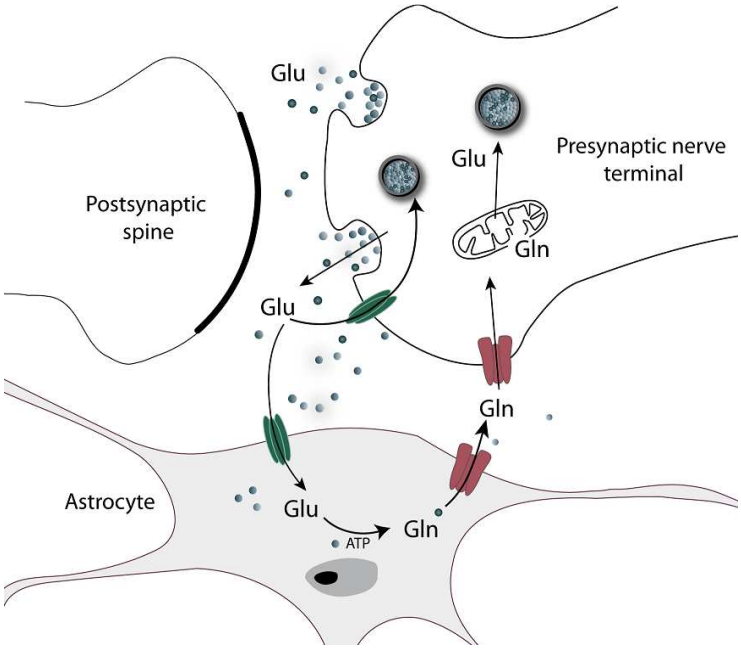

Figure 1. The glutamate-glutamine cycle. Glutamate (Glu) released from presynaptic vesicles is taken up by Glu transporters present on neurons and astrocytes. In astrocytes, Glu is converted into glutamine (Gln) in an ATP-dependent process. Gln is then released into the extracellular cavity by means of Gln transporters, taken up by neurons and converted into Glu by the mitochondrial enzyme phosphate-activated glutaminase. In neuronal presynaptical terminals, Glu is packaged into synaptic vesicles and released on demand by exocytosis.

et al., 2004; Wang et al., 1998), although there are still controversies concerning neuronal protein localisation *in vivo* (Berger et al., 2005; Holmseth et al., 2009). In contrast to GLAST and GLT-1, EAAT3 and EAAT4 are found predominantly in neurons, with EAAT3 being abundant throughout the central nervous system and EAAT4 unique to Purkinje cells of the cerebellum (Furuta et al., 1997a). Lastly, EAAT5 is a retinal glutamate transporter (Rauen et al., 2004).

3.1.3. Role of GLT-1/EAAT2 in disease

Astrocytic GLT-1/EAAT2 is a major molecular determinant of the glutamate removal from the synaptic cleft. Mice deficient in GLT-1 display spontaneous lethal seizures and are highly susceptible to injury-induced edema formation (Tanaka et al., 1997). A number of neurological disorders are linked to glutamate transporter dysfunction such as amyloid lateral sclerosis, Alzheimer's disease, Parkinson's disease, stroke and epilepsy, which is also supported by animal models based on abnormal glutamate neurotransmission (Maragakis and Rothstein, 2004). Moreover, growing evidence supports the role of disrupted glutamate homeostasis in the pathophysiology of clinical depression. Individuals with mood disorders show elevated glutamate levels in cortex (CTX) in nuclear magnetic resonance

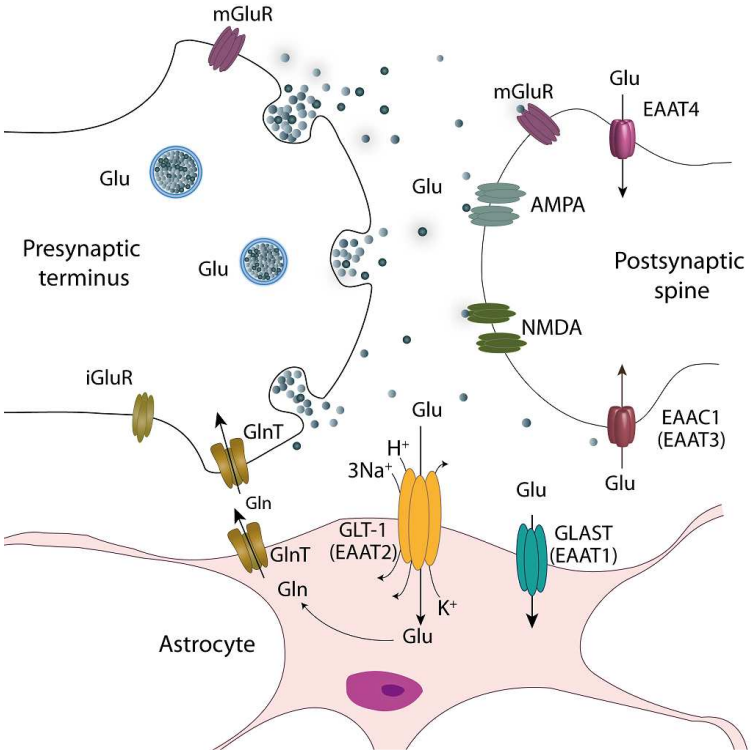

Figure 2. Mechanism of excitatory neurotransmission in the mammalian central nervous system and distribution of excitatory amino acid transporters (EAAT). L-glutamate (Glu) is synthesized in presynaptic terminals. Following release, Glu acts via ionotropic glutamate receptors (iGluR) AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and NMDA (N-methyl-D-aspartate) and metabotropic glutamate receptors (mGluR). The synaptic effects of Glu are terminated by its uptake mediated by several EAATs, primarily GLT-1/EAAT2, and to a lesser extent GLAST/EAAT1. EAAT1 and EAAT2 are predominantly expressed in astrocytes. In astrocytes, Glu is transformed to glutamine (Gln) and shuttled back to presynaptic terminals via glutamine transporters (GlnT). Neuronal EAATs, including EAAT3/EAAC1 (expressed primarily in the forebrain) and EAAT4 (expressed predominantly in Purkinje cells), have a role in synaptic plasticity. Transport of Glu via EAATs is coupled with the inward co-transport of three Na^+ and one H^+ ion and the export of one K^+ ion.

spectroscopy studies (Sanacora et al., 2004; Yildiz-Yesiloglu and Ankerst, 2006) as well as aberrant expression of genes associated with regulation of glutamate clearance and metabolism (Altar et al., 2009). Accordingly, certain areas of the cerebral CTX of patients who suffered from major depressive disorder demonstrate significant down-regulation of EAAT2 levels (Choudary et al., 2005). Furthermore, ketamine and other NMDA receptor antagonists produce fast-acting behavioural antidepressant-like effects in mouse models (Autry et al., 2011). Accumulation of extracellular glutamate also potentially causes cytotoxic damage to neurons and glia (Choi, 1988). Significantly reduced numbers of neuroglia in patients with major depression have been reported (Rajkowska, 2000), evidencing a role of high glutamate levels in depression. Thus, uncovering the mechanisms of GLT-1/EAAT2 gene regulation is critical not only for understanding fundamental cellular processes, but also in finding a new therapeutic approach that aims to promoting glutamate transport in astrocytes.

3.1.4. Regulation of astrocytic GLT-1/EAAT2

Primary cultures of neonatal astrocytes constitute a useful model for elucidation of GLT1/EAAT2 gene regulation. Using primary astrocytes, numerous factors were found to enhance GLT-1/EAAT2 expression including growth factors (Figiel et al., 2003; Rodriguez-Kern et al., 2003), cAMP analogues (Schlag et al., 1998), beta-lactam antibiotics (Rothstein et al., 2005), steroids (Pawlak et al., 2005; Tian et al., 2007; Zschocke et al., 2005), histone deacetylase inhibitors (HDAC-Is) (Allritz et al., 2009), insulin (Ji et al., 2011), dopamine (Brito et al., 2009) and many more. Some of these effects were also reported *in vivo*, in particular the regulation by corticosteroids (Autry et al., 2006; Reagan et al., 2004) and HDAC-Is (Hassel et al., 2001).

Molecular mechanisms of GLT-1/EAAT2 regulation have been partially addressed, especially factors that mediate the effects of epidermal growth factor (EGF), tumour necrosis factor α and cAMP analogues (Li et al., 2006; Sitcheran et al., 2005; Zelenaia et al., 2000). These studies suggest an involvement of phosphatidylinositol 3-kinase- and nuclear transcription factor-kappa B-dependent pathways. Another study described a mechanism of EAAT2 regulation based on translational control through cis-regulatory elements in the 5'-UTR of the EAAT2 mRNA (Tian et al., 2007). Moreover, the role of the 3'-UTR and non-coding RNA in the control of GLT-1/EAAT2 has recently been suggested (Bette et al., 2011).

Furthermore, there is evidence for the involvement of brain-region specific factors which determine GLT-1 regulation. Pharmacological stimulation of primary astrocytes results in increased levels of GLT-1 depending on the brain region investigated. For instance, GLT-1 expression is elevated by pituitary adenylate cyclase-activating polypeptide, tumour growth factor α and EGF in astrocytes from striatum, whereas these factors fail to increase GLT-1 levels in astrocytes from cerebellum (CER), mesencephalon and spinal cord (Schluter et al., 2002). Corticosteroids increase GLT-1 transcription in CTX but not in CER and mesencephalon astroglia (Zschocke et al., 2005). This differential inducibility of GLT-1 in astrocytes from diverse brain regions may have its origin in regional segregation of glutamate transporter subtype expression during pre- and postnatal brain development (Furuta et al., 1997b). In the adult rat brain, GLT-1 transporter subtype is more abundant in CTX than GLAST subtype, the latter being the predominant glutamate transporter in CER astrocytes. Local specialisation of astrocytes during development is also reflected by regionally distinct connexin 43 inducibility (Reuss et al., 1998; Reuss et al., 2000) as well as distinct gene expression patterns in astrocytes from CTX, CER and brainstem (Yeh et al., 2009).

Epigenetic control is a fundamental regulatory mechanism that underpins the establishment of brain-area specific gene activity and reactivity and was proposed to play a role in differential response to the stress hormone dexamethasone (DEX) between CTX and CER astrocytes (Zschocke et al., 2005). Follow-up studies supported this idea by documenting a significant epigenetic component in GLT-1/EAAT2 regulation in glioma cell lines as well as in neonatal astrocytes (Yang et al., 2010; Zschocke et al., 2007). Thus, these studies established an important basis for further investigations of epigenetic mechanisms in regulating GLT-1/ EAAT2.

3.2. Epigenetics

3.2.1. Terminology of epigenetics

The term “epigenetics” was historically used to describe heritable phenotypic changes that do not entail a change in DNA sequence. Cellular differentiation and specialisation represent an example where epigenetic mechanisms come to play through selective activation and silencing of genes across the genome. This is why, in a more narrow sense, epigenetics is used to describe chromatin altering events which lead to changes in gene expression.

3.2.2. Histone modifications and gene regulation

Epigenetic processes use chromatin as a principal substrate. A major building block of the chromatin, called nucleosome, is formed by a 147 bp-long DNA stretch wrapped around one histone octamer comprised of two copies each of histone H2A, H2B, H3 and H4 (Fig. 3). There are other minor histone variants which are involved in the control of chromatin accessibility and specific functions such as DNA repair (Sarma and Reinberg, 2005). Numerous chemical groups can be enzymatically attached to N- and C-termini of histones including acetyl-, methyl-, phospho-, ubiquitin-, ADP-ribosyl-, and small ubiquitin-like modifier groups. Histone modifications do not occur in isolation but appear as combinations of marks which led to the proposal of the existence of a “histone code” (Jenuwein and Allis, 2001). The most investigated modifications are histone acetylation and histone methylation, especially of lysine (K) residues (Fig. 4). Acetylation of histones is catalysed by histone acetyltransferase (HAT) and numerous transcription factors and coregulators have been identified to possess intrinsic HAT activity. Histone acetylation can be reversed by histone deacetylase (HDAC) resulting in a dynamic transition of gene activity (Eberharter and Becker, 2002). Similarly, histone methyltransferases (HMT) and specific histone

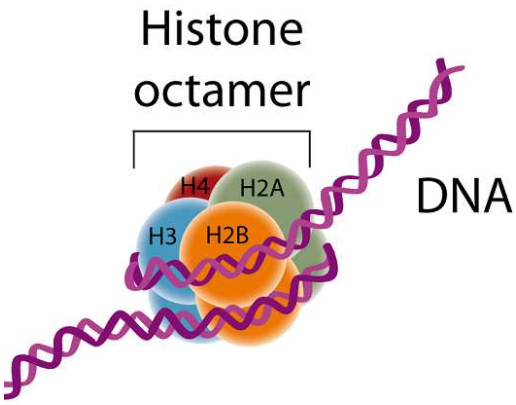

Figure 3. Schematic presentation of a nucleosome. In a nucleosome, the DNA is wrapped around an octamer of histones 1.65 times in a left-handed superhelix. The histone octamer consists of two copies of each core histone (H2A, H2B, H3 and H4).

demethylases (HDM) catalyze the transfer and removal of methyl-groups on histone residues, respectively (Mosammaparast and Shi, 2010; Nimura et al., 2010). Post-translational modifications of histones influence folding and the functional state of the chromatin fiber ultimately affecting gene transcription (Berger, 2001; Grunstein, 1997). Histone acetylation is generally associated with gene activation (Durrin et al., 1991; Pokholok et al., 2005), whereas histone methylation affects gene activity both positively and negatively depending on the number and location of attached methyl-groups (Lachner and Jenuwein, 2002; Spencer and Davie, 1999).

3.2.3. DNA methylation and gene silencing

Another global mechanism which regulates the extent of chromatin accessibility is DNA methylation. DNA methylation is catalysed by DNA methyltransferases (DNMT) and in mammals, it is almost exclusively confined to CpG dinucleotides. Methylation of cytosines is carried out by three types of DNMT: DNMT1, DNMT3a and DNMT3b. The DNMT1 protein function is tightly linked to DNA replication and maintenance of the methylation pattern (Leonhardt et al., 1992), whereas DNMT3a and DNMT3b function as *de novo* methyltransferases (Okano et al., 1999), supported by the activity of the ancillary subtype DNMT3L (Bourc'his and Bestor, 2004; Jurkowska et al., 2011). However, there is also evidence that DNMT3a and DNMT3b are required for the maintenance of certain methylation patterns (Chen et al., 2003; Liang et al., 2002; Jair et al., 2006), and that DNMT1 could participate in *de novo* methylation as well (Jair et al., 2006).

Genomic stretches with a high frequency of the CpG dinucleotides, so called CpG islands, are generally found in gene promoter regions (Bird, 1986). CpG islands are typically maintained in an unmethylated state, whereas the rest of the genome is highly methylated.

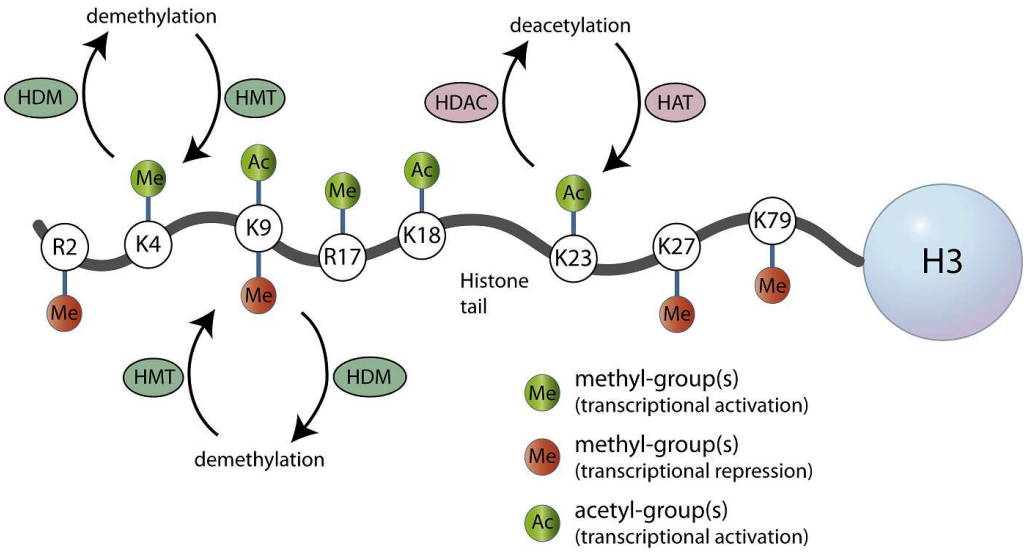

Figure 4. Common covalent modifications of histone H3. Schematic representation of the post-translational modifications of the N-terminal domain of H3 at some lysine (K) and arginine (R) residues. Acetylation is catalysed by histone acetyltransferases (HAT) and reversed by histone deacetylases (HDAC), whereas lysine methylation (linked to either transcriptional activation or repression) is catalysed by histone methyltransferases (HMT) and reversed by histone demethylases (HDM).

There are also examples of CpG islands that become methylated during normal development or tumorigenesis, leading to stable silencing of the associated promoter (Baylin, 2002; Mohn et al., 2008; Stein et al., 1982). However, expression of the gene is not necessarily fully determined by the methylation status of its promoter CpG island (Weber et al., 2007). Genomic regions in the vicinity of CpG islands (termed as CpG island shores) also regulate gene activity in a methylation-dependent manner and may act as cell type-specific enhancers (Irizarry et al., 2009; Schmidl et al., 2009).

In general, DNA methylation prevents binding of transcription factors (Campanero et al., 2000; Iguchiariga and Schaffner, 1989) or recruits methyl-CpG binding (MeCP) proteins which together with numerous chromatin factors establish a repressive chromatin state (Fuks et al., 2003; Jones et al., 1998; Nan et al., 1998) (Fig. 5). However, a growing number of studies suggest that establishment of DNA methylation profiles and gene silencing during early development might be initiated through histone modifications, which serve as primary epigenetic marks (Lock et al., 1987; Ooi et al., 2007; Tamaru and Selker, 2001). For example, DNMT1 targets transcriptionally repressive chromatin through association with HDAC1 and HDAC2 *in vivo*, as well as with the HMT suppressor of variegation 3–9 (SUV39) (Fuks et al., 2000; Rountree et al., 2000). Regardless of the hierarchical order of events, a cross-talk between DNA methylation and histone modifications is supported by both circumstantial and direct evidence (D'Alessio and Szyf, 2006).

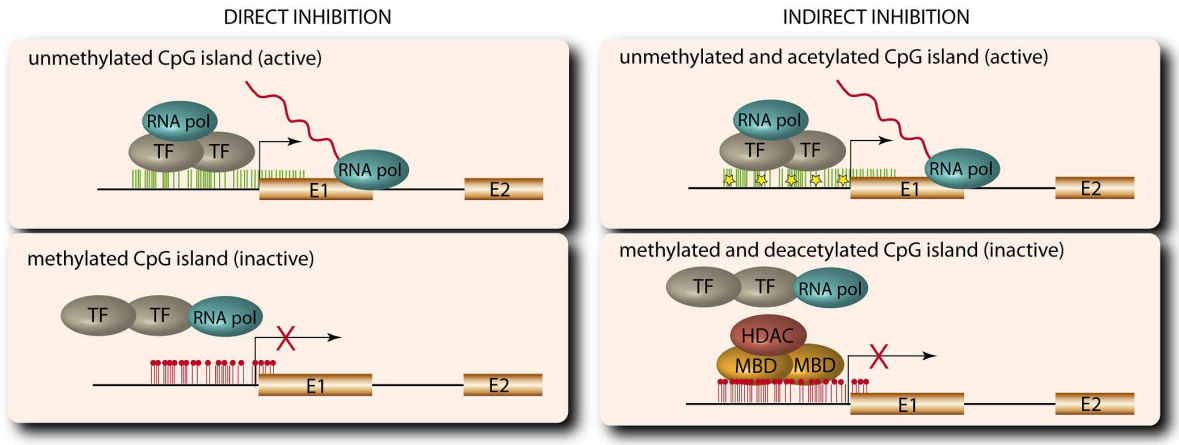

Figure 5. Inhibition of gene activation by CpG methylation. In normal cells, CpG islands are unmethylated and often located in or near the transcription start sites of genes in the proximity of exon 1 (E1). Unmethylated CpGs permit binding of RNA polymerase (RNA pol) and other transcription factors (TF) and thereby enable the initiation of productive transcription. Methylation of CpGs directly hinders the binding of transcriptional machinery. Alternatively, DNA methylation provides recognition signals for the methyl-CpG-binding domain (MBD) protein components of a histone deacetylase complex (HDAC). The HDAC modifies the chromatin by deacetylating histones (depicted as yellow stars) in the region of the CpG island and hence indirectly inactivates the gene.

3.2.4. Dynamics of DNA methylation

In recent years, a number of studies link an active, replication-independent DNA demethylation with gene activation (Barreto et al., 2007; Kim et al., 2009; Milutinovic et al., 2007). In plants, mechanisms of active DNA demethylation are better understood, while the evidence for mammalian demethylases still remains contradictory. It seems that in mammals, depending on the context, this process may be achieved by multiple mechanisms. The initial studies on active DNA demethylation reported an involvement of an RNA-dependent mechanism (Jost, 1993; Weiss et al., 1996), which was questioned later on (Swisher et al., 1998). Active DNA demethylation mediated by methyl-CpG-binding proteins has been demonstrated at promoters of the brain-derived neurotrophic factor (BDNF) and cytochrome p450 27B1 gene (Kim et al., 2009; Martinowich et al., 2003). In the latter study, a DNA repair-dependent mechanism was suggested, which is supported by data from several studies that highlight the role of growth arrest and DNA-damage inducible 45α (GADD45α) protein (Barreto et al., 2007; Schmitz et al., 2009). More recently, the base excision repair pathway was suggested to initiate an oxidative deamination mechanism to actively demethylate DNA (Guo et al., 2011). This process is mediated by ten-eleven translocation 1 (TET1) enzyme and entails formation of 5-hydroxymethylcytosine. Furthermore, there is also a bidirectional cross-talk between DNA demethylation and histone hyperacetylation (Cervoni and Szyf, 2001; D'Alessio et al., 2007; Jackson et al., 2004; Milutinovic et al., 2007). Although details of the demethylation mechanism remain unknown,

many studies evidence that established DNA methylation patterns in non-dividing cells can be erased. Therefore, DNA methylation is a much more dynamic process than previously thought, and pharmacological agents with DNA demethylation properties could be used for reprogramming of not only transformed, but also differentiated cell populations.

