REGULATION OF *FOSB* MRNA ISOFORMS BY DRUGS OF ABUSE

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DEDICATION

I dedicate this work to my parents, Almas and Nizam Alibhai,

for their unending love and support.

REGULATION OF *FOSB* MRNA ISOFORMS BY DRUGS OF ABUSE

by

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ΔFosB, a truncated splice isoform of FosB, is a transcription factor that accumulates within a subset of neurons after chronic administration of drugs of abuse or other chronic stimuli. Due likely to its structure and post-translational modifications, ΔFosB protein is uniquely stable relative to the transiently expressed full-length FosB and all other Fos family proteins. The goal of this study was to determine if the relative expression of the two *fosB* isoforms is regulated at the mRNA level, thereby further contributing to the accumulation of ΔFosB. First, unlike the protein, the half-life of Δ*fosB* mRNA is only slightly longer than that

of full-length *fosB* mRNA both in cultured cells *in vitro* and in the brain *in vivo*. Additionally, similar to *c-fos*, both *fosB* isoforms are induced abundantly in striatum after acute administration of amphetamine and partially desensitize after chronic dosing. Surprisingly, the relative ratio of the *fosB* to Δ*fosB* mRNA (normally 16:1 in saline controls) decreases significantly only after acute doses or at doses that elicit the greatest induction of both transcripts. When acute amphetamine doses are incrementally increased, *fosB* levels are induced to roughly equivalent levels regardless of dose; however, Δ*fosB* levels increase as the drug dose increases. A similar pattern of *fosB* and Δ*fosB* mRNA induction was seen in cell culture. These findings suggest that the splicing of *fosB* RNA may be regulated by the quantity of unspliced transcript available to the splicing machinery. That is, above a certain threshold of full-length *fosB*, the remaining primary transcript is alternatively spliced into Δ*fosB*. This splicing phenomenon is likely regulated by the Polypyrimidine Tract Binding (PTB1) protein. PTB1 protein has been shown to inhibit the U2AF splicing complex and thus prevent alternative splicing of regions in close proximity to where it is bound. It has previously been demonstrated that PTB1 protein binds the *fosB* transcript *in vitro*. Here, it is shown that overexpression of PTB1 in PC12 cells alters the ratios of the *fosB* isoforms by increasing the amount of *fosB* transcript relative to Δ*fosB* transcript. Therefore, this study concludes that under basal conditions PTB1 protein binds the majority of the *fosB* pre-mRNA, thereby inhibiting the generation of the Δ*fosB* transcript. Only when PTB1 protein is saturated with transcript does the ratio of *fosB* to Δ*fosB* decrease significantly, because the unbound pre-mRNA is spliced into Δ*fosB*. These data provide fundamental information

concerning the generation of Δ*fosB* mRNA and indicate the selective accumulation of ΔFosB protein with chronic drug exposure does not involve its preferential generation by splicing.

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PRIOR PUBLICATIONS AND ABSTRACTS

PUBLICATIONS

Alibhai, I.N.; Green, T.A. and Nestler, E.J. *Modulation of FosB mRNA Isoforms via the Polypyrimidine Tract Binding Protein.* In preparation, 2006.

Green, T.A.; **Alibhai, I.N**.; Hommel, J.D.; DiLeone, R.J.; Kumar, A.; Theobald, D.E.; Neve, R.L. and Nestler, E.J. *Induction of ICER Expression in Nucleus Accumbens by Stress or Amphetamine Increases Behavioral Responses to Emotional Stimuli.* J Neurosci (in press), 2006.

Carle, T.L.; **Alibhai, I.N.**; Nestler, E.J. *Absence of Conserved C-Terminal Degron Domain Contributes to _FosB's Unique Stability.* In submission. 2006.

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ABSTRACTS

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"Viral-mediated Overexpression of ICER in the Nucleus Accumbens Increases Behavioral Responses to Emotional Stimuli Program." Society for Neuroscience Meeting, Nov. 2004, San Diego, CA.

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LIST OF DEFINITIONS

AAV – Adeno Associated Virus

AP-1 – Activator Protein 1

BPS – Branch Point Sequence

c-Fos – Cellular oncogene Fos

Cox - Cyclooxygenase

FosB – FBJ (Finkel-Biskis-Jinkins) osteosarcoma oncogene B

ECS – Electroconvulsive shock

EGFP– Enhanced Green Fluorescent Protein

Egr-1 – Early Growth Response -1

CREB – Cyclic AMP Response Element Binding Protein

GAPDH – Glyceraldehyde-3-phosphate Dehydrogenase

hnRNPs – Heterogeneous Nuclear Ribonucleoproteins

Hu – Human embryonic lethal abnormal vision

KH – K Homology RNA binding domain

KSRP – KH-type splicing regulatory protein

IEG – Immediate Early Gene

IP – Intraperitoneal

L-DOPA – 3,4 dihydroxyphenyl-L-alanine

mRNA – messenger RNA

MSK1 - Mitogen- and Stress-activated protein Kinase 1

OG2 - Osteocalcin

PEST – regions rich in proline, glutamate, serine, and threonine

PKA – Protein Kinase A

PMA – Phorbol 12-myristate 13-acetate

PSI – P element Somatic Inhibitor

PTB1 – Polypyrimidine Tract Binding Protein

PTB2 – Neuronal PTB

Puf – Pumilio–Fem-3-binding factor

PY – Polypyrimidine Tract

q-PCR – Quantitative Polymerase Chain Reaction

RBP – RNA Binding Protein

RISC – RNA-Induced Silencing Complex

RRM – RNA binding domains

shRNA – short hairpin RNA

siRNA – small interfering RNA

snRNP – Small Nuclear Ribonucleoproteins

SR – Serine/Arginine-Rich

SRE – Serum Response Element

SRF – Serum Response Factor

Sxl – Sex Lethal

U2AF – U2 small nuclear ribonucleoprotein Auxiliary Factor

UTR – Untranslated Region

CHAPTER ONE
Introduction

Drug Addiction

According to the Office of National Drug Control Policy's 1999 National Drug Control Strategy, there are approximately 4 million chronic drug users in the United States. The National Institute on Alcohol Abuse and Alcoholism report, Improving the Delivery of Alcohol Treatment and Prevention Services, estimates there are 14 million substance abusers. Regardless of the source, a conservative estimate of those in need of substance abuse treatment is between 13 and 16 million people. The estimated societal cost of drug addiction is a staggering 110-160 billion dollars a year. Drug abuse drives some of America's most costly social problems—including domestic violence, child abuse, chronic mental illness, the spread of AIDS, and homelessness. Drug abuse among the homeless and the chronic mentally ill has been conservatively estimated at better than 50 percent. The Centers for Disease Control and Prevention has estimated that 36 percent of new HIV cases are directly or indirectly linked to injecting drug users (Statistics taken from www.nida.com and www.dea.com).

Drug addiction is a biological, psychological and behavioral process driven by the serious compulsive use of a substance despite adverse consequences[1]. Drugs of abuse are particularly dangerous as they are both rewarding and reinforcing. A reward is a stimulus the brain recognizes as positive, or as something to be approached. A reinforcing stimulus is one that increases the likelihood that behaviors paired with it will be repeated[2]. Generally, drug abuse results in tolerance, in which the (behavioral and pharmacological) effect of the drug

decreases after repeated administration at the same dose and consequently the need for increased dose to maintain the same response[1]. Additionally, certain classes of drugs (such as opiates) are marked by a state of physical and/or psychological disturbance (i.e., withdrawal) when the drug is removed[2].

AP-1 Complex

In 1990 and 1991, two groups demonstrated the induction of protein isoforms from the Fos and Jun family of transcription factors in striatal tissue after acute administration of cocaine (or amphetamine)[3, 4]. Using a pan-Fos antibody, c-Fos and Fos-like proteins where transiently induced (2-4 hours) and degraded to basal levels within 12 hours. The induction profile of the Fos protein was concomitant with the appearance of an Activator Protein-1 (AP-1) complex. The AP-1 complex (composed of heterodimeric partnering of Fos and Jun family proteins) is a transcription factor that binds promoter AP-1 sites (consensus sequence: TGAC/GTCA)[5]. Regulation of transcription by the AP-1 complex has been shown to modulate the full spectrum of cellular processes from cell growth to apoptosis[6]. In 1992, the Nestler laboratory tested the effect of chronic cocaine on AP-1 induction[7]. Interestingly, they found the persistence of the AP-1 complex for several days after chronic dosing compared to the transient expression (8-12 hours) after acute administration of cocaine. This long lived complex was aptly named the "chronic AP-1 complex"[7].

ΔFosB Biochemistry

The chronic AP-1 complex was later determined to be composed of JunD and ΔFosB. JunD has a comparable half-life to other Jun family proteins; however, ΔFosB has an unusually long half-life compared to other members of the Fos family of transcription factors[8-10]. Genes encoding Fos proteins are considered immediate early genes (IEGs) due to their rapid and transient expression in the absence of *de novo* protein synthesis. The term was originally used exclusively for viral genes where immediate-early referred to transcription immediately following virus integration into the host cell. It is also used to describe cellular genes that are expressed immediately after resting cells are stimulated by extracellular signals such as growth factors and neurotransmitters[11]. The instability of the Fos isoforms is mediated by motifs found in the mRNA as well as the protein. Both *c-fos* and *fosB* contain AU rich elements (that confer instability to the transcript) in the 3' untranslated region of their respective mRNAs[5]. In addition, both proteins express C- terminal PEST domains (regions rich in proline, glutamate, serine, and threonine), which are polypeptide sequences correlated with targeting proteins for rapid degradation[12].

