Research report

Delayed hypothermia preferentially increases expression of brain-derived neurotrophic factor exon III in rat hippocampus after asphyxial cardiac arrest

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Abstract

Brain-derived neurotrophic factor (BDNF) protein levels increase in rats treated with a regimen of delayed, mild hypothermia that improve neurological recovery after asphyxial cardiac arrest. BDNF transcription in rat brain involves at least five different BDNF exons (exons I–V) that produce four different varieties of mRNA, each containing exon V paired with one of exons I–IV. This study examined whether these different BDNF transcripts are differentially affected by cardiac arrest and by therapeutic hypothermia in rat hippocampus using a reverse transcription and PCR-based method. At 24 h after asphyxial cardiac arrest, transcripts containing exons I and III increased. In rats treated with hypothermia after cardiac arrest, transcripts containing exon III were further increased. No significant alterations in transcripts from exons II or IV were observed, though there was a trend for hypothermia to decrease message from these exons. These data suggest that hypothermia after cardiac arrest produces exon-specific changes in BDNF transcription.

1. Introduction

Alternative promoters are commonly used in mammalian genomes to generate distinctly regulated unique transcripts from a single gene and provide a mechanism that contributes to the complexity of the mammalian phenotype [13]. It is speculated that approximately 9–18% of mammalian genomes utilize alternative promoters to regulate both alternative splicing [25] and tissue and developmental specificity [13]. In many cases, transcripts generated from alternative promoter usage will ultimately encode functionally distinct gene products. Understanding the circumstances that regulate the differential use of alternative promoters for individual genes could elucidate how gene expression responds to environmental cues. Furthermore, if alternative promoters are controlled by different intracellular signaling pathways, the pattern of transcription can provide insight into which signaling pathways are activated in different tissues or conditions.

Brain-derived neurotrophic factor (BDNF) is a gene that utilizes alternative promoters to generate mRNA transcripts that impact translation of the BDNF preprotein [16,22]. The BDNF gene is comprised of four 5′ noncoding exons (exons I–IV) and one coding exon (exon V) which all encode an identical BDNF preprotein [16,22] (Fig. 1). Each of the exons has its own distinct promoter that regulates the expression of BDNF in a tissue-specific manner [2,15,22].
Exons I and II are expressed exclusively in brain, exon III is expressed primarily in the brain, and exon IV occurs in the brain, but is expressed mainly in the heart and lung tissues [22]. Specific response elements for specific transcription factors and specific signaling pathways are associated with the promoters for different BDNF exons [3,5,18,20]. While exons I–IV have been well described, other noncoding exons may exist [2].

In addition to the tissue-specific transcription of the BDNF exons, transcription rates of the various exons are differentially affected by injury. Kainic acid-induced seizures [15,22] and immobilization stress [14] produce time-dependent and exon-dependent increases in BDNF expression in the rat hippocampus. Furthermore, transient forebrain ischemia induced by bilateral common carotid occlusion differentially regulates BDNF exon transcription in the rat hippocampus as measured by in situ hybridization [12,24]. Specifically, ischemia and reperfusion increases exon III transcription 2 h after ischemia, [12], but transcripts of both exons I and III were increased 4 h after ischemia [24].

This study examined the effects of asphyxial cardiac arrest, a model of neurological injury after sudden cardiac arrest, and hypothermia, a clinical treatment strategy to reduce ischemic brain injury, on expression of the BDNF exons in the rat hippocampus using a PCR-based method. Previous studies with this model found that BDNF protein increases 24 h but not 12 h after cardiac arrest in rats that have received a treatment regimen of mild hypothermia (cooling to 33 °C) compared to rats that were kept normothermic (37 °C) [4]. This same regimen of hypothermia improves behavioral recovery and reduces histological signs of brain injury in hippocampus after cardiac arrest [7]. This study tested whether hypothermia preferentially alters the expression of specific BDNF exons within the hippocampus (the most affected region of the brain due to ischemia) after cardiac arrest.