3.2.5. Epigenetic basis of stress-induced behavioural changes

Epigenetic processes are susceptible to changes caused by endogenous as well as by external cues (Fagiolini et al., 2009; Meaney, 2010). Environmental influences are established risk factors for many diseases including psychiatric illnesses. Thus, it is suggested that there may be an epigenetic route to the consolidation of maladaptive behaviour. Among the environmental factors, stress plays a prominent role in the aetiology of psychiatric disorders. Experimental evidence from studies on animals shows how stress modifies behaviour based on epigenetic mechanisms. In one of the pioneering studies in this field, Weaver et al. demonstrated that early life stress in rats elicits long-lasting changes in the sensitivity of the hypothalamic-pituitary-adrenal axis which is associated with altered histone acetylation, DNA hypermethylation and nerve growth factor induced clone A (NGFI-A) transcription factor binding to the promoter of the glucocorticoid receptor gene (Weaver et al., 2004). Interestingly, central infusion of a histone deacetylase inhibitor reverts epigenetic changes and aberrant stress response. Adverse effects of early life stress are also associated with changes in arginine vasopressin (AVP) expression levels in rodent neurons (Murgatroyd et al., 2009). The AVP gene is epigenetically programmed by stress through DNA hypomethylation of the distal promoter sequence which reduces MeCP2 binding. Further support for the possible role of chromatin modifications in development and/or maintenance of certain psychopathological phenotypes was provided by studies reporting that systemic administration of the HDAC-Is sodium butyrate and suberoylanilide hydroxamic acid elicits antidepressant-like effects in animal models of depression (Schroeder et al., 2007; Uchida et al., 2011). Moreover, systemic or intra-hippocampal administration of DNMT inhibitors in rats induces dose-dependent DNA hypomethylation, increased BDNF levels and alleviation of depression-like symptoms in animals (Sales et al., 2011).

3.2.6. Epigenetic mechanism of action of psychoactive drugs

A growing number of studies report that pharmacological effects of certain psychoactive drugs could be epigenetically mediated as well. Chronic treatment with imipramine reverses stress-induced repression of the BDNF gene in the hippocampus (HPC) (Tsankova et al.,

2006). These changes are accompanied by an increase in the level of active histone marks acetyl-histone H3 and methyl-histone H3 Lys4 at BDNF promoters and reversal of the depression-like phenotype. Antipsychotic effects of clozapine are ascribed to DNA hypomethylation at the glutamic acid decarboxylase 67 promoter in the CTX and striatum (Dong et al., 2008). Moreover, chronic administration of haloperidol slightly reduces the content of methyl-cytosines in the brain (Shimabukuro et al., 2009).

Valproic acid (VPA) has been used in the treatment of epilepsy and bipolar disorder. In addition to its known classical actions which include increase of GABAergic activity and negative regulation of voltage gated Na^+ channels (Monti et al., 2009), this drug inhibits HDACs (Gottlicher et al., 2001; Phiel et al., 2001). It has been proposed that therapeutic effects of VPA are partially brought about by reversal of chromatin marks at specific gene targets (Chen et al., 2002; Dong et al., 2008). Moreover, VPA treatment induces widespread epigenetic reprogramming by altering DNA methylation patterns (Milutinovic et al., 2007). Furthermore, chronic treatment of rats with VPA leads to a dose-dependent increase in the level of glutamate transporters GLT-1 and GLAST in the HPC and concomitant increase in glutamate uptake (Hassel et al., 2001). Trichostatin A (TSA), another HDAC-I with broad specificity, also elevates GLT-1 expression in neonatal astrocytes from different brain regions (Allritz et al., 2009). The DNMT inhibitor 5-aza-2'-deoxycytidine, alone or in combination with TSA, also activates GLT-1/EAAT2 (Zschocke et al., 2007), raising the exciting possibility of gene reprogramming by DNA demethylation. Together, these studies allude the possibility that epigenetic drugs induce local alterations of chromatin marks also at the GLT-1 promoter and thereby elevate GLT-1 transcription, but direct evidence is still missing.

3.3. Aims of the thesis

One of the aims of this thesis was to investigate in detail the epigenetic features of the GLT-1 gene promoter in rat primary CTX and CER astrocytes, as well as their susceptibility to alterations upon pharmacological challenge. CTX and CER astrocytes were chosen, because they exhibit differential responsivity to the stress hormone DEX, i.e. DEX evokes up-regulation of GLT-1 transcription in the CTX, but not in the CER (Zschocke et al., 2005). The epigenetic marks to be investigated were acetylated H4, several variants of methylated H3, and level of DNA methylation. Two regions of the GLT-1 promoter were assessed: the CpG island and the CpG island shore located 1.7-1.9 kb upstream of the transcription start site. DEX as well as the HDAC-Is TSA and VPA were used to study GLT-1 transcriptional responses mediated by stress hormone and epigenetic-modifying drugs, respectively. The

goal was to identify GLT-1 promoter element(s) which confer brain-region specific response to stress hormone in an epigenetic fashion.

GLT-1 is also a target of psychoactive drugs other than VPA (Melone et al., 2001; Schneider et al., 1998; Tai et al., 2008). Thus, the further aim was to investigate whether certain antidepressants and mood stabilisers could modify promoter chromatin marks to interfere with GLT-1 transcription. In order to narrow the analysis, global chromatin modifications exerted by psychoactive drugs in astrocytes were first investigated. Detection of large-scale changes allows identification of drugs with pronounced chromatin modifying actions as a basis for more detailed investigation. Some frequently prescribed antidepressants including amitriptyline (AMI), venlafaxine (VEN), citalopram (CIT) and the mood stabilisers VPA, carbamazepine (CBZ) and lamotrigine (LTG) were chosen to evaluate possible epigenetic changes. To gain first insight into molecular processes tentatively involved in epigenomic alterations induced by psychoactive drugs, DNMT as well as DNA repair protein GADD45α expression were to be evaluated.

4 Results

In this section, the results of this cumulative thesis are shortly summarised. They are presented in their full length in the articles included in the Appendix. Here, the publication "Valproate and amitriptyline exert common and divergent influences on global and gene promoter-specific chromatin modifications in rat primary astrocytes" is designated as PI and the publication "The CpG island shore of the GLT-1 gene acts as a methylation-sensitive enhancer" as PII.

An examination of GLT-1 transcriptional activity under basal conditions as well as after DEX, VPA and TSA treatment in CTX and CER astrocytes was undertaken. As shown in Fig. 1A of PII, quantitative real-time analysis revealed no significant differences in GLT-1 mRNA levels between CTX and CER astrocytes under basal conditions. DEX treatment elevated GLT-1 transcription in CTX, but not in CER astrocytes (PII, Fig. 1B). Treatment with VPA/TSA induced an increase of GLT-1 mRNA levels in CTX astrocytes (PI, Fig. 4A and B), contrasting their inhibitory effect in CER (PII, Fig. 1C; TSA data not shown).

Next, a comparative analysis of active and repressive chromatin marks at the GLT-1 promoter between CTX and CER astrocytes under basal conditions was performed. Two regions of the GLT-1 promoter were assessed: the CpG island and the upstream CpG shore. At both investigated regions, chromatin immunoprecipitation analysis revealed that the level of active acetyl-histone H4 (AcH4) mark was higher in CTX than in CER astrocytes (PII, Fig. 2A). In contrast, the level of the repressive tri-methyl-histone H3 Lys27 (3MeH3K27) mark was higher in CER than in CTX astrocytes (PII, Fig. 2B).

The analysis of the histone modifications at the GLT-1 promoter was complemented with examination of DNA methylation levels by using bisulfite sequencing. It was found that the CpG island in untreated astrocytes from both brain regions was unmethylated (not shown). However, at the CpG shore, CpGs were more methylated in CER than in CTX astrocytes (PII, Fig. 3B). Thus, a higher level of repressive marks and H4 hypoacetylation at the CpG shore region in CER astrocytes correlated with GLT-1 unresponsiveness to the stress hormone DEX.

Furthermore, changes of chromatin marks at the GLT-1 promoter in primary astrocytes upon pharmacological stimulation with DEX, VPA and TSA were also elucidated. The results demonstrated that GLT-1 transcriptional response to DEX treatment notably correlated with increased AcH4 levels at the GLT-1 promoter in CTX astrocytes (PII, Fig. 2C). VPA/TSA-induced up-regulation of the GLT-1 transcription in CTX astrocytes was accompanied by DNA demethylation (PI, Fig. 3B) and H4 hyperacetylation (PI, Fig. 3C) of the CpG shore region. Surprisingly, the repression of GLT-1 transcription induced by VPA

treatment was also associated with increased AcH4 levels at the GLT-1 promoter in CER astrocytes (PII, Fig. 2E). For the first time, it could be demonstrated that changes of GLT-1 transcription upon treatment with stress hormone and HDAC-Is are accompanied by alterations in chromatin marks at the gene promoter region.

The transcriptional activity and pharmacological regulation of the GLT-1 promoter sequence was also tested in gene reporter assays. It was demonstrated that DEX and HDAC-Is stimulated the activity of the ectopic promoter in both CTX and CER astrocytes (PII, Fig. 4A and B; TSA data not shown), suggesting that CER cells provide the trans-acting factors necessary for GLT-1 activation. In an attempt to elucidate the contribution of methylation to the observed differences in gene expression, it was found that methylation of the whole promoter notably reduced GLT-1 promoter responsiveness to DEX and HDAC-Is (PII, Fig. 5A; TSA data not shown). In experiments specifically addressing the role of methylation at the CpG shore sequence on the GLT-1 promoter activity, it was shown that this region exhibited methylation-dependent enhancer function (PII, Fig. 5B and Fig. 6)

Epigenetic changes induced by VPA, indicated by H4 hyperacetylation and DNA demethylation, were found locally at GLT-1 gene locus but this drug is also known for its genome-wide effects. Thus, it was important to investigate whether VPA and also other mood stabilizers like CBZ and LTG as well as the antidepressants AMI, VEN and CIT induce global epigenetic changes in rat primary CTX and HPC astrocytes. In addition, epigenetic effects of psychoactive drugs on GLT-1 transcription were assessed as well.

Among the investigated mood stabilisers, only VPA evoked global changes in chromatin marks, including histone H3/H4 hyperacetylation (PI, Fig. 1) and 2MeH3K9 hypomethylation (PI, Fig. 6A and B) as well as DNA demethylation (PI, Table 2) in CTX and HPC astrocytes, as determined by Western blot and luminometric methylation analysis (LUMA), respectively. In the same cell types, TSA induced similar changes in global histone H3/H4 acetylation (PI, Fig. 1) and DNA methylation levels (PI, Table 2). Thus, the effects of VPA and TSA on the global histone acetylation and DNA methylation resembled the effects of these drugs at the GLT-1 locus in CTX astrocytes.

In order to gain some insights into molecular mechanisms of DNA demethylation, two key factors responsible for propagation, maintenance or *de novo* establishment of DNA methylation patterns, DNMT1 and GADD45α were evaluated. The results showed that VPA-associated demethylation at CCpGG sites occurred under unchanged DNMT enzymatic activity (PI, Fig. 5C; contributed by Nicole Zimmermann) or its protein levels (PI, Fig. 5A). Also, DNA demethylation induced by VPA was not associated with global changes in the level of the DNA repair protein GADD45α (PI, Fig. 5A). Since LUMA exclusively monitors the methylation status at CCpGG sites, the level of methylation at all CpG sites was also determined by capillary electrophoresis analysis (contributed by Dr.

Francesca Tuorto, German Cancer Research Centre, Heidelberg). In addition, bisulfite sequencing-based measurement of the level of CpG methylation at long interspersed nuclear elements (LINE-1) sites was performed. Neither the level of total cytosine methylation (PI, Supplementary Table 1) nor the level of CpG methylation at LINE-1 sites (PI, Supplementary Fig. S3) was altered by VPA exposure. In addition, flow cytometry-based analysis of the percentage of dividing cells revealed that a very low percentage of the untreated astrocytes was in the S-phase of the cell cycle and that VPA reduced this number even further, suggesting an involvement of an active DNA demethylation process at CCpGG sites (PI, Supplementary Fig. S2).

Investigation of the impact of antidepressants on global epigenetic modifications in primary astrocytes revealed that the tricyclic antidepressant AMI induced slight global cytosine demethylation (PI, Table 2). The changes of methylation at CCpGG sites were paralleled by a reduction of DNMT enzymatic activity (PI, Fig. 5C; contributed by Nicole Zimmermann), but not by a decrease of DNMT protein levels (PI, Fig. 5B). At the global level, the histone acetylation and methylation status remained unchanged after AMI treatment in CTX and HPC astrocytes (PI, Fig. 1 and Fig. 6). This investigation showed that AMI as well as the mood stabilizer LTG, in contrast to VPA and TSA, fail to stimulate EAAT2 (a human homolog of the GLT-1 promoter) transcriptional activity (PI, Fig. 4C) and induce GLT-1 promoter DNA demethylation (PI, Fig. 3B).

Collectively this thesis emphasises the importance of the epigenetic settings at the CpG shore of GLT-1, which produces different transcriptional outcomes and drug responsiveness. Further, the psychoactive drugs VPA and AMI both change epigenetic parameters leading to DNA demethylation, but they use different pathways and target different genes.

5 Discussion

The thorough examination of the epigenetic mechanisms controlling GLT-1 transcription, including their pharmacological modulation through stress hormone, HDAC-Is and psychoactive drugs, presents the following major contributions: 1. Histone modifications and DNA methylation are relevant in establishing control of GLT-1 transcription in both CTX and CER astrocytes and possibly account for their differential responsiveness to DEX and HDAC-Is. 2. The CpG island shore of the GLT-1 promoter acts as a stress hormone- as well as HDAC-I-responsive enhancer element in a methylation-dependent fashion. 3. While both VPA and AMI promote genome-wide reversible DNA demethylation at CCpGG sites, probably by distinct mechanisms, only VPA targeted the GLT-1 gene.

It is generally accepted that epigenetic mechanisms regulate astrocytic differentiation during neural cell type specification in a temporally- and spatially-controlled manner (Hirabayashi and Gotoh, 2010). Dysregulation of these developmental programs by VPA or TSA treatment has marked effects on global histone acetylation and progenitor cell differentiation both *in vivo* and *in vitro* (Asano et al., 2009; Shen et al., 2005). Nevertheless, on the level of individual genes, only few examples that accompany astrocytic differentiation are characterised. For instance, phenotypic specialisation of astroglial cells is associated with extensive alterations of chromatin at glial fibrillary acidic protein (GFAP) and S100b gene loci in neural precursors (Hatada et al., 2008; Song and Ghosh, 2004; Takizawa et al., 2001). In this study, the CpG island of the GLT-1 was found to be unmethylated in neonatal CTX and CER astrocytes. When the CpG island of the human GLT-1 analogue EAAT2 is hypermethylated the transcription is silenced *in vivo* and *in vitro* (Zschocke et al., 2007). Conversely, the EAAT2 promoter is largely unmethylated in normal human CTX tissue and the protein is expressed. Accordingly, in this study, it was found that GLT-1 gene is transcribed under basal conditions at the same level in CTX and CER astrocytes. In untransformed cells, CpG islands of housekeeping and most of the tissue-specific genes generally remain unmethylated. The presence of specificity protein 1 (Sp1) binding sites at the GLT-1 promoter (Allritz et al., 2010), which seem to inhibit *de novo* methylation during development (Brandeis et al., 1994), may confer protection against DNA methylation. Moreover, within the CpG island of GLT-1, the active AcH4 mark was higher in CTX, whereas the repressive 3MeH3K27 mark was higher in CER astrocytes. Nevertheless, it appears that unmethylated CpGs are strong signals for binding of the transcription machinery in both cell types, irrespective of histone modifications. A few studies show that active histone marks can be present at methylated CpG islands of transcriptionally active promoters (Brinkman et al., 2007; Tshuikina et al., 2008). Thus, signals from DNA

methylation and histone modifications seem not always concordant. At some CpG islands, histone modifications govern transcriptional activity, while DNA methylation dominates at others. However, it is also possible that DNA methylation interferes with repressor binding at promoters associated with active histone marks.

Analysing epigenetic modifications at the shore of the GLT-1 promoter, methylation of several neighbouring CpGs was significantly higher in CER than in CTX astrocytes. Furthermore, the experiments demonstrated that this GLT-1 promoter region exhibits prominent enhancer function after DEX/HDAC-I stimulation, suggesting existence of positive element(s) in this region. *In silico* analysis revealed numerous putative binding sites for transcription factors, of which some have been shown to regulate DEX-mediated response including cAMP response element-binding (CREB) protein and E-twenty six (ETS) (Espinas et al., 1994; van der Laan et al., 2009). Moreover, *in vitro* methylation had an inhibitory effect on transcriptional enhancement by DEX. The data are supporting the hypothesis that brain region-specific GLT-1 transcription in response to stress hormone is controlled by an enhancer element in a methylation-dependent manner. Recently, it was shown that DNA methylation of distal promoter sequences determines cell or tissue type-specific gene expression in untransformed cells and that these regions act as enhancer elements (Schmidl et al., 2009). In epigenetically induced pluripotent stem cells, differentially methylated regions occur more frequently at CpG island shores than at CpG islands, and these sites are tightly linked to genes involved in developmental and regulatory processes (Doi et al., 2009). These studies together with results reported here provide compelling evidence of the importance of CpG island shores in establishment of specific expression patterns and cell phenotypes.

In addition to DNA methylation, histone modifications at distal enhancer elements are also highly correlated with cell type-specific patterns of gene expression (Heintzman et al., 2009). In this thesis, it was found that DNA of the GLT-1 shore region was not only hypomethylated but also accumulated higher level of hyperacetylated H4 in CTX, as opposed to CER astrocytes. It is likely that such combination of chromatin marks in CTX cells conditions a permissive chromatin structure and increased GLT-1 transcription in response to DEX or HDAC-Is. In CER, however, the same region accumulated slightly higher amounts of 3MeH3K27 while AcH4 was underrepresented. The H3K27 mark is thought to be set by DNA methylation (Erhardt et al., 2003; Mendenhall et al., 2010; Vire et al., 2006) and contributes to additional stabilisation of the repressive state. Thus, repressive chromatin at this region could antagonize or even decrease GLT-1 transcription in response to DEX and HDAC-I treatment, respectively. In a study by Bapat et al., it was reported that genes in CP70 cells possessing 3MeH3K27 enrichment at promoters are transcriptionally down-regulated after transition to different culture conditions (Bapat et al., 2010). Since

HDAC-Is exert marked stimulatory effect on ectopic GLT-1 promoter constructs, this supports the role of high order chromatin structure in GLT-1 transcriptional repression. However, it can not be excluded that HDAC-I-induced repression of the GLT-1 in CER astrocytes could be also mediated by some cell type-specific trans-acting factors.

Chromatin is an integral component of mechanisms for transcriptional control but it is also susceptible to epigenetic reprogramming. Early-life stress elicits a reduction of DNA methylation at the AVP enhancer in hypothalamus under elevated corticosterone secretion (Murgatroyd et al., 2009). Also, chronic corticosterone exposure is accompanied by subtle alterations in DNA methylation at the Fkbp5 promoter (Lee et al., 2010). In this study, DEX-induced up-regulation of GLT-1 transcription was not associated with a change of DNA methylation at the distal promoter in CTX astrocytes. However, AcH4 levels at the GLT-1 promoter notably correlated with an increased GLT-1 gene activity after DEX treatment. In line with data presented here, changes in H4 acetylation status elicited by DEX at several gene promoters in a natural killer cell line reflect alterations in expression of respective genes (Krukowski et al., 2011).

Plasticity of chromatin marks at the CpG shore region in response to pharmacological stimulation was even more pronounced in CTX astrocytes exposed to HDAC-Is than to DEX. VPA treatment of CTX glia was accompanied by DNA demethylation of the GLT-1 shore region, enrichment in AcH4 and concomitant increase in GLT-1 gene activity. With this data, previously published findings on increased GLT-1 expression upon HDAC-I treatment were complemented (Allritz et al., 2009; Hassel et al., 2001), demonstrating that HDAC-I-induced GLT-1 gene activity could be mediated, at least partially, by chromatin changes at the promoter region. Surprisingly, VPA-induced hyperacetylation of H4 was also detected at the same GLT-1 region in CER astrocytes despite reduced GLT-1 mRNA levels. Many studies, including this one, show association of AcH4 and gene activity but there are also notable exceptions (Deckert and Struhl, 2001; Kurdistani et al., 2004; Wang et al., 2009). In addition, proteins with bromodomains are essential parts of some repressor complexes and they could mediate their binding to hyperacetylated H4 (Tae et al., 2011; Xhemalce and Kouzarides, 2010). Thus, in CER astrocytes, HDAC-Is might elicit cell type-specific synthesis of a repressor that operates with hyperacetylated AcH4 at GLT-1 endogenous gene locus, but not on the ectopic template.

Previous studies have also demonstrated that HDAC inhibition by VPA triggers hyperacetylation and the loss of the DNA methylation at gene promoters leading to their (re)activation (Detich et al., 2003; Dong et al., 2008). These changes associated with VPA treatment are not confined to small number of gene loci. Rather, they reach genome-wide magnitudes reflected by reprogramming of genome expression and global changes in DNA demethylation as well as H3K9 hyperacetylation (Hezroni et al., 2011; Milutinovic et al.,

2007). The results of this thesis demonstrate that VPA induces strong and reversible global H3/H4 hyperacetylation accompanied by transient demethylation at CCpGG sites in astrocytes from CTX and HPC. The similarity of TSA action on measured epigenetic parameters argues that HDAC inhibitory properties of VPA are attributable to effects of this drug. Moreover, D'Alessio et al. demonstrated that histone acetylation is one of the prerequisites for triggering DNA demethylation (D'Alessio et al., 2007) but the mechanism remains unknown. Several studies reported a functional interaction between HDACs and DNMTs (Deplus et al., 2002; Fuks et al., 2000). Even more, it has been demonstrated that the HDAC-Is VPA, TSA and apicidin down-regulate expression of DNMT subtypes in transformed cells (Arzenani et al., 2011; Kundakovic et al., 2009; You et al., 2008), offering a putative explanation for their demethylating activity. However, the results of this thesis showed that neither VPA nor TSA reduced levels of DNMT1 protein in primary astrocytes. Tumour cells are much more sensitive to HDAC-Is than normal cells (Bolden et al., 2006) which could explain the observed differences. Also, the differences in expression of GADD45α protein between untreated and TSA/VPA-treated CTX astrocytes were not detected. This protein, involved in DNA repair and cell-cycle control, is one of major candidates in replication-independent active DNA demethylation (Barreto et al., 2007; Schmitz et al., 2009). Nevertheless, the data reported here confirm the notion that DNA demethylation in VPA-treated primary astrocytes occurs in cells with very low replicative activity (Detich et al., 2003), but through a mechanism unrelated to decrease in GADD45α levels. However, it can not be excluded that sensitivity of the applied method for determination of protein levels may not suffice to detect subtle alterations in GADD45 levels which could account for slight changes in CCpGG methylation induced by HDAC-Is.

Another mood stabilizer CBZ was reported to inhibit HDAC3 and HDAC7 in a cancer cell line at concentrations typically achieved in patients (Beutler et al., 2005). Changes in levels of global H3/H4 acetylation by applying CBZ at reported therapeutic range for 24 hours in primary astrocytes were not found, suggesting a cell type-specific effect. Similarly, another tested mood stabilizer LTG as well as the antidepressants AMI, VEN and CIT and failed to induce detectable changes in global histone acetylation levels. Excitingly, treatment with the tricyclic antidepressant AMI led to a slight reduction of genome-wide DNA methylation levels at CCpGG sites in CTX astrocytes. Like in the case of VPA, induced DNA demethylation was not accompanied with altered DNMT1 protein levels. However, AMI significantly reduced DNMT activity in treated astrocytes. A follow-up study demonstrated that this inhibitory effect was restricted to DNMT1 subtype and was also observed after treatment with structurally unrelated antidepressant paroxetine (PAR) (Zimmermann et al., submitted). Importantly, diminished DNMT1 activity by AMI and PAR was accompanied by a decrease in levels of G9a, which is a histone H3 Lys9 HMT (Tachibana et al., 2001) and an interaction

partner of DNMT1 (Esteve et al., 2006). In this thesis, a decline in H3K9 levels after 24 hours of treatment with AMI in CTX astrocytes was not detected. In a study by Zimmermann et al., a slight reduction of G9a protein levels by AMI at this time point was measured but this may not be sufficient to decrease global H3K9 methylation. Also, at least several known proteins have H3K9 methyltransferase activity (O'Carroll et al., 2000; Rea et al., 2000; Schotta et al., 2004), thus making it difficult to detect alterations of global H3K9 caused by reduced G9a levels. Nevertheless, these results raise the possibility that AMI might alleviate disease symptoms by interfering with chromatin-remodelling machinery.

