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(54) Title: EPIGENETICAL REGULATION OF BRAIN PLASTICITY

(57) Abstract: The present invention provides an agent for regulating brain plasticity, which comprises a substance affecting an epigenetic state selected from the group consisting of histone acetylation and DNA methylation. Preferably, the substance is a histone deacetylase inhibitor or DNA methyltransferase inhibitor. The agent of the present invention can be used for the remedy or prevention various diseases, disorders and/or conditions wherein the regulation (especially promotion (reactivation)) of brain plasticity is desired.



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DESCRIPTION**EPIGENETICAL REGULATION OF BRAIN PLASTICITY****Field of the Invention**

5 The present invention relates to an agent for regulating brain plasticity, which comprises a substance affecting an epigenetic state including histone modification, such as histone acetylation or DNA methylation, especially a histone deacetylase (HDAC) inhibitor or a DNA methyltransferase (DNMT) inhibitor,
10 as an active ingredient.

Background Art

 Neuronal circuits are shaped by experience during
15 well-defined intervals of early postnatal brain development called critical periods. After the critical period (CP), the brain plasticity is reduced or absent.

 The visual system has been used for the assessment of brain plasticity. In the 1960s and 1970s, Hubel and
20 Wiesel conducted a series of pioneering vision studies using cats and monkeys. In a set of experiments, they deprived kittens of visual stimulation for various periods of time after birth. From these landmark investigations, they deduced that a critical period exists for the proper
25 development of vision. That is, even a brief period (e.g., a few days) of monocular deprivation (MD) at an early stage immediately after birth resulted in permanently impaired vision of the closed eye and a shift of ocular dominance (OD) of the cortical neurons in favor of the
30 non-deprived eye. Thus, the brain has a high degree of plasticity during this period. These findings by Hubel and Wiesel have implications reaching far beyond the field of visual physiology and prove the importance of a richly varied sensory input for the development of the higher
35 functions of the brain.

There are two types of neurons in brain, namely excitatory and inhibitory neurons. Although it had been believed that the plastic change against the visual experience described by Hubel and Wiesel is caused by repetitive stimulation of particular circuits by excitatory neurons, the present inventors revealed this change are driven by inhibitory synapses. The inhibitory neuron releases a transmitter GABA and inhibits neurons connected via a synapse. In mice deficient in GAD65, which is the synapse-specific isoform of GABA synthase, the MD-derived OD plasticity was not detected (non-patent document 1). The administration of a GABA receptor agonist, benzodiazepine, completely rescued the OD plasticity in the GAD65-defective mice not only during the CP but also in adulthood (non-patent document 2). Furthermore, benzodiazepine accelerated the initiation of the plastic change. This is the first report demonstrating the direct control of the timing of CP.

The epigenetic state is known to be regulated by histone and DNA modifications in chromatin, such as histones acetylation, methylation, phosphorylation and the like, and DNA methylation and the like, whereby regulating the gene expression in individuals. As compounds which bring about such modification, inhibitors of HDAC or DNMT are known. Various HDAC inhibitors (for example, patent documents 1-3) and DNMT inhibitors (for example, patent documents 4-5) have been reported, and some of which have been used as pharmaceuticals. For example, valproic acid and salts thereof have been clinically used for the treatment of epilepsy and the like.

There are also some reports on the use of HDAC inhibitors for the treatment of CNS diseases. However, they disclose only a neuroprotective effect and the like of HDAC inhibitors, and does not disclose or suggest

that HDAC and DNMT inhibitors can be used to regulate brain plasticity.

Thus, it has never known that epigenetics is responsible for CP.

5 [patent document 1] JP 2005-272419 A

[patent document 2] JP 2007-001885 A

[patent document 3] WO 2004/103358 A

[patent document 4] WO 2003/012051 A

[patent document 5] WO 2002/067681 A

10 [non-patent document 1] Hensch et al., Science, 282: 1504-1508 (1998)

[non-patent document 2] Fagiolini and Hensch, Nature, 404: 183-186 (2000)

15 Disclosure of the Invention

It is an object of the present invention to provide a method for controlling the timing of brain plasticity and for reactivating brain plasticity even in adulthood.

To accomplish the above-described object, the
20 present inventors focused on the effects of epigenetic on critical period of brain development and investigated global changes that alter the chromatin status, and found that histone hyperacetylation occurs in the pre-critical period of the developing visual cortex. It was
25 also observed that DNA demethylation peaked during the CP. In addition, histone hyperacetylation was confirmed in animal models to delay the onset of plasticity into adulthood. From these results, the present inventors assumed that histone hyperacetylation and/or DNA
30 demethylation might reactivate brain plasticity. To confirm this hypothesis, the present inventors examined the effects of HDAC inhibitors on OD plasticity in adult mice with monocular deprivation during the CP. As a result, these inhibitors remarkably rescued OD
35 plasticity in the mice. The present inventors further

conducted investigations based on these findings, and resulted in the completion of the present invention.

That is, the present invention is following:

- [1] An agent for regulating brain plasticity, which
5 comprises a substance affecting an epigenetic state selected from the group consisting of histone acetylation and DNA methylation;
- [2] The agent according to above [1], wherein the
10 substance induces histone hyperacetylation or DNA demethylation;
- [3] The agent according to above [2], wherein the substance is a histone deacetylase inhibitor or a DNA methyltransferase inhibitor;
- [4] The agent according to above [2], wherein the
15 histone deacetylase inhibitor is selected from the group consisting of a short chain fatty acid or a derivative thereof; a hydroxamic acid derivative; a Benzamide derivative; a cyclic tetrapeptide; an electrophilic ketone derivative; and a psammaplin or Depudecin;
- [5] The agent according to above [4], wherein the
20 histone deacetylase inhibitor is valproic acid or trichostatin A, or a salt thereof;
- [6] The agent according to any of above [1]-[5], which is for treating or preventing one or more diseases or
25 conditions selected from the group consisting of amblyopia, autism spectrum disorders, fragile X syndrome, Rubinstein-Taybi syndrome, mental retardation, Rett's syndrome, schizophrenia, bipolar disorder, Alzheimer's disease, depression, stroke, spinal muscular atrophy,
30 brain lesion and ischemia;
- [7] A method for regulating brain plasticity in a mammal, which comprises regulating an epigenetic state selected from the group consisting of histone acetylation and DNA methylation in said mammal;
- [8] A method for regulating brain plasticity in a
35 mammal, which comprises administering an effective

amount of the agent according to any one of above [1]-
[6] to the mammal;

[9] A use of a substance affecting an epigenetic state
selected from the group consisting of histone

5 acetylation and DNA methylation, for production of an
agent for regulating brain plasticity;

[10] A substance affecting an epigenetic state selected
from the group consisting of histone acetylation and DNA
methylation, for use as an agent for regulating brain

10 plasticity; and

[11] A commercial package comprising the agent according
to any one of above [1]-[6] and a written matter which
states that the agent can or should be used for
regulating brain plasticity.

15

Effects of the Invention

The present invention demonstrates that epigenetic
state is responsible for the CP of brain development for
the first time.

20 A substance regulating histone acetylation or DNA
methylation can alter the chromatin status in a mammal,
thereby regulate brain plasticity even after the CP.

Brief Description of the Drawings

25 Figure 1 shows quantitative analysis of western blot
results. The dashed line represents histones extracted
from hippocampus and the solid line represents histones
extracted from visual cortex.

Figure 2 shows comparison of acetylated H3 and H4
30 histones western blot results. The dashed line represents
acetylated histones H4 and the solid line represents
acetylated H3 histones.

Figure 3 shows result of immunohistochemistry
staining for acetylated histones H3 in the visual cortex.

35 Figure 4 shows proportion of demethylated CpG islands
which represents the status of DNA demethylation.

Figure 5 shows representative blot and the quantitative analysis of western blot results obtained from wild type adult mice aged P56, dark reared mice (DR) and Gad65 knockout mice (GAD KO).

5 Figure 6 shows quantitative analysis of histone deacetylase activity results after injecting wild type adult mice with either vehicle, valproic acid (VPA) or Trichostatin A (TSA).

10 Figure 7 shows shift in ocular dominance and change in CBI values in adult wild-type mice treated daily (>P60) with vehicle (left) or valproic acid (right) during brief monocular deprivation initiated on P60. Black circle indicates deprived eye and white circle indicates undeprived eye.

15 Figure 8 shows that balance between histone acetylation and DNA demethylation predicts transient critical period in visual cortex.

(a) Histone H3 acetylation (ACh3) was observed in the pre-critical period (CP) (<P21) of developing visual cortex, diminished by the peak of the CP (P27) and throughout to adulthood (>P55). Immunofluorescence photomicrographs of ACh3 expressed in different layers of developing visual cortex. Sections of visual cortex obtained from wild-type mice between postnatal ages P11-P56 (n = 4-6 per group) were double-stained with ACh3 (green) and DAPI (blue). Cortical layers (I-VI, WM-white matter) are indicated on the left on DAPI staining. Scale bar, 200 μ m.

(b) Spatial distribution of ACh3 shifted from supragranular to infragranular layer during the course of development in visual cortex. Quantification of the data in a representing percentage of ACh3 against total number of nuclei (average percentage $\pm$ SEM) in the supragranular (II, III) and infragranular (IV, V and VI) layers. Inset, Nuclear staining of ACh3. High magnification of ACh3, DAPI and merged immunofluorescence of P11 neuronal cells. Scale bar, 20 μ m.

(c) Histone acetyltransferase (HAT) activity decreased with age. Total HAT activity was measured in nuclear extracts from visual cortex at P11 and P56. HAT activity values were normalized to P11. The bar graph shows the statistically significant ($P < 0.05$, t-test) decrease of HAT activity from P11 to P56.

(d) Histone acetylation prepares visual cortex during pre-CP and DNA demethylation peaked during CP. Graphical representation of ACH3 (red solid line) and DNA demethylation (black dashed line) levels in developing visual cortex. ACH3 was normalized to H3 histones. DNA demethylation is expressed as percentage of CpG islands that were demethylated. The upward dashed arrow indicates day of eye opening. Area shaded yellow shows range of critical period in the visual cortex ($n = 4$ per age group; average $\pm$ SEM; Two-way ANOVA; $p < 0.05$).

Figure 9 shows that inverted DNA demethylation / histone acetylation balance persists throughout life in the hippocampus.

Downregulation of histone acetylation in developing visual cortex compared to a transient activation of ACH3 during hippocampal development. Immunoblot comparing ACH3 levels in (a) visual cortex and (b) hippocampus during development. Anti-H3 and β -actin antibodies reveal equal amount of protein loaded.

(c) Differential profile of ACH3 distribution in developing hippocampus. Immunofluorescence photomicrographs of ACH3 expressed in developing hippocampus. Sections of hippocampus were obtained from wild-type mice between postnatal ages P11-P16 ($n = 4-6$ per group) and stained with ACH3 (green). Scale bar, 200 μ m.

(d) Maintenance of low levels of histone acetylation and high levels of DNA demethylation in the hippocampus. ACH3 levels were normalized to H3 histones in developing hippocampus (blue) plotted on the same graph as DNA demethylation (black dashed line). The upward dashed

arrow indicates day of eye opening. Area shaded yellow refers to range of critical period in the visual cortex ($n = 4$ per age group; average $\pm$ SEM, $p < 0.05$, t-test).

Figure 10 shows that sensory experience regulates the balance between histone acetylation and DNA demethylation.

(a) Consistent with its latent plasticity, ACh3 was observed in dark reared (DR) mice; exposing DR mice to light quickly diminished ACh3. Immunofluorescence photomicrographs of ACh3 expressed in (from left to right) WT light-reared adult mice (LR), dark-rearing of mice from birth to adulthood (DR), dark-reared adult mice exposed to light for 2-days (+2d) or 7-days (+7d). Cortical layers (I-VI, WM-white matter) are indicated on the left on DAPI staining ($n = 4-6$ per group). Scale bar, 200 μm .

(b) GAD65 deletion mice showed high levels of histone acetylation in adulthood. Immunofluorescence photomicrographs of GAD 65 deletion adult mice (GAD65 KO, $n = 4$). Scale bar, 200 μm .

(c) Spatial distribution of ACh3 is throughout all layers of visual cortex in DR. Quantification of the data in (a) and (b), representing percentage of ACh3 histones against total number of nuclei in the supragranular (white bars) and infragranular (black bars) layers. DR mice maintain high levels of histone acetylation, similar to that of wild-type pre-CP (P16). GAD 65 KO mice also display similar high acetylation levels but in different laminar layers.

(d) Immunoblot comparing ACh3 levels in visual cortex of LR, DR, DR exposed to 2d or 7d of light and GAD65 KO mice ($n = 3$ per group), and its graphical representation in (e).

(f) Concomitant low histone acetylation and low DNA demethylation determines loss of plasticity in DR after light exposure. Graphical representation of ACh3 immunoblot in (d) (red solid line) with DNA demethylation (black dashed line). ACh3 was normalized to histone H3 and DNA demethylation is expressed as percentage of CpG

islands that were demethylated ($n = 4$ per group; average $\pm$ SEM; t-test; $p < 0.05$).

