Histone Deacetylase Inhibitors Exhibit Anti-Inflammatory and Neuroprotective Effects in a Rat Permanent Ischemic Model of Stroke: Multiple Mechanisms of Action

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ABSTRACT

The pathophysiology of cerebral ischemia involves multiple mechanisms including neuroinflammation mediated by activated microglia and infiltrating macrophages/monocytes. The present study employed a rat permanent middle cerebral artery occlusion (pMCAO) model to study effects of histone deacetylase (HDAC) inhibition on ischemia-induced brain infarction, neuroinflammation, gene expression, and neurological deficits. We found that post-pMCAO injections with HDAC inhibitors, valproic acid (VPA), sodium butyrate (SB), or trichostatin A (TSA), decreased brain infarct volume. Postinsult treatment with VPA or SB also suppressed microglial activation, reduced the number of microglia, and inhibited other inflammatory markers in the ischemic brain. The reduction in levels of acetylated histone H3 in the ischemic brain was prevented by treatment with VPA, SB, or TSA. Moreover, injections with HDAC inhibitors superinduced heat-shock protein 70 and blocked pMCAO-induced down-regulation of phospho-Akt, as well as ischemia-elicited up-regulation of p53, inducible nitric oxide synthase, and cyclooxygenase-2. The motor, sensory, and reflex performance of pMCAO rats was improved by VPA, SB, or TSA treatment. The beneficial effects of SB and VPA in reducing brain infarct volume and neurological deficits occurred when either drug was administered at least 3 h after ischemic onset, and the behavioral improvement was long-lasting. Together, our results demonstrate robust neuroprotective effects of HDAC inhibitors against cerebral ischemia-induced brain injury. The neuroprotection probably involves multiple mechanisms including suppression of ischemia-induced cerebral inflammation. Given that there is no effective treatment for stroke, HDAC inhibitors, such as VPA, SB, and TSA, should be evaluated for their potential use for clinical trials in stroke patients.

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vehicle used for VPA and SB was normal saline, and for TSA, the surgery except that the thread was not inserted. Animals were surgery with a heating pad. Sham-operated rats underwent identical body temperature was maintained at 36 to 37°C throughout the approximately 20% of the preischemic basal value (Ren et al., 2003). The cerebral blood flow in the ischemic brain area dropped to approxi-
mately 20 min after MCAO, the reperfusion procedure was omitted. In brief, after incision of

1.5 to 3% halothane during surgery. Focal cerebral ischemia was induced by 3% halothane (70% N2O/30% O2) and maintained with

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Plasma levels used to treat bipolar disorder (50–125 ng/ml). Approximately 300 mm³ in the ipsilateral brain hemisphere were calculated. pMCAO caused a cerebral infarct volume of approximately 300 mm³ in the ipsilateral brain hemisphere within 6 h after VPA injection, and exhibits neuroprotective effects on the ipsilateral hemisphere in a transient rat MCAO model (Ren et al., 2004). Brain damage induced by another injection at 12 h. Rats were sacrificed at 24 h, and brain infarction in coronal sections was detected by TTC staining. A, chemical structure of VPA. B, TTC staining from a typical vehicle (normal saline)- or VPA (300 mg/kg s.c.)-treated rat. C, brain infarction volume was quantified from a group of vehicle- and VPA-treated animals. Data are mean ± S.E.M. from six rats in each group. D, chemical structure of SB. E, TTC staining from a typical vehicle (normal saline)- or SB (300 mg/kg s.c.)-treated rat. F, rats subjected to pMCAO were also injected with various doses of SB as indicated, and infarct volumes were determined in vehicle- or SB-treated animals. Data are mean ± S.E.M. from six rats in each group. G, chemical structure of TSA. H, TTC staining from a typical vehicle (DMSO)- or TSA (0.5 mg/kg s.c.)-treated rat. I, brain infarct volumes were quantified from a group of vehicle- or TSA-treated rats. Data are mean ± S.E.M. from a group of six rats in each group. *** p < 0.001 compared with the respective vehicle-treated group.