Although AMI induced global DNA demethylation, the GLT-1 promoter was not affected and the level of expression remained unchanged. A recent study showed that co-administration of picomolar concentrations of AMI with morphine for 5 days increases GLT-1 expression in the spinal cord dorsal horn (Tai et al., 2008). Authors reported that AMI helped in restoration of decreased GLT-1 levels induced upon morphine infusion. The data presented in this thesis showed that LTG failed to demethylate GLT-1 shore region and enhance GLT-1 transcription. Except for VPA, treatment with other investigated psychoactive drugs also induced no alterations in GLT-1 mRNA levels. These results suggest that only VPA exerts fast-acting effects on GLT-1 transcription probably due to its prominent chromatin-modifying properties. However, it can be that long-term treatment of astrocytes or *in vivo* administration of psychoactive drugs in animals could have an effect on GLT-1 expression, like it was shown for clozapine and haloperidol (Melone et al., 2001; Schneider et al., 1998).

Genome stability is to a great extent determined by a remarkable ability of cells to maintain and faithfully replicate specific epigenetic patterns. This ensures specific cell function and adequate responses to a variety of stimuli. The results of this thesis show that chromatin marks at the GLT-1 promoter in astrocytes may have a significant impact on basal GLT-1 expression as well as on GLT-1 function in different brain regions under conditions of stress and HDAC inhibition. They demonstrate that chromatin modifications at the GLT-1 gene locus are susceptible to specific alterations despite their intrinsic stability. This epigenetic plasticity makes GLT-1 an accessible pharmacological target to drugs with HDAC inhibitory properties. An increase in GLT-1 expression might consecutively lead to modulation of glutamatergic signalling. Thus, normalisation of central neural circuits implicated in mood disorders could be, at last in part, ascribed to epigenetic effects of VPA on GLT-1 regulation, especially knowing the positive effects of this drug on GLT-1 expression in animals (Hassel et al., 2001). According to the result of this thesis, DNA demethylation by VPA and AMI shows gene selectivity. Treatment with AMI may impact multiple targets in astrocytes, other than GLT 1, one example being HMT G9a (Zimmermann et al., submitted). Thus, epigenetic changes elicited by VPA and AMI may have repercussions on the outcome of the therapy by affecting astrocyte-neuron communication.

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Zimmermann N, Zschocke J, Perisic T, Yu S, Holsboer F, Rein T. Mechanisms of antidepressant actions on DNA methyltransferases. *(submitted)*

7 Curriculum Vitae

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Education

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Faculty of Biology, Belgrade University, Serbia

Publication List

Perisic T, Holsboer F, Rein T, Zschocke J (2012) The CpG island shore of the GLT-1 gene acts as a methylation-sensitive enhancer. *Glia (in press)*

Zimmermann N, Zschocke J, **Perisic T**, Yu S, Holsboer F, Rein T. Mechanisms of antidepressant actions on DNA methyltransferases. *(submitted)*

Matic G, Vojnovic Milutinovic D, Nestorov J, Elakovic I, Manitasevic Jovanovic S, **Perisic T**, Dundjerski J, Damjanovic S, Knezevic G, Spiric Z, Vermetten E, Savic D. Lymphocyte glucocorticoid receptor level and hormone binding properties discriminate between war related PTSD and resilience to PTSD. *(submitted)*

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8 Appendix

This section contains reprints of the following articles:

I

Perisic T, Zimmermann N, Kirmeier T, Asmus M, Tuorto F, Uhr M, Holsboer F, Rein T, Zschocke J (2010) Valproate and amitriptyline exert common and divergent influences on global and gene promoter-specific chromatin modifications in rat primary astrocytes. *Neuropsychopharmacology* 3:792-805.

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Valproate and Amitriptyline Exert Common and Divergent Influences on Global and Gene Promoter-Specific Chromatin Modifications in Rat Primary Astrocytes

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Aberrant biochemical processes in the brain frequently go along with subtle shifts of the cellular epigenetic profile that might support the pathogenic progression of psychiatric disorders. Although recent reports have implied the ability of certain antidepressants and mood stabilizers to modulate epigenetic parameters, studies comparing the actions of these compounds under the same conditions are lacking. In this study, we screened amitriptyline (AMI), venlafaxine, citalopram, as well as valproic acid (VPA), carbamazepine, and lamotrigine for their potential actions on global and local epigenetic modifications in rat primary astrocytes. Among all drugs, VPA exposure evoked the strongest global chromatin modifications, including histone H3/H4 hyperacetylation, 2MeH3K9 hypomethylation, and DNA demethylation, as determined by western blot and luminometric methylation analysis, respectively. CpG demethylation occurred independently of DNA methyltransferase (DNMT) suppression. Strikingly, AMI also induced slight cytosine demethylation, paralleled by the reduction in DNMT enzymatic activity, without affecting the global histone acetylation status. Locally, VPA-induced chromatin modifications were reflected at the glutamate transporter (GLT-1) promoter as shown by bisulfite sequencing and acetylated histone H4 chromatin immunoprecipitation analysis. Distinct CpG sites in the distal part of the GLT-1 promoter were demethylated and enriched in acetylated histone H4 in response to VPA. For the first time, we could show that these changes were associated with an enhanced transcription of this astrocyte-specific gene. In contrast, AMI failed to stimulate GLT-1 transcription and to alter promoter methylation levels. In conclusion, VPA and AMI globally exerted chromatin-modulating activities using different mechanisms that divergently precipitated at an astroglial gene locus.

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INTRODUCTION

The vertebrate brain is exposed to a number of environmental cues that are processed in an adaptive manner. These adaptive processes form the basis of neuroplasticity, and are characterized by often-persistent changes in neural gene expression. Epigenetics represents an essential element for transmitting environmental cues into altered neural molecular pathways (Szyf *et al*, 2008). Frequently, epigenetic chromatin modifications affect the acetylation or methylation status of histones and the methylation of cytosine residues within CpG dinucleotides. Histone acetylation and DNA methylation show some degree of interdependency, although a detailed analysis of this interaction is still pending (D'Alessio and Szyf, 2006). Epigenetic modifications subsequently alter chromatin packaging, and, if occurring in the proximity of gene promoters, exons or introns, either promote or suppress gene transcription (Herman and Baylin, 2003). Enzymes catalyzing these epigenetic reactions belong to the classes of histone acetyltransferases (HATs) and methyltransferases (HMTs), histone deacetylases (HDACs), and DNA methyltransferases (DNMTs) (Ng and Bird, 1999).

Recently, it has been shown how external stimuli can be translated into modified epigenetic marks for the example of the brain-derived neurotrophic factor (BDNF) promoter (Chen *et al*, 2003). After membrane depolarization on isolated neurons, specific CpG dinucleotides within the *BDNF* promoter underwent demethylation, accompanied by the upregulation of gene transcription. Similarly, mRNA and

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protein levels of distinct DNMT subtypes were reduced under similar conditions, which resulted in a diminished activity of the respective enzymes (Sharma *et al*, 2008).

The consequence of an intrinsically false or inadequate adaptation process in response to detrimental environmental factors such as stress might go along with the establishment of aberrant epigenetic signatures. In humans, there is mounting evidence that epigenetic mechanisms are involved in the pathophysiology of stress-related disorders, including unipolar and bipolar depression (Mill and Petronis, 2007; Petronis, 2003). Postmortem studies of suicide victims with a history of depression or childhood abuse showed differential expression of *DNMT* subtypes (Poulter *et al*, 2008) and promoter-wide hypermethylation of ribosomal RNA gene promoters (McGowan *et al*, 2008), respectively. Humans who experienced childhood abuse and committed suicide also showed increased methylation of the neuron-specific glucocorticoid receptor (GR) promoter in the hippocampus paralleled by reduced levels of the corresponding mRNA transcripts (McGowan *et al*, 2009). In general, GR has a pivotal role in balancing the hypothalamic pituitary axis (HPA) in response to eg, stressful situations, and a HPA imbalance is observed in various psychiatric disorders. In rodents, it was recently shown that poor maternal care of the offspring increased DNA methylation of the *GR* exon 1_7 promoter in the hippocampus and, thereby impaired negative feedback regulation of HPA axis due to lower GR protein levels (Weaver *et al*, 2004). Interestingly, application of an HDAC inhibitor (HDAC-I) restored normal HPA response to stress in the offspring. In addition, chronic social stress in rodents led to distinct biochemical histone modifications within the *BDNF* gene promoter, paralleled by a reduced *BDNF* transcription (Tsankova *et al*, 2006).

Hence, pharmacological interference with the epigenetic setup of the cell might represent an avenue to normalize the aberrantly installed epigenetic profiles. So far, drug therapy of patients suffering from bipolar and major depression is based on the stabilization of neurotransmitter circuits that involve the serotonergic, noradrenergic, glutamatergic, and GABAergic system. Most of the therapeutic compounds exert their beneficial effects after a latency period, indicating the necessity for preceding adaptive processes. This raises the question, whether psychoactive substances would potentially interfere directly or indirectly with the epigenetic makeup of exposed cells. Indeed, the mood stabilizer, valproic acid (VPA), was the first psychoactive drug shown to exhibit HDAC inhibitory properties (Gottlicher *et al*, 2001; Phiel *et al*, 2001). Another study investigated antiepileptic drugs and identified topiramate as an inhibitor of HDAC activity (Eyal *et al*, 2004).

In this study, we aimed to comprehensively analyze the potential of frequently prescribed antidepressants, including amitriptyline (AMI), venlafaxine (VEN), citalopram (CIT), and mood stabilizers such as VPA, carbamazepine (CBZ), and lamotrigine (LTG) to affect epigenetic parameters in astrocytes. Astrocytes represent the majority of cells within the CNS, and modulate synaptic strength by their association with synapses (Schipke and Kettenmann, 2004). In addition, astrocytes support neuronal function, as they produce neurotrophic factors, including BDNF and glial-derived neurotrophic factor, also in response to VPA exposure (Chen *et al*, 2006; Wu *et al*, 2008c). We monitored drug-induced global epigenetic changes by measuring histone H3 and H4 acetylation/methylation, as well as by DNA methylation. To explore whether global changes would be also reflected at gene loci that are important for astrocytic function, we selected the glutamate transporter (*GLT-1*) promoter as a model gene.

MATERIALS AND METHODS

Primary Astrocyte Cell Culture

Enriched astroglial cultures were prepared from postnatal day 1 rat pups (Sprague–Dawley, Charles River, Sulzfeld, Germany) as described previously (Franke *et al*, 1998). In these cultures, 90% of all cells represent type-1 astrocytes. Briefly, the dissected cortical hemispheres and hippocampus were trypsinized (Invitrogen, Merelbeke, Belgium); trypsin action was terminated by adding Hanks' balanced salt solution (Invitrogen), supplemented with 10% fetal calf serum. The tissue was passed through a serological pipette, spun, and resuspended in modified Eagle's medium (MEM, Invitrogen) supplemented with 10% horse serum (Invitrogen). After the third passage, the cells were maintained in a serum-free (MEM/ Ham's F-12, 1 : 1) N2-supplemented medium.

Pharmacological Treatment of Astrocytes

All experiments were carried out using third-passage astrocytic cells. Sodium butyrate (NaB), VPA, CBZ, LTG, AMI, CIT, and trichostatin A (TSA) were purchased from Sigma (Deisenhof, Germany). VEN was an industrial donation (Wyeth Pharma GmbH, Münster, Germany). Stock solutions of the drugs were prepared by dissolving the substances in distilled water (VPA, NaB, AMI, CIT, VEN), 100% ethanol (TSA), or DMSO (CBZ, LTG). When treating cells for 72 h, the drug-containing medium was renewed after 48 h. For washout experiments, after 72 h of treatment, the cells were rinsed twice with the medium, and thereafter kept in the absence of the drug for an additional 48 h.

Cellular Protein Extraction and Western Blot Analysis

Cells were lysed in the buffer containing 62.5 mM Tris, 2% SDS, and 10% sucrose, supplemented with protease inhibitor cocktail (Sigma). Samples were sonicated and heated at 95°C for 5 min. Proteins were separated by SDS-PAGE and electrotransferred onto nitrocellulose membranes. Blots were placed in Tris-buffered saline, supplemented with 0.05% Tween (Sigma) and 5% non-fat milk for 1 h at room temperature, and then incubated with primary antibody (diluted in TBS/0.05% Tween) overnight at 4°C. Subsequently, the blots were washed and probed with the respective horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The immunoreactive bands were visualized using the ECL detection reagent (Millipore, Billerica, MA, USA). The following primary antibodies were used: anti-acetyl-Histone H3 antibody (recognizes acetylation at lysine 9; 1 : 2000; no. 06–942, Upstate, Temecula, CA, USA), anti-acetyl-Histone H4 antibody (recognizes acetylation at lysines 5, 8, 12, and 16; 1 : 4000; no. 06–866, Upstate), anti-DNMT-1 (1 : 1000; no. IMG-261A, IMGENEX, San Diego, CA, USA), anti-GADD 45α (1 : 250; no. sc-6850, Santa Cruz Biotechnology,

Heidelberg, Germany), anti-dimethyl-Histone H3 antibodies (react with methylated histones on lysine 9 or 27; 1 : 4000; a kind gift from Dr Thomas Jenuwein, Max-Planck-Institute of Immunobiology, Freiburg, Germany), anti-α-actin (1 : 2500; no. A2066, Sigma), and anti-hsp90 (1 : 2000; no. sc-7947, Santa Cruz Biotechnology). Determination of the relative optical density and quantification of band intensities were performed using the Kodak Image Analysis Software.

DNMT Activity Assay

Nuclear extracts from cortical astrocytes were obtained using the EpiQuik Nuclear Extraction Kit (Epigentek, Brooklyn, NY) according to the manufacturer's instructions. DNMT activity was determined by incubating 4 μg of protein nuclear extract with 0.5 μg Poly (deoxyinosinic-deoxycytidylic) acid (Poly(dI-dC) · Poly(dI-dC); Sigma) in reaction buffer (20 mM Tris pH 7.8, 10% glycerol, 5 mM EDTA, 1 mM DTT, 0.2 mM PMSF) containing 3 μM *S*-adenosyl-L-[methyl-3H]methionine (^{3}H-SAM, specific activity: 15 Ci/mmol; GE Healthcare, Munich, Germany) for 3 h at 37°C. Thereafter, DNA was isolated using the GeneClean Kit (MP Biomedicals). To reduce background signals, three washing steps were conducted and the DNA binding step was performed in the presence of a 1000 times excess of unlabeled SAM to reduce nonspecific binding of the labeled SAM. To minimize the loss of DNA, genomic DNA (3 μg per sample) was added to the binding buffer.

Isolation of Genomic DNA

Isolation of genomic DNA was carried out by salting out proteins and precipitating the genomic DNA with isopropanol as described previously (Zschocke *et al*, 2007).

Luminometric Methylation Analysis

Luminometric methylation analysis (LUMA) was performed according to Karimi *et al* (2006), with minor modifications. Briefly, 2 μg of genomic DNA was cleaved with 4 Units of *Eco* RI (Fermentas, St Leon-Rot, Germany) in a buffer containing 66 mM Tris-acetate, 20 mM Mg-acetate, 0.2 mg/ml BSA, 132 mM K-acetate pH 7.9, for 2 h at 37°C. *Eco* RI was inactivated at 65°C for 20 min. Subsequently, the reaction content was diluted by adding an equal amount of dH_2O. In two separate 20 μl reactions, DNA (1 μg each) was subjected to enzymatic digestion by adding 2 Units of *Hpa* II or *Msp* I (New England Biolabs, Frankfurt, Germany), and the mixture was incubated 4 h at 37°C. After heat inactivation of *Hpa* II and *Msp* I at 65°C for 20 min, samples were processed using a pyrosequencer (Biotage, Uppsala, Sweden). The *Hpa* II/*Eco* RI and *Msp* I/*Eco* RI ratios were calculated, and the result of the measurement was expressed as the percentage of CCpGG methylation.

Methylation Analysis by Bisulfite Genomic Sequencing

Bisulfite treatment of genomic DNA (2 μg) was performed using the EpiTech Bisulfite Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Bisulfite-specific primers were designed using Methyl Primer Express Software (Applied Biosystems). The PCR mixture was composed of 1 × PCR Buffer (Invitrogen), 1.5 mM $MgCl_2$ (Invitrogen), 0.2 mM of each dNTP (peqLAB, Erlangen, Germany), 0.5–1 μM of each primer, and 4 Units of DNA Taq polymerase (Invitrogen). Sequences of primers are listed in Table 1. Methylation of individual CpG sites was detected by direct sequencing (Qiagen). All sequencing reaction mixtures were based on the BigDye 3.1 Terminator chemistry (Applied Biosystems). Data collection was carried out on a 3730 × l DNA Analyzer (Applied Biosystems).

Quantitative Real-Time RT-PCR

Total RNA was prepared from astroglial cells according to the manufacturer's protocol for NucleoSpin RNA II (Macherey-Nagel, Düren, Germany). A total of 500 ng of purified RNA was transcribed with random primers and

Table 1 Primer Sequences Used for Bisulfite Sequencing (bs), Quantitative Real-Time PCR (qPCR) and Chromatin Immunoprecipitation (ChIP)

Primer	Primer pair sequences	T_A (°C)	GenBank access. no.
GLT-1 CpG island (bs)	fwd 5′-GGGGTTAAATTTTGTAATTTTT-3′ rev 5′-CCCCTCCTAAATAAACCCTT-3′	52	AY643513
GLT-1 prox. promoter region (bs)	fwd 5′-TTAGATGTTTAGGGAGTGATGG-3′ rev 5′-CTATCCCTCTTCTATTATCCCTC-3′	63	EF017228
GLT-1 dist. promoter region (bs)	fwd 5′-ATTGGTAGGTAAATAAAAAATAAATTT-3′ rev 5′-ATTCAAAAACTATACTATCAAACTCC-3′	56	EF017228
LINE-1 (bs)	fwd 5′-TTAGTTTGAAGAGGTTGTTATTG-3′ rev 5′-TCCTATCAACCCTACACTCTAA-3′	61	M60824
Lambda DNA (bs)	fwd 5′-GTTGGATGTAGAAAGTTGGAAG-3′ rev 5′-TTTATCATACAACCCTATCTCCC-3′	56	J02459
GLT-1 (qPCR)	fwd 5′-CCGAGCTGGACACCATTGA-3′ rev 5′-CGGACTGCGTCTTGGTCAT-3′	60	X67857
Actin (qPCR)	fwd 5′-CTACAATGAGCTGCGTGTGGC-3′ rev 5′-CAGGTCCAGACGCAGGATGGC-3′	60	BC063166
GLT-1 dist. promoter region (ChIP)	fwd 5′-TTCGGTCTCTGAAGCATGTG-3′ rev 5′-TAGCCCAAAGGCACTCTCAT-3′	60	EF017228

Annealing temperatures of the corresponding primer pairs and GenBank accession numbers are provided.

Omniscript Reverse Transcriptase (Qiagen). Quantitative real-time PCR analysis of GLT-1 cDNA was performed using the LightCycler Carousel-Based System (Roche Applied Science, Mannheim, Germany) and SYBR green (QantiFast SYBR Green, Qiagen). β-Actin was used as an endogenous control for normalization. The primers used are listed in Table 1. Relative changes of gene expression were calculated using the comparative $\Delta\Delta C_T$ method.

Transient Transfection and Luciferase Reporter Assay

Approximately $3-5 \times 10^6$ primary cortical rat astrocytes were transiently transfected with human EAAT2 promoter reporter plasmid (a kind gift from Dr Jürgen Engele, University of Leipzig, Leipzig, Germany) using Amaxa's Nucleofector device (Amaxa Biosystem, Cologne, Germany). Simian virus 40 promoter-driven nonsecretory Gaussia luciferase expression vector was cotransfected to correct for transfection efficiency. Twenty-four hours after transfection, drug treatment was carried out for 24 h. Luciferase assays were performed by applying the Dual Luciferase Assay System (Promega, Mannheim, Germany) using the TriStar LB941 Luminometer (Berthold Technologies, Wildbad, Germany).

Chromatin Immunoprecipitation

After pharmacological treatment, 2×10^6 astrocytes were fixed *in vivo* with 1% formaldehyde for 10 min, and the reaction was finally quenched with 0.125 M glycine for 5 min at RT. From then on, all steps were carried out at 4°C, if not stated otherwise. Nuclei were extracted in 1 ml hypotonic buffer composed of 0.5% Igepal-CA630, 10 mM KCl, 1.5 mM $MgCl_2$, and 10 mM HEPES, pH 7.9, followed by 15 min of incubation on ice and brief homogenization. Nuclei were recovered by 5 min by centrifugation at 800 *g*. Nuclear pellets were dissolved in 400 µl lysis buffer (containing 1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) and sonicated using a Branson sonifier (12×20 s bursts, 90% pulse, output control 4). The average length of DNA fragments generated by sonication was 500 bp. Two 200 µl aliquots (for IgG control and 2 µg of anti-acetyl-H4 antibody) were 1 : 10 diluted in ChIP dilution buffer (0.01% SDS, 1.1% Triton, 1.2 mM EDTA, 167 mM NaCl, and 16.7 mM Tris-HCl, pH 8.1) and precleared for 3 h with blocked Dynabeads (Promega, protein A coupled, blocked with 0.1% BSA and 0.05% salmon sperm DNA). Anti-acetyl-H4 antibody was added to the samples, preincubated for another 2 h, and rotated overnight with blocked Dynabeads. Four subsequent washing steps were carried out using low salt (0.1% SDS, 1% Triton, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1), high salt (see low salt, except 500 mM NaCl), LiCl (0.25 M LiCl, 1% NP-40, 1% deoxy-cholate, 1 mM EDTA, 10 mM Tris-HCl, ph 8.1), and TE buffer. Elution and reversion of cross-links were performed in buffer containing 1% SDS, 50 mM $NaHCO_3$, 1 mM EDTA, 50 mM Tris-HCl pH 8.0, for 2 h at 62°C and a final 95°C heating step for 5 min. DNA was purified using the ultra-clean PCR Clean-up Kit according to the manufacturer's instructions (MoBio Laboratories, Carlsbad, CA, USA), and later on used in real-time PCR using primers listed in Table 1.

RESULTS

VPA, but not Other Psychoactive Drugs, Induce Global Histone Hyperacetylation

In this study, we set out to explore the effects of different classes of antidepressants (such as VEN, CIT, AMI) and mood stabilizers (such as VPA, LTG, CBZ) on the epigenetic machinery of primary rat astrocytes, which express the classical targets of antidepressants such as serotonin and noradrenaline transporters, and different 5 HT-receptor subtypes (Azmitia, 2001; Inazu *et al*, 2001; Inazu *et al*, 2003). To further evaluate mechanistic aspects, we also included the established HDAC-I TSA and NaB in some assays.

