Differences in phosphodiesterase 3A and 3B expression after ischemic insult

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List of Abbreviations:

Bcl-2, B-cell leukemia/lymphoma 2 protein;
BDNF, brain derived neurotrophic factor;
cAMP, cyclic adenosine monophosphate;
CBF, cerebral blood flow;
cGMP, cyclic guanosine monophosphate;
COX-2, cyclooxygenase-2;
CREB, cAMP-responsive element binding protein;
GFAP, glial fibrillary acidic protein;
IBZ, ischemic boundary zone;
Iba-1, ionized calcium-binding adapter molecule 1;
IGF-I, insulin-like growth factor I;
MCAO, middle cerebral artery occlusion;
NeuN, neuron-specific nuclear protein;
NGF, nerve growth factor;
OLGs, oligodendrocytes;
OPCs, oligodendrocyte progenitor cells;
PBS, phosphate-buffered saline;
pCREB, phosphorylated cAMP-responsive element binding protein;
PDE, phosphodiesterase;
PDE3, phosphodiesterase type 3;
PKA, protein kinase A;
TH, tyrosine hydroxylase
Abstract

Phosphodiesterase (PDE) exists in the cardiovascular system, adipose tissue and platelets, and its inhibition increases the cellular levels of cAMP, which could activate cAMP-responsive element binding protein (pCREB). The present study was designed to map the expression of PDE3A/B in the forebrain and define the time course of PDE3 expression in the ischemic boundary zone after ischemia. The number of PDE3A-positive cells (neurons and endothelial cells) remained unchanged, while PDE3B-positive cells gradually increased after ischemia/reperfusion. In the corpus callosum, PDE3B was expressed in oligodendrocytes, oligodendrocyte progenitor cells, and astrocytes. PDE3B-expressing astrocytes showed gradual increase after ischemia/reperfusion. In the cortex, the majority of PDE3B-expressing cells before ischemia were neurons, though few were astrocytes. Ischemic insult resulted in gradual increase in PDE3B-expressing astrocytes and neurons, with larger increase in astrocytes. Expression of brain derived neurotrophic factor (BDNF) and B-cell leukemia/lymphoma 2 protein (Bcl-2) was detected in pCREB-positive cells, not in PDE3B-positive cells. Our results demonstrated that ischemic insult increased PDE3B expression, but not PDE3A, and changed the number and type of cells in a time-dependent manner. The variation of PDE3B-expression in the brain might play a crucial pathophysiological role, and regulation of PDE3B production might protect against ischemic brain damage.

Key words: acute cerebral infarction; phosphodiesterase type 3B; ischemic boundary zone, cAMP-responsive element binding protein; oligodendrocyte progenitor cell; astrocyte.
Introduction

Phosphodiesterase type 3 (PDE3) is a superfamily of enzymes involved in the degradation of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) (Jeon et al., 2005). The PDE3 has a relatively high affinity for both cAMP and cGMP, however, based on the lower efficacy of hydrolysis for cGMP, it is essentially considered to affect cAMP (Gresele et al., 2011). The composition of the cAMP-signaling networks plays a key role in target tissues of relevance for energy homeostasis (Degerman et al., 2011). Two different gene products have been identified as part of the PDE3 family, the PDE3A and PDE3B, which are encoded by distinct but related genes and exhibit distinct, but overlapping, patterns of expression (Degerman et al., 2011; Thompson et al., 2007). PDE3A is more highly expressed in the cardiovascular system while PDE3B is more highly expressed in cells involved in the regulation of glucose and lipid metabolism. For example, the PDE3A is localized in smooth muscles, platelets, and cardiac tissues, whereas PDE3B is most abundant in adipocytes and hepatocytes (Beavo, 1995). Together, the PDE are important in several physiological processes such as platelet aggregation, blood pressure regulation, cardiac function, vascular smooth muscle, and insulin action (Degerman et al., 1997; Jeon et al., 2005).

