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Profound molecular changes following hippocampal slice preparation: loss of AMPA receptor subunits and uncoupled mRNA/protein expression

Stephen M. Taubenfeld,† Kimberly A. Stevens,† Gabriella Pollonini,* Jason Ruggiero† and Cristina M. Alberini*

*Department of Physiology and Biophysics, Mount Sinai School of Medicine, New York, New York, USA

†Department of Neuroscience, Brown University, Providence, Rhode Island, USA

Abstract

The acute hippocampal slice preparation is a convenient, *in vitro* model widely used to study the biological basis of synaptic plasticity. Although slices may preserve their electrophysiological properties for several hours, profound molecular changes in response to the injury caused by the slicing procedure are likely to occur. To determine the magnitude and duration of these changes we examined the post-slicing expression kinetics of three classes of genes known to be implicated in long-term synaptic plasticity: glutamate AMPA receptors (GluR), transcription factors and neurotrophins. Slicing resulted in a striking loss of GluR1 and GluR3, but not of GluR2 proteins suggesting that rapid changes in the composition of major neurotransmitter receptors may occur. Slicing caused a significant induction of the transcription factors *c-fos*, *zif268*, CCAAT enhancer binding protein (*C/EBP*) β and δ mRNAs and of the neurotrophin brain-derived neurothophic factor (*BDNF*) mRNA. In contrast, there was no augmentation, and sometimes a decline, in the levels of the corresponding proteins. These data reveal that significant discrepancies exist between the slice preparation and the intact hippocampus in terms of the metabolism of molecular components known to be involved in synaptic plasticity.

Keywords: hippocampus, glutamate receptor, neurotrophin, slice, transcription factor.

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The hippocampal slice preparation has been extensively employed as an *in vitro* model for investigating the biological basis of neural responses. Particularly favored is the use of slices to study long-lasting synaptic changes such as long-term potentiation (LTP) and long-term depression, which are thought to underlie a variety of complex brain functions, including memory (Huang *et al*. 1996; Riedel *et al*. 1996; Hughes *et al*. 1999). However, as tissue sectioning produces somatic injury, which results in axotomy, release of ions, metabolites, and growth factors, profound molecular and structural changes are likely to occur in slices relative to the intact brain (Sheng and Greenberg 1990; Schlingensiepen *et al*. 1994; Zhou *et al*. 1995; Siklos *et al*. 1997; Kirov *et al*. 1999). If these changes are profound and persistent, they likely alter the molecular and metabolic state of the tissue, which therefore no longer reflects native physiological conditions. Therefore, we have investigated the following questions: (i) Can the acute slice preparation provide a suitable model for studying gene regulation that accompanies neuronal functions *in vivo*? and (ii) What are the magnitude and duration of the molecular changes induced by slicing?

We analyzed classes of molecules whose regulation is known to be associated with synaptic plasticity and memory because we contemplated the use of the slice model to characterize molecular changes underlying synaptic responses in long-term memory. These genes included glutamate AMPA receptors, transcription factors, and neurotrophins. A large body of literature reported that these molecules are regulated following learning as well as somatic injury;

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Address correspondence and reprint requests to Cristina M. Alberini, Department of Physiology and Biophysics, Box 1218, Mount Sinai School of Medicine, 1 Gustave L. Levy Place, New York, NY 10029–6574, USA. E-mail: Cristina.Alberini@Physbio.mssm.edu

Abbreviations used: ACSF, artificial cerebrospinal fluid; BDNF, brain-derived neurothophic factor; C/EBP, CCAAT enhancer binding protein; GluR, glutamate receptor; LTP, long-term potentiation; NT-3, neurotrophin 3.

however, the extent to which their expression changes as a consequence of the slicing event alone has not yet been determined (Cole *et al*. 1989; Nikolaev *et al*. 1991; Kaczmarek 1993; Nowak *et al*. 1993; Alberini *et al*. 1994; Hayes *et al*. 1995; Kang and Schuman 1995; Raghupathi *et al*. 1995; Dragunow 1996; Herrera and Robertson 1996; Houpt *et al*. 1996; Lamprecht and Dudai 1996; Akaneya *et al*. 1997; Chaudhuri 1997; Kennis and Holstege 1997; Linnarsson *et al*. 1997; Montkowski and Holsboer 1997; Cammarota *et al*. 1998; Castren *et al*. 1998; Gall *et al*. 1998; Lessmann 1998; Lynch 1998; Ma *et al*. 1998; Nayak *et al*. 1998; Sterneck *et al*. 1998; Berardi and Maffei 1999; Dutcher *et al*. 1999; Hughes *et al*. 1999; Montag-Sallaz *et al*. 1999; Radulovic *et al*. 1999; Schuman 1999; Tischmeyer and Grimm 1999; Walton *et al*. 1999; Wan *et al*. 1999; Zhang *et al*. 1999; Taubenfeld *et al*. 2001). This determination is important when using slice preparations as a model of synaptic plasticity and memory because molecular changes elicited by synaptic plasticity protocols may be superimposed or camouflaged by the modifications produced by slicing.

To compare the molecular steady-state of the acute slice preparation to that of the intact tissue, we assessed the effects of slicing on mRNA and protein expression of several members of the following classes of genes over a 6-h time course: (i) three glutamate AMPA receptor subunits – GluR1, GluR2, and GluR3; (ii) four transcription factors – *c-fos*, *zif 268*, and CCAAT enhancer binding proteins (*C/EBP*) β and δ; (iii) two neurotrophins – *BDNF* and *NT-3*; and (iv) two housekeeping genes – *cyclophilin* and *actin*.

Materials and methods

Animals and hippocampal slicing

All protocols were approved by the Institutional Animal Care and Use Committee and were in accordance with National Institute of Health regulation for the care and use of animals in research.

Young adult Long–Evans rats were anesthetized with Metofane vapors and sacrificed by decapitation. Brains were quickly dissected in an ice-cold oxygenated (95% O_2/5% CO_2) bath of artificial cerebrospinal fluid (ACSF: 124 mM NaCl, 5 mM KCl, 1.23 mM $NaH_2PO_4H_2O$, 26 mM $NaHCO_3$, 10 mM dextrose, 2 mM $CaCl_2$, and 1 mM $MgCl_2$). The hippocampi were removed and either frozen immediately in a dry-ice/ethanol bath and stored at − 80°C (intact) or sliced on a McIlwain tissue chopper (or vibratome, where specified) in ice-cold oxygenated ACSF to a thickness of 400 μm. Slices were either frozen in a dry-ice/ethanol bath and stored at − 80°C (0 min), or placed in a bath of oxygenated ACSF and incubated at 37°C (or 29°C, where specified) for the indicated length of time (15, 30, 60, 120, 240, and 360 min) and then frozen at − 80°C. One entire hippocampus was sliced and used for each single condition. Four hippocampi per timepoint were analyzed. All samples were stored at − 80°C until protein or RNA extractions were performed.

RNA extraction and northern blot analysis

Total RNA was extracted following the method of Chomczynski and Sacchi (1987). One milliliter of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.1 M 2-mercaptoethanol and 0.5% sarcosyl) was added per 100 mg of tissue. The samples were polytron-homogenized, extracted with 1 volume of phenol/chloroform and precipitated with 1 volume of isopropanol. Ten micrograms of total RNA/sample were electrophoresed on 1.2% agarose gels, transferred to Hybond-N + nylon membranes (Amersham, Arlington Heights, IL, USA) and UV-crosslinked. The membranes were hybridized overnight at 42°C with specific probes in 50% formamide, 5 × saline–sodium phosphate–EDTA (SSPE), 0.1% sodium dodecyl sulfate (SDS), 2 × Denhardt's solution, 0.1 mg/mL tRNA and 0.1 mg/mL salmon sperm DNA. Probes were labeled with random oligonucleotide primers (Prime-It II kit, Stratagene, Cedar Creek, TX, USA) and [α-^{32}P]dCTP (Amersham). At the end of the hybridization, the membranes were washed, exposed to BioMax MS film (Eastman Kodak, Rochester, NY, USA) and quantitative densitometric analysis was performed using NIH Image. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post-hoc analysis. The same membrane was stripped and re-hybridized with different rat probes as described in the Results section.

Probes

c-fos: last 700 bp of the 3′ untranslated region (UT); zif268: last 300 bp of the 3′ UT; *C/EBPβ*: last 400-bp *Sma*I/*Pst*I fragment of the 3′ UT; *C/EBPδ*: 493-bp region beginning from base 13 of the open reading frame; *GluR1*, *GluR2*, *GluR3*, *BDNF*, *NT-3*, *cyclophilin* and *β-actin*: entire open reading frames. *β-actin* was utilized as control to which all other hybridizations were normalized.

