Traumatic Brain Injury and Hemorrhagic Hypotension Suppress Neuroprotective Gene Expression in Injured Hippocampal Neurons

Helen Lee Hellmich, Ph.D.,* Jeanna M. Garcia, B.S.,† Megumi Shimamura, M.D., Ph.D.,‡ Syed A. Shah, M.D.,§
Marcela A. Avila, M.D.,§ Tatsuo Uchida, M.S.,‖ Margaret A. Parsley, B.S.,† Bridget A. Capra, B.S.,#
Kristine A. Eidson, B.S.,# Deborah R. Kennedy, B.S.,# John H. Winston, Ph.D.,** Douglas S. DeWitt, Ph.D.,††
Donald S. Prough, M.D.‡‡

Background: After traumatic brain injury, memory dysfunction is due in part to damage to the hippocampus. To study the molecular mechanisms of this selective vulnerability, the authors used laser capture microdissection of neurons stained with Fluoro-Jade to directly compare gene expression in injured (Fluoro-Jade–positive) and adjacent uninjured (Fluoro-Jade–negative) rat hippocampal neurons after traumatic brain injury and traumatic brain injury plus hemorrhagic hypotension.

Methods: Twelve isoflurane-anesthetized Sprague-Dawley rats underwent moderate (2.0 atm) fluid percussion traumatic brain injury followed by either normotension or hemorrhagic hypotension. Animals were killed 24 h after injury. Frozen brain sections were double stained with 1% cresyl violet and 0.001% Fluoro-Jade. RNA from 10 Fluoro-Jade–positive neurons and 10 Fluoro-Jade–negative neurons, obtained from the hippocampal CA1, CA3, and dentate gyrus subfields using laser capture microdissection, was linearly amplified and analyzed by quantitative ribonuclease protection assay for nine neuroprotective and apoptosis-related genes.

Results: In injured CA3 neurons, expression of the neuroprotective genes glutathione peroxidase 1, heme oxygenase 1, and brain-derived neurotrophic factor was significantly decreased compared with that of adjacent uninjured neurons. Superimposition of hemorrhagic hypotension was associated with downregulation of neuroprotective genes in both injured and uninjured neurons of all subregions. Expression of apoptosis-related genes did not vary between injured and uninjured neurons, with or without superimposed hemorrhage.

Conclusions: The authors show, in the first direct comparison of messenger RNA levels in injured and uninjured hippocampal neurons, that injured neurons express lower levels of neuroprotective genes than adjacent uninjured neurons.

PARASAGITTAL, fluid percussion traumatic brain injury (TBI) in rats produces a combination of focal cortical contusion and diffuse subcortical injury[1–4] that repli-cates several clinically relevant features of human TBI. In rats, fluid percussion TBI causes memory dysfunction and scattered loss of neurons in the hippocampus, most prominently in the CA3 subfield.[4–6] In patients surviving TBI, memory dysfunction, likely due to hippocampal damage, is a frequent, disabling complication. After clinical TBI, secondary injury due to hypotension, which frequently accompanies head injury, markedly reduces the likelihood of favorable neurologic outcome.[7]

We previously reported that enhanced expression of neuroprotective genes was associated with the resistance of hippocampal subfields to injury in an *in vivo* model of TBI in rats[8] and that chelation of neurotoxic zinc both increased expression of neuroprotective genes and reduced neuronal cell death in rat brains after TBI.[9] Despite extensive study of the molecular mechanisms of neuronal cell death in animal models of TBI, no neuroprotective treatment has improved outcome in clinical trials of human brain injury.[10] Because many of these treatments involved inhibition of signaling components involved in cell death and degeneration, these failures suggest that alternative therapeutic strategies are needed.

Recent studies of the mechanisms of cancer resistance have suggested that a viable therapeutic strategy would be to boost inhibitors of angiogenesis that occur naturally as part of the endogenous defense against tumor formation.[11] Similarly, a viable therapeutic strategy for patients with TBI could be to augment endogenous neuroprotective responses elicited by brain injury. To that end, in this study, we sought to pursue the identification of the molecular factors (genes) that potentially contribute to injury resistance and survival after TBI. We hypothesized that this could be addressed by examining the effects of TBI on a homogeneous population of brain cells that received an identical injury (same injury level, same time), provided that one could distinguish an injured, degenerating cell from an uninjured cell.

As noted above, experimental TBI causes a distinctive pattern of scattered hippocampal neuronal death that preferentially affects CA3 and the dentate gyrus.[12,13] Because neuronal injury is aggravated by post-TBI hemorrhagic hypotension,[14,15] we used Fluoro-Jade, a poly-anionic fluorescein derivative that sensitively binds to and stains irreversibly injured, degenerating neurons,[16,17] to distinguish injured from uninjured hip-

* Assistant Professor, † Research Associate, § Postdoctoral Fellow, # Research Assistant, †† Professor, ‡‡ Professor and Chair, Department of Anesthesiology, ‖ Senior Biostatistician, Office of Biostatistics, ** Assistant Professor, Division of Gastroenterology, Department of Internal Medicine, University of Texas Medical Branch, Galveston, Texas. ‡ Assistant Professor, Department of Neurology, Yokohama City University School of Medicine, Yokohama, Japan.

Address reprint requests to Dr. Hellmich: Department of Anesthesiology, University of Texas Medical Branch, 301 University Boulevard, Galveston, Texas 77555-0830. Address electronic mail to: hhellmic@utmb.edu. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

806

pocampal neurons after TBI or TBI combined with severe hemorrhagic hypotension (TBI/HH). We then used laser capture microdissection (LCM) to obtain 10 injured and 10 uninjured neurons, identified by Nissl staining with cresyl violet, from the CA1, CA3, and dentate gyrus (DG) subregions of each rat hippocampus. We hypothesized that, in morphologically similar hippocampal neurons, those that were uninjured after TBI would express higher levels of neuroprotective gene expression than adjacent, injured neurons. Although one previous study of Fluoro-Jade–positive degenerating neurons in the rat hippocampus examined expression of the nuclear factor κB p50 subunit in Fluoro-Jade–positive CA1 pyramidal neurons by immunostaining,[18] ours is the first study to examine gene expression in Fluoro-Jade–positive, laser-captured hippocampal neurons. For the current study, we selected a representative group of genes whose expressions we had previously found to be altered by TBI[9] or were known from previous studies to be altered by TBI.[19,20]

Materials and Methods

Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch, Galveston, Texas. Moderate parasagittal fluid percussion TBI was performed in 12 isoflurane-anesthetized rats as previously described.[21] Rats were then randomly assigned to a normotensive group or a hemorrhagic hypotension group, in which was hypotension induced by withdrawing sufficient blood to reduce mean arterial blood pressure to 40 mmHg for 45 min, after which shed blood was returned and animals were permitted to recover from anesthesia. Because in a previous microarray study[9] we observed the greatest number of changes in gene expression 24 h after injury, we chose that time interval for the current study. Therefore, 24 h after TBI, rats were anesthetized with 4% isoflurane and decapitated, and their brains were rapidly removed and frozen on dry ice. Brains were stored at $-80°C$ until they were embedded in OCT (optimal cutting temperature) medium for sectioning on a cryostat.