Cerebral ischemia suppresses the conversion of ATP to cAMP (Moskowitz et al., 2010), and therefore inhibits protein kinase A (PKA) activation and CREB phosphorylation (Miyamoto et al., 2009). Inhibition of PDE3 increases the intracellular levels of cAMP by suppressing its conversion into AMP, which could increase CREB phosphorylation and induce subsequent activation of various target genes related to neuronal survival (Lee et al., 2005) as well as those involved in learning and memory (Watanabe et al., 2006). Several studies found other non-antiplatelet effects for PDE3 inhibitors, such as protection of endothelial cells and brain tissue in focal cerebral ischemic models (Choi et al., 2002; Lee et al., 2003). The main mechanism of this action is increased phosphorylation of CREB through intracellular signaling from the PDE3-cAMP cascade, which induces subsequent activation of the antiapoptotic cascade (Watanabe et al., 2006). CREB phosphorylation could also increase the synthesis of B-cell leukemia/lymphoma 2 protein (Bcl-2), cyclooxygenase-2 (COX-2), tyrosine hydroxylase (TH), brain derived neurotrophic factor (BDNF), and nerve growth factor (NGF) (Tanaka et al., 2010;
In the present study, we used a mouse model of transient focal ischemia first to examine the expression of PDE3A and PDE3B in the forebrain by immunohistochemistry. In the next step, we evaluated the expression profiles of PDE3A and PDE3B in the ischemic boundary zone (IBZ) after focal ischemia, and analyzed the relationship between their expression and CREB phosphorylation.

**Experimental Procedures**

**Experimental protocol.**

All animal procedures described in this report were approved by the Animal Care Committee of Juntendo University. Adult male C57BL/6 mice weighing 20 to 25 g were obtained from the Charles River Japan Institute (Kanagawa, Japan) and maintained on a 12-hour light/dark cycle with free access to food and water.

Transient ischemia was induced by intraluminal middle cerebral artery occlusion (MCAO) using an 8-0 nylon monofilament (Ethicon, Somerville, NJ) coated with silicone resin (Provil novo Light Base; Heraeus Kulzer, Tokyo) and a hardener (Provil Novo Light Catalyst; Heraeus Kulzer), as described previously (Hara et al., 1996). Briefly, the animals were anesthetized with 1.5-2.0% isoflurane in 30% oxygen and 70% nitrogen using a small-animal anesthesia system. Then, the left common carotid artery, the left external carotid artery and the internal carotid artery were exposed via a midline incision. The monofilament was advanced from the external carotid artery stump into the left internal carotid artery to occlude the origin of the left middle cerebral artery. At 60 minutes after occlusion, the monofilament was subsequently removed to allow reperfusion. The body temperature was kept at 37.0±0.5°C (mean±SD) using a heating pad (Unique Medical, Tokyo) throughout the surgical procedure. Mice were sacrificed before the procedure (pre-operation) or at 1, 3, or 7 days after the ischemia/reperfusion (n=5 for each group). Cerebral blood flow (CBF) was recorded through a left temporal window by laser-Doppler flowmetry (Omega Wave, Tokyo) before and after the ischemia/reperfusion, as well as before sacrifice. Neurological function was assessed using a standard scoring system described in detail previously (Hara et al., 1996): grade 0, no observable neurological deficit (normal); grade I, failure to extend the left forepaw on lifting the whole body by the tail (mild); grade II, circling to the contralateral side (moderate); and grade III,
loss of walking or righting reflex (severe). At sacrifice, the mice were deeply
anesthetized with intraperitoneal injection of 50 mg/kg of pentobarbital followed by
transcardial perfusion. The brain was removed immediately en bloc and postfixed for
48 h in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C, then
immersed in 30% sucrose and finally cryopreserved until use. The brain was thawed
and cut into 20-μm-thick consecutive coronal sections of the white matter using a
cryostat (CM 1900, Leica Instruments, Nussloch, Germany). The prepared sections
were used for hematoxylin and eosin (H&E) staining, immunohistochemical analysis
and double immunofluorescence histochemistry.