Figure 11 shows that histone hyperacetylation by valproic acid induces DNA methylation and reignite histone acetylation in parvalbumin-positive cells.

(a) Reduction of histone deacetylase (HDAC) activity by either valproic acid (VPA) or trichostatin A (TSA) measured within 2-5 h of administration. HDAC activity was normalized to vehicle.

(b) Increased levels of histone acetylation after VPA administration. Immunoblot comparing AcH3 levels in adult visual cortex after time of vehicle (Veh) and VPA administration. Anti-H3 and β -actin antibodies revealed equal amount of protein loaded.

(c) Histone hyperacetylation caused DNA methylation. AcH3 level was normalized to H3 histones in adult visual cortex after vehicle (red dashed line) or VPA administration (red solid line). DNA demethylation was expressed as percentage of CpG islands that were demethylated (black dashed line for vehicle, black solid line for VPA).

(d) Colocalization of histone acetylation with parvalbumin-positive (PV) cells after VPA administration. Histone H3 acetylation (AcH3) was observed after 2 h of VPA treatment and diminished by 6d of VPA treatment.

Sections of visual cortex obtained from wild-type mice P60 ($n = 4-6$ per treatment group) were double-stained with AcH3 (green) and PV (red).

(e) VPA treatment reignites high acetylation levels in PV cells to recreate pre-CP in adulthood. Graphical representation of percentage of PV cells colocalized with AcH3 at pre-CP (P18), adulthood (P60), adult treated with 2h, 2d or 6d of VPA ($n = 4$ per group).

Figure 12 shows that histone hyperacetylation by valproic acid reactivates ocular dominance plasticity in adulthood. Brief MD induces a significant CBI reduction after VPA administration. CBI indicates distribution bias

in favor of the contralateral eye (closed circle).

(b) VPA injection showed a significant reduction in CBI in comparison to (a) vehicle-treated mice (CBI = 0.51, $P < 0.0001$, t-test).

5 (c) Orientation selectivity remained the same for both vehicle- and VPA-treated mice.

(d) VPA and diazepam (DZ) are both anti-epileptic drugs. Adult mice with either no MD or MD and treated with VPA or DZ were assessed by their CBI values. While DZ showed no
10 change in CBI values like adult mice with no MD, VPA showed a reduction in CBI value.

Figure 13 shows that valproic acid administration reactivates adult plasticity by induction of non-coding genes and nucleus-localized DNA-binding genes.

15 (a) The number of non-coding genes (blue) was 214 in 796 up-regulated genes (red) and 9 in 480 down-regulated genes (green).

(b) Gene Ontology (GO) analysis of visual cortex-enriched genes highlights the changes in molecular function of the
20 coding genes separated into induced (red gradation) or suppressed (green gradation) groups with the most significant enriched groups in darker shading and least significant groups in lighter shading. The GO terms of the genes were extracted and analyzed for enrichment of
25 functional groups by using DAVID and sorted according to their p-values. Group 1 and 2 are differentiated to indicate that though appearing in the same functional class and group they are different gene sets (refer to supplementary table).

30

Best Mode for Carrying Out the Invention

The present invention provides an agent for regulating brain plasticity, which comprises a substance affecting an epigenetic state selected from the group
35 consisting of histone acetylation and DNA methylation.

Herein, "regulating brain plasticity" generally means

positively regulating (controlling, altering and the like) brain plasticity, and more specifically means, for example, promoting, enhancing, reactivating.

"Epigenetics" refers to reversible, heritable changes
5 in gene expression without changing DNA sequence, and also contributes to individual variation in normal biology and in disease states. Epigenetics is based on modification of histone and/or DNA. Thus, "epigenetic state" means modification (e.g. acetylation, methylation and
10 phosphorylation) pattern of histone, DNA methylation and the like. Particularly, as used herein, "epigenetic state" refers to degree of histone acetylation or DNA methylation.

Histone acetylation/deacetylation is regulated by
15 histone acetyltransferases (HAT) and histone deacetylases (HDAC). It is believed that HAT/HDAC activity is normally in equilibrium, and upon stimulation, histone is acetylated by HAT, activating transcription and the gene expression is induced.

20 "Substance affecting histone acetylation" may be any substance which can affect (e.g., modulate, control, alter, manipulate) the degree of histone acetylation. For example, the substance includes but not limited to HDAC inhibitor, HAT or activator thereof.

25 "Substance affecting DNA methylation" may be any substance which can affect (e.g., modulate, control, alter, manipulate) the degree of DNA methylation. For example, DNA methyltransferase (DNMT) inhibitor, enzymes involved in demethylation of DNA (e.g., 5-methylcytosine DNA
30 glycosylase and DNA repairing enzyme) or activator thereof, and the like can be mentioned.

Preferably, the substance affecting histone acetylation is an HDAC inhibitor. Herein, "HDAC inhibitor" refers any inhibitor which can eventually lead
35 the inhibition of HDAC, whether directly or indirectly. Although many histone deacetylases (e.g., HDAC1-9) are

known in mammals, HDAC targeted by the HDAC inhibitor of the present invention may be any HDAC, and not limited to specific one. Examples of HDAC inhibitor used for the purpose of the present invention include, but not limited to the followings:

- 1) short chain fatty acids (SCFA) and derivatives thereof such as valproic acid, valproate sodium butyrate (Cousens et al., J. Biol. Chem. 254: 1716-1723 (1979)), isovalerate (McBain et al., Biochem. Pharm. 53: 1357-1368 (1997)), valerate (McBain et al., supra); 4-phenylbutyrate (4-PBA) (Lea and Tulsyan, Anticancer Research, 15: 879-873 (1995)), phenylbutyrate (PB) (Wang et al., Cancer Research, 59: 2766-2799 (1999)), propionate (McBain et al., supra), butyramide (Lea and Tulsyan, supra), isobutyramide (Lea and Tulsyan, supra), phenylacetate (Lea and Tulsyan, supra), 3-bromopropionate (Lea and Tulsyan, supra), tributyrin (Guan et al., Cancer Research, 60: 749-755 (2000)), and Pivanex;
- 2) hydroxamic acid derivatives such as trichostatin analogues such as trichostatin A (TSA) and trichostatin C (Koghe et al., Biochem. Pharmacol., 56: 1359-1364 (1998)), suberoylanilide hydroxamic acid (SAHA) (Richon et al., Proc. Natl. Acad. Sci. USA, 95: 3003-3007 (1998)), m-carboxycinnamic acid bishydroxamide (CBHA) (Richon et al., supra), pyroxamide, salicylbishydroxamic acid (Andrews et al., International J. Parasitology 30: 761-768 (2000)), suberoyl bishydroxamic acid (SBHA) (U.S. Patent No. 5,608,108), azelaic bishydroxamic acid (ABHA) (Andrews et al., supra), azelaic-l-hydroxamate-9-anilide (AAHA) (Qiu et al., Mol. Biol. Cell 11: 2069-2083 (2000)), 6-(3-chlorophenylureido) carpoic hydroxamic acid (3C1-UCHA), oxamflatin[(2E)-5-[3-[(phenylsulfonyl)aminolphenyl]-pent-2-en-4-ynohydroxamic acid]] (Kim et al., Oncogene, 18: 2461-2470 (1999)), A-161906, Scriptaid (Su et al., 2000 Cancer Research, 60: 3137-3142), PXD-101 (Prolifix), LAQ-824, CHAP, MW2796 (Andrews et al., supra), MW2996 (Andrews et

al., supra), or any of the hydroxamic acids disclosed in U.S. Patent Nos. 5,369,108, 5,932,616, 5,700,811, 6,087,367 and 6,511,990;

3) benzamide derivatives such as CI-994, MS-275 [N-(2-aminophenyl)-4-[N-(pyridin-3-yl methoxycarbonyl)aminomethyl]benzamide] (Saito et al., Proc. Natl. Acad. Sci. USA, 96: 4592-4597 (1999)) and 3'-amino derivative of MS-275 (Saito et al., supra);

4) cyclic tetrapeptides such as trapoxin A (TPX)-cyclic tetrapeptide (cyclo-(L-phenylalanyl-L-phenylalanyl-D-pipecolinyl-L-2-amino-8-oxo-9,10-epoxy decanoyl)) (Kijima et al., J. Biol. Chem., 268: 22429-22435 (1993)), FR901228 (FK 228, depsipeptide) (Nakajima et al., Ex. Cell Res., 241: 126-133 (1998)), FR225497 cyclic tetrapeptide (H. Mori et al., PCT Application WO 00/08048 (17 February 2000)), apicidin cyclic tetrapeptide [cyclo(N-O-methyl-L-tryptophanyl-L-isoleucinyl-D-pipecolinyl-L-2-amino-8-oxodecanoyl)] (Darkin-Rattray et al., Proc. Natl. Acad. Sci. USA, 93: 13143-13147 (1996)), apicidin Ia, apicidin Ib, apicidin Ic, apicidin IIa, and apicidin IIb (P. Dulski et al., PCT Application WO 97/11366), CHAP, HC-toxin cyclic tetrapeptide (Bosch et al., Plant Cell, 7: 1941-1950 (1995)), WF27082 cyclic tetrapeptide (PCT Application WO 98/48825), and chlamydocin (Bosch et al., supra);

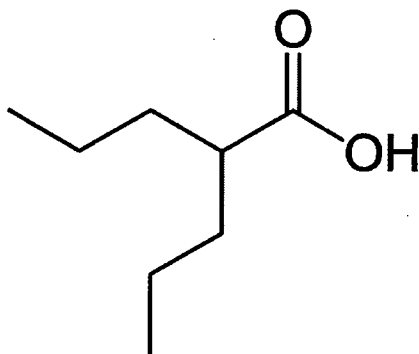
5) electrophilic ketone derivatives such as trifluoromethyl ketones (Frey et al., Bioorganic & Med. Chem. Lett., (2002), 12: 3443-3447, U.S. Patent No. 6,511,990), and α -keto amides such as N-methyl- α -ketoamides; and

6) other HDAC Inhibitors such as psammaplins and Depudecin (Kwon et al., 1998, PNAS 95: 3356-3361).

The above-mentioned HDAC inhibitor may be a free form or a pharmacologically acceptable salt thereof. As the salt, for example, when a compound has an acidic functional group therein such as carboxyl group, inorganic salts such as alkali metal salt (e.g., sodium salt,

potassium salt, lithium salt and the like), alkaline earth metal salt (e.g., calcium salt, magnesium salt, barium salt and the like), ammonium salt and the like can be mentioned, and when a compound has a basic functional group therein such as amino group, salts with inorganic acids such as hydrochloric acid, hydrobromic acid, nitric acid, sulfuric acid, phosphoric acid and the like, and salts with organic acids such as acetic acid, phthalic acid, fumaric acid, oxalic acid, tartaric acid, maleic acid, citric acid, succinic acid, methanesulfonic acid, p-toluenesulfonic acid and the like can be mentioned.

Preferably, the HDAC inhibitor is valproic acid shown by the following formula:



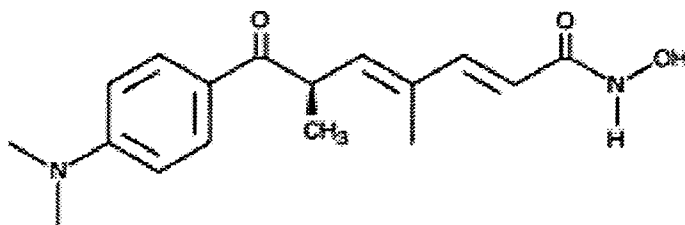
or a salt thereof.

Valproic acid was released in 1967 in Europe and in 1978 in the United States to treat epilepsy. Valproic acid is a chemical compound that has found clinical use as an anticonvulsant and mood-stabilizing drug, primarily in the treatment of epilepsy and bipolar disorder. It is also used to treat migraine headaches and schizophrenia. In epileptics, valproic acid is used to control absence seizures, tonic-clonic seizures (grand mal), complex partial seizures, juvenile myoclonic epilepsy and the seizures associated with Lennox-Gastaut syndrome. It is also used in treatment of myoclonus. In some countries, parenteral (administered intravenously) preparations of valproate are used also as second-line treatment of status epilepticus, alternatively to phenytoin. Related drugs

include the sodium salts sodium valproate, used as an anticonvulsant, and a combined formulation, valproate semisodium, used as a mood stabilizer and additionally in U.S. also as an anticonvulsant.

5 Currently commercially available products of valproic acid include, for example, Depakene (Abbott Laboratories in U.S. & Canada), Valpro (Alphapharm in Australia), Epilim (Sanofi-Aventis in the UK), Convulex (Pfizer in the UK and Byk Madaus in South Africa), Depakine and
10 MicropakineLP by Sanofi-Aventis and Orfiril by Desitin Arzneimittel GmbH in Europe.