LCM and Linear mRNA Amplification

Frozen 10-μm coronal brain sections containing both the ipsilateral and the contralateral hippocampus (3.6–4.5 mm posterior to bregma) were collected on clean uncoated ribonuclease (RNase)-free slides (Fisher Scientific International, Hampton, NH) and kept at $-20°C$ until they were stained. Slides were thawed for 30 s, fixed in 75% ethanol (1 min), rinsed in RNase-free water (1 min), stained with 1% cresyl violet (15 s), rinsed twice with RNase-free water (30 s each), stained for 4 min with

0.001% Fluoro-Jade (diluted 1:10 in 0.1% acetic acid from 0.01% stock solution made with RNase-free water) in a light-tight container, rinsed three times in RNase-free water (1 min each), dehydrated in 95% and 100% ethanol (30 s each) and twice in xylene (3 min each), air-dried in a hood for 10–15 min, and stored with desiccant in a closed slide box until LCM was performed. Neurons were identified by staining with cresyl violet, a Nissl stain that stains only neurons and not glia and that is compatible with preservation of RNA integrity in laser microdissected cells. Fluoro-Jade has a green iridescence with an excitation peak at 480 nm and an emission peak at 525 nm and is visualized using a fluorescein/fluorescein isothiocyanate filter. Adjacent Fluoro-Jade positive and Fluoro-Jade negative cells were only obtained from the ipsilateral (below fluid percussion injury site) hippocampus because there were virtually no injured cells in the contralateral hippocampus of any of the 12 injured rats. Ten Fluoro-Jade–positive neurons and 10 Fluoro-Jade–negative neurons each from each hippocampal subregion (CA1, CA3, and DG) were captured on separate CapSure HS caps (Arcturus Engineering, Mountain View, CA) by LCM using a PixCell II laser capture microscope (Arcturus Engineering). Using an RNAqueous Micro Kit and MessageAmp Kits (both from Ambion, Austin, TX), we performed total RNA isolation and linear amplification of messenger RNA (mRNA) (as previously described[8]) with T7 RNA polymerase, which reproducibly amplifies limited quantities of mRNA while maintaining proportional representation of gene expression.[22]

Ribonuclease Protection Assay, Northern Blots, and Real-time PCR Analyses

To compare mRNA expressions quantitatively, we used ribonuclease protection assay (RPA) analysis, a simple and linear assay sensitive enough to detect even low-abundance transcripts. RPA analysis was performed with 100 ng linearly amplified mRNA samples as previously described[8] using the HybSpeed RPA kit (Ambion). Probes for RPA and Northern blots were cloned using reverse-transcription polymerase chain reaction (PCR) as previously described.[8] Probes for RPA included consensus promoter sequences for T3 (sense strand) and T7 (antisense strand) RNA polymerase. Using reverse-transcription PCR, we cloned nine genes that are neuroprotective (glutathione peroxidase 1 [GPX-1], heme oxygenase 1 [HO-1], brain-derived neurotrophic factor [BDNF], heat shock protein 70 [HSP70]) or proapoptotic or antiapoptotic (Bcl-2, caspase 3, caspase 9, FAS, interleukin 1β [IL-1β]) and prepared two multiprobe sets of sense riboprobes from these templates to hybridize to the antisense amplified mRNA (aRNA) from the neuronal cell samples. We previously reported that one round of linear amplification with T7 RNA polymerase yielded sufficient aRNA (typically 400–1,500 ng) from 600–800 neurons to conduct several RPA experiments.[8] Subse-

Table 1. Normalized Ribonuclease Protection Assay–derived mRNA Expression Values

Gene	Animal's Injury Type	Cell Injury Level	CA1 Mean	CA1 SD	CA3 Mean	CA3 SD	DG Mean	DG SD
GPX-1	TBI	Uninjured	174238	15312	352626	94342	266399	93447
	TBI	Injured	174859	10495	158616	13044	252489	72035
	TBI hemorrhage	Uninjured	109931	15134	81089	6377	83444	9017
	TBI hemorrhage	Injured	107039	12473	79476	3882	84623	13232
IL-1β	TBI	Uninjured	503414	29993	547310	9455	502124	28311
	TBI	Injured	454765	38520	500545	50870	502287	19900
	TBI hemorrhage	Uninjured	194550	13626	194934	10094	157531	9553
	TBI hemorrhage	Injured	176232	10679	178712	19159	170425	15266
BDNF	TBI	Uninjured	366690	31228	484626	86866	413642	55335
	TBI	Injured	366581	39081	327460	23626	398439	78798
	TBI hemorrhage	Uninjured	262008	18624	234058	11783	225201	20100
	TBI hemorrhage	Injured	261126	15564	218686	22971	210149	9638
HO-1	TBI	Uninjured	327326	34215	425807	72372	348704	32117
	TBI	Injured	328502	44870	294087	31433	362198	56204
	TBI hemorrhage	Uninjured	198013	15255	179902	11016	170783	16156
	TBI hemorrhage	Injured	194644	11819	170852	16434	166278	9926
HSP70	TBI	Uninjured	1794150	311135	2050093	404007	1757413	160378
	TBI	Injured	1908188	252019	1870380	215410	2118334	324013
	TBI hemorrhage	Uninjured	513659	53131	395263	32646	397954	60904
	TBI hemorrhage	Injured	424088	66840	300900	82530	284895	24365
CASP3	TBI	Uninjured	454934	54961	500348	66751	453684	29578
	TBI	Injured	476056	44298	495742	26588	518819	56933
	TBI hemorrhage	Uninjured	283157	40196	225953	12201	230606	27559
	TBI hemorrhage	Injured	274484	40804	187944	43351	224783	32975
BCL-2	TBI	Uninjured	557221	45536	540432	56939	510639	25563
	TBI	Injured	559369	49990	571517	38399	605776	54263
	TBI hemorrhage	Uninjured	402080	33017	321799	19563	300637	35197
	TBI hemorrhage	Injured	347779	40400	256850	59058	276109	23814
FAS ligand	TBI	Uninjured	346286	16867	315930	18272	305254	15228
	TBI	Injured	323075	18339	353153	29538	333131	22553
	TBI hemorrhage	Uninjured	321566	20631	299574	18640	265199	20150
	TBI hemorrhage	Injured	307993	25379	231906	44908	275698	25679
CASP9	TBI	Uninjured	367998	9161	348643	16959	333146	14380
	TBI	Injured	343913	13697	385646	23217	367696	21357
	TBI hemorrhage	Uninjured	386135	13107	377530	13694	323854	20838
	TBI hemorrhage	Injured	379415	24077	325503	62658	337607	22449