In contrast to all other members of the Fos family, ΔFosB is a unique product for an immediate early gene. ΔFosB is a truncated isoform that lacks the C-terminal 101 amino acids of the full length FosB protein. Whereas other Fos proteins are induced rapidly and transiently in response to an acute stimulus, and return to basal levels within several hours, isoforms of ΔFosB are stable with *in-vivo* half-lives estimated at weeks[13]. As a result, after repeated stimulation, there is a gradual accumulation of stable 35-37 kDa ΔFosB isoforms (see Figure 1).

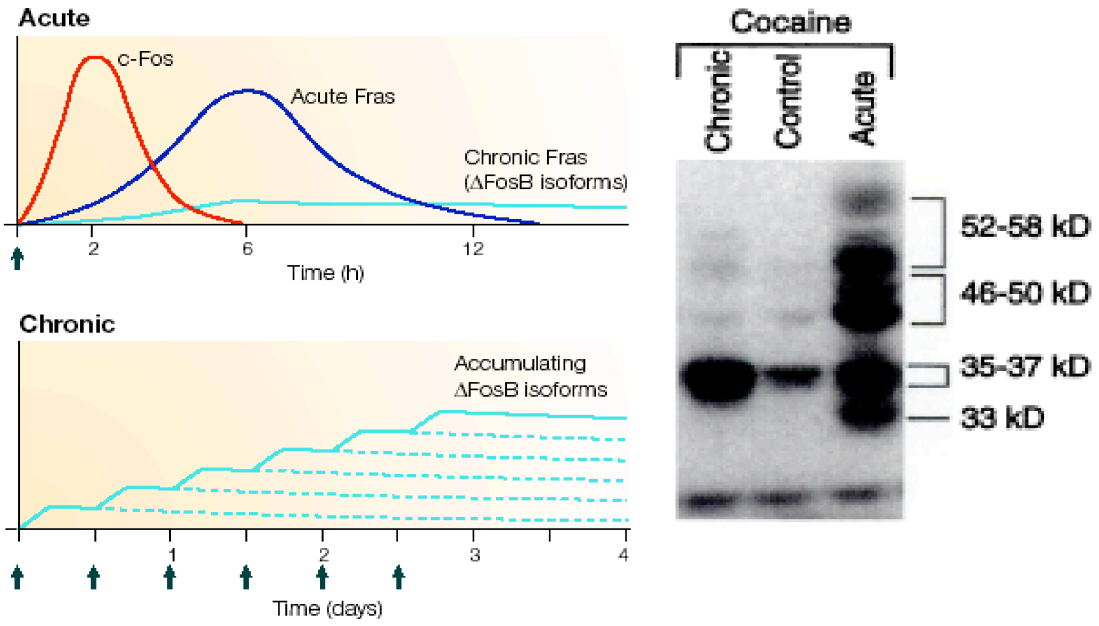


Figure 1. **Regulation of ΔFosB by drugs of abuse.** The top graph shows the several waves of induction of Fos family proteins after a single exposure (arrow) to a drug of abuse. These proteins include c-Fos and acute Fras (FosB), which are relatively unstable. By contrast, isoforms of ΔFosB are highly stable and therefore persist in the brain long after drug exposure. Because of this stability, ΔFosB accumulates with repeated drug exposure, as shown in the bottom graph. Modified from Nestler, 2001[14]. The gel inset shows an example of this phenomenon: the induction of c-Fos (52-58 kDa), FosB (46-50 kDa) and the 33-kDa isoform of ΔFosB in the nucleus accumbens after an acute cocaine exposure, and the switch to the predominant and selective induction of 35– 37 kDa isoforms of ΔFosB after chronic cocaine administration. Modified from Perrotti et al.[15].

Several types of chronic perturbation have been shown to induce ΔFosB in region-specific patterns in the brain (Table 1). A variety of chronic stimuli induce ΔFosB, including drugs of abuse, antidepressant treatment and stress. This induction is seen predominantly in the nucleus accumbens and dorsal striatum, although lower levels of induction occur in several other brain areas, such as prefrontal cortex and amygdala. Interestingly, repetition of naturally rewarding behavior, for example, excessive wheel running or sucrose drinking, also induces ΔFosB[16].

Stimulus	Brain regions[a]
Drugs of abuse	
Cocaine	Nucleus accumbens, dorsal striatum, prefrontal cortex, amygdala, ventral tegmental area
Amphetamine	Nucleus accumbens, dorsal striatum
Morphine	Nucleus accumbens, dorsal striatum
Nicotine	Nucleus accumbens, prefrontal cortex, dorsal striatum
Alcohol	Nucleus accumbens, prefrontal cortex
Phencyclidine	Nucleus accumbens, dorsal striatum
Morphine withdrawal	Locus coeruleus, ventral tegmental area, nucleus accumbens, dorsal striatum
Antidepressant treatments	
ECS	Hippocampus, frontal cortex, parietal cortex, striatum
Tranylcypromine	Hippocampus, frontal cortex
Antipsychotic drugs	
First generation	Nucleus accumbens, dorsal striatum, frontal cortex
Second generation	Frontal cortex
Stress	
Restraint	Prefrontal cortex, nucleus accumbens, amygdala, septum, locus coeruleus
Unpredictable	Prefrontal cortex, nucleus accumbens, amygdala, septum, locus coeruleus
Foot shock	Nucleus accumbens, dorsal raphe
Kainic acid	Hippocampus, striatum
Dopamine denervation	Dorsal striatum

Table 1. **Examples of stimuli that induce ΔFosB.** The listed brain regions are those in which ΔFosB is most highly expressed. Notice that a variety of stimuli outside drugs of abuse induce ΔFosB. Modified from McClung et al.[17].

ΔFosB acts as a functional transcription factor

Initially it was suggested that ΔFosB acted as a repressor to AP-1 transcription by presumably competing with full length Fos proteins at the steps of dimerization with Jun proteins and binding to DNA[18]. A priori, this premise makes sense as ΔFosB retains its dimerization and DNA binding motifs but the 101 amino acid truncation eliminates the transactivation domain. Several lines of evidence have now disproved this hypothesis. First, ΔFosB can drive transcription of a luciferase reporter coupled to AP-1 sites (unpublished results). In addition a dominant negative Jun protein termed ΔcJun can block the action of ΔFosB by presumably preventing heterodimerization with other productive Jun proteins[19].

Further, via studies of candidate genes, ΔFosB has been shown to induce GluR2 (AMPA receptor subunit) in the nucleus accumbens, whereas cocaine induction of GluR2 is blocked by ΔcJun[20]. Finally, using microarray technology McClung et al.[17] demonstrated that early expression of ΔFosB seems to act as transcriptional repressor as many of the genes repressed by ΔFosB are also repressed by ΔcJun. Interestingly, as ΔFosB accumulates a majority (64%) of genes regulated by ΔFosB are regulated in a reciprocal manner to ΔcJun indicating that ΔFosB acts a potent transcriptional activator after long term expression.

Behavioral plasticity regulated by ΔFosB.

The generation of *fosB* knockout mice was the first evidence that ΔFosB mediated complex behaviors[21]. These mutant mice showed a heightened sensitivity to cocaine; however, they failed to show behavioral sensitization to repeated drug administration. Additionally, the FosB knockouts showed abnormal biochemical and behavioral responses to electroconvulsive shock[22]. The interpretation of this phenotype is confounded by the loss of both FosB isoforms in all cells throughout development.

To circumvent this issue, the Nestler laboratory generated transgenic mice that have inducible expression of the ΔFosB protein in a specific subset of neurons in the striatum[20]. Overexpression of ΔFosB was shown to increase an animal's sensitivity and locomotor activity to acute as well as chronic administration of cocaine[20]. They also showed increased preference for cocaine and morphine in place-conditioning assays[20, 23], increased self-administration of low doses of cocaine, and increased motivation for cocaine using a progressive ratio paradigm[20]. In addition, the ΔFosB overexpressing mice show reduced

analgesic responses to morphine, as well as exaggerated development of physical morphine dependence[23]. These results have been replicated with the use of adeno-associated virus (AAV). The Nestler lab has shown that overexpression of ΔFosB in discrete areas of the brain via AAV infection leads to the same behavioral plasticity found in the transgenic mice[24]. Therefore, these data suggest that the accumulation of ΔFosB after chronic stimulation is an important component in the development of addictive behaviors and have lead to the hypothesis that ΔFosB acts as the "molecular switch" to drug addiction[25].