First, we measured the degree of global histone H3 and H4 acetylation (AcH3, AcH4), which is indicative of cellular HAT and HDAC activity, after 24 h of drug exposure at two different concentrations. The lower concentrations are in the range of therapeutic plasma levels, whereas higher concentrations represent a value that might be reached in the brain assuming cumulative processes and enrichment of the drugs at the loci of action. In addition, TSA was used as a pan-inhibitor of HDACs. Western blot analysis showed that out of all psychoactive drugs, only VPA induced hyperacetylation of both histones H3/H4 in a dose-dependent manner (Figure 1). Moreover, there was no profound difference in the pattern of induction when comparing astrocytes from the hippocampus and cortex.

Dynamic Changes of Histone Hyperacetylation After VPA Exposure

To evaluate the dynamics of VPA-mediated histone acetylation during and after drug exposure, we conducted a time-course analysis of 24 and 72 h VPA treatment, followed by a drug-removal step for 48 h. In both, cortical and hippocampal astrocytes, VPA-induced hyperacetylation of H3K9 was stronger after 24 h than after 72 h, whereas this difference was less pronounced in the case of H4 acetylation (Figure 2). A similar trend was observed in cells treated with 0.2 µM TSA. Forty-eight hours after VPA withdrawal, AcH3K9 and AcH4 returned to basal levels. Our results indicate that the effects of VPA on histone acetylation are only transient, peak during the first hours of treatment, and are fully reversible after drug removal.

DNA Demethylation at CCpGG Sites Exerted by VPA is Reversible

Histone acetylation and DNA methylation operate in a concerted manner. In agreement with this hypothesis, HDAC-Is are capable of inducing DNA demethylation in human cancer cell lines (Detich *et al*, 2003; Ou *et al*, 2007) and *in vivo* (Dong *et al*, 2007). Therefore, we examined whether VPA/TSA trigger CpG demethylating in nontransformed primary astrocytes. In total, three different methods were used to determine the degree of global CpG methylation. LUMA measures the average methylation status of all CCpGG sites by *Hpa* II/*Msp* I restriction analysis, coupled to a Taq polymerase-dependent luminometric reaction. The second, capillary electrophoresis technique depicts the average methylation status of all cytosines. Third, we

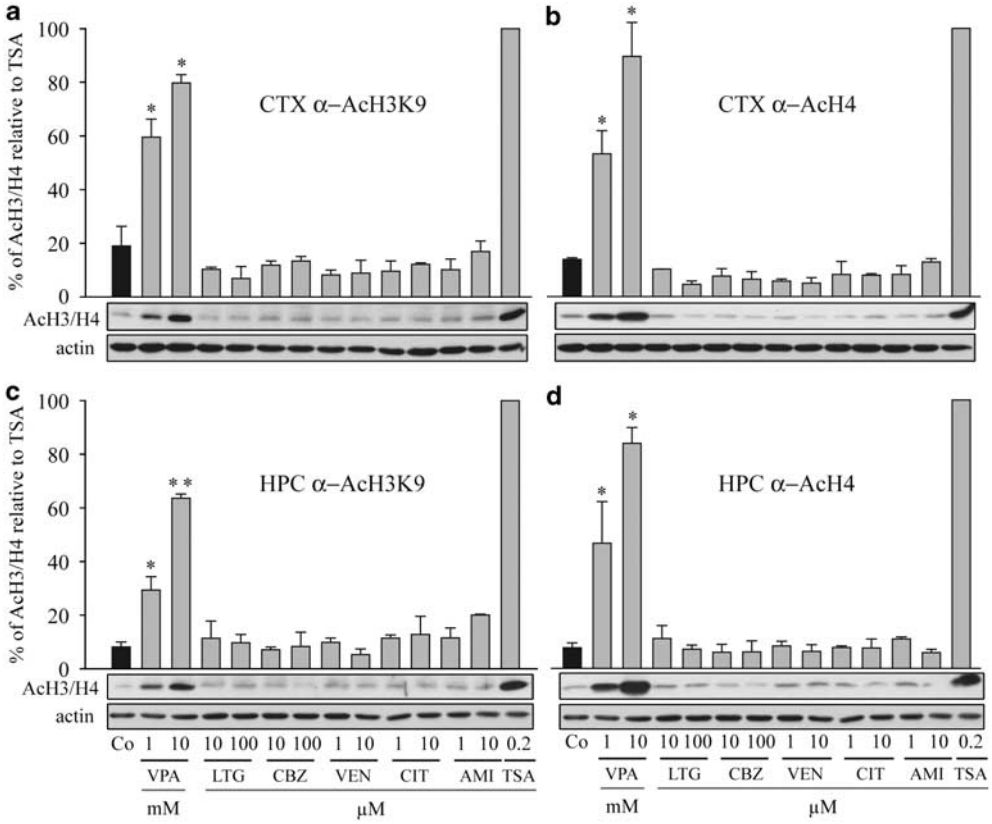

Figure 1 Screen of psychoactive drugs for their global effects on histone acetylation. Cortical (CTX; a, b) and hippocampal astrocytes (HPC; c, d) were treated with VPA (1 and 10 mM), LTG (10 and 100 μM), CBZ (10 and 100 μM), VEN (1 and 10 μM), CIT (1 and 10 μM), AMI (1 and 10 μM), or with the HDAC inhibitor TSA (0.2 μM) for 24 h. After drug treatment, cell lysates were prepared and subjected to western blot analysis using polyclonal antibodies against AcH3K9 and AcH4. AcH3K9 and AcH4 band intensities were normalized to α-actin. The bars are depicted as the percentage of optical band densities (mean ± SE, $n = 2$) calculated relative to the value that corresponds to histone acetylation induced by TSA (set to 100%); *t*-test: $*p < 0.05$ *vs* untreated cells, $**p < 0.005$ *vs* untreated cells.

analyzed long interspersed nuclear elements (LINE-1), serving as surrogate markers of global DNA methylation changes (Yang *et al*, 2004), by applying direct bisulfite sequencing of a certain stretch of the LINE-1 ORFII.

First, we validated the accuracy of LUMA and bisulfite sequencing with respect to CpG methylation. There was a high degree of similarity between results obtained by LUMA and direct bisulfite sequencing ($r^2 = 0.98$, Supplementary Figure S1a).

As determined by LUMA, untreated cortical and hippocampal astrocytes displayed 64 ± 1% and 67 ± 1% methylation of CCpGG sites, respectively (Supplementary Figure S1b). In cells from the cortex, treatment with 1 mM VPA induced significant demethylation after 72 h (−4.9 ± 1.8%), whereas 48 h after drug withdrawal, the level of CCpGG methylation returned to baseline (Table 2). At a concentration of 10 mM, VPA triggered DNA demethylation by −7.5 ± 1.3% already after 24 h, and reached −17.2 ± 2.0% after 72 h. Again, demethylation was reversed after 48 h of drug washout. In cells from the hippocampus, results obtained by LUMA were slightly less coherent. After 24 and 72 h of 1 mM VPA exposure, significant hypomethylation (−9.7 ± 2.4% and −5.2 ± 0.6%, respectively) was observed. Higher doses of VPA of 10 mM induced −5.0 ± 1.4% hypomethylation after 72 h. Although the reversibility of methylation changes was not as pronounced as in the cortex, it appeared to be in this direction. Finally, 0.2 μM TSA induced significant DNA hypomethylation at CCpGG sites in the cortex and hippocampus. Collectively, our results suggest that VPA-induced hypomethylation of CCpGG profiles are present in both brain regions, although magnitude and kinetics seem to differ slightly.

DNA demethylation processes can occur passively by eg, retention of DNMT enzymes in the cytoplasm during cell-cycle transitions. In addition, active mechanisms of DNA demethylation occurring in quiescent cells in short periods of time are discussed, requiring a putative demethylating enzyme or DNA repair machinery. To determine the percentage of cells present in different stages of the cell cycle, we conducted flow cytometry analysis (Supplementary Figure S2). Prolonged treatment with 10 mM VPA for 72 h increased the percentage of G_1/G_0 cells by 9% (92 ± 1% of the total cells), paralleled by a reduction of cells in the S- and G_2/M-phase (2 ± 1% and 6 ± 1% of the total cells, respectively). The cell-cycle data illustrate that very few of the untreated astrocytes were in the S-phase and VPA reduced this number even further. Thus, it seems very unlikely that the VPA-mediated reduction in DNA methylation by 18% (LUMA) can be accounted for by the few dividing cells. Thus, VPA might rather trigger active demethylation processes as already claimed by other groups for eg, HEK293 cells (Detich *et al*, 2003).

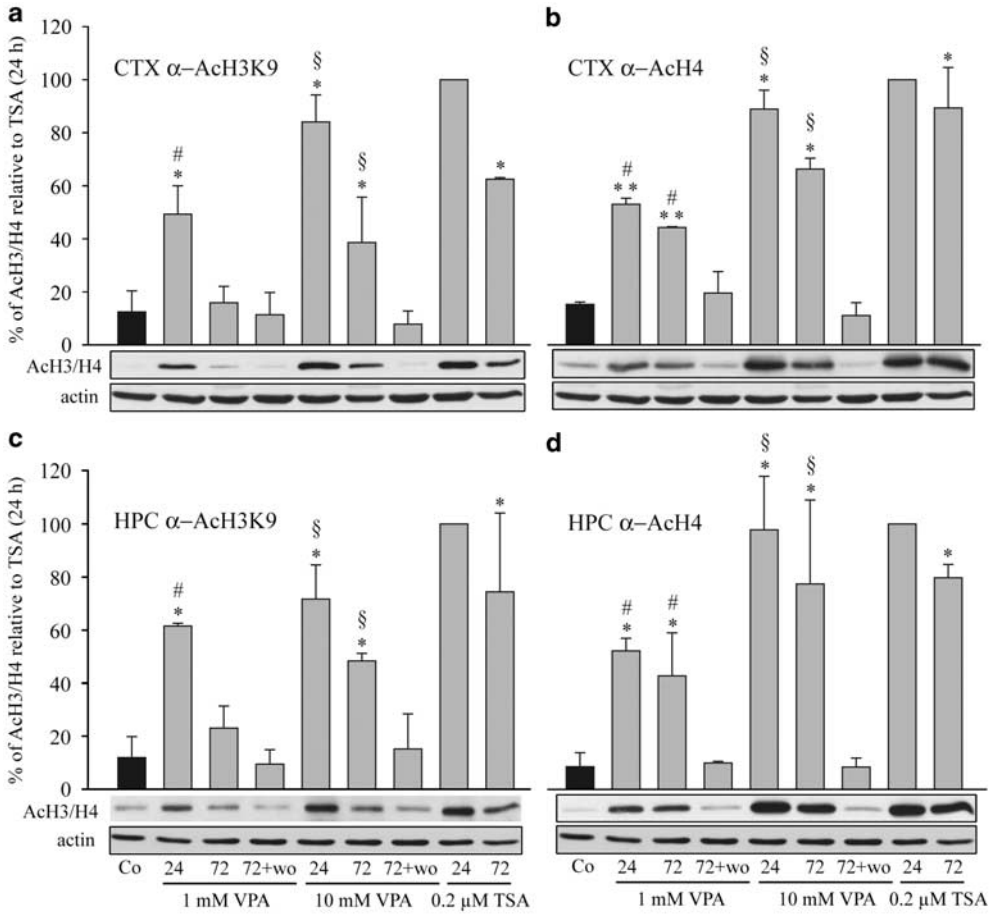

Figure 2 VPA induces dynamic changes of AcH3K9 and AcH4 patterns in astrocytes from different brain regions. Cortical (a, b) and hippocampal (c, d) astrocytes were exposed to 1 and 10 mM VPA for 24, 72, and 72 h, followed by a 48 h washout period, whereas a single dose of TSA (0.2 μM) was applied for 24 and 72 h. The level of AcH3K9 and AcH4 was monitored by western blot analysis and detected with AcH3K9- and AcH4-specific antibodies. α-Actin immunoreactivity served as a control for protein loading. Optical densities of AcH3K9- and AcH4-specific bands were normalized to α-actin. Data represent mean ± SE ($n = 2$) of the percentage of optical band densities calculated relative to the value that corresponds to histone acetylation induced by 24 h treatment with 0.2 μM TSA (set to 100%); *t*-test: $^{*}p < 0.05$ *vs* untreated cells, $^{**}p < 0.005$ *vs* untreated cells, $^{\#}p < 0.05$ *vs* 1 mM VPA depletion treatment, $^{\S}p < 0.05$ *vs* 10 mM VPA depletion treatment.

Table 2 VPA/TSA and AMI Trigger CCpGG DNA Hypomethylation

Treatment	Change in DNA methylation analyzed by LUMA					
Drug	24 h		72 h		72 h+48 h washout	
	CTX	HPC	CTX	HPC	CTX	HPC
1 mM VPA	−4.2 ± 3.3	−9.7 ± 2.4*	−4.9 ± 1.8*	−5.2 ± 0.6*	−0.6 ± 2.8	−6.7 ± 2.4
10 mM VPA	−7.5 ± 1.3*	−4.0 ± 2.7	−17.2 ± 2.0*	−5.0 ± 1.4*	−2.6 ± 4.1	−6.0 ± 3.1
100 μM LTG	n.d.	n.d.	+0.5 ± 3.0	n.d.	n.d.	n.d.
1 μM AMI	−7.4 ± 4.2*	n.d.	−9.7 ± 4.0*	n.d.	−4.4 ± 1.3	n.d.
10 μM AMI	−4.3 ± 2.8	n.d.	−9.2 ± 2.0*	n.d.	−6.5 ± 3.5	n.d.
0.2 μM TSA	−7.1 ± 3.7*	−9.6 ± 1.0*	−11.6 ± 5.0*	−10.4 ± 2.3*	n.d.	n.d.

Abbreviation: n.d., not determined.

Primary astrocytes were treated with different concentrations of VPA, LTG, AMI, and TSA as specified in the table. Levels of DNA methylation were measured after 24 and 72 h, as well as after the washout period. For all experiments, genomic DNA was isolated from the cells and 2 μg was subjected to luminometric methylation analysis (LUMA). The results are expressed as mean ± SE of the percentage of the change in CCpGG DNA methylation compared with untreated cells. The experiments were performed in two to four independent trials, each with six technical replicates; *t*-test: $^{*}p < 0.05$ *vs* untreated cells.

Amitriptylin Induces Genome-Wide CCpGG Hypomethylation Independent of HDAC Inhibition

We further examined whether drugs that do not possess HDAC inhibitory activities might also alter the degree of DNA methylation. For that purpose, we chose AMI from the group of antidepressants, and LTG as a mood stabilizer, and treated cortical astrocytes with 100 μM LTG for 72 h and with 1 or 10 μM AMI (for 24 and 72 h). Surprisingly, AMI (1 μM) exposure led to a significant decrease of methylation at CCpGG sites after 24 and 72 h ($-7.4 \pm 4.2\%$ and $-9.7 \pm 4.0\%$, respectively) (Table 2). A higher dose was also effective after 72 h of the treatment ($-9.2 \pm 2.0\%$). In contrast, LTG exposure did not produce changes of DNA methylation. Thus, AMI induced significant DNA hypomethylation (Table 2) in the absence of global histone hyperacetylation (Figure 1), which was partially reversible after drug withdrawal.

Total Cytosine Methylation is not Altered by Drug Exposure

Of all CpG sites in the genome, CCpGG tetranucleotides represent only a minor part of approximately 7–8%, where 35.25% of these sites are located in transposable elements, 64% in unique sequences, from which 14% are in CpG islands, as calculated for the mouse genome (Fazzari and Greally, 2004). As LUMA exclusively detects CCpGG sites, we additionally performed capillary electrophoresis analysis covering the methylation status of all CpG sites. The equivalent LUMA samples of (1) the time course of high dose VPA and (2) the 72 h time point of high dose AMI were subjected to capillary electrophoresis analysis. Strikingly, differences in total cytosine methylation levels between control and treated astrocytes could not be detected by capillary electrophoresis (Supplementary Table 1). Possibly, smaller changes in cytosine methylation that might not be evenly distributed over the whole genome, but rather located at certain CCpGG-rich DNA regions, could be better resolved by LUMA than by the capillary electrophoresis technique.

Finally, genomic DNA derived from cortical astrocytes treated with VPA (10 mM), TSA (0.2 μM), AMI (10 μM), and LTG (100 μM) for 72 h showed no differences in the average methylation levels of total LINE-1 CpGs sites compared with control cells (Supplementary Figure S3a-c). Together with the results obtained from capillary electrophoresis and LUMA analysis, we propose that VPA, TSA, and AMI do not cause ample DNA hypomethylation randomly at any CpG site of the genome, but rather at distinct regions that are likely enriched in CCpGG tetranucleotides and prone to DNA demethylating events.

Modulation of the Epigenetic Signature of an Astrocyte-Specific Promoter by VPA and TSA

On the basis of our observations that VPA triggers global histone hyperacetylation and DNA hypomethylation events, and that AMI, to a certain extent, influences the genomic DNA methylation status as well, we speculated whether genes crucial for the physiology of astrocytes are among the affected loci. As a paradigm, we chose the glutamate transporter subtype, GLT-1 (EAAT2, human homolog), the gene product ensuring low resting glutamate concentrations in the synaptic cleft (Choi, 1988; Rothstein *et al*, 1996). Previously, we collected data of an epigenetic component of human *EAAT2* regulation (Zschocke *et al*, 2007), and there is increasing evidence that a disturbed glutamatergic neurotransmission in psychiatric disorders might also be affected by pharmacological interventions (Hashimoto *et al*, 2007; Javitt, 2004).

Hence, we determined the methylation status of the rat *GLT-1* gene covering a substantial part of the 5′-UTR and two parts of distinct promoter regions by bisulfite sequencing. The 5′-UTR of *GLT-1* is composed of a classical CpG island (Zschocke *et al*, 2005). Sequencing of approximately two-thirds of the CpG island (Figure 3a) showed that all inspected CpG dinucleotides were unmethylated in untreated cortical astrocytes. Furthermore, a region outside the CpG island located ~900 bp upstream of the ATG start codon was also completely unmethylated (data not shown). Nevertheless, a region ~1950 bp relative to the ATG start codon that contains putative binding sites for two chromatin-associated transcriptional regulators, proofed to be differentially methylated before and after drug exposure. Three CpG sites at positions −1978, −1958, and −1929 were methylated to $34 \pm 1\%$, $33 \pm 1\%$, and $36 \pm 2\%$, respectively, in untreated cells, as measured by direct bisulfite sequencing (data not shown). A 72 h treatment with 10 mM VPA led to a reduction of methylation at all three sites ($-18.3 \pm 7.8\%$, $-16.8 \pm 0.3\%$, and $-27.7 \pm 4.6\%$, respectively) (Figure 3b). Similarly, TSA noticeably decreased cytosine methylation by −27.7 to −32.5%. In contrast to VPA/TSA, AMI (10 μM, 72 h) did not induce significant changes at any of the examined CpG dinucleotides. Similarly, LTG (100 μM, 72 h) did not change the methylation at positions −1978 and −1958, although a small increase in cytosine methylation was detected at position −1929 ($+9.2 \pm 1.8\%$). Our findings show that global CCpGG DNA hypomethylation induced by VPA, TSA, and AMI have differential impacts at the single gene level.

To further characterize the epigenetic signature at the *GLT-1* promoter after drug exposure, we investigated the composition of acetylated histone H4 at the differentially methylated promoter stretch by chromatin immunoprecipitation (ChIP). Indeed, we observed a 3.5-fold enrichment of acetylated H4 at the *GLT-1* promoter in VPA (10 mM, 72 h) treated cortical astrocytes, as compared with control cells (Figure 3c).

HDAC-Is Increase GLT-1 Gene Transcription in Cortical Astrocytes

As VPA and TSA treatment resulted in an altered epigenetic composition of the *GLT-1* promoter that potentially leads to a more relaxed chromatin structure, we tested whether these compounds are able to enhance *GLT-1* mRNA transcription. In untreated cortical astrocytes, *GLT-1* mRNA was detected at fairly moderate levels as determined by real-time PCR. After stimulation with VPA (low millimolar range) or TSA (low nanomolar range) for 24 h, we observed a dose dependent ~4-fold increase of *GLT-1* mRNA, respectively (Figure 4a and b). Moreover, neither AMI nor LTG influenced *GLT-1* mRNA expression

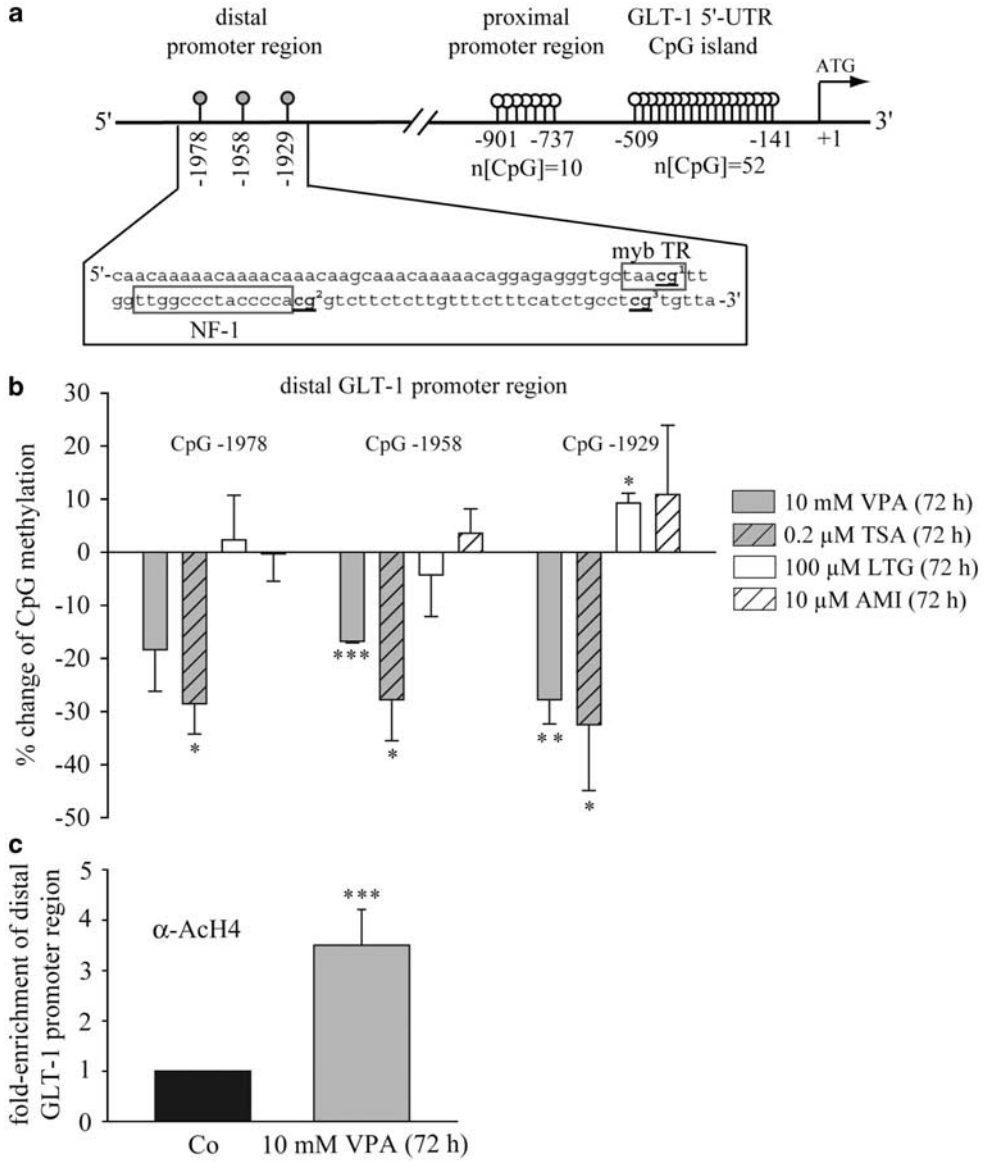

Figure 3 Epigenetic modification of the GLT-1 promoter of rat cortical astrocytes by VPA and TSA treatment. (a) The scheme depicts the 5′-UTR of GLT-1 and adjacent promoter region. Empty circles represent unmethylated CpG regions, whereas filled circles represent moderately methylated individual CpGs (~34%), as measured in untreated cortical astrocytes. The genomic sequence of GLT-1 distal promoter region is shown and putative binding sites for myb-like transcriptional regulator (myb TR) and for nuclear factor-1 (NF-1) are depicted. (b) CpG methylation changes were monitored at individual CpG sites (−1978, −1958, and −1929) after drug exposure. Cortical astrocytes were treated for 72 h with 10 mM VPA, 0.2 μM TSA, 10 μM AMI, and 100 μM LTG. After treatment, genomic DNA was isolated, bisulfite-converted, PCR amplified, and submitted to direct sequencing. Bars represent percentage change of CpG methylation (mean ± SE, $n = 3$) compared with untreated cells; *t*-test: $^{*}p < 0.05$ *vs* control, $^{**}p < 0.005$ *vs* control, $^{***}p < 0.0005$ *vs* control. (c) Cortical astrocytes were exposed to 10 mM VPA for 72 h, genomic DNA was isolated, fragmented and immunoprecipitated using an anti-AcH4 antibody or IgG control. Precipitated DNA was subjected to real-time PCR analysis with a GLT-1 promoter-specific set of primers. AcH4 enrichment is depicted as fold-increase ± SE ($n = 2$) compared with untreated cells (set to 1). The results are normalized to input; *t*-test: $^{***}p < 0.0005$ *vs* control.