Western blot analysis

Extracts from rat hippocampi were obtained by polytron homogenization in cold lysis buffer with protease inhibitors [0.2 M NaCl, 0.1 M HEPES, 10% glycerol, 2 mM NaF, 2 mM $Na_4P_2O_7$, 5 mM EDTA, 1 mM EGTA, 2 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM benzamidine, 10 mg/mL leupeptin, 400 U/mL aprotinin, 1 mM microcystin]. After 10 min on ice, the samples were centrifuged at 16 000 *g* for 15 min at 4°C. The total protein concentration in each lysate was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of total protein corresponding to 25 μg/lane were resolved on denaturing 10% SDS-PAGE gels and transferred to Hybond-P membranes (Amersham) by electroblotting. Membranes were pre-treated with 5% BLOTTO buffer and then incubated with primary antiserum in Tris-buffered saline overnight at 4°C. The membranes were then washed, treated with a secondary horseradish peroxidase (HRP)-labeled donkey anti-rabbit antibody (1/4000) for 1 h, washed again and incubated with HRP–streptavidin complex and ECL detection reagents (Amersham). Membranes were exposed to ECL Hyperfilm (Amersham) and quantitative densitometric analysis was performed using NIH Image. Each immunostained sample was normalized against the corresponding actin signal. Actin stainings were performed on the same membranes. Concentrations were expressed as mean percentage ± SEM of the intact hippocampal control mean values (100%). Statistical analysis was performed

using one-way ANOVA followed by Dunnett's multiple comparison test.

Primary antisera

Anti-c-Fos (1 : 2000), anti-Zif268 (1 : 2000), anti-C/EBPβ (1 : 4000) anti-C/EBPδ (1 : 500), anti-GluR3 (1 : 2000), anti-BDNF (1 : 1000), anti NT-3 (1 : 1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-GluR1 (1 : 2000), anti-GluR2 (1 : 2000), anti-actin (1 : 2000; Chemicon, Temecula, CA, USA); and anti-cyclophilin (1 : 2000; Upstate Biotechnology, Lake Placid, NY, USA).

Electrophysiological recordings

Four hundred-micrometer hippocampal slices were cut on a McIlwain tissue chopper or vibratome and incubated in oxygenated ACSF for 360 min at 37°C or 29°C, as indicated. Slices were then transferred to a submersion-type recording chamber and continually perfused with 29–30°C ACSF at 2 mL/min. Synaptic responses were evoked by stimulating Schaffer collaterals with 0.2-ms pulses and then recorded extracellularly in the CA1 stratum radiatum. Baseline responses were obtained by stimulating at 0.033 Hz using an intensity that yielded a half-maximal field potential (FP) slope. Long-term potentiation (LTP) was induced after 15 min with four 0.1 Hz episodes of theta burst stimulation, using the same stimulation intensity as for baseline. A single stimulation is comprised of 10 stimulus trains delivered at 5 Hz, and each train consists of four pulses at 100 Hz. Field potentials were simultaneously recorded in control slices and LTP slices in the same recording chamber.

Results

Northern and Western blot time course analyses were performed on rat hippocampal extracts to examine the mRNA and protein levels of members from the following classes of genes: (i) AMPA glutamate receptors – GluR1, GluR2, and GluR3; (ii) transcription factors – *c-fos*, *zif268*, *C/EBPβ*, and *C/EBPδ*; (iii) neurotrophins – *BDNF* and *NT-3*; (iv) housekeeping genes – *cyclophilin* and *actin*. Rat hippocampi were dissected and either immediately frozen (intact) or quickly sliced in ice-cold ACSF buffer. The sliced hippocampi were either frozen at − 80°C (0 min) or incubated at 37°C for 15, 30, 60, 120, 240 or 360 min. Independent extractions were performed to analyze either total RNA or protein as described in the Methods section.

Slicing results in a severe loss of GluR1 and GluR3 but not GluR2 AMPA glutamate receptor subunits

Figure 1 and Table 1a depict the mRNA expression profiles of the three AMPA receptor subunits *GluR1*, *GluR2*, and *GluR3* revealed by northern blot analysis over a 6-h post-slicing time course. The same membrane was sequentially hybridized with all three GluR probes and also re-hybridized with probes specific for the housekeeping genes *cyclophilin* and *β-actin*. The expression levels of housekeeping genes are generally used as a loading reference since they likely reflect

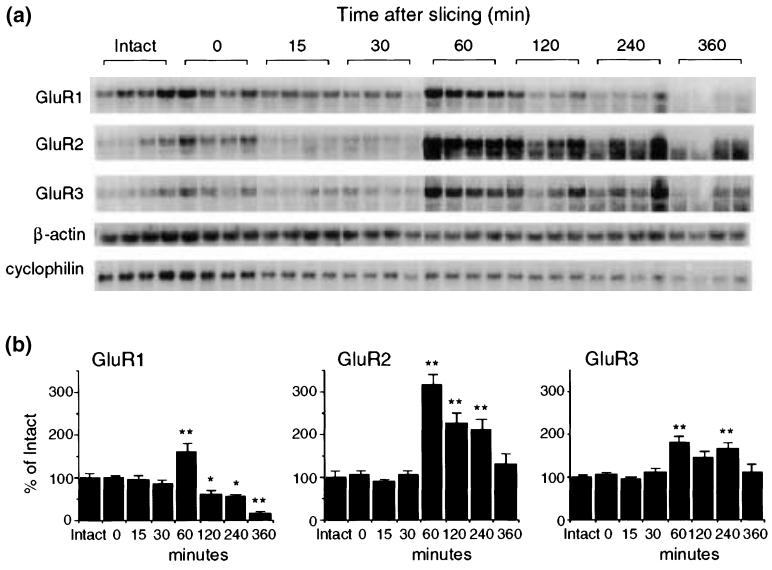

Fig. 1 Changes in GluR subunit mRNA levels following hippocampal slice preparation. (a) Northern blot analyses of extracts from intact or sliced hippocampi over a 6-h time course. Autoradiographs show *GluR1*, *GluR2*, *GluR3*, *β-actin* and *cyclophilin* mRNA levels of individual hippocampi ($n = 4$ per group) that were either dissected and frozen (intact) or sliced and incubated at 37°C in ACSF for the indicated period of time. (b) Densitometric analysis of the northern blots shown in (a). Significant increases compared to intact hippocampal levels in *GluR1*, *GluR2* and *GluR3* mRNA began at 60 min post-slicing and remained significantly elevated for *GluR2* and *GluR3* at later timepoints (indicated by asterisks). *GluR1* mRNA levels significantly declined by 120 min. *β-actin* was used as a control probe for normalization of all GluR hybridizations. Data are expressed as mean percentage ± SEM of the intact (100%) control mean values. GluR subunit values were normalized against those of β-actin to account for variations in gel loading.

Table 1 GluR subunit mRNA and protein levels following hippocampal slicing

	Intact	0 min	15 min	30 min	60 min	120 min	240 min	360 min
a								
mRNA								
GluR1	100.0	101.6	96.4	83.5	157.6	59.0	54.9	16.9
± SEM	9.6	3.8	8.1	10.9	21.3	11.6	7.1	4.6
p					< 0.01	< 0.05	< 0.05	< 0.01
GluR2	100.0	106.6	90.2	103.8	310.4	220.6	208.8	126.7
± SEM	12.7	8.4	6.1	11.6	28.2	29.0	26.6	28.3
p					< 0.01	< 0.01	< 0.01	
GluR3	100.0	106.7	95.4	108.1	178.8	145.8	164.2	107.9
± SEM	3.7	5.0	6.1	9.8	12.8	16.0	18.0	19.9
p					< 0.01		< 0.01	
b								
protein								
GluR1	100.0	98.7	88.4	60.4	45.9	37.4	19.0	14.5
± SEM	8.2	2.8	2.1	4.8	5.8	7.6	1.6	2.3
p				< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
GluR2	100.0	106.7	108.0	100.9	113.0	114.0	127.9	114.3
± SEM	6.5	3.1	4.1	7.9	5.2	5.4	10.7	13.1
GluR3	100.0	106.5	110.5	107.4	102.3	53.6	38.3	30.8
± SEM	8.5	8.6	9.9	9.4	11.7	2.1	3.2	2.6
p						< 0.01	< 0.01	< 0.01
actin	100.0	108.5	109.1	112.3	98.6	112.4	95.1	97.4
± SEM	4.2	2.3	7.1	2.1	4.2	4.7	9.1	8.1
cyclophilin	100.0	89.6	108.0	106.6	111.4	88.8	93.5	91.2
± SEM	6.8	4.5	4.6	6.0	5.7	4.0	4.7	5.2

Concentrations are expressed as mean percentage ± SEM of the intact hippocampal control mean values (100%). Hippocampi were either rapidly dissected and immediately frozen (intact) or sliced with a tissue chopper and incubated at 37°C for the assigned timepoint. Statistical analyses were performed using one-way ANOVA followed by Dunnett's multiple comparison tests.