The role of ΔFosB is not restricted to drug addiction. In both rodent and monkey models of Parkinson's disease, in which midbrain dopamine neurons are lesioned, chronic treatment with 3,4 dihydroxyphenyl-L-alanine (L-DOPA, a precursor to dopamine) gradually induced dyskinetic side-effects. Consequently, the use of L-DOPA in Parkinson's disease patients is limited by these side effects, which are thought to be linked to underlying changes in striatal gene expression. ΔFosB may modulate this expression as its gradual appearance correlates temporally with the abnormal movements in animal models[26, 27]. Similar to drugs of abuse, an acute administration of L-DOPA to a dopamine denervated animal induced several Fos family proteins in the striatum. In contrast, chronic dopamine replacement failed to induce these proteins, but instead caused a selective increase in ΔFosB, which was restricted to dynorphin-containing neurons of the striatum[26, 27].

Finally, FosB expression is not restricted to the brain as both isoforms are ubiquitously expressed in varying degrees at both the mRNA and protein level. It has been demonstrated that in non-neuronal cells, ΔFosB plays unique roles distinct from its apparent function in the brain. Transgenic mice overexpressing ΔFosB, under the control of the

neuron-specific enolase promoter show both markedly increased bone formation and decreased adipogenesis[28]. To determine whether the two phenotypes were linked, Kveiborg et al.[29] targeted overexpression of ΔFosB in mice to the osteoblast by using the osteocalcin (OG2) promoter. OG2-ΔFosB mice demonstrated increased osteoblast numbers and an osteosclerotic phenotype but normal adipocyte differentiation. This result suggests that the skeletal phenotype is cell autonomous to the osteoblast lineage and independent of adipocyte formation[29].

The fosB Gene

As the descriptive literature on the behavioral plasticity of ΔFosB protein grows it becomes increasingly evident that this transcription factor plays an important role as the molecular switch to drug addiction. Unlike the other Fos transcription factors, ΔFosB is able to exert long-term adaptive changes on neural circuitry because of its longevity. The structure and post-translational modification state of ΔFosB protein presumably play key roles in its uniquely stability relative to the transiently expressed full-length FosB. The Nestler laboratory has studied both the phosphorylation and ubiquitination states of ΔFosB in an effort to understand if these post-translational changes contribute to the longevity of the protein (the result of these efforts are found in the Discussion). The goal of this work is to determine if the relative expression of the two *fosB* isoforms is regulated at the mRNA level, thereby further contributing to the accumulation of ΔFosB protein.

The *fosB* gene consists of four exons (see Figure 1 in the Results). Exons I, II and III are constitutively expressed or included in both the final *fosB* and Δ*fosB* mRNA. Exon IV

has an unique structure. Embedded in exon IV is an intron that is expressed in the final *fosB* transcript. This phenomenon of an intron found in the open reading frame of the mRNA which codes for protein is rare and is known as intron retention[30]. To this point there are approximately 25 predicted genes in the human genome that have evolutionarly conserved introns located in the open reading frame of the mRNA and consequently code for protein[31]. Though the fourth intron of the *fosB* gene is only 140 nucleotides long it contains all the motifs found in much larger constitutively excised introns: a 5' splice site, a branch point, a polypyrimidine tract and a 3' splice site. For clarity's sake, the exons found upstream and downstream of intron 4 are labeled as exons IVa and IVb. Δ*fosB* mRNA is generated by the excision of the 140 nucleotide intron 4. In addition, this splice event results in a one nucleotide frameshift in which a stop codon (TGA) is generated. Except for the exon/exon junction of IVa and IVb, Δ*fosB* mRNA has no unique sequence that is not found in *fosB* mRNA. Based on this information and the manipulation of *in vivo* and *in vitro* systems it is the goal of the present work to determine the regulation of *fosB* mRNA isoforms by drugs of abuse.

CHAPTER TWO
Methodology

Animals

Male Sprague-Dawley rats (Harlan, Houston), 250-350 g, were used for all experiments. Rats were pair-housed in an AAALAC-approved colony and all experiments conformed to the NIH Guide for the use and care of laboratory animals (1995). Rats were housed on a 12hr/12hr light/dark cycle with lights on at 7:00 a.m. All procedures were conducted during the light phase of the cycle.

Amphetamine injection

Amphetamine hemi-sulfate (Sigma, St. Louis, MO) was administered at a dose of 4 mg/kg (IP). For all experiments, animals received 7 daily injections of saline or amphetamine. In other words, "acute" amphetamine animals received 6 daily injections of saline, followed by an amphetamine injection on day 7, while "7 doses" amphetamine animals received 7 daily amphetamine injections. Additionally, animals were pre-treated with 3 daily injections of saline for habituation resulting in 10 total IP injections. These drug doses and treatment regimens were based on earlier studies of amphetamine induction of ΔFosB and other Fos family proteins[32].

Restraint Stress

Rats were placed in a plastic conical sleeve (DecapiCone, Braintree; model DC200) for up to 60 min. Rats were removed from the cone and placed back into their home cage for time points exceeding 60 min. Immobilization occurred for 1 hr daily over a period of 1-9 consecutive days. Control rats were either not handled or were handled daily but not

subjected to stress. This stress paradigm was used, since it was recently shown to robustly induce ΔFosB in striatum[15].

Rats were killed by rapid decapitation, whole striatum was dissected and placed in RNA Stat-60 reagent (Tel-Test, Friendswood, TX). The tissue was briefly sonicated to homogeneity by a tabletop Ultrasonic Processor (Cole Parmer, Vernon Hills, IL) set at an amplitude of 40.

Cell culture and transfections

PC12 cells (Clontech, Mountain View, CA) were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% horse serum and 5% fetal bovine serum (Invitrogen) with antibiotics at 37°C and 95% O_2-5% CO_2. Cells were transiently transfected with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Transfection efficiency was determined by co-expression of GFP with all plasmids. The total amount of DNA within experiments was kept constant by adding empty vector to the transfection mixture when necessary. PMA (Sigma) and Actinomycin D (Sigma) were dissolved in DMSO and ethanol, respectively.

Design and construction of shRNA

Hairpin RNA was designed to target specific regions of *PTB1* mRNA. Five 24-nucleotide sequences were identified within the coding region of *PTB1* using published criteria[33]. The hairpins were designed such that the antisense strand came before the sense strand during transcription. Six sets of oligonucleotides (5 against *PTB1* and 1 control) (Qiagen) were synthesized for cloning:

shPTBI(Top:5'TTTGAATAAAGAGGCTTTGGGGTGTGACTTCCTGTCATCACACCCC
AAAGCCTCTTTATTATTTTT 3',

Bottom:5’CTAGAAAAATAATAAAGAGGCTTTGGGGTGTGATGACAGGAAGTCACA
CCCCAAAGCCTCTTTATTC 3’),
shPTBII(Top:5’TTTGAGAATAAAGAGGCTTTGGGGTGTCTTCCTGTCAACACCCCA
AAGCCTCTTTATTCTATTTTT 3’,
Bottom:5’CTAGAAAAATAGAATAAAGAGGCTTTGGGGTGTTGACAGGAAGACACC
CCAAAGCCTCTTTATTCTC 3’),
shPTBIII(Top:5’TTTGCATTGTAGATGTTCTGGCCATCCCTTCCTGTCAGGATGGCC
AGAACATCTACAATGATTTTT 3’,
Bottom:5’CTAGAAAAATCATTGTAGATGTTCTGGCCATCCTGACAGGAAGGGATG
GCCAGAACATCTACAATGC 3’),
shPTBIV(Top:5’TTTGAACTGGATGTAGATGGGCTGTCCCTTCCTGTCAGGACAGCC
CATCTACATCCAGTTATTTTT 3’,
Bottom:5’CTAGAAAAATAACTGGATGTAGATGGGCTGTCCTGACAGGAAGGGACA
GCCCATCTACATCCAGTTC 3’),
shPTBV(Top:5’TTTGCTATCATTTCCGTTGGCTGCTGACTTCCTGTCATCAGCAGCC
AACGGAAATGATAGATTTTT 3’,
Bottom:5’CTAGAAAAATCTATCATTTCCGTTGGCTGCTGATGACAGGAAGTCAGCA
GCCAACGGAAATGATAGC 3’) and
shCON(Top:5’TTTGTGGAGGCGACATTCATAATTCCGCTTCCTGTCACGGAATTAT
GAATGTCGCCTCCAATTTTT 3’,
Bottom:5’CTAGAAAAATTGGAGGCGACATTCATAATTCCGTGACAGGAAGCGGAA
TTATGAATGTCGCCTCCAC 3’). The oligonucleotides had *Sap*I and *Xba*I overhangs to

allow for ligation downstream of the mU6pro promoter in the pAAV plasmid and all final clones were verified by sequencing. The pAAV plasmid was designed to co-express EGFP under the control of an independent RNA polymerase II promoter and terminator[34]. The constructs were transfected into cells using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, cells were sorted by FACS based on EGFP fluorescence and positive cells were re-plated. After 24 hours the cells were stimulated with 100nM PMA for 1 hour and harvested for RNA.

