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Genes Preferentially Induced by Depolarization after Concussive Brain Injury: Effects of Age and Injury Severity

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ABSTRACT

Fluid percussion (FP) brain injury leads to immediate indiscriminate depolarization and massive potassium efflux from neurons. Using Northern blotting, we examined the post-FP expression of primary response/immediate early genes previously described as induced by depolarization in brain. RNA from ipsilateral and contralateral hippocampus was harvested from immature and adult rats 1 h following mild, moderate, or severe lateral fluid percussion injury and compared against age-matched sham animals. C-fos gene expression was used as a positive control and showed marked induction in both pups (6–25-fold with increasing severity) and adults (9.7–17.1-fold). Kinase-induced-by-depolarization–1 (KID-1) and salt-inducible kinase (SIK) gene expression was increased in adult (KID-1 1.5–1.6-fold; SIK 1.3–3.9-fold) but not developing rats. NGFI-b RNA was elevated after injury in both ages (pups 1.8–6.1-fold; adults 3.5–5-fold), in a pattern similar to that seen for c-fos. Secretogranin I (sec I) demonstrated no significant changes. Synaptotagmin IV (syt IV) was induced only following severe injury in the immature rats (1.4-fold). Our results reveal specific severity- and age-dependent patterns of hippocampal immediate early gene expression for these depolarization-induced genes following traumatic brain injury. Differential expression of these genes may be an important determinant of the distinct molecular responses of the brain to varying severities of trauma experienced at different ages.

Key words: depolarization; development; fluid percussion; gene expression; hippocampus; immediate-early genes; traumatic brain injury

INTRODUCTION

Concussive brain injury leads to neurological dysfunction in the absence of significant anatomical damage. Neurological symptoms and cognitive deficits (often transient) are the acute hallmarks of clinical concussion, while persistent neurobehavioral symptoms make up what has been termed the "postconcussive syndrome" (Bernstein, 1999; Bazarian et al., 1999). How do such neurobehavioral impairments exist in a setting of little, if any, cerebral atrophy or cell death? Likely mechanisms involve alterations of molecular and cellular processes in injured neurons.

Significant derangements in neuronal function and cerebral physiology seen following concussive brain injury include abrupt excitatory neurotransmitter release

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(Faden et al., 1989; Katayama et al., 1990), accelerated glycolysis (Kubota et al., 1989; Yoshino et al., 1991), inhibition of oxidative metabolism (Hovda et al., 1991; Xiong et al., 1997), changes in blood flow (Yamakami et al., 1989; Dietrich et al., 1996; Ginsberg et al., 1997), impaired neurotransmission (Miller et al., 1990; Hayes et al., 1992; Gorman et al., 1996), and axonal damage (Povlishock et al., 1995; Pettus et al., 1996). The initial response to traumatic brain injury is an indiscriminate release of neurotransmitters (Faden et al., 1989; Hayes et al., 1992). The release of excitatory neurotransmitters leads to widespread neuronal depolarization and unchecked ionic shifts, particularly efflux of potassium (Takahashi et al., 1981; Hubschmann and Kornhauser 1983; Katayama et al., 1990) and influx of calcium (Cortez et al., 1989; Fineman et al., 1993; Nilsson et al., 1993; Osteen et al., 2001). These pathophysiological changes likely result in altered cognition and behavior. In addition, they may manifest as deficits in subsequent neural plasticity, thereby affecting recovery of function and normal cerebral maturation (Fineman et al., 2000).

Following experimental brain concussion, adult animals develop severity-dependent neurological and cognitive deficits in tasks such as beam walking and spatial learning (Dixon et al., 1987; McIntosh et al., 1989; Prins et al., 1996). Additionally, experimental concussive brain injury leads to impairment in neuroplastic mechanisms such as long-term potentiation in adult animals (D'Ambrosio et al., 1998; Reeves et al., 1995; Sanders et al., 2000; Sick et al., 1998). Immature animals show minimal cognitive deficits following mild to moderate traumatic brain injury (Adelson et al., 1997; Prins et al., 1996; Prins et al., 1998), but develop dysfunctional hippocampal ionic homeostasis (D'Ambrosio et al., 1999) and impaired neuroplasticity (Fineman et al., 2000). Given this postconcussive attenuation of plasticity in the absence of substantial morphological change, we have turned our attention to investigating potential molecular mechanisms of trauma-induced neurological and developmental deficits.

Traumatic brain injury undoubtedly leads to alterations in the expression of many genes, resulting from the myriad of pathophysiological changes associated with biomechanical cell disruption. Each wave of gene expression and subsequent protein synthesis may, in turn, trigger secondary and tertiary waves of molecular change in the brain, both as a direct response to damage and to effect repair of injured neural systems. This molecular response includes, but is not limited to, changes in gene products such as the glucose transporter (Cornford et al., 1996), neurotransmitter transporters and receptors (Gong et al., 1999; Rao et al., 1998), inflammatory mediators (Cook et al., 1998; Gourin et al., 1997; Posmantur et al., 1996; Strauss et al., 2000; Whalen et al., 2000), cytoskeletal proteins (Posmantur et al., 1996; Sahin et al., 1999), neurotrophic factors (DeKosky et al., 1994; Hicks et al., 1999; Oyesiku et al., 1999), and enzymes involved in programmed cell death (Beer et al., 2000; Clark et al., 1999).

From this broad array of postinjury genetic signals, we limited our current study to immediate early genes, with the aim of investigating the critical *initial* changes in cellular function induced by injury. We further narrowed the number of candidate genes by selecting only those that show preferential induction by a depolarizing stimulus versus a growth factor stimulus in cell culture. The rationale was that many neural genes are induced by both depolarization and trophic signals, but genes induced preferentially by depolarization may play specific roles in synaptic activity-dependent plasticity that are distinct from more generalized postinjury regenerative responses.

Five genes preferentially induced by depolarization rather than by growth factors were selected for study: (1) kinase-induced-by-depolarization–1, (2) salt-inducible kinase, (3) NGFI-b, a nuclear orphan receptor, and two synaptic vesicle-associated proteins, (4) secretogranin I and (5) synaptotagmin IV.

Kinase-Induced-by-Depolarization–1

Kinase-induced-by-depolarization–1 (KID-1) was recently described (Feldman et al., 1998) as having significant sequence homology with the PIM-1 proto-oncogene, which itself codes for a serine/threonine protein kinase (Hoover et al., 1991; Padma et al., 1991; Saris et al., 1991). *In vitro* studies demonstrate that KID-1 also exhibits kinase activity, and is capable of phosphorylating other proteins and of autophosphorylation (Feldman et al., 1998).

The relationship of KID-1 transcription to depolarization has been described both in cell culture and *in vivo*. In PC12 cells, KID-1 is induced by both membrane depolarization and forskolin, and not by neurotrophins. This suggests that KID-1 expression is mediated by a cyclic AMP dependent pathway (via activation of adenylyl cyclase and/or cyclic AMP response element-binding protein [CREB]). *In vivo*, kainic acid–induced seizures clearly increase KID-1 expression in both cerebral cortex and hippocampus, as demonstrated by *in situ* hybridization (Feldman et al., 1998).