Results

Treatment with HDAC Inhibitors Reduces pMCAO-Induced Brain Infarct Volume. Three HDAC inhibitors, VPA, SB, and TSA, were examined for their effects on pMCAO-induced brain infarction, and their chemical structures are shown (Fig. 1, A, D, and G). To examine the neuroprotective effects of VPA against ischemia-induced brain damage, rats subjected to pMCAO were treated with 300 mg/kg VPA by s.c. injection every 12 h starting immediately after the onset of insult. Our previous study has shown that VPA at this dose inhibits HDAC activity, as shown by an increase in histone H3 acetylation in the intact contralateral brain hemisphere within 6 h after VPA injection, and exhibits neuroprotective effects on the ipsilateral hemisphere in a transient rat MCAO model (Ren et al., 2004). Brain damage was detected by TTC staining, and the infarct volume was calculated. pMCAO caused a cerebral infarct volume of approximately 300 mm³ in the ipsilateral brain hemisphere 24 h after ischemic onset, and postinsult VPA treatment decreased the infarct size by approximately 37% (Fig. 1, B and C). At this time, the plasma levels of VPA were 54.2 ± 3.5 µg/ml (n = 12), which is in the lower range of the therapeutic plasma levels used to treat bipolar disorder (50–125 µg/ml). Treatment with SB (300 mg/kg), another fatty acid HDAC inhibitor, by s.c. injection immediately after pMCAO followed by another injection 12 h later also decreased brain infarction determined at 24 h (Fig. 1E). The effect was dose-dependent with an approximately 50% decrease in infarct volume at the dose of 200 to 300 mg/kg, whereas the protective effect was less pronounced at higher doses, notably 700 mg/kg, which induced only a 22% decrease (Fig. 1F). These effective doses of SB were comparable with those used in mice by other investigators (Ferrante et al., 2003; Ryu et al., 2003). Likewise, postinsult injections with TSA (0.5 mg/kg s.c. immediately after pMCAO followed by another injection 12 h later), a potent hydroxamic acid HDAC inhibitor, decreased pMCAO-induced brain infarction by 34% (Fig. 1, H and I), compared with the vehicle (DMSO) control. The dose of TSA was also comparable with those used in mice and rats (Korzus et al., 2004; Leng and Chuang, 2006).

Treatment with VPA Exhibits Anti-Inflammatory Properties in the Ischemic Brain. We assessed effects of
VPA treatment on microglia activation associated with pMCAO, which plays a critical role in ischemia-induced neurodegeneration (Gregersen et al., 2000). Microglia express complement type 3 receptors (CD11b/CD18 complex), which are recognized by the monoclonal antibody against OX-42 in the rat (Stoll et al., 1998). OX-42 immunostaining of microglia revealed the presence of activated microglia characterized by larger, amoeboid morphology in the ischemic corpus callosum 24 and 48 h after pMCAO (Fig. 2, A–C). Postinsult VPA administration suppressed pMCAO-induced microglia activation in the corresponding brain area of treated rats at both time points. The numbers of OX42-positive microglia at the anterior, middle, and posterior sections of the ipsilateral brain hemisphere were all significantly reduced by VPA treatment at both 24 and 48 h (Fig. 2, D and E). We also examined the effects of VPA on the number of cells stained positively by an antibody to ED1, a marker for monocytess/macrophages and, to some extent, for activated microglia (Stoll et al., 1998). Monocytes/macrophages contribute to neuroinflammation by infiltrating into the damaged brain area due to disruption of the blood-brain barrier after cerebral stroke (Gregersen et al., 2000). ED1-positive cells, characterized by a larger round shape, were detected in the ischemic frontal cortex, and this ED1 immunostaining determined 24 and 48 h after pMCAO was suppressed by VPA treatment in the corresponding brain area (Fig. 3, A and B). Quantified results showed that the numbers of ED1-positive cells in the anterior, middle, and posterior sections of the ipsilateral hemisphere were all markedly decreased by VPA treatment determined at both time points (Fig. 3, C and D).

It has been reported that iNOS is induced in the brain after ischemic stroke (Nogawa et al., 1998). Immunohistochemical staining of iNOS confirmed that its immunoreactivity was undetectable in the striatum of the contralateral hemisphere but abundantly present in cells as well as vascular structures in the ischemic striatum 24 h after pMCAO (Fig. 4, A, C, and E). VPA treatment essentially depleted iNOS immunoreactivity in the ischemic striatum (Fig. 4, D and F). Similar inhibitory effects of VPA on iNOS immunoreactivity were found in the ischemic frontal cortex (Fig. 4, G–J).