(data not shown). A reporter gene assay with the human *GLT-1* promoter ortholog coupled to a luciferase gene confirmed the activation of the promoter by VPA and TSA, but not by LTG or AMI treatment (Figure 4c). This alludes to a role of transacting factors that might be triggered by VPA/TSA, as chromatin modifications at the reporter plasmids might be less relevant. We further tested whether the HDAC-I NaB also exerts an effect on *GLT-1* expression in cortical astrocytes. Application of 1 mM NaB increased *GLT-1* mRNA transcript levels after 72 h, but not at 24 h of treatment as determined by real-time PCR analysis (Figure 4d). Compared with VPA and TSA, this regulatory effect was weaker with regard to the kinetics of gene activation. In conclusion, CpG demethylation and histone hyperacetylation of a distinct region of the *GLT-1* promoter induced by VPA or TSA was associated with an increased transcriptional activity of the gene. Conversely, AMI and LTG, failing to modify epigenetic marks at the promoter, did not enhance the basal transcription of *GLT-1*.

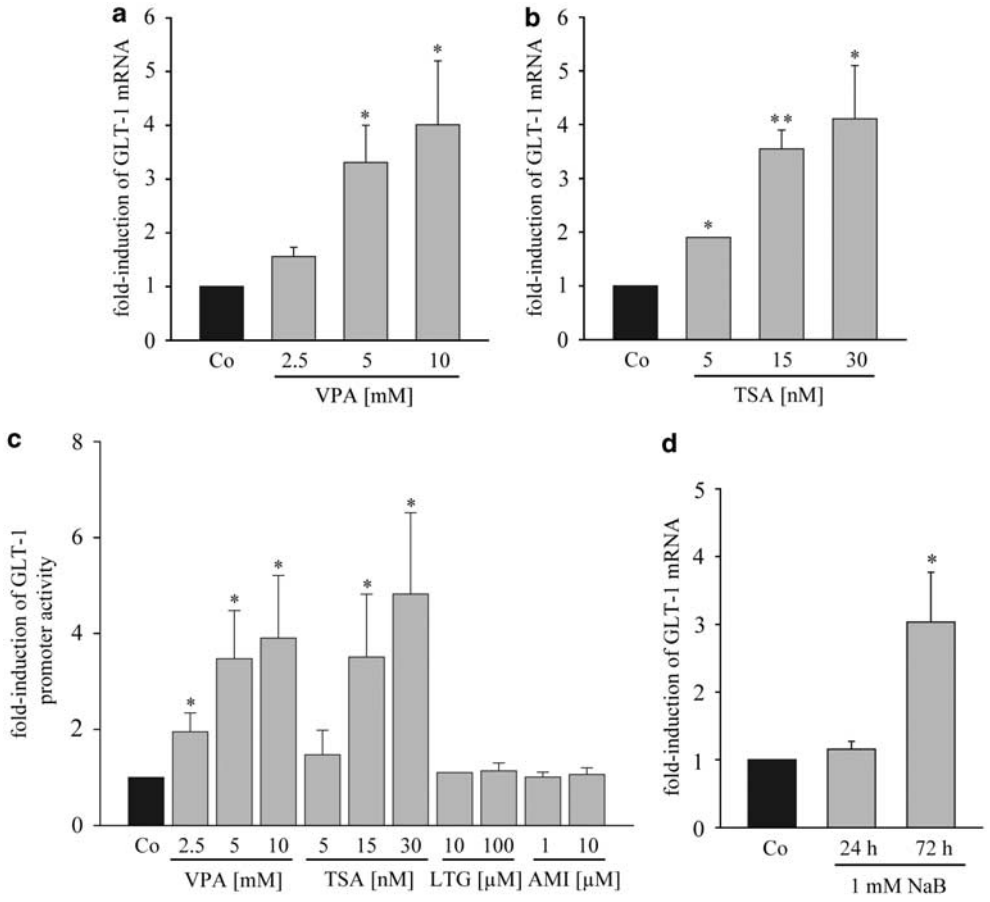

Figure 4 HDAC inhibitors VPA and TSA increase *GLT-1* gene transcription in a dose-dependent manner. *GLT-1* transcript levels were quantified by real-time PCR after 24 h treatment with 2.5, 5, and 10 mM VPA (a) and with 5, 15, or 30 nM TSA (b) and 24 and 72 h after 1 mM NaB treatment (d). Real-time PCR analysis was carried out with total RNA derived from treated and untreated (Co) cortical astrocytes and with *GLT-1*- and actin-specific primers. (control is set to 1; bars depict mean ± SE ($n = 3$) of the fold-increase; *t*-test: $^*p < 0.05$ *vs* control, $^{**}p < 0.005$ *vs* control.) (c) Reporter gene assays were conducted to directly assess promoter activity. Cortical primary astrocytes were transiently transfected with reporter plasmid containing a homolog of the human *GLT-1* promoter coupled to a luciferase gene. The cells were cotransfected with nonsecretory Gaussia luciferase expression vector for normalization. The luciferase activity was assayed 24 h after applying drugs at the indicated concentrations. Bars present mean ± SE ($n = 3$–5) of the fold-increase compared with untreated cells (set to 1); *t*-test: $^*p < 0.05$ *vs* control.

Cellular DNMT-1 and GADD 45α Protein Levels are Unaffected by VPA/TSA and AMI

The molecular mechanisms of DNA demethylation are still unclear. We focused our further analysis on two key factors that are responsible for propagation, maintenance, or *de novo* establishment of DNA methylation patterns, namely DNMT-1 and GADD 45α (growth arrest and DNA damage-inducible protein 45α). In cortical astroglial cells, treatment with 1 and 10 mM VPA or with 200 nM TSA for 24 and 72 h, did not significantly decrease the levels of DNMT-1 protein under either condition (Figure 5a). In addition, AMI did not influence DNMT-1 levels under the specified treatment regiment (Figure 5b). Recently, it has been proposed that GADD 45α might have an active role in demethylating DNA (Barreto *et al*, 2007). As shown in Figure 5a and b, VPA/TSA and AMI did not robustly alter GADD 45α expression levels, although TSA slightly reduced protein levels after prolonged exposure. Our data imply that global changes in DNA methylation are not correlated with major differences in DNMT-1 or GADD 45α protein levels after VPA/TSA and AMI administration.

DNMT Activity is Differentially Affected by VPA and AMI

To test the possibility that DNMT activity is directly modified by VPA or AMI, we performed an *in vitro* methylation assay using poly dIdC DNA as template and tritium-labeled *S*-adenosyl-methionine (^{3}H-SAM) as substrate. Nuclear extracts of VPA (10 mM, 72 h)- and AMI (1, 10 μM; 72 h)-treated cortical astrocytes were subjected to methylation reaction, and the amount of incorporated ^{3}H-SAM was quantified (Figure 5c). In parallel, aliquots of nuclear extracts were analyzed for DNMT-1 levels by western blot (data not shown). VPA did not reduce the amount of incorporated SAM, nor were nuclear DNMT protein levels significantly reduced in the nuclear extracts, indicating that VPA does not impair DNMT function. Conversely, 10 μM AMI led to a dramatic reduction in DNMT enzymatic activity of ~55% (Figure 5c), concomitantly with unchanged DNMT-1 levels (data not shown). Application of the specific DNMT inhibitor 5-aza-deoxycytidine (8 μM; 3–11 days) reduced the activity of DNMT by approximately 50–90%, which proofs the reliability of the

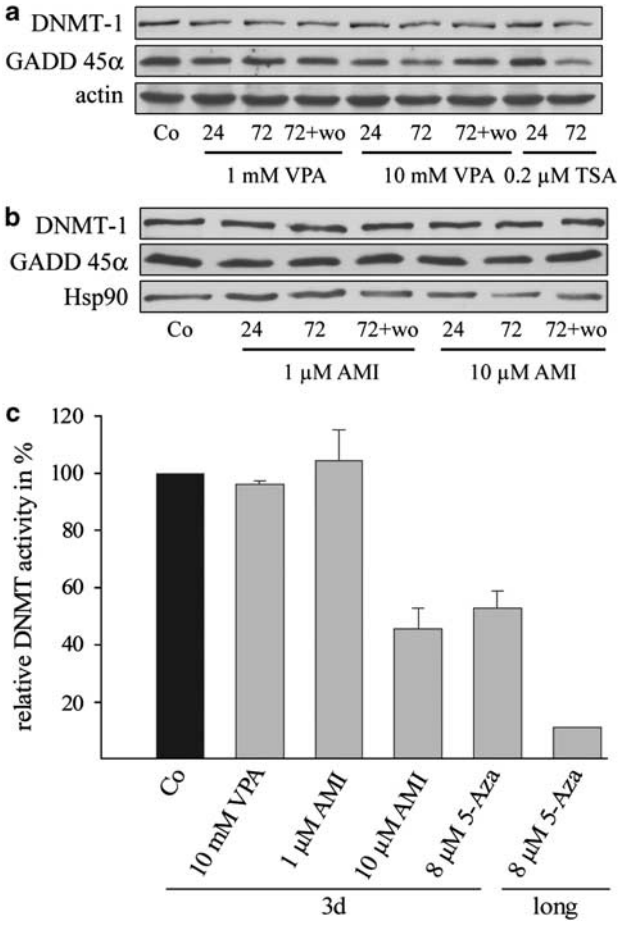

Figure 5 AMI, but not VPA, reduces DNA methyltransferase activity of cortical astrocytes independent of DNMT-1 downregulation. (a, b) Drug treatment has no impact on DNMT-1 and GADD 45α expression levels. Cortical astroglial cells were treated with VPA, AMI, and TSA ranging from 24 to 72 h, including drug washout as specified. Western blot analysis was carried out using cellular extracts prepared from cortical astrocytes, which were probed with DNMT-1-, GADD 45α-, α-actin-, or hsp90-specific antibodies. (c) Cells were treated with VPA (10 mM), AMI (1,10 μM), and 5-Aza (8 μM) for 3d or 6–11d (5-Aza, long-term). Nuclear extracts were isolated and supplemented with the respective drugs. DNA methyltransferase activity was quantified by measuring the incorporation of *S*-adenosyl-L-[methyl-3H]methionine into the substrate Poly(dI-dC) · Poly(dI-dC). Data are presented as relative DNA methyltransferase activity ± SE ($n = 2$ for VPA/5-Aza, $n = 4$ for AMI). Control is set to 100%.

assay to monitor DNMT activity changes. Altogether, our results indicate different modes of actions by VPA and AMI to reduce DNA methylation.

VPA and AMI Differentially Modulate Histone H3K9 Methylation Marks

So far, our results endorsed the idea of differing actions of VPA and AMI with regard to histone acetylation patterns and induction of GLT-1 expression. To corroborate these findings with other putative differences, we focused on further histone marks, the dimethylation of histone H3 at lysine residues 9 (2MeH3K9) and 27 (2MeH3K27). Psychoactive drugs possess the potential to influence the level of histone methylation *in vivo* (Huang *et al*, 2007; Tsankova *et al*, 2006; Wilkinson *et al*, 2009). We exposed cortical and hippocampal astrocytes to VPA (1 and 10 mM) and AMI (1 and 10 μM) for 24 h, and performed western blot analysis using 2MeH3K9- and 2MeH3K27-specific antibodies. VPA slightly, but significantly, decreased the levels of 2MeH3K9, whereas AMI showed a minor and nonsignificant increase of this repressive histone mark (Figure 6a and b). The applied drugs exerted no effect on 2MeH3K27 in both brain regions (Figure 6c and d). It is noteworthy that although the slight increase in 2MeH3K9 levels induced by NaB (applied at 0.1 and 1 mM for 24 h) was not significant compared with controls, these levels were significantly different from the reduced levels in response to the structurally related drug, VPA (Figure 6a and b). In this context, it is worth mentioning that NaB exposure did not result in global CCpGG methylation changes, although both substances VPA and NaB increased acetylation of H3 and H4 to a similar extent (data not shown). Collectively, our data support the idea that VPA and AMI affect different chromatin components as reflected at the level of 2MeH3K9 or AcH3/4. Furthermore, also structurally similar HDAC-I like VPA and NaB do not necessarily match in their epigenetic modes of action. This is in agreement with other studies showing various HDAC-I differentially affecting target genes (Hauke *et al*, 2009).

DISCUSSION

There is mounting evidence that psychiatric illnesses, major depression in particular, are associated with compromised glial function. For example, a reduced number of glial cells was reported in the prefrontal, orbitofrontal, and cingulate cortex (Cotter *et al*, 2001; Rajkowska *et al*, 1999). A number of animal studies replicated similar findings, for example, describing a 25% reduction of glial cells in the hippocampal formation of psychosocially stressed male tree shrews (Czeh *et al*, 2006). It is noteworthy that fluoxetin treatment for 28 days prevented the numerical decrease in astrocytes of the animals. Hence, abnormal astrocytic function might add to the pathogenesis and progression of mood disorders by deteriorating neuroplasticity and related processes. It has become increasingly evident, that beside neurons being the primary targets of antidepressant/mood stabilizer action, astrocytes also respond to these treatments (Manev *et al*, 2003; Pavone and Cardile, 2003).

In this study, we evaluated the effects of CIT, AMI, VEN, as well as VPA, LTG, and CBZ on various aspects of astroglial epigenetic parameters. For mechanistic insight, we also applied broad-spectrum HDAC-I, including TSA and NaB. With regard to HDAC subtypes, TSA is generally considered to inhibit both class I and class II HDACs, whereas VPA and NaB are preferentially class I inhibitors (Guardiola and Yao, 2002; Gurvich *et al*, 2004). Of all psychoactive drugs tested, only VPA led to a pronounced and transient global hyperacetylation of histones H3 and H4, paralleled by a small and transient demethylation of genomic CCpGG tetranucleotides. These results are consistent with previous studies showing that VPA directly inhibits the catalytic center of HDAC 2 with an EC_{50} value of 0.52 mM and of HDAC 5/6 with an EC_{50} of 2.4 mM (Gottlicher *et al*, 2001). HDAC-Is such as MS-275 and TSA also diminish DNMT-1, −3a and −3b protein levels and

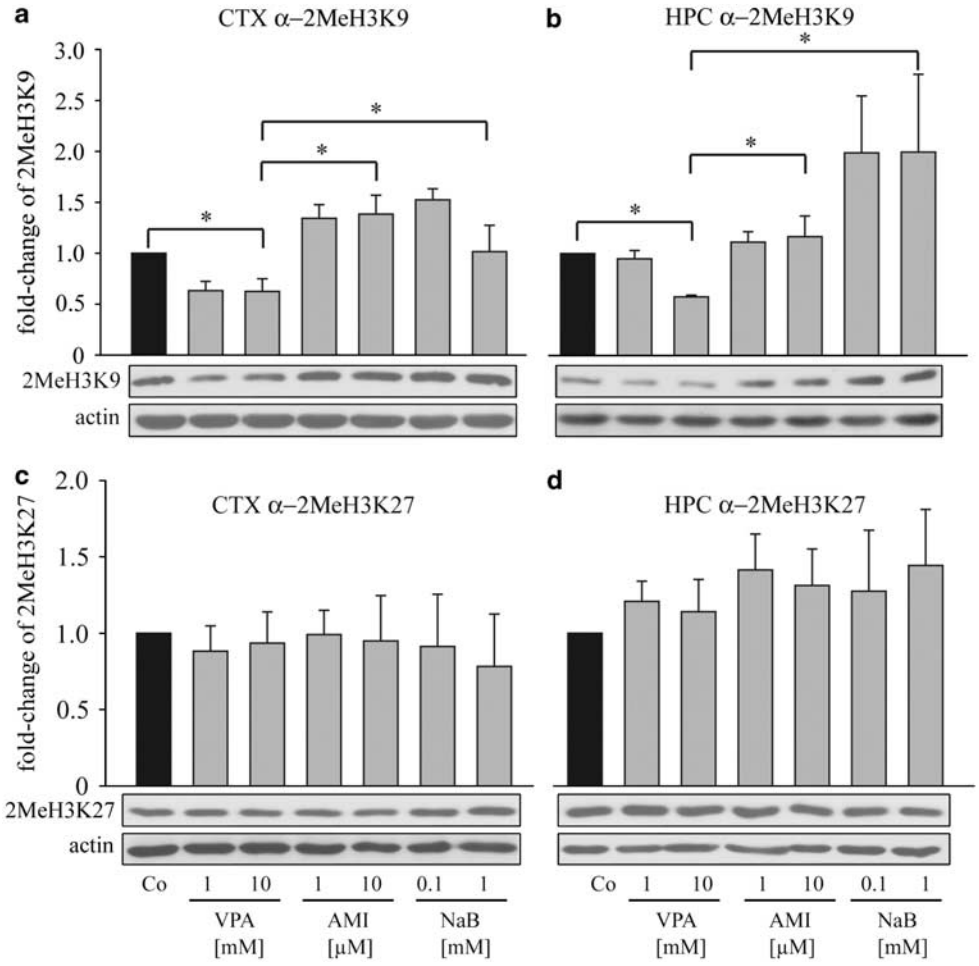

Figure 6 Differential effects of VPA and AMI on H3K9 dimethylation levels. Cortical (CTX) and hippocampal (HPC) astrocytes were treated with VPA (1 and 10 mM), AMI (1 and 10 μM) and NaB (0.1 and 1 mM) for 24 h. Western blot analysis was performed using antibodies directed against (a, b) dimethyl-H3K9 (α-2MeH3K9) or (c, d) dimethyl-H3K27 (α-2MeH3K9). As a control for protein loading, α-actin-specific antibody was used. Data represent mean ± SE ($n = 3$) of the percentage of optical band densities normalized to α-actin immunoreactivity. Control is set to 1; *t*-test: $^{*}p < 0.05$.

enzymatic activity in NT-2 precursor cells, tentatively explaining the mechanism of DNA demethylation events (Kundakovic *et al*, 2009). In primary astrocytes, we found no changes in DNMT-1 levels upon VPA administration. GADD 45α, a protein involved in cell-cycle control and DNA repair, represents a candidate as a mediator for active DNA demethylation as the overexpression of GADD 45α activates methylation-silenced reporter plasmids and promotes global DNA demethylation. VPA upregulates GADD 45α in N1E-115 neuroblastoma cells, thereby inducing growth arrest (Yamauchi *et al*, 2007), whereas in astroglial cells, GADD 45α levels were not elevated after VPA and TSA exposure in our experimental setup. Hence, we conclude that mechanisms other than DNMT-1 downregulation and GADD 45α upregulation are accountable for VPA-mediated CCpGG demethylation in quiescent cells. It is noteworthy that LINE-1 retrotransposons were spared from demethylation events, and methylation changes were not measurable at total cytosine residues, possibly due to resolution limits of capillary electrophoresis. In that context, VPA presumably does not reactivate silenced retrotransposons, which would harbor a mutagenic risk for the cell, and also does not mediate the demethylation of the gross of genomic CpG sites. We further observed small brain region-specific differences in responsiveness to DNA demethylation triggered by VPA, with generally lower magnitudes in the hippocampus.

Although it has been shown that CBZ inhibits representatives of class I and class II HDACs leading to slightly increased AcH4 levels in human HepG2 and HEK cell lines (Beutler *et al*, 2005), CBZ did not exert similar effects in primary astrocytes. Possibly, cells treated with CBZ need to actively proliferate to be more susceptible for these minor acetylation changes, as opposed to VPA. Moreover, LTG did not mediate any changes in the global histone acetylation profile in astrocytes. Similarly, acutely administered antidepressants CIT, AMI, and VEN did not show any significant influence on the global H3 and H4 acetylation status in hippocampal and cortical astrocytes. At this point in time, we cannot exclude the possibility that chronic administration of the drugs over several weeks might, nevertheless, change the degree of histone acetylation. Daily i.p. injections of fluoxetine, a substance related to CIT, over 10 days leads to an increase in HDAC2 immunoreactivity in coronal tissue sections, accompanied by reduction in global acetylated histone H3 (Cassel *et al*, 2006). Furthermore, chronic, but not acute imipramine treatment of socially

stressed animals induces hyperacetylation of H3 at distinct regions of the *BDNF* gene potentially mediated by the downregulation of HDAC5 expression.

Surprisingly, although AMI did not display any HDAC inhibitory properties, it induced DNA hypomethylation at CCpGG sites in cortical astrocytes, similarly to VPA. This observation raised the possibility that DNA hypomethylation does not depend on histone hyperacetylation. Indeed, DNMT enzymatic activity was markedly reduced in AMI-treated astroglial cells from the cortex. To our knowledge, only very few studies deal with psychoactive drug-induced changes of the global degree of DNA methylation. For instance, 3-weeks administration of haloperidol, an antipsychotic drug, to rats was documented to result in a small, but significant reduction in total methyl-cytosine content (Shimabukuro *et al*, 2009).

