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GENE EXPRESSION FOLLOWING TRAUMATIC BRAIN INJURY

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A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science at
Virginia Commonwealth University

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Acknowledgment

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List of Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole-4-propionic acid
BBB	blood-brain barrier
BDNF	brain derived neurotrophic factor
CaBP	calcium binding protein
CAMK	Ca^{2+}-calmodulin kinase
CNS	central nervous system
CREB	CAMP response element protein
DAG	diacylglycerol
DAI	diffuse axonal injury
DNA	deoxyribonucleic acid
EAA	excitatory amino acid
GCS	Glasgow Coma Scale
GFAP	glial fibrillary acidic protein
GRP	glucose related protein
HSP	heat shock protein
ICP	intracranial pressure
IEG	immediate early gene
IL	interluekin
IP3	inositol 1,4,5-trisphosphate
mRNA	messenger ribonucleic acid
NGF	nerve growth factor
NMDA	N-methyl D-aspartate
PCCK	procholecystokinin
PKC	protein kinase C
PLA_2	phospholipase-A_2
TBI	traumatic brain injury
TNF	tumor necrosis factor

ABSTRACT

GENE EXPRESSION FOLLOWING TRAUMATIC BRAIN INJURY

By. Rajiv Malhotra, B.S.

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science at
Virginia Commonwealth University.

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The pathology which results from traumatic brain
injury (TBI) have long been believed to be immediate and
irreversible. However, recently it has been shown that,
although the primary effects are virtually unavoidable, the
secondary effects manifest themselves through biochemical
processes set in motion at the time of the injury. These
events are frequently mediated through the process of
excitotoxicity, which results from a widespread release of
excitatory neurotransmitters. These neurotransmitters go
on to activate both ionotropic and metabotropic receptors.
The signal transduction initiated through these receptor
populations gives rise to changes in gene expression.

One result of this release of neurotransmitter is an influx of calcium by means of excitatory receptors on the cell. The neurotransmitters upon which most research is focused are glutamate, aspartate, and acetylcholine. Current research is aimed at investigating antagonists to this process as well as elucidating steps within the process. Antagonists primarily function to reduce the calcium toxicity through modulation of receptor activity. However, the therapeutic window for effective antagonist usage is short. Therefore, although they may represent a viable treatment option, they need to be administered as early as possible following the injury to have the greatest effect.

The purpose of this paper is to provide a summary of the available literature on TBI and excitotoxicity with a focus on changes in gene regulation. This paper will summarize information on the steps involved in the intracellular signaling cascade following brain injury and provide insight to further sites for regulation and treatment. This will also allow for development hypotheses on the possible roles of some of the genes whose expression is already known to be altered.

I. INTRODUCTION

Traumatic injuries are a very common yet preventable problem. The best prevention for TBI lies in taking proper precautions such as wearing a seatbelt, a helmet or having an air bag equipped vehicle. Trauma is the fourth leading cause of death for all ages and is the leading cause of death for children and young adults in the United States (Bledsoe et al., 1994). Trauma is also the most costly public health problem with an estimated $180 billion dollars expended in 1988. This total represents a cost per death that is more than twice that of cardiovascular disease and cancer combined. TBI alone is responsible for over half a million hospital admissions every year (Rockswold and Pheley, 1993).

A traumatic insult to the central nervous system has both primary and secondary effects. The primary effects include loss of consciousness, loss of balance, confusion, apnea, concussion and short-term loss of memory (Young, 1985; Evans, 1992). The secondary effects refer to any changes that are of longer duration and can be attributed to molecular and cellular changes, as opposed to symptoms seen

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as a direct result of the physical insult. The secondary

effects include cellular and vascular changes such as

diffuse axonal injury and blood brain barrier breakdown,

long-term memory loss, learning disability, post-traumatic

epilepsy, delayed amnesia, psychosis, dementia and neuronal

death (Young, 1985; Evans, 1992).

These secondary effects of TBI are known not to be a

direct result of the insult itself. At the time of injury,

physiological cascades are
set in motion which play a
role in the development of
the secondary effects of
TBI (Olney, 1974). A flow
diagram representing this
is shown in Figure 1.
Once a head injury has
occurred, there is little
that can be done for the
primary effects of TBI due
to the immediate and
unsuspected nature of
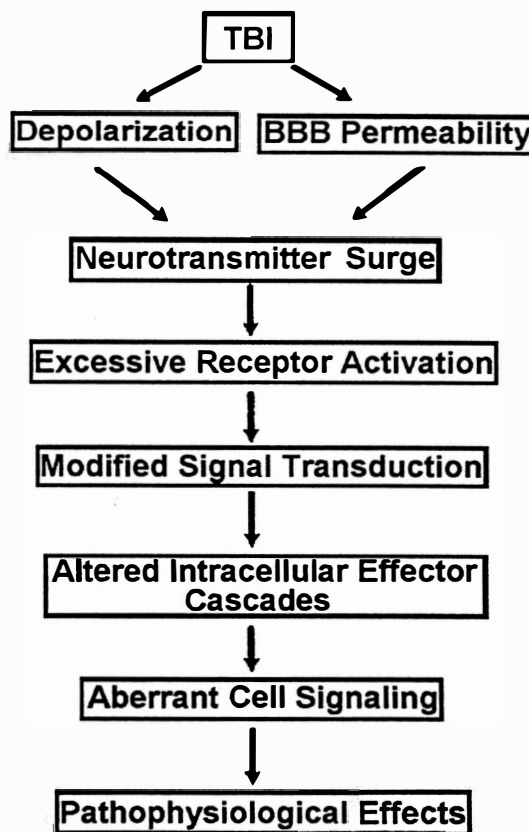
Figure 1. **TBI Physiology**

injury. Because the secondary effects of TBI can take days to evolve and manifest themselves, they represent a more viable option for treatment.

The majority of current research on TBI focuses on excitotoxicity. One common occurrence seen with TBI is the presence of elevated levels of excitatory neurotransmitters, particularly glutamate and aspartate (Hayes et al., 1992). When these neurotransmitters bind to excitatory receptors they cause an influx of calcium ions into the cytosol which sets in motion a variety of intracellular responses, including activation of intracellular protein kinases, calcium toxicity, altered signal transduction, and alterations in gene expression (Farooqui and Horrocks, 1994).

Excitotoxicity and the role it plays in gene regulation have been studied as a source of altered neuronal function secondary to TBI (Bazan et al., 1995). It would seem that one source of physical change in cells might involve a change in gene regulation and/or expression. This connection between TBI and the secondary effects has prompted studies examining the expression of genes encoding

proteins such as c-fos, nerve growth factor (NGF), and stress proteins (Hayes et al., 1995; Phillips and Belardo, 1992; Bazan et al., 1995; Belluardo et al., 1995; Hayes et al., 1995).

A greater understanding of the molecular pathophysiology of neurotrauma will result in clues that will identify therapeutic approaches. Discovering what genes are activated and examining their roles in the injury process is imperative in defining treatments for the secondary effects of neurotrauma. Certain chemical mediators may accumulate after gene activation following neurotrauma and drive pathways that point to either neural damage and cell death or repair and regeneration. Determining the roles of activated genes and finding which genes support repair and regeneration and which cause cell damage and death is the first step toward identifying treatments which are based on inhibiting and promoting gene expression.

This paper will serve as a summary of the current research on excitotoxicity and TBI, and will concentrate on changes in gene expression. It will begin with a background

on TBI and its effects on the CNS and the patient. This discussion will lead into a summary of neuroexcitation with emphasis on links to TBI, the neurotransmitter systems involved and the pathophysiology of neuroexcitation. Next, specific studies will be cited linking the alteration in gene regulation following TBI to neuroexcitation. These will demonstrate how a cascade of gene transcription could begin following TBI, as well as generate hypotheses on possible roles of these activated genes in the injury process. The conclusion will consist of a discussion of possible therapeutic interventions, including the use of antagonists and other treatment options.

II. TRAUMATIC BRAIN INJURY

Blunt closed-head injuries are a leading cause of death and disability in trauma patients. These injuries can present in any car, motorcycle, or pedestrian trauma. Although most closed-head injuries do not require operative intervention, aggressive diagnostic management and prompt treatment are necessary to insure good outcomes. These treatments are aimed primarily at reducing intracranial pressure (ICP) in order to preserve vital brain function.

Signs of head injury include loss of consciousness, disorientation, coma, unequal pupils, and hemiparesis (Bledsoe et al., 1994). The Glasgow Coma Scale (GCS) is depicted in Table 1 (National Association of Emergency Medical Technicians, 1994). It is a useful tool for evaluating patients with head injuries, and for determining the severity of the injury. Generally a GCS score of eight or less is indicative of severe head trauma.

Table 1.

Glasgow Coma Scale

Eye Opening		Best Verbal Response		Best Motor Response	
Natural	4	Oriented	5	Obeys Commands	6
To Command	3	Confused	4	Localizes Pain	5
To Pain	2	Inappropriate	3	Withdraws	4
No Response	1	Incomprehensible	2	Flexion	3
		No Response	1	Extension	2
				No Response	1

There are three major types of neurological injuries associated with TBI. The first type of neurological injury is the contusion. Contusions are focal changes in the brain that are most similar to bruising. These patients usually do not require operative treatment, but may require ICP monitors and intensive care. Contusions usually cause edema which is directly related to the size of the contusion, and is the reason for the ICP monitoring (as reviewed in Bledsoe et al., 1994).

