

A Model to Induce Low Temperature Trauma for *in vitro* Astrogliosis Study*

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Astrogliosis is an inevitable and rapid response of astrocytes to physical, chemical and pathological injuries. To study astrogliosis, we developed a reproducible *in vitro* model in which low temperature injury to cultured astrocytes could be induced by placing the culture dish onto a copper pipe pre-cooled by liquid nitrogen. Using this model, the relationship between the temperature decline and the severity of cellular damage was analyzed. An increase in the expression of some known injury-related proteins, such as glial fibrillary acidic protein (GFAP), immediate early response genes (IEGs), and heat shock proteins 70 (HSP70), was demonstrated in astrocytes after low temperature trauma. With the use of this low temperature trauma model, the flexibility in the temperature control and injury area may allow researchers to evaluate cryotherapy and cryosurgery, which could be applicable to future development of quality health care.

KEY WORDS: astrocytes; glial fibrillary acidic protein (GFAP); gliosis; heat shock protein; immediate early gene; injury model; low temperature trauma.

INTRODUCTION

Many types of insult, including chemically and metabolically induced hypoxia or ischemia, bacterial or viral infection, radiation and mechanical trauma

may temporarily or permanently damage the central nervous system (CNS) (1). Injury to the CNS activates resting astrocytes by forming a glial scar through astrogliosis, which seals off the injured areas to restore the structural and functional integrity of the neural parenchyma (2–4). However, rapid astrogliotic response may interfere with the functions of the residual neuronal circuits by preventing remyelination and inhibiting axonal regeneration (2). In order to assess trauma-related behavioral and systematic effects, investigations on the pathophysiological mechanism of post-traumatic damage to astrocytes have been performed using animal models. However, *in vivo* models have many drawbacks, such as difficulties in reproducing the same extent of injuries in each experiment, and interference of systemic effects with the traumatic effects on specific cells.

To study astrogliosis more efficiently, we previously established an *in vitro* mechanical injury mod-

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el—scratch wound in primary culture of astrocytes (5). Similar to other *in vitro* models (6), the scratch wound model provides an efficient way to study morphology and other events on cellular and molecular levels, without the interference of other cell types. However, response from uninjured cells would dilute the scratch-induced effects on the damaged cells along the scratch, making the model moderate for gene expression study. Here, we introduce an *in vitro* physical injury model induced by low temperature using a copper pipe pre-cooled with liquid nitrogen onto a culture dish. This model provides a homogeneous physical damage in the contact area, making it well suited for gene expression study of injury to astrocytes and astrogliosis. The relationship between temperature decline and severity of cell injury was determined after low temperature trauma. Changes in GFAP, HSP70, and selected IEG products, including c-FOS, c-MYC and c-JUN, were studied using immunostaining.

MATERIALS AND METHODS

Astrocyte Cultures

Preparation of astrocytes from cerebral cortices of newborn mice was carried out as previously described (7). Cells were cultured at 37°C in a 5% CO₂ humidified atmosphere. Culture medium was changed twice weekly. Cultures that were at least four weeks old were used for experiments.

Low Temperature Trauma Model

Low temperature trauma was induced by placing the bottom of the culture dish in direct contact with a copper pipe that was pre-cooled in liquid nitrogen for 20 min (Fig. 1A). All experiments were performed at room temperature (15–18°C). The copper pipe used in this study had an outer diameter of 2.3 cm, an inner diameter of 0.8 cm and a height of 4 cm. Contact of the pipe with a 35-mm culture dish would introduce injury to ~38% area of the culture dish. Prior to inducing low temperature injury, culture medium was discarded. Direct contact between the dish bottom and pre-cooled copper pipe was made for 5 s. Pre-warmed medium (2 ml, 37°C) was added to the culture dish after-