DNA methylation and histone acetylation are complemented by combinatorial monomethylation, dimethylation, or trimethylation of histones H3/H4 to form a complex epigenetic code-controlling gene transcription (Lachner and Jenuwein, 2002). There exists a functional interplay of enzymes establishing various histone and DNA modifications. For instance, HDAC and H3K9 HMT G9a are recruited to chromatin sites together with transcriptional coactivators (Roopra *et al*, 2004), and DNMT-1 was reported to interact with SUV39H1, a histone H3K9 methyltransferase (Fuks *et al*, 2003). Our data show that VPA exposure caused a slight but specific reduction in the levels of dimethyl-H3K9, leaving dimethyl-H3K27 unchanged. In line with our results, HDAC-I desipeptide decreases the level of H3K9 methylation and induces DNA demethylation by reducing G9A and SUV39H1 activity and DNMT-1 binding, respectively (Wu *et al*, 2008b). In contrast to VPA, AMI did not change dimethyl-H3K9 levels.

Global epigenetic alterations caused by VPA hit a selected gene promoter, the activity of which is crucial for astrocytic functionality, *GLT-1*. At glutamatergic synapses, GLTs recycle released extracellular glutamate back into astrocytes to terminate postsynaptic and presynaptic receptor activation (Schousboe and Waagepetersen, 2006; Vandenberg, 1998). Distinct parts of the *GLT-1* promoter were demethylated and enriched in acetylated histone H4 after VPA treatment, whereas the CpG island in proximity of the translational start site was completely unmethylated in treated and untreated cells. Concomitantly, the activity of the *GLT-1* gene was profoundly increased, analogously to the actions of TSA on *GLT-1* gene transcription, indicating that HDAC inhibitory properties of VPA are attributable to the documented effects. The differentially methylated promoter region contains putative consensus sequences (as predicted by MatInspector software) for the recruitment of two chromatin regulatory proteins, namely nuclear factor 1 and myb-like transcriptional regulator (Chikhirzhina *et al*, 2008; Ko *et al*, 2008). A number of epigenetically regulated genes such as *myoD* are differentially methylated at distal enhancer elements, whereas CpG islands close to transcription start sites are protected from methylation (Brunk *et al*, 1996) and, hence, are less susceptible to epigenetic alterations.

Chronic VPA exposure increases GLT-1 expression *in vivo* in the rodent hippocampus (Hassel *et al*, 2001). In addition, I-phenylpyridinium-challenged primary astrocytes, simultaneously treated with TSA, showed improvement in glutamate uptake as reported by Wu *et al* (2008a). Very recently, Allritz *et al* (2009) showed stimulatory effects of TSA on GLT-1 mRNA and protein levels in rat astrocytes of various brain regions. We complement the findings that HDAC-I likely induces *GLT-1* expression in part by chromatin changes at the promoter region, probably in concert with the induction of transacting factors. Conversely, AMI neither targeted *GLT-1* gene transcription nor the respective CpG sites within the *GLT-1* promoter.

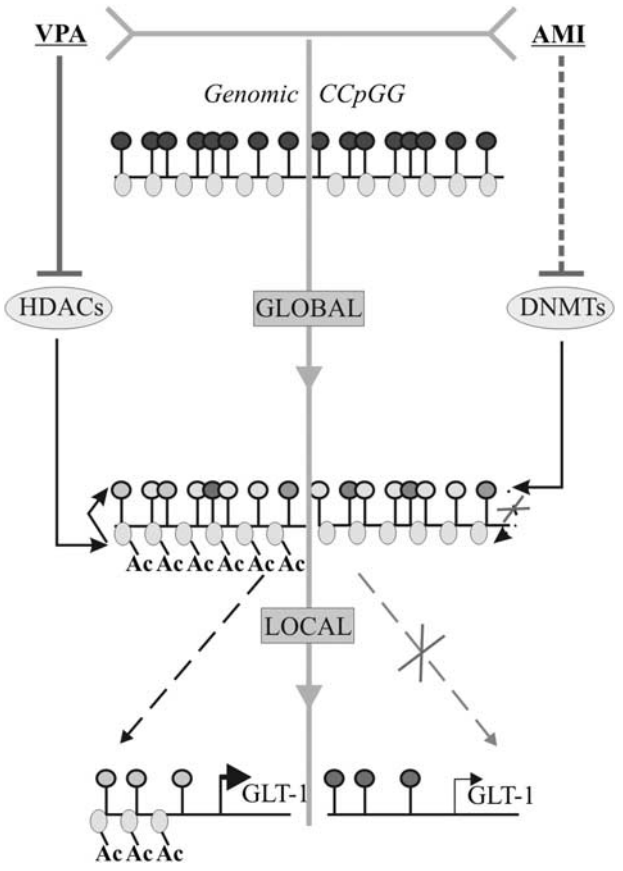

Figure 7 Proposed model for VPA- and AMI- mediated epigenetic alterations in astrocytes. VPA and AMI display an input on global DNA demethylation, although through different mechanisms. As VPA inhibits HDACs, and AMI impairs DNMT function, the measurable difference in global epigenetic marks consists of hyperacetylation of histones in the case of VPA, which are not affected by AMI. Locally, downstream pathways further diverge as exemplified for the GLT-1 gene. The biochemical composition of the promoter is markedly altered by VPA exposure by means of histone hyperacetylation and DNA demethylation, resulting in an enhanced gene transcription. AMI does not possess similar features, and subsequently leaves gene activity unaffected. Round circles represent CpG sites; shading reflects the degree of methylation; oval circles depict core histones.

Taken together, we could show for the first time that of all tested psychoactive substances, VPA exerted the strongest influence on chromatin remodeling events in astrocytes involving CpG demethylation and histone modifications, targeting *GLT-1*, an astroglial gene important for glutamate homeostasis in the CNS. Similarly, AMI also brought about changes in global DNA methylation, possibly by functional impairment of DNMTs, without targeting the *GLT-1* gene (Figure 7). The underlying molecular mechanisms and biological conditions including different neural cell types

and brain regions of AMI-affected DNMT-1 function are yet to be determined. It will be interesting to uncover the common and divergent sites affected by the global demethylating actions by these two drugs. It also remains to be seen whether other tricyclic and SNRI/SSRI drugs show similar effects on the DNA methylation machinery, and whether these effects might contribute to the recovery of disturbed neural processes.

In conclusion, a promising new approach for the pharmacological treatment of psychiatric illnesses could be based on the reversal of aberrantly established epigenetic patterns. Two principle strategies could guide to the future objective: first, pharmacological intervention directly interferes with epigenetic machinery as shown in this study for VPA and AMI, resulting in overall changes in the epigenetic pattern of chromatin. In fact, HDAC-I NaB exerts antidepressant-like effects (Schroeder *et al*, 2007) and improves cognitive performance in mice (Fischer *et al*, 2007). Apparently, this strategy holds the risk of reactivating genes in a false spatial-temporal context. The parameter ensuring that only a subset of crucial genes are reactivated might rely on the composition of transcription factors governing the transcriptome characteristic for a specific cell type, such as an astrocyte or neuron. The second strategy comprises the indirect modulation of epigenetic marks either globally or locally by interfering with neurotransmitter and signal transduction pathways, ultimately resulting in epigenetically based and permanently changed target gene expression patterns. In general, identifying novel HDAC-I- or DNMT-1-regulated genes acting in support of neural circuits might provide a starting point for the development of new pharmacological treatment designs.

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DISCLOSURE

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)

Supplementary Materials and Methods

Flow cytometry - Cell cycle analysis

Flow cytometry was performed with VPA-treated cortical astrocytes. The cells were fixed with PBS/0.2% formaldehyde on ice for 30 min, washed with PBS and resuspended in 100 µL 100% (v/v) ice-cold ethanol. After incubation on ice and subsequent centrifugation, RNAse (Sigma) in PBS was added and incubated for 30 min at 37 ºC. Finally, staining with propidium iodide (50 µg/mL in PBS; Sigma) was conducted. The stained cells were immediately subjected to cell cycle analysis using a Beckman Coulter flow cytometer.

Capillary electrophoretic analysis of DNA methylation level

Global DNA methylation levels were determined by capillary electrophoresis, as described (Stach et al., 2003). Briefly, 5 µg of genomic DNA was enzymatically hydrolyzed to single nucleotides by using an enzyme mixture consisting of micrococcal nuclease (150 mU/µl; Sigma) and calf spleen phosphodiesterase (2.5 mU/µl; Calbiochem-Novabiochem, Darmstadt, Germany). Subsequently, single nucleotides were labeled with the fluorescent marker BODIPY (Molecular Probes, Eugene, OR, USA). Labeled nucleotides were separated and analyzed in a Beckman P/ACE MDQ Molecular Characterization System.

Supplementary Results

Fig. S1

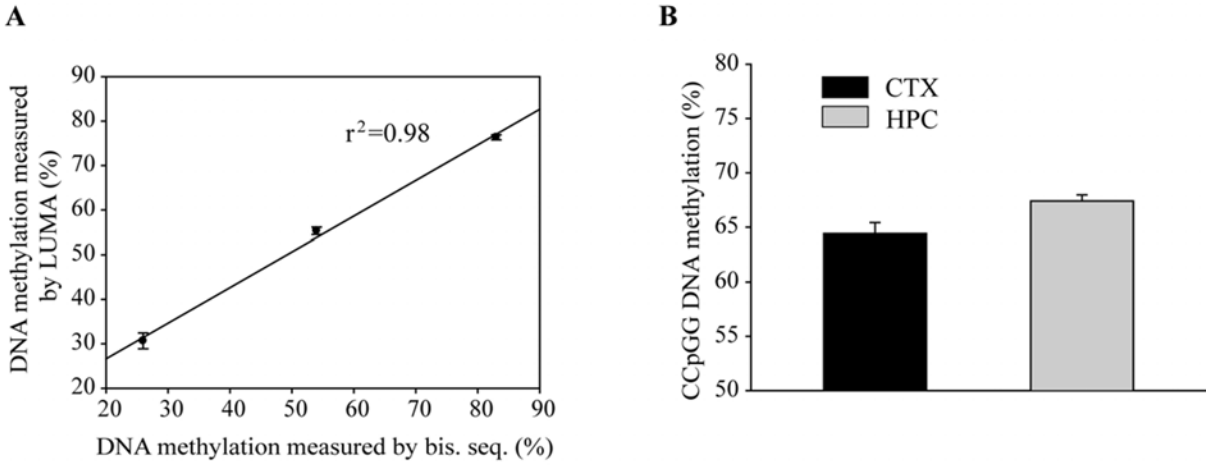

Fig. S1. Level of CCpGG DNA methylation in untreated astrocytes. (A) Validation of Luminometric Methylation Analysis (LUMA) and direct bisulfite sequencing techniques with respect to CpG methylation. M.SssI methylated and fully unmethylated Lambda control DNA were mixed to obtain 25% , 50% and 75% methylated Lambda DNA that was subsequently subjected to both methods. Applying direct bisulfite sequencing, the degree of CpG methylation of a randomly selected, 248 bp CpG-rich sequence within lambda DNA was determined. The sequence of the bisulfite-specific primer pair is listed in Table 1. For LUMA, lambda DNA was cleaved with two combinations of restriction enzymes, either *Hpa* II + *Eco* RI or *Msp* I + *Eco* RI, and digested DNA samples were assayed on a pyro-sequencer. The data points present mean values ± S.E. of three independent LUMA measurements against a single measurement of lambda DNA methylation performed by direct sequencing. A curve constructed to fit the data points revealed a Pearson's correlation coefficient of 0.98. **(B)** LUMA-determined level of CCpGG DNA methylation in untreated cortical and hippocampal rat primary astrocytes.

Fig. S2

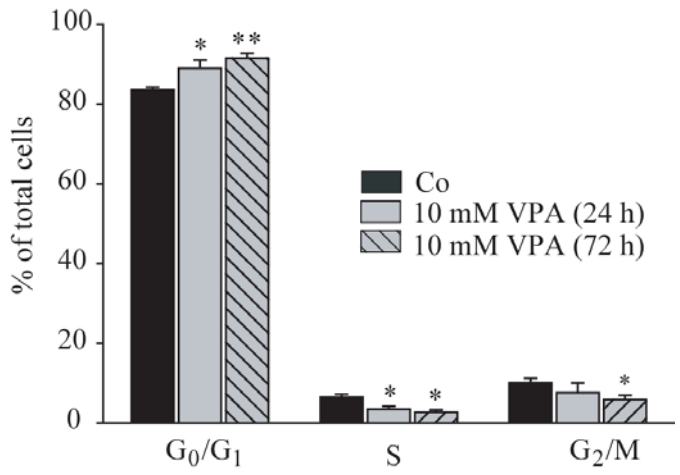

Fig. S2. Distribution of cortical astrocytes in different cell cycle stages. Astrocytes from the cortex were treated with 10 mM VPA for 24 h and 72 h. The cells were harvested, fixed and then stained with PI. The relative abundance of cells at different stages of the cell cycle is shown for untreated and VPA-treated cells. Bars present the mean ± S.E. (n=3); t-test: * $p<0.05$ vs. control, ** $p<0.005$ vs. control. Twenty-four hours after treatment with 10 mM VPA, the percentage of cells in G_1/G_0-phase was slightly, but significantly increased (89 ± 2% of the total cells), compared to untreated cells (83 ± 1% of total cells). At the same time, the percentage of cells in S- and G_2/M-phase decreased (3 ± 1% and 8 ± 2% of total cells, respectively) compared to control cells (6 ± 1% and 10 ± 1% of the total cells, respectively).

Fig. S3

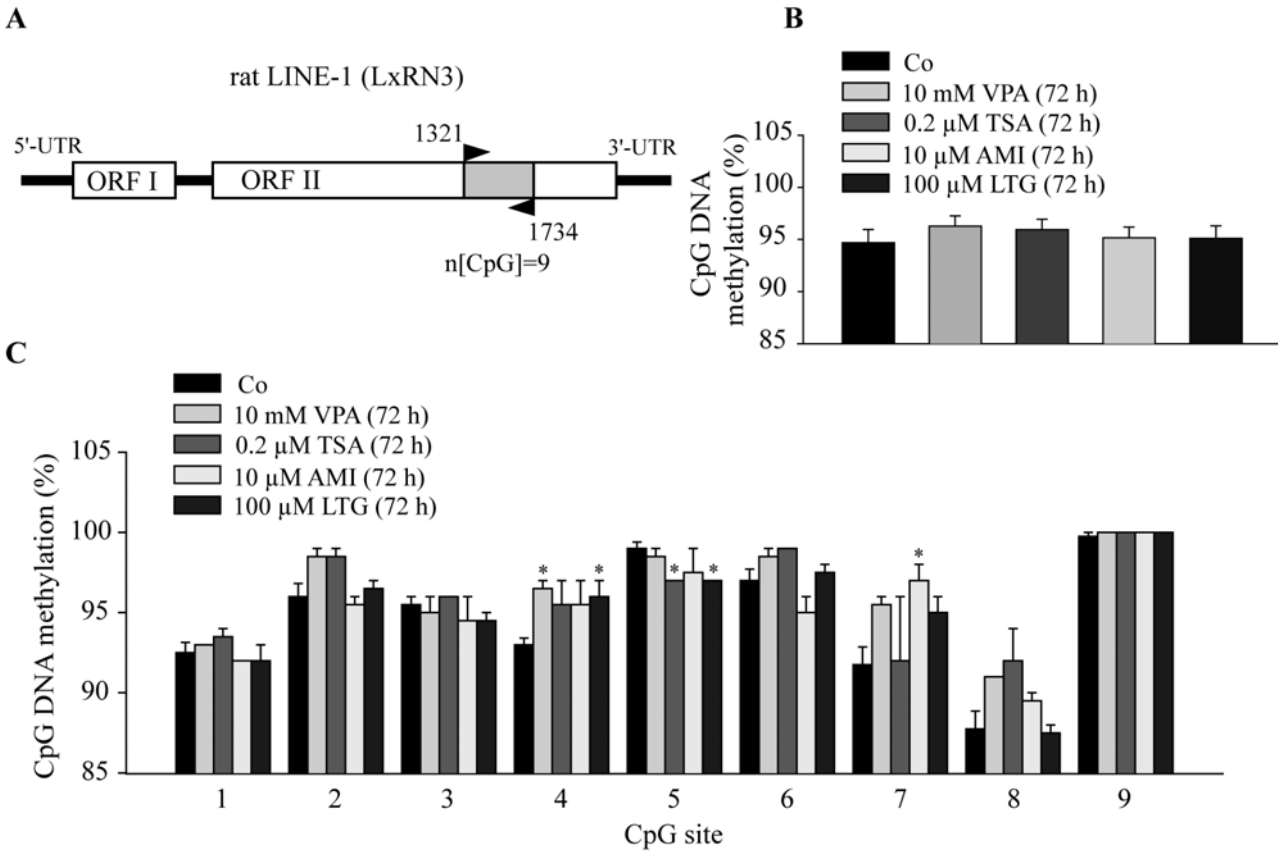

Fig. S3. Methylation of repetitive LINE-1 sequences in cortical astrocytes is not affected by drug treatment. (A) Schematic presentation of rat LxRN3 LINE-1 sequence (GeneBank accession number M60824). Primers were designed for direct bisulfite sequencing of a 413 bp region in the ORF II of LINE-1 containing 9 CpG sites. The annealing sites of primers are indicated by arrow heads. Cells were treated with VPA (10 mM), TSA (0.2 µM), AMI (10 µM) or LTG (100 µM) and harvested 72 h later. Isolated genomic DNA was bisulfite-converted, PCR amplified and submitted to direct sequencing (Qiagen). **(B)** The graph represents methylation levels of all CpGs of the analyzed nucleotide stretch within rat LINE-1 repetitive sequence. Bars present the mean value ± S.E. of the average CpG methylation level of all nine analyzed CpGs from two to three independent experiments. **(C)** Representation of the percentage of CpG methylation at each individual CpG site before and after drug treatment. Each bar represents the mean value ± S.E. (n=2-3) of the methylation degree at a given CpG site; t-test: *$p<0.05$ vs. control. More detailed analysis of individual CpG sites unveiled minor differences existing at CpG #4, CpG #5 and CpG #7 in the range of ± 3% (Fig. S2C). Of note, all the analyzed LINE-1 CpGs were not embedded in CCpGG tetranucleotides.

Supplementary Table 1

Drug / Unit	10 mM VPA				10 µM AMI	
	Co	24 h	72 h	72 h + wo	Co	72 h
5mC/5mC+C	4.1±0.1	4.3±0.1	4.4±0.2	4.0±0.4	4.2±0.2	4.5±0.2

Analysis of cytosine methylation levels in cortical rat astrocytes treated with 10 mM VPA (24 h, 72 h and 72 h followed by a 48 h washout period (wo)) and 10 µM AMI (72 h). 5 µg of genomic DNA was enzymatically hydrolysed. After hydrolysis, nucleotides were labelled with BODIPY and analysed for 5-methylcytosine (5mC) genomic content by capillary electrophoresis. The result presents mean ± S.E. of the ratio of 5-methyl cytidine to total cytidine of a single experiment done with four to five technical replicates.

The CpG Island Shore of the GLT-1 Gene Acts as a Methylation-Sensitive Enhancer

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KEY WORDS
EAAT2; epigenetics; histones; astrocytes; DNA methylation

ABSTRACT
Astrocytic lineage commitment and brain region-dependent specialization of glia are partly ascribed to epigenetic processes. Clearance of glutamate is an essential task, which astrocytes assume in a temporal-spatial fashion by distinct glutamate transporter expression. Glutamate transporter subtype 1 (GLT-1) is predominant in cortex (CTX), while it plays an inferior role in cerebellum (CER). Here, we set out to identify regulatory elements that could account for the differences in brain region-specific activity as well as response to dexamethasone (DEX) or epigenetic factors. We found a distal promoter element at the shore of the CpG island exhibiting enhancer function in response to DEX in reporter gene assays. This shore region showed slight enrichment in repressive trimethyl-histone H3 (Lys27) and underrepresentation of acetyl-histone H4 (H4ac) marks in DEX nonresponsive CER astrocytes as determined by chromatin immunoprecipitation. In addition, CpG sites of the shore region displayed higher methylation in CER than in CTX cells. Targeted *in vitro* methylation of CpG sites within the shore abrogated the stimulatory effects of DEX. Interestingly, the shore was characterized by a pronounced epigenetic plasticity in CTX cells since DEX exposure elicited an increase of H4ac in CTX in comparison to DEX nonresponsive CER. The transcriptional activity of this region was also affected by histone deacetylase inhibitors in a methylation- and brain region-dependent manner. Together, our study highlights the impact of an epigenetically adaptive DNA element of the GLT-1 promoter being decisive for brain region-specific activity and reactivity.

INTRODUCTION

Epigenetic modifications are implicated in the regulation of mammalian brain development and function, and are important for the integration of external stimuli in gene activity (Meaney, 2010). Methylation of cytosines within CpG dinucleotides of the DNA and posttranslational modifications of histone proteins are major epigenetic marks that determine chromatin structure. DNA methylation either directly interferes with the binding of transcription factors (Campanero et al., 2000; Iguchiariga and Schaffner, 1989) or attracts methyl-CpG binding proteins, which recruit chromatin modifiers to establish a repressive chromatin structure (Jones et al., 1998; Nan et al., 1998). DNA methylation involved in gene regulation typically occurs at CpG-dense DNA stretches designated as CpG islands (Bird, 1986). Intergenic and intragenic CpG sites (Maunakea et al., 2010) as well as CpGs at island shores (Irizarry et al., 2009) also exhibit regulatory properties.

Covalent modifications of histones on the N-terminal tails and the histone fold domain influence their interaction with DNA and neighboring nucleosomes. Histone acetylation is generally associated with gene activation (Pokholok et al., 2005), whereas histone methylation affects transcription both positively and negatively depending on the number and location of attached methyl-groups (Lachner and Jenuwein, 2002). DNA methylation and histone modifications act in concert to establish sustainable epigenetic programs (D'Alessio and Szyf, 2006).

Functional specialization of brain regions goes along with the establishment of region-specific gene expression profiles (Letwin et al., 2006). Epigenetic mechanisms appear crucial in the unfolding and stabilization of these patterns. Pronounced differences between cerebellum (CER) and cortex (CTX) have been mapped to CpG sites of genes relevant for brain function (Ladd-Acosta et al., 2007). Examples of brain area-specific epigenetic signatures and corresponding gene activity include the Na^+, K^+-ATPase modulator Fxyd1, adenosine A_{2A} receptor, and glutamate receptors (Banine et al., 2011; Buira et al., 2010; Stadler et al., 2005). Glutamate transporters terminate glutamatergic signaling by clearing glutamate from the synaptic cleft. Glutamate transporter subtype 1 (in rodents: GLT-1) and glutamate aspartate transporter (in rodents: GLAST) are predominately expressed in astrocytes; however, they are also localized in cultured neurons as well as in neurons of the rat brain (Anderson and Swanson, 2000; Chen et al, 2002; Plachez et al., 2000). GLT-1 and GLAST are expressed in a brain-region specific fashion during development and in the adult (Furuta et al., 1997). GLT-1 is present early in fetal brain and spinal cord, and its expression progressively increases leaving the CER spared until postnatal day 10. The expression of GLAST is low prenatally, becomes activated in CER early postnatally and later on also in the forebrain. In the adult rat brain, the expression of glutamate transporters is partitioned between different regions,

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with GLT-1 being dominant in forebrain, brainstem, spinal cord, and GLAST in the CER. Accordingly, both genes respond differently to stimulatory cues depending on the spatial-temporal context. For instance, stress hormones up-regulate GLT-1 expression in primary CTX but not in CER astrocytes (Zschocke et al., 2005). Previous studies provided first indications for an epigenetic control of GLT-1 transcription in human glioma cells and rat astrocytes (Allritz et al., 2009; Perisic et al., 2010; Yang et al., 2010; Zschocke et al., 2007).