Salt-Inducible Kinase

Salt-inducible kinase (SIK) shares many similarities with KID-1. Like KID-1, SIK is also an immediate early gene that codes for a protein with kinase activity (Wang et al., 1999). It is capable both of phosphorylating a synthetic peptide and of autophosphorylation (Feldman et al., 2000). SIK also demonstrates depolarization-induced

transcription in cell culture and *in vivo*. In PC12 cells, SIK is preferentially induced by membrane depolarization versus trophic factor administration (Feldman et al., 2000). *In vivo*, SIK is also increased in cortex and hippocampus at 1–8 h following kainic acid–induced seizures (Feldman et al., 2000).

NGFI-b (or nur77, TIS-1)

NGFI-b codes for a nuclear orphan receptor with zinc-finger domains. Zinc-finger proteins are induced by many stimuli, including seizures (Dragunow et al., 1992; Honkaniemi et al., 1999), ischemia (Honkaniemi et al., 1997; Wang et al., 1995), and trophic factors (Hazel et al., 1988; Milbrandt, 1988). Proteins with these structural motifs are capable of sequence-specific DNA binding and can thus regulate gene expression.

Like other zinc-finger proteins, NGFI-b is actually induced by both depolarization (Honkaniemi et al., 1999) and neurotrophins (Hazel et al., 1988; Milbrandt, 1988). However, the mechanism of its induction may have a significant contribution to its ultimate function. A study by Katagiri et al. (1997) reports that only when NGFI-b is induced by depolarization does it demonstrate transcriptional activity. This implies a distinct role for NGFI-b in synaptic activity-dependent neuroplasticity, as it is only after depolarization that NGFI-b is able to bind DNA and directly modulate further gene expression. Thus, NGFI-b may exert its trophin-induced effects by a different pathway.

Secretogranin I (or Chromogranin B)

Secretogranin I (Sec I) is an immediate early gene coding for a synaptic vesicle-associated protein that appears to play a role in vesicular sorting (Natori et al., 1996). Sec I expression is upregulated following forskolin treatment in PC12 cells (Thompson et al., 1992). This induction appears to be due to activation of adenylyl cyclase by forskolin treatment, as treatment with 8-bromo-cAMP also led to an increase in sec I and treatment with a cAMP-dependent protein inhibitor attenuated the effect of forskolin (Thompson et al., 1992). *In vivo*, sec I expression was also induced by cortical spreading depression in rat cortex but not in hippocampus (Shen et al., 1998).

Synaptotagmin IV

Synaptotagmin IV (Syt IV) is one member of a family of 11 isoforms of the synaptotagmins (Schiavo et al., 1998). However, syt IV is unusual among the synaptotagmins in that it lacks one of the two typical calcium-binding domains (Ullrich et al., 1994). In fact, syt IV has been implicated in control of calcium sensitivity of synaptic vesicle fusion, as overexpression of syt IV attenuates synaptic release in *Drosophila* (Littleton et al., 1999), resulting in an impairment of synaptic transmission. Interestingly, syt IV null transgenic mice also demonstrate deficits, particularly in motor control and hippocampal-based memory functions (Ferguson et al., 2000). These two studies suggest that syt IV levels modulate synaptic plasticity. Interestingly, syt IV demonstrates a normal developmental pattern of expression that peaks soon after birth and declines into adulthood in the rat (Berton et al., 1997).

Syt IV mRNA levels are generally increased by depolarization. In cell culture with PC12 cells, syt IV expression was induced by application of potassium, forskolin, ATP, or calcium ionophore A23187 (Vician et al., 1995). Syt IV expression can also be elevated in vivo by a depolarizing stimulus such as kainic acid–induced seizures (Vician et al., 1995).

Nonspecific depolarization and potassium efflux are pathophysiological hallmarks of traumatic brain injury (Hubschmann et al., 1983; Katayama et al., 1990; Takahashi et al., 1981). Several recent studies demonstrate that brain concussion alters plasticity in both mature (Phillips et al., 1994; Sanders et al., 2000) and developing animals (Fineman et al., 2000; Shieh, 2002). Since genes preferentially induced by depolarization are likely to modulate activity-dependent neuroplasticity, we investigated the effects of traumatic brain injury on this set of immediate-early response genes. Both injury severity and age-at-injury were studied.

MATERIALS AND METHODS

Subjects

Animals studied were either postnatal day 17 (P17, $n = 26$) or adult (approximately P60, $n = 22$) male Sprague-Dawley rats (Charles River, Hollister, CA). All pups were housed as litters with access to mothers until the time of surgery. P17 rats are the youngest age with sufficient skull thickness to support the plastic fluid percussion (FP) injury cap (Prins et al., 1996). There were no significant weight differences between injury severity groups for pups (one-way ANOVA, $F[3,21] = 0.476$, $p = 0.70$) or adults ($F[3,17] = 2.13$, $p = 0.15$; Table 1). The UCLA Chancellor's Committee for Animal Research approved all animal studies.

Lateral Fluid Percussion Injury

P17 pups and adults were anesthetized with enflurane (1.0–1.5 and 1.5–2.0 mL/min, respectively, in 100% O_2 via facemask). All animals were placed into a stereotaxic frame. Body temperature was maintained at 37–38°C with a thermostatically controlled heating pad. FP animals underwent lateral FP injury as previously described

TABLE 1. GROUP CHARACTERISTICS[a]

Group	n	Weight (g)	Pressure (atm)	Apnea (sec)	Unconsciousness (sec)
Pup sham	5	47.2 ± 1.5	NA	NA	NA
Pup mild	7	45.3 ± 0.7	2.65 ± 0.06	15.0 ± 3.0	20.3 ± 3.5
Pup moderate	5	46.8 ± 1.3	2.81 ± 0.14	50.6 ± 6.6	66.8 ± 8.9
Pup severe	5	46.6 ± 1.8	3.2 ± 0.25	287.0 ± 36.8	473.4 ± 105.7
Effects in pups		**NS, $p = 0.71$**	**$p = 0.06$**	**$p < 0.001$**	**$p < 0.001$**
Adult sham	5	257.7 ± 4.4	NA	NA	NA
Adult mild	5	233.2 ± 14.2	1.87 ± 0.1	10.8 ± 2.6	22.2 ± 6.1
Adult moderate	5	256.2 ± 7.3	2.18 ± 0.03	23.0 ± 3.5	66.2 ± 3.0
Adult severe	5	292.2 ± 26.7	2.52 ± 0.19	38.0 ± 11.5	184.4 ± 15.0
Effects in adults		**NS, $p = 0.15$**	**$p < 0.05$**	**$p = 0.06$**	**$p < 0.001$**

[a]Unconsciousness represents duration of time from injury until recovery of hindpaw withdrawal reflex. Values ± SEM. Separate comparisons were made for pups and adults (one-way ANOVA, p values for main effect of injury-severity group).