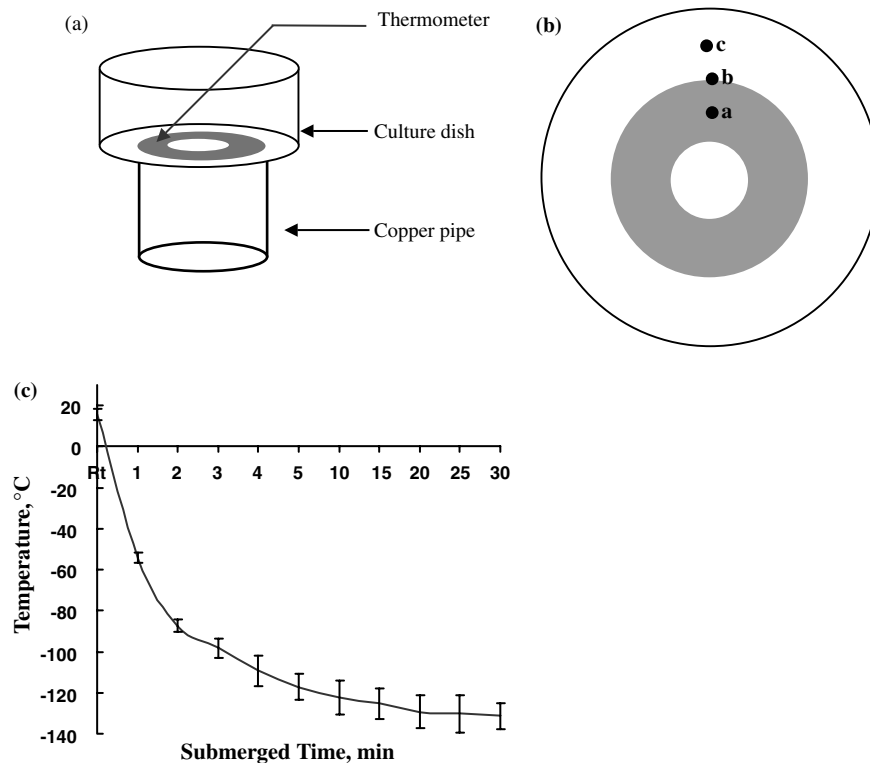


Fig. 1. Schematic representation of the low temperature trauma apparatus. (a) The bottom of culture dish in contact with the hollow copper pipe. The grey area is the contact point of the copper pipe with the plate. (b) Overhead view of the dish. The grey area indicates the zone in direct contact with the hollow copper pipe below. Point "a" is in direct contact with the pipe; point "b" is at the border of the direct contact zone, and point "c" is the farthest from the direct contact. The two views (a and b) are not drawn to the same scale. (c) Change in temperature of the upper surface of the copper pipe after immersion in liquid nitrogen against time. The temperature change was measured using a digital thermometer. Data were collected from three separate experiments and expressed as mean \pm SEM.

wards and culture was returned to the CO₂ incubator. Changes in temperature at the contact surface were monitored with a digital thermometer (Model DM6801A, Shenzhen Instrument Inc., Shenzhen, China), supplemented with a surface probe. Temperature changes were expressed as means \pm S.E.M. from three separate measurements and statistical significance was calculated using Students' *t*-test.

Immunocytochemistry

Cultured astrocytes were washed three times with 1 \times phosphate-buffered saline (PBS) before fixation in 4% paraformaldehyde for 30 min at room temperature. Cells were permeabilized with 0.2% Triton X-100 for 10 min. Primary antibodies against GFAP (polyclonal, 1:200; Sino-American Biotechnology Company, China), HSP70 (monoclonal, 1:5,000; Gene Company Ltd., Shanghai, China), c-FOS (polyclonal, 1:200; Santa Cruz Biotechnology, USA), c-MYC (polyclonal, 1:200; Santa Cruz Biotechnology, USA), or c-JUN (polyclonal, 1:200; Santa Cruz Biotechnology, USA) were added and incubated for 1 h at room temperature. Excess antibody was removed by washing three times with 1 \times PBS, after which the culture cells were treated with FITC-conjugated goat anti-rabbit immunoglobulin secondary antibody (1:400; Sino-American Biotechnology, China) for 30 min. Cells were mounted in Mowiol 4-88 and observed with an OLYMPUS fluorescence microscope.