**VPA and TSA Regulate Levels of Proteins Related to Apoptosis.** Western blotting was performed to assess changes in levels of cytoprotective and apoptotic proteins. The levels of acetylated histone H3 were decreased 24 h after pMCAO in the ipsilateral brain hemisphere, and this reduction was blocked by postinsult VPA injections (Fig. 5, A and B), suggesting VPA-induced inhibition of HDAC activity. The levels of cytoprotective HSP70 in the ischemic, ipsilateral hemisphere were increased by pMCAO compared with the sham-operated control, and this increase was potentiated by VPA treatment (Fig. 5, A and C). The levels of p53, a transcription factor and proapoptotic protein negatively regulated by HDACs (Juan et al., 2000), were also markedly increased in the ischemic hemisphere compared with the sham group, and this p53 up-regulation was suppressed by VPA treatment (Fig. 5, A and D). The protein levels of β-actin used as loading control were unchanged under these experimental conditions.

Similar to VPA, TSA treatment blocked the loss of acetylated histone H3 and potentiated HSP70 induction but suppressed p53 up-regulation in the ischemic brain hemisphere (Fig. 5E). In addition, levels of two cytoprotective factors, Bcl-2 and phospho-Akt, were decreased after pMCAO in the ischemic brain, compared with the sham-operated control, and these effects were inhibited by TSA injections.

**SB Also Shows Anti-Inflammatory and Antiapoptotic Effects in Ischemic Rats.** Similar to the effects of VPA, SB treatment suppressed the activation and number of microglia detected by OX42-immunostaining in the infarcted corpus callosum (Fig. 6A). Quantification of OX42-positive cells revealed that their numbers were decreased by almost 30% in the anterior, middle, and posterior sections of the ischemic hemisphere (Fig. 6B). Western blots showed that...
**Discussion**

The present study employed a rat pMCAO model to investigate the neuroprotective effects of VPA, SB, and TSA. Compared with transient MCAO, pMCAO produces a more severe and rapid brain infarction with a smaller and more short-lived penumbra. The process of pMCAO also favors the mechanisms of necrotic-type cell death, presumably due to rapid loss of glucose utilization and protein synthesis following permanent ischemia (for review, see Lo et al., 2003). Despite the severity of brain pathology induced by pMCAO, we found that postinsult treatment with VPA, SB, or TSA effectively reduced brain infarction and suppressed neurological deficits (Figs. 1 and 8). The dose of VPA (300 mg/kg s.c. twice daily) produced a level of 54 μg/ml (or 0.33 mM) in the plasma. The

**The Beneficial Time Window for VPA and SB Is at Least 3 h after Ischemic Onset.** We next examined whether beneficial effects of VPA and SB were observed by delayed administration of either drug. When VPA (300 mg/kg) was first injected 3 h after pMCAO, a significant decrease (by 32.7%) in infarct volume determined at 24 h was observed (Fig. 9A). This protective effect in the pMCAO rats was accompanied by a significant increase in the neurological scores on the eight-point test (Fig. 9B). The Rotorod test has been validated as a means of assessing motor coordination in rats, and impaired Rotorod performance is significantly correlated with increasing brain infarct volume (Rogers et al., 1997). Our results showed that the retention time on the accelerating Rotorod of VPA-treated rats was increased from 20 to almost 50 s (Fig. 9C). Similar effects on the reduction of brain infarction and improvement of neurological performance were observed in pMCAO rats first injected with SB (300 mg/kg) 3 h after the ischemic onset (Fig. 10, A–C). The pMCAO-induced up-regulation of p53 and iNOS was also blocked by SB (Fig. 7, A and E). Furthermore, pMCAO-induced increase of COX-2 protein, the rate-limiting enzyme for prostanooid synthesis and an inflammatory marker, was robustly suppressed by SB treatment (Fig. 7, A and F).