2. Methods

2.1. Animals and experimental design

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Male Sprague–Dawley rats (Harlan Sprague–Dawley, Inc., Indianapolis, IN, USA) (N = 28) were individually housed with access to food and water ad libitum, and were kept on a 12-h light/dark cycle. Rats weighed 300–350 g at the time of the experiment. Rats were randomly assigned to sham operation with no cardiac arrest, or 8 min of asphyxia and cardiac arrest. Rats were separately assigned to have temperature controlled at 37 °C (normothermia), or 33 °C (beginning 60 min after resuscitation or immediately after sham operation). Rats were sacrificed at 12 or 24 h after sham operation or cardiac arrest. Subjects assigned to the cardiac arrest treatment that could not be resuscitated were excluded.

2.2. Asphyxial Cardiac Arrest

At least 3 days prior to the asphyxia, rats were anesthetized with 1.5% halothane in oxygen, and a 4.5 mm × 20 Gauge stainless-steel guide cannula was stereotactically placed over the parietal cortex (AP-2.0 mm, ML + 2.0 mm relative to bregma, and 1.0 mm below skull surface). Dental cement and skull screws (#080, Small Parts Inc., Miami Lakes, FL) were used to secure the cannula and a plastic protective cap which protected the temperature probe. Battery-operated, wireless temperature probes (XHF-BP, MiniMitter, Sun River, OR) were inserted on the day prior to ischemic insult. Brain probes were calibrated against a 0.1 °C graduated mercury thermometer (Fisher Scientific, Pittsburgh, PA) after each battery change, and were checked for accuracy between rats. The 6 mm × 25
Gauge temperature probe extended 1.5 mm beyond the guide cannula into the epidural space. The temperature at the tip of the brain probe was transmitted to an FM receiver and recorded every 3 s by a PC-compatible computer using commercial software (Vital View, MiniMitter, Sun River, OR). This arrangement allowed continuous temperature monitoring in the freely moving rat as well as thermostatic control of temperature via software-driven relays connected to a 100-W infrared heating lamp and a cooling fan.

Asphyxial cardiac arrest was induced as described previously [4,7,8]. Briefly, rats were anesthetized using 0.8% halothane in oxygen, endotracheally intubated with a 14-Gauge intravenous catheter, and mechanically ventilated at a tidal volume of 9 ml/kg, 40 respirations/min and a positive end-expiratory pressure of 5 cm H2O (Harvard Rodent Ventilator, Harvard Apparatus, South Natick, MA). The left femoral vein and artery were exposed via an incision and cannulated with polyethylene catheters (PE-50 tubing, Fisher Scientific, Pittsburgh, PA). The arterial catheter was connected to a pressure transducer for continuous arterial blood pressure recording and blood gas analysis (I-STAT, Heska Corporation., Ft. Collins, CO). Ventilation was adjusted to maintain eucapnia ($\rho\text{CO}_2 = 35–45$ mm Hg) and normal pH prior to asphyxia.

After reducing the fraction of inspired oxygen to 0.21 (room air) for 2 min, rats were chemically paralyzed with intravenous vecuronium (2 mg/kg). Halothane was weaned and discontinued during this period of normoxic ventilation to minimize anesthetic concentrations during ischemia. In separate experiments, rats that were not paralyzed did not regain consciousness or move spontaneously during similar washout periods. Asphyxia was induced by disconnecting the ventilator at end-expiration for 8 min. Complete circulatory arrest reliably occurred 180–200 s after onset of asphyxia and was recognized by a fall of central arterial blood pressure to equal central venous pressure. After 8 min, ventilation with oxygen was restarted at a rate of 60 respirations/min. Intravenous epinephrine (0.005 mg/kg) and bicarbonate (1.0 mEq/kg) were administered, and external chest compressions were performed at a rate of 200 compressions/min. Rats without return of spontaneous circulation within 2 min were considered out of protocol and were excluded from analyses. After stabilization for at least 60 min and confirming adequate spontaneous respirations, rats were extubated and weaned from oxygen to room air.