RESULTS AND DISCUSSION

A reproducible *in vitro* trauma model to study astrogliosis has been demonstrated. This model simply employs a pre-cooled copper pipe to induce trauma in astrocytes cultured in 35 mm culture dishes. The degree of induced injury varied with the temperature change at the contact surface, while the degree of cooling differed on different regions of the culture dish. Three areas of contact were studied—the area of direct contact (point a in Fig. 1b), the area at the edge of direct contact (point b in Fig. 1b), and the area farthest from the contact zone (point c in Fig. 1b). In the experiments, the temperature was routinely monitored at points a–c at designated room temperature since the degree of cooling in the culture dish could readily be affected by ambient temperature fluctuation.

The temperature of the upper surface of the copper pipe fell rapidly when the lower part was immersed in liquid nitrogen (Fig. 1c), with a rate of $25.6 \pm 1.2^\circ\text{C}/\text{min}$ in the first 5 min, after which the rate leveled off. After 20 min, the temperature of the upper surface of the pipe reached -120°C , which was maintained thereafter. The consistent rate of temperature decline on the upper surface of the copper pipe allowed reproducibility of low tem-

perature trauma introduced to the astrocytes for each experiment. At points a, b and c, the rates of cooling after 5 s of contact were $42.6 \pm 6.8^\circ\text{C}/\text{min}$, $27.3 \pm 4.7^\circ\text{C}/\text{min}$ and $9.2 \pm 1.4^\circ\text{C}/\text{min}$, respectively. The lowest temperatures recorded at points a and b were $5.1 \pm 1.2^\circ\text{C}$ and $9.2 \pm 0.8^\circ\text{C}$, respectively. As the lowest temperature in the culture dish was always above freezing point, cell death was likely due to causes other than the formation of intracellular ice crystals.

All cells at point a suffered severe temperature shock. Trypan blue revealed that injured astrocytes became swollen, indicating severe injury to cell membrane. These cells subsequently ruptured after returning to the incubator for 10 min (Fig. 2b). Most cells at point b along the edge, however, suffered moderate temperature shock, and they were also swollen. But some cells sent out pale, flat processes while microglia appeared at this edge and migrated into the area with direct contact (Fig. 2c). The pale processes extending into the injured area became longer and more hypertrophic at 24 h after injury. On day 3, cell bodies with nuclei at point b started to migrate into the denuded area. The morphology of the cells at point c, however, remained normal throughout the experiment (Fig. 2d).

Changes of GFAP, HSP70 and IEG products (c-FOS, c-JUN and c-MYC) at different points of contact and duration of injury were followed by immunostaining. In the present model, GFAP was used as a characteristic marker of astrogliosis since it is the major component of the intermediate filaments in differentiated astrocytes (8), and is extensively synthesized adjacent to and within sites of injury (5,9,10). The use of an antisense GFAP mRNA demonstrated an increase in GFAP and morphological changes in scratch-injured astrocytes (11). Similar studies involving an antisense GFAP mRNA also showed blocking of dBcAMP-induced GFAP content in primary culture of astrocytes (5) and the morphological changes in co-cultures following mechanical lesion (12).

The change in GFAP content was mainly measured at point b because all the cells at point a died immediately while those at point c were not affected very much by the low temperature trauma. In agreement with the morphological changes, many astrocytes were stained heavily for GFAP, indicating higher GFAP content. The intensity of GFAP immunostaining at point b increased with the time after low temperature trauma (data not shown). GFAP content on day