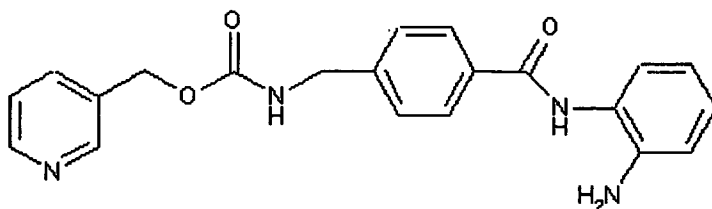
Another preferable HDAC inhibitor is trichostatin A shown by the following formula:



15

Trichostatin A is an organic compound that serves as an antifungal antibiotic and selectively inhibits the mammalian histone deacetylases enzyme. TSA inhibits the eukaryotic cell cycle during the beginning of the growth
20 stage. TSA can be used to alter gene expression by interfering with the removal of acetyl groups from histones and therefore altering the ability of DNA transcription factors to access the DNA molecules inside chromatin. Thus, TSA has some uses as an anti-cancer drug.
25 By promoting the expression of apoptosis-related genes, it may lead to cancerous cells surviving at lower rates, thus slowing the progression of cancer.

Another preferable HDAC inhibitor is MS-275 shown by the following formula:



The formula name of MS-275 is N-(2-Aminophenyl)-4-[N-(pyridine-3-ylmethoxy-carbonyl)aminomethyl]benzamide.

5 It is a potent, long-lasting brain region-selective inhibitor of histone deacetylases. It preferentially inhibits HDAC1 (IC₅₀=300nM) over HDAC3 (IC₅₀=8μM). It has no inhibitory activity towards HDAC8 (IC₅₀>100μM).

Preferably, the substance affecting DNA methylation
10 is a DNMT inhibitor. Herein, "DNMT inhibitor" refers any inhibitor which can eventually lead the inhibition of DNA methyltransferase, i.e. inhibition of DNA methylation, whether directly or indirectly. Although four DNA methyltransferases (DNMT1, 2, 3A and 3B) are known in
15 mammals, DNMT targeted by the DNMT inhibitor of the present invention may be any DNMT, and not limited to specific one. Examples of DNMT inhibitor used for the purpose of the present invention include, but not limited to the followings: RG108, DNMTI 5-azacytidine, DNMTI
20 zebularine (these compounds can be available from Calbiochem) and the like.

The above-mentioned DNMT inhibitor may be a free form or a pharmacologically acceptable salt thereof. As the salt, for example, when a compound has an acidic
25 functional group therein such as carboxyl group, inorganic salts such as alkali metal salt (e.g., sodium salt, potassium salt, lithium salt and the like), alkaline earth metal salt (e.g., calcium salt, magnesium salt, barium salt and the like), ammonium salt and the like can be
30 mentioned, and when a compound has a basic functional group therein such as amino group, salts with inorganic acids such as hydrochloric acid, hydrobromic acid, nitric

acid, sulfuric acid, phosphoric acid and the like, and salts with organic acids such as acetic acid, phthalic acid, fumaric acid, oxalic acid, tartaric acid, maleic acid, citric acid, succinic acid, methanesulfonic acid, p-toluenesulfonic acid and the like can be mentioned.

The above-mentioned HDAC and DNMT inhibitors can be produced by methods known in the art or described in the references cited above.

The substance affecting histone acetylation or DNA methylation can alter the epigenetic state (chromatin status) in a mammal, thereby regulating brain plasticity of the mammal. More specifically, the substance bringing about histone hyperacetylation or DNA hypermethylation in chromatin, preferably HDAC or DNMT inhibitor, can reactivate brain plasticity even after the CP. Therefore, the agent for regulating brain plasticity of the present invention can be used for the remedy or prevention of diseases, disorders or conditions wherein the regulation (preferably promotion (reactivation)) of brain plasticity is necessary or suitable for the remedy or prevention thereof. Such diseases, disorders or conditions include, but not limited to, amblyopia, autism spectrum disorders, fragile X syndrome, Rubinstein-Taybi syndrome, mental retardation, Rett's syndrome, schizophrenia, bipolar disorder, Alzheimer's disease, depression, stroke, spinal muscular atrophy, brain lesion and ischemia.

When the agent of the present invention is used as a pharmaceutical, the active ingredient, substance affecting histone acetylation or DNA methylation, can be safely administered orally or parenterally (e.g., topically, intravenously, intracerebrally, intraperitoneally and the like) as it is or as a preparation containing a pharmaceutical composition containing a pharmacologically acceptable carrier admixed according to a method known per se, such as

tablets (including sugar-coated tablets and film-coated tablets), powder, granule, capsule (including soft capsule), orally disintegrating tablet, liquid, injectables, suppository, sustained-release preparation, 5 plaster and the like.

The content of an active ingredient in the pharmaceutical composition of the present invention is about 0.01 to 100% by weight relative to the entire composition.

10 The pharmacologically acceptable carrier that may be used to produce the pharmaceutical composition of the present invention includes various organic or inorganic carrier substances in common use as pharmaceutical materials, including excipients, lubricants, binders, 15 disintegrants, water-soluble polymers and basic inorganic salts for solid preparations; and solvents, dissolution aids, suspending agents, isotonizing agents, buffers and soothing agents for liquid preparations and the like. Other conventional additives such as 20 preservatives, anti-oxidants, coloring agents, sweetening agents, souring agents, bubbling agents and flavorings may also be used as necessary.

Such "excipients" include, for example, lactose, sucrose, D-mannitol, starch, cornstarch, crystalline 25 cellulose, light anhydrous silicic acid, titanium oxide and the like.

Such "lubricants" include, for example, magnesium stearate, sucrose fatty acid esters, polyethylene glycol, talc, stearic acid and the like.

30 Such "binders" include, for example, hydroxypropyl cellulose, hydroxypropylmethyl cellulose, crystalline cellulose, starch, polyvinylpyrrolidone, gum arabic powder, gelatin, pullulan, low-substituted hydroxypropyl cellulose and the like.

35 Such "disintegrants" include (1) crosspovidone, (2) what is called super-disintegrants such as

crosscarmellose sodium (FMC-Asahi Chemical) and
carmellose calcium (Gotoku Yakuhin) etc, (3)
carboxymethyl starch sodium (e.g., product of Matsutani
Chemical), (4) low-substituted hydroxypropyl cellulose
5 (e.g., product of Shin-Etsu Chemical), (5) corn starch,
and so forth. Said "crosspovidone" may be any
crosslinked polymer having the chemical name 1-ethenyl-
2-pyrrolidinone homopolymer, including
polyvinylpyrrolidone (PVPP) and 1-vinyl-2-pyrrolidinone
10 homopolymer, and is exemplified by Colidon CL (produced
by BASF), Polyplasdon XL (produced by ISP), Polyplasdon
XL-10 (produced by ISP), Polyplasdon INF-10 (produced by
ISP) and the like.

Such "water-soluble polymers" include, for example,
15 ethanol-soluble water-soluble polymers [e.g., cellulose
derivatives such as hydroxypropyl cellulose (hereinafter
also referred to as HPC) etc., polyvinylpyrrolidone and
the like], ethanol-insoluble water-soluble polymers
[e.g., cellulose derivatives such as hydroxypropylmethyl
20 cellulose (hereinafter also referred to as HPMC) etc.,
methyl cellulose, carboxymethyl cellulose sodium and the
like, sodium polyacrylate, polyvinyl alcohol, sodium
alginate, guar gum and the like] and the like.

Such "basic inorganic salts" include, for example,
25 basic inorganic salts of sodium, potassium, magnesium
and/or calcium. Preferred are basic inorganic salts of
magnesium and/or calcium. More preferred are basic
inorganic salts of magnesium. Such basic inorganic
salts of sodium include, for example, sodium carbonate,
30 sodium hydrogencarbonate, disodium hydrogenphosphate and
the like. Such basic inorganic salts of potassium
include, for example, potassium carbonate, potassium
hydrogencarbonate and the like. Such basic inorganic
salts of magnesium include, for example, heavy magnesium
35 carbonate, magnesium carbonate, magnesium oxide,
magnesium hydroxide, magnesium aluminometasilicate,

magnesium silicate, magnesium aluminate, synthetic hydrotalcite $[\text{Mg}_6\text{Al}_2(\text{OH})_{16}\cdot\text{CO}_3\cdot 4\text{H}_2\text{O}]$, and aluminum magnesium hydroxide. Preferred are heavy magnesium carbonate, magnesium carbonate, magnesium oxide, magnesium hydroxide and the like. Such basic inorganic salts of calcium include, for example, precipitated calcium carbonate, calcium hydroxide, etc.

Such "solvents" include, for example, water for injection, alcohol, propylene glycol, macrogol, sesame oil, corn oil, olive oil and the like.

Such "dissolution aids" include, for example, polyethylene glycol, propylene glycol, D-mannitol, benzyl benzoate, ethanol, trisaminomethane, cholesterol, triethanolamine, sodium carbonate, sodium citrate and the like.

Such "suspending agents" include, for example, surfactants such as stearyltriethanolamine, sodium lauryl sulfate, laurylaminopropionic acid, lecithin, benzalkonium chloride, benzethonium chloride, glyceryl monostearate etc; hydrophilic polymers such as polyvinyl alcohol, polyvinylpyrrolidone, carboxymethyl cellulose sodium, methyl cellulose, hydroxymethyl cellulose, hydroxyethyl cellulose, hydroxypropyl cellulose etc., and the like.

Such "isotonizing agents" include, for example, glucose, D-sorbitol, sodium chloride, glycerol, D-mannitol and the like.

Such "buffers" include, for example, buffer solutions of phosphates, acetates, carbonates, citrates etc., and the like.

Such "soothing agents" include, for example, benzyl alcohol and the like.

Such "preservatives" include, for example, p-oxybenzoic acid esters, chlorobutanol, benzyl alcohol, phenethyl alcohol, dehydroacetic acid, sorbic acid and the like.

Such "antioxidants" include, for example, sulfites, ascorbic acid, α -tocopherol and the like.

Such "coloring agents" include, for example, food colors such as Food Color Yellow No. 5, Food Color Red
5 No. 2, Food Color Blue No. 2 etc.; food lake colors, red ferric oxide and the like.

Such "sweetening agents" include, for example, saccharin sodium, dipotassium glycyrrhizinate, aspartame, stevia, thaumatin and the like.

10 Such "souring agents" include, for example, citric acid (citric anhydride), tartaric acid, malic acid and the like.

Such "bubbling agents" include, for example, sodium bicarbonate and the like.

15 Such "flavorings" may be synthetic substances or naturally occurring substances, and include, for example, lemon, lime, orange, menthol, strawberry and the like.

The active ingredient may be prepared as a preparation for oral administration in accordance with a
20 commonly-known method, for example, by compression-shaping with a carrier such as an excipient, a disintegrant, a binder, a lubricant, or the like, and subsequently coating the preparation as necessary by a commonly known method for the purpose of taste masking,
25 enteric dissolution or sustained release. For an enteric preparation, an intermediate layer may be provided by a commonly known method between the enteric layer and the drug-containing layer for the purpose of separation of the two layers.

30 For preparing the active ingredient as an orally disintegrating tablet, available methods include, for example, a method in which a core containing crystalline cellulose and lactose is coated with the active ingredient and, where necessary, a basic inorganic salt,
35 and then further coated with a coating layer containing a water-soluble polymer to give a composition, which is

coated with an enteric coating layer containing polyethylene glycol, further coated with an enteric coating layer containing triethyl citrate, still further coated with an enteric coating layer containing
5 polyethylene glycol, and finally coated with mannitol to give fine granules, which are mixed with additives and shaped.

The above-mentioned "enteric coating layer" includes, for example, a layer consisting of a mixture
10 of one or more kinds from aqueous enteric polymer substrates such as cellulose acetate phthalate (CAP), hydroxypropylmethyl cellulose phthalate, hydroxymethyl cellulose acetate succinate, methacrylic acid copolymers (e.g., Eudragit L30D-55 (trade name; produced by Rohm),
15 Colicoat MAE30DP (trade name; produced by BASF), Polyquid PA30 (trade name; produced by San-yo Chemical) etc.), carboxymethylethyl cellulose, shellac and the like; sustained-release substrates such as methacrylic acid copolymers (e.g., Eudragit NE30D (trade name),
20 Eudragit RL30D (trade name), Eudragit RS30D (trade name), etc.) and the like; water-soluble polymers; plasticizers such as triethyl citrate, polyethylene glycol, acetylated monoglycerides, triacetin, castor oil and the like; and the like, and the like.

25 The above-mentioned "additive" includes, for example, water-soluble sugar alcohols (e.g., sorbitol, mannitol, maltitol, reduced starch saccharides, xylitol, reduced palatinose, erythritol, etc.), crystalline cellulose (e.g., Ceolas KG 801, Avicel PH 101, Avicel PH
30 102, Avicel PH 301, Avicel PH 302, Avicel RC-591 (crystalline cellulose carmellose sodium) etc.), low-substituted hydroxypropyl cellulose (e.g., LH-22, LH-32, LH-23, LH-33 (Shin-Etsu Chemical), mixtures thereof etc.) and the like. Furthermore, binders, souring
35 agents, bubbling agents, sweetening agents, flavorings,

lubricants, coloring agents, stabilizers, excipients, disintegrants etc. are also used.

The active ingredient may be used in combination with one or more (preferably 1 to 3) other

5 pharmaceutically active ingredients at a suitable ratio.