the amount of total RNA present in each sample. Both *cyclophilin* and *β-actin* appeared to decrease over time, however, cyclophilin seemed to decay more rapidly. Therefore, β-actin was chosen as a loading reference to which all the other hybridizations were normalized. All three GluR subunits showed a detectable concentration in the intact hippocampus (intact) that changed after slicing. All the GluR subunit mRNAs moderately, but significantly increased at 60 min after slicing (157.6 ± 21.3%, 310.4 ± 28.2% and 178.8 ± 12.8% for *GluR1*, *GluR2*, and *GluR3*, respectively) compared to intact hippocampi, as shown in Table 1a. This peak decreased at later timepoints with different slopes for the various subunits. *GluR1* levels significantly decreased starting at 120 min and continued to fall at later timepoints, reaching a level of 16.9 ± 4.6% at 360 min. *GluR2* and *GluR3* mRNAs remained elevated until 240 min and returned to baseline at 360 min after slicing. A one-way ANOVA revealed a significant main effect of time ($F = 14.58$, $p < 0.0001$ for *GluR1*; $F = 14.24$, $p < 0.0001$ for *GluR2*; $F = 6.32$, $p < 0.001$ for *GluR3*) and Dunnett's post-hoc comparisons confirmed that these changes were significant compared to the control values (intact tissue; Table 1a).

In striking contrast to the mRNA concentration profile, the levels of GluR1 and GluR3 proteins following slicing decreased dramatically over the 6-h time course, while the levels of GluR2 remained unchanged (Fig. 2 and Table 1b). A significant decrease of GluR1 was evident starting at 30 min after slicing (60.4 ± 4.8%) compared to the expression levels of the intact hippocampi. GluR1 continued to decline at later timepoints and reached its lowest level at 360 min post-slicing (14.5 ± 2.3%). GluR3 was significantly diminished at 120 min after slicing (53.6 ± 2.1%) and reached its lowest concentration at 360 min (30.8 ± 2.6%). A one-way ANOVA revealed a significant main effect of time ($F = 47.55$, $p < 0.0001$ for GluR1; $F = 18.96$, $p < 0.0001$ for GluR3), and Dunnett's post-hoc comparisons confirmed that these decreases were significant compared to the control values (intact tissue; Table 1b). Unlike GluR1 and GluR3, GluR2 protein did not show any significant change throughout the entire time course (Fig. 2 and Table 1b).

We then determined the concentration of two housekeeping proteins, cyclophilin and actin. Both cyclophilin and actin levels remained stable across the 6-h time course

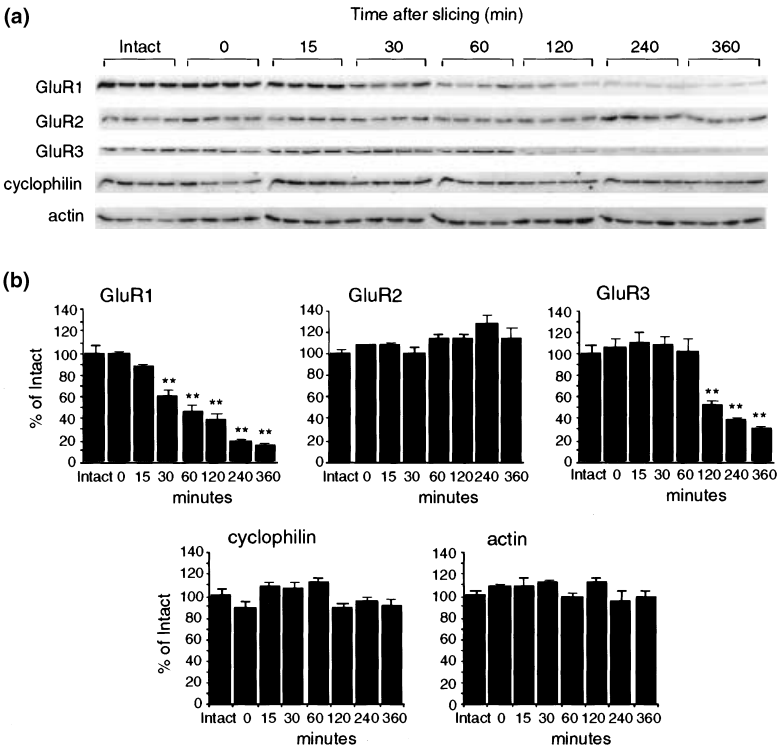

Fig. 2 Decrease of GluR1 and GluR3 protein subunits following hippocampal slice preparation. (a) Western blot time course analysis of extracts from intact or sliced hippocampi ($n = 4$ per group). (b) Densitometric analysis of western blots shown in (a). GluR1 protein steadily decreased beginning at 30 min after slicing compared to intact control values. GluR3 protein levels also decreased by 120 min post-slicing and continued to decline at 240 and 360 min. GluR2 protein levels, however, did not significantly change. Cyclophilin and actin were stable throughout the 6-h time course. All data are normalized against actin and expressed as mean percentage ± SEM of the intact (100%) control mean values.

(Table 1b). Thus, it appears that the loss of GluR1 and GluR3 subunits is selective and not due to a non-specific, general decline in protein levels as the expression of GluR2 and the two housekeeping genes remained unchanged throughout the entire time course.

Although controversial (Watson *et al.* 1997), some authors have suggested that slices generated with a McIlwain tissue chopper are more damaged than slices prepared with a vibratome (Aitken *et al.* 1995). In addition, although we incubated slices at 37°C in order to closely reproduce physiological conditions, most of the electrophysiological experiments that measure changes in synaptic plasticity are performed at 29–30°C. To determine whether the changes we observed were linked either to the slicing apparatus or the temperature, or both, we repeated the slice preparations using a vibratome and incubated the slices at 29°C. The time course of this experiment included intact hippocampi (intact) and sliced hippocampi incubated for 0, 60 and 360 min. We analyzed four hippocampi per condition and determined the concentration of GluR1 with western blot analysis. As shown in Fig. 3(a), a similar profile of GluR1 protein was detected in the preparations obtained with the vibratome/29°C condition, compared to the tissue chopper/37°C condition previously used. Immediately following slicing (0 min), the concentration of GluR1 was 90.2 ± 6.8% of intact control levels (100 ± 5.4%). By 60 min, GluR1 levels decreased to 56.0 ± 3.6%, and finally reached 35.6 ± 6.8% of intact levels at 360 min. A significant main effect of time ($F = 26.56$, $p < 0.0001$) was revealed by a one-way ANOVA, and Dunnett's post-hoc comparisons confirmed that both decreases at 60 min ($p < 0.01$) and 360 min ($p < 0.01$) were significant when compared to control values (intact). In addition, also the changes of both GluR2 and GluR3 at 29°C were very similar to those found at 37°C; GluR2 protein remained stable, while GluR3 protein strongly decreased (data not shown).

Finally, we determined whether the slices incubated at 37°C/tissue chopper or 29°C/vibratome for the 6-h time course maintained their functional responsiveness. We compared LTP at the Schaffer collateral CA1 synapse in hippocampal slices randomly chosen from both 360-min/37°C and 360-min/29°C incubations. Four slices per condition were analyzed, and Fig. 3(b) shows the responses of one representative slice under each condition. Four 0.1 Hz episodes of theta burst stimulation elicited a strong and stable potentiation in both slice preparations, which was sustained for the entire time of testing (40 min). As expected, under both conditions slices were viable, electrophysiologically responsive and exhibited plasticity following application of a tetanus.