Postinsult treatment with TSA also increased the neurological activity measured in the eight-point test (Fig. 8B). The long-term effects of postinsult SB treatment on functional outcome were further assessed by the eight-point behavioral test. There was a significant increase in the neurological performance score with time after pMCAO in the saline-treated group (*p < 0.05 from days 2–7 or 2–11), and daily SB treatment significantly improved upon this neurological function at 2, 4, 7, and 11 days after ischemia (Fig. 8C). The neurological functions seemed to be normal at any of these time points in the sham-operated group.

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**Discussion**

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plasma levels of SB and TSA were not determined in our experimental conditions. However, based on the assumption that the pharmacokinetic parameters of both drugs are similar to those of VPA, the doses of SB (300 mg/kg s.c. twice daily) and TSA (0.5 mg/kg s.c. twice daily) can be projected to have estimated plasma levels of 54 (or approximately 0.5 mM) and 0.9 μM (or approximately 3 μM), respectively.

Importantly, the beneficial effects were evident when VPA or SB was administrated at least 3 h after the ischemic onset (Figs. 9–11). This time window, together with the long-lasting neurological improvement (Fig. 8), suggests that HDAC inhibitors might have utility in treating acute stroke.

One striking feature of our results is that the neuroprotective effects of VPA and SB are associated with inhibition of pMCAO-induced neuroinflammation. It is now generally accepted that neuroinflammation mediated by microglia and/or monocyte/macrophages has a prominent role in ischemia-induced brain injury. Inflammatory cytokines (TNF-α and IL-1β), iNOS and COX-2 are overexpressed to induce the inflammatory response. After stroke, many of the leukocytes including monocytes and macrophages move from the blood vessels and accumulate in the infarct zone to contribute to neuroinflammation and neurodegeneration (for review, see Zheng and Yenari, 2004). Our results showed that VPA treatment significantly reduced the number of both OX42-positive microglia and ED1-positive monocytes/macrophages following pMCAO (Figs. 2 and 3). A similar decrease in the number

![Image](https://example.com/image1.png)

**Fig. 4.** pMCAO-induced iNOS immunohistochemical staining was blocked by postinsult VPA treatment. Experimental conditions are as described in the legend to Fig. 1B. Immunohistochemistry of iNOS-positive cells was examined in the striatum and frontal cortex of the ipsilateral hemisphere. A and B, lack of iNOS-positive cells in the striatum of the contralateral hemisphere of vehicle- and VPA-treated rats, respectively (magnification, ×40). C and D, iNOS-positive cells in the corresponding area of the striatum of the ipsilateral hemisphere of vehicle- and VPA-treated rats, respectively (magnification, ×40). E and F, same as C and D except that the magnification was ×100. Arrow, iNOS-positive cell; arrowhead, iNOS-positive vessel. G and H, iNOS-positive cells in the corresponding area of the frontal cortex of the ipsilateral hemisphere of vehicle- and VPA-treated animals, respectively (magnification, ×40). I and J, same as G and H except that the magnification was ×100. Bar, 30 μm.

![Image](https://example.com/image2.png)

**Fig. 5.** Effects of postinsult VPA or TSA treatment on levels of Ac-H3 and antiapoptotic/proapoptotic proteins in the ischemic brain hemisphere of pMCAO rats. Experimental conditions are as described in the legend to Fig. 1. Animals were sacrificed 24 h after pMCAO, and the lysates of the ipsilateral hemisphere were prepared for Western blotting analysis. A, Western blots of acetylated histone H3 (Ac-H3), HSP70, p53, and β-actin from sham-operated rats and pMCAO rats treated with vehicle or VPA. B to D, quantified results of Ac-H3, HSP70, and p53, respectively. Data are mean ± S.E.M. of percentage of sham-operated control with three to four animals in each group. *, p < 0.05; **, p < 0.01 between the indicated groups. E, Western blots of Ac-H3, HSP70, p53, Bcl-2, P-Akt, and β-actin from sham-operated and pMCAO rats treated with vehicle (DMSO) or TSA. Results shown are from a representative experiment that included three to four rats in each group.
of microglia in the ischemic brain was also observed in pMCAO rats treated with SB (Fig. 6). In rat midbrain microglia-enriched cultures, treatment with VPA, SB, or TSA has been shown to inhibit LPS-induced microglial activation, which is associated with a decrease in microglial number (Peng et al., 2005; Chen et al., 2006). Our preliminary data showed that postinsult VPA treatment induced a marked increase in the population of microglia that was stained positive for caspase-3 in the ischemic cortex, consistent with the notion that HDAC inhibition induces apoptosis of microglia. Thus, HDAC inhibitor-induced apoptosis of microglia and macrophages probably contributes to antineuroinflammation in the ischemic brain of pMCAO rats. On the other hand, it has been suggested that VPA may suppress LPS-induced production of inflammatory cytokines via inhibition of NF-κB transcriptional activity (Ichiyama et al., 2000).

