Published in final edited form as:

J Neurosci Res. 2009 February 1; 87(2): 545–555. doi:10.1002/jnr.21865.

9-Cis-Retinoic acid reduces ischemic brain injury in rodents via bone morphogenetic protein

Hui Shen, Yu Luo, Chi-Chung Kuo, Xiaolin Deng, Chen-Fu Chang, Brandon K. Harvey, Barry J Hoffer, and Yun Wang

National Institute on Drug Abuse, Intramural Research Program, Baltimore, MD, 21224

Abstract

Retinoic acid (RA), a biologically active derivative of vitamin A, has protective effects against damage caused by H₂O₂ or oxygen-glucose deprivation in mesangial and PC12 cells. In cultured human osteosarcoma cells, RA enhances the expression of bone morphogenetic protein -7 (BMP7), a trophic factor that reduces ischemia- or neurotoxin –mediated neurodegeneration *in vivo*. The purpose of this study is to examine whether RA reduces ischemic brain injury through a BMP7 mechanism. We found that intracerebroventricular administration of 9-cis-retinoic acid (9cRA) enhanced BMP7 mRNA expression, detected by RTPCR, in rat cerebral cortex at 24 hours after injection. Rats were also subjected to transient focal ischemia induced by ligation of the middle cerebral artery (MCA) at one day after 9cRA injection. Pretreatment with 9cRA increased locomotor activity and attenuated neurological deficits 2 days after MCA ligation. 9cRA also reduced cerebral infarction and TUNEL labeling. These protective responses were antagonized by BMP antagonist noggin given at one day after 9cRA injection. Taken together, our data suggest that 9cRA has protective effects against ischemia -induced injury and these effects involve BMPs.

Keywords

retinoic acid; BMP-7; stroke; neuroprotection

INTRODUCTION

Retinoic acid (RA) is a biologically active derivative of vitamin A. Two isomers of RA, 9-cis-RA (9cRA) and all-trans-RA (atRA) have been identified. atRA is normally present at high levels in the developing spinal cord and at low levels in the forebrain of mouse embryos (Horton and Maden, 1995). 9cRA is present in xenopus embryos (Kraft et al., 1994), but not in mouse embryos (Horton and Maden, 1995; Mic et al., 2003; Ulven et al., 2001) or tissue extracts from adult rats (Werner and Deluca, 2001). RAs interact with two major groups of nuclear receptors: retinoic acid receptors (RAR) and retinoid X receptors (RXR). RXR forms heterodimers with RAR (RXR/RAR). 9cRA binds with high affinity to RXR (Levin et al., 1992), whereas both 9cRA and atRA activate RAR (Allenby et al., 1994; Mangelsdorf et al., 1990).

Several physiological responses to RA have been identified. RA is important in development and regeneration (Maden and Hind, 2003; McCaffery et al., 2003). RA inhibited H₂O₂-induced apoptosis via suppression of c-fos/c-jun expression and JNK activation in mesangial cells (Kitamura et al., 2002) and increased survival during anoxia/glucose deprivation in PC12 cells

(Boniece and Wagner, 1995). These data suggest that RA can induce protective responses in cultured cells. The function of RA in the mature nervous system is less well known. The expression of cytosolic cellular RA binding proteins increased more than 10 fold after sciatic nerve injury (Zhelyaznik et al., 2003) or transient middle cerebral artery occlusion (MCAo) in adult rats (Raghavendra, V et al., 2002). Pretreatment with docosahexaenoic acid, a candidate ligand for RXR, reduced cerebral infarction induced by MCAo (Glozman et al., 1998; Umemura et al., 1995). Taken together, these data suggest that retinoids are involved in neuronal protection in vivo.

RA is known to control the expression of some proteins in the transforming growth factor (TGF) β superfamily. In the human osteosarcoma cell line U-2 OS cells, pretreatment with RA enhances bone morphogenetic protein-7 (BMP7) production (Paralkar et al., 2002). Knocking out the RA receptor induces interdigital webbing and down regulation of BMP7 (Dupe et al., 1999). Previous studies have demonstrated that BMP7 may play an important role during neuronal damage. The expression of BMP-7 transcripts is enhanced following transient forebrain ischemia (Chang et al., 2003). BMP7 reduces H₂O₂ –mediated toxicity in primary cortical cultures through activation of the mitogen-activated protein kinase pathway (Cox et al., 2004). Intracerebral administration of BMP7 reduces cerebral infarction after a transient middle cerebral artery occlusion (MCAo) in adult rats (Lin et al., 1999). Similarly, intracerebral transplantation of fetal kidney tissue, which contains high levels of BMP7 protein, reduces caspase-3 activity and cerebral infarction in stroke rats (Chang et al., 2002). These data suggest that anti-apoptotic mechanisms are involved in BMP7 –mediated protection from ischemic injury. Since RA modulates BMP7 expression in vitro, it is possible that RA reduces ischemic insults through the activation of BMP7 production in vivo.

In this study, we therefore examined the protective effect of 9cRA in an animal model of stroke. We found that pretreatment with 9cRA enhanced BMP7 expression and reduced brain damage after MCAo in vivo. RA-mediated protection was antagonized by the BMP antagonist noggin. Our data suggest that 9cRA induces neuroprotection against stroke through the upregulation and signaling of BMPs.

MATERIALS AND METHODS

Experimental design

Adult male Sprague-Dawley rats (3 months old, purchased from the Charles River Laboratory, Inc) were used for this study. Animals were subjected to intracerebroventricular administration of 9cRA or vehicle and, one day later, a 60-min MCAo (see below). A summary of experimental timeline is presented in Fig1.