The second type of neurological injury is shear injury (Bledsoe et al., 1994). These injuries represent an actual disruption of tissue and include diffuse axonal injury (DAI). They are often the result of severe injury and have a worse prognosis than contusions. Shear injuries are associated with widespread dysfunction, and may result in an immediate prolonged coma. ICPs are elevated along with

blood pressure and temperature. These injuries often require ICP monitoring and care, to detect changes in level of consciousness, which indicate further intracranial swelling. Surgery is often the route taken to relieve the ICP.

The third type of neurological injury is the hematoma which can be further divided into epidural and subdural. These are usually caused by direct blows to the skull (Bledsoe et al., 1994). Because subdural hematomas and diffuse axonal injury are attributed to the same forces, they are frequently seen together. These often produce focal neurological signs and coma. The only chance these patients have for survival is surgical intervention to relieve the increasing ICP. If left unchecked, the brain eventually begins to herniate through the foramen magnum resulting in pressure upon the brain stem. This causes symptoms often seen in patients with head injuries, including hypertension, bradycardia, and an irregular respiration rate.

The above injuries arise from forces exerted on the brain. The two forces seen are either contact forces or acceleration forces. Contact forces are the result of

direct contact with the skull. It is rare to see a contact injury without acceleration injuries due to movement of the head following the contact, but it can be simulated in a lab with immobilization of the head prior to contact as shown in Figure 2. Contact forces result in focal injuries such as coup contusions, skull fractures, and epidural hematomas (Gennarelli and Thibault, 1985).
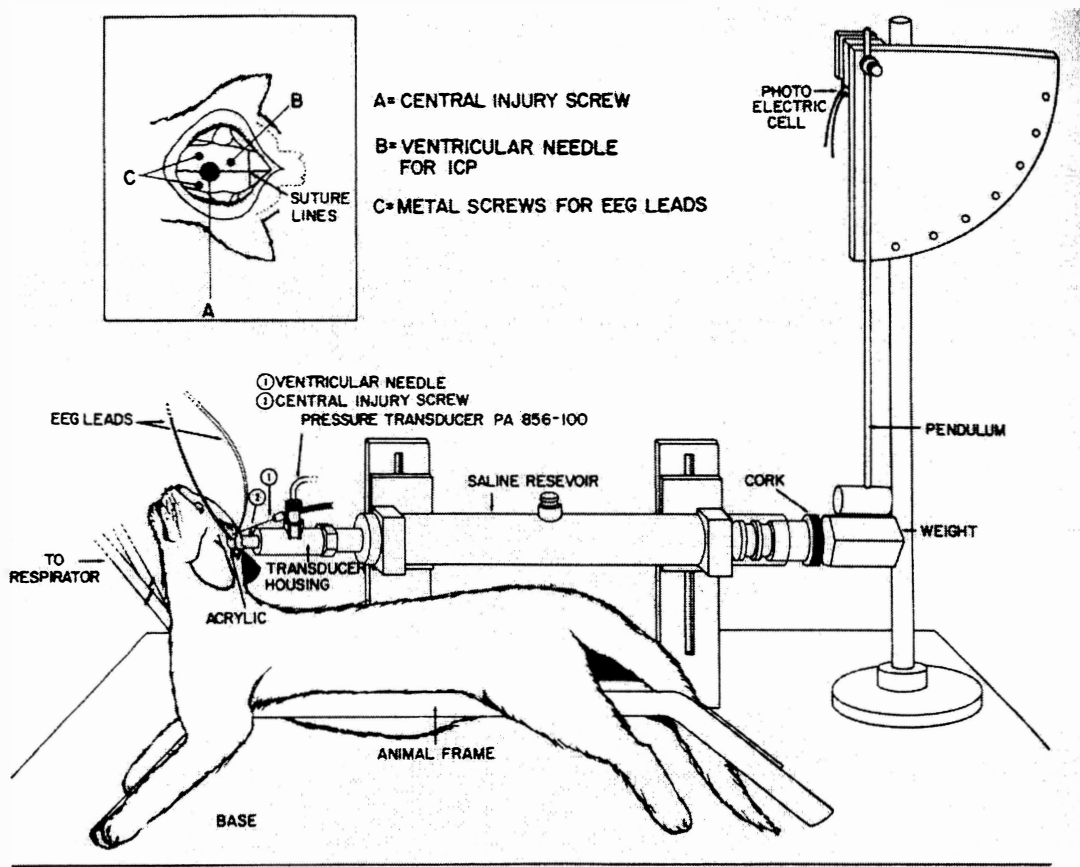
Figure 2. **Model of Fluid Percussion Injury**

Acceleration forces result from inertial effects caused by impact. Acceleration forces cause injuries within the brain in two ways. First, intracranial collisions between the brain and the skull result from differential acceleration of the two. This also results in subdural hematomas because of strain imparted between the dura and the brain. The dura moves with the skull because it is attached to the periosteum of the inside of the skull. Second, the acceleration changes cause diffuse brain injuries such as DAI and concussion syndromes (Gennarelli and Thibault, 1985).

Povlishock and Kontos (1985) studied axonal and vascular changes following brain trauma in cats using fluid-percussion injury. To determine axonal integrity, anterograde peroxidase transport was tested with HRP, and vascular changes were observed using implanted cranial windows. With regard to axonal changes, the researchers witnessed axonal swelling and separation following fluid-percussion injury. This axonal injury pattern demonstrates what is referred to as DAI.

The above described injuries are responsible for the primary effects of a traumatic insult to the CNS. The secondary effects may be attributed to cellular events set in motion at the time of injury and can take days to manifest themselves. Most current research in TBI is centered on abnormal agonist-receptor interactions that are believed to play a role in the secondary effects (Hayes et al., 1992).

As mentioned earlier, due to the immediate nature of the primary effects, treatments options are limited. However, if the cellular pathways that mediate the secondary effects are determined, they represent a viable means of modulating these effects. Given that the major pathway through which injury progresses from the initial insult and primary effects to these secondary injuries is thought to be through excessive neuroexcitation, the next section of the paper will first address the pathophysiology of neuroexcitation in relationship to TBI.

III. NEUROEXCITATION

The cellular cascades responsible for the secondary effects of TBI are believed to be activated in large part through excitotoxicity. This has been observed following mild to moderate brain injury and involves the over-stimulation of neurons via their excitatory amino acid (EAA) receptors and the excitatory subtype of muscarinic acetylcholine receptors (Olney, 1994). Excitatory receptors are broadly divided into two categories, ionotropic and metabotropic. Ionotropic receptors are coupled to ion channels and thus permit an instant signal transduction, while metabotropic receptors exert their actions through G-protein and thus have a slower transduction rate (Burt, 1993). Metabotropic receptors frequently function to modulate neuronal excitability.

Each of the receptors has a specific distribution pattern within the brain. The highest density of NMDA receptors are found within the hippocampus, striatum and thalamus (Farooqui and Horrocks, 1994). Kainate receptors are also concentrated within the hippocampus, and AMPA receptors follow the same pattern of density as NMDA

receptors (Farooqui and Horrocks, 1994). Because of the high density of these receptors seen within the hippocampus, an amplification of effects is often observed in this region. For this reason, most current research is concentrated on changes in the hippocampus and other areas of the brain where a high density of these receptors are found.

Ionotropic receptors are further divided based upon specificity for certain synthetic agonists (i.e., NMDA, AMPA and kainate). Excitatory receptors bind and are stimulated by excitatory neurotransmitters such as glutamate and aspartate (Lodish et al., 1995). The dicarboxylic amino acids glutamate and aspartate are normally present in the central nervous system and act as neurotransmitters for the NMDA class of receptors (Burt, 1993). These receptors are known to be permeable primarily to Ca^{2+}, and all of them contain phosphorylation sites for Ca^{2+}-calmodulin kinase (CAMK) and protein kinase C (PKC) (Farooqui and Horrocks, 1994). Of the three subtypes of ionotropic receptors, extensive information is available on the NMDA receptor and its subtypes.

Metabotropic receptors bind and are stimulated by presence of acetylcholine and function through a G-protein to cause polyphosphoinositide hydrolysis (Lodish et al., 1995). This process leads to intracellular increases in diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), which are the products of hydrolysis. Both of these molecules function to increase intracellular Ca^{2+} concentration through different pathways (Lodish et al., 1995). Thus, both metabotropic and ionotropic receptors have a common pathway of activation involving increased intracellular Ca^{2+}. This can prove to be an important concept if it is shown that loss of Ca^{2+} homeostasis is a key in neurotoxicity. This also represents an important site for therapeutic intervention which will be discussed in section five.