Such "other active ingredients" include, for example, cebutolol, acetylcysteine, acetylsalicylic acid, acyclovir, alprazolam, alfacalcidol, allantoin, allopurinol, ambroxol, amikacin, amiloride, aminoacetic
10 acid, amiodarone, amitriptyline, amlodipine, amoxicillin, ampicillin, ascorbic acid, aspartame, astemizole, atenolol, beclomethasone, benserazide, benzalkoniumhydrochloride, benzocaine, benzoic acid, betamethasone, bezafibrate, biotin, biperiden,
15 bisoprolol, bromazepam, bromhexine, bromocriptine, budesonide, bufexamac, buflomedil, buspirone, caffeine, camphor, captopril, carbamazepine, carbidopa, carboplatin, cefachlor, cefalexin, w cefadroxil, cefazolin, cefixime, cefotaxime, ceftazidime,
20 ceftriaxone, cefuroxime, selegiline, chloramphenicol, chlorhexidine, chlorpheniramine, chlortalidone, choline, cyclosporin, cilastatin, cimetidine, ciprofloxacin, cisapride, cisplatin, clarithromycin, clavulanic acid, clomipramine, clonazepam, clonidine, clotrimazole,
25 codeine, cholestyramine, cromoglycic acid, yanocobalamin, cyproterone, desogestrel, dexamethasone, dexpanthenol, dextromethorphan, dextropropoxiphen, diazepam, diclofenac, digoxin, dihydrocodeine, dihydroergotamine, dihydroergotoxin, diltiazem, diphenhydramine,
30 dipyridamole, dipyrone, disopyramide, domperidone, dopamine, doxycycline, enalapril, ephedrine, epinephrin, ergocalciferol, ergotamine, erythromycin, estradiol, ethinylestradiol, etoposide, Eucalyptus globulus, famotidine, felodipine, fenofibrate, fenoterol, fentanyl,
35 flavin mononucleotide, fluconazole, flunarizine, fluorouracil, fluoxetine, flurbiprofen, furosemide,

gallopamil, gemfibrozil, gentamicin, Ginkgo biloba,
glibenclamide, glipizide, clozapine, Glycyrrhiza glabra,
griseofulvin, guaifenesin, haloperidol, heparin,
hyaluronic acid, hydrochlorothiazide, hydrocodone,
5 hydrocortisone, hydromorphone, ipratropium hydroxide,
ibuprofen, imipenem, indomethacin, iohexol, iopamidol,
isosorbide dinitrate, isosorbide mononitrate,
isotretinoin, ketotifen, ketoconazole, ketoprofen,
ketorolac, labetalol, lactulose, lecithin, levocarnitine,
10 levodopa, levoglutamide, levonorgestrel, levothyroxine,
lidocaine, lipase, imipramine, lisinopril, loperamide,
lorazepam, lovastatin, medroxyprogesterone, menthol,
methotrexate, methyldopa, methylprednisolone,
metoclopramide, metoprolol, miconazole, midazolam,
15 minocycline, minoxidil, misoprostol, morphine,
multivitamin mixtures or combinations and mineral salts,
n-methylephedrine, naftidrofuryl, naproxen, neomycin,
nicardipine, nicergoline, nicotinamide, nicotine,
nicotinic acid, nifedipine, nimodipine, nitrazepam,
20 nitrendipine, nizatidine, norethisterone, norfloxacin,
norgestrel, nortriptyline, nystatin, ofloxacin,
omeprazole, ondansetron, pancreatin, panthenol,
pantothenic acid, paracetamol, penicillin G, penicillin
V, phenobarbital, pentoxifylline, henoxymethylpenicillin,
25 phenylephrine, phenylpropanolamine, phenytoin, piroxicam,
polymyxin B, povidone-iodine, pravastatin, prazepam,
prazosin, prednisolone, prednisone, propafenone,
propranolol, proxyphylline, pseudoephedrine, pyridoxine,
quinidine, ramipril, ranitidine, reserpine, retinol,
30 riboflavin, rifampicin, rutoside, saccharin, salbutamol,
salcatonin, salicylic acid, simvastatin, somatropin,
sotalol, spironolactone, sucralfate, sulbactam,
sulfamethoxazole, sulfasalazine, sulpiride, tamoxifen,
tegafur, teprenone, terazosin, terbutaline, terfenadine,
35 tetracycline, theophylline, thiamine, ticlopidine,
timolol, tranexamic acid, tretinoin, triamcinolone

acetonide, triamterene, trimethoprim, troxerutin, uracil, vancomycin, verapamil, vitamin e, folinic acid, zidovudine, zotepine.

The agent of the present invention can be administered to any mammal (e.g., mouse, rat, hamster, guinea pig, rabbit, cat, dog, bovine, sheep, monkey, human, etc.), and more preferably human. While the dose of the agent varies depending on subject of administration, administration route, target disease, symptoms and the like, it is generally about 10 to about 1000 mg/kg/day, and preferably about 100 to about 500 mg/kg/day, based on the active ingredient, which is administered once or several times a day.

The agent of the present invention can also be used as a reagent for controlling the timing of the onset of critical period in brain research and the like.

Examples

The present invention is only illustrated by, but not limited to the following Examples in any way. All experiments in the Examples below were carried out according to "METHODS AND MATERIALS" below.

METHODS AND MATERIALS

1) Animals

C57BL/6 mice were used during the WT development period postnatal day 11 (P11) to adulthood P56. During dark rearing (DR), animals were kept in a darkroom till P56 and feeding or cage cleaning was performed wearing an infrared visor. Some animals were exposed to light at P56 for a 2 (DR to 2d) or 7-day (DR to 7d) period. Fast photographic film was exposed in the darkroom for several days to monitor effectiveness of the light seal before use. Mice carrying a functional disruption of GAD65 were generated as described previously (Asada et al., 1996). Animals were maintained on a 12 hr light/dark (LD) cycle (except when noted for dark-

adaptation experiments) and had access to food and water *ad libitum*.

2) Immunofluorescence for hyperacetylated histones and parvalbumin

5 Mice were perfused transcardially with 0.9% saline and 4% paraformaldehyde. The brains were removed, post-fixed and immersed in buffered 30% sucrose solution overnight at 4°C. Brain slices were sectioned at 40µm thickness by a cryostat and detected for either histone
10 H3 acetylated at lysines K9 and K14 (AcH3; 1:300; Upstate) or histone H4 acetylated at lysines K5, K8, K12 and K16 (AcH4; 1:300; Upstate) double stained with DAPI (1:200; Nacalai Tesque) for nuclear staining and/or parvalbumin antibody (1:1000, Swant). The secondary
15 antibody was Alexa Fluor 488 conjugate (Molecular Probes, Eugene, Oregon) or mouse Cy3 (Sigma) diluted to 1:200. Immunofluorescence imaging was performed with a confocal laser-scanning microscope (Olympus IX81 with Fluoview FV1000 scanner). Wavelengths 408 nm and 488 nm were used
20 for detection. Images were captured using Fluoview ver 1.3a and processed with Adobe Photoshop 7.0. The number of acetylated histones localized in the nucleus was quantified using Image Pro Plus 5.0 software. The binocular area of the visual cortex was selected and
25 separated into the supragranular layer (layers I, II and III) and the infragranular layer (layers IV, V and VI) first on the DAPI image detected at 408 nm to count the number of outlined nuclei of each layer. The same area of interest was then used to count the number of
30 acetylated histones. The percentage of acetylated histones was measured as the number of acetylated histones over the number of nuclei in each layer. The percentage of parvalbumin-positive (PV) interneurons colocalized with AcH3 was expressed against total number
35 of PV cells.

3) Immunoblot

For histone extraction, tissue homogenates were centrifuged to remove the supernatant fraction. The nuclear fraction was sonicated in 0.2N H₂SO₄ and kept on ice to extract histones. The acid extracts were further
5 centrifuged and TCA/ 4mgml⁻¹ DCA was add to the supernatant. The pellet obtained after centrifugation was washed with acidified acetone followed by acetone. Purified histone proteins were resuspended in 10mM Tris (pH 8.0) and denatured in loading buffer. Histone protein
10 (5µg) was loaded and separated by 15% SDS-PAGE followed by electrophoretic transfer onto polyvinylidene difluoride membrane (Millipore, MA, USA). The membranes were probed with rabbit polyclonal anti-AcH3 (1:1000), anti-histone H4 acetylated at lysines K5, K8, K12 and K16 (anti-AcH4,
15 1:1000, Upstate), anti-H3 (1:500, Upstate) and anti-H4 (1:500, Upstate) antibodies. The secondary antibody Alexa Fluor 680 conjugate (Molecular Probes, Eugene, Oregon) diluted to 1:3000. The membranes were reprobed with anti-β-actin antibody (1:3000, Sigma) and detected with
20 IRDye800 (1:3000, Rockland) as secondary antibody to ensure equal protein loading. The bands were visualized with Odyssey (Aloka, Japan) and analyzed with Quantitative One software. Statistical analysis were performed using n=3 or n=4 and expressed as a ratio of acetylated histones
25 over the loading amount of its respective histones.

4) Drug administration

Valproic acid (VPA; 200mgkg⁻¹, i.p; Sigma-Aldrich) was dissolved in sterile saline. Trichostatin A (TSA; 1mgkg⁻¹; Wako) was dissolved in vehicle solution (25%
30 DMSO, 5% propylene glycol in saline). Both solutions were freshly prepared and administered to WT P56 mice every 12 hr for 2 or 6 days. The same volume of vehicle solution was injected into control animals. Mice were anesthetized with halothane and killed by cervical
35 dislocation at 2h for VPA and 5h for TSA for HDAC activity. VPA or vehicle solution (Veh) was injected

into WT P56 adult mice and the mice were sacrificed at 2h or 12h after 2nd day or 6th day of injection for DNA methylation or ACh3 immunoblot assay.

5) HDAC and HAT activity

5 HDAC activity was measured using HDAC Fluorometric Assay/Drug Discovery kit (BIOMOL research Laboratories Inc.), according to manufacturer's instructions. Briefly, nuclear proteins were extracted from visual cortex (Kawabata H, et al. 2002). Nuclear extract containing
10 75µg proteins was incubated with acetylated substrate for 1 hour at room temperature. The reaction was stopped by addition of developer solution, which produces a fluorophore.

HAT activity was analyzed using a non-radioactive
15 HAT assay kit (Upstate) according to manufacturer's protocol, without or with the modification that 100 µl H3 or H4 (4 µg/ml) were immobilized in each well. The acetylation reaction occurred for 45 minutes with 100 µg protein lysate. HDAC and HAT activity was measured in a
20 microtiter plate reader.

6) Global CpG island methylation

Cytosine-extension assay to detect alteration in DNA methylation was performed as previously described in Progribny and James (1999). The isoschizomers HpaII (DNA
25 methylation sensitive) and MspI (DNA methylation unsensitive) were used to digest 2 µg gDNA. The digested DNA was purified with QiaexII kit (Qiagen) and eluted with 25 µl EB buffer. The single nucleotide extension reaction was performed on all elute, using Ex Taq
30 polymerase (Takara BIO Inc.) and α P³² labeled dCTP, in a 25 µl reaction mixture. Duplicate 10 µl aliquots from each reaction were applied on Whatman DE-81 paper and washed 4 times with 0.5 M Na-phosphate buffer (pH 7.0) and processed for scintillation counting. The
35 proportion methylated CpG island correlate with the ratio between the HspII and MspI digested samples.

7) Monocular deprivation and drug administration

Prior to monocular deprivation (MD) experiments, VPA was injected into wild-type adult mice every 12 hourly for 2 days. After the 2nd day, eyelid margins were trimmed and sutured under halothane anaesthesia for 4 days (brief MD). VPA was administered i.p. every 12 hourly over the 4 days. All recordings were obtained contralateral to the deprived eye and blind to drug treatment.

10 8) Electrophysiology

Electro-physiological recordings were performed under Nembutal (50 mg kg⁻¹; Abbot)/chlorprothixene (0.2 mg; Sigma) anaesthesia using standard techniques. For each animal, 5 to 8 single units (> 75 µm apart) were recorded in each of 4 to 6 vertical penetrations spaced evenly (> 200 µm intervals) across the medio-lateral extent of primary visual cortex to map the monocular and binocular zones and avoid sampling bias. Cells were assigned ocular dominance scores using a 7-point classification scheme. For each binocular zone, a CBI was calculated according to the formula: $CBI = [(n1 - n7) + (2/3)(n2 - n6) + (1/3)(n3 - n5) + N]/2N$, where N = total number of cells, and n_x = number of cells of ocular dominance score equal to x . This weighted average of the bias toward one eye or the other takes values from 0 to 1 for complete ipsilateral or contralateral eye dominance, respectively.