BDNF, brain-derived neurotrophic factor; CASP3 = caspase 3; CASP9 = caspase 9; DG = dentate gyrus; GPX-1 = glutathione peroxidase 1; HO-1 = heme oxygenase 1; HSP-70 = heat shock protein 70; IL-1β = interleukin 1β; mRNA = messenger RNA; TBI = traumatic brain injury.

quently, we demonstrated equivalent detection of a moderately expressed gene BDNF using 100 ng amplified mRNA from 5, 10, or 25 hippocampal neurons in an RPA analysis with a sense BDNF riboprobe. Consequently, we chose to study 10 injured neurons and 10 uninjured neurons from the three subfields of each rat hippocampus. In previous experiments in which we examined the differences in gene expression between young and aged rat brains with and without TBI, we found that normalizing mRNA levels to β-actin or glyceraldehyde-3-phosphate dehydrogenase (G3PDH) or both gave the same results.[8] β-Actin has been shown to be unchanged after injury in the rat retina,[23] after fluid percussion TBI in rat brain,[24] and after cerebral ischemia in mice.[25] Because these two commonly accepted "housekeeping genes" are completely different and serve different functions, we concluded that using G3PDH as an internal control was justified in our experimental paradigm. Further, a recent study of the use of housekeeping genes in quantitative gene expression

studies[26] showed that genes such as G3PDH are good single housekeeping genes for any given tissue type. Therefore, in the current study, we used G3PDH to normalize mRNA expression because we were comparing mRNA levels in adjacent neurons of homogeneous populations of hippocampal cells and the size of the protected fragment fit more appropriately with the multiprobe gene sets.

The first sense, multiprobe, riboprobe set was composed of GPX-1 (GenBank acc# ×12367), BDNF (GenBank acc# NM_012513), HO-1 (GenBank acc# J02722), IL-1β (GenBank acc# NM_031512), and G3PDH (GenBank acc# NM_017008); the second set consisted of caspase 3 (GenBank acc# NM_012922), caspase 9 (GenBank acc# NM_031632), FAS ligand (GenBank acc# U03470), Bcl-2 (GenBank acc# L14680), HSP70 (GenBank acc# Z27118), and G3PDH. Quantitative real-time PCR with rat BDNF primers[27] was performed with the Applied Biosystems 5700 thermal cycler (Foster City, CA) and Applied Biosystems kits and reagents, according

Table 2. Log-transformed Normalized Gene Expression Values

Log Data Gene	Animal's Injury Type	Cell's Injury Level	CA1 Mean	CA1 SD	CA3 Mean	CA3 SD	DG Mean	DG SD
GPX-1	TBI	Uninjured	5.233	0.036	5.475	0.110	5.339	0.109
	TBI	Injured	5.239	0.026	5.194	0.034	5.333	0.103
	TBI hemorrhage	Uninjured	5.022	0.056	4.902	0.034	4.914	0.046
	TBI hemorrhage	Injured	5.016	0.048	4.898	0.021	4.913	0.062
IL-1β	TBI	Uninjured	5.698	0.027	5.738	0.007	5.697	0.025
	TBI	Injured	5.649	0.041	5.685	0.052	5.699	0.018
	TBI hemorrhage	Uninjured	5.283	0.032	5.287	0.024	5.195	0.026
	TBI hemorrhage	Injured	5.242	0.027	5.238	0.050	5.226	0.040
BDNF	TBI	Uninjured	5.557	0.035	5.653	0.074	5.600	0.051
	TBI	Injured	5.552	0.045	5.510	0.030	5.570	0.067
	TBI hemorrhage	Uninjured	5.413	0.029	5.367	0.021	5.347	0.041
	TBI hemorrhage	Injured	5.413	0.025	5.327	0.049	5.321	0.021
HO-1	TBI	Uninjured	5.504	0.044	5.597	0.076	5.533	0.039
	TBI	Injured	5.497	0.057	5.457	0.045	5.536	0.062
	TBI hemorrhage	Uninjured	5.291	0.032	5.251	0.027	5.227	0.040
	TBI hemorrhage	Injured	5.285	0.027	5.222	0.042	5.219	0.026
HSP70	TBI	Uninjured	6.212	0.091	6.275	0.079	6.236	0.038
	TBI	Injured	6.259	0.064	6.254	0.058	6.301	0.065
	TBI hemorrhage	Uninjured	5.700	0.042	5.588	0.042	5.581	0.076
	TBI hemorrhage	Injured	5.599	0.071	5.299	0.234	5.449	0.041
CASP3	TBI	Uninjured	5.638	0.063	5.680	0.059	5.652	0.028
	TBI	Injured	5.667	0.043	5.692	0.024	5.700	0.052
	TBI hemorrhage	Uninjured	5.429	0.065	5.351	0.023	5.352	0.058
	TBI hemorrhage	Injured	5.412	0.070	5.119	0.219	5.335	0.073
BCL-2	TBI	Uninjured	5.738	0.038	5.721	0.045	5.705	0.022
	TBI	Injured	5.738	0.041	5.753	0.027	5.773	0.040
	TBI hemorrhage	Uninjured	5.597	0.034	5.503	0.029	5.469	0.052
	TBI hemorrhage	Injured	5.525	0.055	5.254	0.221	5.436	0.041
FAS ligand	TBI	Uninjured	5.537	0.021	5.496	0.026	5.482	0.022
	TBI	Injured	5.505	0.027	5.540	0.036	5.517	0.033
	TBI hemorrhage	Uninjured	5.503	0.027	5.473	0.026	5.420	0.033
	TBI hemorrhage	Injured	5.480	0.039	5.231	0.206	5.434	0.044
CASP9	TBI	Uninjured	5.565	0.011	5.540	0.022	5.521	0.019
	TBI	Injured	5.535	0.018	5.582	0.026	5.561	0.027
	TBI hemorrhage	Uninjured	5.586	0.014	5.576	0.016	5.507	0.030
	TBI hemorrhage	Injured	5.574	0.030	5.367	0.219	5.526	0.027

BDNF = brain-derived neurotrophic factor; CASP3 = caspase 3; CASP9 = caspase 9; DG = dentate gyrus; GPX-1 = glutathione peroxidase 1; HO-1 = heme oxygenase 1; HSP-70 = heat shock protein 70; IL-1β = interleukin 1β; TBI = traumatic brain injury.

to the manufacturer's protocols, using pooled uninjured CA3 aRNA and pooled injured CA3 aRNA from all six TBI rats. Reaction conditions for first strand synthesis were 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. The 50-cycle, two-step PCR conditions were 95°C for 10 min followed by 50 cycles of 15 s at 94°C and 1 min at 60°C. BDNF expression was normalized to protein gene product (PGP9.5). The protein gene product 9.5 primer sequences were forward 5'-CCTGGGCTCAGTGCCA-TCT-3' and reverse 5'-CCTCAATTTGTTTTTTCCTGAA-GTTT-3', and the probe was 5'-ATTCTCTGCATCCGTC-CTCACCGG-3'. Northern blot analysis of injured and uninjured CA3 aRNA samples (pooled samples from six TBI rats) was performed according to standard protocols and as previously described[8] using a random primer [α-^{32}P]deoxycytidine triphosphate–labeled GPX-1 probe. RPA and Northern blots were exposed to phosphoimaging screens, scanned on a Cyclone PhosphorImager (Perkin Elmer Life Sciences, Downers Grove IL), analyzed with OptiQuant software (Perkin Elmer), and imported into an Excel (Microsoft, Redmond, WA) spreadsheet for further analysis.