The signaling pathways involved in excitotoxicity by either NMDA or muscarinic receptors both result in increased intracellular Ca^{2+} (Burt, 1993). The NMDA receptors accomplish this by becoming permeable to Ca^{2+} and Na^+ once they bind an excitatory neurotransmitter and the neuron becomes sufficiently depolarized, as shown in Figure 3 (Olney, 1994). The resulting increased intracellular Ca^{2+}

serves to activate the membrane localized enzyme
phospholipase A_2, which produces such intracellular
secondary messengers as DAG, IP3, and arachidonic acid
(Burt, 1993). These second messengers go on to further
mobilize intracellular Ca^{2+} as well as to activate other
enzymes listed in Figure 4 (Farooqui and Horrocks, 1994).
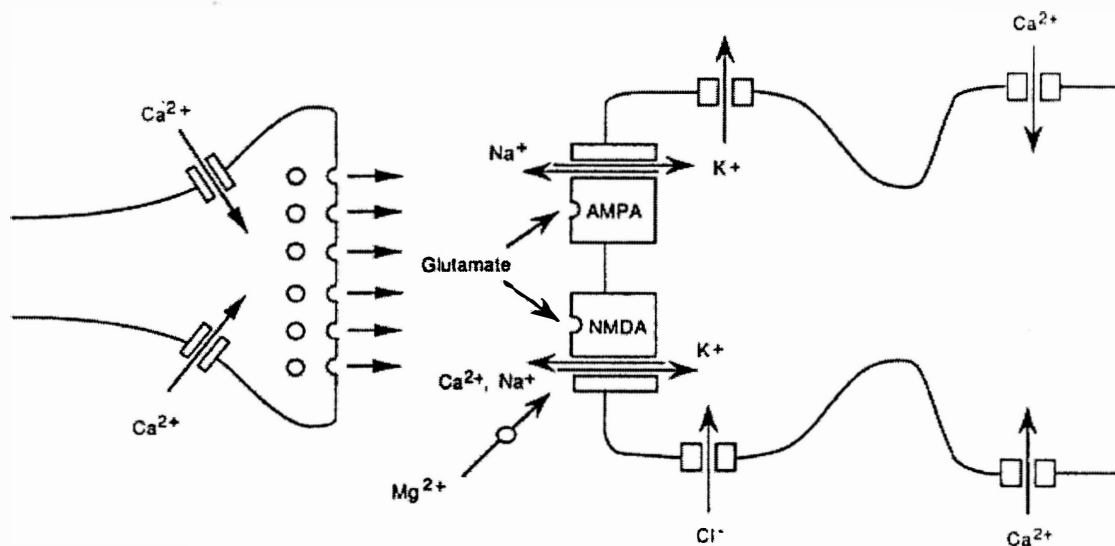
Figure 3. **Model of Neuroexcitation**

Endonuclease	Phospholipase C	Diacylglycerol lipase
Plasmalogenase	Phospholipase A_2	Calmodulin kinase
Calpain	Guanylate cyclase	Nitric oxide synthase
Calcineurin	Protein Kinase C	

Figure 4. **Enzymes Activated by a Rise in Intracellular Ca^{2+}**

The muscarinic acetylcholine receptors have a variety of subtypes based on the different responses they induce through G-proteins. There are inhibitory subtypes which inhibit depolarization or adenylate cyclase and there are stimulatory subtypes such as those thought to participate in excitotoxicity (Lodish et al., 1995). These receptors are coupled to G_p-proteins which activate phospholipase C and subsequently lead to mobilization and release of intracellular Ca^{2+} stores through the same pathways as metabotropic receptors (Burt, 1993).

The increase in intracellular Ca^{2+} can serve to activate the Ca^{2+}/calmodulin pathway, which involves activation of protein kinases and subsequent activation of many secondary responses. It is believed that the signals communicated by these receptors can regulate neuronal survival, synaptogenesis, neuronal plasticity, gene regulation, learning and memory.

The opening of voltage-gated and receptor-gated Ca^{2+} channels can produce a transient rise in intracellular Ca^{2+}; however, these channels are rapidly inactivated through either sustained depolarization or desensitization (Mody and MacDonald, 1995). Next, researchers looked for another

method of increased intracellular Ca^{2+} levels following

stimulation which led to the postulation of release Ca^{2+}

from intracellular Ca^{2+} stores. The endoplasmic reticulum

within CNS neurons possess receptors for both IP_3 and

ryanodine, which provides a link between voltage or

receptor-gated channels and intracellular Ca^{2+} release (Mody

and MacDonald, 1995).

This hypothesis was further substantiated using

antagonists to intracellular Ca^{2+} release following TBI as a

route of ameliorating excitotoxic damage. The same authors

chose to use the muscle relaxant dantrolene, which is a

known ryanodine receptor antagonist. The results supported

the hypothesis of an additional release of Ca^{2+} from

intracellular stores. Further study showed that the

effects of dantrolene are only protective in certain EAA

induced toxicities. Lower levels of protection were

conferred in kainate and AMPA activation as compared to

NMDA and quisqualic acid induced toxicity. This may be

explained by different routes of Ca^{2+} entry between the two

systems.

When levels of EAAs are abnormally high, these

neurotransmitters represent a potential source of

excitotoxicity by causing an alteration in normal signal transduction processes in the receptors. The etiology of the increased levels of neurotransmitter is yet unknown. One possible hypothesis is based on a widespread depolarization at the time of the insult. This can be likened to the depolarization that occurs in cardiac muscle when a precordial thump or chest thump is delivered. If a widespread depolarization were to occur, it could cause a massive release of stored neurotransmitter from pre-synaptic vesicles of excitatory nerves. This could be a possible trigger for excitotoxicity.

Another mechanism of excitotoxicity lies in the activation of the lipolytic enzymes phospholipase A_2 and stimulation of phospholipase C. These enzymes function to activate the arachidonic acid cascade which results in an increase in prostaglandins, leukotrienes, and thromboxanes (Burt, 1993; Lerea et al., 1995). The accumulation of the these byproducts inhibit the uptake of glutamate and thereby result in exposure of the neurons to increased EAA levels which leads to even further receptor activation (Lerea et al., 1995).

Several possible mechanisms for excitotoxicity following stimulation have been mentioned. The mechanisms of cell injury caused by excitotoxicity are still not known, however, two major possibilities are under investigation. First, neuronal swelling from the influx of Na^+ and Ca^{2+} followed by water could lead to acute neurotoxicity. Secondly, the influx of Ca^{2+} via the NMDA channels serves to modulate the activity of a number of intracellular enzymes (see Figure 4).

The possible link between the intracellular signaling mechanisms induced by neurotoxicity and gene regulation will be the focus of the remainder of this paper. In the next section, data will be presented from several sources linking gene activation following TBI. This section will examine the genes shown to be activated following TBI and present hypotheses on their possible roles following injury. The data will be discussed in terms of whether targeting gene regulation represents a viable treatment option for traumatic brain injury, as well as offer a current summary of the salient experimental data in a rapidly evolving area of TBI research.

IV. GENE ACTIVATION

The hypothesis that physical changes within cells might be attributed to alterations in gene expression led researchers to examine changes in gene transcription. It was found that a large number of genes were modulated following traumatic brain injury. The major classes of genes seen to be transcribed are the immediate early genes (IEGs), cytokines, heat shock proteins (HSPs) and glucose related proteins (GRPs). In addition to these classes of genes, changes in transcription have been examined in glial fibrillary acidic protein (GFAP), procholecystokinin (PCCK), brain derived neurotrophic factor (BDNF), and the apoptosis suppressor gene bcl-2.

A. Immediate Early Genes

One class of genes under investigation is the early inducible genes also known as IEGs. IEGs such as c-fos and c-jun are proto-oncogenes that serve as transcription factors (Lewin, 1990). However, due to their ability to form transcription factors and promote transcription of other genes, they also have they ability to transform cells if their expression is not regulated. This is not the case

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for all transcription factors, but c-fos and c-jun are known to possess the ability to transform cells if overexpressed (Lewin, 1990). These genes are transcribed when a cell receives a message to initiate transcription of other genes. It is possible that the second messengers created following TBI may directly act to stimulate transcription of these genes to yield their protein products, Fos and Jun.

The individual gene products of c-fos and c-jun possess complementary leucine zipper binding motifs that allow the formation of an amphipathic α-helix or a coiled coil, which can be comprised of either the heterodimer of fos/jun or a homodimer of jun/jun (Lewin, 1990). The dimeric complex of fos/jun is termed AP-1. AP-1 is a transcription factor which has the ability to bind DNA at specific regulatory sites called AP-1 binding sites in promoter regions of various genes (Lewin, 1990). AP-1 binding sites are common and have been localized to the glial fibrillary acidic protein (GFAP) gene (Hayes et al., 1995) and on the nerve growth factor (NGF) gene (Zheng and Heinrich, 1988). Examination of these IEGs and

consideration of their roles in cellular processes will provide insight on the secondary insults in TBI attributed to an alteration in gene expression.

Fineman et al. found increased intracellular Ca^{2+} levels in rat brain after a lateral fluid percussion (1993). These high Ca^{2+} levels may serve to activate phosphorylation of cAMP response element binding protein (CREB) by the protein kinases activated by Ca^{2+} influx, such as PKA and CAMK (Du and Montminy, 1998). CREB protein was shown to exhibit increased phosphorylation following TBI (Dash et al., 1995). This phosphorylated form of CREB has been shown to increase expression of IEGs and genes encoding stressor proteins (Greenberg et al., 1985). The physiological roles of stressor proteins will be discussed in a later section. The response protein could accomplish this by aiding in the assembly of the transcription complex. Some enhancers and transcription factors function by binding the DNA in areas of the promoter and causing the DNA to undergo a conformational change which aides in assembly of the transcription complex (Lewin, 1990).