RNA Isolation

RNA was harvested following the manufacturers protocol. Briefly, the organic layer was extracted with chloroform and precipitated with isopropanol in the presence of Linear Acrylamide (Ambion, Austin TX). The RNA pellet was washed with 70% ethanol and re-suspended in DEPC water. The purified total RNA was DNAse treated (Ambion) and reverse transcribed to cDNA with random hexamers using a first-strand synthesis kit (Invitrogen). The amount of cDNA synthesized from a target mRNA was quantified using q-PCR. Primer sets are listed in Table 1. Real-time PCR was performed in triplicate using an Applied Biosystems 7500 Real-Time PCR System (95°C- 10 min, 1cycle; 95°C-20 sec, variable annealing temperatures, see Table 1- 30 sec, 72°C- 33 sec, 35 cycles; melt curve from 60°C-95°C) with SYBR Green Master Mix (Applied Biosystems, Foster City, CA). GAPDH quantification was used as an internal control for normalization. Fold differences of mRNA levels over control values were calculated using the $\Delta\Delta C_t$ method as described in the Applied Biosystems manual.

Protein Isolation

Method 1: Striatum was isolated from amphetamine- and saline-treated rats by gross dissection from 1-mm-thick coronal sections. Brain samples were sonicated to homogeneity in a buffer containing 20 mM HEPES, 0.4 M NaCl, 20% (v/v) glycerol, 5 mM MgCl, 0.5 mM EDTA, 0.1 mM EGTA, 1% (v/v) NP-40, 1 M DTT, 1 M PMSF, and 5 µl/ml of a protease inhibitor mixture. Protein concentration was determined by the DC protein assay method (Bio-Rad) using bovine serum albumin as a standard[9].

Method 2: Protein was precipitated from the organic layer of RNA samples following the manufacturers protocol supplied by Tel-test with the following modifications. After isopropanol precipitation, the protein pellet was washed with 0.3M guanidine hydrochloride (Sigma) in 95% ethanol. Previous to centrifugation, the samples were placed in a VWR Model 50HT water bath sonicater (West Chester, PA) to resuspend the pellet into a heterogeneously uniform mixture. The samples were washed twice more in 0.3M guanidine hydrochloride in 95% ethanol and once in 100% ethanol. The protein pellet was dried in a Savant SC110A Speed Vac (Thermo Electron Corporation, Waltham, MA) and re-suspended in 1% (w/v) SDS. The samples were heated for 10 minutes at 55 °C and then sonicated briefly with an Ultrasonic Processor set at an amplitude of 40. Finally, the samples were spun at maximum speed for 10 minutes at room temperature in an Eppendorf 5417R table-top centrifuge (Brinkmann Instruments) to pellet insoluble material. Protein concentration was determined by the DC protein assay method (Bio-Rad) using bovine serum albumin as a standard.

Fractionation

Rats were administered 4 mg/kg amphetamine or saline for 1 or 7 days by IP. Animals were killed 1 hour after the last injection. After gross dissection striatal tissue was homogenized by a Dounce homogenizer in 500 µl of 0.32 M sucrose, 1 mM $MgCl_2$. The homogenate was diluted with 0.32 M sucrose to 2 mL before centrifugation (2000 x g, 10 min), to obtain the crude nuclear fraction (P1). The supernatant (S1) was saved. The pellet was re-suspended in PBS and washed twice. The resulting P1 pellet was homogenized in 0.32 M sucrose, and pelleted again (20,000 x G, 10 min) to obtain the soluble P1 fraction. The S1 fraction was pelleted, (100,000 x G, 1 hr) and the supernatant saved to obtain the crude cytosolic fraction (S3). Protein concentration was determined by the DC protein assay method (Bio-Rad) using bovine serum albumin as a standard.

Western Blot

Equal amounts of protein (50 µg) were separated by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide gels), and transferred to PVDF membranes (0.2 µm) (Bio-Rad). Membranes were blocked for 60 minutes in TBST (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% (v/v) TWEEN-20) containing 5% (w/v) nonfat dry milk and immunoblotted using a 1:1,000 dilution of a polyclonal rabbit FosB antibody (generated by Biosource, International, Camarillo, CA) or a polyclonal rabbit N-terminal PTB1 antibody (gift of Dr. Douglas Black). A monoclonal mouse GAPDH antibody (Pierce, Rockford, IL) was used at a dilution of 1:10,000 to verify equal loading and transfer. After washing 4 times for 20 minutes in TBST, antibody binding was revealed by incubation with a 1:10,000 dilution of goat anti-rabbit horseradish peroxidase-linked IgG (Pierce) and the SuperSignal West Dura immunoblotting detection system (Pierce). A 1:20,000 dilution of rabbit anti- mouse

horseradish peroxidase-linked IgG (Pierce) was used to identify GAPDH.

Chemiluminescence was detected by autoradiography using Kodak autoradiography film.

TABLE

q-PCR PRIMERS

Primer Pair	Oligonucleotide Sequence	Annealing Temp.
c-Fos	For:5'- 5'-GGAATTAACCTGGTGCTGGA-3' Rev:5'-TGAACATGGACGCTGAAGAG-3'	59°C
ΔFosB	For:5'-AGGCAGAGCTGGAGTCGGAGAT-3' Rev:5'-GCCGAGGACTTGAACTTCACTCG-3'	61°C
FosB	For:5'-GTGAGAGATTTGCCAGGGTC-3' Rev:5'-AGAGAGAAGCCGTCAGGTTG-3'	61°C
GAPDH	For:5'-AGGTCGGTGTGAACGGATTTG-3' Rev:5'-TGTAGACCATGTAGTTGAGGTCA-3'	59-61°C
PTB1	For:5'-AACGGTGGTGTGGTCAAAGG-3' Rev:5'-GTGGACTTGGAAAAGGACACTC-3'	61°C
PTB2	For:5'-CATCCCTCTTAGCTGTTCCAGG-3' Rev:5'-GGGCGTAACCATCTCTTCATTTA-3'	60°C

CHAPTER THREE
Results

Overall Goals

Due likely to its structure and post-translational modification state, ΔFosB protein is uniquely stable relative to full-length FosB. The goal of this work was to determine if the relative expression of the two isoforms is regulated at the mRNA level, thereby further contributing to the accumulation of ΔFosB. Initially, RNA from amphetamine treated Sprague Dawley rats was quantified by q-PCR to characterize the induction of the *fosB* mRNA isoforms *in vivo*. After which, a cell culture system was designed to recapitulate endogenous expression of the *fosB* transcripts. The *in vivo* and *in vitro* paradigms identified the mRNA half-lives as well as relative ratios of the two isoforms after acute and chronic stimulation. Using overexpression and siRNA knockdown strategies an RNA binding protein was identified that regulates the relative levels of *fosB* to Δ*fosB* mRNA.

Experimental Design

Figure 1B depicts the 3' ends of both *fosB* isoforms. Δ*fosB* mRNA is generated by a 140 nucleotide excision of "intronic" sequence found in the open reading frame of full-length *fosB* mRNA. In addition, the splice event results in a one nucleotide frameshift in which a stop codon (TGA) is recognized. Consequently, the only region unique to *fosB* is this 140 nucleotide sequence (colored in yellow) because the 3' UTR of Δ*fosB* is identical to the coding region of exon IVb and subsequent 3' UTR of *fosB*. Primers for amplification of *fosB* were directed to this unique intronic region. A variety of primers were tested to amplify Δ*fosB* because its only distinguishing characteristic from *fosB* is the exon-exon junction

between IVa and IVb. It was determined that a reverse primer (see Table 1 for primer sequences) spanning this junction was specific and most efficient at amplifying Δ*fosB* (Figure 1B). Additionally, assuming that both isoforms have identical 3' UTRs, then the two primer pairs are almost the same distance from their respective poly A tails. This is an important consideration when using oligo dT primers in the reverse transcription reaction as the polymerase becomes less efficient at producing accurate first strand cDNAs - which are several kb in length farther - from the poly A tail.

Figure 2 is a diagram of how tissue and cell culture samples were processed for data collection. The extraction of total RNA (and subsequent cDNA synthesis) from the aqueous layer of a triazole reagent (RNA STAT 60) was performed following manufacturers protocols. By modifying a protocol for protein precipitation from the remaining organic layer of the processed samples, both protein and total RNA could be harvested from the same samples. The western blot in Figure 2 demonstrates that brain tissue from one hemisphere processed by protein precipitation from the organic layer of a RNA sample yields the same signal as tissue from the other hemisphere processed in a homogenizing buffer used in more conventional methods[9].

Endogenous expression of fosB isoforms in vitro

Initially, a main focus of this study was to generate a method to endogenously express both FosB isoforms in cell culture because previously the laboratory used overexpression to study protein half-lives. A range of stimuli induced both protein isoforms. Figure 3A is a representative western blot of a time course of serum stimulation in PC12 cells. After 18

hours of serum starvation, PC12 cells were treated with 20% serum and harvested 0 to 12 hours later. The resulting immunoblot was incubated with an N-terminal anti-FosB antibody that recognizes both FosB and ΔFosB. ΔFosB is initially induced as a 33 kDa protein, whose mass increases to 35-37 kDa. These bands co-migrate with protein extracts from ΔFosB overexpression in PC12 cells. FosB (45-48 kDa), which is partially obscured by a non-specific protein, also increases in molecular mass; however, in contrast to the persistent ΔFosB bands, FosB has begun to degrade at the 8-hour time point. In a similar fashion to serum, PMA, forskolin, and nicotine also induced expression of both protein isoforms.