9) RNA preparation and GeneChip hybridization

Total RNA was isolated from visual cortex using QIAzol reagent (Qiagen, Crawley, UK.) and the aqueous phase was removed after centrifugation through a Phase-Lock Gel column (Eppendorf). Total RNA was purified with RNeasy Micro Kit MinElute Spin columns (Qiagen) and eluted into 50 µl of RNase-free water. The concentration and purity of the total RNA samples were first assessed by spectrophotometry (Nanodrop,

Wilmington, DE). Only samples with a sufficiently high yield (1 µg of total RNA from five animals at a minimum concentration of 50 ng/µl) and purity (an A260:A280 ratio of close to 2) were further analyzed for quantity and integrity with denaturing gel. Samples (each n=4 for vehicle and VPA groups) that met the quality control criteria were used as templates for cRNA synthesis and biotin-labeling, incorporating a single round of linear amplification by using the Message Amp II-biotin enhanced kit (Ambion, Huntingdon, U.K.). The quantity and size distribution of purified aRNA was assessed by Nanodrop and denaturing gel to ensure that the aRNA amplification was successful. Target fragmentation was achieved by incubation at 94°C for 35 min in fragmentation buffer (40 mM Tris-acetate, pH 8.1/100 mM KOAc/30 mM MgOAc). The size distribution of the fragmented labeled transcripts was assessed by denaturing gel. After fragmentation and quality confirmation with the Affymetrix Test-2 Array, 20 µg of the biotinylated aRNA were hybridized to Affymetrix Murine Genome 430 GeneChips (45 101 probe sets) (Affymetrix, Inc., Santa Clara, CA, USA). The chips were washed, stained with streptavidin-phycoerythrin and scanned with a probe array scanner (HP GeneArray Scanner, Hewlett-Packard Company, Palo Alto, CA, USA).

10) GeneChip data analysis

Microarray experiments were performed at the Research Resource Centre, RIKEN Brain Science Institute. Eight independent microarray experiments were performed (n = 4 per treatment). Data were analyzed with Affymetrix Microarray Suite 5.0 software (Affymetrix, Inc.) and GeneSpring 5.0 software (Silicon Genetics, Redwood City, CA, USA). When comparing the data, the Suite 5.0 software normalized the values of expression levels using all probes. Statistical comparisons of expression levels between Veh and VPA mice were

performed by using the Mann-Whitney U-test. After normalization, the software scaled the data for each chip and then generated a change P-value, a change call and a signal log ratio using Wilcoxon's signed-rank test.

5 A pairwise analysis was performed using Affymetrix software. Genes that showed an appropriate absence or presence call in all 4 repeats of each sample were selected. Fold changes were calculated using the formula: 2 signal log ratio. During the comparison
10 analysis, each probe set on the experiment array was compared with its counterpart on the Veh baseline array to calculate the change P-value that was used to generate the change call of increase ($P < 0.0025$), marginal increase ($0.0025 < P < 0.003$), decrease ($P > 0.9975$),
15 marginal decrease ($0.997 < P < 0.9975$) or no change ($0.003 < P < 0.997$). Two sets of gene list were produced and genes were recited as potential candidates from an increase/decrease or marginal increase/decrease in 5 or more of the 16 comparisons between the 4 pairs. The
20 genes were sorted into non-coding RNAs and coding RNAs by using the absence or presence of Swiss Protein. The coding RNAs from the increase and decrease gene list were categorized into gene ontology (GO) terms by using DAVID, an online tool for identification of enriched
25 functional groups within gene lists. GO molecular function (level 2) was selected and the functional groups were ranked according to their threshold of EASE Score, which is a modified Fisher Exact p-value. The functional groups with p-value smaller or equal to 0.05
30 were considered as strongly enriched in the annotation categories.

Example 1: Quantitative analysis of acetylated histones

In the hippocampus and visual cortex, the ratio of
35 acetylated histone to non-acetylated histone was analyzed.

The results are shown in Fig. 1. Although histone

acetylation profiles somewhat differ between visual cortex and hippocampus, histone acetylation seems to prepare the visual cortex to enter the critical period.

5 Example 2: Comparison of acetylated H3 and H4 histones

 In the visual cortex, the ratio of acetylated histone to non-acetylated histone was analyzed for histone H3 and H4, respectively.

 The results are shown in Fig. 2. Values of
10 acetylated histones were expressed over their respective histones values. Histone H4 acetylation is also higher at P11 and P16 than at later stages of development but its fold change is not as dramatic as acetylated histone H3.

 Histone H3 and H4 hyperacetylation were observed in
15 the pre-critical period of the developing visual cortex. This phenomenon diminished during the peak of CP (P27) till adulthood P56. This result indicated that histone hyperacetylation prepares the visual cortex before entering the CP.

20

Example 3: Immunohistochemistry staining for acetylated histones

 The visual cortex was stained for the presence of acetylated histone H3.

25 The results are shown in Fig. 3. Histone acetylation was observed at the early stages of development, strengthening the need of histone acetylation to prepare the visual cortex before entering the critical period.

30 Example 4: Determination of proportion of unmethylated CpG islands

 Proportion of unmethylated CpG islands, i.e. the status of DNA demethylation was determined in DNA obtained from visual cortex.

35 The results are shown in Fig. 4. The peak of DNA demethylation coincided with the peak of critical period.

Example 5: Effect of dark-rearing on histone acetylation

To further investigate if the environment can impact the epigenome during the CP of brain development, just as sensory experiences shape neuronal function, two models were used to delay the onset of plasticity into adulthood: dark-rearing of mice from birth to adulthood (DR P56) and Gad 65 knockout (GAD KO) mice.

The results are shown in Fig. 5. Histone hyperacetylation was observed in the visual cortex in both DR P56 and GAD KO mice, indicating latent plasticity even in adulthood.

Example 6: Quantitative analysis of histone deacetylase activity

To confirm our findings, two HDAC inhibitors, valproic acid (VPA) and trichostatin A (TSA) were injected i.p. to adult mice and their effects on histone deacetylase (HDAC) activity were tested in visual cortex.

The results are shown in Fig. 6. VPA and TSA reduced HDAC activity within hours of administration, that is, both HDAC inhibitors can manipulate histone acetylation/deacetylation equilibrium significantly.

Example 7: Effects of HDAC inhibitors on brain plasticity

To investigate effects of HDAC inhibitors on brain plasticity, monocular deprivation (MD) was used as a model of experience-dependent plasticity. Occlusion of one eye during CP results in a shift of ocular dominance (OD) of the cortical neurons in favor of the non-deprived eye. This shift does not occur in adult mice after MD.

The results are shown in Fig. 7. Functional analysis showed that VPA-injected adult mice with MD showed a shift in OD. That is, no plasticity was

expressed with vehicle, but plasticity was expressed with VPA drug treatment showing a shift in ocular dominance and change in CBI values.

5 Example 8: Balance between histone acetylation and DNA demethylation predicts transient critical period in visual cortex

We first investigated the global distribution of histone acetylation in the binocular region of primary visual cortex. High levels of histone H3 acetylation (ACh3) was observed in the pre-critical period (P11 and P16) and was downregulated upon entering the critical period (CP) throughout to adulthood (Fig. 9a), consistent with Putiagano et al (2007). We confirmed that both histone H3 and H4 (ACh4) acetylation occurred in similar fashion using immunoblot of acid-extracted histones (Fig. 9a). Both ACh3 and ACh4 were observed in the pre-CP (<P21) of developing visual cortex, diminished by the peak of the CP (P27) and throughout to adulthood (>P55) (Fig. 8d and 9a). However, ACh3 was more pronounced in intensity than ACh4 (Fig. 8d), which could be a natural occurrence in development.

We next investigated the spatial distribution of histone acetylation in the developing visual cortex by immunofluorescence. Consistent with our western blot results, high ACh3 was observed in early postnatal ages and its expression was diminished at the peak of CP (P27) throughout to adulthood (Fig. 8a). On closer inspection of the immunosections, we found that there was layer-specificity of ACh3 at different stages of development. During the early stages of visual cortical development, ACh3 was observed more in the supragranular layer than the infragranular layer (Fig. 8a and b bottom panel). By P16, the pattern of histone acetylation distribution penetrated further into the infragranular layer and the staining in the supragranular layer was

reduced. This continued to just before the start of critical period at P20 and diminished completely at the peak of CP (P27) and throughout to adulthood (P56). Histone AcH3 staining was nucleated in neurons when
5 double-stained with DAPI (Fig. 8b top panel).

Histone acetyltransferase (HAT) activity is a direct measurement of histone acetylation level. Histone acetyltransferase enzymes are responsible for acetylation of histones while histone deacetylases
10 (HDACs) are for deacetylation of histones. Equilibrium between HATs and HDACs is necessary for controlled levels of gene transcription. Nuclear extracts taken from early postnatal age at P11 have considerably higher HAT activity than adults (Fig. 8c), further supporting
15 our observation.

This was a surprisingly unexpected result as we expected during the peak of CP, where there is high plasticity in the visual cortex, there is a sudden shutdown of histone acetylation. Previous microarray
20 studies have shown that during the peak of CP, there is high expression of gene transcription factors (Sur and Shatz). Thus, there must be another mechanism that governs the chromatin status and we chose to investigate DNA methylation, which is an opposing global chromatin
25 alteration to histone acetylation. There was a switch between low DNA demethylation pre-CP, followed by a peak of DNA demethylation during the CP and reverting to low DNA demethylation at adulthood (Fig. 8d). Taken together, high histone acetylation and low DNA
30 demethylation may prepare the visual cortex to enter the CP. During the peak of CP, low histone acetylation and high DNA demethylation may drive the expression of the CP. At adulthood, low histone acetylation and low DNA demethylation lead to no expression or plasticity in the
35 visual cortex. A balance between these two global

epigenomic changes may be essential to create the critical period window in the visual cortex.

Example 9: Inverted DNA demethylation / histone

5 acetylation balance persists throughout life in the
hippocampus

We wondered the specificity of this balance of DNA methylation and histone acetylation creating plasticity. To test this idea, we investigated another region of the
10 brain, the hippocampus. Histone acetylation has been reported to increase with aberrant neural activity but stay low at basal levels (Huang et al 2002, Tsankov et al 2005, and Sng et al 2006). Tissue-specific patterns of DNA methylation are established early during
15 development as a consequence of cellular differentiation (Monk et al 1987, Levenson et al 2006) and are usually very high during early development (Moshe Szyf et al). Levenson et al has shown that both histone acetylation and DNA methylation have important roles in long term
20 memory formation.

To our surprise, we found that ACH3 status in the hippocampus exhibited a profile distinct from that of visual cortex with close to unacetylated levels of histone H3 at all other ages, except a transient rise in
25 P16 (Fig. 9b, c and d). On the other hand, DNA demethylation rises steadily throughout life (Fig. 9d). This was consistent with hippocampus's lifelong plasticity and ruled out the correlation of both chromatin modifications and their being an age-dependent
30 phenomenon.

Example 10: Sensory experience regulates the balance
between histone acetylation and DNA demethylation

We then investigated whether the environment
35 impacts the epigenome during the CP of visual development, just as sensory experience shapes neuronal

function. We assayed two mouse models of delayed CP onset: dark-rearing from birth to adulthood (DR) and GAD65 deletion (GAD65 KO). In our findings, we see a pattern of low histone acetylation and high DNA demethylation at the peak of critical period, P27. We wanted to understand whether the findings are an age-dependent phenomenon or experience-dependent resultant. Thus, the use of both DR and GAD65 KO can resolve this question. Dark rearing creates no sensory experience for such animals and GAD65 KO have sensory experience but the inability to translate this experience to a critical period window.

First, we investigated the global status of AcH3 for both models. As predicted, both DR and GAD KO models showed significantly higher levels of AcH3 even at adulthood consistent with their latent plasticity (Fig. 10d, lanes 2 and 5). We confirmed the same data with immunofluorescence showing high levels of AcH3 in DR and GAD65 KO mice (Fig. 10a DR, b GAD KO). However, animals showed different laminar profiles. Histone H3 acetylation was observed in adult DR (Fig. 10a) with almost equal distribution of AcH3 in both supragranular and infragranular layers (Fig. 10c). On closer examination, its profile resembled our observation in postnatal P16-P20 staining (Fig. 8a). Meanwhile, GAD65 KO adult mice were also observed to maintain high levels of AcH3 but the distribution of AcH3 was more in the supragranular layer than the infragranular layer (Fig. 10b and c). We also checked the subsequent sequential sections of hippocampus of GAD65 KO mice and found no staining (data not shown).

We previously found that DR adults exposed to light for two or seven days (+2d and +7d respectively) began the onset of CP. If exposure to light triggers a change in visual cortical plasticity, we should see a change in AcH3 and DNA demethylation after light exposure. As

expected, immunoblot analysis revealed light exposure of dark-reared animals significantly reduced H3 acetylation levels (Fig. 10d and e). We also observed a rapid reduction of AcH3 staining in the visual cortex within 5 days in both supra- and infragranular layers (Fig. 10a and c). Two days of light exposure matured the visual cortex to an intermediary of P20 and P27 (Fig. 10a and Fig. 8b). Seven days of light exposure resembled the low levels seen in adults P56, consistent with no 10 plasticity. We next focused on the window in which we recreate CP in dark-reared animals by exposure to light with a consistent low histone acetylation and high DNA demethylation to represent the peak of plasticity. In support to what we observe at P27 (Fig. 8d), we see the 15 same results of an increase in DNA demethylation and reduction in AcH3 when dark reared animals are exposed to light (Fig. 10f). This evidence strengthened our hypothesis that the high plasticity during the critical period window is expressed when the balance of low 20 histone acetylation and high DNA demethylation is achieved.