Statistical Analysis

As a result of heterogeneous variances, levels of mRNA were logarithmically transformed (original normalized data and log-transformed data are shown in tables 1 and 2). The transformed data were analyzed using analysis of variance for a split-plot design with a main unit factor of treatment (TBI or TBI/HH) and subunit factors of region (CA1, CA3, and DG) and injury (uninjured and injured) (PROC MIXED in SAS®, release 8.2; SAS Institute Inc., Cary NC).[28] Main effects and interactions were assessed at the 0.05 level of significance. The Fisher least significant difference procedure with Bonferroni adjustment was used for multiple comparisons.

Results

Coronal sections containing the ipsilateral and contralateral hippocampi of all experimental rat brains were
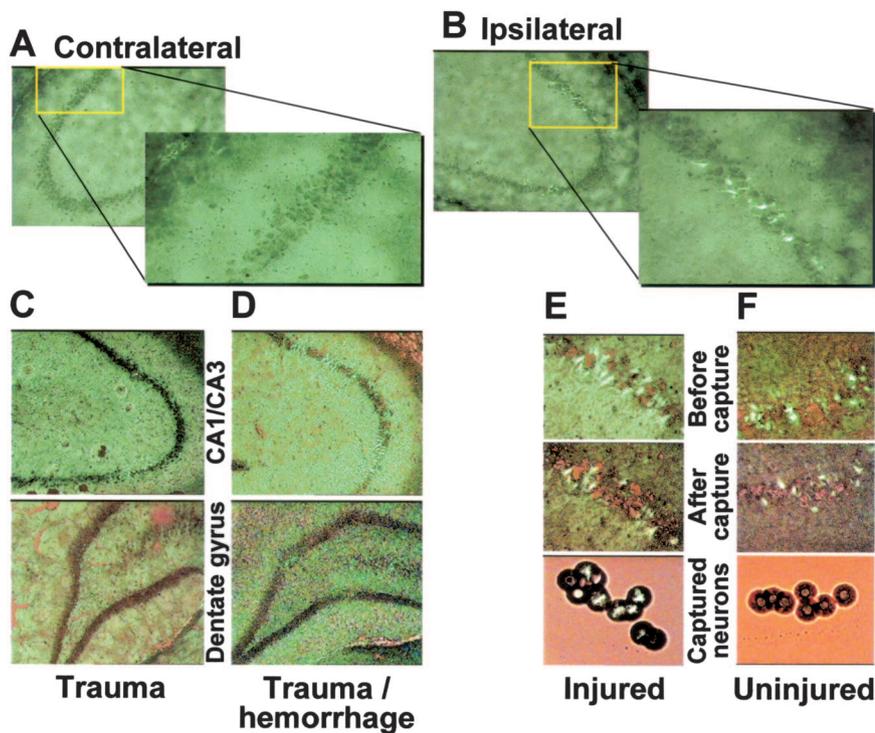
Fig. 1. Fluoro-Jade–positive neurons in the CA1, CA3, and dentate gyrus subfields of the rat hippocampus. (*A*) After double staining with Fluoro-Jade and cresyl violet, no injured (Fluoro-Jade–positive) neurons were found in the contralateral hippocampus of any of the injured rats. The *lower panel* is a higher magnification view of the CA1 pyramidal neurons shown in the *inset*. (*B*) Fluoro-Jade–stained neurons were always observed in the ipsilateral injured hippocampus. The *lower panel* is a higher magnification view of the Fluoro-Jade–positive neurons in the CA1 pyramidal layer shown in the *inset*. (*C*) Distribution of Fluoro-Jade–positive neurons in hippocampus of trauma-alone animals. Note the greater number of Fluoro-Jade–positive cells in the CA3 region, which is more vulnerable to fluid percussion injury. (*D*) Distribution of Fluoro-Jade–positive neurons in hippocampus of trauma/hemorrhage animals. Note the large number of Fluoro-Jade–positive neurons in the CA1 subfield (in comparison to trauma alone), which is particularly susceptible to ischemic injury. (*E*) Fluoro-Jade–positive injured pyramidal neurons in CA1 before and after laser capture microdissection and as seen on the HS CapSure caps. (*F*) Uninjured, Fluoro-Jade–negative pyramidal neurons immediately adjacent to the injured, Fluoro-Jade–positive neurons in CA3 before and after laser capture microdissection and on the laser capture microdissection caps.

all double-stained with Fluoro-Jade and cresyl violet. We first examined the sections under bright field illumination, which clearly showed the cresyl violet–stained neurons, and after we found the hippocampal neurons, we switched to the fluorescein isothiocyanate filter to find and visualize the Fluoro-Jade–positive neurons and perform the laser capture. We examined the ipsilateral and contralateral hippocampal regions of all sections of all experimentally injured rat brains after double staining with Fluoro-Jade and cresyl violet, and we did not find any injured, Fluoro-Jade–positive neurons in the contralateral hippocampus of any of the injured rats (fig. 1A). In the ipsilateral hippocampus, 24 h after TBI, we found a consistent and distinctive distribution of Fluoro-Jade–positive neurons in the CA1 and CA3 subregions (fig. 1B), as well as scattered positive neurons in the DG and dentate hilus (fig. 1C). These results confirm previous reports of Fluoro-Jade staining in TBI animals.[17] Fluoro-Jade staining has not been used previously to assess the influence of adding a secondary injury, such as hemorrhagic hypotension, after TBI. In TBI/HH rats, we consistently observed more Fluoro-Jade–positive neurons in all three subregions (fig. 1D), especially the CA1 subregion, which supports previous observations that the CA1 subfield is vulnerable to ischemic injury.[29] Because our study focused on differences in gene expression between adjacent injured and uninjured neurons, we did not quantify differences in the numbers of Fluoro-Jade–positive neurons.