(Prins et al., 1996). A 3-mm-diameter craniotomy was placed 2 mm posterior to bregma and 6 mm lateral (left) of the midline, using a high-speed drill (Dremel, Racine, WI). A plastic injury cap was then fixed in place over the craniotomy using silicone adhesive, cyanoacrylate, and, finally, dental cement. When the cement had hardened, the injury cap was filled with saline, anesthesia briefly discontinued, and the animal attached to the FP device. Hind paw withdrawal reflex was determined by pinching the middle toe of the rear paw every 15 sec and noting whether the animal retracted the limb. At the first sign of the hind paw withdrawal reflex, a fluid pulse was administered. After the injury, apnea time was determined by the resumption of spontaneous respiration and the unconsciousness time by the return of the hind paw withdrawal reflex. If no spontaneous respirations were evident by 45 sec, manual chest compressions and 100% O2 were administered until spontaneous respirations returned. Upon return of the hind paw withdrawal reflex, the animal was placed back under anesthesia for removal of the injury cap and closure of the surgical wound. Sham animals ($n = 5$/age group) were subjected to anesthesia and incision but no injury.

Severity was determined by post-FP duration of unconsciousness (time until return of hind paw withdrawal reflex) which has been shown to correlate with fluid pulse pressure (atm) and functional outcome in adults (Dixon et al., 1987). Mild injury was defined as unconsciousness of <45 sec, moderate injury as 45–120 sec, and severe injury as >120 sec.

RNA Collection and Preparation

One-hour post-FP, all animals were sacrificed and brains removed. Right and left whole hippocampi were dissected on ice, weighed, and RNA prepared using a modification of the acid-guanidinium-phenol-chloroform protocol (Chomczynski et al., 1987; Feldman et al., 1998). Immediately after harvest, hippocampal tissue was placed in 10 volumes of ice-cold denaturing solution (Trizol©; Gibco, BRL, Gaithersburg, MD), then homogenized before freezing and storage at −80°C. RNA was thawed and extracted by adding chloroform, followed by precipitation using isopropanol. RNA was pelleted, washed with ethanol and resuspended in TE (10 mM Tris, 1 mM EDTA, pH 8.0) before spectrophotometric determination of RNA yield. RNA was then stored at −80°C until Northern blotting.

Northern Blotting

All samples (20 μg total RNA/lane) for a given age group (pup vs. adult) and brain region (right vs. left hippocampus) were loaded and run on 1.2% agarose, 6% formaldehyde gels. Gels were photographed briefly under UV light. RNA was transferred to (0.45 μm) nitrocellulose blots via upward capillary transfer (overnight) and then crosslinked to the blot by UV light. The locations of the 18S and 28S bands were marked on the blot before hybridization or storage at −20°C. Duplicate gels and blots were made for each side (ipsilateral, contralateral) and age group.

c-fos Gene Expression

c-fos expression was measured as a positive control, since significant upregulation of c-fos mRNA has been reported in hippocampus following FP injury in adults (Dragunow et al., 1990; Phillips et al., 1992; Raghupathi et al., 1996; Yang et al., 1995), as well as after other brain injury such as ischemia (Honkaniemi et al., 1997; Wang et al., 1995), hypoglycemia (Gass et al., 1995), and seizures

(Dragunow et al., 1992; Labiner et al., 1993). However, c-fos upregulation is not depolarization-specific, as stimulation with neurotrophins may also induce its expression (Engele et al., 1996; Ip et al., 1993; Marsh et al., 1996).

Probe Preparation and Hybridization

Blots were incubated in prehybridization buffer (100 μg/mL salmon sperm DNA, 50% (v/v) deionized formamide, 0.28 M sodium phosphate, 1 mM EDTA, 0.56 M sodium chloride, 1% SDS) at 42°C for 4 h (Vician 1995).

Probes were synthesized using either random priming (c-fos, GAPDH, NGFI-b, sec I, syt IV) or specific primers (KID-1, SIK). All probes were rat. Full-length cDNAs were used for c-fos, GAPDH, NGFI-b, sec I, and syt IV. For random priming, cDNA for each of the genes of interest was incubated with nucleotides, ^{32}P-labeled dCTP (50 (Ci), Klenow (8 U), and reagent mix (Oligolabelling Kit; Amersham, Arlington Heights, IL) for at least 1 h. The KID-1 probe is a 718-bp fragment consisting of nucleotides 935–1652 from the full-length cDNA. The SIK probe is a 422-bp fragment consisting of nucleotides 942–1363 from the full-length cDNA. KID-1 and SIK were isolated from the product of a representative difference analysis procedure and were bound on either end to the NBgl primer (Lisitsyn et al., 1993; Vician et al., 1997). Therefore, cDNA for each of these two genes was incubated with nucleotides, ^{32}P-labeled dCTP (50 μCi), Klenow (8 U, Amersham), Klenow buffer (Amersham), and NBgl (0.4 (M) for at least 1 h. Unincorporated nucleotides were removed by centrifugation through a Chromaspin 30 column (Clonetech, Palo Alto, CA). Radiolabeled probe was then mixed with hybridization buffer (same recipe as prehybridization buffer, above) and added to the blots, which were hybridized at 42°C overnight.

Following hybridization, blots were washed with 2 × SSPE/0.5% SDS at room temperature 3 × 5 min and at 65°C 3 × 15 min. Moist blots were then placed into heat-sealed plastic bags and exposed to Kodak (Rochester, NY) phosphorimager screens for varying exposure times depending upon the probe. Screens were analyzed using either a Molecular Devices (Sunnyvale, CA) or Bio-Rad (Hercules, CA) phosphorimager. After analysis, blots were incubated twice at 95°C for 2 × 15 min in stripping buffer (10 mM Tris pH 7.5, 1 mM EDTA, 0.5% SDS), then reprobed with the next gene of interest. Each blot was also incubated with probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to serve as a within-sample RNA loading control.

Quantitation and Statistics

Phosphorimager bands were optimized for visualization using ImageQuant software (for the Molecular Devices machine) or Quantity One software (for the Bio-Rad machine). Four blots analyzed on both phosphorimagers showed no significant differences between machines. No saturated pixels were detected on any blot. Each band of interest was quantified after subtracting background signal from the same lane. The identical background subtraction was performed for GAPDH. The signal for the gene of interest was normalized to the GAPDH signal from the same lane to determine the relative amount of RNA of interest for each sample. The injured values were then normalized to sham values from the same blot, with the average sham value set at 1.0. For each gene, one-way ANOVA was utilized to determine if there was a main effect of injury. Post-hoc comparisons (Bonferroni) of injury severity group versus sham controls were performed only if a statistically significant main effect was found. All data from genes of interest are presented as fold-induction over shams. Differences were considered significant at $p < 0.05$. All statistical analyses were performed using SPSS software version 9.0.