Transcription factors: uncoupled mRNA-protein expression of *c-fos*, *zif268*, *C/EBPβ*, and *C/EBPδ*

A northern blot time course analysis was performed to examine mRNA levels of four transcription factors: *c-fos*,

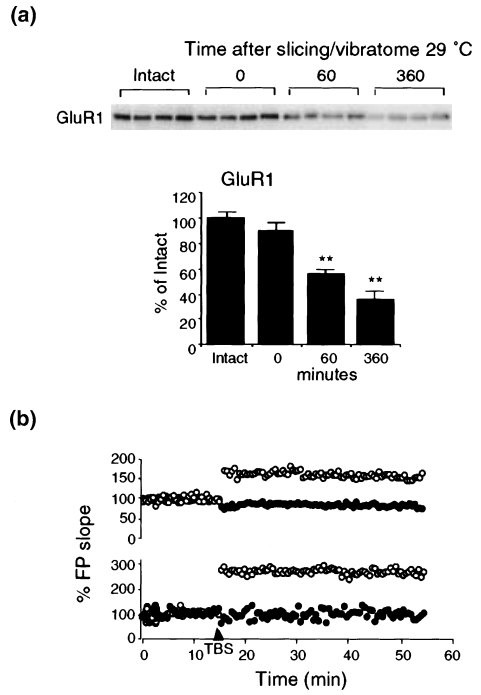

Fig. 3 Hippocampal slices with decreased GluR1 subunits exhibit normal synaptic plasticity in the CA1 region. (a) Western blot immunostaining of extracts from intact or vibratome-sliced hippocampi ($n =$ 4 per group) incubated at 29°C in ACSF for 0, 60 or 360 min. Significant decreases in GluR1 were evident at both 60 and 360 min compared to intact control levels. All data are normalized against actin and expressed as mean percentage ± SEM of the intact (100%) control mean values. (b) LTP induction in the CA1 region of hippocampal slices cut with either a tissue chopper (top) or vibratome (bottom) and incubated for 360 min at 37°C or 29°C, respectively. Simultaneous recording of field potentials in the tetanized (○) and control (●) slices prepared using either method show normal induction and maintenance of LTP. One representative experiment is shown for each slicing/incubating condition. Arrow indicates time of tetanic stimulation (15 min).

zif268, *C/EBPβ* and *C/EBPδ*. The housekeeping genes *cyclophilin* and *β-actin* were again used as control probes. Autoradiographs and their corresponding densitometric analyses are shown in Fig. 4 and Table 2a. Slicing caused a significant induction of all transcription factors at the mRNA level, but not at the protein level.

The mRNA levels of the immediate early gene (IEG) *c-fos* increased remarkably and steadily after slicing, reaching an initial peak of 931.9 ± 233.2% of control conditions (intact) at 30 min and continued to increase to 2594.3 ± 397.1% at 60 min and 4130.6 ± 267.5% of control at 120 min. Induction was sustained until 360 min (7169.6 ± 660.0%). A one-way ANOVA showed a main effect of time ($F = 71.95$, $p < 0.0001$) and Dunnett's post-hoc analysis revealed that all timepoints starting from 60 min were significantly increased over the control group (intact).

Like *c-fos*, *zif268* mRNA followed a similar induction profile after slicing. Levels were increased starting at 30 min (234.9 ± 25.4% of control) and continued to significantly increase at 60 min (427.3 ± 50.0%), 120 min (666.7 ± 16.0%), 240 min (784.8 ± 84.7%) and at 360 min (983.3 ± 149.4%).

The *C/EBP* isoforms *β* and *δ* also exhibited significant increases after slicing, although at a lesser degree and with a different kinetic profile. While the induction of *c-fos* and *zif 268* mRNA was relatively rapid, the increase of *C/EBPβ* and *C/EBPδ* became significant at later time points, beginning at 120 min after slicing. *C/EBPβ* increased to 144.5 ± 14.8% of control levels at 120 min, 203.8 ± 17.3% at 240 min, and 273.7 ± 41.9% at 360 min. *C/EBPδ* was elevated by 153.6 ± 17.8% of control levels at 120 min, 326.6 ± 11.4% at 240 min and 394.8 ± 41.0% at 360 min. The same membrane was hybridized sequentially with all transcription factor and housekeeping gene probes. Again, *β-actin* was used as a reference control to normalize all the other hybridizations. Thus, all four transcription factors were significantly induced after slicing, although with different kinetic profiles.

We then performed western blot analyses to determine the corresponding protein levels for each transcription factor throughout the time course. Autoradiographs and densitometric analyses are shown in Fig. 5 and Table 2b. The expression profiles of the proteins along the time course did not reflect the changes observed at the mRNA level. To the contrary, protein levels remained stable or decreased over time. c-Fos and Zif268 remained constant for the entire time course. C/EBPβ levels did not change significantly for 240 min after slicing, however, exhibited a significant decrease of 61.6 ± 8.6% at 360 min. C/EBPδ protein levels were only quantified for intact and 360 min sliced conditions because this experiment needed to be carried out independently. In fact, the C/EBPδ western blot was performed on nuclear extracts because the anti-C/EBPδ antiserum gave a nearly undetectable signal in total cell lysates. Similar to C/EBPβ, the concentration of C/EBPδ dramatically dropped at 360 min after slicing to a level of 17.0 ± 3.7% of control (intact).

Changes in NT-3 and BDNF levels following hippocampal slicing

As illustrated in Fig. 6 and Table 3a, levels of both 4.2 and 1.6 kb *BDNF* mRNA transcripts significantly increased starting at 60 min post-slicing (253.3 ± 12.5% and 277.7 ± 7.5%, respectively). This increase was sustained for the entire time course. In contrast, *NT-3* mRNA levels appeared stable up to 240 min, but significantly decreased to 45.1 ± 5.6% of control levels at 360 min after slicing.

Both BDNF and NT-3 proteins remained stable throughout the 6-h time course (Fig. 7 and Table 3b). Thus, like the other classes of proteins, namely transcription factors and

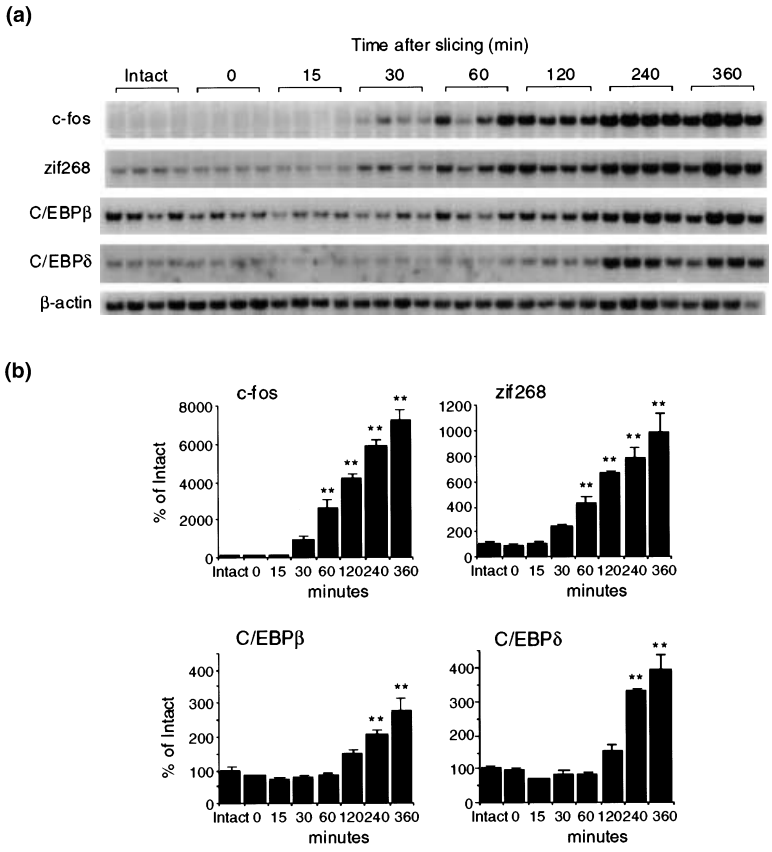

Fig. 4 *c-fos*, *zif268*, *C/EBPβ* and *C/EBPδ* mRNA levels are markedly induced following hippocampal slicing. (a) Northern blot time course analysis of extracts from intact or sliced hippocampi. The membrane was sequentially hybridized with probes specific for *c-fos, zif268, C/EBPβ, C/EBPδ, β-actin* and *cyclophilin*. (b) Densitometric analysis of the northern blot shown in (a). *c-fos* and *zif268* were significantly induced starting at 60 min, while *C/EBPβ* and *C/EBPδ* significantly increased starting at 240 min ($p < 0.01$) post-slicing compared to intact controls. *β-actin* was used as a reference control probe for normalization. Data are expressed as mean percentage ± SEM of the intact (100%) control mean values. Each transcription factor densitometric value was normalized to that of actin to account for variations in gel loading.

GluR subunits, the mRNA induction of *BDNF* was not coupled to an increase in corresponding protein levels.

Discussion

Our studies show that the expression levels of several classes of mRNAs and proteins dramatically change following acute slice preparation compared to the intact brain tissue. First, while *GluR1*, *GluR2* and *GluR3* mRNAs significantly increased beginning at 60 min after slicing, GluR1 and GluR3 proteins dramatically diminished throughout the entire time course. Unlike GluR1 and GluR3, GluR2 protein levels remained stable. This suggests that changes in the composition of AMPA receptors may occur following slicing and implies that the overall excitability and synaptic response of a slice preparation may be profoundly and persistently altered. Second, a remarkable induction of *c-fos*, *zif 268*, *C/EBPβ*, *C/EBPδ* and *BDNF* mRNAs occurred after slicing. The induction profile varied for each of these factors. Like the GluR changes, none of these mRNA inductions correlated with an increased expression of the corresponding proteins. In fact, these proteins either decreased or remained stable over time, indicating that slicing causes uncoupling of mRNA and protein expression. Finally, *NT-3* mRNA and protein levels remained relatively constant throughout the entire time course. As expected, the housekeeping proteins actin and cyclophilin appeared to be stable confirming that the changes observed in the other classes of proteins were selective.