Although this pathway linking high extracellular EAA levels to increased expression of IEGs is feasible, the researchers fail to show support for one step. The activated CREB protein is not shown to directly increase expression of IEGs. Examining the primary structure of the CREB protein and determining which amino acid is phosphorylated and subsequently preventing phosphorylation could be accomplished by mutating the amino acid. This would allow for examination of c-fos expression following TBI. Another possibility would be to examine c-fos expression in a cell with a CREB+ protein mutation in the presence and absence of TBI. It would be interesting to see if a constitutively active CREB protein would result in an increased level of c-fos.

1. C-fos

It was previously established that c-fos expression increases in response to kindling (Dragunow and Robertson, 1987) and seizures (Morgan et al., 1987). Phillips and Belardo (1992) examined the effects of central fluid percussion-induced brain injury on c-fos expression. Mild

to moderate central fluid percussion had been shown to induce neuronal pathophysiology (Hayes et al., 1992). Because NMDA and muscarinic receptors are also known to play a role in the injury process (Olney, 1994) and are known to increase transcription of c-fos (Chiarugi et al., 1989; Dragunow et al., 1990), it is reasonable to suggest that c-fos transcription is increased by trauma. The objective of the Phillips and Belardo study was to investigate c-fos expression within the hippocampus at different post-injury time points and varying traumatic intensities, and then determine if there was a correlation between trauma and increased expression of c-fos. If c-fos expression could be spatially and temporally related to patterns of TBI pathophysiology, a link between trauma and gene expression for c-fos would be established.

Phillips and Belardo found that c-fos protein, identified with immunohistochemical methods, was present in pyramidal and granule cells of the CA1 region of the hippocampus as early as fifteen minutes following central fluid percussion injury. This is compared to sham-operated animals (control) which showed minor induction of c-fos

expression. The researchers attributed this slight increase to surgical trauma, because it was not seen in the non-operated controls. It would seem that trauma can be directly linked to increasing c-fos expression.

The results depart from those seen after other insults when the intensities seen within individual neurons are considered. The labeling intensity within the CA1 neurons of the hippocampus was not homogenous, as was seen with seizures, kindling, and ischemia (Dragunow, 1990). This result could be attributed to differential vulnerability to excitotoxicity between the cell layers of the hippocampus due to different concentrations of the NMDA receptors through which excitotoxicity proceeds. However, it is believed that damage caused by seizures and ischemia proceed through similar pathways as TBI, and so does not explain why this phenomenon seems to be unique to TBI. A nonuniform distribution was also seen in control hippocampal neurons. However, the range of intensities seen in the control hippocampal neurons was not as prominent as that seen in the injured neurons.

Another interesting finding was that the time course of c-fos protein expression was longer lasting for the mild injury as compared to the moderate injury. The authors stated that the numbers of c-fos positive cells from moderate injury had decreased to control levels at one hour post-injury, while the c-fos levels from mild injury remained elevated until almost twenty-four hours post-injury. If increasing injury levels yield a greater agonist surge, it would seem that a direct correlation does not exist between injury intensity and c-fos expression. This led the authors to postulate the presence of other mechanisms acting in a modulatory sense with the agonist surge to yield the actual level of c-fos expression seen with immunohistochemistry. Modulation of neurotransmitter release to prevent cell death would take place at moderate levels of injury, while at mild injury levels, less modulation takes place. However, as injury levels increase in severity it is possible that modulation is ineffective resulting in cell death. This could account for the disparity seen between injury severity and c-fos production.

Dave et al. (1997) used northern blot analysis to examine whether TBI produces activation of c-fos in cortex, cerebellum, and hippocampus and to examine to what degree secondary hypoxia affects activation. With regard to clinical applications, head injured patients brought to emergency rooms are also frequently in a hypoxic state, with post-traumatic hypoxia being identified as a complication which significantly affects morbidity and mortality (Lutz et al., 1982). Post-traumatic hypoxia has also been shown clinically to exacerbate the pathogenesis of TBI (Ishige et al., 1987). This data points to the importance of identifying what pathophysiological changes occur following post-traumatic hypoxia.

Injury was performed using fluid percussion through craniotomies centered over the right parietal cortex. Hypoxemia was achieved following substitution of oxygen with halothane anesthesia. The results with fluid percussion TBI and normoxic states demonstrated widespread elevations in c-fos mRNA (Dave et al., 1997). The induction of hypoxia alone produced no significant change in c-fos mRNA levels. However, when hypoxia was induced

concurrently with TBI through fluid percussion, a large increase in c-fos mRNA levels was seen. Levels were increased in hippocampus, cerebellum, and frontal cortex by 100%, 88%, and 57%, respectively.

This increase in c-fos mRNA combined with data showing pathophysiological changes with hypoxia and TBI demonstrate the necessity for the further investigation of c-fos following TBI. One significant observation which can be made is that hyperventilation of the traumatically head injured patient could serve to attenuate c-fos levels and might serve as a therapeutic intervention once the role of c-fos in pathophysiology of TBI is determined.

2. AP-1

As mentioned earlier, c-fos and c-jun have the ability to form complexes similar in structure and function to the AP-1 transcription factors (Lewin, 1990). For this reason the expression of c-fos was further examined by Yang et al. (1994) in conjunction with the expression and association with DNA of the AP-1 transcription factors after lateral fluid percussion injury.

Increased expression of c-fos was seen ipsilateral to the impact site within the hippocampus and cortex. In addition, the distribution of c-fos was dispersed throughout the cortex while it was limited to the cell layers within the hippocampus. Yang et al. also showed that c-fos was increased not only in the ipsilateral cortex and hippocampus, but also in the contralateral hippocampus restricted to certain cell layers. The intensity of the contralateral hippocampus was comparable to the intensity seen in the ipsilateral hippocampus in the absence of direct injury on the contralateral side. This finding supported the hypothesis of the hippocampus' vulnerability to injury, which may be a function of increased presence or concentration of NMDA receptors (Farooqui and Horrocks, 1994).

Yang et al. also used gel mobility shift assays to examine the change in transcription factor AP-1 binding with DNA at specific time points after injury. An increase in binding activity was detected as early as one hour and

increased steadily to remain elevated for 24 hours post injury. However a direct link between c-fos and AP-1 was not established. It was later shown that AP-1 binding activity increases after c-fos mRNA levels and persists for a longer duration of time (Hayes et al., 1995). The increased time of expression of AP-1 might seem to indicate a role in mediating the intracellular cascades that occur following injury. AP-1 is known to activate transcription of other genes, its longer time of expression could serve that function by allowing for activation of expression of genes needed later in the cascade which begins following injury. Previous studies had shown that increased c-fos levels led to increased formation of the AP-1 transcription factor complex (Curran and Franza, 1988) which led Hayes et al. to postulate that c-fos levels and AP-1 levels could be directly related and perhaps linked after a neurological insult.

AP-1 transcription factors have a variety of binding sites in the promoter regions of genes and can serve to activate their transcription (Hayes et al., 1995; Zheng and Heinrich, 1988). When this evidence is combined with the

longer duration of expression of AP-1 seen by Yang et al. (1994) and Hayes et al. (1995) it points towards a central role of AP-1 and c-fos in the injury process which follows TBI. It is possible that AP-1 serves as the main signal to activate transcription of other genes. Whether these genes are recruited to heal the cells or mark them for death remains to be seen. This evidence would support future study examining c-fos and c-jun gene expression since they are the monomers of the AP-1 complex.

3. Nerve Growth Factor Expression (NGF)

NGF is a neurotrophic factor that can stimulate and direct neuronal growth and survival (Burt, 1993). Previous studies have shown that administration of NGF to injured CNS neurons serves to spare these neurons from death and degeneration (Hayes et al., 1992). Therefore, the control over increased expression of the NGF gene and other neurotrophic factors as well as the mechanisms regulating these changes are important in the consideration of head trauma.

As stated earlier, NGF is known to have an AP-1 binding site (Zheng and Heinrich, 1988), therefore its expression was examined in conjunction with that of c-fos by Yang et al. (1995). Rats were injured parasagitally using a controlled cortical impact model. Localization of c-fos and NGF mRNA was performed using *in situ* hybridization.

First, these authors found that levels of c-fos mRNA were seen to increase as early as 30 min post-injury using *in situ* hybridization and NGF expression did not increase until one hour post-injury. Secondly, Yang et al. saw that certain areas of the brain such as the neocortex showed only an increase in c-fos and no change in NGF above levels of sham controls. This result suggested that certain injuries follow separate pathways that involve differential activation of gene expression.

4. Nerve Growth Factor Induced Gene Expression (NGFI-A)

Nerve growth factor induced gene encodes a subclass of IEGs which are activated by the NGF family (Milbrandt J., 1987) and encode transcription factors containing zinc

finger motifs (Honkaniemi et al., 1995). Very little is known about them as compared to fos and jun. The most frequently studied of these genes is NGFI-A. To study the induction of this gene, Honkaniemi et al. (1995) simulated brain injury with intracerebral injections of saline and ibotenic acid into the hippocampus. Ibotenic acid is a glutamate analogue and serves to imitate a localized release of glutamate. Patterns of gene expression were determined using *in situ* hybridization and Northern blotting. The IEGs examined were NGFI-A, NGFI-B, NGFI-C, egr-3, and Nurr1.

The results showed that injection of saline or ibotenic acid was sufficient to induce expression of the five zinc finger IEGs examined. The researchers observed activation in the cortex, caudate-putamen, and hippocampus with the greatest increase seen within the dentate gyrus. All of these increases were seen in the ipsilateral hemisphere which would be expected since the route of excitotoxicity is not from widespread depolarization and release, but rather from a local increase in EAA or in this case, the glutamate analogue, ibotenic acid.