Example 11: Histone hyperacetylation by valproic acid reactivate plasticity in adulthood

25 Our next question is whether our hypothesis is true, we should be able to reopen plasticity in adults by recreating this low histone acetylation and high DNA demethylation profile. We decided to manipulate the equilibrium between histone acetylation and 30 deacetylation using available pharmacological drugs inhibiting the enzyme that catalyses histone deacetylation reaction. This in turn causes massive histone hyperacetylation. There are two histone deacetylase (HDAC) inhibitors, valproic acid and 35 trichostatin A. Valproic acid (VPA) is a clinically used as antiepileptic drug and has recently been found

to have HDAC inhibitory actions. It has been used already in humans for several decades and we wonder how its use can affect plasticity in the brain. Trichostatin A (TSA) was used in a previous study and showed that

5 with its inhibitory action on HDAC, it can affect histone acetylation status (Putiagno et al). However, our concern is that TSA is still under clinical trials and may take years before it can be clinically available to be used in humans. First, we checked whether both

10 drugs administered intraperitoneally can cause histone hyperacetylation in the adult visual cortex. VPA worked as well as TSA in decreasing HDAC activity within hours after administration (Fig. 11a). VPA, similar to TSA, significantly reduced HDAC activity by 30% within hours

15 after administration. Conversely, we checked the HAT activity by direct measurement of the levels of histone acetylation by western blot. VPA administration simultaneously induced histone H3 hyperacetylation (Fig. 11b and c solid red line with closed triangles) and

20 decreased in DNA demethylation (Fig. 11c, solid black line with closed squares). This confirmed our finding of high histone acetylation balanced with low DNA demethylation in pre-CP and the administration of VPA re-created a transient pre-CP at 2h after administration.

25 Continual administration of VPA then restored DNA demethylation as histone acetylation returned to its low level. There was no change in histone acetylation or DNA demethylation status in vehicle-treated mice (Fig. 11c, red/black dotted lines with open triangles/squares).

30 We tested our findings by causing histone hyperacetylation using valproic acid (VPA) in adult mice experiencing monocular deprivation (MD) as a model of experience-dependent plasticity. Occluding one eye during the CP results in a shift of ocular dominance

35 (OD) of cortical responsiveness in favor of the open eye, which is not observed after the CP. Functional analysis

revealed that adult mice injected continually with VPA for 6d had shifted their OD after brief 4-day of MD. Brief MD induces a significant CBI reduction after VPA administration. CBI indicates distribution bias in favor of the contralateral eye and VPA injection showed a significant reduction in CBI in comparison to vehicle-treated mice (Fig. 12a and b). VPA did not affect the orientation bias preference of the mice (Fig. 12c). VPA and diazepam (DZ) are both anti-epileptic drugs. We wanted to show that both work on different mechanistic pathways to manipulate visual cortical plasticity. Adult mice with either no MD or MD and treated with VPA or DZ were assessed by their CBI values. While DZ showed no change in CBI values as in adult with no MD, VPA showed a reduction in CBI value (Fig. 12d). Putigano et al (2007) also demonstrated ocular dominance plasticity in WT adult mice with TSA injection which supports our work that the use of HDAC inhibitor can reactivate adult plasticity.

20

Example 12: Histone hyperacetylation occurs in all cell types, including parvalbumin cells

GABAergic neurons contain high levels of parvalbumin, both in their soma and neurites. Parvalbumin is a slow Ca^{2+} buffer that may affect the amplitude and time course of intracellular Ca^{2+} transients in terminals after an action potential, and in turn regulate short-term synaptic plasticity (Caillard et al, PNAS 2000).

We wanted to investigate what cell types are responsible for the recreation of critical period in adulthood. We focused on parvalbumin cells as they are known to be the trigger of critical period and GABA. We visualized the extent of the histone acetylation after two and six days of 12 hourly administration of VPA in the visual cortex. The sections were double-stained

with AcH3 (green) and parvalbumin (red) antibodies.

Histone H3 acetylation were observed in all laminar layers of the visual cortex after 2h of VPA

administration but disappeared at 6d (Fig. 11d, top

5 panel). Under high magnification, many cell types, including parvalbumin cells were colocalized with AcH3 after 2h of VPA administration (Fig. 11d, bottom panel)

but there was little or no colocalization in the

parvalbumin cells at 6d. The number of colocalization

10 of histone H3 hyperacetylation occurring in parvalbumin cells is higher in P18 than adults. After 2h of VPA administration, the number of colocalization of PV cells with AcH3 positive cells increased more than at P18 (Fig.

11e). This indicates that the VPA treatment reignited

15 the histone acetylation levels in parvalbumin cells in adulthood.

Example 13: Histone hyperacetylation upregulates non-coding genes and reactivates adult plasticity

20 Taking a step further, we wanted to examine the mechanism behind reactivation of plasticity. We also wanted to confirm that the change in epigenetic status is a causal effect and not a consequence for the critical period plasticity. We were also able to

25 identify whether VPA is truly working epigenetically on the gene expression states. We used commercial genechip analysis to see the changes in gene expression after 2h of VPA treatment. A total of 796 were up-regulated and 480 were down-regulated in VPA-treated mice compared to

30 vehicle-treated mice. Interestingly, from the transcripts that were induced, 27% were non-coding genes and only 2% of the suppressed transcripts were non-

coding. In addition, we analyzed the data comparing

development data of mice postnatal ages P16 and P27, and

35 found very few age-specific overlap of gene sets (data not shown) with some non-coding overlaps after VPA

increase with P16. Thus, we hypothesized the non-coding genes have a role in either directly or indirectly via suppression of plasticity-related genes on the ocular dominance plasticity.

5 We separated the gene lists into up- or down-regulated and categorized them into their functional groups, focusing on the molecular functional aspects. From that, we can tell that VPA at 2h increases gene expression that act mainly in the cellular nucleus and
10 protein binding. Several genes are responsible for chromatin remodeling such as histone acetyltransferases (N-myristoyltransferase 2), RNA II polymerase (TAF4A, Kruppel-like factor 13), SWI/SNF. DNA-directed beta polymerase and upstream transcription factor 2 were
15 upregulated indicative of transcriptional activation. Not surprisingly, we see another gene subset that was downregulated nuclearly possibly to assist the DNA methylation process. They are mainly DNA binding protein chromodomain helicase, another family of SWI SNF
20 and we see a reduction in CREB binding protein (which has known HAT activity). But we see a reduction of myelin-related expression: myelin transcription factor, myelin basic protein and myelin-associated oligodendrocytic basic protein. We also see a decrease
25 in expression of an inhibitor of tissue protein metalloproteinase. We proposed that VPA reduce myelination and tissue process and allow plasticity and restructuring of the visual cortex to occur.

We found that there are some splicing of 3 genes
30 that were up and down regulated at the same time, namely NCAM1, calcium/calmodulin dependent protein kinase II and activated leukocyte adhesion molecule.

Lastly, we see a decrease in myocyte enhancer factor 2C which was reported to suppress excitatory
35 synapse number (Flavell et al, 2006). We also see an increase of ionotropic glutamate receptors AMPA2 alpha

and kainate 2 beta and a decrease in GABA receptor 1, thus we see a change in excitatory-inhibitory balance after VPA treatment.

5 While this invention has been described with an emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that variations of the preferred embodiments may be used and that it is intended that the invention may be practiced otherwise
10 than as specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the claims.

 All of the references cited herein, including
15 patents, patent applications, and publications, are hereby incorporated in their entireties by reference.

Industrial applicability

 The present invention provides a novel use of HDAC
20 inhibitor and DNMT inhibitor for regulating brain plasticity by altering the epigenetic state. Using the agent according to the present invention, brain plasticity can be reactivated even in adulthood. Although in this document we have used mice, epigenetic
25 modifications are general mechanisms that are generally conserved through evolution: therefore, modifications observed in mice represent modifications that are applicable to all mammals including human subjects. Therefore, the agent of the present invention is useful
30 for the remedy or prevention of various diseases, disorders and/or conditions wherein the reactivation of brain plasticity is necessary or suitable for the remedy or prevention. Since some of the active ingredient of the present invention including valproic acid and the
35 like has been clinically used as a medicament with

safety for a long time, they have a high possibility to be put into practice.

This application is based on JP patent application
5 No. 2007-102150 filed on April 9, 2007, the contents of which are incorporated in full herein by this reference.

CLAIMS

1. An agent for regulating brain plasticity, which comprises a substance affecting an epigenetic state
5 selected from the group consisting of histone acetylation and DNA methylation.
2. The agent according to claim 1, wherein the substance induces histone hyperacetylation or DNA
10 demethylation.
3. The agent according to claim 2, wherein the substance is a histone deacetylase inhibitor or a DNA methyltransferase inhibitor.
15
4. The agent according to claim 2, wherein the histone deacetylase inhibitor is selected from the group consisting of a short chain fatty acid or a derivative thereof; a hydroxamic acid derivative; a Benzamide
20 derivative; a cyclic tetrapeptide; an electrophilic ketone derivative; and a psammaplin or Depudecin.
5. The agent according to claim 4, wherein the histone deacetylase inhibitor is valproic acid or trichostatin A,
25 or a salt thereof.
6. The agent according to any of claims 1-5, which is for treating or preventing one or more diseases or conditions selected from the group consisting of
30 amblyopia, autism spectrum disorders, fragile X syndrome, Rubinstein-Taybi syndrome, mental retardation, Rett's syndrome, schizophrenia, bipolar disorder, Alzheimer's disease, depression, stroke, spinal muscular atrophy, brain lesion and ischemia.

35

7. A method for regulating brain plasticity in a mammal, which comprises regulating an epigenetic state selected from the group consisting of histone acetylation and DNA methylation in said mammal.

5

8. A method for regulating brain plasticity in a mammal, which comprises administering an effective amount of the agent according to any one of claims 1-6 to the mammal.

10

9. A use of a substance affecting an epigenetic state selected from the group consisting of histone acetylation and DNA methylation, for production of an agent for regulating brain plasticity.

15

10. A substance affecting an epigenetic state selected from the group consisting of histone acetylation and DNA methylation, for use as an agent for regulating brain plasticity.

20

11. A commercial package comprising the agent according to any one of claims 1-6 and a written matter which states that the agent can or should be used for regulating brain plasticity.

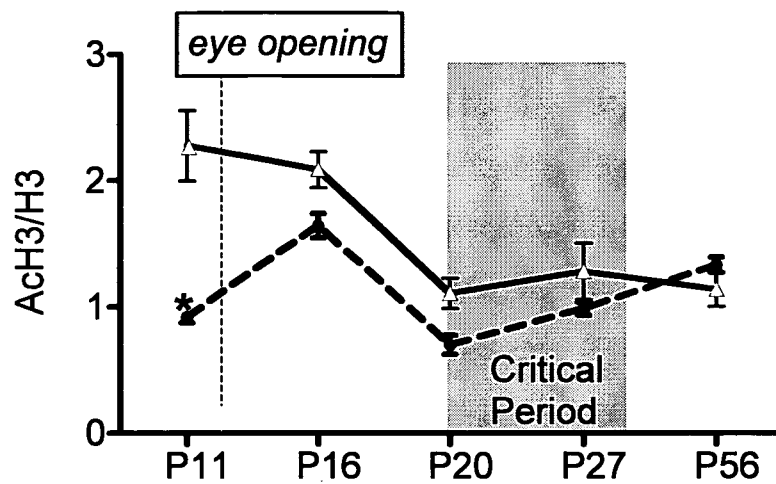
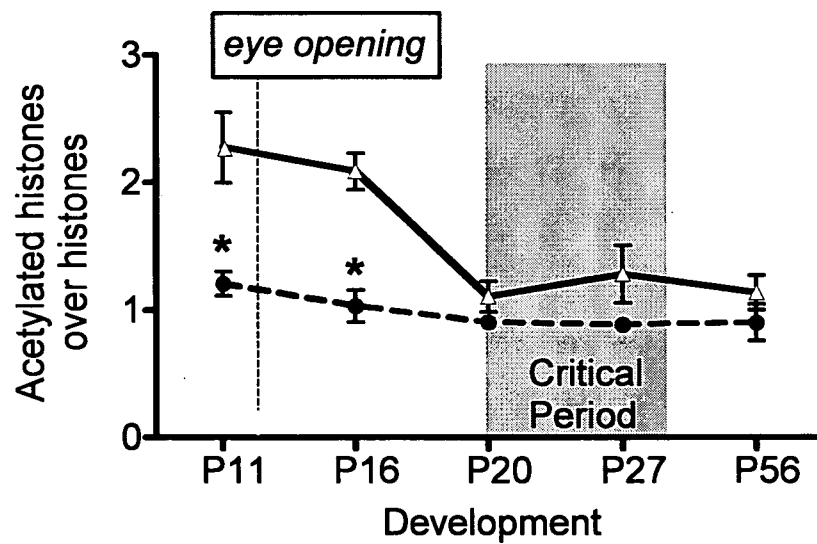
FIG. 1**FIG. 2**