These data together demonstrate a strong and persistent change in the molecular metabolism of hippocampal slices compared to the intact tissue. Our data do not invalidate cellular and molecular studies performed with slice preparations, rather they suggest the need for careful interpretation of gene expression regulation results when using the acute slice as a model to study physiological responses. Persistent changes in slice preparation have also been found at the morphological level. Kirov *et al*. (1999) reported that slices have an increase in spines and synapses number compared to the state *in vivo*, which becomes evident within a couple of hours after preparing the slices and persist for up to 13 h *in vitro*.

Profound loss of AMPA glutamate receptor proteins

The majority of the proteins analyzed exhibited stability throughout the post-slicing time course. GluR1 and GluR3 were the exception: they decreased dramatically starting at 30 min after slicing and continued to decrease further with time. The mechanism underlying this decrease is unclear. One possibility is that their synthesis is blocked. However,

Table 2 Transcription factor mRNA and protein levels following hippocampal slicing

	Intact	0 min	15 min	30 min	60 min	120 min	240 min	360 min
a								
mRNA								
c-fos	100.0	98.5	115.5	931.9	2594.3	4130.6	5832.5	7169.6
± SEM	4.8	7.9	7.1	233.2	397.1	267.5	395.5	660.0
p					< 0.01	< 0.01	< 0.01	< 0.01
zif268	100.0	89.5	104.8	234.9	427.3	666.7	784.8	983.3
± SEM	16.1	11.3	13.7	25.4	50.0	16.0	84.7	149.4
p					< 0.01	< 0.01	< 0.01	< 0.01
C/EBPβ	100.0	80.8	70.0	78.9	84.4	144.5	203.8	273.7
± SEM	10.9	8.4	8.1	7.9	5.9	14.8	17.3	41.9
p							< 0.01	< 0.01
C/EBPδ	100.0	95.9	69.1	78.9	81.6	153.6	326.6	394.8
± SEM	4.4	6.8	3.9	15.8	11.5	17.8	11.4	41.0
p							< 0.01	< 0.01
b								
protein								
c-Fos	100.0	104.8	114.4	98.7	104.2	100.2	103.7	92.8
± SEM	11.6	6.9	1.3	4.6	6.5	1.1	1.9	9.4
Zif268	100.0	106.4	100.1	100.2	106.3	104.1	92.3	89.8
± SEM	8.4	10.1	5.9	2.6	4.1	9.6	3.7	4.4
C/EBPβ	100.0	94.4	104.9	95.9	85.0	84.6	71.3	61.6
± SEM	3.4	4.3	4.1	11.0	14.7	10.4	8.9	8.6
p								< 0.05
C/EBPδ	100.0							17.0
± SEM	10.6							3.7
p								< 0.001

Concentrations are expressed as mean percentage ± SEM of the intact hippocampal control mean values (100%). Hippocampi were either rapidly dissected and immediately frozen (intact) or sliced with a tissue chopper and incubated at 37°C for the assigned timepoint. Statistical analyses were performed using one-way ANOVA followed by Dunnett's multiple comparison tests.

because the metabolic half-lives of GluR subunits have been calculated to range from 20 to as long as 100 h (Huh and Wenthold 1999; Kjoller and Diemer 2000), their post-slicing decline may not be merely explained by a general mechanism of protein synthesis inhibition. Thus, this rapid decrease may also result from the activation of a fast and selective degradation pathway. In contrast, GluR2 was protected from such a rapid decrease, indicating that GluR2 subunits may use degradation or targeting pathways distinct from those of GluR1 and GluR3.

The stability of the GluR2 pool over that of GluR1 or GluR3 after slicing indicates that this procedure may lead to a change in the abundance of GluR2 subunits in surface AMPA receptors and, consequently, a change in the AMPA receptor-mediated excitotoxicity of the preparations. Perhaps, as the presence of GluR2 in AMPA glutamate receptors decreases their permeability to Ca^{2+}, this modification may represent a protective mechanism against cell death. It is known, in fact, that Ca^{2+} influx is a major player in injury-induced cell death (Morley *et al*. 1994; Zipfel *et al*. 2000).

The magnitude of AMPA receptor subunit loss was similar whether slices were incubated at the physiological temperature of 37°C or at 29°C, the temperature at which most acute slice studies measuring electrophysiological responses are performed. At both temperatures, despite the molecular changes described, the slices appeared electrically viable and electrophysiologically responsive. In fact, under both temperature conditions, 6 h after slicing, tetanization produced LTP. Hence, in the acute slice preparation, LTP is induced under an altered cellular and molecular state, very different from that existing *in vivo*. In fact, as discussed below, slicing also produces a large induction of several mRNAs without an increase in their corresponding proteins. These profound changes likely affect the physiological metabolic state of hippocampal neurons.

Dissociation between mRNA and protein expression levels

The procedure required to prepare an acute brain slice causes profound injury and ischemia to the tissue. In fact, slices have often been used as a model of ischemic damage (Lipton and Wittingham 1979; Clark and Rothman 1987; Vornov and Coyle 1991). Our results document that the injury inherent in the procedure causes major changes in the mRNA

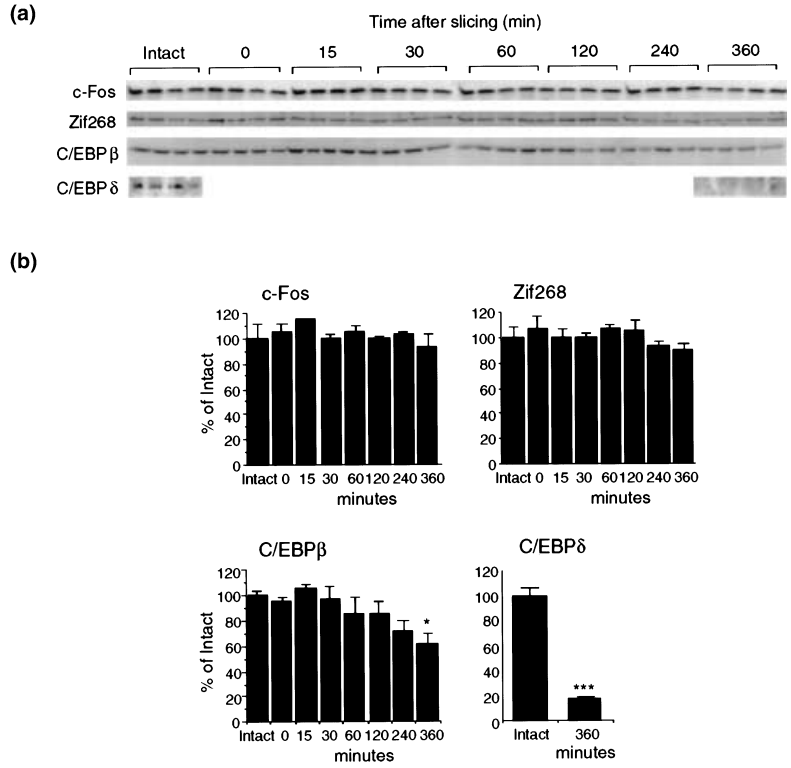

Fig. 5 Expression profiles of c-Fos, Zif268, C/EBPβ and C/EBPδ proteins following hippocampal slicing. (a) Western blot time course analysis of extracts from intact or sliced hippocampi ($n = 4$ per group). Nuclear extracts from intact and 360 min sliced hippocampi were analyzed using anti-C/EBPδ antiserum due to undetectable signals using total cell lysates. (b) Densitometric analysis of the western blots shown in (a). c-Fos or Zif268 protein levels did not significantly change over time following slicing compared to control levels. C/EBPβ levels also remained stable throughout most of the time course and significantly decreased only at 360 min post-slicing. Nuclear C/EBPδ significantly decreased 360 min after slicing compared to control levels. All data, normalized against actin, are expressed as mean percentage ± SEM of the intact (100%) control mean values.

concentrations of several classes of genes, including *c-fos*, *zif268*, *C/EBPβ*, *C/EBPδ*, *GluR1*, *GluR2*, *GluR3* and *BDNF*. These inductions, however, are not accompanied by increases in corresponding proteins, which appear to be stable or decrease significantly throughout the time course.