In midbrain neuron-glial cocultures, VPA treatment robustly suppresses LPS-induced secretion of proinflammatory TNF-α and production of NO (Peng et al., 2005). The present study showed that the expression levels of iNOS in the ischemic brain of pMCAO rats were blocked by VPA or SB treatment (Figs. 4 and 7). The role of iNOS in ischemia-induced pathology is supported by the observation that iNOS gene knockout attenuates ischemia-induced brain damage and neurological deficits (Iadecola et al., 1997). pMCAO-induced COX-2 overexpression was blocked by postinsult treatment with SB when examined 24 h after the onset of ischemia (Fig. 7). In rodents and humans, cerebral ischemia causes COX-2 overexpression in neurons, glia, vascular cells, and inflammatory cells that invade the ischemic area (Iadecola and Gorelick, 2005). COX-2 catalyzes the production of prostanooids and free radicals, both of which contribute to the deleterious effects of neuroinflammation and excitotoxicity resulting from cerebral ischemia. It has also been suggested that NO, produced by iNOS after focal cerebral ischemia, regulates the activity of COX-2 (Nogawa et al., 1998), possibly via nitrosylation and activation of this enzyme (Kim et al., 2005). Moreover, inhibition of COX-2 leads to attenuation of ischemic injury (Sugimoto and Iadecola, 2003), whereas transgenic COX-2 overexpression exacerbates cerebral infarction (Doré et al., 2003). Collectively, our results strongly...
suggest that anti-inflammation induced by HDAC inhibitors contributes to neuroprotection against cerebral ischemia, rather than being the result of neuroprotection induced by these drugs.

It is becoming clear that chromatin structural remodeling via changes in levels of histone acetylation is a key mechanism underlying the regulation of gene expression (for review, see Drummond et al., 2005). Acetylation of histones is catalyzed by histone acetyltransferase, whereas their deacetylation is mediated by HDACs. Following pMCAO, there was a marked decrease in the acetylation levels of histone H3 in the ischemic brain (Figs. 5 and 7), similar to results from our previous study using a rat transient MCAO model (Ren et al., 2004). This decrease in acetylated histone could be related to inhibition of histone acetyltransferase and/or activation of HDAC following ischemic insult. In any event, MCAO-induced loss of histone acetylation was prevented by postinsult treatment with VPA, SB, or TSA (Figs. 5 and 7). Together, these results suggest that HDAC activity is inhibited in the rat brain by treatment with these three drugs under our experimental conditions. VPA is a short-chain fatty acid structurally similar to SB and has been shown to inhibit multiple HDAC subtypes from classes I and II (but not HDAC6 and HDAC10) (Gurvich et al., 2004),...
HSP70, a molecular chaperone assisting in proper protein folding, HSP70 provides protection from cerebral ischemia in an animal model (Yenari et al., 2005). HSP70 is induced in ischemic brain by postinsult VPA, SB, or TSA treatment is possibly due to HDAC inhibition, which results in altered chromatin to a more open conformation, which then favors HSP70 transcriptional initiation. This notion is supported by our observation that HDAC70 levels are also increased by VPA treatment in normal rats (Ren et al., 2004). VPA, SB, and TSA have also been shown to induce HSP70 in Xenopus and Drosophila (for review, see Langley et al., 2005). In addition to being a molecular chaperone assisting in proper protein folding, HSP70 has several critical effects against apoptotic and necrotic cell death. Overexpression of HSP70 in mice has been shown to provide protection from cerebral ischemia in an animal model of stroke (Rajdev et al., 2000; Hoehn et al., 2001). HSP70 up-regulation also leads to increased expression of antiapoptotic Bcl-2 and suppresses microglia/monocyte activation following experimental stroke (Yenari et al., 2005). HSP70 and Bcl-2 were recently reported to be increased in the brain of ischemic mice treated with another HDAC inhibitor, suberoylanilide hydroxamic acid, which also displayed neuroprotective effects (Faraco et al., 2006). The levels of phospho-Akt, which has suggested involvement in the pathophysiology of and neuroprotection against cerebral ischemia (Zhao et al., 2005), were decreased by pMCAO, and this reduction was blocked by TSA injections (Fig. 5E). Moreover, the down-regulation of cytoprotective Bcl-2 in the ischemic brain was prevented by TSA injections (Fig. 5E).