Here, we addressed the question whether epigenetic events form the basis for the brain region-specific expression and responsiveness of the GLT-1 gene in rat primary astrocytes from CTX and CER. We aimed at functionally characterizing promoter elements that show brain region-specific differences in epigenetic signatures and confer DNA methylation-dependent response to the synthetic stress hormone dexamethasone (DEX).

MATERIALS AND METHODS

Primary Astrocytic Cell Cultures

Enriched astroglial cultures were prepared from postnatal day 1 rat pups (Sprague-Dawley, Charles River, Germany) as described (Franke et al., 1998). After the third passage, cells were maintained in serum-free (MEM/Ham's F-12, 1:1) N2-supplemented medium and treated with DEX, valproic acid (VPA) or trichostatin A (TSA), all from Sigma.

RT and Real-Time PCR

Preparation of total RNA and real-time PCR analysis were as described (Perisic et al., 2010). For detection of GLT-1 and β-actin in case of semi-quantitative measurements, cDNA was amplified with P1 and P2 primers, respectively (Table 1). Generation of PCR products by quantitative real-time PCR was performed using P2 and P3 primers (Table 1). Here, relative levels of gene expression were calculated using the formula $2^{-}(Ct_{GLT\text{-}1} - Ct_{Actin})$ according to the comparative $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001).

Methylation Analysis by Bisulfite Genomic Sequencing

Extraction of genomic DNA from primary astrocytes was as described (Perisic et al., 2010). Bisulfite treatment of genomic or plasmid DNA was performed using the EpiTech Bisulfite Kit (Qiagen) according to the manufacturer's instructions. Primer pair P4 was used for amplification of the CpG island of the GLT-1 gene and primers P5 and P6 for amplification of the shore GLT-1 promoter region (Table 1). In addition to direct sequencing, PCR products were also subcloned using the pGEM-T Easy Vector System (Promega, Mannheim, Germany), followed by Sanger sequencing.

Construction of reporter plasmids

A 1.8-kb genomic fragment containing the rat GLT-1 gene promoter sequence (from −2,106 to −253, relative to TSS; GenBank: EF017228) was amplified using P7 primers (Table 1) and inserted into the CpG-free luciferase-reporter pCpGL-basic vector (Klug and Rehli, 2006), kind gift from Michael Rehli, Department of Haematology and Oncology, University Hospital, Regensburg, Germany. By using pCpGL-basic vector as well as P8 oligonucleotides (which contain canonical TATA-box element), a pTATA-Luciferase vector was obtained (Table 1). Furthermore, P9 primers (Table 1) were used to amplify the shore region of the GLT-1 gene promoter which was inserted upstream of the TATA-box to generate the pTATA-GLT1−1867/−1515 vector. The pCpGL-CMV/EF1α (pCMV/EF1α) vector (kind gift from Michael Rehli), which harbors a CpG-free constitutive human elongation factor 1α (EF1α) promoter driven by a TATA-box, was used for construction of pEF1α-GLT1−1867/−1515 (employing P10 primers, Table 1). The identity of all clones was verified by sequencing.

In Vitro DNA Methylation of Reporter Plasmids

The respective vector (25–30 μg) was subjected to *in vitro* methylation at 37°C overnight using 10 units of CpG SssI DNA methyltransferase in a reaction supplemented with 650 μM *S*-adenosyl methionin (SAM). All reagents were from NEB. The extent of DNA methylation was evaluated employing HpaII restriction analysis or genomic bisulfite sequencing showing close to 100% efficiency. Mock controls were without SssI DNA methyltransferase or SAM in the reaction mixture.

Sequence-Specific Methylation ("Patch Methylation")

PCR-amplified GLT-1 promoter shore region was either methylated or mock-methylated as described above. The fragments were ligated into the reporter construct and gel-purified. Efficiency of the methylation reaction was confirmed by combined bisulfite restriction analysis (Xiong and Laird, 1997). The methylation status of a single CpG site, as an indicator of the methylation of the whole sequence, was inspected in at least 20 independent subclones. The percentage of DNA methylation was between 95 and 100%.

Transient Transfection and Luciferase Reporter Assay

Primary astrocytes (2×10^6) were transiently transfected with 2 μg of reporter plasmids using Amaxa's Nucleofector device (Lonza) according to the manufacturer's protocol. The SV40 promoter-driven nonsecretory Gaussia luciferase expression vector of 15 ng (Schülke

TABLE 1. Primer Sequences Used for Semiquantitative PCR (sqPCR), Quantitative Real-Time PCR (qPCR), Bisulfite Sequencing (BS), Cloning Procedures, and Chromatin Immunoprecipitation (ChIP)

Primer	Application	Sequences	T_A (°C)
P1	sqPCR	fw 5′-GGACACCATTGACTCCCAAC-3′ re 5′-CCTCAACACTGCAGTCAGC-3′	58
P2	sqPCR, qPCR	fw 5′-CTACAATGAGCTGCGTGTGGC-3′ re 5′-CAGGTCCAGACGCAGGATGGC-3′	60
P3	qPCR	fw 5′-CCGAGCTGGACACCATTG A-3′ re 5′-CGGACTGCGTCTTGGTCAT-3′	60
P4	BS	fw 5′-GGGGTTAAATTTTGTAATTTTT-3′ re 5′-CCCCTCCTAAATAAACCCTT-3′	52
P5	BS	fw 5′-GGAGTTTGATAGTATAGTTTTTGAAT-3′ re 5′-AACAAACTCTACTAAAAAACTTACAAA-3′	58
P6	BS	fw 5′-ATTGGTAGGTAAATAAAAAATAAATTT-3′ re 5′-ATTCAAAAACTATACTATCAAACTCC-3′	56
P7	Cloning	fw 5′-CGACTAGTTAGGAGCTGCCAAGAGGGTGC-3′ re 5′-ATATGCATAAGCTTCGTACCACGAGTGGCCGTC-3′	68
P8	Cloning	5′-ACGACTAGTGACTCTAGAGGGTATATAAAGCTTAAG-3′ 5′-CTTAAGCTTTATATACCCTCTAGAGTCACTAGTCGT-3′	/
P9	Cloning	fw 5′-ACGACTAGTCGTTGGTTGGCCCTACC-3′ re 5′-CTTAAGCTTTATATACCCTCTAGAGTCCGGGTTCCTCTGAGCAG-3′	62
P10	Cloning	fw 5′-ACGCTGCAGCGTTGGTTGGCCCTACC-3′ re 5′-CTTACTAGTCGGGTTCCTCTGAGCAG-3′	68
P11	ChIP	fw 5′-GGGCTAAACCTTGCAATCC-3′ re 5′-GCAGACCCGTACCACGAG-3′	60
P12	ChIP	fw 5′-CAAAACCAAAAACCAAACCAAACC-3′ re 5′-AGCCAAGAAACACGAGGCAG-3′	60

Experimentally determined optimal annealing temperatures (T_A) of the respective primer pairs are provided.

et al., 2010) served as control for transfection efficiency. Posttransfection for 24 h, the medium was changed to N2 supplement-containing medium, and the pharmacological treatment started. Cells were assayed 48 h posttransfection. Luciferase assays were as described (Wochnik et al., 2005). Luminescent readouts were obtained with a TriStar LB941 Luminometer (Berthold Technologies, Bad Wildbad, Germany). In Figs. 4 and 5B, normalized reporter activity was expressed as fold-induction relative to the activity in untreated cells. In Figs. 5A and 6A,B, the reporter activity is depicted in arbitrary units of Gaussia-normalized Firefly luciferase to avoid misleading data presentation due to differential activities of reporter constructs in untreated cells.

Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) analysis was as described (Perisic et al., 2010). Antibodies were antiacetyl-histone H4 (H4ac) (06-866, Millipore; recognizes H4 acetylation at lysines K5, K8, K12, and K16) and anti-H3 antibodies (kind gift from Prof. T. Jenuwein, Max Planck Institute for Immunology and Epigenetic, Freiburg, Germany): anti-dimethyl-histone H3 (Lys9) (H3K9me2), anti-trimethyl-histone H3 (Lys9) (H3K9me3), and anti-trimethyl-histone H3 (Lys27) (H3K27me3). Relative DNA enrichment was quantified by real-time PCR using P11 and P12 primers for amplification of the CpG island and the shore region of the GLT-1 promoter, respectively. ChIP-qPCR data are initially calculated as percent input. Percent input was obtained using the formula $100 \times 2\text{^}(Ct_{\text{Adjusted input}} - Ct_{\text{Enriched}})$. Adjusted input is the Ct value corrected for differences in the amount of input and amount of DNA added into the reaction. The fraction that bound nonspecifically to protein G Dynabeads was subtracted. Data are presented as percent input (Fig. 2A,B) or fold-enrichment of immunoprecipitated DNA relative to control values (control set to 1; Fig. 2C–E) to minimize variations in immunoprecipitation efficiency between the experiments.

Statistical analysis

Determination of statistical significance of differences between two data sets was performed using two-tailed Student's *t*-test. We also applied three-factor analysis of variance with repeated measure design for the analysis of data obtained in patch methylation experiments.

RESULTS

Induction of GLT-1 Expression by DEX and HDAC-I Differs Between CTX and CER Astrocytes

To study the responsiveness of the GLT-1 promoter to stress hormones and epigenetic factors, we used the synthetic stress hormone DEX as well as the histone deacetylase inhibitors (HDAC-I) VPA and TSA as established inducers of GLT-1 (Allritz et al., 2009; Hassel et al., 2001; Perisic et al., 2010; Zschocke et al., 2005). Under basal conditions, the mRNA levels of the GLT-1 gene did not differ between CTX and CER astrocytes (Fig. 1A). The amount of GLT-1 mRNA of untreated astrocytes was relatively low and did not translate into detectable levels of GLT-1 protein (Western blotting, not shown). Consistent with our previous observations (Zschocke et al., 2005), 100 nM DEX induced a marked time-dependent increase of GLT-1 mRNA levels in primary CTX

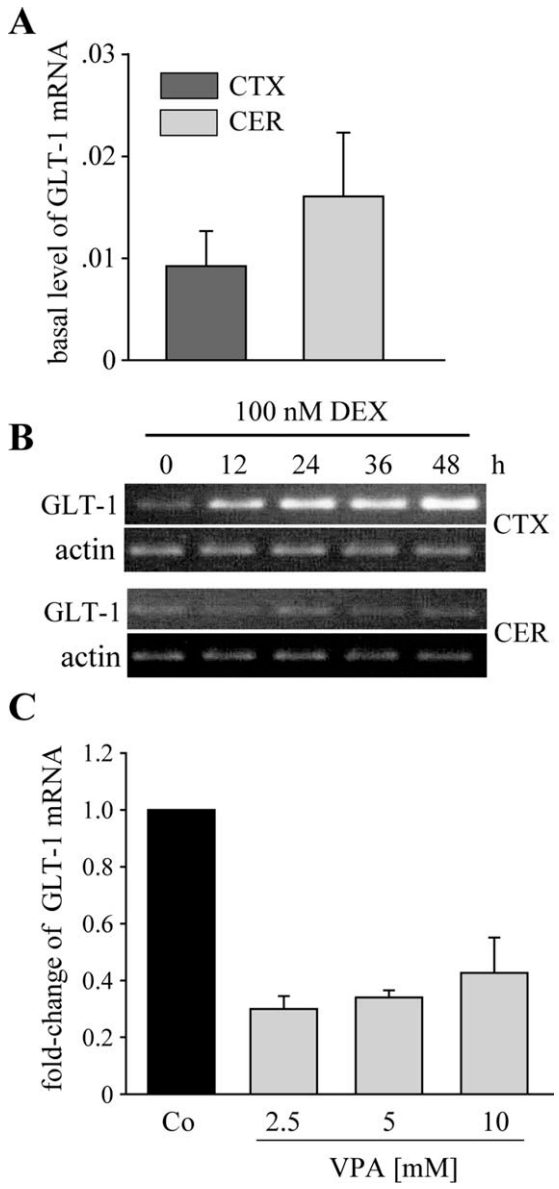

Fig. 1. Expression of the GLT-1 gene under basal conditions and after DEX or HDAC-I treatment in rat primary astrocytes. **A**: Comparison of basal levels of GLT-1 mRNA in untreated CTX and CER astrocytes by quantitative real-time PCR. The GLT-1 transcript level was normalized to that of β-actin. Relative levels of GLT-1 mRNA are depicted as mean + SE ($n = 3$). **B**: Representative agarose gels show PCR products of GLT-1 from amplified cDNA derived from CTX and CER astrocytes after treatment with 100 nM DEX at the time points indicated. **C**: Levels of GLT-1 mRNA in VPA-treated CER astrocytes were quantified by real-time PCR. Control (Co) is set to 1 and bars depict mean + SE ($n = 3$) of the fold-change of mRNA transcripts relative to untreated cells.

astrocytes, but not in CER astrocytes (Fig. 1B). Surprisingly, VPA reduced GLT-1 mRNA levels in CER astrocytes after 24 h of treatment (Fig. 1C), contrasting the stimulatory effect in the CTX (Perisic et al., 2010). Treatment of CER astrocytes with TSA (3–250 nM) exerted a similar repressive effect on GLT-1 expression (not shown).

Brain Region-Specific Abundance of H4ac and H3K27me3 at the GLT-1 Promoter Before and After Treatment with DEX or HDAC-I

Based on previous findings suggesting epigenetic regulation of the GLT-1/EAAT2 gene (Allritz et al., 2009; Perisic et al., 2010; Yang et al., 2010; Zschocke et al., 2005, 2007), we screened for epigenetic factors causing the differential DEX/VPA responsiveness in astrocytes from CTX and CER. First, we employed ChIP to decipher the composition of the following histone marks at the 5′-flanking region of the GLT-1 gene: acetylated histone H4 (H4ac) as well as methylated histones H3K9me2, H3K9me3, and H3K27me3. We investigated the CpG island as well as the shore region. CTX astrocytes accumulated more H4ac at both the shore and island region than CER astrocytes (Fig. 2A). In contrast, the level of H3K27me3 was higher in CER than in CTX astrocytes at the GLT-1 promoter (Fig. 2B). The histone marks H3K9me2 and H3K9me3 could not be detected at these regions (not shown). This was not due to antibody inefficiency, because anti-H3K9me2 and anti-H3K9me3 antibodies captured substantially higher amounts of DNA at the muscle creatinine kinase enhancer element (Tao et al., 2011) as compared with the control without antibody (not shown).

Since DEX and VPA differentially regulate GLT-1 gene transcription in CTX and CER astrocytes, we tested whether this is also reflected by concomitant alterations in H4ac association with the investigated DNA regions. Exposure to 100 nM DEX induced an increase of the level of H4ac at the CpG island as well as at the shore region in CTX (Fig. 2C), but not in CER cells (Fig. 2D). Previously, we already demonstrated a similar effect of VPA at this particular region in CTX cells (Perisic et al., 2010). Intriguingly, in CER astrocytes, 5 mM VPA increased the amount of H4ac at both investigated promoter regions after 24 h (Fig. 2E), despite its repressive effect on gene transcription.

CpG Island Shore Methylation at the GLT-1 Promoter is Higher in CER Than in CTX Astrocytes

To test whether DNA methylation profiles correlate with brain region-specific regulation of the GLT-1 gene, we subjected the CpG island and the shore region of the GLT-1 promoter to bisulfite sequencing (Fig. 3A). All inspected CpGs of the island (from −32 to −398) were unmethylated in both brain regions (not shown). In contrast, the CpGs at the shore region (positions −1,867, −1,847, −1,660, −1,643, −1,626, −1,570, and −1,516) were significantly more methylated in CER astrocytes (Fig. 3B). Treatment of CTX and CER astrocytes with 100 nM DEX or treatment of CER astrocytes with 5 mM VPA for 72 h did not evoke changes of CpG shore methylation (not shown). The level of methylation could not be determined for the CpG dinucleotides at positions −1,750 and −1,714 due to technical difficulties in the sequencing process.

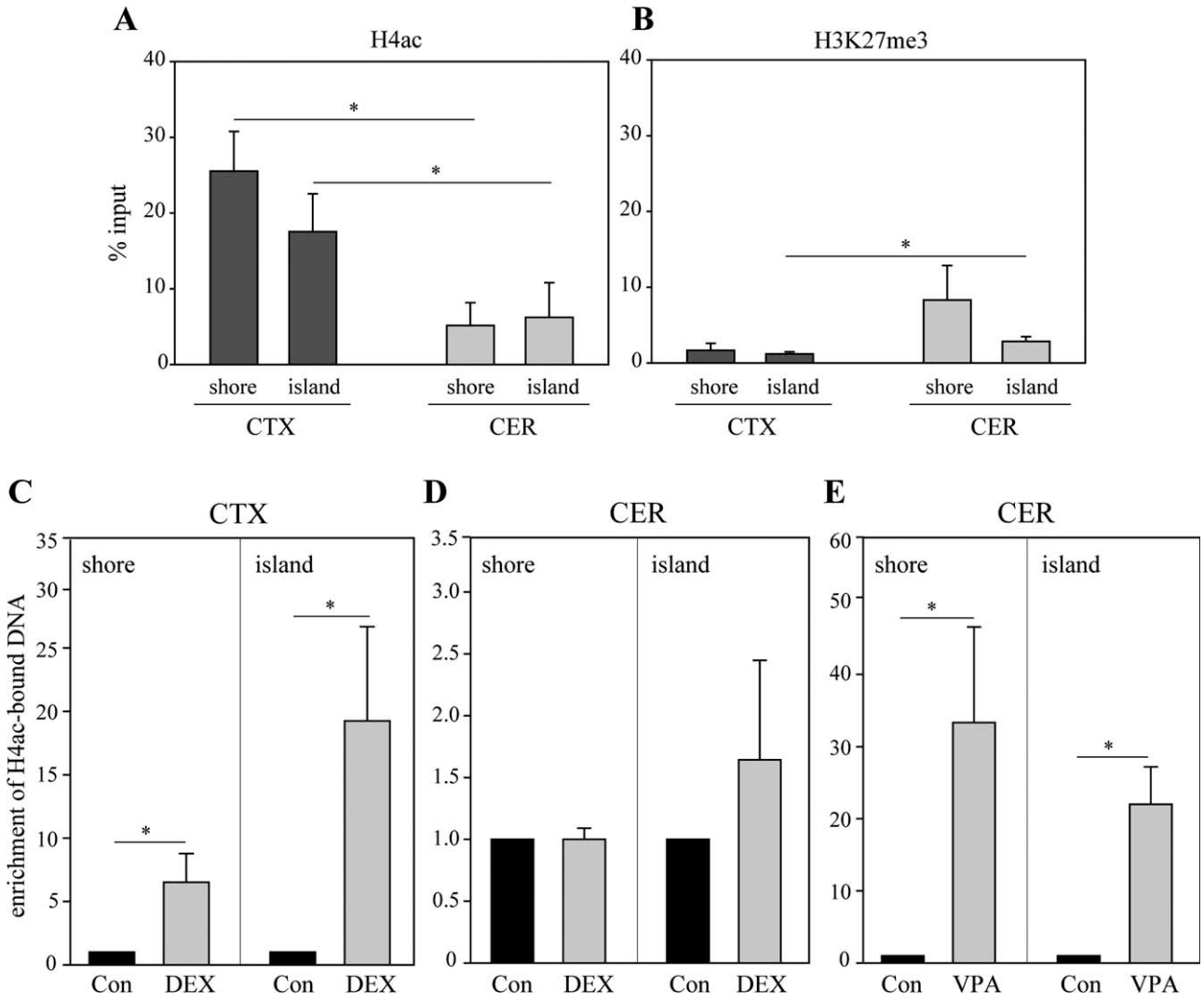

Fig. 2. Active and repressive histone marks at the promoter region of the GLT-1 gene in astrocytes. Comparison of H4ac (**A**) and H3K27me3 (**B**) levels at the CpG island and the CpG island shore region of the GLT-1 promoter in untreated CTX and CER astrocytes by ChIP analysis (see Fig. 3A for positions of analyzed regions). The occupancy of the H4ac histone mark at the CpG island and shore region was also determined in CTX cells (Co) before and after exposure to 100 nM DEX (48 h) (**C**), as well as in CER after treatment either with 100 nM DEX (72 h) (**D**) or with 5 mM VPA (24 h) (**E**). Data are presented as mean + SE ($n = 3 - 5$, $^*P < 0.05$) of the input percentage or of enrichment of the DNA bound to histones relative to control (set to 1).

Ectopic GLT-1 Promoter is Active in Both CTX and CER Astrocytes

In addition to chromatin modifications, brain region specific composition of transcription factors might also contribute to differential gene activity in CTX and CER. To evaluate whether CER cells contain the necessary transacting factors for GLT-1 transcription, we tested the transcriptional activity of an ectopic promoter sequence (pGLT1−2106/−253) in CTX and CER astrocytes applying luciferase reporter gene assays. In agreement with equal basal transcription of GLT-1, the basal activity of the reporter gene did not differ between untreated CTX and CER astrocytes (not shown). After exposure to DEX or VPA for 24 h, a concentration-dependent increase in luciferase activity was detected in astrocytes from both brain regions (Fig. 4A,B). In addition, we observed a stimulatory effect of TSA on ectopic GLT-1 promoter activity in both CTX and CER astrocytes (not shown). The results of these experiments demonstrate that CER astrocytes also provide transacting factors required for induction of the GLT-1 promoter upon DEX treatment, suggesting that the endogenous chromatin structure is critical for the differential promoter responsiveness between CTX and CER.

DNA Methylation Reduces GLT-1 Promoter Responsiveness

We next used reporter gene assays to elucidate whether promoter methylation is involved in GLT-1 gene regulation. We methylated either all CpGs of the full-length GLT-1 promoter or only the CpGs at the shore (positions −1,867,

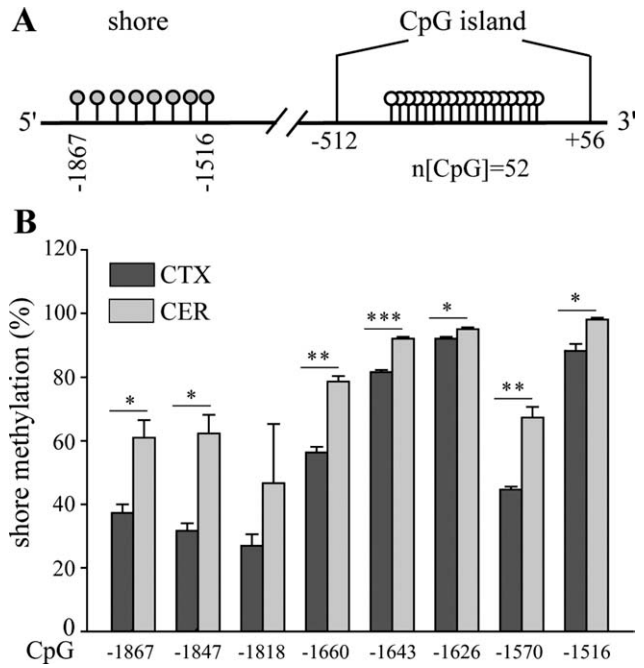

Fig. 3. DNA methylation levels at the GLT-1 promoter in CTX and CER astrocytes. **A**: The scheme depicts positions (relative to the transcription start site) and the methylation status of analyzed CpGs at the GLT-1 promoter. Open circles depict unmethylated, filled circles methylated cytosines. **B**: DNA methylation levels of the CpGs at the shore region in CTX and CER astrocytes were determined by direct sequencing of the PCR products, which were obtained from bisulfite-converted DNA. Positions of cytosines at the shore region are also indicated and given relative to the transcription start site. Data present mean + SE ($n = 3$; $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$) of the percentage of CpG methylation at individual cytosine residues.