Why is there a massive mRNA induction without a parallel increase in proteins? One possible explanation is that a general impairment of new protein synthesis results from the severe injury produced by slicing. A large body of literature reports that a persistent protein synthesis failure is a hallmark of ischemic damage to neurons in the hippocampus. A marked decline in the rate of protein synthesis has been documented in models of brain injury, ischemia and hypoxia, or in response to anoxia and glutamate in hippocampal slices (Thilmann *et al*. 1986; Vornov and Coyle 1991; Hossmann 1993; Raley-Susman and Murata 1995; Lipton and Raley-Susman 1999; Monje *et al*. 2000). Lipton and Raley-Susman (1999) reported that protein synthesis rates in rat cortical slices were an order of magnitude lower than those *in vivo* (0.07% vs. 0.62% replacement of protein per hour). The

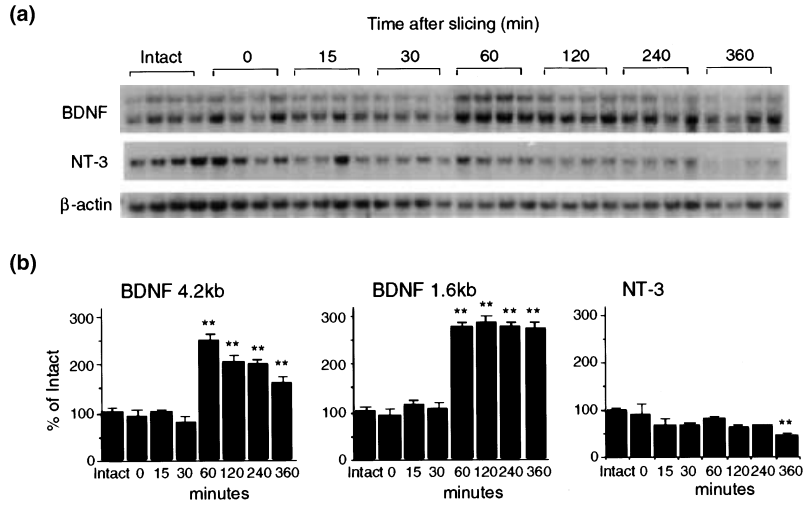

Fig. 6 *BDNF* and *NT-3* mRNA expression time course following hippocampal slice preparation. (a) Northern blot time course analysis of extracts from intact or sliced hippocampi ($n = 4$ per group). (b) Densitometric analysis of the northern blots shown in (a). Both 4.2 and 1.6 kb *BDNF* transcripts significantly increased beginning at 60 min post-slicing compared to control level and remained elevated for the entire time course. *NT-3* mRNA significantly increased only at 360 min ($p < 0.01$) post-slicing. *β-actin* was used as a reference control probe for normalization. Data are expressed as mean percentage ± SEM of the intact (100%) control mean values.

Table 3 Neurotrophin mRNA and protein levels following hippocampal slicing

	Intact	0 min	15 min	30 min	60 min	120 min	240 min	360 min
a								
mRNA								
BDNF (4.2)	100.0	92.6	102.6	79.4	253.3	206.7	200.6	1622.2
± SEM	11.4	13.2	5.2	14.4	12.5	12.2	10.5	15.1
p					< 0.01	< 0.01	< 0.01	< 0.01
BDNF (1.6)	100.0	93.8	116.9	108.0	277.7	285.6	279.8	274.3
± SEM	12.9	12.9	7.3	10.7	7.5	15.8	9.4	12.1
p					< 0.01	< 0.01	< 0.01	< 0.01
NT-3	100.0	92.8	71.3	68.4	82.7	64.0	67.5	45.1
± SEM	3.4	22.4	10.9	7.7	5.4	4.3	2.3	5.6
p								< 0.01
b								
protein								
BDNF	100.0	94.0	111.3	108.7	106.8	119.0	119.6	115.2
± SEM	12.0	9.0	6.1	4.3	13.3	8.8	8.0	2.6
NT-3	100.0	97.9	103.8	94.4	108.6	89.1	93.9	87.8
± SEM	12.3	7.1	10.2	4.9	4.2	4.5	6.6	5.7

Concentrations are expressed as mean percentage ± SEM of the intact hippocampal control mean values (100%). Hippocampi were either rapidly dissected and immediately frozen (intact) or sliced with a tissue chopper and incubated at 37°C for the assigned timepoint. Statistical analyses were performed using one-way ANOVA followed by Dunnett's multiple comparison tests.

mechanism by which protein synthesis is inhibited following such stresses is still under investigation. However, it appears that alteration of Ca^{2+} homeostasis plays a major role (Hossmann 1999; Paschen and Doutheil 1999) and that the disruption occurs at the translational level due to selective inhibition of polypeptide chain initiation (Hossmann 1993). This agrees with our results reporting strong mRNA induction without protein increase. Another possibility, which is not mutually exclusive with the former one, yet still controversial because of its complexity (Feig and Lipton 1993; Djuricic *et al*. 1994; Raley-Susman and Barnes 1998), is that the slicing-induced responses are mediated by glutamate. We do not address this aspect in the present paper and further experiments are required to test this hypothesis.

The hippocampus is known to be particularly sensitive to injury/hypoxia; however, distinct hippocampal cell types exhibit different levels of vulnerability to stress: neurons are more susceptible to injury than glia, and CA1 neurons are the most sensitive cell population. Thus, the slice should not be considered as a preparation in which all the cells have an impaired and irreversible protein synthesis disruption, but rather a condition in which protein synthesis is largely altered. Indeed, the injury-induced protein synthesis inhibition state can be reversed, and this reversibility correlates with the degree of stress or ischemia (Sharp *et al*. 1993; Raley-Susman and Barnes 1998; Monje *et al*. 2000).

Similar to our findings, several authors have described an uncoupled mRNA/protein induction following brain injury/

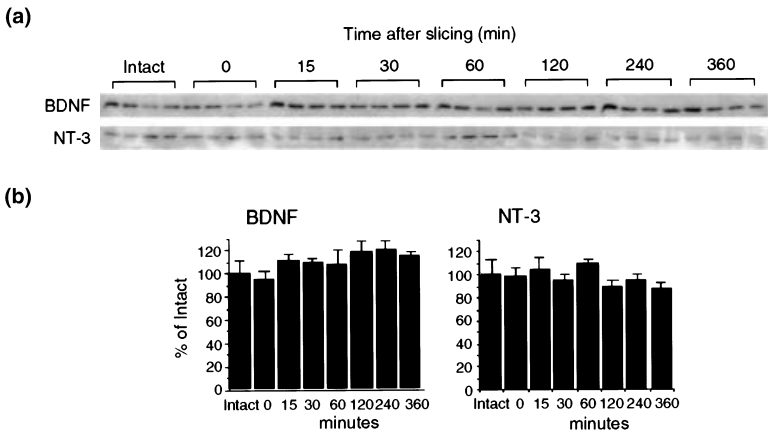

Fig. 7 BDNF and NT-3 protein levels remain stable following hippocampal slicing. (a) Western blot time course analysis of extracts from intact or sliced hippocampi ($n = 4$ per group). (b) Densitometric analysis of the western blots shown in (a). BDNF and NT-3 did not significantly change following slicing compared to intact control levels. All data are normalized against actin and expressed as mean percentage ± SEM of the intact (100%) control mean values.

ischemia. Oorschot *et al*. (2000) showed that hypoxic–ischemic brain injury in rat pups strongly induced *c-fos* mRNA without an increase in Fos protein level. Kiessling *et al*. (1993) found that transient global ischemia in the gerbil hippocampus results in a remarkable increase in transcription of both *c-fos* and *zif268*, which was not followed by translation into protein in the vulnerable CA1 neurons. Instead, induction of protein was restricted to other neuronal populations less vulnerable to brief ischemia. Likewise, injury caused by treatment of neonatal rat cortical slices with toxic concentrations of NMDA led to increased expression of *c-fos* and *hsp70* mRNAs without subsequent protein induction (Hasegawa *et al*. 1998).

One study, however, suggested that Fos and Jun proteins are induced following slicing. Zhou *et al*. (1995) reported that, in the brain slice preparation, mRNA induction of *c-fos* and *jun* is paralleled by an increase in their corresponding proteins. These protein elevations, however, were significantly delayed compared to their mRNA inductions (described at 1 h after slicing) and became evident only at 6 h. An explanation for this finding may be that under these experimental conditions inhibition of protein synthesis was reversed at later time points. Indeed, the magnitude and duration of the injury/hypoxia insult appear to be important parameters for the correlation with the injury response. Hughes *et al*. (1999) reported that *in vivo* hippocampal focal injury correlates with an induction of *zif268* at both mRNA and protein levels. However, while induction of transcription occurred immediately, peaked at 1 h, and returned to baseline 4 h following injury, protein expression was delayed and reached its highest levels 2 h after injury. Similar conclusions were drawn by several other authors (Nowak *et al*. 1993; Takemoto *et al*. 1995; Tomimoto *et al*. 1999), which showed that c-Fos and c-Jun were induced selectively in reversibly but not in irreversibly damaged neurons.