RESULTS

Pathophysiological Group Characteristics

Among subjects injured at P17, duration of unconsciousness averaged (mean ± standard error of the mean [SEM]) 20.3 ± 3.5 sec in the mild injury group, 66.8 ± 8.9 sec in the moderate group, and 473 ± 105.7 sec in the severe group (one-way ANOVA, $F[2,16] = 20.8$, $p < 0.001$; Table 1). Differences in apnea times between groups were also highly significant ($F[2,16] = 58.7, p < 0.001$), while fluid pulse pressure (atm) between groups approached statistical significance ($F[2,16] = 3.6$, $p = 0.06$). Four pups did not survive the injury. Among surviving rat pups, there were seven mild, five moderate, and five severe injuries. All pups were ambulating freely at the time of sacrifice.

In the adult groups, unconsciousness times were 22.2 ± 6.1 sec in the mild group, 66.2 ± 3.0 sec among the moderately injured, and 184.4 ± 15.0 sec in the severe group (one-way ANOVA, $F[2,14] = 77.6$, $p < 0.001$; Table 1). Duration of apnea group differences approached significance ($F[2,14] = 3.7$, $p = 0.06$), and fluid pulse pressure (atm) differed significantly between groups ($F[2,14 = 6.6, p < 0.05$). One adult died acutely after the injury, and one adult died during the 1-h recovery period between injury and harvest. Excluding these two, among the adults there were five animals in each injury severity group. Like pups, all adults were ambulatory at the time of sacrifice.

During the harvest, subarachnoid and occasional sub-

Table 2. Variance Among Shams[a]

Age group	*c-fos*	*KID-1*	*SIK*	*NGFI-b*	*Sec I*	*Syt IV*
Ipsi pup	±0.42	±0.002	±0.19	±0.29	±0.31	±0.02
Contra pup	±0.10	±0.11	±0.25	±0.11	±0.31	±0.09
Ipsi adult	±0.35	±0.11	±0.11	±0.14	±0.10	±0.04
Contra adult	±0.11	±0.04	±0.03	±0.13	±0.05	±0.03

[a]For each region/age group, the average signal for sham animals ($n = 5$) was normalized to a value of 1.0-fold. The variance (SEM) for each gene is presented in the table. All sham variances were less than ± 0.5-fold.

dural blood was noted, primarily on the side ipsilateral to injury. No intraparenchymal lesions were found, and the dura was intact in all animals. There were no complications in the tissue harvesting.

Quality of Northern Blots, Sham Variances, and GAPDH Controls

No problems occurred during RNA purification. For both age groups, the transfer of RNA to the blot appeared uniform, when viewed by UV light.

For each region (ipsilateral vs. contralateral), age (pup vs. adult), and gene of interest, the average of the signals from the five shams was set at 1.0-fold. From this, a normalized signal was calculated for each individual, and variance among shams determined by SEM of the normalized individual signals (for each gene, see Table 2). All of the sham groups had a variance (SEM) less than ±0.5-fold and most were greater than ±0.15-fold. In general, sham SEM in the pups was greater than adults, probably reflecting normal developmental variability.

Analysis of GAPDH signals was performed within each blot. Using one-way ANOVA, GAPDH expression showed no significant main effect of group (sham vs. FP) on any blot, substantiating the use of GAPDH as a normalization control.

FP Injury Induces c-fos Expression in Both Pups and Adults

Following FP in preweanling rats, c-fos expression was increased in ipsilateral and contralateral hippocampus (ipsilateral $F[3,19] = 12.8$, $p < 0.001$; contralateral $F[3,21] = 21.5$, $p < 0.001$; Fig. 1A). Compared with sham pups, mild injury induced c-fos 6.0-fold ipsilaterally (NS), and 1.5-fold contralaterally, while moderate injury led to a 6.2-fold increase ipsilaterally (post-hoc Bonferroni $p < 0.01$) and only 1.3-fold contralaterally. After severe FP, c-fos was elevated 25.9-fold within the ipsilateral hippocampus (post-hoc $p < 0.05$) and 3.7-fold within the contralateral hippocampus (post-hoc $p < 0.001$).

Like the pups, adults also showed significant injury increases in c-fos expression bilaterally (ipsilateral $F[3,19] = 14.4$, $p < 0.001$; contralateral $F[3,19] = 4.7$, $p < 0.05$; Fig. 1B). Mild injury induced a 9.7-fold increase over shams in ipsilateral hippocampus (post-hoc $p < 0.01$). In the same region, moderate and severe FP triggered 17- and 14-fold increases, respectively (post-hoc both $p < 0.001$). Contralaterally, c-fos mRNA levels were increased 6.7-, 7.2-, and 6.6-fold in the mild, moderate, and severe injury groups (post-hoc mild $p < 0.001$, moderate $p < 0.01$, severe $p < 0.05$), respectively.

Overall, both pups and adults demonstrated significant induction of c-fos expression. The magnitude of this increase was greater ipsilateral to injury than contralaterally, and, in general, the more severe the injury, the greater the induction of c-fos.

NGFI-b and syt IV Are Induced Following FP in Developing Animals

Developing rats showed specific injury severity-dependent patterns of depolarization-induced gene expression 1 h after FP. There was no significant induction of KID-1 or SIK in either hippocampus, although average signal for SIK was increased compared to sham bilaterally (1.3–2.5-fold) for all injury severities (ipsilateral, $F[3,20] = 2.1$, $p = 0.14$; contralateral, $F[3,21] = 1.1$, $p = 0.395$; Fig. 2A,B).

NGFI-b was significantly elevated bilaterally (ipsilateral, $F[3,20] = 13.8$, $p < 0.001$; contralateral, $F[3,21] = 5.1$, $p = 0.01$). After severe injury, NGFI-b demonstrated a 6.1-fold increase over sham ipsilaterally (post-hoc $p < 0.01$) and a 1.8-fold increase contralaterally (post-hoc $p < 0.01$). Increases in NGFI-b were also seen ipsilaterally after mild (2.4-fold, NS) and moderate (2.2-fold, $p < 0.05$) injury.