It was stated that the exact targets of these transcription factors are unknown. However, there was speculation that these transcription factors serve to activate members of the NGF family. One possibility to consider is the joint interactions of fos/jun and NGFI genes. The spatial distribution of the NGFI genes corresponds to the location of fos/jun families following trauma. This would further support the possibility of interactions between fos/jun family members and the zinc finger transcription factors in regulating NGF transcription (Honkaniemi et al., 1995). However, it is important to consider that if both gene families are activated through the same pathway at the same time, it would be expected to find them in the same brain areas after trauma. This could be clarified by examining the temporal profile in regard to activation of these families.

Before this hypothesis can be tested it would require direct proof of interactions between NGFI-A and AP-1. Localization of adjacent DNA binding sites for NGFI-A and AP-1 on genes known to be activated would be an important first step in determining if they act cooperatively.

Another possibility would be to determine if the two protein products have sites that allow them to bind together and form a complex similar to other transcription factors. It is also possible that one protein might act as a negative regulator for the other. If NGF is activated through an AP-1 site which has a low binding affinity, it would only be transcribed at high AP-1 levels. Binding of NGFI at adjacent sites to AP-1 sites could cause steric hindrance and prevent further gene activation by AP-1.

In an effort to elucidate the cellular mechanisms responsible for induction of c-fos and NGFI-A via NMDA receptors, Lerea et al. (1995) examined the arachidonic acid cascade. The study by Lerea et al. focused on mechanisms by which NMDA activation might play a role in neuronal plasticity through IEG activation. It had previously been established that the induction of c-fos mRNA in dentate gyrus granule cells by NMDA requires increases in Ca^{2+} ion concentration. Additionally, the concurrent activation of phospholipase-A_2 (PLA_2) and cyclooxygenase was seen (Lerea et al., 1993). Given these

observations, these enzymes were examined for their roles in activation of NGFI-A.

In order to test whether activation proceeds through PLA_2, two distinct inhibitors of PLA_2, quinacrine and aristolochic acid were used. They prevented induction of c-fos but still allowed for the intracellular increases in Ca^{2+} (Lerea et al., 1993). This shows that PLA_2 has no direct role in increasing Ca^{2+} concentration, but probably acts later in the cascade to stimulate c-fos expression. Both of these inhibitors also markedly reduced NMDA-mediated increases in NGFI-A mRNA. This evidence indicates that PLA_2 activation lies upstream in the cascade from NMDA activation to c-fos and NGFI-A induction.

The next enzymes in the cascade, cyclooxygenase and lipoxygenase, were examined for their roles in activation of NGFI-A. Cyclooxygenase is an enzyme which metabolizes arachidonic acid to prostaglandins and thromboxanes (Garrett and Grisham, 1995). Lipoxygenase is an enzyme which metabolizes arachidonic acid to leukotrienes and other fatty acids (Garrett and Grisham, 1995). It was previously shown that inhibitors of cyclooxygenase blocked

NMDA directed increases of c-fos (Lerea et al., 1993). No inhibition was seen when the effects of cyclooxygenase inhibitors on NGFI-A expression were examined (Lerea et al., 1995). Because inhibition was seen with PLA_2 inhibitors and not cyclooxygenase inhibitors, NGFI-A activation must proceed through a pathway following PLA_2.

When lipoxygenase inhibitors were used, an inhibition of NGFI-A was seen (Lerea et al., 1994). The lipoxygenase inhibitors NDGA and esculetin exhibited inhibition of 82% and 88%, respectively, of NGFI-A expression. The inhibitors had little or no affect on c-fos mRNA levels. This further supported the hypothesis of divergent pathways from PLA_2. The above data indicate that cyclooxygenase and lipoxygenase play distinct roles in activation of c-fos and NGFI-A, respectively. This hypothesis must be further tested with different inhibitors and activators of the enzymes to examine the roles of these enzymes on c-fos and NGFI-A levels after trauma.

B. Cytokines

Cytokines are proteins released by immune system cells that mediate biochemical activities which can be beneficial or harmful depending on the circumstances. They are established mediators and modulators of inflammation and tissue damage. The major cytokines examined after TBI are IL-1, IL-6, and TNF-α due to their presence within the CNS in several pathological conditions. TNF-α had been previously shown to induce apoptosis in neurons (Westmoreland et al., 1996). Taupin et al. (1993) reported regional increases in these cytokines following lateral fluid-percussion injury, which was later localized to specific brain regions which were susceptible to TBI.

IL-1, IL-6, and TNF-α were shown to be robustly expressed in the ipsilateral cortex and hippocampus following closed head injury (Shohami et al., 1997). Weak TNF-α expression was seen in the contralateral hippocampus following closed head injury (Shohami et al., 1997). This expression occurred within the first hour following injury during which time no inflammatory response is present.

This suggests that the cytokines were produced by CNS cells as opposed to invading inflammatory cells.

It was also shown that HU-211, a noncompetitive NMDA antagonist, inhibited TNF-α production in the brain following TBI, and this control was exerted at a post-transcriptional level (Shohami et al., 1997). HU-211 improved clinical outcome of test subjects, reduced cell death, and preserved blood brain barrier (BBB) integrity (Shohami et al., 1997).

The cytokine IL-1 has also been shown to be increased following brain injury and excitotoxic damage and is believed to be the pathway through which damage is conferred (Rothwell, 1996). Inhibitors of IL-1, such as IL-1 receptor antagonists, have been assessed in rodent models of traumatic brain injury with promising results. Administration of these antagonists at the time of injury or immediately afterwards have demonstrated a remarkable degree of protection by reducing tissue damage (Rothwell, 1996).

Rothwell (1996) also found that the therapeutic window for head trauma is greater than that of cerebral ischemia.

This fact combined with increased awareness in the pre-hospital field leading to faster transport times have made head trauma an attractive target for the neuroprotective effects of IL-1 antagonists (Rothwell and Dantzar, 1992).

C. Heat Shock Protein and Stress Protein Expression

Heat shock proteins are a family of stress-induced proteins that may play a role in cellular repair and protection (as reviewed in Mayer and Brown, 1994). They are induced by hypothermia, hyperthermia, ischemia, and tissue injury. Initially they were called "heat shock proteins" (hsp) because they were first found to respond to thermal stress. Since then it has been seen that these proteins respond to a variety of stresses including chemical, ischemic, and infectious, they have also been called "stress proteins". It is believed that these proteins might function in cell repair. This supports the hypothesis of their stimulation by the same events that cause cell damage such as hypothermia, hyperthermia, ischemia, and TBI.

The heat shock proteins are currently known as molecular chaperones that function to mediate the folding of proteins but are not part of the mature protein product. Some examples of these chaperone proteins include cytosolic hsp70 (heat shock protein, 70 kDa), matrix hsp70, and matrix hsp60. These proteins function to bind nascent unfolded proteins destined for the mitochondria within the cytosol of the cell (Lodish et al., 1995). This prevents these proteins from folding and keeps them linear allowing for their entry into the mitochondria. The unfolded protein is first bound by cytosolic hsp70 and escorted to the mitochondria. Once inside the mitochondria the protein chain is bound by the matrix hsp70 which stabilizes the protein chain until matrix hsp60 can bind and mediate proper folding within the matrix of the mitochondria. This exemplifies one of the known roles of stress proteins (as reviewed in Lodish et al., 1995).

Tissue injuries such as surgical lesions have been shown to induce hsp70 gene expression during their early stages of response (Tanno et al., 1993). These same results have been seen with peripheral axotomy (New et al.,

1989), concussive spinal cord injury (Gower et al., 1989),

crush injury (Xue and Grossfield, 1993), and mild fluid

percussive injury (Tanno et al., 1993).

One model proposed by Tanno et al. (1993) to account

for the activation of heat shock proteins from mild fluid

percussive injury includes the parallel breakdown of the

blood-brain barrier. The basis for this rationale lies in

previous experiments by Tanno et al. (1992) that focused on

the spatial profile of blood-brain barrier breakdown as

related to mild fluid percussive head injury. The

breakdown resulted in the unrestricted influx of plasma

proteins and humoral agents which are thought to play a

role in the activation of a heat shock response. The

presence of these extracellular proteins activating heat

shock genes would support the role of these heat shock

proteins in binding and stabilizing proteins. Through this

process the heat shock proteins would prevent the influx of

extracellular plasma proteins from binding with any

intracellular proteins and interfering with their function.

The authors speculated that heat shock proteins might play

a role in endothelial stabilization of the blood brain

barrier (Tanno et al., 1993).

Tanno et al. (1993) decided to examine whether

extravasation of plasma proteins played a direct role in

expression of hsp72 by examining the distribution of hsp72

at various time points after mild fluid percussive brain

injury. These patterns were then compared to known

patterns of blood-brain barrier breakdown seen after mild

fluid percussive brain injury. The expression of hsp72 was

examined at the impact site, the parasagittal cortex and

the CA3 region of the hippocampus which are areas known to

exhibit blood-brain barrier breakdown. Table 2 shows the

percentage of brains expressing hsp72 in indicated areas at

various time points.