Temperature was maintained at 37 °C during operation and for 60 min after resuscitation. Rats assigned to hypothermia treatment after cardiac arrest were cooled to 33 °C at 60 min using misting with a water spray and cooling with a fan. This cooling required 7–10 min to reach the target temperature. Because conscious rats are distressed by the cooling procedure, rats assigned to hypothermia after sham operation were cooled while still under anesthesia. Maintenance of hypothermia after discontinuing anesthesia was not overtly distressing to these rats.

Neurological scores were measured for all rats at baseline and at the times of sacrifice (12 and 24 h) using a scale customized for this model that has been previously reported [7]. Briefly, points are scored for ability to perform specific behaviors including moving each limb (8 points), demonstrating sensation in each limb (4 points), righting reflex (2 points), grooming (2 points), forepaw grasp (2 points), body position (3 points), locomotion (2 points), rearing (1 point), detecting a ledge (1 point), and traversing a beam (1 point). A score of 26 points is normal, whereas a score of 0 points would represent complete absence of neurological response.

2.3. RNA Extraction

At 12 or 24 h after resuscitation from asphyxial cardiac arrest, rats ($n = 3–4$ per group) were anesthetized with halothane and decapitated. Hippocampi were dissected onto dry ice and frozen until RNA extraction. Total RNA was extracted from frozen hippocampi by homogenization in a phenol-based reagent (RNAsiz, Ambion Inc., Austin, TX) using a pestle to aid in tissue disruption. After addition of chloroform, RNA was collected from the aqueous phase, which had been separated by centrifugation. RNA was precipitated by adding isopropanol and resuspended in a citrate buffer (RNAstorage solution, Ambion Inc., Austin, TX). Total RNA concentration was determined from a measure of absorbance at 260 nm (A260). RNA purity was revealed by a measure of the A260/A280 ratio, and integrity assessed by appearance on a denaturing agarose gel.

2.4. RT-PCR and semi-quantitative PCR

Initially, the RNA from individual rats was pooled according to group (i.e., normothermic sham 12 h, hypothermic cardiac arrest 24 h, etc.) for a total concentration of 2 μg. For the main experiment, the RNA for only the 24-h time points were reverse transcribed individually. Reverse transcription of 2 μg of total RNA extract from the hippocampi of either the pooled samples or each rat was conducted in a final reaction volume of 20 μl using an Omniscript RT Kit (Qiagen Inc.) containing 2.0 μl 10X RT Buffer, 2.0 μl of a 5 mM dNTP Mix, 2.0 μl of 10 μM Oligo-dT primer, 1.0 μl RNase inhibitor (10 units/μl), 1.0 μl Omniscript Reverse Transcriptase (4 units/μl), and RNase-free H2O as the master mix. The total RNA was denatured at 94 °C for 5 min before the master mix was added. The mixture was then incubated at 37 °C for 1 h 15 min. After the incubation period, 30 μl of PCR-grade water was added to each reaction tube for a working volume of 50 μl. Serial dilutions of the cDNA for each RT-PCR reaction were prepared ranging from 100 to 0.01 nl of original cDNA in a final volume of 25 μl as described previously [9].

The primers used to detect BDNF exons I–IV were those used by Bishop et al. [2]. The sense sequences were for exon I, 5'-TGACCTCAAACAAGCACACATTAC-3'; exon II, 5'-GTTGGTATACTGGGTACACTTCGGG-3'; exon III, 5'-
GTGCGAGTATTACCTCCGCA-3'; and exon IV 5'-CGTGACAAATGTGACTCCACTG-3'. The common antisense sequence was exon V, 5'-CCCTCAAGATTGTCAGACATGC-3'; antisense 5'-GTCCGCAGTGAGCCAGATG-3' [23]; cyclophilin sense 5'-CGTCGCTTGAGCACTGAGGGAGAAA-3'; antisense 5'-CATGCCTTCTTTCACTTCCCAGAAGAC-3' [14]. The product sizes for GAPDH and cyclophilin were 410 and 299 base pairs, respectively. All primers were synthesized by University of Pittsburgh DNA Synthesis Center.