Using LCM, we obtained 10 injured neurons and 10 immediately adjacent uninjured neurons from each subregion (CA1, CA3, and DG) for comparative molecular analysis (figs. 1E and F). In preliminary experiments using a sense BDNF riboprobe, we determined that the amplified RNA signals from 5, 10, and 25 neurons were equivalent (fig. 2A). After TBI and TBI/HH, we found prominent regional differences in gene expression in injured and uninjured neurons. In hippocampal subregion CA3 of the TBI group, the mean mRNA levels of the neurotrophin BDNF and the antioxidants GPX-1 and HO-1 in uninjured cells were significantly higher than in adjacent injured cells ($P < 0.009$; figs. 2B [confirmed by Northern Blot and real-time PCR analysis, Figs. 2C,D] and 3A–C). Although there were no differences between injured and uninjured neurons of the CA1 and DG subfields in TBI rats, the mean mRNA levels of BDNF, GPX-1, and HO-1 in the TBI/HH rat neurons (all subregions) were significantly lower than in TBI rats alone ($P \leq 0.0005$). Expression of IL-1β, HSP70, caspase 3, and Bcl-2 was not significantly different in injured *versus* uninjured neurons (all subregions), but mean mRNA levels of all four genes in the TBI/HH group were significantly lower than in the TBI group ($P \leq 0.0001$; figs. 3D–G). FAS ligand and caspase 9 were similarly expressed in injured and uninjured neurons in all hippocampal regions in both experimental groups (figs. 3H–I).

To confirm the results obtained using RPA, we performed a limited set of experiments using Northern Blot analysis and reverse-transcription PCR. Using Northern Blot analysis of pooled aRNA from uninjured and injured
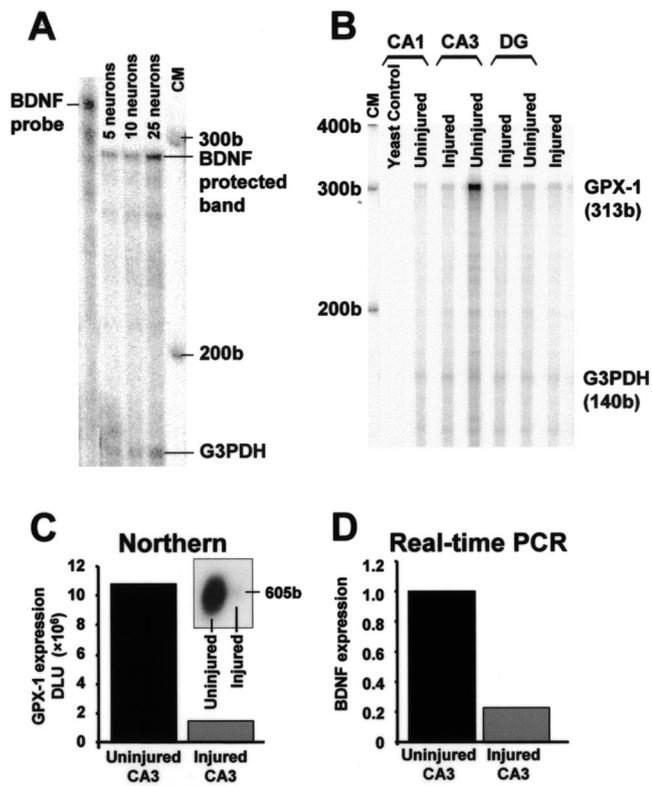
Fig. 2. Quantitative analysis of linearly amplified messenger RNA (mRNA) in injured and uninjured hippocampal neurons. (*A*) Ribonuclease protection assay analysis of 100 ng linearly amplified hippocampal neuron mRNA with brain-derived neurotrophic factor (BDNF) riboprobe. mRNA expression in 5, 10, and 25 neurons is equivalent when normalized to the glyceraldehye-3-phosphate dehydrogenase (G3PDH) signal. CM = century marker (Ambion). (*B*) Ribonuclease protection assay analysis of glutathione peroxidase 1 (GPX-1) expression in uninjured (Fluoro-Jade–negative) and injured (Fluoro-Jade–positive) neurons in hippocampal subfields CA1, CA3, and dentate gyrus (DG) of one traumatic brain injury rat. (*C*) Expression of GPX-1 signal in Northern Blot analysis (*inset*) of pooled uninjured (from six rats) and pooled injured CA3 neurons with GPX-1 probe (from six rats) in digital light units (DLU) normalized to G3PDH expression. (*D*) Real-time polymerase chain reaction (PCR) analysis of BDNF expression in pooled uninjured and pooled injured CA3 neurons (all six traumatic brain injury rats).

neurons (amplified from 60 CA3 pyramidal neurons from six TBI rats), we confirmed substantially higher expression of GPX-1 in uninjured neurons (fig. 2C). Real-time PCR analysis of BDNF expression in injured and uninjured CA3 neurons confirmed higher levels (4.4-fold) of BDNF in the uninjured cells (fig. 2D).

Discussion

Although two previous studies compared gene expression in injured neurons (identified by staining with terminal deoxynucleotidyl transferase–mediated biotinylated deoxyuridine triphosphate nick end labeling) from rats after TBI to uninjured neurons from control animals that had not undergone TBI,[30] ours is the first study to

directly compare mRNA levels in injured and adjacent uninjured neurons from the same animals. Ours is also the first study to use samples obtained using LCM (which is a rapid, precise technique for isolating single cells or populations of cells[31]) to directly compare gene expression in injured and uninjured neurons after TBI.

With the select group of genes examined in this study, there were significant differences in neuroprotective gene expression between injured and uninjured neurons, particularly in the CA3 region, which has been shown to be selectively vulnerable to fluid percussion injury.[6] On the other hand, we found a striking lack of detectable differences in proapoptotic gene expression in either group. We interpret this unexpected result with caution because we examined only two members of the proapoptotic gene family and there is no reason, *a priori*, to assume that the expression of all apoptosis-related genes would be altered by TBI. Furthermore, a recent study showed that some apoptosis-related genes are also critical for cell survival; *e.g.*, in an *in vitro* model of ischemic preconditioning, neuroprotection from a severe injury seemed to depend on sublethal activation of caspase 3 expression and subsequent up-regulation of downstream neuroprotective pathways.[32] Future studies involving comprehensive global gene chip analysis ongoing in our laboratory are expected to shed light on TBI-induced changes in apoptotic gene expression. Nonetheless, the results of our study suggest that increases in neuroprotective gene expression are associated with neuronal survival in the CA3 subregion after TBI rather than down-regulation of proapoptotic gene expression. Furthermore, after TBI in rats, severe hemorrhagic hypotension dramatically suppressed expression of both neuroprotective and potentially harmful genes (seven of the nine genes studied) in both injured and uninjured neurons. Although unexpected, this may be the result of the overall suppression of transcription after TBI and hemorrhage, due to, for example, injury-induced suppression of transcription factors such as activating transcription factor 2.[33] The lack of differences in neuroprotective gene expression in the injured and surviving neurons of rats subjected to TBI/HH suggests the involvement of additional factors critical to survival of individual neurons, at least in the first 24 h after TBI. Future studies in our laboratory will address these issues.