**Immunohistochemistry.**

After incubation in 3% H₂O₂ followed by blocking in 10% normal goat
serum (Dako Corporation, Carpentaria, CA) in PBS, the sections were immunostained
overnight at 4°C with antibodies against anti-PDE3B (dilution, 1:25; Santa Cruz
Biotechnology, Santa Cruz, CA), anti-PDE3A (dilution, 1:25; Santa Cruz
Biotechnology), anti-GSTπ (dilution, 1:100; Medical & Biological Laboratories Co.,
Nagoya, Japan), anti-NG2 (chondroitin sulfate proteoglycan, dilution, 1:100;
Millipore, Bedford, MA), anti-glial fibrillary acidic protein (GFAP, dilution, 1:500;
Medical & Biological Laboratories), ionized calcium-binding adapter molecule 1
(Iba-1, dilution, 1:500; Wako Pure Chemicals, Osaka, Japan), anti-neuron-specific
nuclear protein (NeuN, dilution, 1:100; Millipore), and anti-pCREB (dilution, 1:100;
Upstate Biotechnology, Lake Placid, NY). The sections were then treated with
secondary antibodies (Vector Laboratories, Burlingame, CA). Immunoreactivity was
visualized by the avidin-biotin complex method (Vectastatin ABC kit, Vector
Laboratories) and developed with diaminobenzidine.

**Double immunofluorescence histochemistry.**

Double immunofluorescence histochemical staining was performed to
determine the origin of PDE3B- and 3A-positive cells. Brain sections were washed
with PBS and incubated in a blocking solution; 2% Block-Ace (Yukijirushi, Sapporo,
Japan) in PBS, for 30 minutes at room temperature. Double-immunofluorescence
staining was performed by simultaneous incubation of sections overnight with
anti-PDE3B (dilution, 1:25; Santa Cruz Biotechnology), anti-PDE3B (dilution, 1:200;
Abcam Cambridge, MA), anti-PDE3A (dilution, 1:25; Santa Cruz Biotechnology),
anti-PDE3A (dilution, 1:200; Abcam), anti-NeuN (dilution, 1:100; Millipore),
anti-GSTπ (dilution, 1:100; MBL), anti-NG2 (dilution, 1:100; Millipore), anti-GFAP (dilution, 1:500; Medical & Biological Laboratories Co.), Mac-1(CD11b, dilution, 1:100; Millipore), anti-type IV collagen antibody (dilution, 1:100; Millipore), anti-pCREB (dilution, 1:100; Upstate Biotechnology), anti-BDNF (dilution, 1:100; Santa Cruz Biotechnology), anti-CREB (dilution 1:1,000, Cell Signaling Technology, Beverly, MA), anti-Bcl-2 (dilution, 1:50; Santa Cruz Biotechnology), and TUNEL (Roche, Indianapolis, IN) at 4°C. For double labeling, the primary antibodies were detected by incubation with Cy3- or fluorescein isothiocyanate-conjugated secondary antibody (1:500; Jackson Immunoresearch Laboratories, West Grove, PA) for 90 minutes at room temperature. The sections were washed with PBS and mounted on microslide glass with Vectorshield Mounting Medium (Vector Laboratories).

**Western blotting.**

Mice of each group were decapitated at before and 1, 3, or 7 days after ischemia/reperfusion (n=3 for each group). Samples were taken from two regions: the ischemic region (comprising the cortex and striatum) and the contralateral region (comprised the cortex and striatum on the contralateral side). Aliquots containing 20 μg of protein were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein bands were transferred onto polyvinylidene fluoride membrane (Millipore). The membranes were blocked with 1% bovine serum albumin (BSA) in 0.05% Tween 20, and sequentially incubated with the primary antibody: anti-PDE3B (dilution, 1:100; Santa Cruz Biotechnology), anti-pCREB (dilution 1:1,000; Upstate Biotechnology), and anti-CREB (dilution 1:1,000, Cell Signaling Technology). After incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (dilution, 1:5,000; Amersham Life Science, Buckinghamshire, UK) for 1 hr at room temperature, the immunoreactive bands were visualized in the linear range with enhanced chemiluminescence ECL Western blotting system (Amersham Biosciences, Piscataway, NJ). The western blots were evaluated quantitatively using a computerized digital image system (LAS-1000plus, Fujifilm, Tokyo). Equal protein loading was confirmed by measuring α-tubulin.