PCR was carried out in a reaction volume of 25 μl using a Dyad Disciple thermal cycler (MJ Research, Inc.). The master mix contained 2.5 μl 10X PCR Buffer (100 mM Tris–HCl, pH 8.3 at 25 °C, 500 mM KCl, 15 mM MgCl2 and 0.01% gelatin; Sigma-Aldrich Co.), 0.5 μl of 10 mM PCR Nucleotide Mix (Promega Corporation), 2.0 μM of GAPDH and cyclophilin sense and antisense primers and 12.5 μM of the BDNF primers, RNase-free H2O and 1.25 μl Jumpstart RedTaq DNA polymerase (Sigma-Aldrich Co.). The master mix was then added to 1 μl of template cDNA. The cDNA templates were denatured for 5 min at 94 °C, and then cDNA was amplified for 35 cycles for GAPDH and the BDNF exons. Cyclophilin was amplified for 40 cycles. Each cycle consisted of 30 s denaturation at 94 °C for the BDNF primers, 54 °C for the cyclophilin primers and 30 s extension at 72 °C. A final extension step was implemented for 10 min at 72 °C. Cycle numbers and annealing temperatures were optimized for each primer using temperature gradients.

The amplified cDNA was subjected to electrophoresis (75 V and 300 mA for 30 min using a BioRad PowerPac 3000 electrophoresis system) in a 2% agarose gel containing 0.02 μg/l ethidium bromide under 1 × TAE buffer. Stained gels were then placed on a UV Transilluminator (Spectroline) and photographed using Kodak DC290 Macro Photography kit (Eastman Kodak Co.). The resulting images were then subjected to densitometric analysis using NIH-Image software analysis program (version 1.52). The average of the density/volume ratios of each PCR product within the linear range of each exon (3 or more points) was determined and they were divided by the average of the density/volume ratios of the corresponding PCR products in the linear range of GAPDH. For comparison, levels of the cyclophilin cDNA were also compared to GAPDH. All of the linear ranges for the genes of interest were compared to GAPDH on the same gel to eliminate inter-gel variability [9].

2.5. Statistical analysis

Baseline characteristics and neurological scores were compared using ANOVA with treatment groups (sham vs. cardiac arrest, normothermia vs. hypothermia, and time of sacrifice) taken as independent factors. Hemodynamic and blood gas values were compared between using a general linear ANOVA model with repeated measures across time. Hypothermia treatment was taken as an independent factor. When justified by a significant main effect, values were compared between normothermia and hypothermia groups by t test with a Bonferroni correction.

The expression of each transcript was expressed as the average (optical density/cDNA template volume) relative to GAPDH. These values are reported as mean ± standard deviation (SD) for 3–4 animals per group. Relative gene transcription levels were compared between groups using ANOVA with treatment (sham versus cardiac arrest) and temperature regimen (normothermia versus hypothermia) as independent factors. When justified by significant main effects, post hoc t tests with Bonferroni correction were performed to compare individual groups. For all comparisons, significance level was designated as P < 0.05. Analyses were conducted with SPSS 11.0 for PC.

3. Results

3.1. Asphyxial cardiac arrest

A total of 14 rats underwent cardiac arrest with a corresponding 14 rats that were subjected to the same surgical procedures without asphyxia followed by normothermia or hypothermia for 12 or 24 h. The baseline weights, blood gases, and hemodynamics did not differ between the rats in any of the groups. All sham rats exhibited normal neurological scores (26) at the time of sacrifice. After cardiac arrest, there were no significant differences between normothermia and hypothermia groups for total anesthesia duration or duration of chest compressions. The expected time-dependent changes in blood pressure, heart rate, and blood gas values occurred after resuscitation as has been described extensively previously [4,7,8]. Thus, there were significant main effects of time on all of the variables in Table 1, reflecting an initial hyperdynamic state with metabolic acidosis at 10 min after resuscitation followed by a return to more normal physiology by 60 min after resuscitation.