In a previous study, we showed that higher endogenous expression of other neuroprotective and survival genes in the CA1 subfield correlated highly with resistance to injury.[8] In that study, we laser dissected intact groups of cells containing several hundred neurons and glia from each region of the hippocampus. Although it is known that more than 95% of the cells in the CA1 region, for example, are neurons,[34] the laser-captured cells in that study were, nevertheless, a mixed population of neurons, interneurons, and a few glia. In the
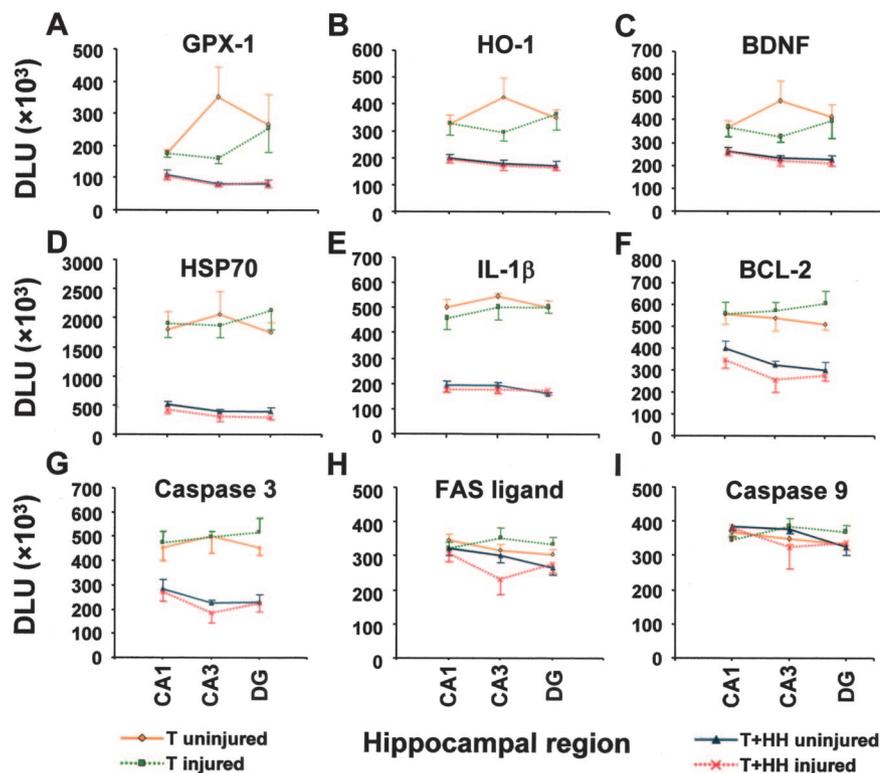
Fig. 3. Comparative ribonuclease protection assay analysis of messenger RNA (mRNA) levels of neuroprotective, proapoptotic, and antiapoptotic genes in hippocampal subregions. (A–I), Mean mRNA levels of glutathione peroxidase 1 (GPX-1), heme oxygenase 1 (HO-1), brain-derived neurotrophic factor (BDNF), heat shock protein 70 (HSP70), interleukin 1β (IL-1β), Bcl-2, FAS ligand, caspase 9, and caspase 3 in CA1, CA3, and dentate gyrus (DG). Significant increases in GPX-1, HO-1, and BDNF expression were observed in uninjured CA3 neurons of traumatic brain injury (TBI) rats as compared with injured CA3 neurons ($P < 0.009$). Secondary hemorrhagic hypotension (HH) significantly suppressed gene expression (seven of nine genes) in all hippocampal regions as compared with TBI-alone rats (TBI, n = 6; TBI/HH, n = 6; $P \leq 0.0005$ for GPX-1, HO-1, and BDNF; $P \leq 0.0001$ for HSP70, IL-1β, Bcl-2, and caspase 3). mRNA levels are expressed in digital light units (DLU; ×1,000) normalized to internal glyceraldehye-3-phosphate dehydrogenase expression with SD bars. Normalized raw and log-transformed mRNA expression data are shown in tables 1 and 2.

current study, individual Nissl-stained neurons were captured singly by LCM; therefore, the gene expression data obtained from these cells are representative of a much finer resolution than previously achieved.

We propose several possible explanations for the differences between injured and uninjured neurons in the CA3 subfield and the dramatic suppression of gene expression by hemorrhagic hypotension after TBI. The mammalian brain has 2,500–5,000 specific neuronal cell types,[35] and morphologically similar but functionally distinct neurons may differentially express certain genes. Even within apparently homogeneous populations of cells, there is substantial heterogeneity,[36] so perhaps uninjured neurons were differentially capable of increasing neuroprotective gene expression after TBI, whereas adjacent, apparently similar neurons were not. Another possible explanation is that differences between injured and uninjured neurons are the result of stochastic, or randomly varying, gene expression[37] (*i.e.*, at the time of injury, expression of neuroprotective genes was randomly increased in some neurons, which then were better able to survive an injury, but not in others). Finally, the results of our study could suggest that injury-induced signals may actively suppress neuroprotective gene expression in some neurons, which would render them vulnerable to traumatic injuries. Further comparative gene expression studies of injured and uninjured neurons will help to identify other injury-induced genes that may play a key role in the transcriptional regulation of neuroprotective gene expression.

Our data suggest that higher endogenous expression

of neuroprotective genes 24 h after TBI is associated with protection of neurons from irreversible injury. Antioxidants have provided prolonged and robust neuroprotection in animal models of brain injury[38] and *in vitro* models of injury,[39] which are all associated with oxidative stress.[40] Transgenic mice overexpressing GPX-1 have been shown to be resistant to ischemia–reperfusion heart and brain injury as well as various types of oxidant tissue injury.[41] Furling *et al.*[42] reported improved recovery of synaptic transmission in CA1 pyramidal cells of GPX-1 transgenic mice after a short period of hypoxia, suggesting that a moderate increase in endogenous antioxidant levels was sufficient to prevent irreversible damage. Increases in antioxidant activity are also positively associated with several biologic phenomena, including lifespan extension[43] and stress resistance.[44] We showed previously that up-regulation of HO-1 was significantly associated with the protective effects of zinc chelation after experimental TBI.[9] Induction of HO-1 was cytoprotective in pathologic states associated with oxidative stress (HO-1 catalyzes the rate-limiting reaction in the degradation of heme, a prooxidant that is released from hemoglobin after brain injury).[45] Increases in expression of neurotrophins such as BDNF have been shown to be associated with neuroprotection in brain injury models.[27,46-48] In immortalized mouse hippocampal HT22 cells, the neuroprotective effects of BDNF have been shown to be mediated by activation of the extracellular signal–regulated protein kinase and phosphatidylinositol 3-kinase survival pathways.[49] Finally, a recent report on a new therapeutic

approach to amyotrophic lateral sclerosis showed that enhancement of the neuroprotective heat shock response slowed motor neuron degeneration and extends survival in a transgenic mouse model of the disease,[50] suggesting that selective enhancement of neuroprotective responses is sufficient to decrease neuronal cell death.