**Cell counts and statistical analysis.**

In the double immunofluorescence histochemical analysis, positively stained cells at the IBZ were counted using three sections per animal (Figure 1A, 0.25 mm²). Values presented in this report are expressed as mean±SEM. ANOVA (analysis of variance) and subsequent *post hoc* Fisher protected least significant difference test
were used to determine the statistical significance of differences in various indices among the groups. A $P$ value less than 0.05 denoted the presence of a statistically significant difference.

**Results**

**Changes in the CBF and number of glial/neuronal cells after ischemic insult.**

All mice of this model were confirmed to have developed an area infarct in the cortex and striatum after MCAO. The neurological deficit scores are summarized in Figure 1B. In all mice, the deficit after ischemia/reperfusion was at least of grade II, confirming successful placement of the intraluminal suture. Figure 1C plots the mean CBF values, indicating a decrease in CBF by about 50% immediately after the occlusion, followed by a gradual increase in CBF until 7 days. However, the CBF level was always significantly lower than the pre-operation flow level ($P<0.001$). We also evaluated brain damage by pathologically using H&E staining, to compare normal area, IBZ, and ischemic core in the cortex and corpus callosum (Figure 1D). In this staining, necrotic cell stained small and densely (pyknotic nuclei), and tissue damage was observed. In cortex, only necrotic cells were found in the ischemic core (Figure 1D-iii, arrow), whereas many normal cells were detected in the IBZ (Figure 1D-ii). In corpus callosum, cell number in core area was decreased compare to IBZ (Figure 1D-v) and normal area (Figure 1D-iv), and some vacuoles (tissue damage) were observed in core area (Figure 1D-vi, arrowhead). Those results also confirmed successful placement of the intraluminal suture of MCAO.

Next, we checked the serial changes in glial and neuronal cell counts in IBZ after MCAO. In the IBZ of the cortex, the number of NeuN-stained cells (i.e., neurons) decreased significantly in a time-dependent manner ($P<0.001$). On the other hand, the numbers of GFAP-(i.e., astrocytes) and Iba-1-stained cells (i.e., microglia) were significantly higher compared with the pre-operation ($P<0.001$, Figure 1E-F). In the IBZ of the corpus callosum, the number of GSTπ-stained cells (i.e., oligodendrocytes) was lower at day 1 ($P<0.05$) compared with the pre-operation, but increased at day 3 and recovered to the control level at day 7 (Figure 1G-H). On the other hand, the number of NG2-stained cells (i.e., OPCs) increased marginally though significantly after the ischemia/reperfusion ($P<0.05$, Figure 1G-H). In comparison, GFAP- and Iba-1-stained cells increased greatly and significantly in a time-dependent
manner \((P<0.001, \text{Figure 1G-H})\). The above results were similar to those described in previous reports (Stoll et al., 1998; Tanaka et al., 2003; Zhang et al., 2005).

**Distribution of PDE3A- and PDE3B- in normal mouse forebrain.**

Next, we examined the presence of PDE3 in normal mice brain by immunohistochemistry (Figure 2A). PDE3A- and PDE3B- positive neuron-like cells were found in the cortex and putamen. PDE3B were detected in glial cells whereas PDE3A were detected in endothelial cells in the medial and lateral portions of the corpus callosum (Figure 2B-C).