ICV injection

Animals were anesthetized with chloral hydrate (0.4 g/kg, i.p.). 9cRA (1 μ g/1 μ l $\times$ 20 μ l, dissolved in 10% DMSO, Sigma, pH 7.0) or vehicle (10% DMSO in saline pH 7.0, 20 μ L) was given intracerebroventricularly, contralateral to the ischemic hemisphere, through a 25 μ l Hamilton syringe 1 day before MCAo. The coordinates for intracerebroventricular injections were: 0.8 mm posterior to the bregma, 1.5 mm lateral to the midline; 3.5 mm below dura surface. The speed of injection was controlled by a syringe pump at a rate of 2.5 μ l/min. The needle was retained in place for 5 min after injection. After injection, a piece of bone wax (W810, Ethicon) was applied to the burr hole in the skull to prevent efflux of the solution. In some animals, the BMP antagonist noggin (1 μ g/10 μ l, i.c.v., R & D Systems, Inc) or saline (10 μ l, i.c.v.) was administered 5–10 min before MCAo.

Ligation of the middle cerebral artery (MCA)

Animals were anesthetized with chloral hydrate (initially 400 mg/kg, i.p. followed with 100 mg/kg every hour). The bilateral common carotids (CCAs) were ligated with nontraumatic arterial clips. A craniotomy of about $2 \times 2 \text{ mm}^2$ was made in the right squamosal bone. The right MCA was ligated with a 10-0 suture as previously described to generate focal infarction in the cerebral cortex (Chen et al., 1986; Wang et al., 2003). The ligature and clips were removed after 60-min ischemia to generate reperfusion injury. Core body temperature was monitored with a thermistor probe and maintained at 37°C with a heating pad during anesthesia. After recovery from the anesthesia, body temperature was maintained at 37°C using a temperature-controlled incubator. Immediately after the recovery from anesthesia, an elevated body swing test was used to evaluate the success of MCAo surgery. All animals used for this study demonstrated prominent motor bias contralateral to the lesion side.

Behavioral assay

i. locomotor behavior—Locomotor activity was measured using an Accuscan activity monitor (Columbus, OH). Animals were individually placed in a $42 \times 42 \times 31 \text{ cm}$ plexiglass open box which contained horizontal and vertical infrared sensors spaced 2.5 cm apart. Motor activity was calculated using the number of beams broken by the animals every 10 min for 60 min. The following variables were measured: (A) horizontal activity (the total number of beam interruptions that occurred in the horizontal sensors), (B) horizontal movement time, (C) total distance traveled (the horizontal distance traveled by the animals), and (D) vertical movement time.

ii. Body asymmetry—Body asymmetry was analyzed using an elevated body swing test (Borlongan and Hida, 1998). Rats were examined for lateral movements/turning when their bodies were suspended 20 cm above the testing table by lifting their tails. The frequency of initial turning of the head or upper body contralateral to the ischemic side was counted in 20 consecutive trials. The maximum impairment in body asymmetry in stroke animals is 20 contralateral turns/20 trials. In normal rats, the average body asymmetry is 10 contralateral turns/20 trials (i.e. the animals turn in each direction with equal frequency).

iii. Modified Bederson's test—Neurological deficits were evaluated using Bederson's score (Bederson et al. 1986). In a postural reflex test, rats were examined for the degree of abnormal posture when suspended by 20–30 cm above the testing table. They were scored according to the following criteria.

0. Rats extend straight both forelimbs. No observable deficit.
1. Rats keep the one forelimb to the breast and extend the other forelimb straight.
2. Rats show decreased resistance to lateral push in addition to behavior in score 1 without circling.
3. Rats twist the upper half of their body in addition to behavior in score 2.

Triphenyltetrazolium chloride (TTC) staining

Two days after MCA ligation, some animals were killed by decapitation. The brains were removed, immersed in cold saline for 5 minutes, and sliced into 2.0 mm thick sections. The brain slices were incubated in 2% triphenyltetrazolium chloride (Sigma), dissolved in normal saline, for 10 minutes at room temperature, and then transferred into a 5% formaldehyde solution for fixation. The area of infarction on each brain slice was measured double blind using a digital scanner and the Image Tools program (University of Texas Health Sciences

Center, San Antonio). The total infarction volume in each animal was obtained from the product of average slice thickness (2 mm) and sum of the area of infarction in all brain slices.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) histochemistry

Animals were decapitated at 24 hours after ischemia. The brains were removed, frozen in isopentane on dry ice and stored -80°C . For sectioning, frozen brains were cut into 30 μm sections using a cryostat. The sections were mounted onto Superfrost/Plus microscopy slides (Cat. 12-550-15; Fisher, PA). Subsequently, the sections were fixed in 4% paraformaldehyde at 4°C for 30 min. They were then washed in tap water to eliminate the fixative solution and air dried (Deng et al., 1999). A standard TUNEL procedure for frozen tissue sections with minor modifications was performed. Briefly, slide-mounted sections from rat brain were rinsed in 0.5% Triton X-100 in 0.01M PBS for 20 min at 80°C to increase permeability of the cells. To label damaged nuclei, 50 μL of the TUNEL reaction mixture was added to each sample in a humidified chamber followed by a 60 min incubation at 37°C . Procedures for negative controls were carried out as described in the manufacturer's manual (Roche, IN) and consisted of not adding the label solution (terminal deoxynucleotidyl transferase) to the TUNEL reaction mixture. Material was examined by fluorescence microscopy. The average value from 3 sections with a visualized anterior commissure (near - 0.24mm to bregma) from each brain was used for analysis.