Interestingly, the lack of differences in proapoptotic gene expression between injured and uninjured neurons suggests that antiapoptotic strategies might be expected to exert little therapeutic effect after TBI. Even more important, further suppression of proapoptotic genes by subsequent hemorrhage suggests that the worsening of the outcome by a secondary injury is not mediated by proapoptotic factors. However, the lower levels of neuroprotective genes in all neurons after the combination of TBI and hemorrhagic hypotension suggest that enhancing the normal endogenous neuroprotective response may be a rational therapeutic strategy for patients with TBI. We interpret our results with caution because we did not examine the corresponding protein expression levels in the identified injured and uninjured neurons. However, for some of these genes, we and others have shown up-regulation in brain tissue after injury, though not at the single-cell level.[9,38,42] Future studies in our laboratory will examine the expression of some of these proteins at the single-cell level using immunohistochemical methods. Nonetheless, although our study provides only an association of increased neuroprotective gene expression with survival, we suggest that our results support the value of future studies of neuroprotective molecular mechanisms.

We have also demonstrated in the current study that quantitative mRNA analysis of small numbers of laser capture microdissected neurons is eminently straightforward and practical if current linear mRNA amplification methods are combined with sensitive RPA analysis. In principle, these methods can be readily applied to any neurodegenerative disease (*e.g.*, Parkinson or Alzheimer disease) in which dying or degenerating cells can be identified histologically. The transcriptional profile of these cells can be directly compared with adjacent cells that are not injured (or not yet injured) at that time point, allowing identification of factors that may contribute to neuronal survival in these neurodegenerative disorders. Furthermore, this approach allows facile comparison of the expression of any gene of interest in differentially identifiable cells in any type of tissue.

Finally, the results of this study illustrate the importance of studying gene expression at the single-cell level. In a previous microarray study using whole brain RNA, we found that HSP27 and HSP70 gene expression increased significantly after TBI and further increased after a neuroprotective zinc chelation treatment.[9] However, analysis of HSP70 expression in individual hippocampal neurons showed that there were no significant differ-

ences in mRNA levels between injured and uninjured neurons. We conclude that, at least in hippocampal neurons, increased HSP expression is not associated with neuronal survival. Further studies of injury-induced gene expression in discrete populations of neurons are necessary for better understanding of the molecular determinants of neuronal survival after TBI.

References

1. Gennarelli TA: Mechanisms of brain injury. J Emerg Med 1993; 11(suppl 1):5-11
2. Gennarelli TA: Animate models of human head injury. J Neurotrauma 1994; 11:357-68
3. Smith DH, Chen X-H, Pierce JES, Wolf JA, Trojanowski JQ, Graham DI, McIntosh TK: Progressive atrophy and neuron death for one year following brain trauma in the rat. J Neurotrauma 1997; 14:715-27
4. Hamm RJ, Temple MD, Pike BR, O'Dell DM, Buck DL, Lyeth BG: Working memory deficits following traumatic brain injury in the rat. J Neurotrauma 1996; 13:317-23
5. Floyd CL, Golden KM, Black RT, Hamm RJ, Lyeth BG: Craniectomy position affects morris water maze performance and hippocampal cell loss after parasagittal fluid percussion. J Neurotrauma 2002; 19:303-16
6. Grady MS, Charleston JS, Maris D, Witgen BM, Lifshitz J: Neuronal and glial cell number in the hippocampus after experimental traumatic brain injury: analysis by stereological estimation. J Neurotrauma 2003; 20:929-41
7. Chesnut RM, Marshall LF, Klauber MR, Blunt BA, Baldwin N, Eisenberg HM, Jane JA, Marmarou A, Foulkes MA: The role of secondary brain injury in determining outcome from severe head injury. J Trauma 1993; 34:216-22
8. Shimamura M, Garcia JM, Prough DS, Hellmich HL: LCM and analysis of amplified antisense RNA from distinct cell populations of the young and aged rat brain: effect of traumatic brain injury on hippocampal gene expression. Brain Res Mol Brain Res 2004; 17:47-61
9. Hellmich HL, Frederickson CJ, DeWitt DS, Saban R, Parsley MO, Stephenson R, Velasco M, Uchida T, Shimamura M, Prough DS: Protective effects of zinc chelation in traumatic brain injury correlate with upregulation of neuroprotective genes in rat brain. Neurosci Lett 2004; 355:221-5
10. Maas AI: Neuroprotective agents in traumatic brain injury. Expert Opin Investig Drugs 2001; 10:753-67
11. Folkman J, Kalluri R: Cancer without disease. Nature 2004; 427:787
12. Dietrich WD, Alonso O, Halley M: Early microvascular and neuronal consequences of traumatic brain injury: A light and electron microscopic study in rats. J Neurotrauma 1994; 11:289-301
13. Dietrich WD, Alonso O, Busto R, Prado R, Zhao W, Dewanjee MK, Ginsberg MD: Posttraumatic cerebral ischemia after fluid percussion brain injury: an autoradiographic and histopathological study in rats. Neurosurgery 1998; 43:585-94
14. Matsushita Y, Bramlett HM, Kuluz JW, Alonso O, Dietrich WD: Delayed hemorrhagic hypotension exacerbates the hemodynamic and histopathologic consequences of traumatic brain injury in rats. J Cereb Blood Flow Metab 2001; 21:847-56
15. Kroppenstedt S-N, Kern M, Thomale U-W, Schneider G-H, Lanksch WR, Unterberg AW: Effect of cerebral perfusion pressure on contusion volume following impact injury. J Neurosurg 1999; 90:520-6
16. Schmued LC, Albertson C, Slikker W Jr: Fluoro-Jade: A novel fluorochrome for the sensitive and reliable histochemical localization of neuronal degeneration. Brain Res 1997; 751:37-46
17. Sato M, Chang E, Igarashi T, Noble LJ: Neuronal injury and loss after traumatic brain injury: Time course and regional variability. Brain Res 2001; 917:45-54
18. Pennypacker KR, Kassed CA, Eidizadeh S, Saporta S, Sanberg PR, Willing AE: NF-kappaB p50 is increased in neurons surviving hippocampal injury. Exp Neurol 2001; 172:307-19
19. Dash PK, Kobori N, Moore AN: A molecular description of brain trauma pathophysiology using microarray technology: An overview. Neurochem Res 2004; 29:1275-86