On the other hand, although evidence shows that the process of protein synthesis is inherently affected by injury/ischemia, we cannot exclude that the absence of protein increase, despite significant induction of mRNA, is only apparent. In fact, if slicing induces protein degradation to an extent that overcomes the rate of new protein synthesis, the result will be a net decrease in the protein levels. Our studies cannot determine whether increases or decreases in protein levels, despite mRNA induction, are the result of protein synthesis inhibition, increased protein degradation, or a combination of the two. Similarly, the mRNA increases may be due to increased mRNA synthesis, decreased degradation, or both.

Damage to death?

We investigated whether the profound molecular changes produced by slicing were related to cell death. While it is known that the superficial layers of brain slices are profoundly damaged and show morphological features of necrosis within a few hours post-slicing, it seems that the inner layers of the tissue, if maintained under 'healthy' conditions, remain reasonably intact for several hours. In agreement, our data suggest that slices are generally healthy; in fact, during a 6-h time course, they show strong molecular responses, including profound induction of mRNAs and selective changes in the levels of certain proteins and, 6 h post-slicing, LTP is normally induced. However, cell death can also occur through a programmed process, known as apoptosis, which requires a phase of gene expression. In results not shown (unpublished data) we measured the activation of caspase 3, a specific protease activated during apoptosis (Martin and Green 1995; Tewari *et al*. 1995; Whyte and Even 1995; Takahashi and Earnshaw 1996). Caspase 3 was present in its inactive form across the entire time course of our experiment. These data were confirmed by a second set of experiments that determined the presence of DNA fragmentation, another hallmark of apoptosis (McConkey *et al*. 1988). No evidence of nucleosomal fragmentation was observed. These results suggested that apoptosis did not occur within 6 h after hippocampal slicing. Thus, we concluded that the molecular changes we found are likely to occur in viable cells that are in the process of responding to the injury and hypoxia produced by the slicing procedure.

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References

Aitken P. G., Breese G. R., Dudek F. F., Edwards F., Espanol M. T., Larkman P. M., Lipton P., Newman G. C., Nowak T. S. Jr, Panizzon K. L. *et al.* (1995) Preparative methods for brain slices: a discussion. *J. Neurosci. Methods* **59**, 139–149.

Akaneya Y., Tsumoto T., Kinoshita S. and Hatanaka H. (1997) Brain-derived neurotrophic factor enhances long-term potentiation in rat visual cortex. *J. Neurosci.* **17**, 6707–6716.

Alberini C. M., Ghirardi M., Metz R. and Kandel E. R. (1994) C/EBP is an immediate-early gene required for the consolidation of long-term facilitation in *Aplysia*. *Cell* **76**, 1099–1114.

Berardi N. and Maffei L. (1999) From visual experience to visual function: roles of neurotrophins. *J. Neurobiol.* **41**, 119–126.

Cammarota M., Bernabeu R., Levi De Stein M., Izquierdo I. and Medina J. H. (1998) Learning-specific, time-dependent increases in hippocampal Ca^{2+}/calmodulin-dependent protein kinase II activity and AMPA GluR1 subunit immunoreactivity. *Eur. J. Neurosci.* **10**, 2669–2676.

Castren E., Berninger B., Leingartner A. and Lindholm D. (1998) Regulation of brain-derived neurotrophic factor mRNA levels in hippocampus by neuronal activity. *Prog. Brain Res.* **117**, 57–64.

Chaudhuri A. (1997) Neural activity mapping with inducible transcription factors. *Neuroreport* **10**, v–ix.

Chomczynski P. and Sacchi N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chlorophorm extraction. *Anal. Biochem.* **162**, 156–159.

Clark G. D. and Rothman S. M. (1987) Blokade of excitatory amino acid receptors protects anoxic hippocampal slices. *Neuroscience* **21**, 665–671.

Cole A. J., Saffen D. W., Baraban J. M. and Worley P. F. (1989) Rapid increase of an immediate early gene messenger RNA in hippocampal neurons by synaptic NMDA receptor activation. *Nature* **340**, 474–476.

Djuricic B., Berger R. and Paschen W. (1994) Protein synthesis and energy metabolism in hippocampal slices during extended (24 hours) recovery following different periods of ischemia. *Metab. Brain Dis.* **9**, 377–389.

Dragunow M. (1996) A role for immediate-early transcription factors in learning and memory. *Behav. Genet.* **26**, 293–299.

Dutcher S. A., Underwood B. D., Walker P. D., Diaz F. G. and Michael D. B. (1999) Patterns of immediate early gene mRNA expression following rodent and human traumatic brain injury. *Neurol. Res.* **21**, 234–242.

Feig S. and Lipton P. (1993) Pairing the cholinergic agonist carbachol with patterned Schaffer collateral stimulation initiates protein synthesis in hippocampal CA1 pyramidal cell dendrites via a muscarinic, NMDA-dependent mechanism. *J. Neurosci.* **13**, 1010–1021.

Gall C. M., Hess U.S. and Lynch G. (1998) Mapping brain networks engaged by, and changed by learning. *Neurobiol. Learn. Mem.* **70**, 14–36.

Hasegawa K., Litt L., Maryceline T. E., Sharp F. R. and Chan P. H. (1998) Expression of *c-fos* and *hsp70* mRNA in neonatal rat cerebrocortical slices during NMDA-induced necrosis and apoptosis. *Brain Res.* **785**, 262–278.

Hayes R. L., Yang K., Raghupathi R. and McIntosh T. K. (1995) Changes in gene expression following traumatic brain injury in the rat. *J. Neurotrauma* **12**, 779–790.

Herrera D. G. and Robertson H. A. (1996) Activation of c-fos in the brain. *Prog. Neurobiol.* **50**, 83–107.

Hossmann K. A. (1993) Ischemia-mediated neuronal injury. *Resuscitation* **26**, 225–235.

Hossmann K. A. (1999) The hypoxic brain: insight from ischemia research. *Adv. Exp. Med. Biol.* **474**, 155–169.

Houpt T. A., Philopena J. M., Joh T. H. and Smith G. P. (1996) c-Fos induction in the rat nucleus of the solitary tract correlates with the retention and forgetting of a conditioned taste aversion. *Learn. Mem.* **3**, 25–30.

Huang Y. Y., Nguyen P. V., Abel T. and Kandel E. R. (1996) Long-lasting forms of synaptic potentiations in the mammalian hippocampus. *Learn. Mem.* **3**, 74–85.

Hughes P. E., Alexi T., Walton M., Williams C. E., Dragunow M., Clark R. G. and Gluckman P. D. (1999) Activity and injury-dependent expression of inducible transcription factors, growth factors and apoptosis-related genes within the central nervous system. *Prog. Neurobiol.* **57**, 421–450.

Huh K. H. and Wenthold R. J. (1999) Turnover analysis of glutamate receptors identifies a rapidly degraded pool of the *N*-methyl-D-aspartate receptor subunit, NR1, in cultured cerebellar granule cells. *J. Biol. Chem.* **274**, 151–157.

Kaczmarek L. (1993) Molecular biology of vertebrate learning: is c-fos a new beginning? *J. Neurosci. Res.* **34**, 377–381.

Kang H. and Schuman E. M. (1995) Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus. *Science* **267**, 1658–1662.

Kennis J. H. and Holstege J. C. (1997) A differential and time-dependent decrease in AMPA-type glutamate receptor subunits in spinal motorneurons after sciatic nerve injury. *Exp. Neurol.* **147**, 18–27.

Kiessling M., Stumm G., Xie Y., Herdegen T., Aguzzi A., Bravo R. and Gass P. (1993) Differential transcription and translation of immediate early genes in gerbil hippocampus after transient global ischemia. *J. Cereb. Blood Flow Metab.* **13**, 914–924.

Kirov S. A., Sorra K. E. and Harris K. M. (1999) Slices have more synapses that perfusion-fixed hippocampus from both young and mature rats. *J. Neurosci.* **19**, 2876–2886.

Kjoller C. and Diemer N. H. (2000) GluR2 protein synthesis and metabolism in rat hippocampus following transient ischemia and ischemic tolerance induction. *Neurochem. Int.* **37**, 7–15.

Lamprecht R. and Dudai Y. (1996) Transient expression of c-Fos in rat amygdala during training is required for encoding conditioned taste aversion memory. *Learn. Mem.* **3**, 31–41.

Lessmann V. (1998) Neurotrophin-dependent modulation of glutamatergic synaptic transmission in the mammalian CNS. *Gen. Pharmacol.* **31**, 667–674.

Linnarsson S., Bjorklund A. and Ernfors P. (1997) Learning deficit in BDNF mutant mice. *Eur. J. Neurosci.* **9**, 2581–2587.