An apparent respiratory acidosis in the hypothermia group at 60 min was reflected by a significant main effect of hypothermia treatment on pH (F(1,13) = 6.12; P = 0.028) and pCO2 (F(1,13) = 9.37; P = 0.009). As observed previously [7], this acidosis is an artifact of the fact that the rat is being cooled to 33 °C at 60 min, but the blood samples are warmed in the blood gas analyzer blood to 37 °C. Correcting for temperature, there was less difference between normothermia and hypothermia groups at 60 min for pH (7.42 ± 0.02 vs. 7.36 ± 0.04) and pCO2 (48 ± 4 mm Hg vs. 54 ± 5 mm Hg). The hypothermia group also...
Table 1

Physiological variables for rats with cardiac arrest

<table>
<thead>
<tr>
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<th>Baseline</th>
<th>Time after reperfusion (min)</th>
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<tr>
<td></td>
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<td>10</td>
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<tr>
<td>Mean arterial pressure (mm Hg)</td>
<td>80 ± 21</td>
<td>180 ± 15</td>
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<tr>
<td>Hypothermia</td>
<td>86 ± 16</td>
<td>177 ± 11</td>
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<tr>
<td>Heart Rate (beats per minute)</td>
<td>259 ± 84</td>
<td>420 ± 72</td>
</tr>
<tr>
<td>Normothermia</td>
<td>313 ± 31</td>
<td>440 ± 12</td>
</tr>
<tr>
<td>pH</td>
<td>7.39 ± 0.04</td>
<td>7.31 ± 0.04</td>
</tr>
<tr>
<td>Hypothermia</td>
<td>7.42 ± 0.03</td>
<td>7.30 ± 0.03</td>
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<tr>
<td>pCO₂ (mm Hg)</td>
<td>41 ± 5</td>
<td>46 ± 4</td>
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<tr>
<td>Hypothermia</td>
<td>42 ± 4</td>
<td>50 ± 4</td>
</tr>
<tr>
<td>pO₂ (mm Hg)</td>
<td>359 ± 124</td>
<td>374 ± 145</td>
</tr>
<tr>
<td>Hypothermia</td>
<td>403 ± 138</td>
<td>331 ± 163</td>
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<tr>
<td>Base excess (mEq/L)</td>
<td>0.2 ± 1.6</td>
<td>-3.2 ± 2.2</td>
</tr>
<tr>
<td>Hypothermia</td>
<td>2.4 ± 1.4</td>
<td>-2.4 ± 1.3</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>342 ± 51</td>
<td>321 ± 57</td>
</tr>
<tr>
<td>Hypothermia</td>
<td>267 ± 44</td>
<td>269 ± 60</td>
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* Significantly different from Normothermia, P < 0.05.

demonstrated lower serum glucose values at 30 min after resuscitation (F(1,13) = 13.54; P = 0.003). Neurological scores improved over time (F(1,11) = 12.49; P = 0.005). The hypothermia-treated rats exhibited better neurological scores compared to normothermia rats at both 12 h (7.8 ± 2.2 vs. 3.7 ± 1.2) and 24 h (22 ± 4.8 vs. 11.5 ± 10.2) (F(1,11) = 5.45; P = 0.040).

3.2. Expression of BDNF exon mRNA by PCR

A range of concentrations of cDNA template was determined for each primer pair for each rat wherein the amount of PCR product was linearly related to the amount of template. For each sample, the correlation between template concentration and optical density of product on the gel was excellent (all correlation coefficients > 0.97). The results of a comparative gel can be seen in Fig. 2. These data were used to calculate the expression of each BDNF exon relative to GAPDH.