**Ischemia alters PDE3B but not PDE3A expression.**

The number of PDE3A-positive cells in the IBZ area of the cortex and corpus callosum did not change throughout the study period (Figure 3A-C). Furthermore, there were no PDE3A-positive cells in the ischemic core area (Figure 3A). Western blot analysis also demonstrated the lack of change in the density of the PDE3A-positive band (Figure 3D, E). To identify the type of PDE3A-positive cells, we carried out double immunofluorescence studies using PDE3A and markers that identify oligodendrocytes (GST\(\pi\)), OPCs (NG2), astrocytes (GFAP), microglia (Iba-1), neurons (NeuN), and endothelial cells (collagen type IV). PDE3A was coexpressed with NeuN in the cortex (Figure 3B-day 1). In both the cortex and corpus callosum, collagen type IV was coexpressed with PDE3A (Figure 3B-day 1). However, the number of PDE3A-NeuN double-positive cells remained unchanged throughout the experiment (Figure 3C). In agreement with these results, the PDE3A-positive band (110 kDa) in western blotting did not change at all time points (Figure 3D, E).

Next, we investigated the expression of PDE3B in the MCAO model. Ischemia/reperfusion resulted in a gradual and time-dependent increase in the number of PDE3-positive cells in the IBZ area of the cortex and corpus callosum at days 1, 3, and 7 after reperfusion \((P<0.05, \text{Figure 4A-B})\). However, no PDE3B-positive cells were detected in the ischemic core area (Figure 4A). Western blot analysis confirmed that the density of the PDE3B-positive band (135 kDa) increased in a time-dependent manner \((P<0.001, \text{Figure 4C, D})\).

We also examined the type of cells that expressed PDE3B in the brain, using the aforementioned cell-type markers. In the corpus callosum, GST\(\pi\), NG2, and GFAP were coexpressed with PDE3B (Figure 4E-day 1). The number of PDE3B-GST\(\pi\) double-positive cells almost did not change at all time points. In comparison, PDE3B-NG2 double-positive cells decreased at day 1 \((P<0.05)\), followed by a gradual
increase at days 3 and 7. The number of these cells at day 7 was significantly higher than in the pre-operation group ($P<0.05$, Figure 4Ba). For GFAP, the number of double-positive cells gradually increased in a time-dependent manner ($P<0.001$, Figure 4Ba).

In the cortex, the majority of PDE3B cells coexpressed NeuN, although a few coexpressed GFAP at pretreatment and day 1. At days 3 and 7 after MCAO insult, both positive cells gradually increased in IBZ, but the increase was larger for PDE3B-GFAP-positive cells ($P<0.05$, Figure 4Bb and 4F).

**Relationship between PDE3B and CREB phosphorylation.**

Based on the above results, the effect of ischemia/reperfusion was limited to changes in the number of PDE3B-positive cells and type of cells in a time-dependent manner. Previous studies indicated that PDE3 inhibitors protect the brain against cerebral ischemia through CREB phosphorylation (Lee et al., 2003). Next, we analyzed the relationship between PDE3B and CREB phosphorylation. The number of pCREB-positive cells and band (43 kDa) increased mostly at day 1 after reperfusion, and then gradually decreased in a time-dependent manner ($P<0.05$, Figure 5A-D). The majority of PDE3B-positive cells coexpressed CREB at all time points (Figure 5E). However, the ratio of PDE3B-pCREB-double positive cells was decreased in time-dependent manner, and the ratio of those cells was only about 50% at day 7 after reperfusion compared to pre-operation ($P<0.001$, Figure 5E-F).

We also analyzed the type of pCREB expressing cells, and the trophic factor that was activated by CREB signaling pathway, such as BDNF and Bcl-2 (Tao et al., 1998) by immunohistochemistry. The pCREB-positive cells coexpressed NeuN in the cortex, GSTπ and GFAP in the corpus callosum, but did not coexpress Mac-1 (microglial maker) in the corpus callosum (Figure 5G). The majority of BDNF-positive cells coexpressed pCREB at all time points, but the ratio of PDE3B-BDNF-double positive cells decreased significantly in a time-dependent manner (Figure 6A-day 7, B). Furthermore, the majority of Bcl-2-expressing cells coexpressed the pCREB, but not PDE3B at all time points (Figure 6C-day 7, D).