HDAC inhibitor-induced neuroprotection in the ischemic brain also seems to involve changes in the expression of proapoptotic proteins. For example, p53, which is negatively regulated by HDACs (Juan et al., 2000), was decreased by pMCAO (Figs. 5 and 7). Moreover, the down-regulation of cytoprotective Bcl-2 in the ischemic brain was prevented by TSA injections (Fig. 5E). The inhibition of p53 protein levels by VPA, SB, and TSA probably contributes to the neuroprotective effects of both HDAC inhibitors against ischemic brain damage.

Fig. 10. SB injected 3 h after ischemia still shows a reduction in brain infarction and improvement of neurological performance. Experimental conditions are as described in the legend to Fig. 1E, except that SB or vehicle was first injected 3 h after ischemic onset. At 24 h after pMCAO, animal were assessed for neurological performance by the eight-point behavioral test and accelerating Rotorod test. Animals were then immediately sacrificed for the determination of brain infarct volume. A, TTC staining of brain infarction in six coronal sections was performed, and the quantified results of the infarct volume are shown. Data are mean ± S.E.M. from six rats in each group, n = 6 in the vehicle group; n = 10 in the SB-treated group). C, quantified results of the Rotorod test. Data are mean ± S.E.M. of the retention time on the accelerating Rotorod (n = 11 in sham-operated group as shown Fig. 9D; n = 6 in pMCAO-vehicle group; n = 11 in pMCAO-SB group). * p < 0.05; **, p < 0.01; ***, p < 0.001 compared with the respective vehicle group.

Fig. 11. Effects of SB injected 6 h postinsult on pMCAO-induced brain infarction and neurological deficits. Experimental conditions are as described in the legend to Fig. 1E, except that SB or vehicle was first injected 6 h after ischemic onset. A, TTC staining of brain infarction in six coronal sections was performed, and the quantified results of the infarct volume are shown. Data are mean ± S.E.M. from six rats in each group. ***, p < 0.001 compared with the vehicle group. B, quantified results of the eight-point behavioral test. Data are mean ± S.E.M. of the performance score (n = 8 in the vehicle group; n = 8 in the SB-treated group; p = 0.1633 between groups). C, quantified results of the Rotorod test. Data are mean ± S.E.M. of the retention time on Rotorod (n = 9 in the vehicle group; n = 8 in the SB-treated group; p = 0.2268 between groups).
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It should be noted that VPA, SB, and/or TSA also have other neuroprotective and neurotrophic actions. These include their ability to acetylate the neuroprotective transcription factor, Sp-1 (Ryu et al., 2003), and to induce neuroprotective proteins such as Bcl-2 (Chen et al., 1999), and glucose-regulated protein 78 (Bown et al., 2000). Conversely, these drugs inhibit the excitotoxicity-induced nuclear accumulation of glyceraldehyde-3-phosphate dehydrogenase (Kanai et al., 2004), which has been shown to have a pro-apoptotic role in neurons (for review, see Chuang et al., 2005). HDAC inhibitors also induce brain-derived neurotrophic factor in rat brain neurons (Fukumoto et al., 2001) and brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor in cultured astrocytes from rat midbrain, causing robust neurite outgrowth (Chen et al., 2006). Collectively, our present results together with these reports suggest that HDAC inhibitor-induced neuroprotection following ischemia probably involves multiple mechanisms of action, including their ability to markedly inhibit ischemia-induced cerebral inflammation.

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References


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