All BDNF exons were detected in rat hippocampus. After the same number of PCR cycles, 2- to 25-fold higher concentrations of cDNA template were required to visualize BDNF products than were required to visualize GAPDH products. Under normothermic control conditions, the relative expression was greatest for BDNF exon II and least for exon I.

Within the pooled samples, qualitatively there appeared to be an increase in expression of exons I, II, and III within the animals sacrificed 24 h after cardiac arrest. No significant change was observed for pooled samples from the 12-h groups (data not shown). Therefore, the individual RNA samples were reverse transcribed for the 24-h groups in order to thoroughly examine the effects of cardiac arrest on BDNF exon expression.

The pattern of relative gene expression remained similar with the pooled samples and the individually reverse transcribed groups (Fig. 3). The relative expression for exon IV in normothermia sham rats differed between pooled samples and individual samples. This difference was primarily due to an extreme value for an individual rat as reflected in the large variance for that group in Fig. 3. This subject did not differ from the other rats in any other parameter.

Twenty-four hours after cardiac arrest, expression of exons I and III was increased, as reflected by a significant main effect of ischemia on the expression of exon I (F(1,9) = 9.89, P = 0.012) and exon III (F(1,9) = 10.7, P = 0.010) (Fig. 3). There were no significant effects of cardiac arrest on exon II or exon IV.

Induction of hypothermia augmented the increase in expression of exon III after cardiac arrest, as reflected by a significant interaction between ischemia and hypothermia (F(1,9) = 6.12, P = 0.035) (Fig. 3). There was no effect of hypothermia by itself on expression in the rats that did not undergo cardiac arrest. In contrast, there was a trend toward hypothermia decreasing expression of exon II (F(1,9) = 4.84, P = 0.055) and exon IV (F(1,9) = 3.69, P = 0.087) after both cardiac arrest and sham operation (Fig. 3).

3.3. Expression of cyclophilin mRNA by PCR

Expression of another housekeeping gene, cyclophilin, required 40 cycles to obtain a detectable linear range. With the extra 5 cycles GAPDH and cyclophilin required approximately the same cDNA concentration. Comparison of cyclophilin to GAPDH revealed that cyclophilin expression did not change with respect to GAPDH under any of the experimental conditions (Fig. 4), suggesting no effect of these interventions on global transcription. However, there was considerable variation within the groups. Extrapolating the relative expression of the BDNF exons to GAPDH and comparing that data with the results obtained from the cyclophilin data revealed the same pattern of relative expression of the BDNF exons to cyclophilin (data not shown).

4. Discussion

This study reveals that there is increased expression of BDNF exons I and III relative to GAPDH in rat hippocampus 24 h after asphyxial cardiac arrest. In addition, a regimen of hypothermia after cardiac arrest that improves behavioral recovery selectively increased BDNF exon III expression. Confirming previous work, all rats in this study exhibited neurological deficits after resuscitation from cardiac arrest [7]. Rats treated with hypothermia exhibited improved neurological scores for the first 24 h. Thus, the
increase in exon III transcription is associated with improved functional recovery. The effect on exon III was specific because neither exon II nor IV was significantly altered after ischemia or hypothermia treatment. In fact, there appeared to be a trend toward diminished expression of exons II and IV in the both the sham and cardiac arrest hypothermia groups. Furthermore, the fact that there was no alteration in the expression of cyclophilin relative to GAPDH indicates that the observed effects of cardiac arrest and hypothermia on BDNF exons were specific rather than global changes in transcription. The present data did not examine another 5' noncoding exon for BDNF that has been described by one group [2].