We also analyzed the relationship between cell death and PDE3B expression. Ischemia/reperfusion increased in the number of TUNEL-positive cells in the IBZ area of the cortex and corpus callosum at days 1, 3, and 7 after reperfusion and such increase showed a time-dependent pattern ($P<0.05$, Figure 6E-day 3). PDE3B-TUNEL-double positive cells also gradually increased in a time-dependent manner (Figure 6F-day 3, G).
Discussion

PDE3 inhibitors prevent inactivation of intracellular second messenger cyclic AMP, resulting in increased phosphorylation of CREB via the PDE3-cAMP signaling cascade in platelets and blood vessels, and irreversible inhibition of platelet aggregation, vasodilatation and proliferation of vascular smooth muscle cells (Kambayashi et al., 2003; Lee et al., 2005). The inhibitors also activate various tropic factors and anti-apoptotic signaling pathways (Choi et al., 2002; Shin et al., 2010). Previous studies demonstrated that transient focal ischemia does not induce significant changes in CREB expression but reduces the levels of phosphorylated CREB (Gao et al., 2006). In the present study, induction of cerebral ischemia resulted in a gradual increase in PDE3B-positive cells, and the majority of these cells coexpressed CREB, though some coexpressed pCREB. These findings suggest that PDE3B might suppress CREB phosphorylation via cAMP-depending signaling pathway.

Interestingly, our study showed increased expression of PDE3B in astrocytes. The number of astrocytes is known to increase during acute brain ischemia (i.e., reactive astrocytes) (Eddleston and Mucke, 1993). Moreover, in the acute phase of ischemia, astrocytes and microglia play an important role in the inflammatory reaction (Stoll et al., 1998), and are subjected to oxidative stress, similar to microglia (Zhang et al., 2005). On the other hand, an increase in BDNF-positive astrocytes was reported in a recent study (Tanaka et al., 2010). Furthermore, inhibition of PDE3 induces upregulation of BDNF through the CREB signaling cascade, even in the acute phase (Tanaka et al., 2010). The results of our study suggest that ischemia induces local PDE3B-expressing astrocytes, and that these astrocytes did not express BDNF due to impaired CREB phosphorylation. Considered together with the results of the above previous studies, astrocytes seem to play a dual role; providing neurovascular protection and playing a crucial role in inflammation.

Our results showed a transient decrease in the number of PDE3B-expressing OPCs in the acute phase ischemia, but these cells gradually increased in the chronic phase. In the peri-infarct area, an increase in OPCs was observed after ischemia, which was associated with their proliferation (Tanaka et al., 2003). Furthermore, OPCs respond to short and sublethal ischemic injury that causes myelin damage (Dewar et al., 2003). Other studies reported that the survival of OPCs and
maintenance of myelination are closely associated with CREB phosphorylation (Miyamoto et al., 2010; Tanaka et al., 2001). Moreover, CREB phosphorylation promotes Bcl-2 and COX-2 transcription in OLG, and protects against ischemic white matter damage in chronic cerebral hypoperfusion in rats (Watanabe et al., 2006). Similar to astrocyte and oligodendrocyte lineages, the expression of PDE3B increases the number of neurons during the late phase of ischemia. Several studies have shown that the CREB/cAMP response element (CRE) transcriptional pathway regulates the expression of both Bcl-2 and BDNF (Tao et al., 1998; Walton et al., 1996). With respect to the role of the CREB/CRE transcriptional pathway in neuroprotection, CRE-mediated gene expression is necessary for the induction of ischemic tolerance (Hara et al., 2003). Thus, our data may expand these findings; - PDE3B inhibition may play a critical role in brain tissue regeneration, since PDE3B can also regulate CREB cascade (Degerman et al., 2011; Thompson et al., 2007). In our study, the number of pCREB-BDNF-double positive cells did not change under ischemic condition, rather, the number of PDE3B-BDNF-double positive cells was decreased. Based on these results, the expression of PDE3 after ischemic insult may suppress CREB cascade and lead to a decrease in BDNF production. The same theory could also be true for Bcl-2 expression.