Quantitative reverse transcription-PCR (qRT-PCR)

Rats were euthanized at 8, 12, and 24 hours after injection of 9cRA or vehicle; brains were immediately harvested and chilled on ice. The cerebral cortex (4 mm to 8 mm from the rostral end) of the injected side was dissected out and total RNA was extracted according to manufacturers' instructions (RNAqueous, Ambion). Total RNA (2 μg) was treated with RQ-1 Rnase-free Dnase I and reverse transcribed into cDNA using random hexamers by AMV reverse transcriptase (Roche). cDNA levels for hypoxanthine phosphoribosyltransferase 1 (HPRT1), PGK1, BMP2, BMP4, BMP6 and BMP7 were determined by specific universal probe Library primer probe sets (Roche) by quantitative RT-PCR using standard curves for each primer sets as described previously (Chou et al., 2008b). Specificity of the primer sets was confirmed by a single band of predicted size on 2% agarose gel using serial dilution of cDNA templates. The quantity of each gene was determined by standard curves for each gene. For each sample, duplicates were measured with real-time PCR and the results were repeated at least once with similar results. Primers and FAM-labeled probes used in the quantitative RT-PCR for each gene are as follows:

HPRT1 : forward primer (5' –gaccggttctgtcatgtcg); reverse primer (5' – acctggttcatcatcactaatcac); probe (rat universal probe Library #95, Roche)
PGK1 (phosphoglycerate kinase 1): forward primer (5' – tacctgctggtggtggatgg); reverse primer (5' – cacagcctcgcatatttct); probe (rat universal probe Library #108, Roche)

BMP7 : forward primer (5' – gagggctggttggtatttga); reverse primer (5' – aactgggggttgatgctctg); probe (rat universal probe Library #76, Roche)

BMP2 : forward primer (5' – cggactgcggtctcctaa); reverse primer (5' – gggaagcagcaactaga); probe (rat universal probe Library #49, Roche)

BMP4 : forward primer (5' – cgggttgagtaccctgag); reverse primer (5' – tgggatgttccagatgttc); probe (rat universal probe Library #21, Roche)

BMP6 : forward primer (5' – gtgacaccgcagcacaac); reverse primer (5' – tcgtaaggccgctctctg); probe (rat universal probe Library #78, Roche)

Blood pressure and blood gas measurements

Physiological parameters were measured as previously described (Wang et al., 2003). Rats were injected intracerebroventricularly with 9cRA or vehicle. One day after the injection, animals were anesthetized and a polyethylene catheter was inserted into the femoral artery. Mean arterial pressure was recorded through a strain gauge transducer and recorded on a strip chart recorder. Arterial blood (<0.3 mL) was withdrawn from the artery for blood gas analysis using standard methods. In some animals, blood gas was measured at 30 min after 60-min MCAo.

Statistics

Two tailed Student's t-test and ANOVA were used for statistical comparison. Student Newman-Keuls and Fisher test were used for post-hoc analysis. Data are presented as mean $\pm$ s.e.m. P values <0.05 are considered significant.

RESULTS

1. Expression of BMPs

The effects of 9cRA on BMP genes were directly measured by qRT-PCR. Animals were injected intracerebroventricularly with 9cRA (n=20) or vehicle (n=15). Cortical tissue was harvested at 8, 12, and 24 hours after injection and subjected to qRT-PCR. Expression levels for each gene were determined by standard curves. The expression of the housekeeping gene HPRT1 or PGK1 was not altered by 9cRA or vehicle injection ($p>0.05$, t test, data not shown). The expression of each BMP gene was normalized by comparison to the expression of HPRT1 in all the samples. The expression of BMP2, 4, 6, 7 was not altered from 8 to 24 hours after injection of vehicle; these data were thus pooled and used as a control. 9cRA significantly increased BMP7 mRNA expression at 24 hours after injection (Fig 2A, $p=0.002$, $F_{3,31}=6.154$, one-way ANOVA; $p<0.05$, post-hoc Newman-Keuls test). RA did not alter the expression of BMP2 ($p=0.419$, $F_{3,31}=0.971$, one-way ANOVA), BMP4 ($p=0.725$, $F_{3,31}=0.442$, one-way ANOVA), and BMP6 ($p=0.517$, $F_{3,31}=0.775$, one-way ANOVA) from 8 to 24 hours after injection (Fig 2B–D).

2. Behavior tests

Since 9cRA increased BMP7 expression at 24 hours after injection of 9cRA, we next pretreated 32 rats with 9cRA or vehicle at 1 day before MCAo (n=17) or sham (n=15) surgery. Three behavioral tests were performed 2 days later. (i) Locomotor activity: Horizontal (i.e. horizontal activity, total distance traveled, horizontal movement time) and vertical movement (vertical movement time) were recorded every 10 min for 60 min. 9cRA or vehicle did not significantly alter any locomotor parameters after sham surgery (Fig 3A–D). MCAo, compared to sham surgery, significantly reduced all locomotor activities in animals treated with vehicle (Fig 3). Using a two-way ANOVA and post-hoc Newman-Keuls analysis, we found that pretreatment with 9cRA significantly increased horizontal activity (Fig 3A, $p<0.05$, $F_{3,168}=15.424$), total distance traveled (Fig 3B, $p<0.05$, $F_{3,168}=10.957$), and movement time (Fig 3C, $p<0.05$, $F_{3,168}=17.258$) in stroke rats. These horizontal movement parameters were similar between rats receiving MCAo + 9cRA and those with sham surgery + vehicle. Vertical movement time was also significantly increased by 9cRA at 2 days after MCAo (Fig 3D, $p<0.05$, $F_{3,168}=26.647$). However, the vertical movement time in rats receiving MCAo + 9cRA was still significantly less than those with sham surgery + vehicle (Fig 3D, $p<0.05$, two-way ANOVA + post-hoc Newman-Keuls test). (ii) Body asymmetry: The elevated body swing test was used to examine body asymmetry. All MCAo animals that received vehicle showed body asymmetry. 9cRA significantly reduced the frequency of swinging the upper body to the side contralateral to the lesion in 20 trials in stroke rats (Fig 3E, $p<0.001$, $F_{3,28}=60.614$, one-way