Among the genes coding for vesicle-associated proteins, sec I expression did not change significantly following FP injury (ipsilateral, $F[3,20] = 2.46$, $p = 0.1$; contralateral, $F[3,21] = 1.19$, $p = 0.34$). Syt IV, however, showed a modest but statistically significant effect of injury severity ($F[3,20] = 11.6$, $p < 0.001$) ip-

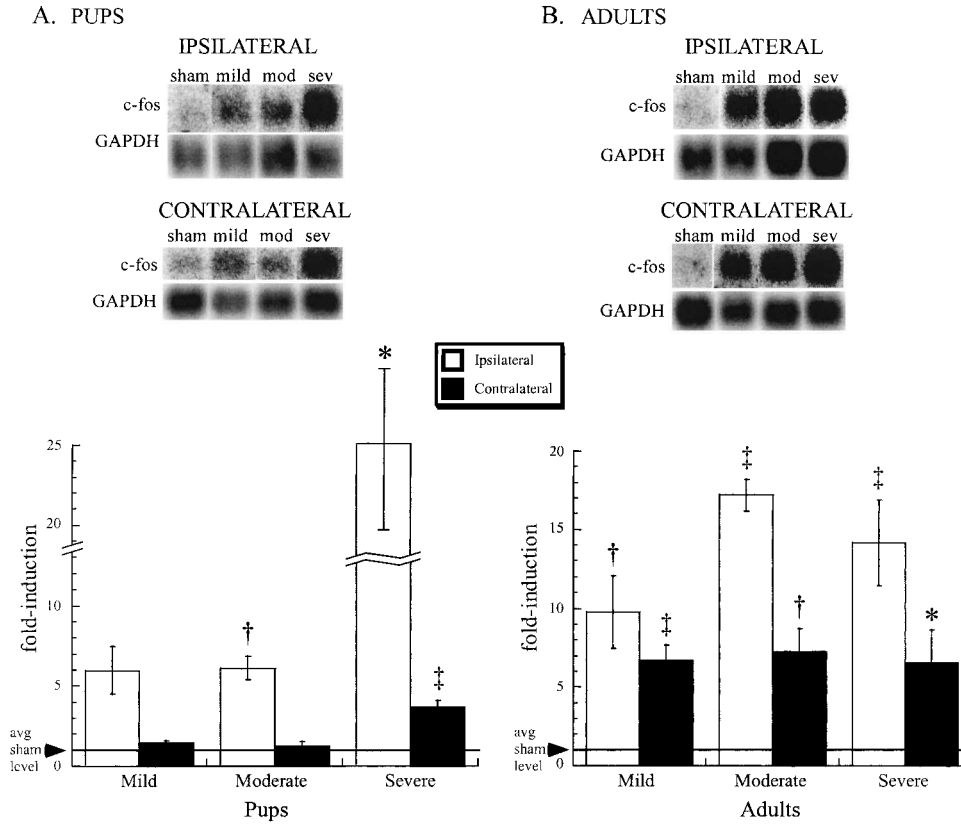

FIG. 1. Postinjury expression of c-fos. Both age groups show prominent c-fos upregulation, with pups showing generally less induction than adults, except at the most severe level of injury. Relative fold-induction of c-fos gene expression (average sham level = 1-fold) following increasing injury severity (values ± SEM) in rat pups (**A**) and adults (**B**). Difference from sham by one-way post-hoc Bonferroni, $*p < 0.05$, $^{\dagger}p < 0.01$, $^{\ddagger}p < 0.001$). Insets show representative bands from the respective Northern blots (each c-fos band is shown with its corresponding GAPDH band from the same lane of the same blot).

silaterally. Post hoc analysis revealed a 1.4-fold elevation in syt IV mRNA levels ipsilaterally following severe injury only ($p < 0.01$). No significant syt IV induction was seen contralaterally ($F[3,21] = 2.31$, $p = 0.11$).

KID-1, SIK, and NGFI-b Are Induced after FP in Adults

In general, increases in depolarization-inducible genes 1 h following FP in adults were more robust over sham than in pups (Fig. 3A,B). KID-1 expression was significantly increased over sham in ipsilateral hippocampus ($F[3,19] = 3.6$, $p < 0.05$) by a factor of 1.5 following mild injury (NS), 1.6 after moderate injury (post-hoc $p = 0.001$), and 1.7 after severe injury (post-hoc $p = 0.001$). The contralateral hippocampus also showed increased KID-1 mRNA ($F[3,19] = 4.1$, $p < 0.05$). Induction of KID-1 contralaterally was seen after moderate (1.7-fold, post-hoc $p < 0.001$) and severe injuries (1.5-fold, post-hoc, $p < 0.001$).

SIK levels increased after all injury severities ipsilaterally ($F[3,19] = 3.4$; $p < 0.05$). Compared to shams, SIK was elevated 3.7-fold after mild (post-hoc $p < 0.05$), 3.9-fold after moderate (post-hoc $p < 0.05$), and 2-fold (NS) following severe injury. Contralaterally, no definitive changes were found ($F[3,19] = 0.662$, $p = 0.59$).

NGFI-b showed the most dramatic induction of the depolarization-induced genes studied in adults, with increases in expression in all severity groups bilaterally. The major changes were found in ipsilateral hippocampus ($F[3,19] = 6.1$, $p < 0.01$), where mild FP triggered a 3.5-fold elevation (post-hoc $p < 0.05$), moderate a 4.9-fold increase (post-hoc $p < 0.01$), and severe FP a 5-fold induction (post-hoc $p < 0.01$). Contralateral levels went up 1.5-, 2.1-, and 2.8-fold, respectively, but these values failed to achieve statistical significance ($F[3,19] = 2.2$, $p = 0.13$).