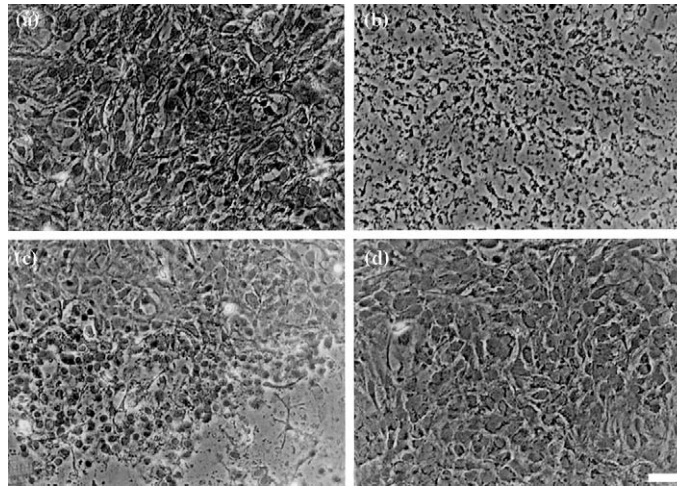


Fig. 2. Morphological changes in cultured mouse astrocytes at different contact points after low temperature trauma. The culture dish was in contact with the copper pipe for 5 s. (a) control; (b) point a in Fig. 1B; (c) point b in Fig. 1B; and (d) point c in Fig. 1B. Scale bar = 50 μ m.

6 after low temperature trauma remained high in the parallel arrays of the slender elongated processes (Fig. 3b).

The induction of HSP genes such as HSP90, HSP70 and small HSPs (HSP27) represents a universal response of cells to stressful conditions (13,14). In the HSP gene family, HSP70 has been shown to protect the CNS and its induction serves as a marker of protection against cellular damage and prompt recovery from the inducing stress (15,16). Although heat stress, or hyperthermia, is a typical trigger of heat shock responses, HSP70 has found to be induced by cold injury in bacterial and human cells (17,18). In this model, HSP70 content was studied and the intensity of HSP70 immunostaining became concentrated around the nuclei at 4 h (Fig. 3c) and 24 h after injury. In agreement with other published reports, this model demonstrated that cold injury could induce the expression of HSP70 in CNS cells.

IEG products (c-FOS, c-Jun and c-MYC) are also representative markers for increased neural activity and early-activated changes during injury. Stress in the CNS, including ischemia (19–21), traumatic injury (22), and seizures (23,24) induces expression of IEGs. We have previously shown the induction of IEG by scratch wound and ischemia in primary culture of astrocytes (6,25). Although the roles of these IEG products are still unclear, many, like c-FOS, are characterized by their rapid synthesis-independent activation and their role as transcription factors. It has been reported that

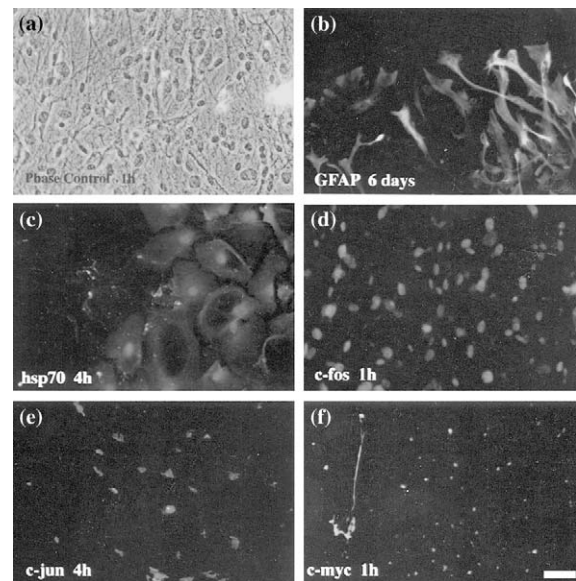


Fig. 3. Immunostaining of GFAP, HSP70 and IEG products at different time points after low temperature trauma. After incubation with corresponding primary antibodies, the cells were treated with FITC-conjugated goat anti-rabbit antibody. Cells were then mounted in Mowiol 4-88 and observed under a fluorescence microscope. (a) Phase control of astrocytes after low temperature trauma; (b) immunostaining of GFAP on day 6 after injury; (c) immunostaining of HSP70 at 4 h after injury; (d) immunostaining of c-FOS at 1 h after injury; (e) immunostaining of c-JUN at 1 h after injury; (f) immunostaining of c-MYC at 1 h after injury. Scale bar = 50 μ m.

c-FOS may be involved in the regulation of the expression of AP-1 complex, which subsequently affected GFAP expression (26,27).