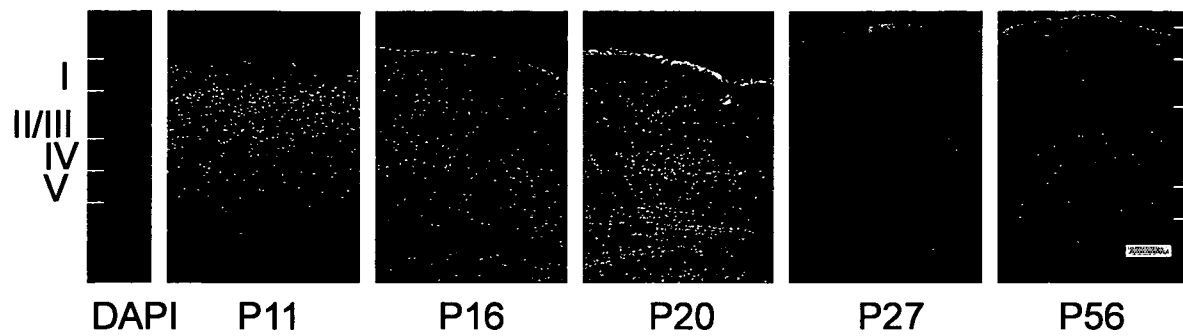
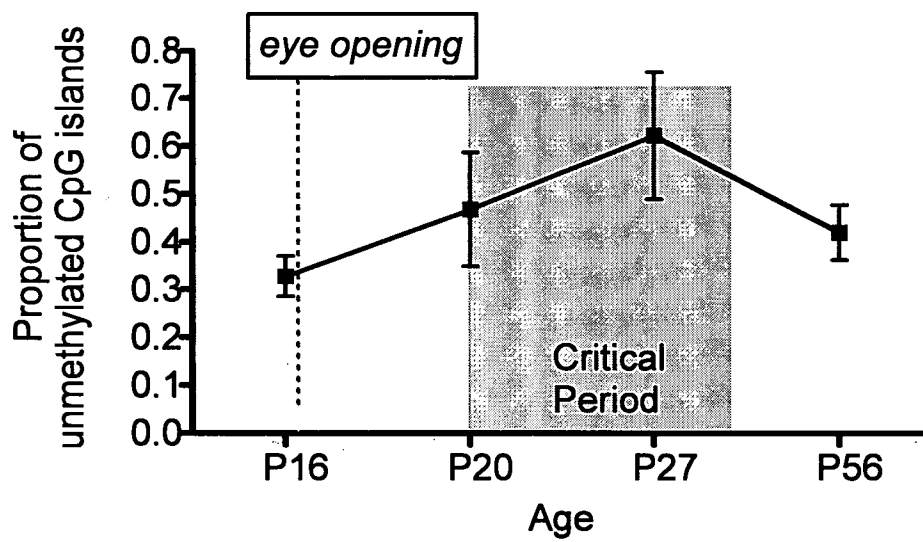
FIG. 3**FIG. 4**