Although our study demonstrated that cerebral ischemia increases PDE3B expression, with subsequent suppression of CREB phosphorylation, there are some important caveats that need to be carefully discussed here for future studies. First, we focused on the PDE3-cAMP-CREB cascade only, although CREB phosphorylation is known to be regulated by various signaling pathways (Ballif and Blenis, 2001). However, signal reductions may fail to activate CREB phosphorylation during ischemic stress. Differences between other signaling pathway-induced and PDE3-induced reduction of the CREB phosphorylation need to be carefully considered in future studies. Second, in this study, we only examined changes in the immunohistological and protein expression of PDE3. Future studies need to analyze whether the enzymatic activity of PDE3 is really increased in parallel with the increased in immunohistological and protein expression. Third, PDE3 is an important factor in the regulation of energy metabolism. With regard to PDE3B as a possible target for the treatment of cerebral infarction, one has to keep in mind the dynamic interplay among various tissues expressing PDE3B. PDE3B inhibition in hepatocytes, adipocytes and hypothalamus may result in worsening of glucose disposal, glucotoxicity and weight gain (Degerman et al., 2011). Recently, PDE3 inhibitors
have been used therapeutically. However, their side effects of tachycardia and arrhythmia sometimes pose a clinical risk (Yan et al. 2007). Since PDE3A is mostly expressed in cardiac tissue compared with PDE3B, selective PDE3A inhibitors also increase the heart rate by around 10% (Nikpour et al. 2010). With regard to our result, PDE3B inhibition, which was induced by PDE3B-related substances within 3 days after ischemia (before any increase in TUNEL-PDE3B-double positive cells), could be viewed as a new treatment strategy for ischemic brain damage. Further studies need to analyze the beneficial and side effects of PDE3B-related substances in cerebral ischemia.

Conclusion

The present results identified the expression of PDE3A/B in neurons, astrocyte and oligodendrocyte lineage cells in the normal brain, and increased number of PDE3B-expressing cells after focal transient brain ischemia. Our findings suggest that the pattern of PDE3B brain localization could play a crucial pathophysiological role in ischemic brain damage, and that regulation of PDE3B production could protect against the progression of ischemic brain damage.
References


**Figure legends**

**Figure 1. Serial changes in glial cells and neuronal cells after middle cerebral artery occlusion (MCAO).**

(A) Schematic representation of the distribution of neuronal damage in mice brain after ischemia/reperfusion. The light shaded area represents the ischemic boundary zone (IBZ), dark shaded area represents the ischemic core, and cortex (a), white matter (b). (B) Neurological deficit scores measured at immediately after reperfusion...
Temporal changes in cerebral blood flow (CBF). pre: before MCA occlusion; during: just before reperfusion; post: immediately after reperfusion, and at days 1, 3 and 7 after reperfusion. (D) Photomicrographs (at day 1) of hematoxylin-eosin stained section showing normal area (i, iv), IBZ (ii, v), and ischemic core (iii, vi) in the cortex (i-iii), and corpus callosum (iv-vi). arrow; necrotic cell, arrow head; vacuoles. Bars = 1 mm (a), 20 μm (b).

Photomicrographs (at day 3, E) and results of quantitative analysis of the number of NeuN-, GFAP-, and Iba-1- positive cells (F) in the cortex before and at days 1, 3, and 7 days after reperfusion. (G-H) Photomicrographs (at day 3, G) and results of quantitative analysis of the number of GSTπ-, NG2-, GFAP-, and Iba-1- positive cells (H) in the white matter before and at days 1, 3, and 7 days after reperfusion. Bars = 20 μm. Data are mean±SEM of five mice in each group. pre; pre-operation.

Figure 2. PDE3A/B expression in the normal forebrain.
(A) Schematic representation of the cortex (i), lateral (ii) and medial (iii) portions of the corpus callosum and putamen (iv). (B-C) Photomicrographs of PDE3A- (B) and PDE3B-positive cells (C) in the cortex (a, b), putamen (c, d), medial (e, f) and lateral portions (g, h) of the corpus callosum in the normal brain. Bars = 200 μm (a, c, e, g, i), Bars = 50 μm (b, d, f, h, j, k, l).