Using quantitative PCR (q-PCR), PMA (100 nM) and forskolin (10 μM) induction of *fosB* mRNA transcripts was measured in PC12 cells (Figure 4A). The left-hand graph shows both isoforms are induced rapidly and peak at 1 hour of stimulation. After 8 hours, Δ*fosB* and *fosB* return to near baseline levels. The right hand graph depicts the relative ratio of *fosB* to Δ*fosB* mRNA. Normally, the ratio is 12:1 under basal conditions. The relative ratio shifts to 2:1 at 2 hours of drug treatment and rebounds to near control levels by 8 hours. In decreasing effectiveness, serum, PMA, forskolin and nicotine induce both *fosB* transcripts. Though induction values varied for each of the above treatments the relative ratios at maximal induction do not differ. This result suggests expression levels of the 2 isoforms are linked and that none of the tested stimuli can selectively induce one transcript relative to the other.

Induction levels were also measured using the above stimuli in non-neuronal cell lines (HEK293, HeLa and NIH 3T3 cells). Figure 3 depicts the similar FosB /ΔFosB protein induction in PC12 (Figure 3A) and HeLa (Figure 3B) cells after serum stimulation. Different

stimuli induced both mRNA transcripts in these cell lines; however, as shown in Figure 4B, the relative ratios of *fosB* to Δ*fosB* differ from neuronal cells *in vitro* (PC12 cells) and *in vivo* (Striatal tissue) to the tested non-neuronal cell lines. This result was an early indication that PC12 cells were the only easily cultured cell line that could recapitulate *in vivo* neuronal conditions.

In vivo accumulation of fosB isoforms after amphetamine treatment

To measure the relative expression of *fos* isoforms *in vivo* after drug treatment, male Sprague-Dawley rats were treated once daily with amphetamine (4 mg/kg) over a period of 1-7 days. This regimen is known to maximally induce ΔFosB protein in striatum[32]. Animals were killed 1, 3, or 24 hours (Figure 5A, B, C, respectively) after the last injection. RNA was harvested from the striatum and quantified by RT-PCR. The left hand panels depict induction of each isoform relative to its saline control. In agreement with previously published work from the Nestler laboratory[7], *c-fos* transcript is induced acutely and diminishes over the course of treatment (Figure 5A). Similar to *c-fos*, both *fosB* isoforms are induced after acute administration of amphetamine and return to control levels after chronic dosing. Interestingly, the relative ratio (right hand panel, Figure 5A) of *fosB* to Δ*fosB* mRNA (normally 16:1 in saline controls) decreases significantly only after acute doses. A similar pattern of acute induction and subsequent desensitization is seen in Figure 5B; however, at 3 hours, Δ*fosB* levels continue to rise whereas *fosB* levels have begun to decrease. This change is reflected in the relative ratios (right hand panel, Figure 5B) of the isoforms; doses on days 3 through 7 are significantly decreased relative to day 1. By 24 hours (Figure 5C), both *fosB*

isoforms have returned to control levels. To measure accumulation of the *fosB* transcripts, animals were injected acutely or chronically with 4 mg/kg amphetamine and killed over a 12-hour period. Relative to their respective saline controls, *fosB* transcript reaches maximal induction at 1 hour whereas the Δ*fosB* transcript reaches a peak at 3 hours (Figure 6). Both transcripts degrade to control levels by 12 hours. Animals injected chronically with 4 mg/kg of amphetamine showed a similar rate of accumulation and degradation as acutely treated animals, albeit at reduced levels of induction. These data supports previous literature from the Nestler laboratory, which suggested that the *in vivo* half lives of the *fosB* mRNA isoforms were similar[8].

Induction of fosB isoforms by restraint stress

Perrotti et al.[15] demonstrated recently that, like drugs of abuse, chronic restraint stress induces ΔFosB protein in discrete areas of the brain including the striatum. Therefore, this study sought to expand on those experiments to determine if the chronic restraint stress paradigm showed a similar pattern of *fosB* isoform mRNA expression to chronic administration of drugs of abuse. To this end, male Sprague Dawley rats were restrained in plastic cones for a maximum of one hour (Figure 7A). For the 2 and 4- hour time points, animals were removed from the cone and placed back into their home cage after 1 hour of restraint followed by a recovery period of 1 or 3 hours, respectively. Similar to amphetamine treatment, *fosB* induction is greatest at 1 hour, whereas, Δ*fosB* levels continue rise until the 2-hour time point. Figure 7B depicts a time course of restraint stress in which animals were restrained for 1 hour once daily over a period of 1 to 9 days. Once again, similar to

amphetamine treatment, both *fosB* mRNA isoforms are induced after acute restraint stress and desensitize to near control levels after chronic treatment. As expected, amphetamine produced a more robust induction of the Δ*fosB* isoform relative to restraint stress. Under acute conditions the Δ*fosB* transcript was induced approximately 9-fold with amphetamine treatment versus approximately 5-fold with restraint stress. These findings agree with the amphetamine data, where the Δ*fosB* to *fosB* mRNA ratio increases only in the acute situation.

In vivo dose response of fosB induction by amphetamine

The above experiments indicated that Δ*fosB* mRNA was preferentially spliced relative to *fosB* only at acute doses (Figures 5A and 7B) or at doses that elicited the greatest induction of both transcripts. This outcome suggested that the generation of Δ*fosB* transcript was directly proportional to the stimulus applied to the system. To test this hypothesis, animals were acutely administered increasing doses of amphetamine (1-4 mg/kg) and killed 1 (Figure A) or 3 (Figure B) hours after the last injection. As seen in Figure 8, *fosB* levels are induced to roughly equivalent levels regardless of dose; however, Δ*fosB* levels increase as the drug dose increases. The shift in the *fosB* to Δ*fosB* mRNA is, therefore, directly proportional to the strength of the inducing stimulus.

In vitro recapitulation of in vivo findings

To determine an accurate half-life of the *fosB* isoforms, quiescent PC12 cells (serum starved for 18 hours) were re-exposed to 20% serum. After 1 hour of stimulation, the cells were treated with 5 μM actinomycin D and harvested for RNA 0-8 hours later. Figure 9A

shows that Δ*fosB* and *fosB* (graphed relative to their respective controls) degrade at approximately the same rate. Along with the data from Figure 6, with respect to accumulation and degradation, both *fosB* isoforms seem to behave similarly *in vivo* and *in vitro*.

To replicate the *in vivo* dose response, quiescent PC12 cells were treated with increasing amounts of PMA (Figure 9B). Similar to Figure 8, increasing the strength of the stimulus applied to the cell increases the induction of *fosB* mRNA isoforms and shifts the ratio of isoforms further from control levels. These *in vivo* and *in vitro* findings suggest that the splicing of *fosB* pre-mRNA may be regulated by the quantity of unspliced transcript available to the splicing machinery. That is, above a certain threshold of full-length *fosB*, the remaining unspliced transcript is alternatively spliced into Δ*fosB*.

PTB levels in vivo

Lou et al.[35] first described that the polypyrimidine tract-binding (PTB1) protein could compete against the U2AF splicing complex to bind polypyrimidine rich regions in introns, thereby negatively regulating splicing of nearby exons. These data and the presence of a PTB1 consensus binding site in the expressed "intron" flanked by exons IVa and IVb in the *fosB* gene suggested the PTB1 protein as an attractive candidate to regulate the generation of the *fosB* transcript. cDNA from striatum generated for the 1 hour amphetamine time course (Figure 5A) was subjected to real time PCR with primers specific to the *PTB1* gene. Figure 10A demonstrates that *PTB1* mRNA levels do not vary over the course of a 4 mg/kg amphetamine treatment. Similarly, cDNA generated from PC12 cells treated with 100 nM

PMA over a 6-hour time course also do not show differences in *PTB1* transcript levels (Figure 10B). Finally, protein extracted from the organic layer of the experimental samples from Figure 5A (by the protocol described in Figure 1B) illustrate that PTB1 protein levels also remain unchanged through a course of acute and chronic amphetamine administration (Figure 10C.). Together, these data show that PTB1 mRNA and protein stay constant while *fosB* and Δ*fosB* transcripts fluctuate in the same samples.

In vivo PTB1 shuttling

The Black group has shown that PKA phosporylation of PTB1 on a conserved Serine 16 site results in shuttling of the PTB1 protein from the nucleus to the cytoplasm[36]. Additionally, it is well known that PKA activity is upregulated, including in striatum, after chronic administration of drugs of abuse[14, 37]. Therefore, though PTB1 levels do not change after the amphetamine treatment (Figure 10C), the possibility existed that shuttling of the PTB1 protein regulates *fosB* isoform ratios. To test this hypothesis, rats were treated with either saline or acute or chronic doses of amphetamine (4 mg/kg). The animals were killed one hour after their last injection and striatum was obtained by gross dissection. The tissue was homogenized in a sucrose buffer and fractionated into cytoplasmic and nuclear fractions. Figure 11 is a representative immunoblot of the fractions probed with an N-terminal PTB1 antibody. The Western blot indicates that PTB1 levels remain unchanged in the nucleus as well as the cytoplasm over the course of the amphetamine administration.