An important technical aspect of the present study is that BDNF gene expression is reported relative to the expression of GAPDH. This approach is consistent with prior studies in which certain housekeeping genes, like GAPDH, are assumed to have constant transcription across experimental conditions. Recent studies recognize that expression of housekeeping genes varies after different insults. For example, a middle cerebral artery occlusion model of focal ischemia alters the commonly used control genes β-actin [1,6] and GAPDH [19]. Furthermore, changes in GAPDH and β-actin expression were noted using a high-density oligonucleotide probe array in a model of global cerebral ischemia and reperfusion [11]. That study suggested that cyclophilin as well as 27 other genes did not vary and could be used as adequate controls for relative gene expression. In the present study, altered GAPDH expression is less likely because the relative cyclophilin/GAPDH expression did not differ among our experimental groups, although there was significant between-subject variability in cyclophilin/GAPDH expression. Ideally, total gene expression analysis needs to be characterized in this model of asphyxial cardiac arrest in order to adequately determine proper housekeeping genes.

An alternative explanation for these data is that alterations in transcript stability may account for differences in BDNF exon expression. While hypothermia might slow the degradation of the exon III/V transcript, it is unclear why transcripts of similar size (exon II/V, exon IV/V) would be oppositely affected. Likewise, there is no obvious mechanism whereby hypothermia would increase degradation of transcripts containing exons II and IV. Distinguishing these possibilities would require further experiments with in vivo labeling of newly transcribed RNA.

The increases in exons I and III after cardiac arrest supports previous reports that transcription of exons I and III increase in the hippocampus after kainic acid-induced seizures [12,15,22] and transient forebrain ischemia [12,24]. However, in those experiments, the largest increases in expression were found 3–4 h after the experimental manipulation, and the expression levels of the exons I and III returned to baseline by 24 h. In this study, the expression of exon I remained significantly elevated 24 h after the insult in the normothermia ischemic rats. This difference may be related to the severity of the insult induced in this experiment. Asphyxia lasting for 8 min produces whole brain

![Figure 2](image-url)
ischemia with severe neurological injury, whereas the bilateral common artery occlusion models for 2 or 10 min used in the prior studies produces only transient forebrain reductions in blood flow [12,24]. Therefore, the greater extent of the insult after cardiac arrest could account for the temporal differences in BDNF transcription between the two models.

Increased expression of exon III was observed when delayed hypothermia treatment was imposed after asphyxial cardiac arrest. These data suggest that the previously reported increase in the mature BDNF protein 24 h after cardiac arrest in the delayed hypothermia treated rats is due largely to the increased expression of exon III [4]. Since all of the transcripts produce the same protein and exon I was also increased in the normothermic rats, an increase in transcription of exon III could account for the hypothermia-induced increase in BDNF protein. The fact that expression of exons II and IV were decreased by hypothermia minimizes the role of those exons in the therapeutic effect of cooling. Therefore, exon III transcriptional regulation

Fig. 3. Histograms representing mean (+ standard deviation) expression of each exon relative to GAPDH. Expression levels were calculated as mean (optical density/cDNA template volumes) as in Fig. 2. Ratios of expression levels for exons to expression levels of GAPDH were multiplied by 1000 for ease of graphing. Cardiac arrest resulted in increased expression of Exons I and III. Hypothermia after cardiac arrest increased expression of Exon III in particular. Hypothermia after cardiac arrest tended to reduce expression of Exons II and IV. NS, Normothermic Sham (n = 3); HS, Hypothermic Sham (n = 3); Norm, Normothermia after ischemia (n = 3); Hypo, Hypothermia after ischemia (n = 4); (a) Significant effect of cardiac arrest relative to sham (P < 0.05); (*) Significantly different from Norm, P < 0.05; (!) Significantly different from HS, P < 0.05.

Fig. 4. Histograms representing mean ± standard deviation cyclophilin expression relative to GAPDH multiplied by 1000. There were no significant differences between the groups in the expression of cyclophilin relative to GAPDH. Abbreviations are the same as Fig. 3.
may provide the primary control of increased BDNF expression during post-resuscitation hypothermia.