20. Matzilevich DA, Rall JM, Moore AN, Grill RJ, Dash PK: High-density microarray analysis of hippocampal gene expression following experimental brain injury. J Neurosci Res 2002; 67:646–63

21. Mathew BP, DeWitt DS, Bryan RM Jr, Bukoski RD, Prough DS: Traumatic brain injury reduces myogenic responses in pressurized rodent middle cerebral arteries. J Neurotrauma 1999; 16:1177–86

22. Van Gelder RN, von Zastrow ME, Yool A, Dement WC, Barchas JD, Eberwine JH: Amplified RNA synthesized from limited quantities of heterogeneous cDNA. Proc Natl Acad Sci U S A 1990; 87:1663–7

23 Humphrey MF, Chu Y, Mann K, Rakoczy P: Retinal GFAP and bFGF expression after multiple argon laser photocoagulation injuries assessed by both immunoreactivity and mRNA levels. Exp Eye Res 1997; 64:361–9

24. Egan TD, Kuramkote S, Gong G, Zhang J, McJames SW, Bailey PL: Fentanyl pharmacokinetics in hemorrhagic shock. ANESTHESIOLOGY 1999; 91:156–66

25. Asahi M, Wang X, Mori T, Sumii T, Jung JC, Moskowitz MA, Fini ME, Lo EH: Effects of matrix metalloproteinase-9 gene knock-out on the proteolysis of blood-brain barrier and white matter components after cerebral ischemia. J Neurosci 2001; 21:7724–32

26. Szabo A, Perou CM, Karaca M, Perreard L, Quackenbush JF, Bernard PS: Statistical modeling for selecting housekeeper genes. Genome Biol 2004; 5:R59.1–R59.10

27. Wu A, Molteni R, Ying Z, Gomez-Pinilla F: A saturated-fat diet aggravates the outcome of traumatic brain injury on hippocampal plasticity and cognitive function by reducing brain-derived neurotrophic factor. Neuroscience 2003; 119:365–75

28. SAS Institute I: SAS/STAT User's Guide. Version 8. Cary, NC, SAS Institute, 1999

29. Benveniste H: The excitotoxin hypothesis in relation to cerebral ischemia. Cerebrovasc Brain Metab Rev 1991; 3:213–45

30. Marciano PG, Brettschneider J, Manduchi E, Davis JE, Eastman S, Raghupathi R, Saatman KE, Speed TP, Stoeckert CJ Jr, Eberwine JH, McIntosh TK: Neuron-specific mRNA complexity responses during hippocampal apoptosis after traumatic brain injury. J Neurosci 2004; 24:2866–76

31. Emmert-Buck MR, Bonner RF, Smith PD, Chuaqui RF, Zhuang Z, Goldstein SR, Weiss RA, Liotta LA: Laser capture microdissection. Science 1996; 274:998–1001

32. McLaughlin B, Hartnett KA, Erhardt JA, Legos JJ, White RF, Barone FC, Aizenman E: Caspase 3 activation is essential for neuroprotection in preconditioning. Proc Natl Acad Sci U S A 2003; 100:715–20

33. Martin-Villalba A, Winter C, Brecht S, Buschmann T, Zimmermann M, Herdegen T: Rapid and long-lasting suppression of the ATF-2 transcription factor is a common response to neuronal injury. Brain Res Mol Brain Res 1998; 62:158–66

34. Zeng Y-C, Bongrani S, Bronzetti E, Cadel S, Ricci A, Valsecchi B, Amenta F: Effect of long-term treatment with L-deprenyl on the age-dependent microanatomical changes in the rat hippocampus. Mech Ageing Dev 1995; 79:169–85

35. Bota M, Dong HW, Swanson LW: From gene networks to brain networks. Nat Neurosci 2003; 6:795–9

36. Kamme F, Salunga R, Yu J, Tran DT, Zhu J, Luo L, Bittner A, Guo HQ, Miller N, Wan J, Erlander M: Single-cell microarray analysis in hippocampus CA1: Demonstration and validation of cellular heterogeneity. J Neurosci 2003; 23: 3607–15

37. Blake WJ, Kaern M, Cantor CR, Collins JJ: Noise in eukaryotic gene expression. Nature 2003; 422:633–7

38. Sheng H, Batinc-Haberle I, Warner DS: Catalytic antioxidants as novel pharmacologic approaches to treatment of ischemic brain injury. Drug News Perspect 2002; 15:654–65

39. Ahlemeyer B, Junker V, Huhne R, Krieglstein J: Neuroprotective effects of NV-31, a bilobalide-derived compound: Evidence for an antioxidative mechanism. Brain Res 2001; 890:338–42

40. Beni SM, Kohen R, Reiter RJ, Tan DX, Shohami E: Melatonin-induced neuroprotection after closed head injury is associated with increased brain antioxidants and attenuated late-phase activation of NF-kappaB and AP-1. FASEB J 2004; 18:149–51

41. Ho YS, Magnenat JL, Gargano M, Cao J: The nature of antioxidant defense mechanisms: A lesson from transgenic studies. Environ Health Perspect 1998; 106(suppl 5):1219–28

42. Furling D, Ghribi O, Lahsaini A, Mirault ME, Massicotte G: Impairment of synaptic transmission by transient hypoxia in hippocampal slices: Improved recovery in glutathione peroxidase transgenic mice. Proc Natl Acad Sci U S A 2000; 97:4351–6

43. Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisielewski A, Zhang LL, Scherer B, Sinclair DA: Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. Nature 2003; 425:191–6

44. Longo VD, Fabrizio P: Regulation of longevity and stress resistance: A molecular strategy conserved from yeast to humans? Cell Mol Life Sci 2002; 59:903–8

45. Maines MD: Heme oxygenase 1 transgenic mice as a model to study neuroprotection. Methods Enzymol 2002; 353:374–88

46. Mocchetti I, Wrathall JR: Neurotrophic factors in central nervous system trauma. J Neurotrauma 1995; 12:853–70

47. Cheng Y, Gidday JM, Yan Q, Shah AR, Holtzman DM: Marked age-dependent neuroprotection by brain-derived neurotrophic factor against neonatal hypoxic-ischemic brain injury. Ann Neurol 1997; 41:521–9

48. Mattson MP, Scheff SW: Endogenous neuroprotection factors and traumatic brain injury: Mechanisms of action and implications for therapy. J Neurotrauma 1994; 11:3–33

49. Rossler OG, Giehl KM, Thiel G: Neuroprotection of immortalized hippocampal neurones by brain-derived neurotrophic factor and Raf-1 protein kinase: Role of extracellular signal-regulated protein kinase and phosphatidylinositol 3-kinase. J Neurochem 2004; 88:1240–52

50. Kieran D, Kalmar B, Dick JR, Riddoch-Contreras J, Burnstock G, Greensmith L: Treatment with arimoclomol, a coinducer of heat shock proteins, delays disease progression in ALS mice. Nat Med 2004; 10:402–5