Intense c-FOS immunostaining, mainly located in the nuclei, was detected at 1 h after low temperature injury (Fig. 3D), whereas untreated cells showed no cFOS signal. The c-FOS signal remained high at 24 h after injury. Unlike c-FOS, c-JUN immunostaining was moderate and distributed evenly throughout the nuclei and the cytoplasm in the control astrocytes. The intensity of c-JUN immunostaining in the nuclei increased at 1 h and peaked at 4 h after low temperature trauma (Fig. 3E). The c-MYC immunostaining, however, was concentrated in the nuclei of cells at the edge of the low temperature trauma. Although the intensity of c-MYC immunostaining was comparatively weaker than that of c-FOS or c-JUN, its expression peaked at 1 h after low temperature trauma (Fig. 3F) and remained detectable at 24 h after injury. These demonstrated an involvement of IEG products in the progress of astrogliosis.

Different *in vitro* trauma models have been designed for studying astrogliosis, including laser microdissection of neurites (28), deformation of a silastic membrane upon which neurons and/or glia have been plated (29), rapid acceleration/deceleration of culture flasks (30), elevation of pressure for extended time periods (31), propagation of fluid-pulses in brain slices (32) or cultured cells (33), induction of shear forces (34), scoring of culture plates with needles (35) or mechanical punch devices (36), and scratching cultured cells with plastic pipette tips (5,37). The injury induced by our low temperature trauma model resembles cryosurgical incision (38), which inevitably induces a cellular response along the cryoinjured edge. Other similar *in vitro* physical methods to induce reactive gliosis involve mechanical scraping with a specially designed motorized punch device (36) and mechanical stretching model using cells cultured on an elastic membrane (29). However, limitations in some of these injury models include producing regional injury only to the cells along the injury sites, and difficulties in reproducing the physical injury for each experiment.

This low temperature trauma model allows the measurement and observation of temperature effects on astrocytes by morphological and immunostaining means. The most important feature of this model is its flexibility since a defined area of injury can be easily and directly modified by varying the size and shape of the copper pipe according to the designs of the experiments. For instance, a solid copper block may be used instead of a copper pipe

to increase the injury area to the whole culture plate, the diameter of the copper pipe may be varied, and the rate of temperature change can be adjusted to monitor the degree of cellular damage. With the aid of laser dissection microscope to isolate injured and unharmed cells, the study of astrogliosis can be performed at cellular and molecular levels, focusing on gene and protein expression patterns in these cells after injury.

In conclusion, this paper describes a reproducible *in vitro* model to study astrogliosis and temperature effects on astrocyte injury. The flexibility in the temperature control and injury area in this model may allow researchers to evaluate cryotherapy and cryosurgery, which could be applicable to future development of quality health care.

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REFERENCES

1. Trump, B. F., and Berezsky, I. K. 1992. The role of cytosolic Ca^{2+} in cell injury, necrosis and apoptosis. *Cur. Opin. Cell Biol.* 4:227–232.
2. Eng, L. F., Yu, A. C. H., and Lee, Y. L. 1992. Astrocytic response for injury. pages 353–365. in Yu, A.C. H. Hertz, L., Norenberg, M. D., Sykova, E., and Waxman S. G. (eds.), *Prog. Br. Res.* Vol. 94. Amsterdam: Elsevier.
3. Khanolkar-Young, S., Young, D. B., Colston, M. J., Stanley, J. N., and Lockwood, D. N. 1994. Nerve and skin damage in leprosy is associated with increased intralosomal heat shock protein. *Clin. Exp. Immunol.* 96:208–213.
4. Reier, P. J., and Houle, J. D. 1988. The gliar scar: Its bearing on axonal elongation and transplantation approaches to CNS repair. Pages 87–138 in: *Physiological basis for functional recovery in neurological disease.* Waxman, S. G. (ed), New York: Raven Press.
5. Yu, A. C. H., Lee, Y. L., and Eng, L. F. 1993. Astrogliosis in culture: I. The model and the effect of antisense oligonucleotides on glial fibrillary acidic protein synthesis. *J. Neurosci. Res.* 34:295–303.
6. Eng, L. F., Lee, Y. L., Murphy, G. M., and Yu, A. C. H. 1995. A RT-PCR study of gene expression in a mechanical injury model. *Prog. Br. Res.* 105:219–229.
7. Yu, A. C., Yung, H. W., Hui, M. H., Lau, L. T., Chen, X. Q., and Collins, R. A. 2003. Cyclohexamide and actinomycin D delay death and affect bcl-2, bax, and Ice gene expression in astrocytes under *in vitro* ischemia. *J. Neurosci. Res.* 15:318–325.
8. Eng, L. F., Reier, P. J., and Houle, J. D. 1987. Astrocyte activation and fibrous gliosis: Glial fibrillary acidic protein