Table 2. **Percentage of Brains Demonstrating hsp 72 Expression in Areas Susceptible to BBB Breakdown**

Brain Area	1 h	3 h	6 h	24 h	3 days	7 days
Impact site	0	100	100	100	100	67
Parasagittal cortex	0	0	0	43	40	0
Cortical layer VI	0	0	0	86	60	0
CA3 hippocampus	0	0	0	71	20	0

(data taken from Tanno et al, 1993)

The results in table 2 show that hsp72 expression is

seen in areas known to exhibit blood brain barrier

breakdown. However, these results still do not show any direct evidence for the extravasation of plasma proteins as a means to activate expression of hsp72. Additionally, the researchers need to examine hsp72 expression at sites where blood brain barrier breakdown is not seen. This would allow for better interpretation of dependence on hsp72 expression on blood brain barrier breakdown.

In the Tanno et al. study (1992) a correlation existed between the periods of permeability in the blood-brain barrier and the time points of hsp72 expression. Areas which are known to show prolonged permeability, such as the impact site, also exhibited prolonged expression of hsp72, while other regions known to exhibit transient barrier breakdown showed corresponding intermittent hsp72 expression. However, no direct link between blood brain barrier permeability and hsp expression is shown.

Whether the heat shock proteins are activated by direct physical insult, influx of proteins, or increased intracellular Ca^{2+} levels is still unknown. The etiology of direct physical insult would support the proposed pathway for TBI and result in increased intracellular Ca^{2+}. It may

be a pathway which progresses through all of the above mechanisms before leading to heat shock stimulation.

Further experiments *in vivo* to examine the effects of humoral agents and extracellular proteins on heat shock gene regulation need to be performed. The stimulating factor which affects gene expression might not be the presence of the proteins themselves, but the breakdown of the barrier or a signal transduction mechanism set in motion upon loss of blood brain barrier integrity. If the stimulating factor is indeed the presence of the extracellular proteins, it would make sense that the proteins would bind molecular chaperones such as hsp70 already present and stimulate the transcription of further molecular chaperones.

Creating a loss in blood brain barrier integrity through methods which did not inflict traumatic injury to the brain would help identify whether or not the presence of the extracellular proteins alone were the stimulating factors towards gene activation. This would allow elimination of the BBB permeability variable and may directly link extravasation of plasma proteins to hsp

activation. Use of drugs which open the blood brain barrier or examining pathological conditions where blood brain barrier integrity is compromised, such as infiltrating tumors, would offer conditions in which hsp activation could be studied.

The effects of trauma on CNS tissue have also been examined by inflicting injury to the spinal cord at the thoracic level after a single laminectomy (Gower et al., 1989). Injury was performed by removing the lamina at the thoracic level and leaving the dura intact. A plunger was placed over the exposed dura and a 1.0-gram weight was dropped from a known height upon the plunger, which impacted on the dura-enclosed spinal cord. Localization of stress protein was performed using immunohistochemistry with a monoclonal antibody against SP-70, which is the 70-kDa stress protein known to be most frequently induced.

Gower et al. found that there was an increase in the levels of SP-70 in the area surrounding the injury and that the cells at the site of impact showed no SP-70 expression. Over the 12 hours following the injury there was a gradual decrease in the levels of SP-70 until none remained at 18

hours post-injury. The actual site of injury contained minimal SP-70 and was surrounded by a ring of cells expressing high levels of the protein. This is similar to results reported by Tanno et al. where hsp72 was detected in the blood vessels surrounding the impact zone or necrotic core. Radiating away from the impact zone the level of SP-70 gradually decreased until it reached levels similar to control animals where only a laminectomy was performed.

It had been shown that pretreatment of yeast cells with a minor shift in temperature allowed the cells to tolerate much higher temperature than "non-primed" cells (Mayer and Brown, 1994). This temperature tolerance can be transferred by transfection of other cells with the stress protein gene. If the role of stress proteins as molecular chaperones is considered, it is possible that this heat protection might be mediated by association of the stress proteins with proteins within the cell increasing stability and resistance to temperature. A relevant and important discovery by Lowenstein et al. in 1991 that further supported the "heat priming" finding was a resistance to

glutamate toxicity by heat primed cerebellar granule cells. The protection from glutamate was only present at lower levels and could be overwhelmed as concentrations of glutamate increased. Lowenstein et al. did not test other EAAs such as aspartate to examine the effects on heat priming. Similar results of protection from excitotoxicity by heat shock were observed by Rordorf et al. in 1991. Their study shows another possible role of heat shock proteins in an injured neuron and a possible treatment through up-regulation. This points to a possible protective role of increased heat shock protein expression after trauma.

An increased level of SP-70 was seen in the axonal retraction balls generated by TBI (Gower et al., 1989). Although transport of the SP-70 is possible, the amount of protein seen in the retraction balls was much greater than could be accounted for by the normal rate of axonal transport. The source of the protein might be from neighboring glial cells due to the lack of de novo synthesis ability in the axons. This hypothesis would

support the role of glia as a supportive matrix for the neurons after trauma.

One consideration, which warrants further study, is the possible role of astrocytes in this increased resistance to abnormal glutamate concentrations. Theoretically, if the astrocytes function to translocate SP-70 to the axons during a heat-shock response, it is also possible that they may, in some manner, act to increase the resistance of the neurons to toxic glutamate levels. One possibility might be astrocytic uptake of glutamate from the surrounding media. An experiment could be performed to investigate the effects of varying numbers of astrocytes and different concentrations of glutamate in the presence of heat-primed neurons. It would be interesting to determine what potential pathway is used by the astrocyte to protect the neurons.

Raghupathi et al. (1995) examined the coexpression of heat-shock protein and c-fos following brain injury by fluid-percussion and focal microvascular brain damage. *In situ* hybridization was performed to examine the induction of both c-fos and hsp72 following lateral fluid-percussion

injury. A coexpression of c-fos and hsp72 was detected in the cortex ipsilateral to the side of impact. The levels of expression were significantly higher for c-fos compared to hsp72 at two and six hours post-injury. Additionally, the distribution of c-fos expression was more prevalent than hsp72. Once again, increased c-fos expression was seen bilaterally in the hippocampus in addition to ipsilaterally in the cortex, thalamus, and subcortical white matter. Hsp72 expression was seen only in ipsilateral cortex at significantly lower levels.

Based upon the results of Raghupathi et al. (1995), there exists the possibility that expression of c-fos and hsp 72 are linked as was postulated above for c-fos and NGF. However, it was also observed in the Raghupathi et al. (1989) study, that the levels of hsp72 were normal at six hours post injury, while certain areas of the brain were still exhibiting c-fos. Therefore, it seems unlikely that c-fos is tightly coupled to hsp72 expression because hsp72 mRNA was not increased at six hours when elevated levels of c-fos were still detectable. This suggests a differential induction of c-fos and hsp72 by excitotoxicity

and degraded proteins from dying cells, respectively. The fact that hsp 72 was only seen ipsilaterally also lends support to this hypothesis, although it must be tested further.

Another perspective that has been examined concerning induction of gene expression in CNS injury is the effect of an ischemic insult. Although ischemia is known to occur during instances of decreased cerebral blood flow such as cardiac arrest (global ischemia) or during a stroke (focal ischemia), it is also known that ischemia can result from traumatic brain injury (Young, 1985). As ischemia persists, there is a progressive loss of neurons over time (Young, 1985). It is also known that cells respond by producing a class of proteins with the characteristics of the heat shock proteins (Mayer and Brown, 1994). If a link between changes in gene expression and cell loss due to secondary ischemia can be established after TBI it may be possible to design treatments limiting the amount of neuronal loss.

The neurons that are especially sensitive to ischemia include those located in the hippocampus. These changes

are manifested as increases in expression of hsp72, which
Simon et al. (1991) examined by inducing global ischemia
through bilateral cauterization of the vertebral arteries
and subsequent clamping of the carotids. Localization was
performed using immunocytochemistry and monoclonal antibody
against hsp72. The majority of staining was seen in cells
in the hippocampus and thalamus due to their increased
vulnerability to ischemia. This vulnerability was also
shown to be graded as a function of the responsiveness of
the cell groups to ischemia. Those cell groups which were
more susceptible to ischemia, such as CA1 neurons in the
hippocampus, showed a more robust production of hsp72,
while cell groups resistant to ischemia, such as the
dentate granule cells, showed less expression of hsp72.
These observations would seem to support a hypothesis of
induction of hsp72 by a cell once it has reached an
ischemic threshold.

D. Glucose Related Proteins (GRPs)

When changes in intracellular Ca^{2+} are being considered
it is important to look at proteins which function in

mediating intracellular Ca^{2+} reactions. The glucose related proteins (grp78 and grp94) and Ca^{2+} binding protein calbindin-D28K have been examined for expressional changes following TBI. The GRPs are similar in sequence to HSPs and are believed to function in the same manner. Their expression can be increased following treatment with Ca^{2+} ionophores (Hayes et al., 1995), linking their activation to changes in calcium homeostasis. Ca^{2+} binding proteins (CaBPs) such as calbindin-D28K are believed to be one of the physiological buffers of cytoplasmic Ca^{2+} (Baimbridge et al., 1982).

Lowenstein et al. (1994) demonstrated increases in calbindin-D28K, grp78, and grp 94 in the hippocampus and cortex following lateral fluid percussion injury. This increase was found to be part of the late transcription phase following IEG activation. Because these proteins are similar in function to HSPs (Hayes et al., 1995), the GRPs are likely to help the cell withstand stress while the CaBPs would be acting as a buffer to modulate Ca^{2+} entry and activity. These results further support a gene

expressional reaction to altered Ca^{2+} homeostasis following brain injury.