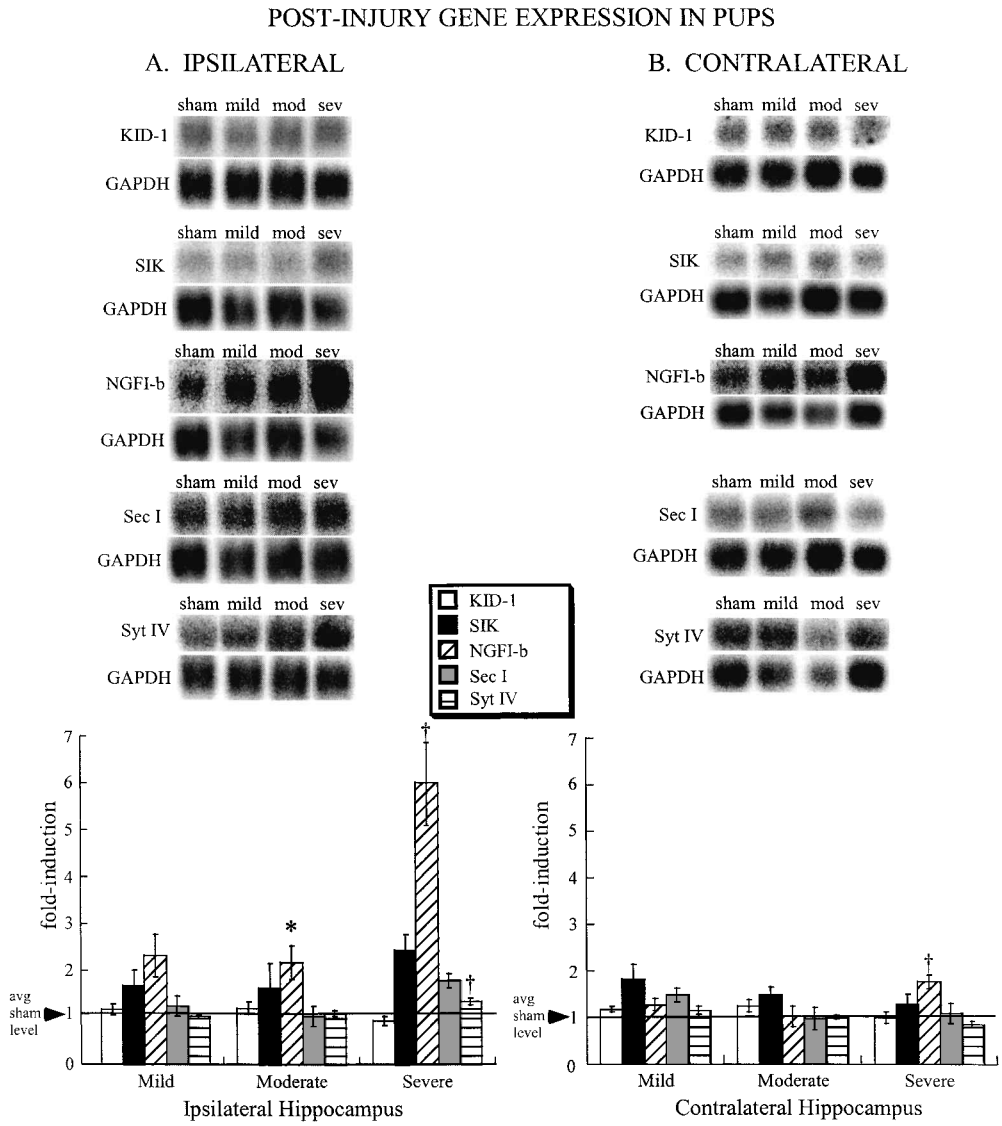

FIG. 2. Postinjury expression of depolarization-induced genes in pups following lateral fluid percussion injury. Pups show significant induction of NGFI-b and syt IV only at the more severe levels of injury. Relative fold-induction of gene expression (average sham level = 1-fold) with increasing injury severity (values ± SEM) in ipsilateral (**A**) and contralateral (**B**) hippocampus. (Difference from sham by one-way ANOVA, post-hoc Bonferroni, $*p < 0.05$, $^{\dagger}p < 0.01$). Insets show representative bands from the respective Northern blots (each gene of interest band is shown with its corresponding GAPDH band from the same lane of the same blot).

The vesicle-associated protein genes did not demonstrate major alterations after injury in the adults. Sec I expression showed a trend toward an increase ipsilaterally ($F[3,19] = 3.1$, $p = 0.06$) and no change contralaterally ($F[3,16] = 0.836$, $p = 0.5$). Syt IV demonstrated no significant alteration after FP in the adult subjects (ipsilateral, $F[3,19] = 1.8$, $p = 0.2$; contralateral, $F[3,19] = 1.1$, $p = 0.37$). Complete results for pups and adults are summarized in Table 3.

DISCUSSION

Northern analysis demonstrated significant severity- and age-dependent increases in immediate early gene expression following FP injury in rats. Expression of c-fos was induced bilaterally in both pups and adults, although the pattern of induction was different. In pups, increases in c-fos were seen after moderate injury, but by far the largest upregulation was seen after severe injury, bilat-

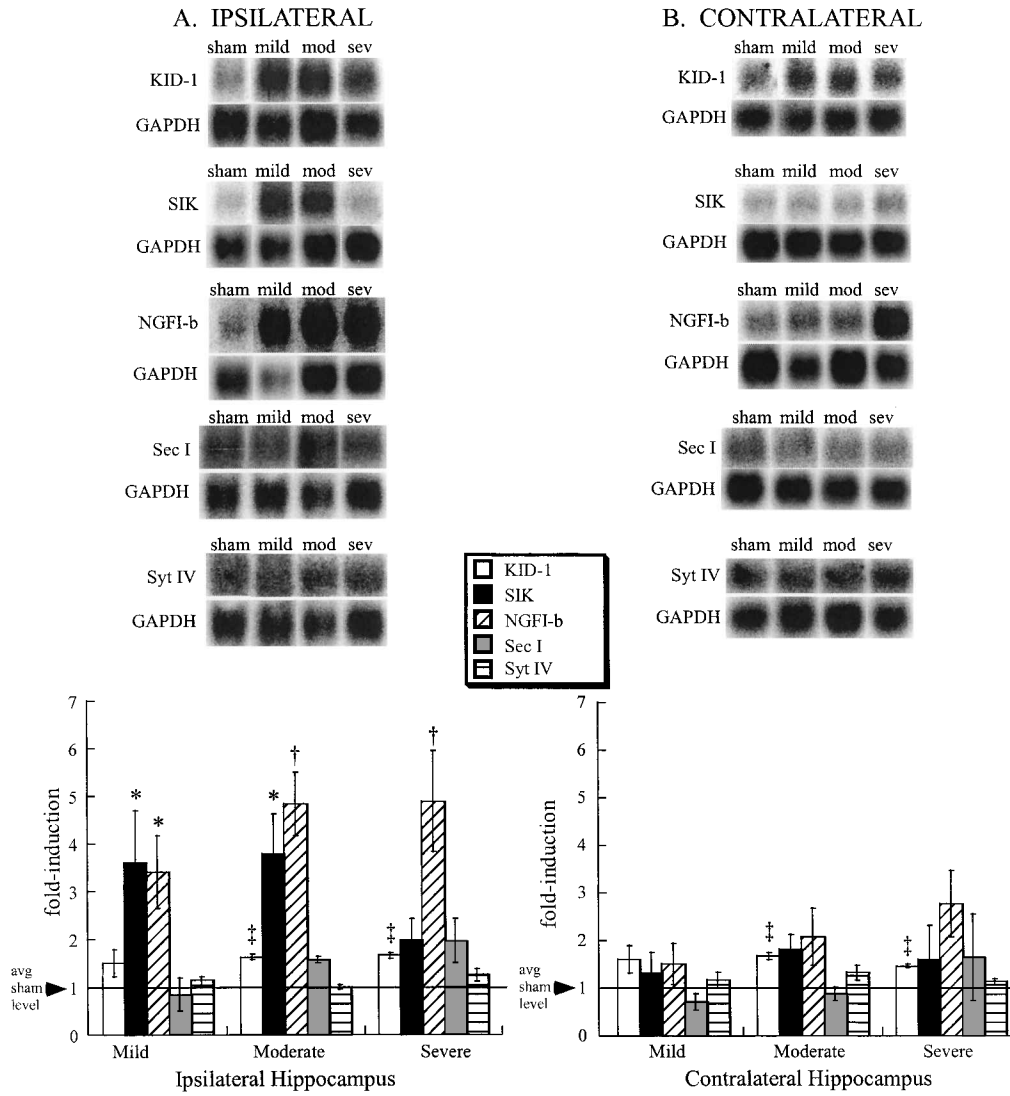

FIG. 3. Postinjury expression of depolarization-induced genes in adults following lateral fluid percussion injury. Note the broader pattern of induction, with increases of KID-1, SIK, and NGFI-b expression in all injury severity groups (compare with Fig. 2). Relative fold-induction of gene expression (average sham level = 1-fold) with increasing injury severity (values ± SEM) in ipsilateral (**A**) and contralateral (**B**) hippocampus. (Difference from sham by one-way ANOVA, post-hoc Bonferroni, $^{*}p < 0.05$, $^{\dagger}p < 0.01$, $^{\ddagger}p < 0.001$). Insets show representative bands from the respective Northern blots (each gene of interest band is shown with its corresponding GAPDH band from the same lane of the same blot).

erally. Adults showed more uniform c-fos induction over all severities, with ipsilateral increases consistently greater than contralateral.