FIG. 5

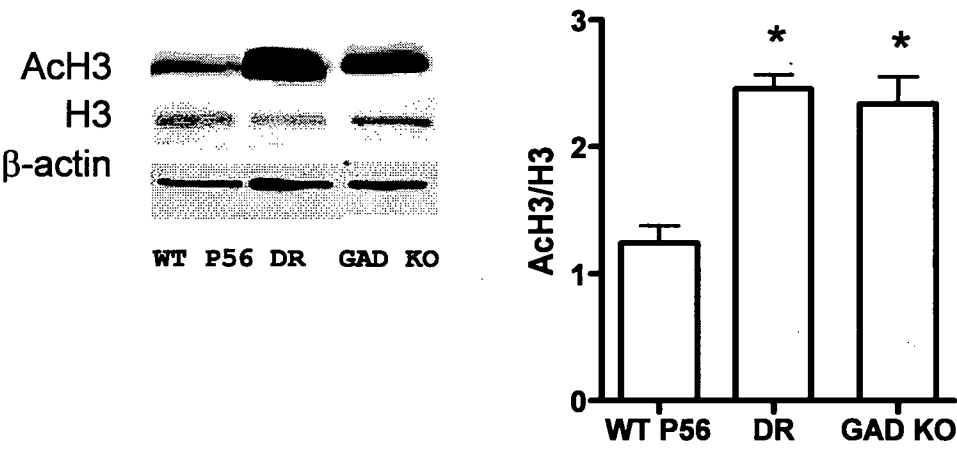


FIG. 6

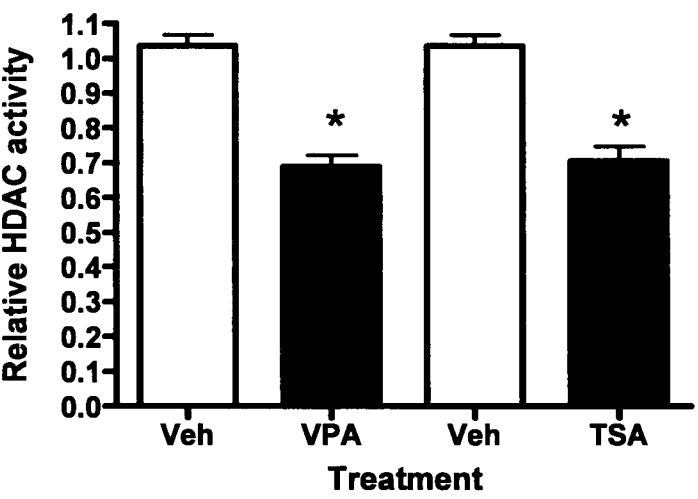


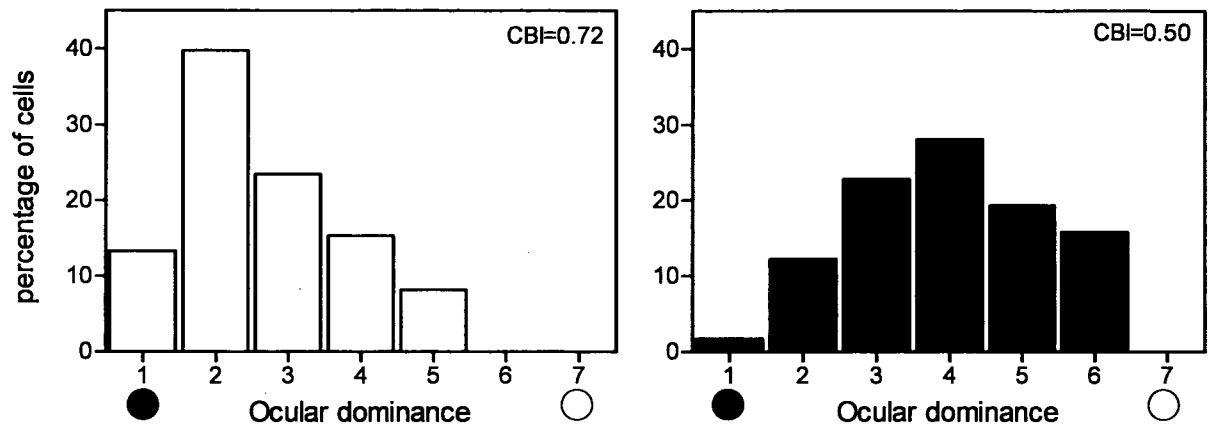
FIG. 7

FIG. 8

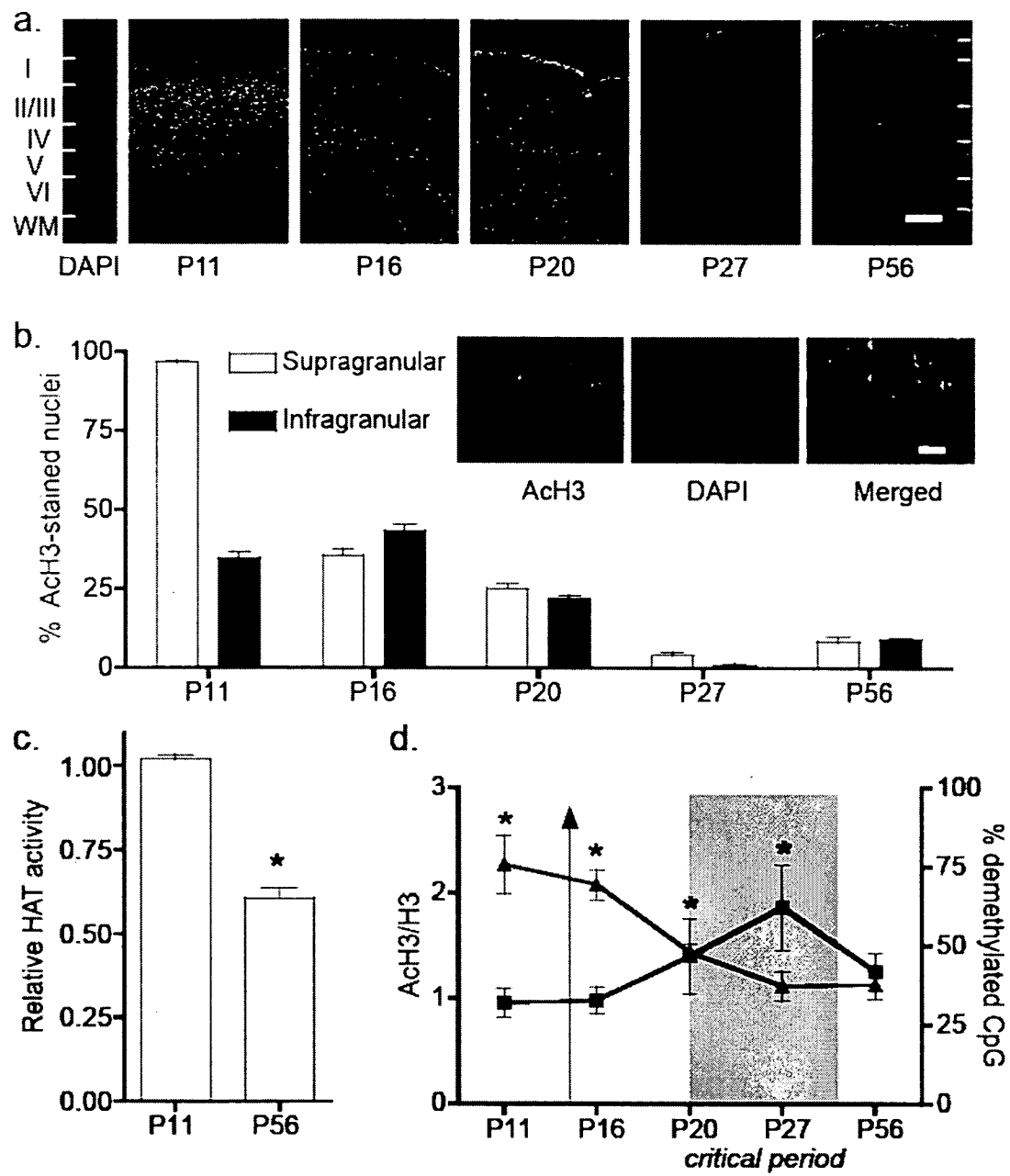


FIG. 9

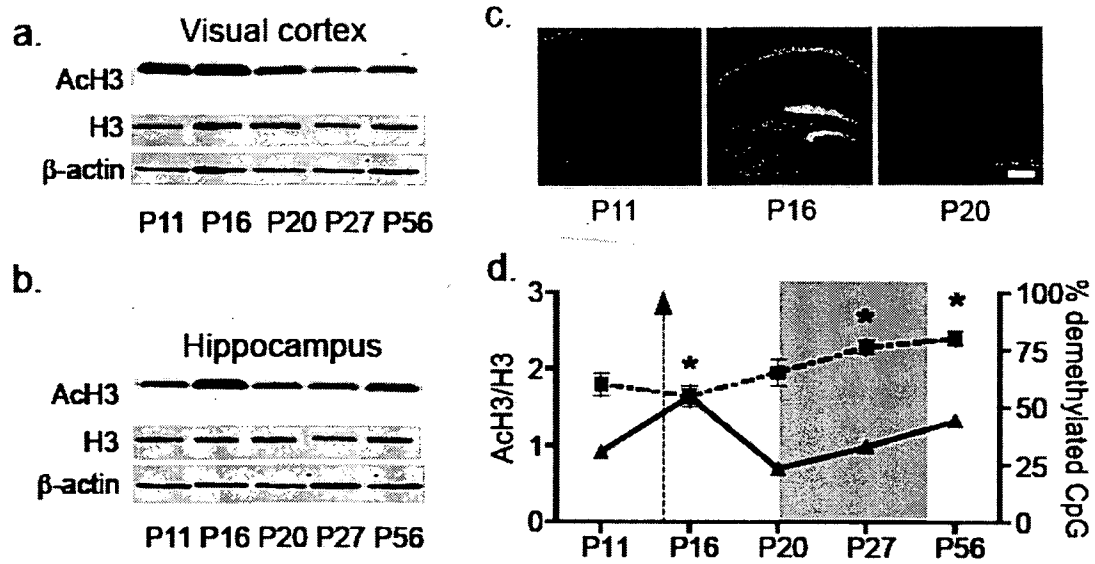


FIG. 10

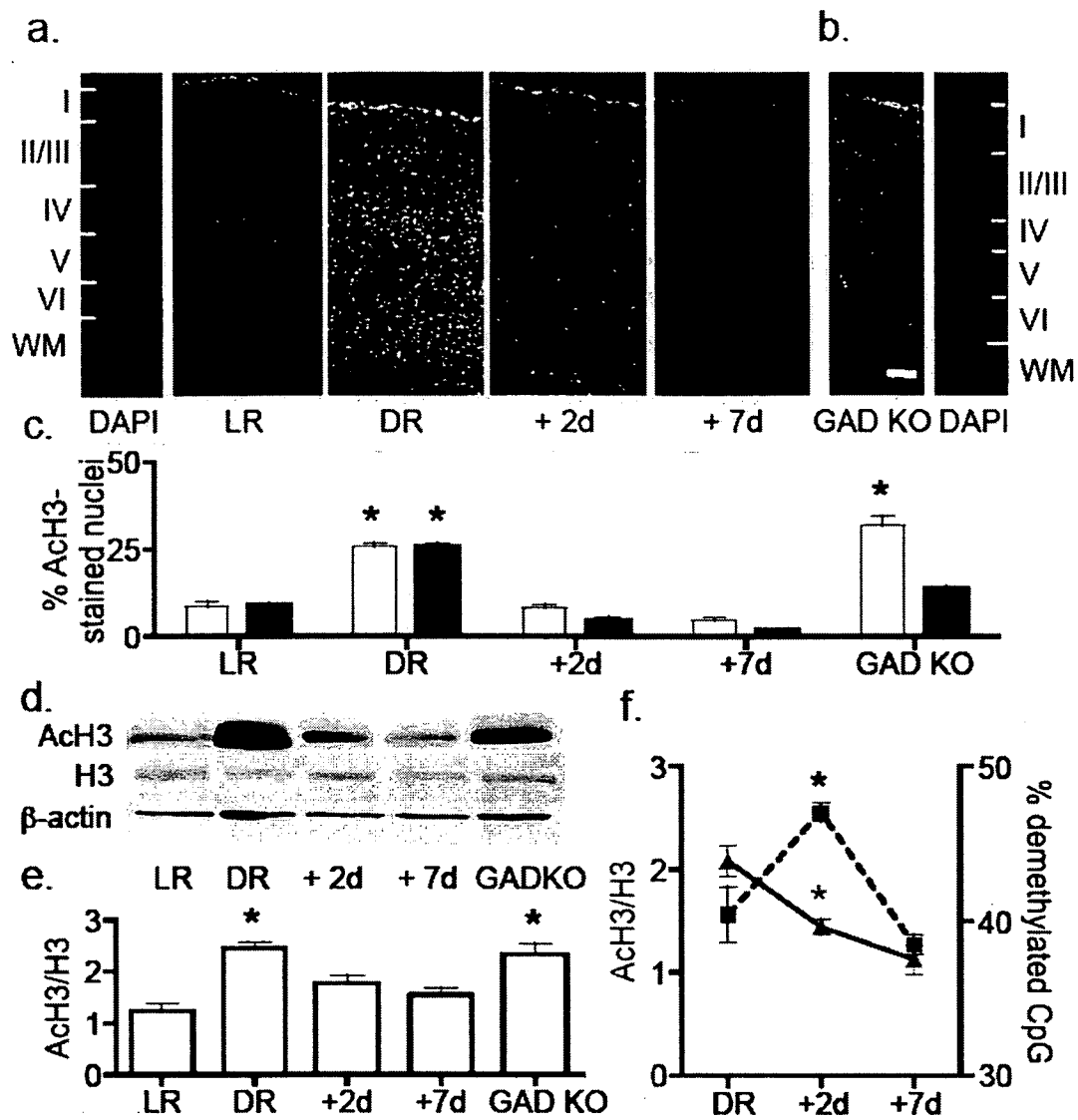


FIG. 11

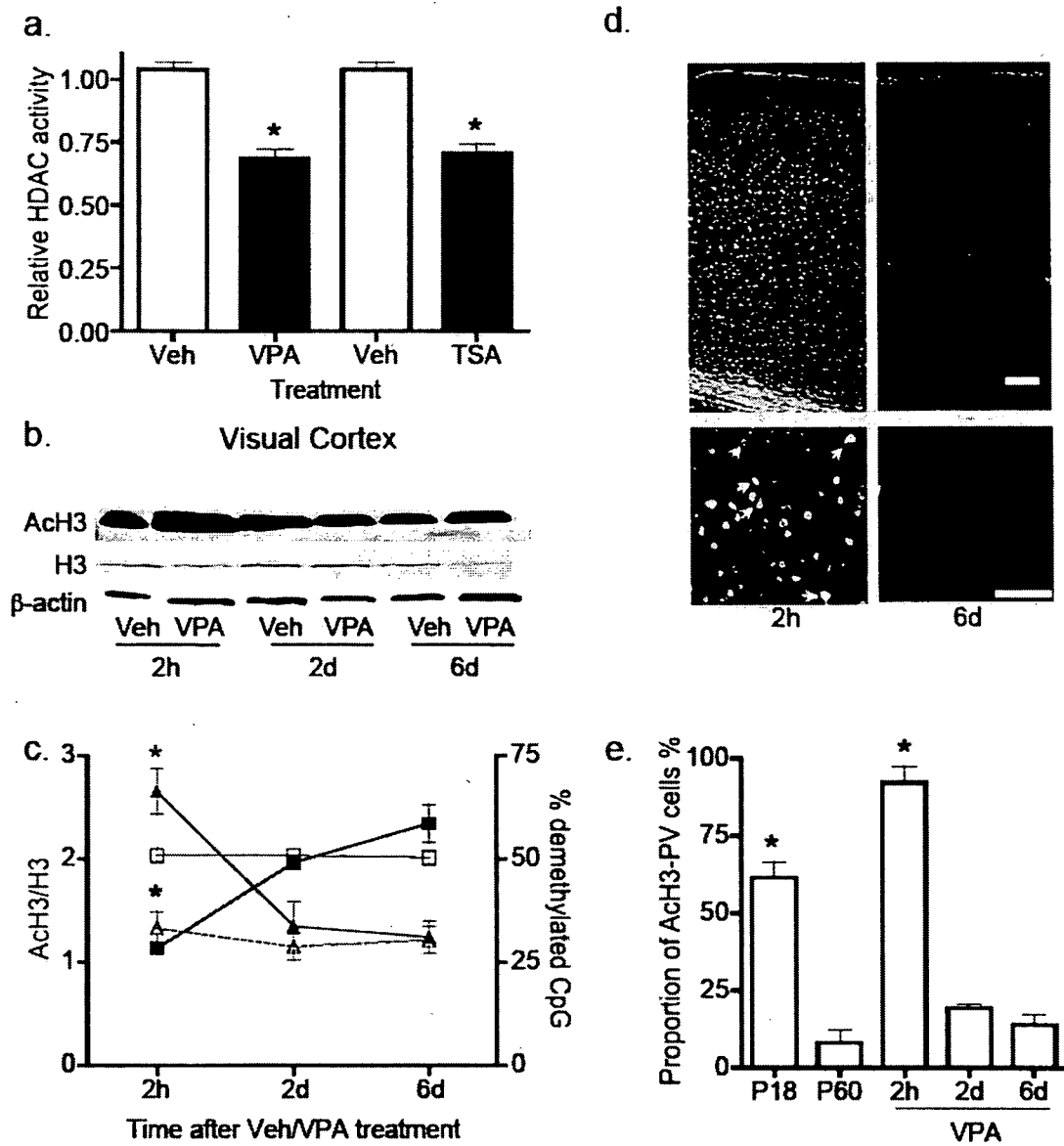


FIG. 12

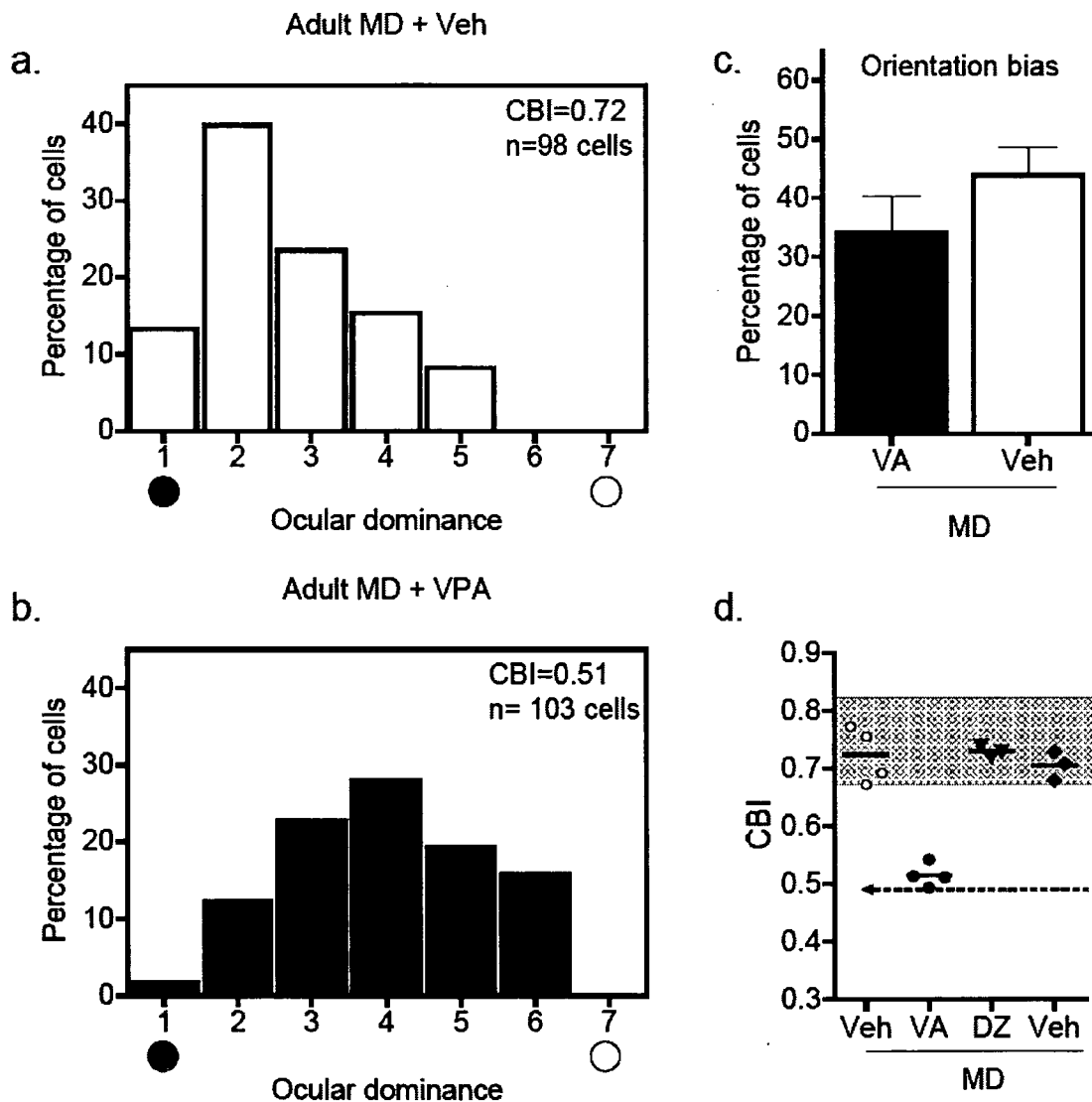
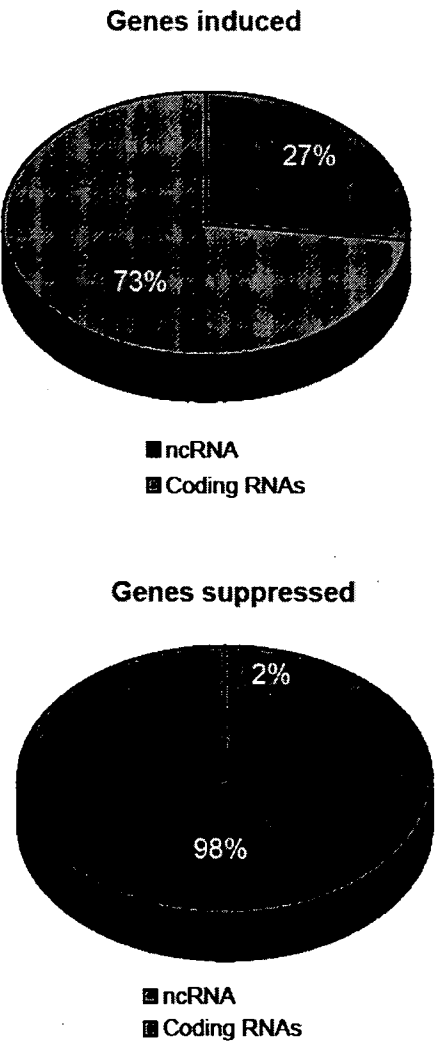


FIG. 13

a.



b.