PTB overexpression

To determine if PTB1 protein could alter the ratio of *fosB* to Δ*fosB*, PC12 cells were transfected with a GFP plasmid plus an empty plasmid, a plasmid encoding Human PTB1 or a plasmid encoding Human PTB2. PTB2, or neuronal PTB, is enriched in the brain and shares high sequence similarity (84% at the amino acid level) to PTB1[38]. PTB2 binds polypyrimidine regions and acts as a repressor (although weaker than PTB1) of splicing *in vitro*[39]. Quantification by real time PCR shows that *PTB1* and *PTB2* mRNAs are at nearly equivalent levels in PC12 cells as well as in striatal tissue. After transfection, cells were serum starved for 18 hours and then stimulated with 100 nM PMA. Relative to the empty vector, cells overexpressing PTB1 showed an increased induction of *fosB* and a concomitant decrease in Δ*fosB* (right hand panel, Figure 12). The right hand panel depicts this shift in isoform ratio. After stimulation, there is 2:1 ratio of *fosB* to Δ*fosB* in the empty vector cells. In contrast, PTB1 overexpressing cells show a significant shift in the relative ratio of *fosB* to Δ*fosB* to 4:1. Overexpression of PTB2 had no effect on the induction or the ratio of the *fosB* isoforms. Similarly, overexpression of another homolog of PTB1, hnRNP A1, had no measurable effect (data not shown).

PTB1 knockdown

Initially, knockdown experiments in PC12 cells were conducted with double strand small interfering RNAs (siRNAs) purchased from Ambion and Dharmacon. These efforts were unsuccessful partially due to poor transfection rates and the inability to differentiate which cells had been transfected. To circumvent these issues, five short hairpin RNA (shRNA) constructs against distinct regions of the *PTB1* open reading frame were designed

following the protocol described by Hommel et al.[34]. An AAV vector (Figure 13A) was designed to express both enhanced green fluorescent protein (EGFP), allowing for detection of transfected cells, and a U6 promoter-driven shRNA. Forty-eight hours after transfection, cells were sorted by FACS scan based on EGFP fluorescence and positive cells were re-plated. After 24 hours the cells were stimulated with 100 nM PMA for one hour and harvested for RNA. Figure 13B (left hand panel) shows the knockdown efficiency of the five constructs (PTBI-V) relative to a control shRNA (CON). Cells expressing the shRNA construct PTBIV, which knocked down *PTB1* mRNA levels by approximately 70 percent, were unable to induce any of the *fos* isoforms after PMA treatment. Even *c-fos*, which has no PTB1 consensus sites, stayed near control levels suggesting that the PTB1 protein perhaps has a functional role in proteins upstream of *fos* transcription. Possible explanations for why this experimental approach did not work are provided in the Discussion.

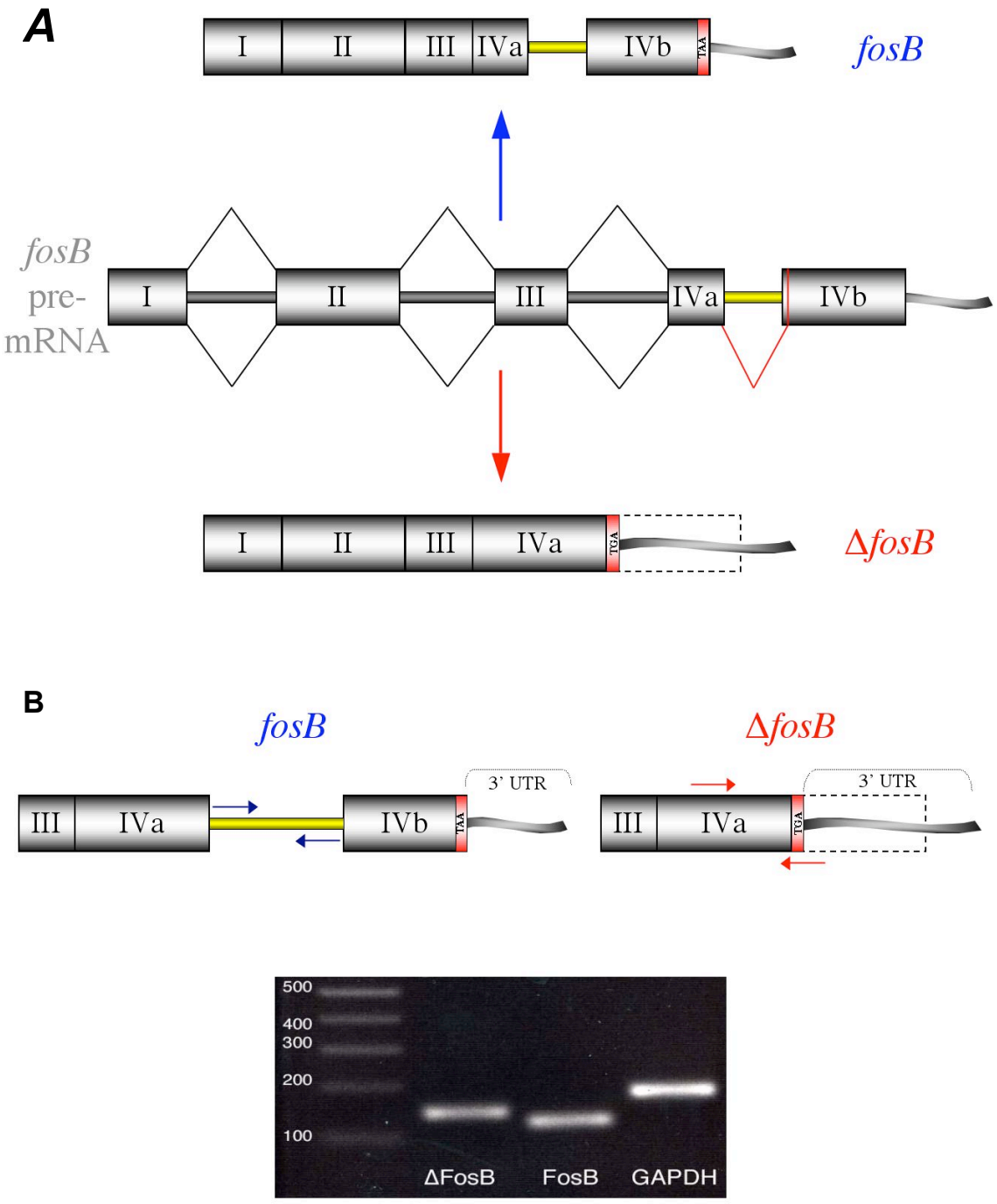


Figure 1. Alternative Splicing of *fosB* RNA. A. Δ*fosB* is generated by a 140 nucleotide excision of "intronic" sequence found in the open reading frame of full length *fosB*. In addition, the splice event results in a one nucleotide frameshift in which a stop codon (TGA) is recognized. **B.** 3' ends of the *fosB* and Δ*fosB*. Primers used in this study amplify the "intronic" region (colored in yellow) specific to full length *fosB* (blue arrows) and the unique exon-exon junction specific to Δ*fosB* (red arrows). Also shown is the specificity of the primer pairs.

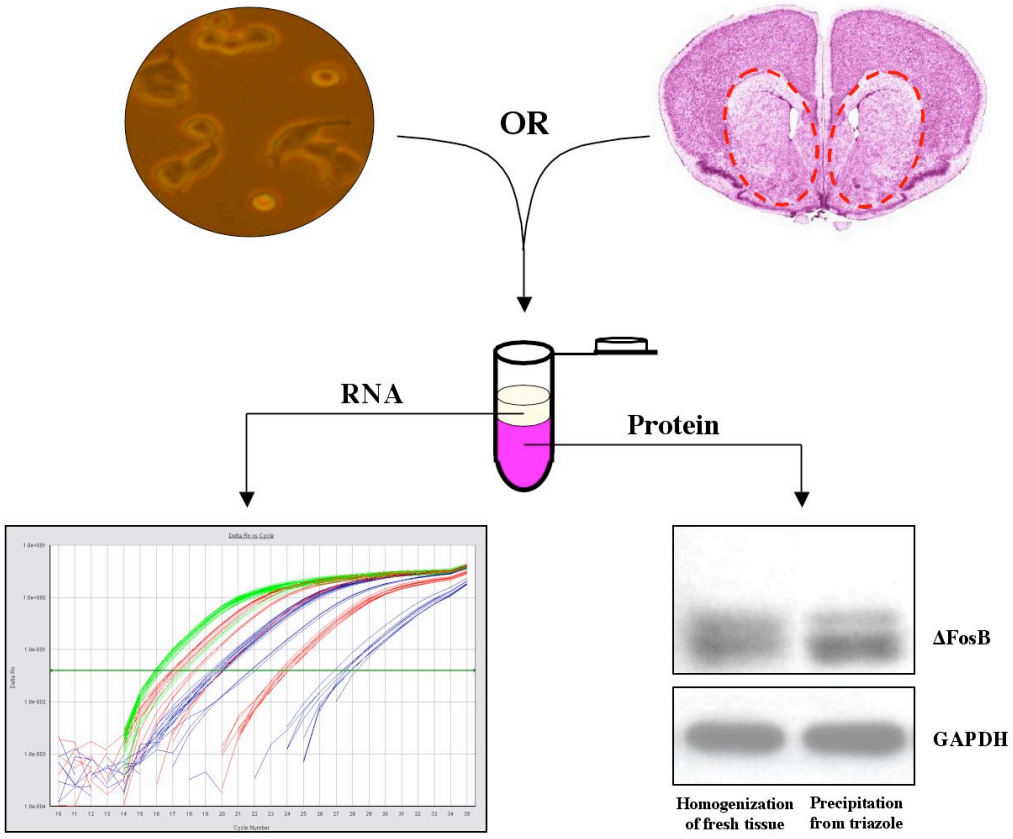


Figure 2. Scheme of Methods. PC12 cells or striatal tissue was homogenized in RNA STAT-60. RNA was extracted, DNAse treated, reverse transcribed into cDNA and quantified by q-PCR. By modifying a protocol for protein precipitation from the remaining organic layer of the processed samples, both protein and total RNA could be harvested from the same samples. The Western blot demonstrates that brain tissue from one hemisphere processed by protein precipitation from the organic layer of a RNA sample yields the same signal as tissue from the other hemisphere processed in a homogenizing buffer used in more conventional methods[9].