Analysis of exon-specific transcription provides insight into the signaling pathways that are activated in response to ischemia and hypothermia. In the particular case of BDNF, each exon-specific promoter has unique sets of response elements. Thus, an increase of exon-specific transcripts suggests the activation of intracellular signals that converge on the promoters for that exon. For example, the promoter for exon III includes a cAMP/Ca²⁺-response element (CRE) in rats [20] and humans [5] and two distinct Ca²⁺-response elements (CaRE1 and CaRE2) immediately 5’ to the CRE [3,21]. The CRE binding protein (CREB) is a requisite factor for the activity-dependent transcription of BDNF exon III, but it is not sufficient for transcriptional activation [17,20]. Concomitant activation of the CaRE1 by CaRF [21] and CaRE2, a Ca²⁺-responsive E-box, by Ca²⁺-activated upstream stimulatory factors (USF’s) 1 and 2 also appear to be required for transcription of BDNF exon III [3]. Both promoters for BDNF exon I and exon III are activated by Ca²⁺ influx, but these exons are differentially affected depending on the Ca²⁺ channels involved [18]. Promoter I is activated mainly by L-type voltage-dependent Ca²⁺ channels (L-VDCCs) while promoter III is activated by both L-VDCCs and N-methyl-D-aspartate (NMDA) glutamate receptors, but mainly by L-VDCCs [18].

Taken together with the present data, these features of the exon III promoter region suggest that hypothermia stimulates BDNF transcription by increasing CREB activation and Ca²⁺-influx via either L-VDCC or NMDA receptors. Unfortunately, little is known about the calcium influx during this time frame after cardiac arrest. Excitatory amino acid-mediated Ca²⁺-influx is unlikely to contribute directly because it has been shown in this same model that peak excitatory amino acid levels occur immediately after reperfusion and decrease within 2 h [10]. Therefore, it is plausible other factors beside glutamate are arbitrating the opening of NDMA receptors or L-VDCCs and the activation of USF 1 and 2. Increased activation of mitogen-activated protein kinases occurs in hippocampus after cardiac arrest, and these kinases are further activated by hypothermia treatment [8]. These signaling pathways may activate CREB. Further study is required to determine the upstream events directing the transcription of BDNF exon III.

Although localization of BDNF exon expression was not conducted in this study, it has been reported previously. Following kainic acid-induced seizures, maximal increases in exons I and III were seen in the dentate gyrus with minor increases in CA1 and CA3 neurons [12,15,16,22]. Likewise, after transient focal ischemia, the majority of alterations of exons I and III expression were in the dentate gyrus as measured by in situ hybridization [12,24]. However, slight increases were found for exon I in CA1 neurons and for exon III in CA3 and CA4 neurons as well as within the piriform cortex [24]. Within the hippocampus, the granule cells of the dentate gyrus are resistant to ischemic damage, whereas the cells within the CA1 region are extremely sensitive to ischemia [7]. At 14 days after cardiac arrest, normothermic rats lose 50% of their CA1 neurons compared to a 25% loss of CA1 neurons in rats receiving delayed hypothermia [7]. Thus, while BDNF expression is expected in dentate gyrus, increased expression of BDNF in the CA1 field could hypothetically account for the attenuation in CA1 neuronal loss after hypothermia compared to normothermic treatment after asphyxial cardiac arrest. Further studies using anatomically precise techniques are necessary to determine the sub-regional localization of these changes in gene expression.

This study provides evidence for specific transcriptional regulation of different BDNF exons after cardiac arrest. Therapeutic hypothermia after asphyxial cardiac arrest increases BDNF transcription primarily via an increase in transcription of exon III. These data are an example of a specific effect on intracellular signaling and gene expression in response to a very nonspecific injury (ischemia) and therapy (hypothermia). While these data do not establish a causal connection between BDNF and improved functional recovery, they do provide a more complete picture of how induced hypothermia affects cellular signaling after ischemia in a vulnerable brain region. These findings do demonstrate that induced hypothermia can increase specific signaling events in addition to slowing deleterious processes. Future studies can examine what transcription factors are involved in the exon-specific increases in expression, as well as their contribution to the therapeutic benefit afforded by hypothermia treatment.

Acknowledgments

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References