E.   Glial Fibrillary Acidic Protein (GFAP) Expression

In a study conducted by Kost-Mikucki and Oblinger (1991), the effects of corticospinal axotomy in adult hamster on the levels of glial fibrillary acidic protein (GFAP) expression were examined to determine if the level of control exerted on GFAP expression occurred at the mRNA level. The pyramidal tract was incised just rostral to the pyramidal decussation. The areas just rostral to the incision that experienced retrograde degeneration and the areas caudal to the incision, which experienced Wallerian degeneration, were examined for GFAP expression.

Results of immunocytochemistry and immunoblotting confirmed an increase in GFAP levels on the side of the lesion compared to the same side in control animals. This increased presence of GFAP mRNA was seen rostral to the lesion, caudal to the lesion, and at the lesion site as early as 48 hours and lasted up to one week post-injury. The results of the *in situ* hybridization were correlated

with increases in GFAP mRNA seen with immunocytochemistry and immunoblotting.

One interesting fact that Kost-Mikucki and Oblinger (1991) did not address was a disparity seen between the protein levels and mRNA levels of GFAP. They found a greater change in mRNA levels than in protein levels. Explanations such as post-transcriptional modification and protein stability could account for this result. It would seem that a further level of control exists beyond transcriptional activation in the regulation of GFAP. In either case, more information must be obtained regarding the regulation of GFAP expression before any conclusions can be drawn regarding the role of GFAP in the scarring process within the CNS. It can be said however, that GFAP levels increase following TBI (Kost-Mikucki and Oblinger, 1991).

Scarring after injury in the CNS is a cellular process as opposed to the extracellular process seen in normal scar formation that involves connective tissue cells releasing collagen into the extracellular matrix (Kost-Mikucki and Oblinger, 1991). The scar seen in the CNS is composed of a

large number of astrocytic processes loaded with GFAP intracellular filaments (Bignami and Dahl, 1976). GFAP is an intermediate filament protein and it is believed that the GFAP-related scarring that occurs in the CNS impedes axonal regeneration (Bignami and Dahl, 1976). Reduction in the upregulation of GFAP following traumatic insult might serve to allow for faster healing.

It can be argued that corticospinal axotomy does not accurately reflect what happens in a traumatic situation. However, it was shown that diffuse axonal injury (DAI) is a result of TBI (Povlishock, 1983). Povlishock also found that axotomy could be accounted for by both the direct insult and secondary effects stemming from a breakdown in the axonal cytoskeleton (1993). The same phenomenon of axonal cytoskeleton breakdown and Wallerian degeneration seen in DAI was seen following axotomy.

F. Procholecystokinin (PCCK)

Procholecystokinin is a gene for which not much information is available. The protein product for this

gene is the prohormone procholecystokinin. Cleavage by
endopeptidases yield cholecystokinin.

Cholecystokinin is a non-opioid neuropeptide which is
known to be found in GABAergic neurons (Olenik and Meyer,
1990). Such neuropeptides can act alone as
neurotransmitters or concurrently with neurotransmitters as
neuromodulators. The principle location of CCK within the
CNS is in the amygdala where it participates in modulation
of autonomic function (Burt, 1993). It is possible that
PCCK might have an antagonistic effect to the excess
excitation present during TBI.

It first was shown by Olenik and Meyer (1990) that a
meningio-cortical injury enhances expression of the PCCK
gene. Subsequent studies examined both the role of c-fos in
PCCK induction, since c-fos is known to be enhanced through
the same injury, and the effect of administration of the
NMDA antagonist MK-801 on IEG expression at varying time
points relative to injury.

An early rise in IEGs was first observed, followed by
a rise of PCCK mRNA (Olenik et al., 1994). To determine
the relationship between the IEGs and the PCCK expression,

MK-801 was administered at varying timepoints. Results showed that IEG expression was inhibited and the PCCK expression persisted. This expression of one protein with the concurrent inhibition of the other suggests that the injury-induced expression of the IEGs c-fos and c-jun and the enhancement of PCCK mRNA are not directly coupled. Although this research failed to show a link, it did demonstrate that activation of PCCK does not follow the NMDA mediated pathway of activation as IEG activation does, nor is it triggered by IEG expression. It is possible that once the alternative mode of activation for PCCK is found it will provide insight into activation of other genes following TBI.

G.   Brain Derived Neurotrophic Factor (BDNF)

BDNF is a member of the family of proteins known as neurotrophins. It has been shown to increase neuronal survival in culture and prevents cell death *in vivo* (Burt, 1993). BDNF has chemical characteristics close to those of NGF, however its biological spectrum of activity is different than that of NGF (Thoenen et al., 1987).

It is believed that the neurotrophic factor BDNF is a target for upregulation by the protein products of IEGs such as c-fos and c-jun since it is related to NGF (Hughes et al., 1993). Induction of BDNF coincided with induction of IEGs such as c-fos, jun-B, krox 24, and c-jun (Hughes et al., 1993). Additionally, administration of MK-801 blocked induction of the above IEGs and BDNF. This suggests that BDNF follows the same route of activation as the IEGs, namely NMDA receptor mediated activation.

An increase in BDNF following lateral fluid percussion in the rat was also seen by Hicks et al. (1997). This upregulation was seen in the dentate gyrus and the CA1-CA3 regions of the hippocampus. The dentate gyrus exhibited the greatest increase in BDNF and also showed the highest resistance to cell death following lateral fluid percussion.

Neurotrophins such as BDNF exert their effects through binding of transmembrane protein-tyrosine kinase receptors (trks). Hicks et al. (1998) examined rat hippocampus for changes in expression of trkB, the BDNF receptor, following experimental brain trauma due to the previously established

link between neurotrophin/trk interactions and

neuroprotection and recovery (Mattson and Scheff, 1994).

It was found that an increase in trkB mRNA was seen within

the same tempero-spatial profile as BDNF mRNA in the

dentate gyrus.

Determining the actual pathway of gene activation for

BDNF is the next step. A potential route of

experimentation would lie in examining the DNA sequence of

BDNF and looking for homology with activation sequences

recruited by TBI within the promoter region. Finkbeiner et

al. (1997) demonstrated the role of CREB in activating

BDNF. This was concurrently correlated by Hayes et al.

(1997) through elucidation of an AP-1 site and a CRE site

within the promoter region of the mouse BDNF gene after

TBI.

This gene represents another potential avenue for

treatment in "rescuing" cells following insult. The result

of BDNF upregulation following traumatic brain injury

should be examined for changes in cell viability. A cell

with a constitutively active BDNF gene would be an ideal

model since interference from other genes such as c-fos, jun-B and krox 24 would be eliminated.

H. Apoptosis-suppressor bcl-2

The apoptosis suppressor gene bcl-2 is a gene which not only suppresses apoptosis but also can protect neurons from stimuli which normally induce apoptotic cell death. It was found to be present in cells which survived a prolonged epileptic attack (Graham et al., 1996). Because epilepsy and neuronal death following TBI may share similar pathways, the expression of bcl-2 following TBI has been examined after brain trauma.

It was previously shown that the ipsilateral cortex, CA3 hippocampus, and dentate gyrus are susceptible to apoptotic death following TBI (Rink et al., 1995). The researchers found that bcl-2 and its translated protein are induced in these regions following TBI, and is increased in spared neurons. Some differences in survivability were seen with regard to different hippocampal regions but these might be due to degrees of injury susceptibility for a particular NMDA receptor population. Further

experimentation using NMDA blockers such as MK-801 or Ca^{2+} release inhibitors would aid in determining if excitotoxic pathways are used for bcl-2 activation.

The mode of action of bcl-2 in "rescuing" cells from apoptosis is as yet unknown. Several possible mechanisms are suggested by researchers, but the most provocative seems to lie in maintenance of Ca^{2+} homeostasis. It had previously been shown that induction of bcl-2 following an excitotoxic challenge served to protect rat hippocampal neurons in culture (Lam et al., 1994). If this proves to be a mechanism of protection it would further support Ca^{2+} imbalance as a primary mediator of injury.

V. ANTAGONISTS & POSSIBLE TREATMENTS

Many of the therapeutic approaches today deal with
modulating neuroexcitation due to the large body of
evidence associating TBI and neuroexcitation (Hayes et al.,
1992; Bazan et al., 1995; Lerea et al., 1995). The most
common treatments are receptor antagonists (Nowak, 1991;
Von Euler, 1997; Okiyama, 1997), modulation of
neuroexcitation (Mody and MacDonald, 1995), and induction
of a heat shock response (Rordorf et al., 1991; Lowenstein,
1991).

The use of EAA receptor antagonists is based on the
known associations between TBI and neuroexcitation.
Because glutamate and aspartate play a central role in
trauma-induced neuroexcitation (Costa, 1994), the NMDA
receptor is a potential site for antagonistic treatment.
It has been shown that MK-801, a noncompetitive NMDA
antagonist, can significantly attenuate hsp70 induction
when administered to animals prior to induction of
ischemia. It was also seen that a single bolus dose did
not prevent the reappearance of hsp70 in the CA1 neurons of
the hippocampus at 24 - 48 hs. These are the same neurons

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which showed the highest susceptibility to ischemia so it is not surprising that these neurons are still induced to express hsp70. What remains to be determined is whether hsp and other genes mentioned in this paper serve to help or hurt the cell. Global inhibition such as MK-801 would actually be harmful if it served to inhibit genes which are activated to help the cell. Data reviewed in this paper further support the connection between excitatory amino acid release and the stimulation of the pathophysiology following trauma or ischemia and definitely provides a strong basis for further study.