Figure 3. PDE3A expression after middle cerebral artery occlusion (MCAO).
(A) Photomicrographs of PDE3A-positive cells in the corpus callosum and cortex in the ischemic boundary zone (IBZ) and ischemic core at day 1 after reperfusion. Bars = 100 μm. (B) Double immunofluorescence staining for PDE3A (green), NeuN (red), collagen IV (red) and merged images in the cortex and corpus callosum at day 1 after reperfusion. Bars=20 μm. (C) Number of PDE3A-NeuN double-positive cell in the cortex. (D-E) Western blotting (D) and densitometric analysis (E) of PDE3A. (B)-(E) show in the IBZ. Data are mean±SEM of five mice in each group. pre; pre-operation.

Figure 4. PDE3B expression after MCAO.
(A) Photomicrographs of PDE3B-positive cells in the corpus callosum and cortex in the ischemic boundary zone (IBZ) and ischemic core at day 1 after reperfusion. Bars = 100 μm. (B) Number of PDE3B-GSTπ, -NG2, -GFAP, -NeuN double-positive cell in the (a) corpus callosum and (b) cortex. (C-D) Western blotting (C) and densitometric analysis (D) of PDE3B. (E, F) Double immunofluorescence staining for PDE3B (green), GSTπ (red), NG2 (red), GFAP (red), NeuN (red) and merged images in the
corpus callosum (E) at day 7 and in cortex (F) at days 1 and 7 after reperfusion. (B)-(F) show in the IBZ. Bars=20 μm. Data are mean±SEM of five mice in each group. *P<0.05, **P<0.001, compared with the pre-operation group (pre).

**Figure 5. Relationship between PDE3B and CREB phosphorylation.**

(A, C) Photomicrographs (A, day1) and number (C) of pCREB-positive cells in the corpus callosum and cortex. Bars = 10 μm. (B, D) Western blotting (B) and densitometric analysis (D) of pCREB and CREB. (E) Double immunofluorescence staining of PDE3B (green), CREB (red), pCREB (red) and merged images at days 1 and 7 after reperfusion in the corpus callosum. (F) The ratio of PDE3B-pCREB and PDE3B-CREB double positive cell in the IBZ. (G) Double immunofluorescence staining for pCREB (green), NeuN (red), GSTπ (red), GFAP (red), Mac-1 (red) and merged images at day 1 after reperfusion in the cortex and corpus callosum. Bars=20 μm. Data are mean±SEM of five mice in each group of IBZ. *P<0.05, **P<0.001, compared with same time points, pre; pre-operation.

**Figure 6. Relationship between pCREB, PDE3B and BDNF, Bcl-2 expression.**

(A) Double immunofluorescence staining of pCREB (green), PDE3B (green), BDNF (red) and merged images at day 7 after reperfusion in the cortex and corpus callosum. Bars=20 μm. (B) The ratio of pCREB-BDNF and PDE3B-BDNF double positive cell in the IBZ. (C) Double immunofluorescence staining of pCREB (green), PDE3B (green), Bcl-2 (red) and merged images at day 7 after reperfusion in the cortex and corpus callosum. Bars=20 μm. (D) The ratio of pCREB-Bcl-2 and PDE3B-Bcl-2 double positive cell in the IBZ. (E-F) Photomicrographs (at day 3, E) and number of TUNEL-positive cells (F) in the cortex and corpus callosum. Bars=50μm. (G) Double immunofluorescence staining of PDE3B (green), TUNEL (red) and merged images at days 3 after reperfusion in the IBZ. Bars=20 μm. Inserted image; high magnificated image of merged cell. (H) The ratio of PDE3B-TUNEL double positive cells in the IBZ. Data are mean±SEM of five mice in each group of IBZ. *P<0.05, **P<0.001, compared with same time points, pre; pre-operation.