ANOVA; $p < 0.05$, post-hoc Newman-Keuls test). No body asymmetry was found in rats receiving sham surgery and pretreatment of either 9cRA or vehicle (Fig 3E). (iii) Bederson's test. Neurological symptoms were measured using Bederson test. Administration of 9cRA significantly reduced the Bederson's score in MCAo rats ($p < 0.001$, $F_{3,28} = 20.871$, one-way ANOVA; $p < 0.05$, post-hoc Newman-Keuls test; Fig 3F).

3. Cerebral infarction

Animals were sacrificed 2 days after MCAo or sham surgery for TTC staining. No infarction was found in any rats with sham surgery. Pretreatment with 9cRA ($n = 23$) reduced the size of cortical infarction compared to vehicle pretreatment ($n = 19$). A typical TTC staining is shown in Fig 4A. Three parameters were used to analyze the degree of lesioning: (A) the volume of infarction, i.e. thickness of the slice $\times$ sum of the infarction area in all brain slices, (B) the area of the largest infarction in a slice and (C) the number of infarcted slices from each rat. All three parameters were significantly reduced by pretreatment with 9cRA ($p < 0.05$, t test; Fig 4B–D).

4. Interactions with the BMP antagonist noggin in cerebral infarction

To determine whether BMPs are involved in 9cRA –induced neural protection, the BMP antagonist noggin ($n = 7$) or saline ($n = 6$) was given to animals pretreated with 9cRA at 5 to 10 min before MCAo (Fig 1). These animals were killed for TTC staining 2 days after MCAo. We found that noggin significantly attenuated 9cRA –mediated protection in volume of infarction (Fig 5A, $p = 0.007$, $F_{(3,30)} = 4.877$, one-way ANOVA; $p < 0.05$, post-hoc Fisher LSD test), area of the largest infarction slice (Fig 5B, $p = 0.007$, $F_{(3,30)} = 4.842$, one-way ANOVA; $p < 0.05$, post-hoc Fisher LSD test), and number of slices that had infarction (Fig 5C, $p < 0.001$, $F_{(3,30)} = 10.475$, one-way ANOVA; $p < 0.05$, post-hoc Fisher LSD test). Noggin ($n = 5$) itself, however, did not alter the size of infarction (Fig 5, $p > 0.05$, one-way ANOVA).

5. TUNEL staining

The stroke animals that received vehicle ($n = 12$), 9cRA ($n = 12$), and noggin + 9cRA ($n = 6$) pretreatment were examined for TUNEL labeling at 24 hours after MCAo. Representative photomicrograms of TUNEL histochemistry are shown in Fig 6 (A–C). TUNEL (+) cells were found in the lesion core and penumbra after MCAo (Fig 6A). Pretreatment with 9cRA reduced TUNEL labeling (Fig 6A: vehicle; Fig 6B: 9cRA), which was antagonized by noggin (Fig 6C).

The density of TUNEL (+) cells was further quantified from sections with a visualized anterior commissure (near -0.24 mm to bregma) in each brain. Total number of TUNEL (+) cells per section was counted. The density of TUNEL positive cells was normalized by comparison to the vehicle controls. 9cRA, compared to vehicle, significantly reduced TUNEL (+) cell density in the stroke animals (Fig 6D, $p < 0.001$, $F_{(2,27)} = 24.239$, one-way ANOVA; $p < 0.05$, post-hoc Student-Newman-Keuls test). 9cRA –mediated reduction in TUNEL labeling was significantly antagonized by noggin (Fig 6D, $p < 0.001$, $F_{(2,27)} = 24.239$, one-way ANOVA; $p < 0.05$, post-hoc Student-Newman-Keuls test).

6. Blood gas and blood pressure

Blood pressure and blood gas were examined in 13 stroke and 12 non-stroke rats to evaluate systemic effects. Animals were treated with 9cRA or vehicle intracerebroventricularly. In the stroke rats, blood gas was analyzed at 30 min after MCAo. Administration of 9cRA did not significantly alter the mean blood pressure, arterial PaO_2 , PaCO_2 before or after MCAo (Table 1, $p > 0.05$, one-way ANOVA).

DISCUSSION

We found that 9cRA pretreatment reduced ischemia/reperfusion –mediated cerebral infarction. The volume of infarction, the area of largest infarction in a brain slice, and the total number of infarcted slices were all reduced by 9cRA treatment. 9cRA pretreatment also attenuated neurological symptoms and body asymmetry as well as permitted maintenance of a higher level of locomotor activity after MCAo. These data suggest that 9cRA is neuroprotective against cerebral ischemia.