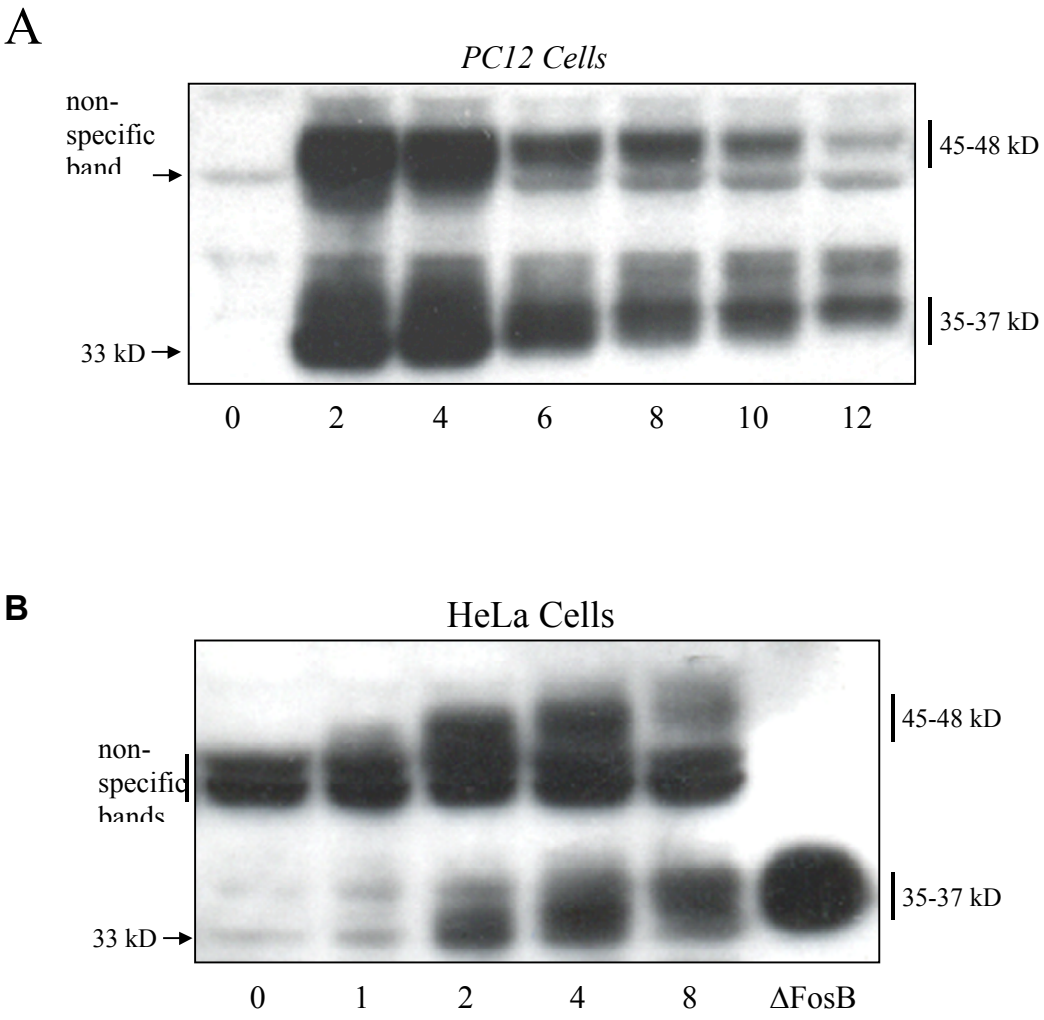


Figure 3. In vitro Expression of FosB Protein Isoforms. Quiescent PC12 (**A**) and HeLa (**B**) cells were stimulated for 0-12 hours with 20% serum. Both sets of cells induce the FosB isoforms. The M_r of both proteins increases over time suggesting the presence of post-translational modifications. ΔFosB in **B.** is protein extracts from cells transiently transfected with a plasmid driving ΔFosB expression.

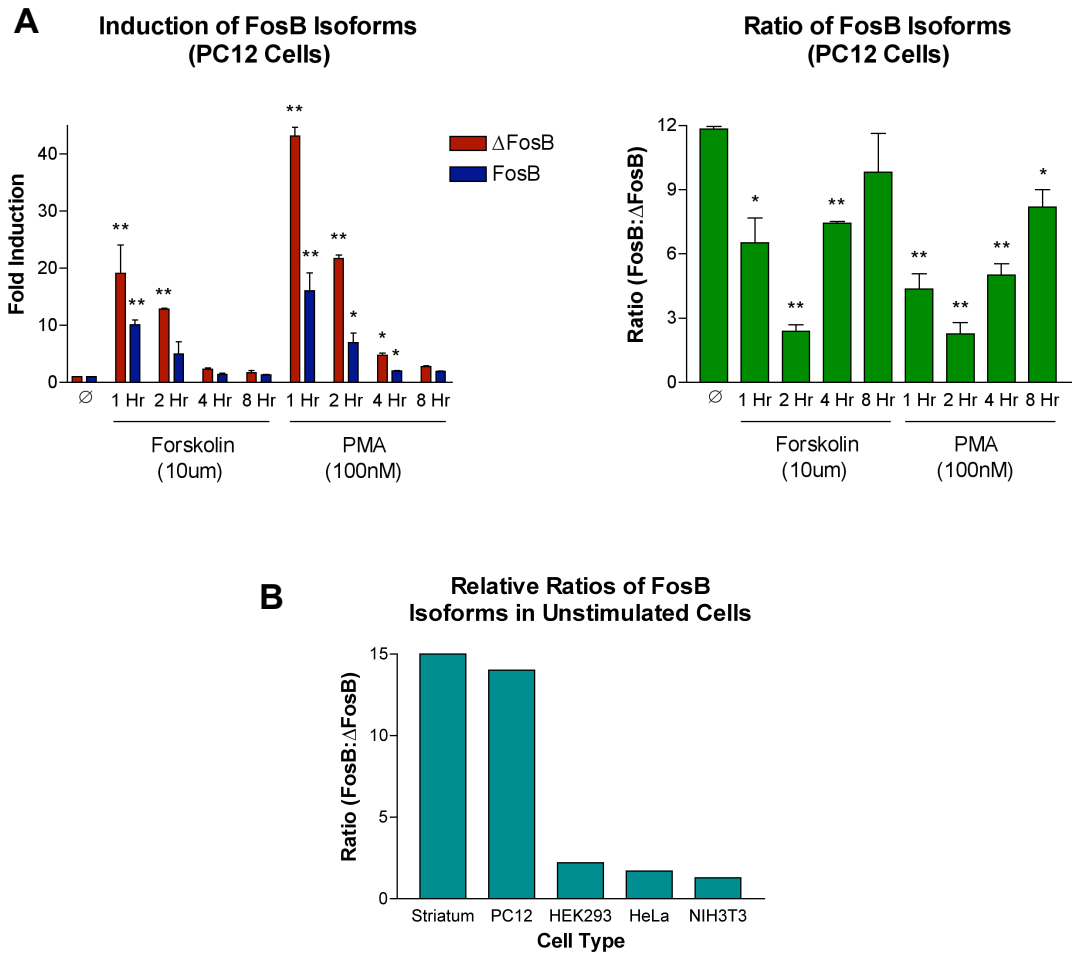


Figure 4. ***In vitro*** **expression of mRNA Isoforms.** **A.** Induction of *fosB* isoforms with PMA and Forskolin. Though induction levels vary, both stimuli induce the isoforms in similar ratios. **B.** Relative ratio of *fosB* isoforms in neuronal vs. non-neuronal cells.