Other NMDA antagonists under investigation include memantine (Von Euler, 1997) and a class of novel NMDA antagonists (Okiyama, 1997). One potential problem with the use of NMDA antagonists lies in their side effects and the fact that antagonism can not be directed at injured CNS areas. Therefore the systemic effects of the antagonists must be considered and tend to limit the effectiveness of treatment.

Altering postinjury inflammation following TBI is a rapidly growing field. Antagonists to interleukin-1 receptors have been used and have shown promise in patient

outcome (Relton and Rothwell, 1992). Administration of these receptor antagonists following fluid percussion in rats close to and at the time of injury has resulted in neuroprotection and decreased level of tissue damage (Rothwell, 1996).

Other pharmacological agents that confer protection are also being examined. Most of these treatments exert their effects on neuroexcitation at points in the cascade downstream from initial simulation of the excitatory receptor. One example is seen in the case of inhibition of intracellular Ca^{2+} release by dantrolene (Mody and MacDonald, 1995) which antagonized the ryanodine receptor on neuronal endoplasmic reticulum thereby reducing further increases in intracellular Ca^{2+}. The main problem with these treatments would be comparable to concerns with antagonist treatments. These treatments also cannot be directed specifically at injured area. Similarly, they will have systemic effects and these must be considered before they can be used effectively.

Dantrolene is being currently used for neuroleptic malignant syndrome (Tanaka et al., 1998) and spasticity of cerebral origin such as stroke and traumatic brain injury

(Gracies et al., 1997). Therefore it offers promise in treatment of excitotoxicity since its efficacy and safety has already been studied.

Hyperthermia has been used to induce a heat shock response (Rordorf et al., 1991) in cultured neurons. This protective effect of heat shock proteins to glutamate toxicity was also observed by Lowenstein et al., 1991. The overall evidence would seem to point towards a beneficial effect of gene activation of heat shock proteins. As mentioned earlier, the mechanism through which protection is conferred is unknown but probably lies in protein stabilization by heat shock proteins. Conversely, the use of hypothermia with the intent of lowering metabolism and reducing the posttraumatic response as a means to attenuate the secondary injury cascade is being tested(McIntosh et al., 1998).

Clinical trials are currently underway in the use of EAA antagonists and are primarily focusing on the safety of these agents in humans. Studies examining neuroprotection have not been performed in humans as of yet, but should be the next step if the EAA antagonists prove to be safe.

VI. CONCLUSIONS

The above papers provide information on the possible mechanisms and associations between the acute injury process and the neurochemical cascade that follows. Most of the research points to the pathway of increased excitatory amino acid release resulting in excessive cellular stimulation. This stimulation is known to be mediated in large part through NMDA and muscarinic receptors. A variety of pathways can ensue from this stimulation involving the activation of intracellular cascades either directly from the receptors or indirectly by opening of Ca^{2+} channels increasing intracellular Ca^{2+}. Increases in c-fos and c-jun expression have been correlated with increased intracellular Ca^{2+} levels and could cause induction of the AP-1 transcription factor which can mediate expression of any genes with an AP-1 binding site in their promoter region.

It remains to be discovered what types of cellular connections there are between the TBI-induced genes, as well as the elucidation of the function of the genes recruited by TBI. The IEGs seem to function primarily as

transcription factors regulating the expression of other genes. However, since they posses the ability to affect a wide variety of genes they represent an important step in understanding TBI pathology. It is possible that the genes activated by c-fos/c-jun can serve either a protective or degenerative function. This imposes a serious limitation on the use of AP-1 as a treatment, thus more specific details in the pathways are needed, as exemplified by the determination of the specific steps with cyclooxygenase and lipoxygenase.

NGF has been shown to stimulate growth (Burt, 1993) and spare injured neurons (Hayes et al., 1992). BDNF has also been shown to increase neuronal survival in culture and prevent death in vivo (Burt, 1993). The genes for both of these proteins have been shown to be activated following TBI. They represent proteins which might serve a protective function in TBI and as such, they warrant further research. Their upregulation might provide a treatment for "rescuing" cells following TBI.

The BDNF gene data indicates an increase in cell survivability and neuroprotection (Hicks et al., 1997). It is evident that the protective effects of BDNF and other

neurotrophins are exerted through interactions with their receptors which also show an increased transcription in the same areas as BDNF (Hicks et al, 1998).

The data on cytokines demonstrate their concurrent expression in brain areas susceptible to TBI and they are known to participate in apoptosis (Westmoreland et al., 1996). NMDA antagonism with HU-211 showed a reduction in the cytokine TNF-α production, as well as improved clinical outcome of test subjects (Shohami et al., 1997). Interleukin-1 receptor antagonism has also been shown to decrease neuronal damage and offer neuroprotection (Rothwell, 1996). This would indicate a potentially harmful role of an acute increase in cytokine expression following TBI.

It is important to note that while most studies indicate a pathological role for cytokines, there is some belief that their presence might be beneficial for the cell. A study by Strijbos and Rothwell (1995) indicated a neurotrophic effect of IL-1 against excitotoxic damage. Further studies are definitely warranted with inhibitors of cytokine function to further determine the clinical efficacy in head injury.

Heat shock proteins have been known to play a role in cellular repair and protection (as reviewed in Mayer and Brown, 1994). Although different mechanisms of activation have been proposed such as blood brain barrier compromise, direct physical insult, and increased intracellular Ca^{2+}, it is known that activation occurs following TBI (New et al., 1989; Xue and Grossfield, 1993; and Tanno et al., 1993). These proteins have also been shown to provide protection from glutamate toxicity (Lowenstein et al., 1991 and Rordorf et al., 1991). Heat shock genes are genes for which a lot of information has been gathered and should represent a viable option for treatment through their induction.

The increases seen in GRP expression are still inconclusive with regard to treatment options. If these proteins serve to modulate and buffer calcium levels they may be helpful. However, if increased calcium levels serve to increase gene transcription of potentially beneficial genes, such as heat shock proteins, gross modulation of calcium levels may not be helpful to the cell. More studies need to be performed aimed at investigating clinical outcome of TBI patients with regard to GRP

expression. Using specific inhibitors similar to what was performed to investigate TNF-α might be helpful (Shohami et al., 1997).

The data concerning GFAP indicates an increase following trauma (Kost-Mikucki and Oblinger, 1991). Since it is believed that GFAP-related scarring impedes axonal regeneration, further studies on GFAP gene expression are needed, and might provide a site for therapeutic intervention (Bignami and Dahl, 1976).

The apoptosis-suppressor bcl-2 is another gene which represents a potential for therapeutic intervention. The gene expression and translation of bcl-2 was shown to be activated in surviving neurons found within regions which are susceptible to apoptotic death following TBI (ipsilateral cortex, hippocampus, and dentate gyrus) (Rink et al., 1995).

Do these genes whose transcription is altered merely serve to mark cells that are injured or do they also serve to function in cellular repair? There is much work still left in this field especially in examining the regulatory mechanisms associated with gene expression. If glutamate concentrations do stimulate expression of genes which cause

the resistance of lethal glutamate concentrations, it would seem that glutamate would have a dual effect on the cell. Although it is neurotoxic to the cell, it is also necessary for the cell to launch mechanisms to help resist the effects of the glutamate. It will be interesting to see what future experiments tell us about the varying roles of excitatory amino acids such as glutamate and aspartate.

The fact remains that many possible routes exist for intervention in the process of excitotoxicity. The amount of information on the NMDA receptor is vast and constantly growing, putting us closer to designing therapeutic solutions. One possibility is to design a noncompetitive NMDA antagonist that will bind specifically or preferentially to neural NMDA receptors. The glycine site offers an excellent blockade site which would permit antagonism but still allow the channel to open and preserve function. One route for this would be to exploit the greater concentration of these receptors found within certain areas of the CNS and thus the stronger probability of binding. For example, a heavy concentration of receptors exists within the hippocampus and most of the effects of TBI such as memory and learning deficits are

known to be processes in which the hippocampus plays an important role (Burt, 1993).

Another aspect to consider would be the use of multiple receptor antagonists. Using a single antagonist only serves to show the results of a single receptor as opposed to excitotoxicity which is the function of multiple transmitter receptors. Use of antagonists to other potentially excitatory receptors, such as scopolamine muscarinic receptors, is currently under investigation (Hamm et al., 1995 and Phillips et al., 1997). Synergistic approaches with antagonists and other pharmacological therapies such as dantrolene are also currently under investigation.

The research on TBI-induced changes in gene expression to date is tempered with the consideration that the data only indicates change in gene expression. The reader must recall that there are several additional sites for modulation following transcription such as message stability, translational efficiency, and post-translational modification. A direct 1:1 relationship does not necessarily exist between increase in message and protein.

As with the early phases of any research, the data addressing gene expression and TBI is mostly descriptive and presents a variety of genes which are shown to be modulated following injury. Future research will definitely focus on these genes and their contributions to the injury mechanism or recovery processes.

VII. BIBLIOGRAPHY

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