RA has been found to up-regulate BMP7 mRNA expression in the human osteosarcoma cell line U-2 OS cells (Paralkar et al., 2002). Using RT-PCR, we found that 9cRA also increased BMP7 expression in mammalian brain tissue. 9cRA, at the dose of 20 ug, increased the expression of BMP7, but not BMP2, BMP4 and BMP6, in rat cerebral cortex. The upregulation of BMP7 took place at 24 hours after injection, suggesting that 9cRA selectively and time-dependently increases the expression of BMP7 in the cortex.

Both in vitro and in vivo experiments have shown that BMP7 is neuroprotective against injuries induced by neurotoxins, free radicals, and ischemia. BMP7 reduces neurotoxicity mediated by 6-hydroxydopamine and methamphetamine in dopaminergic neurons (Chou et al., 2008b; Harvey et al., 2004a). BMP7 protein or overexpression of BMP7 by adenovirus protects reduces H₂O₂ –mediated toxicity in primary cortical cultures (Cox et al., 2004; Tsai et al., 2007). In vivo, administration of BMP7 reduces cerebral infarction after a transient MCAo in adult rats (Lin et al., 1999) or brain damage after general hypoxia in neonatal rats (Perides et al., 1995). Transplantation of fetal kidney tissues, which contain high levels of BMP7, to the ischemic cortex reduced cerebral infarction in rodents (Chang et al., 2002). The protective responses of fetal kidney transplants were antagonized by the pretreatment with noggin (Chang et al., 2002). These data suggest that exogenous BMP7 is protective against brain injury.

Endogenous BMP7 may also play a protective role during brain injury. Deficiency in endogenous BMP7 signaling increases vulnerability to toxic dose of methamphetamine in BMP7 –/+ mice or BMPRII dominant negative (BMPRIIDN) mice (Chou et al., 2008b; Chou et al., 2008a). To examine if upregulation of endogenous BMP7 is required for 9cRA-mediated protection in stroke rats, the BMP antagonist noggin was given at 24 hours after injection of 9cRA. Noggin inhibited 9cRA –mediated protection; the reduction in cerebral infarction and TUNEL labeling induced by 9cRA were all antagonized by noggin. Although noggin antagonizes other BMPs besides BMP7 in several physiological responses, its action on 9cRA –mediated protection in ischemic rats may derive mainly from the suppression of BMP7 since only BMP7 was upregulated by 9cRA within 24 hours in this study. Taken together, these data further support the hypothesis that 9cRA –mediated protection involves upregulation of endogenous BMPs.

RA can act as a pro-apoptotic agent in neoplastic and developing cells (Herget et al., 1998; Zheng et al., 1997). On the other hand, RA exerts anti-apoptotic and antioxidant activity in neuronal and kidney cells at lower concentrations. RA, at 10 nM, reduced staurosporine-induced oxidative stress and apoptosis by preventing the decrease in the levels of Cu,Zn-superoxide dismutase (SOD-1) and Mn-superoxide dismutase (SOD-2) in primary hippocampal cultures (Ahlemeyer et al., 2001) and facilitating NGF-induced protection in chick embryonic neurons (Ahlemeyer et al., 2000). RA suppressed hydrogen peroxide –induced apoptotic nuclear condensation and membrane blebbing in rat glomeruli mesangial cells. Similar to RA, BMP7 has anti-apoptotic properties. BMPs reduced caspase-3 activation and DNA fragmentation in the ischemic brain (Chang et al., 2002; Wang et al., 2001). BMP7 inhibited TUNEL labeling induced by high doses of methamphetamine in primary cortical culture (Chou et al., 2008b). In current study, RA reduced the density of TUNEL labeling, a

marker for apoptosis/necrosis in ischemic cortex. This protective effect was antagonized by the BMP antagonist noggin. Taken together, our data suggest that 9cRA inhibits apoptosis and/or necrosis in ischemic brain through a BMP signaling mechanism.

RA analogs, such as 9cRA and atRA, are agonists for RXR and RAR receptors. Compared to atRA, 9cRA is a more selective agonist for the RXR. Although both atRA and 9cRA are neural protective against oxygen-glucose deprivation in hippocampal neurons (Shinozaki et al., 2007), a differential sensitivity of these two ligands have been seen in other brain regions. Animals pretreated with 9cRA had lesser cortical infarction than those treated with atRA after MCAo (Harvey et al., 2004b). Using RTPCR, we also found that 9cRA is more potent than atRA in inducing BMP7 expression in primary cortical cultures (unpublished observation). These differential responses of 9cRA suggest that neuroprotection induced by 9cRA in cerebral cortex involves activation of RXR.

Although 9cRA has a high affinity to RXR, it is not detectable in adult brain tissue and, thus, may not be a candidate endogenous ligand for RXR. Studies have indicated that the polyunsaturated fatty acids, such as linolenic and docosahexaenoic acid, activate RXR (de Urquiza et al., 2000; Goldstein et al., 2003). These polyunsaturated fatty acids are enriched in the adult mouse brain and can also reduce ischemic brain damages (Blondeau et al., 2002; Heurteaux et al., 2006). Future experiments are required to investigate the mechanism of protection induced by these polyunsaturated fatty acids and their effects on RXR and BMP expression.

In conclusion, our data suggest that 9cRA has neuroprotective effects against CNS injury in a transient focal ischemia model. The protective response of 9cRA may involve the activation of BMPs. The beneficial effects of RA in other ischemia models require further investigation. The effectiveness of 9cRA pretreatment in stroke may be clinically useful for patients susceptible to ischemic events, for example, for those suffering from transient ischemic attacks.