Developmental Distinctions

Age proved to be an important variable in the expression of depolarization-induced genes 1 hour following FP injury. Overall, increases in depolarization-induced genes in pups following FP were prominent only for NGFI-b (and c-fos). Syt IV increases also achieved statistical significance, but only after severe injury and only in ipsilateral hippocampus. This suggests that, at least for the group of genes studied, FP injury does not trigger a broad, early molecular response in the developing brain.

While increases in NGFI-b mRNA are triggered by

Table 3. Fold Increase in mRNA Compared to Shams

	Mild		*Moderate*		*Severe*	
Gene	*Pup*	*Adult*	*Pup*	*Adult*	*Pup*	*Adult*
c-fos	6/1.5	9.7†/6.7‡	6.2†/1.3	17‡/7.2†	26*/3.7‡	14‡/6.6*
KID-1	1.2/1.2	1.5/1.6	1.2/1.2	1.6‡/1.7‡	1.0/1.0	1.7‡/1.5‡
SIK	1.7/1.8	3.7*/1.3	1.7/1.5	3.9*/1.8	2.5/1.3	2.0/1.6
NGFI-b	2.4/1.3	3.5*/1.5	2.2*/1.0	4.9†/2.1	6.1†/1.8†	5.0†/2.8
Sec I	1.3/1.5	0.8/0.7	1.1/1.0	1.6/0.9	1.8/1.0	2.0/1.7
Syt IV	1.0/1.1	1.1/1.2	1.1/1.0	1.0/1.3	1.4†/0.8	1.3/1.1

Ipsilateral/contralateral: *$p < 0.05$; †$p < 0.01$; ‡$p < 0.001$.

both neurotrophins and synaptic activation, one study reports that only depolarization-induced NGFI-b is capable of DNA binding and transcriptional regulation (Katagiri et al., 1997). This suggests that depolarization-induced NGFI-b will trigger molecular pathways distinct from neurotrophin-induced NGFI-b. At this time, however, the actual genetic targets for NGFI-b are not well characterized.

Syt IV is the second gene whose expression was induced by FP injury in pups, and the only gene we studied whose transcription was increased more in the pups than in the adults. This may represent an age-dependent difference in the molecular response to brain trauma. However, the relatively small increase seen in syt IV suggests this result be interpreted cautiously.

Altered expression of genes such as NGFI-b and syt IV is a viable mechanism for changes in experience-dependent plasticity seen following FP in the immature brain (Fineman et al., 2000; Shieh, 2002). Increases in these genes have the potential to affect subsequent development, as NGFI-b can act as a transcription factor (Katagiri et al., 1997; Wilson et al., 1991) and syt IV overexpression may lead to diminished synaptic activity (Littleton et al., 1999).

A broader early pattern of post-FP gene expression was seen in adults as compared with the developing animals. Overall, adults upregulated KID-1, SIK, and NGFI-b more readily and usually to a higher level above shams than in pups. After severe injury, there were also increases in sec I, but these did not achieve statistical significance. Changes in syt IV expression were not statistically significant in adults 1 h postinjury. Following FP injury, many metabolic parameters also demonstrate greater severity in adults than in pups. Hyperglycolysis is more severe and longer-lasting in adults (Thomas et al., 2000; Yoshino et al., 1991), as is calcium accumulation (Osteen et al., 2001). Histologic evidence of injury and cell death is also more prominent (Osteen et al., 2001). Preweanling (P17) rats tend to have more sensitive excitatory receptors than adults (Insel et al., 1990; Jin et al., 1997; Miller et al., 1990). Thus, these differences in post-FP pathophysiology are not likely due simply to increased synaptic release of excitatory amino acids. They are more plausibly related to a fundamentally distinct response to injury in young animals. This response is most certainly mediated on some level by differences in gene expression. Our demonstration of specific patterns of immediate early gene induction in pups versus adults represents a reasonable place to begin dissecting the molecular signals leading to posttraumatic age-specific derangements in cellular function.

Effects of Injury Severity

The effects of FP injury appear to have both severity-dependent and severity-independent components. Studies of the pathophysiology of FP injury suggest an all-or-none threshold-like response with regards to the magnitude of K^+ efflux (Katayama et al., 1990). Duration and amplitude of spreading depression also appear to be independent of FP severity, although the frequency of these negative DC shifts does increase with increasing injury (Kubota et al., 1989). Previous studies have demonstrated correlations between FP pulse pressure, duration of unconsciousness and subsequent motor and cognitive deficits (Dixon et al., 1987; McIntosh et al., 1989; Sanders et al., 1999). Nonetheless, some outcomes appear independent of injury severity. Sanders et al. (2000) report similar degrees of impairment in maintaining long-term potentiation 8 weeks after all severities of FP injury when compared with sham animals.

KID-1 was elevated both ipsilaterally and contralaterally after moderate and severe injury in adults. Its level of induction was also relatively constant (1.5–1.7-fold), suggesting that only the presence and not the degree of injury triggers its upregulation. A similar pattern of severity-independent upregulation was seen for SIK, which showed increased mRNA ipsilaterally after all injuries.

This effect, however, was also seen only in adults. Such a response would indicate that, at least in adult rats after FP injury, a threshold is breached, leading to an all-or-none response in KID-1 and SIK transcription. Extracellular K^+ concentrations are also known to rise in a threshold-dependent manner following brain trauma (Katayama et al., 1990), presumably due to nonspecific depolarization overcoming physiological potassium reuptake by glia (Ballanyi et al., 1987; Kuffler, 1967) and/or triggering waves of spreading depression (Kubota et al., 1989; Sugaya et al., 1975). This would suggest either that pups are somehow more resistant to K^+ efflux or that the mechanism for induction of these two kinases is still immature at the time of weaning, in any case exposing a potentially important distinction in the molecular response to developmental brain trauma.

NGFI-b expression correlates with severity in both age groups. In pups, there were small increases ipsilaterally after mild-moderate injury. After severe injury, NGFI-b mRNA was elevated significantly in both ipsilateral and contralateral hippocampus. In adults, the magnitude and statistical significance of NGFI-b induction increased with increasing severity, plateauing between moderate and severe FP ipsilaterally.