Lipton P. and Raley-Susman K. M. (1999) Autoradiographic measurements of protein synthesis in hippocampal slices from rats and guinea pigs. *Methods* **18**, 127–143.

Lipton P. and Wittingham T. S. (1979) The effect of hypoxia on evoked potentials in the *in vitro* hippocampus. *J. Physiol.* **287**, 427–438.

Lynch G. (1998) Memory and the brain: unespected chemistry and a new pharmachology. *Neurobiol. Learn. Mem.* **70**, 82–100.

McConkey D. J., Hartzell P., Nicotera P., Wyllie A. H. and Orrenius S. (1988) Stimulation of endogenous endonuclease activity in hepatocytes exposed to oxidative stress. *Toxicol. Lett.* **42**, 123–130.

Ma Y. L., Wang H. L., Wu H. C., Wei C. L. and Lee E. H. (1998) Brain-derived neurotrophic factor antisense oligonucleotide impairs memory retention and inhibits long-term potentiation in rats. *Neuroscience* **82**, 957–967.

Martin S. J. and Green D. R. (1995) Protease activation during apoptosis: death by a thousand cuts? *Cell* **82**, 349–352.

Monje M. L., Chatten-Brown J., Hye S. E. and Raley-Susman K. M. (2000) Free radicals are involved in the damage to protein synthesis after anoxia/aglycemia and NMDA exposure. *Brain Res.* **857**, 172–182.

Montag-Sallaz M., Welzl H., Kuhl D., Montag D. and Schachner M. (1999) Novelty-induced increased expression of immediate-early genes c-fos and arg 3.1 in the mouse brain. *J. Neurobiol.* **38**, 234–246.

Montkowski A. and Holsboer F. (1997) Intact spatial learning and memory in transgenic mice with reduced BDNF. *Neuroreport* **8**, 779–782.

Morley P., Hogan M. J. and Hakim A. M. (1994) Calcium-mediated mechanisms of ischemic injury and protection. *Brain Pathol.* **4**, 37–47.

Nayak A., Zastrow D. J., Lickteig R., Zahniser N. R. and Browning M. D. (1998) Maintenance of late-phase LTP is accompanied by PKA-dependent increase in AMPA receptor synthesis. *Nature* **394**, 680–683.

Nikolaev E., Tischmeyer W., Krug M., Matthies H. and Kaczmarek L. (1991) c-fos protooncogene expression in rat hippocampus and entorhinal cortex following tetanic stimulation of the perforant path. *Brain Res.* **560**, 346–349.

Nowak T. S. J., Osborne O. C. and Suga S. (1993) Stress protein and proto-oncogene expression as indicators of neuronal pathophysiology after ischemia. *Prog. Brain Res.* **96**, 195–208.

Oorschot D. E., Black M. J., Rangi F. and Scarr E. (2000) Is Fos protein expressed by dying striatal neurons after immature hypoxic-ischemic brain injury? *Exp. Neurol.* **161**, 227–233.

Paschen W. and Doutheil J. (1999) Disturbance of endoplasmic reticulum functions: a key mechanism underlying cell damage? *Acta Neurochir. (Suppl.)* **73**, 1–5.

Radulovic J., Kammermeier J. and Spiess J. (1999) Relationship between fos production and classical fear conditioning: effects of novelty, latent inhibition, and unconditioned stimulus pre-exposure. *J. Neurosci.* **18**, 7452–7461.

Raghupathi R., McIntosh T. K. and Smith D. H. (1995) Cellular responses to experimental brain injury. *Brain Pathol.* **5**, 437–442.

Raley-Susman K. M. and Barnes J. R. (1998) The effects of extracellular pH and calcium manipulation on protein synthesis and reponse to anoxia/aglycemia in the rat hippocampal slice. *Brain Res.* **782**, 281–289.

Raley-Susman K. M. and Murata J. (1995) Time course of protein changes following *in vitro* ischemia in the rat hippocampal slice. *Brain Res.* **694**, 94–102.

Riedel G., Wetzel W. and Reymann K. G. (1996) Comparing the role of metabotropic glutamate receptors in long-term potentiation and in learning and memory. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **20**, 761–789.

Schlingensiepen R., Terlau H., Brysch W. and Schlingensiepen K. H. (1994) Differential expression of c-jun, junB and junD in rat hippocampal slices. *Neuroreport* **30**, 101–104.

Schuman E. M. (1999) Neurotrophin regulation of synaptic transmission. *Curr. Opin. Neurobiol.* **9**, 105–109.

Sharp F. R., Kinouchi H., Koistinaho J., Chan P. H. and Sagar S. M. (1993) HSP70 heat shock gene regulation during ischemia. *Stroke* **24**, 172–175.

Sheng M. and Greenberg M. E. (1990) The regulation and function of c-fos and other immediate early genes in the nervous system. *Neuron* **4**, 477–485.

Siklos L., Kuhnt U., Parducz A. and Szerdahelyi P. (1997) Intracellular calcium redistribution accompanies changes in total tissue Na^+, K^+ and water during the first two hours of *in vitro* incubation of hippocampal slices. *Neuroscience* **79**, 1013–1022.

Sterneck E., Paylor R., Jackson-Lewis V., Libbey M., Przedborski S., Tessarollo L., Crawley J. N. and Johnson P. F. (1998) Selective enhanced contextual fear conditioning in mice lacking the transcriptional regulator CCAAT/enhancer binding protein δ. *Proc. Natl. Acad. Sci. USA* **95**, 10908–10913.

Takahashi A. and Earnshaw W. C. (1996) ICE-related proteases in apoptosis. *Curr. Opin. Genet. Dev.* **6**, 50–55.

Takemoto O., Tomimoto H. and Yanagihara T. (1995) Induction of c-fos and c-jun gene products and heat shock protein after brief and prolonged cerebral ischemia in gerbils. *Stroke* **26**, 1639–1648.

Taubenfeld S. M., Wiig K. A., Monti B., Dolan B., Pollonini G. and Alberini C. M. (2001) Fornix-dependent induction of hippocampal CCAAT enhancer-binding protein β and δ co-localizes with phosphorylated cAMP response element binding protein and accompanies long-term memory consolidation. *J. Neurosci.* **21**, 84–91.

Tewari M., Quan L. T., O'Rourke K., Desnoyers S., Zeng Z., Beidler D. R., Poirier G. G., Salvesen G. S. and Dixit V. M. (1995) Yama/CPP32β, a mammalian homologue of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly (ADP-ribose) polymerase. *Cell* **81**, 801–809.

Thilmann R., Xie Y., Kleihues P. and Kiessling M. (1986) Persistent inhibition of protein synthesis precedes delayed neuronal death in postischemic gerbil hippocampus. *Acta Neuropathol.* **71**, 88–93.

Tischmeyer W. and Grimm R. (1999) Activation of immediate early genes and memory formation. *Cell. Mol. Life Sci.* **55**, 564–574.

Tomimoto H., Takemoto O., Akiguchi I. and Yanagihara T. (1999) Immunoelectron microscopic study of c-Fos, c-Jun and heat shock protein after transient cerebral ischemia in gerbils. *Acta Neuropathol.* **97**, 22–30.

Vornov J. J. and Coyle J. T. (1991) Glutamate neurotoxicity and the inhibition of protein synthesis in the hippocampal slice. *J. Neurochem.* **56**, 996–1006.

Walton M., Connor B., Lawlor P., Young D., Sirimanne E., Gluckman P., Cole G. and Dragunow M. (1999) Neuronal death and survival in two models of hypoxic–ischemic brain damage. *Brain Res. Brain Res. Rev.* **29**, 137–168.

Wan H., Aggleton J. P. and Brown M. W. (1999) Different contributions of the hippocampus and perirhinal cortex to recognition memory. *J. Neurosci.* **19**, 1142–1148.

Watson P. L., Weiner J. L. and Carlen P. L. (1997) Effects of variations in hippocampal slice preparation protocol on the electrophysiological stability, epileptogenicity and graded hypoxia responses of CA1 neurons. *Brain Res.* **775**, 134–143.

Whyte M. and Even G. (1995) Apoptosis: the last cut is the deepest. *Nature* **376**, 17–18.

Zhang Y., Widmayer M. A., Zhang B., Cui J. K. and Baskin D. S. (1999) Suppression of post-ischemic-induced fos protein expression by an antisense oligonucleotide to c-fos mRNA leads to increased tissue damage. *Brain Res.* **832**, 112–117.

Zhou Q., Abe H. and Nowak T. S. Jr (1995) Immunocytochemical and in situ hybridization approaches to the optimization of brain slice preparations. *J. Neurosci. Methods* **59**, 85–92.

Zipfel G. J., Babcock D. J., Lee J. M. and Choi D. W. (2000) Neuronal apoptosis after CNS injury: the roles of glutamate and calcium. *J. Neurotrauma* **17**, 857–869.