ACKNOWLEDGEMENTS

This research was supported by the IRP of NIDA, NIH, DHHS. The authors would like to thank Dr. Jean Lud Cadet for his comments and suggestions.

Reference

- Ahlemeyer B, Bauerbach E, Plath M, Steuber M, Heers C, Tegtmeier F, Krieglstein J. Retinoic acid reduces apoptosis and oxidative stress by preservation of SOD protein level. *Free Radic. Biol. Med* 2001;30:1067–1077. [PubMed: 11369496]
- Ahlemeyer B, Huhne R, Krieglstein J. Retinoic acid potentiated the protective effect of NGF against staurosporine-induced apoptosis in cultured chick neurons by increasing the trkA protein expression. *J. Neurosci. Res* 2000;60:767–778. [PubMed: 10861789]
- Allenby G, Janocha R, Kazmer S, Speck J, Grippo JF, Levin AA. Binding of 9-cis-retinoic acid and all-trans-retinoic acid to retinoic acid receptors alpha, beta, and gamma. Retinoic acid receptor gamma binds all-trans-retinoic acid preferentially over 9-cis-retinoic acid. *J. Biol. Chem* 1994;269:16689–16695. [PubMed: 8206989]
- Blondeau N, Widmann C, Lazdunski M, Heurteaux C. Polyunsaturated fatty acids induce ischemic and epileptic tolerance 5. *Neuroscience* 2002;109:231–241. [PubMed: 11801360]
- Boniece IR, Wagner JA. NGF protects PC12 cells against ischemia by a mechanism that requires the N-kinase. *J. Neurosci. Res* 1995;40:1–9. [PubMed: 7714916]
- Borlongan CV, Hida H. Early assessment of motor dysfunctions aids in successful occlusion of the middle cerebral artery. *Neuroreport* 1998;9:3615–3621. [PubMed: 9858369]

- Chang CF, Lin SZ, Chiang YH, Morales M, Chou J, Lein P, Chen HL, Hoffer BJ, Wang Y. Intravenous administration of Bone morphogenetic protein-7 after ischemia improves motor function in stroke rats. *Stroke* 2003;34:558–564. [PubMed: 12574575]
- Chang CF, Morales M, Chou J, Chen HL, Hoffer BJ, Wang Y. Bone morphogenetic proteins are involved in fetal kidney tissue transplantation - induced neuroprotection in stroke rats. *Neuropharmacology* 2002;43:418–426. [PubMed: 12243771]
- Chen ST, Hsu CY, Hogan EL, Maricq H, Balentine JD. A model of focal ischemic stroke in the rat: reproducible extensive cortical infarction. *Stroke* 1986;17:738–743. [PubMed: 2943059]
- Chou J, Harvey BK, Ebendal T, Hoffer BJ, Wang Y. Nigrostriatal alterations in bone morphogenetic protein receptor II dominant negative mice. 2008aIn
- Chou J, Lu Y, Kuo CC, Powers K, Shen H, Harvey BK, Hoffer BJ, Wang Y. Bone morphogenetic protein-7 reduces toxicity induced by high doses of methamphetamine in rodents. *Neuroscience* 2008b;151:92–103. [PubMed: 18082966]
- Cox S, Harvey BK, Sanchez JF, Wang JY, Wang Y. Mediation of BMP7 neuroprotection by MAPK and PKC in rat primary cortical cultures. *Brain Res* 2004;1010:55–61. [PubMed: 15126117]
- de Urquiza AM, Liu S, Sjoberg M, Zetterstrom RH, Griffiths W, Sjovall J, Perlmann T. Docosahexaenoic acid, a ligand for the retinoid X receptor in mouse brain. *Science* 2000;290:2140–2144. [PubMed: 11118147]
- Deng X, Ladenheim B, Tsao LI, Cadet JL. Null mutation of c-fos causes exacerbation of methamphetamine-induced neurotoxicity. *J. Neurosci* 1999;19:10107–10115. [PubMed: 10559418]
- Dupe V, Ghyselinck NB, Thomazy V, Nagy L, Davies PJ, Chambon P, Mark M. Essential roles of retinoic acid signaling in interdigital apoptosis and control of BMP-7 expression in mouse autopods. *Dev. Biol* 1999;208:30–43. [PubMed: 10075839]
- Glozman S, Green P, Yavin E. Intraamniotic ethyl docosahexaenoate administration protects fetal rat brain from ischemic stress. *J. Neurochem* 1998;70:2484–2491. [PubMed: 9603213]
- Goldstein JT, Dobrzyn A, Clagett-Dame M, Pike JW, DeLuca HF. Isolation and characterization of unsaturated fatty acids as natural ligands for the retinoid-X receptor 1. *Arch. Biochem. Biophys* 2003;420:185–193. [PubMed: 14622989]
- Harvey BK, Mark A, Chou J, Chen GJ, Hoffer BJ, Wang Y. Neurotrophic effects of bone morphogenetic protein-7 in a rat model of Parkinson's disease. *Brain Res* 2004a;1022:88–95. [PubMed: 15353217]
- Harvey BK, Shen H, Chen GJ, Yoshida Y, Wang Y. Midkine and retinoic acid reduce cerebral infarction induced by middle cerebral artery ligation in rats. *Neurosci. Lett* 2004b;369:138–141. [PubMed: 15450683]
- Herget T, Specht H, Esdar C, Oehrllein SA, Maelicke A. Retinoic acid induces apoptosis-associated neural differentiation of a murine teratocarcinoma cell line. *J. Neurochem* 1998;70:47–58. [PubMed: 9422346]
- Heurteaux C, Laigle C, Blondeau N, Jarretou G, Lazdunski M. Alpha-linolenic acid and riluzole treatment confer cerebral protection and improve survival after focal brain ischemia 4. *Neuroscience* 2006;137:241–251. [PubMed: 16289892]
- Horton C, Maden M. Endogenous distribution of retinoids during normal development and teratogenesis in the mouse embryo. *Dev. Dyn* 1995;202:312–323. [PubMed: 7780180]
- Kitamura M, Ishikawa Y, Moreno-Manzano V, Xu Q, Konta T, Lucio-Cazana J, Furusu A, Nakayama K. Intervention by retinoic acid in oxidative stress-induced apoptosis. *Nephrol. Dial. Transplant* 2002;17:84–87. [PubMed: 12386300]
- Kraft JC, Schuh T, Juchau M, Kimelman D. The retinoid X receptor ligand, 9-cis-retinoic acid, is a potential regulator of early *Xenopus* development. *Proc. Natl. Acad. Sci. U. S. A* 1994;91:3067–3071. [PubMed: 8159708]
- Levin AA, Sturzenbecker LJ, Kazmer S, Bosakowski T, Huselton C, Allenby G, Speck J, Kratzseisen C, Rosenberger M, Lovey A. 9-cis retinoic acid stereoisomer binds and activates the nuclear receptor RXR alpha. *Nature* 1992;355:359–361. [PubMed: 1309942]
- Lin SZ, Hoffer BJ, Kaplan P, Wang Y. Osteogenic protein-1 protects against cerebral infarction induced by MCA-ligation in adult rats. *Stroke* 1999;30:126–133. [PubMed: 9880400]
- Maden M, Hind M. Retinoic acid, a regeneration-inducing molecule. *Dev. Dyn* 2003;226:237–244. [PubMed: 12557202]