- immunostaining of astrocytes following intraspinal cord grafting of fetal CNS tissue. Pages 439–455 in: Seil, F. J., Herbert, E., and Carson B. M. (eds.), Prog. Br. Res. Vol 71. Amsterdam: Elsevier.
9. Li, R., Fujitani, N., Jia, J. T., and Kimura, H. 1998. Immunohistochemical indicators of early brain injury: an experimental study using the fluid-percussion model in cats. *Am. J. Forensic. Med. Pathol.* 19:129–136.
 10. Wu, V. W., and Schwartz, J. P. 1998. Cell culture models for reactive gliosis: new perspectives. *J. Neurosci. Res.* 51:675–681.
 11. Ghirnikar, R. S., Yu, A. C. H., and Eng, L. F. 1994. Astrogliosis in culture. III. Effect of recombinant retrovirus expressing antisense glial fibrillary acidic protein RNA. *J. Neurosci. Res.* 48:376–385.
 12. Lefrancois, T., Fages, F., Peschanski, M., and Tardy, M. 1997. Neuritic outgrowth associated with astroglial phenotypic changes induced by antisense glial fibrillary acidic protein (GFAP) mRNA in injured neuron-astrocytes cocultures. *J. Neurosci.* 17:4121–4128.
 13. Jaattela, M., and Wissing, D. 1992. Emerging role of heat shock proteins in biology and medicine. *Ann. Med.* 24:249–258.
 14. Li, G. C., Mivechi, N. F., and Weitzel, G. 1995. Heat shock proteins, thermotolerance, and their relevance to clinical hyperthermia. *Int. J. Hyperthermia.* 11:459–488.
 15. Kogure, T., and Kogure, K. 1997. Molecular and biochemical events within the brain subjected to cerebral ischemia (targets for therapeutic intervention). *Clin. Neurosci.* 4:179–183.
 16. Massa, S. M., Swanson, R. A., and Sharp, F. R. 1996. The stress gene response in brain. *Cerebrovasc. Brain Metab. Rev.* 8:95–158.
 17. Yiangou, M., Tsapogas, P., Nikolaidis, N., and Scouras, Z. G. 1997. Heat shock gene expression during recovery after transient cold shock in *Drosophila auraria* (Diptera: Drosophilidae). *Cytobios* 92:91–98.
 18. Liu, A. Y., Bian, H., Huang, L. E., and Lee, Y. K. 1994. Transient cold shock induces the heat shock response upon recovery at 37 degrees C in human cells. *J. Biol. Chem.* 269:14768–14775.
 19. Kim, Y., Truettner, J., Zhao, W., Busto, R., and Ginsberg, M. D. 1998. The influence of delayed postischemic hyperthermia following transient focal ischemia: alterations of gene expression. *J. Neurol. Sci.* 159:1–10.
 20. Welsh, F. A. 1994. Regional expression of immediate-early genes and heat-shock genes after cerebral ischemia. *Ann. NY Acad. Sci.* 723:318–327.
 21. Nowak, T. S. Jr., Osborne, O. C., and Suga, S. 1993. Stress protein and proto-oncogene expression as indicators of neuronal pathophysiology after ischemia. *Prog. Br. Res.* 96:95–208.
 22. Raghupathi, R., Welsh, F. A., Lowenstein, D. H., Gennarelli, T. A., and McIntosh, T. K. 1995. Regional induction of c-fos and heat shock protein-72 mRNA following fluid-percussion brain injury in the rat. *J. Cereb. Blood Flow Metab.* 15:467–473.
 23. Del Bel, E. A., Silveira, M. C., Graeff, F. G., Garcia-Cairasco, N., and Guimaraes, F. S. 1998. Differential expression of c-fos mRNA and Fos protein in the rat brain after restraint stress or pentylenetetrazol-induced seizures. *Cell. Mol. Neurobiol.* 18:339–346.