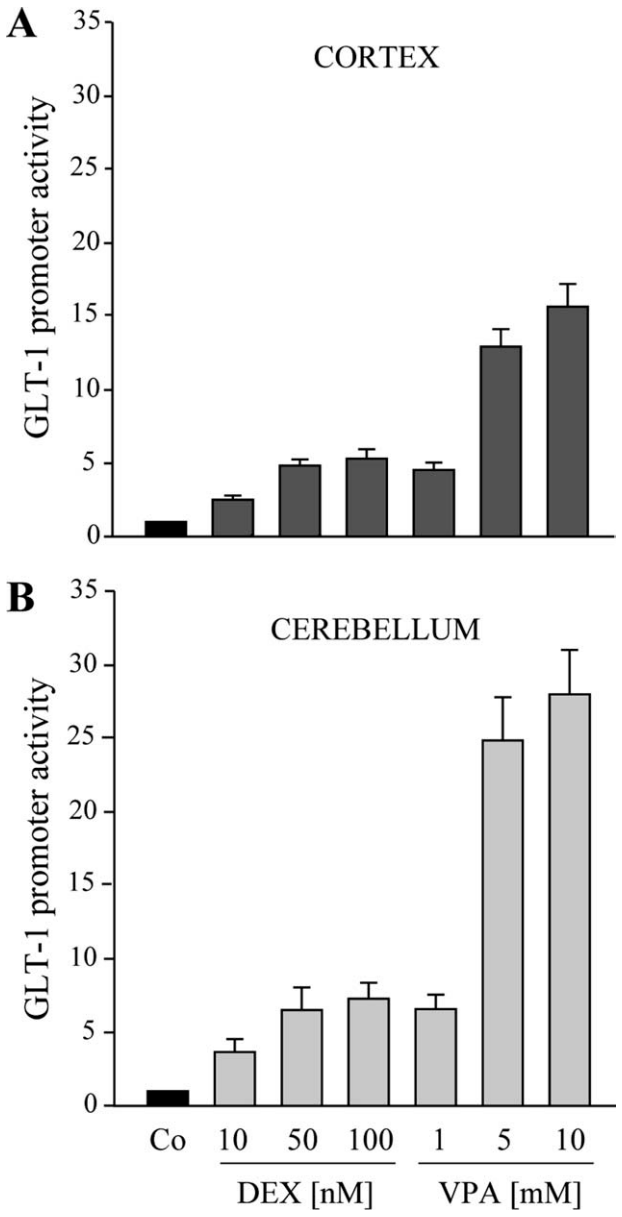

Fig. 4. Activity of the ectopic GLT-1 promoter in primary astrocytes. CTX (**A**) and CER (**B**) astrocytes were transiently transfected with equimolar amounts of CpG—free luciferase reporter plasmid containing a 1.8 kb—long sequence of the rat GLT-1 promoter (pGLT1–2106/–253). Luciferase activity was assayed 24 h after treatment with the indicated concentrations of DEX or VPA. Data are presented as mean + SE ($n = 9$ for CTX; $n = 3$ for CER) of the reporter activity in relation to the activity in untreated cells.

−1,847, −1,818, −1,750, −1,714, and −1660). Mock-methylated and methylated GLT-1 promoter-driven reporter plasmids were transiently transfected into CTX astrocytes and cells were treated with increasing concentrations of DEX or VPA for 24 h. Methylation of the full-length pGLT1−2106/−253 promoter abolished the reporter activity in both untreated and pharmacologically challenged astrocytes (Fig. 5A). Selective methylation of the shore region of the GLT-1 promoter did not change the reporter activity in untreated cells (not shown) but significantly reduced the stimulatory effects of DEX or VPA (Fig. 5B). The repressive effect of DNA methylation on the activity of both GLT-1 promoter-luciferase constructs was also observed in astrocytes treated with TSA (not shown). These results point to a methylation-dependent function of the shore region.

The CpG Island Shore Region of the GLT-1 Promoter Exhibits DNA Methylation-Dependent Enhancer Function

The concept of an epigenetically controlled enhancer function of the CpG island shore was further substantiated by the *in silico* identification of several transcription factors that putatively bind to this promoter region in a methylation-dependent manner. Some of them have been reported to participate in DEX-mediated response including interferon regulatory factor-1, E-twenty-six factors, and cAMP response element binding protein (CREB) (Espinas et al., 1994; Franchimont et al., 2000; van der Laan et al., 2009). In order to test methylation-dependent enhancer function experimentally, we cloned the shore region of the GLT-1 promoter upstream of either a TATA-box element or a constitutively active EF1α element in a CpG-free luciferase reporter vector. Like the TATA promoter, the EF1α promoter is CpG-free

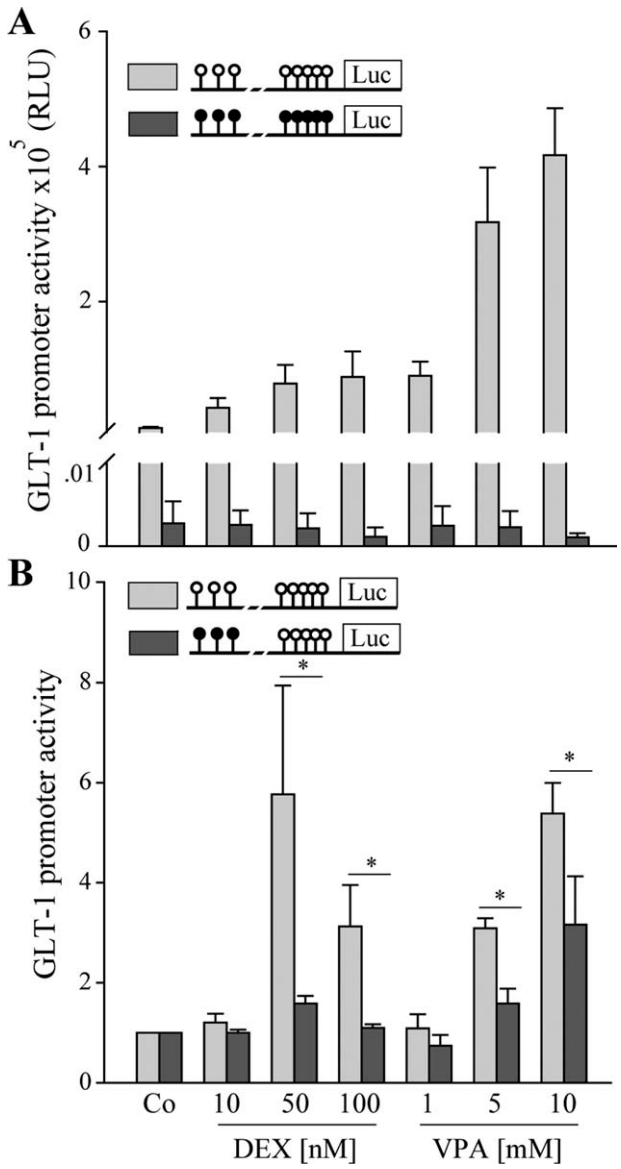

Fig. 5. CpG methylation influences GLT-1 promoter activity in reporter gene assays. **A**: Primary CTX astrocytes were transiently transfected with *in vitro* methylated or mock methylated full length pGLT1–2106/–253. Luciferase activity was determined 24 h after pharmacological treatment for both constructs. Data are presented as mean + SE ($n = 3$) of the GLT-1 promoter activity expressed in relative light units (RLU). **B**: pGLT1–2106/–253 was methylated only at the distal shore region (−1867/−1638) and the activity was compared with its mock methylated analog in transiently transfected CTX astrocytes. Measurement of luciferase activity was done following the treatment with indicated concentrations of DEX or VPA (24 h). Data analysis was performed using four measurements of each condition and GLT-1 promoter activity is presented relative to untreated cells ($^*P < 0.05$).

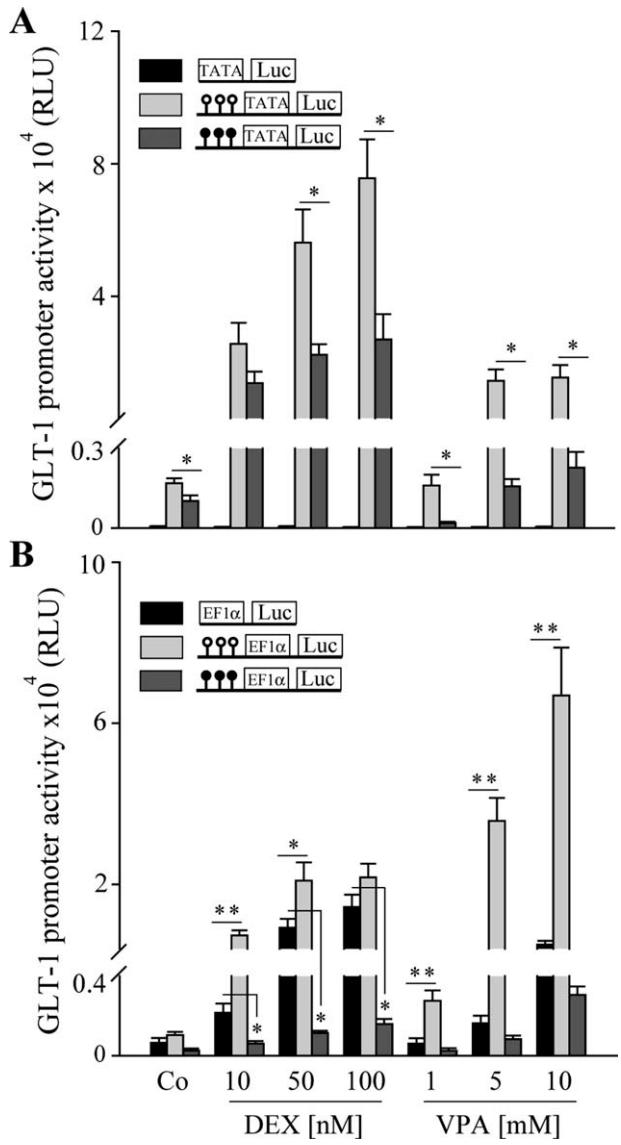

Fig. 6. The shore region of the GLT-1 promoter acts as methylation-dependent enhancer. Plasmids used in gene reporter assays contain either a short sequence with a TATA-box (**A**) or a constitutively active EF1α promoter (**B**). The shore region of the GLT-1 promoter (−1867/−1515) was cloned upstream of these sequences. CTX astroglial cells were transiently transfected with equimolar amounts of plasmids. Luciferase activity was determined 24 h after pharmacological treatment (DEX or VPA, with indicated concentrations) for both *in vitro* methylated and mock-methylated constructs. Data are presented as mean + SE ($n = 4 - 6$; $^*P < 0.05$; $^{**}P < 0.005$) of the GLT-1 promoter activity expressed in relative light units (RLU).

allowing selective methylation of the shore region of the GLT-1 promoter. As shown in Fig. 6A, the pTATA-Luciferase vector displayed neither basal nor pharmacologically induced activity. Addition of the shore region of the GLT-1 promoter upstream of the TATA-box element significantly increased luciferase activity in untreated and in particular in DEX or VPA exposed cells. Upon methylation of the CpG dinucleotides at the shore sequence, a reduction of the drug-induced luciferase activity was observed. In contrast to the pTATA-Luciferase vector, the activity of the pEF1α-luciferase vector was increased by DEX or VPA also in the absence of the shore region (Fig. 6B). Insertion of the GLT-1 promoter shore sequence further potentiated the transcription of the pEF1α vector upon DEX or VPA exposure for 24 h. After methylation, reporter activity of the pEF1α-GLT1–1867/–1515 was greatly reduced. Even more, methylation appears to commute the shore region to a suppressive mode of action under conditions of DEX but not

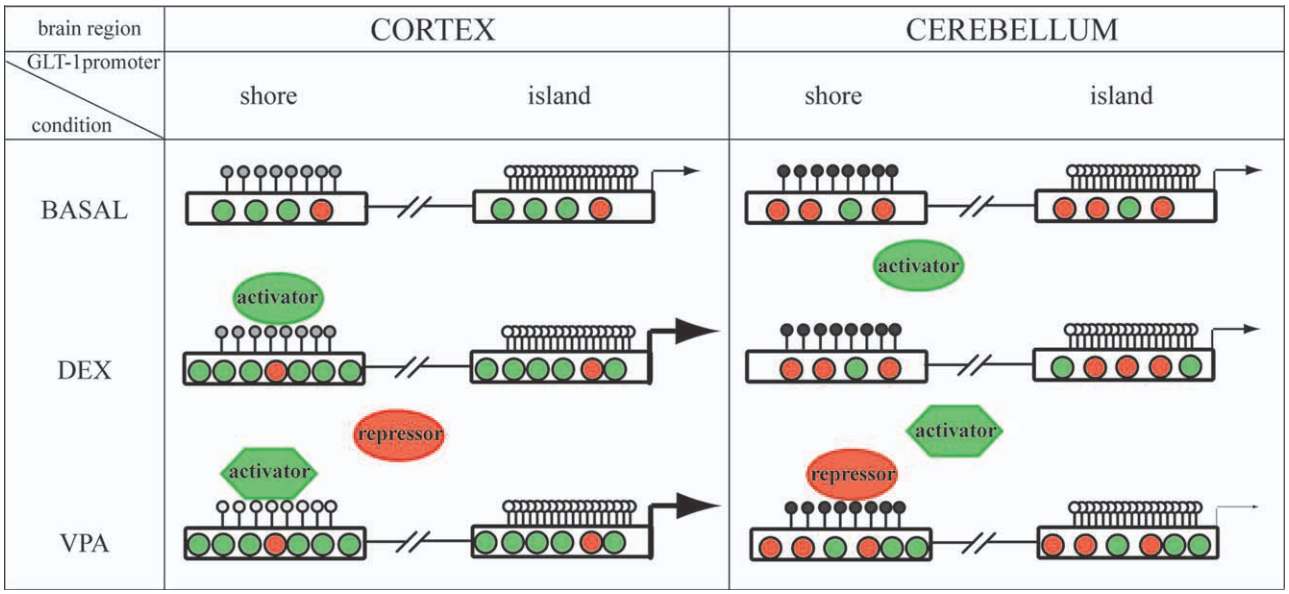

Fig. 7. Model for brain-region specific expression and responsiveness of the GLT-1 gene. Chromatin modifications at the shore and CpG island of the GLT-1 promoter are depicted comparing CTX and CER astrocytes. The degree of shading of the lollipops corresponds to the intensity of CpG methylation at the respective sites. Arrows illustrate transcription start while thickness of arrow heads indicates the level of transcription. The extent of enrichment of activatory acetylated-H4 (green circles) and inhibitory tri-methylated H3 (red circles) is denoted. Under basal conditions, the unmethylated CpG island allows leaky GLT-1 transcription in astrocytes from both brain regions, despite differences in histone marks at this region. Upon DEX and HDAC-I exposure, the permissive chromatin structure at the island shore enables binding of putative transactivator(s) and up-regulation of GLT-1 transcription in CTX astrocytes. This goes along with reshaping the epigenetic set-up of the promoter, that is, introduction of acetylated H4 as well as CpG demethylation in case of HDAC-I. In contrast, repressive chromatin marks or the underrepresentation of activatory marks in CER glia prevent binding of DEX-induced trans-activator(s) or even support binding of repressor(s) that mediate HDAC-I-initiated down-regulation of GLT-1 expression.

VPA exposure. In addition, TSA showed similar effects to those of VPA (not shown). This set of experiments suggests that the shore region of the GLT-1 promoter contains crucial methylation-sensitive cis-active elements with enhancer function.

DISCUSSION

During mammalian nervous system development, epigenetic modifications of the chromatin play a pivotal role in the acquisition of the astroglial cell fate (Freeman, 2010). The molecular switch from neural progenitor cells to astrocytes is accompanied by a marked reduction of DNA methylation at numerous astrocyte-specific gene loci, including glial fibrillary acidic protein (GFAP) and S100β (Namihira et al., 2004; Takizawa et al., 2001). In addition to DNA methylation, histone modifications at the GFAP promoter are subject to developmental changes (Song and Ghosh, 2004). Epigenetic events not only determine basal promoter activity, but also prepare glial cells for future events by setting the responsiveness to regulatory inputs. In addition to astrocytic lineage commitment, epigenetic factors are thought to define the spatial functional specialization of differentiated astroglial cells. Such epigenetic, brain region-specific differences presumably exert an impact on the transcriptome and the molecular response to regulatory factors as shown here for the GLT-1 gene. While CER glia fail to up-regulate GLT-1 transcription, CTX astrocytes showed a strong response to DEX [Fig. 1; (Zschocke et al., 2005)]. In this study, we provide evidence that the epigenetic signature of the GLT-1 promoter accounts for divergent responsiveness to stress hormone and HDAC-I in primary astrocytes from two brain regions, CTX and CER.

The rat GLT-1 gene harbors a canonical CpG island upstream of the annotated transcription start site (Zschocke et al., 2005). In CTX and CER astrocytes, the CpG island was completely unmethylated [Fig. 3; (Perisic et al., 2010)]. In parallel, the GLT-1 gene was transcribed at equally low basal levels in astrocytes from both regions. Most CpG island-associated promoters of housekeeping and tissue-specific genes are protected from *de novo* methylation in untransformed cells and Sp1 sites seem to play a key role in this process (Brandeis et al., 1994). The rat GLT-1 promoter also contains Sp1 sites within the CpG island (Allritz et al., 2010) which might, in concert with other cis-active and transactive elements, account for the absence of methylation in this region. Nevertheless, the presence of unmethylated CpG islands *per se* is not indicative of an active or responsive gene. Proximal CpG-dense promoters are largely free of methylation, whereas CpG-poor promoters are mostly methylated (Mohn et al., 2008). Distal promoter regions at the shore of CpG islands are also less CpG-dense, and therefore preferentially targeted by DNA methylation. Indeed, we detected DNA methylation at the shore region of the GLT-1 promoter ranging from 30 to 100% [Fig. 3; (Perisic et al., 2010)]. The

degree of DNA methylation was higher in CER than in CTX astrocytes. Moreover, this region exerted a strong enhancer function on reporter gene expression and proofed to be responsive to stress hormone in dependence of its DNA methylation status. *In silico* analysis of the CpG island shore revealed consensus sequences for a number of transcription factor binding sites functionally related to the enhancement of GLT-1 gene activity such as CREB (Schluter et al., 2002). As demonstrated in T-cell lineages, most of the differentially methylated DNA regions are also located at distal promoter sites and display DNA methylation-dependent enhancer activity (Schmidl et al., 2009).

Cellular DNA methylation processes are intimately linked to histone modifications. DNA methyltransferases partly interact with histone modifying enzymes including histone deacetylates, suppressor of variegation 3–9 homologue 1 and enhancer of zeste homolog 2 (Fuks et al., 2000; Vire et al., 2006). Targeted DNA methylation might depend upon preestablished histone methylation (Johnson et al., 2002) or vice versa, DNA methylation might constitute a cue by which histone modifications are brought about. Here, we could demonstrate that CTX astrocytes assembled more H4ac at the CpG island and shore region of the GLT-1 gene than CER astrocytes, whereas the repressive H3K27me3 mark at the shore region was slightly more abundant in CER glia. Thus, this pattern of active and repressive epigenetic marks at the shore region is in line with the state of responsiveness of the GLT-1 gene to hormonal factors. A concomitant presence of both active and repressive chromatin at promoters was reported, for example, for developmentally important genes in embryonic stem cells (Bernstein et al., 2006). Assuming a similar performance of the H4ac and H3K27me3 antibodies in ChIP assays, the repressive H3K27me3 mark was much less abundant at the GLT-1 promoter than H4ac, suggesting that GLT-1 does not present a gene with bivalent chromatin structure.

The epigenetic signature of the shore region might not only govern responsiveness to regulatory factors, but might also itself be subject to changes subsequent to drug exposure. Such reprogramming has already been described (Murgatroyd et al., 2009). Rat embryonic neural stem cells undergo heritable changes paralleled by a decrease in global DNA methylation when exposed to DEX (Bose et al., 2010). In mice, chronic administration of corticosterone for 4 weeks results in DNA demethylation of the FKBP5 gene in the hippocampus and hypothalamus (Lee et al., 2010). In our study, the DEX-mediated increase of GLT-1 expression was not associated with a change of DNA methylation at the shore of the GLT-1 promoter in CTX astrocytes. However, we observed DEX-induced elevation of the levels of H4ac at the shore in CTX, but not in CER astrocytes. Of note, epigenetic plasticity of the shore region after HDAC-I exposure reflected by a marked reduction of methylated CpG levels was monitored in CTX astrocytes, as shown previously (Perisic et al., 2010), but not in CER glia. These data point to clear differences between these two brain regions not only in the epigenetic signature of the shore region, but also in its reprogramming after drug exposure.

Unexpectedly, VPA and TSA repressed basal GLT-1 mRNA expression in CER astrocytes, while in parallel a prominent H4 hyperacetylation at the shore and the CpG island was detected. On an unmethylated ectopic reporter gene template, HDAC-I showed a clear stimulatory effect (Fig. 4). The presence of H3K27me3 at the endogenous promoter might stabilize a hypermethylated state of the DNA even under conditions of elevated H4ac levels. Wong et al. recently demonstrated that H3K27me3 marks are rather stable and connected to DNA methylation recovery processes (Wong et al., 2011). Adding another layer of complexity, HDAC-I might elicit the synthesis of coregulatory factors that, depending on the context of surrounding modifications, operate differently on the endogenous and ectopic gene loci. In addition, an important issue relates to the limitation of plasmid-based reporter gene assays for assessing the functional role of modified histone species, since the protein decoration of plasmid DNA is less multifarious as compared with endogenous chromatin. However, reporter gene assays have been shown to be useful for the focused investigation of regulatory activities of transacting factors as well as cytosine methylation marks.

As demonstrated by Hassel et al., long-term treatment of 3-month-old rats with VPA results in elevated levels of hippocampal GLT-1 protein while CTX and CER regions were not affected (Hassel et al., 2001). Moreover, stimulatory effects of HDAC-I on GLT-1 protein levels in primary CER astrocytes from neonatal rats have been reported recently (Allritz et al., 2009). However, regulation of protein levels may be completely uncoupled from gene transcription and hence does not allow extrapolating for transcriptional outcome. Thus, studies suggest that GLT-1 regulation by HDAC-I varies depending on the brain region and developmental phase as well as on the stage of gene expression.

In summary, our data add to the emerging concept that CpG island shores, rather than CpG islands, are targets of epigenetic (re)programming (see Fig. 7 for summary and model) and important determinants of the spatiotemporal pattern of gene activity in health and disease (Doi et al., 2009; Feber et al., 2011; Irizarry et al., 2009).

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