- Mangelsdorf DJ, Ong ES, Dyck JA, Evans RM. Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature* 1990;345:224–229. [PubMed: 2159111]
- McCaffery PJ, Adams J, Maden M, Rosa-Molinar E. Too much of a good thing: retinoic acid as an endogenous regulator of neural differentiation and exogenous teratogen. *Eur. J. Neurosci* 2003;18:457–472. [PubMed: 12911743]
- Mic FA, Molotkov A, Benbrook DM, Duester G. Retinoid activation of retinoic acid receptor but not retinoid X receptor is sufficient to rescue lethal defect in retinoic acid synthesis. *Proc. Natl. Acad. Sci. U. S. A* 2003;100:7135–7140. [PubMed: 12782789]
- Paralkar VM, Grasser WA, Mansolf AL, Baumann AP, Owen TA, Smock SL, Martinovic S, Borovecki F, Vukicevic S, Ke HZ, Thompson DD. Regulation of BMP-7 expression by retinoic acid and prostaglandin E(2). *J Cell Physiol* 2002;190:207–217. [PubMed: 11807825]
- Perides G, Jensen FE, Edgecomb P, Rueger DC, Charness ME. Neuroprotective effect of human osteogenic protein-1 in a rat model of cerebral hypoxia/ischemia. *Neurosci. Lett* 1995;187:21–24. [PubMed: 7617293]
- Raghavendra RV, Bowen KK, Dhodda VK, Song G, Franklin JL, Gavva NR, Dempsey RJ. Gene expression analysis of spontaneously hypertensive rat cerebral cortex following transient focal cerebral ischemia. *J. Neurochem* 2002;83:1072–1086. [PubMed: 12437578]
- Shinozaki Y, Sato Y, Koizumi S, Ohno Y, Nagao T, Inoue K. Retinoic acids acting through retinoid receptors protect hippocampal neurons from oxygen-glucose deprivation-mediated cell death by inhibition of c-jun-N-terminal kinase and p38 mitogen-activated protein kinase 1. *Neuroscience* 2007;147:153–163. [PubMed: 17521827]
- Tsai MJ, Weng CF, Shyue SK, Liou DY, Chen CH, Chiu CW, Yang TH, Pan HA, Liao RI, Kuo HS, Huang MC, Huang WC, Hoffer BJ, Cheng H. Dual effect of adenovirus-mediated transfer of BMP7 in mixed neuron-glia cultures: neuroprotection and cellular differentiation. *J Neurosci. Res* 2007;85:2950–2959. [PubMed: 17628501]
- Ulven SM, Gundersen TE, Sakhi AK, Glover JC, Blomhoff R. Quantitative axial profiles of retinoic acid in the embryonic mouse spinal cord: 9-cis retinoic acid only detected after all-trans-retinoic acid levels are super-elevated experimentally. *Dev. Dyn* 2001;222:341–353. [PubMed: 11747070]
- Umemura K, Toshima Y, Asai F, Nakashima M. Effect of dietary docosahexaenoic acid in the rat middle cerebral artery thrombosis model. *Thromb. Res* 1995;78:379–387. [PubMed: 7660354]
- Wang Y, Chang CF, Morales M, Chiang YH, Harvey BK, Su TP, Tsao LI, Chen SY, Thiernemann C. Diadenosine tetraphosphate protects against injuries induced by ischemia and 6-hydroxydopamine in rat brain. *J. Neurosci* 2003;23:7958–7965. [PubMed: 12944527]
- Wang Y, Chang CF, Morales M, Chou J, Chen HL, Chiang YH, Lin SZ, Cadet J, Deng X, Wang JY, Chen SY, Kaplan PL, Hoffer BJ. Bone morphogenetic protein-6 reduces ischemia-induced brain damage in rats. *Stroke* 2001;32:2170–2178. [PubMed: 11546913]
- Werner EA, Deluca HF. Metabolism of a physiological amount of all-trans-retinol in the vitamin A-deficient rat. *Arch. Biochem. Biophys* 2001;393:262–270. [PubMed: 11556813]
- Zhelyaznik N, Schrage K, McCaffery P, Mey J. Activation of retinoic acid signalling after sciatic nerve injury: up-regulation of cellular retinoid binding proteins. *Eur. J. Neurosci* 2003;18:1033–1040. [PubMed: 12956703]
- Zheng A, Savolainen ER, Koistinen P. All-trans retinoic acid induces apoptosis in acute myeloblastic leukaemia cells. *Apoptosis* 1997;2:319–329. [PubMed: 14646545]