Both genes coding for vesicle-associated proteins showed small increases only after severe injury. Sec I tended to be upregulated after severe injury ipsilaterally in pups and bilaterally in adults, but levels did not reach statistical significance. Syt IV showed an increase ipsilaterally in pups after severe injury. This elevation of syt IV, albeit small, provides further evidence that developmental injury is not just a milder form of injury, but a unique entity with its own specific pathophysiology. These differences may also be somewhat limited by sample size.

Experimental Concerns and Limitations

Fluid percussion injury appears to be qualitatively distinct at different ages. For example, immature rats demonstrate longer apnea, lower blood pressure, less sustained increases in intracranial pressure and shorter unconsciousness times than adults subjected to the same FP pulse pressure (Prins et al., 1996). One critical problem is the 100% mortality among pups following "severe" injury as defined by pulse pressure (Prins et al., 1996). Investigations in adults have demonstrated severity-dependent correlations between pulse pressure, unconsciousness (loss of hind paw withdrawal reflex; Dixon et al., 1987) and neurological outcome (McIntosh et al., 1989). In this study, injury severity in each age group was defined by duration of unconsciousness. Thus, both young and adult rats could be subjected to graded injuries.

Molecular markers may also be used to characterize injury severity. Postinjury changes in c-fos expression have been described by many authors (Hayes et al., 1995; Phillips et al., 1992; Raghupathi et al., 1996), but this work represents the first description of its induction in developing animals following traumatic brain injury. While both ages demonstrate severity-dependent increases in c-fos expression, the pattern of induction appears different between the two age groups. The magnitude of c-fos mRNA elevation is generally less in pups than in adults, for all injury regions/groups except ipsilateral severe (Fig. 1). This would suggest that the pups have less profound alterations in immediate-early gene expression after mild to moderate injury and may represent an inherent difference in the responsiveness of the developing brain to traumatic injury.

An alternative explanation for the dramatic effects seen with both c-fos and NGFI-b following severe injury in pups is that a secondary hypoxic injury is being superimposed upon the initial biomechanical injury. Longer apnea times are seen postinjury in pups compared to adults (Prins et al., 1996; Table 1), so this apneic response can be considered part of the post-FP pathophysiology in the immature rat. Interestingly, within the severely injured group, there was no direct correlation between duration of apnea and level of c-fos expression (data not shown), suggesting that the induction seen is not solely a manifestation of apnea-induced hypoxia. Nonetheless, the likelihood that prolonged apnea contributes significantly to the gene induction seen in severely injured pups must be considered when interpreting these results.

Although immediate-early genes are classically expressed with a rapid time course, they often take hours to reach peak expression in hippocampus after depolarizing stimuli (Vician et al., 1995 [sytIV]; Feldman et al., 1998 [KID-1]; Feldman et al., 2000 [SIK]). Therefore, a limitation of the 1-h time point used in this study is potentially missing the peak post-FP expression of these genes. Furthermore, while increases in mRNA levels are usually interpreted as increases in gene expression, it is also possible for mRNA levels to change if the stability of a particular mRNA is altered without a concomitant change in transcription. And, of course, changes in gene expression do not always reflect protein changes, particularly functional alterations in proteins that may occur following injury.

A final factor to consider is the possibility of significant hippocampal cell death. By harvesting only 1 h after injury, the effects of significant delayed cell death were minimized. In addition, the FP injury used in this study characteristically produces little, if any, histological damage, particularly in rat pups (Osteen et al., 2000; Prins et al., 1996; Thomas et al., 2000).

Patterns of Posttraumatic Gene Expression

From this study, three separate patterns of post-FP gene expression can be described. First are genes induced only in adult animals after injury, such as KID-1 and SIK. Both are kinases, and both are upregulated only in the adult animals. Kinases may have widespread effects on cellular processes, including mediating the function of other proteins (Blanquet, 2000; Girault et al., 1990; Imahori et al., 1997; Ouyang et al., 1999; Schreibmayer, 1999) and modulating receptor activity (Konietzko et al., 1999; Levine et al., 1995; Lin et al., 1998; Popoli et al., 2000). Early necrosis and later programmed cell death occur consistently in adults, while pups rarely show significant anatomic damage. Perhaps the substrates of these kinases include proteins active in pathways that lead to delayed apoptosis.

Mitochondrial dysfunction and impaired energy production are clearly associated with delayed cell death (Green et al., 1998; Lee et al., 1999). SIK is a member of the SNF1/AMPK family of kinases that are implicated as sensors of metabolic state (Hardie et al., 1998). Expression of SIK may thus be important in determining the metabolic health or energy state of the cell.

A second pattern of post-FP gene expression is an injury severity-dependent induction in both adults and pups. Genes like NGFI-b exhibited this pattern and are candidates to be involved with age-independent neuroplastic mechanisms. NGFI-b is capable of transcriptional activation of other genes following induction by a depolarizing stimulus (Katagiri et al., 1997). However, the specific targets of NGFI-b are not well characterized at this time. It is interesting to note that c-fos, an archetypal transcription factor, has a similar pattern of gene expression following FP injury and is implicated in many diverse molecular pathways, including those modulating neuroplasticity (Herdegen et al., 1998; Kaminska et al., 1997).

The third pattern of transcriptional regulation was that seen only in the P17 animals, in this case, the upregulation of syt IV. Granted, the increase in syt IV expression is small but there are reasons to suspect that syt IV does mediate developmental differences in plasticity. In rats, syt IV demonstrates a developmental pattern of expression that is highest immediately after birth and gradually declines into adulthood (Berton et al., 1997). Increases in syt IV have previously been shown to impair synaptic transmission (Littleton et al., 1999), suggesting a role for syt IV in the postinjury derangement of development-specific neuroplasticity recently described by Fineman, et al. (2000). In particular, injured immature rats reared in an enriched environment fail to develop the expected cognitive enhancement in spatial learning as tested by Morris water maze acquisition (Fineman et al., 2000).

In conclusion, by studying a select group of immediate early genes preferentially induced by depolarization we can gain insight into the early molecular responses to concussive brain injury. By investigating transcriptional changes following different injury severities as well as in animals of different ages, we identified three distinct patterns of early post-FP gene expression in hippocampus. One set of genes, both kinases, was induced primarily in adults and may signal early molecular changes that lead to cell death. A second pattern was seen in the transcriptional regulators, NGFI-b and c-fos, that are induced in both pups and adults and may play a role in plasticity mechanisms operant during recovery from injury. Although induction occurs in both ages, the effect of injury severity in this pattern appears age-specific, with adults showing increases after any injury and pups demonstrating the most marked upregulation only after severe injury. The final pattern described is that of syt IV, which is increased only in developing hippocampus and may be involved in the loss of age-specific neuroplasticity. It is clear that unique transcriptional changes occur following concussive brain injury and that these patterns are dependent both on severity and age-at-injury. Further studies directed at the time course of transcriptional changes, protein expression, and regional localization will permit more precise correlation of altered gene expression with postinjury pathophysiology and neurological outcome. Targeted studies of injury-induced gene expression will play an important role in untangling the interwoven web of molecular changes and cellular dysfunction following traumatic brain injury.

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