 24. Tanaka, K., and Simon, R. P. 1994. The pattern of neuronal injury following seizures induced by intranigral kainic acid. *Neurosci. Lett.* 176:205–208.
 25. Yu, A. C. H., Lee, Y. L., Fu, W. Y., and Eng, L. F. 1995. Gene expression in neural cells during and after ischemia. *Prog. Br. Res.* 105:245–253.
 26. Barnett, S. C., Rosario, M., Doyle, A., Kilbey, A., Lovatt, A., and Gillespie, D. A. 1995. Differential regulation of AP-1 and novel TRE-specific DNA-binding complexes during differentiation of oligodendrocyte-type-2-astrocyte (O-2A) progenitor cells. *Development* 121:3969–3977.
 27. Pennypacker, K. R., Thai, L., Hong, J. S., and McMillian, M. K. 1994. Prolonged expression of AP-1 transcription factors in the rat hippocampus after systemic kainate treatment. *J. Neurosci.* 14:3998–4006.
 28. Lucas, J. H., Gross, G. W., Emery, D. G., and Gardner, C. R. 1985. Neuronal survival or death after dendrite transection close to the perikaryon: correlation with electrophysiologic, morphologic and ultrastructural changes. *CNS Trauma* 2:231–255.
 29. Ellis, E. F., Mckinney, J. S., Willoughby, K. A., Liang, S., and Povlishock, J. T. 1995. A new model for rapid stretch-induced injury of cells in culture: Characterization of the model using astrocytes. *J. Neurotrauma* 12:325–339.
 30. Lucas, J. H. and Wolf, A. 1991. *In vitro* studies of multiple impact injury to mammalian CNS neurons: prevention of perikaryal damage and death by ketamine. *Brain Res.* 543:181–193.
 31. Murphy, E. J. and Horrocks, L. A. 1993. A model for compression trauma: pressure-induced injury in cell culture. *J. Neurotrauma* 10:431–444.
 32. Girard, J., Panizzon, K., and Wallis, R. A. 1996. Azelastine protects against CA1 traumatic neuronal injury in the hippocampal slice. *Eur. J. Pharmacol.* 300:43–49.
 33. Shepard, S. R., Ghajar, J. B. G., Giannuzzi, R., Kupferman, S., and Hariri, R. J. 1991. Fluid percussion barotrauma chamber: a new *in vitro* model for traumatic brain injury. *J. Surg. Res.* 51:417–424.
 34. Laplaca, M. C., Lee, V. M. Y., and Thibault, L. E. 1997. An *in vitro* model of traumatic neuronal injury: loading rate-dependent changes in acute cytosolic calcium and lactate dehydrogenase release. *J. Neurotrauma* 14:355–368.
 35. Regan, R. F. and Choi, D. W. 1994. The effect of NMDA, AMPA/kainate, and calcium channel antagonists on traumatic cortical neuronal injury in culture. *Brain Res.* 633:236–242.
 36. Mukhin, A. G., Ivanova, S. A., Allen, J. W., and Faden A. I. 1998. Mechanical injury to neuronal/glial cultures in microplates: role of NMDA receptors and pH in secondary neuronal cell death. *J. Neurosci. Res.* 51:748–758.
 37. Yu, A. C. H. and Lau, L. T. 2000. Expression of interleukin-1 alpha, tumor necrosis factor alpha and interleukin-6 genes in astrocytes under ischemic injury. *Neurochem. Internat.* 36:369–377.
 38. He, X., and Bischof, J. C. 2003. Quantification of temperature and injury response in thermal therapy and cryosurgery. *Crit. Rev. Biomed. Eng.* 31:355–422.