Fig 1.

Experimental time line. Rats were injected with 9cRA or vehicle intracerebroventricularly. One day later, animals were subjected to a 60- min MCAo. In some animals, the BMP antagonist noggin, or saline, was given at 5 to 10 min before MCAo. Behavioral analysis and TUNEL or TTC staining were performed 2 days after MCAo.

A. BMP7

B. BMP2

C. BMP4

D. BMP6

Fig 2.

9cRA increases BMP7 mRNA expression at 24 hours after injection. Cerebral cortex was harvested at 8 to 24 hours after i.c.v. of 9cRA or vehicle. Vehicle did not alter the expression of any BMP genes, measured by qRT-PCR. 9cRA-mediated changes in BMP mRNA were normalized by comparison to the mean levels of each mRNA after vehicle injection. (A) BMP7 mRNA levels were significantly increased at 24 hours after injection of 9cRA (* $p < 0.05$ one-way ANOVA+ Newman-Keuls test). (B–D) 9cRA did not alter BMP2, BMP4, or BMP6 expression at 8–24 hours after injection.

Fig 3.

9cRA improved locomotor activity and decreased neurological deficits in stroke rats. Locomotor activity (A–D) was recorded every 10 min for 60 min at 2 days after MCAo. Pretreatment with 9cRA significantly increased (A) horizontal activity, (B) total distance traveled, (C) horizontal movement time, and (D) vertical movement time in animals with MCAo. (* $p < 0.05$, A–D, two-way ANOVA, 9cRA+MCAo vs. vehicle+MCAo.) (E) Elevated body swing and (F) Bederson's tests were also examined 2 days after MCAo. (E) 9cRA significantly reduced the frequency of swinging the upper body to the side contralateral to the lesion in 20 trials in stroke rats. (F) Administration of 9cRA reduced the Bederson's score in MCAo rats. (* $p < 0.05$, E–F, one-way ANOVA)

Fig 4.

9cRA pretreatment reduced infarction in stroke animals. (A) TTC staining demonstrating that administration of 9cRA reduced cortical infarction in stroke animals. (B) The volume of infarction was significantly decreased in the animals pretreated with 9cRA, as compared to that in the vehicle control. The volume of infarction equals 2 mm [thickness of the slice] $\times$ [sum of the infarction area in 7 consecutive brain slices (mm²)]. (C) The area of the largest infarction in a slice and (D) the number of infarcted slices, from each rat, were also significantly diminished after 9cRA exposure (* $p < 0.05$, t-test).

Fig 5.

Noggin antagonized 9cRA-mediated reduction in cerebral infarction in stroke rats. Noggin or saline was given to animals pretreated with 9cRA at 5 to 10 min before MCAo. These animals were killed for TTC staining 2 days after MCAo. Noggin significantly attenuated 9cRA – mediated protection in (A) volume of infarction, (B) area of the largest infarction slice, and (C) number of slices that had infarction ($p < 0.05$, one-way ANOVA).

Fig 6.

Noggin antagonized 9cRA-mediated reduction in TUNEL. TUNEL histochemistry was examined at 24 hours after MCAo in animals pretreated with (A) vehicle (A1: low magnification; A2: high magnification), (B) 9cRA (B1: low magnification; B2: high magnification), and (C) 9cRA and noggin (C1: low magnification; C2: high magnification). Marked reduction in TUNEL (+) cells can be seen in the 9cRA -treated animal (A vs. B). Noggin antagonized 9cRA – induced reduction in TUNEL (+) cells (C). All sections with a visualized anterior commissure were used to quantify the density of TUNEL (+) cells (D). TUNEL was normalized to the mean of TUNEL in the stroke animals receiving vehicle. 9cRA

pretreatment significantly reduced the TUNEL (+) cells. Noggin significantly attenuated the effects of 9cRA on TUNEL. Calibration: 100 μm . * $p < 0.05$, one-way ANOVA.

Table 1

Arterial blood gas and blood pressure in animals treated with 9cRA or vehicle in stroke and non-stroke rats

	Non-stroke rats		Stroke* rats	
	9cRA	Vehicle	9cRA	Vehicle
n	6	6	6	7
pH	7.441 ±0.026	7.430 ±0.017	7.450 ±0.015	7.457 ±0.010
PaCO ₂ (mmHg)	43.0 ±4.6	42.0 ±3.7	40.2±1.5	42±1.2
PaO ₂ (mmHg)	88.3 ±4.5	87.3 ±8.2	91.5±2.9	87±2.0
B.P. (mmHg)	120.2 ±10.2	116.0 ±17.5	117.8±2.8	110.6±2.5

* Blood gas was measured at 30 min after MCAo.

p>0.05, one-Way ANOVA