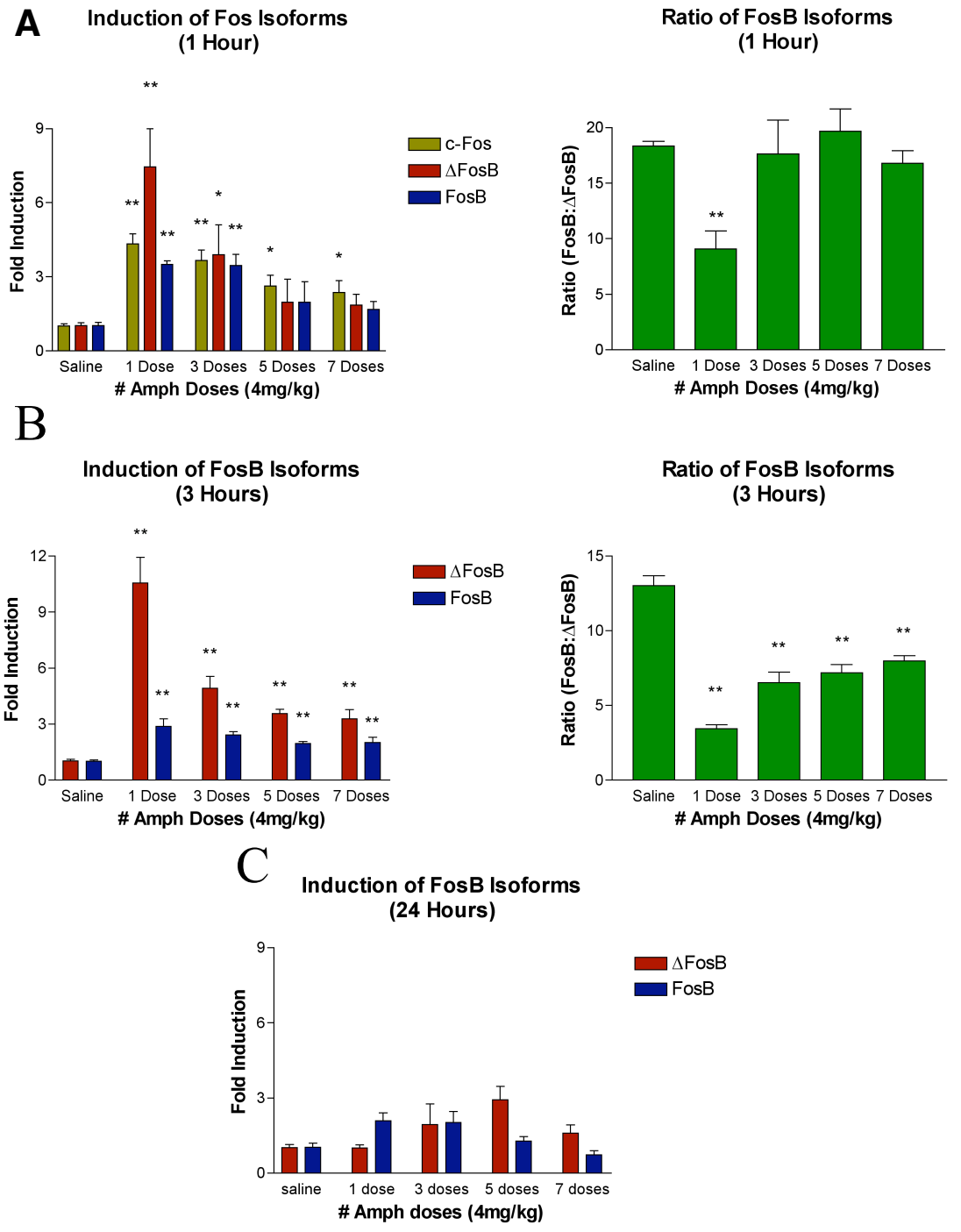


Figure 5. ***In vivo*** **Accumulation of** ***fosB*** **Isoforms.** Sprague-Dawley rats were treated once daily with amphetamine (4 mg/kg) over a period of 1-7 days. Animals were killed 1, 3, or 24 hours (**A**, **B**, **C**, respectively) after the last injection. The left hand panels depict induction of each isoform relative to its saline control. The right hand panels depict the relative ratio of *fosB* to Δ*fosB*. Note the largest shift in isoforms correlates with the greatest overall induction of both transcripts.

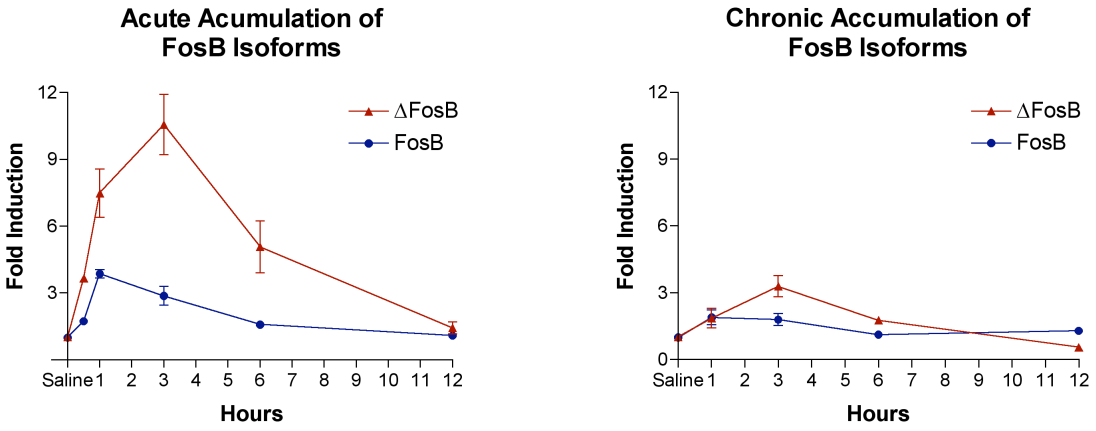


Figure 6. ***In vivo*** **Accumulation of** ***fosB*** **Isoforms.** Animals were administered saline or 4 mg/kg of amphetamine acutely (**A**) or chronically (**B**) and killed 0-12 hours later. Graphs depict induction of each isoform relative to its saline control. No change in accumulation is seen between isoforms induced acutely or chronically.

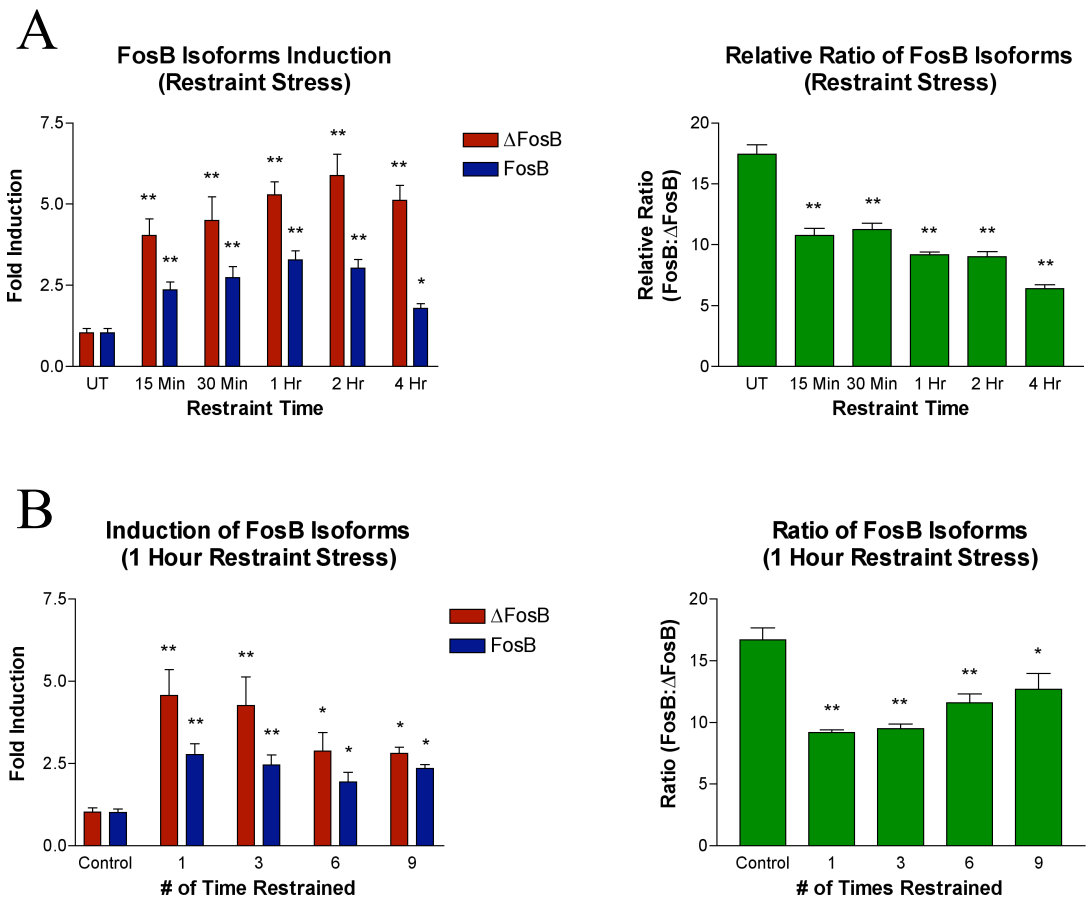


Figure 7. Restraint Stress. A. Rats were acutely restrained in plastic cones for a maximum of one hour. For the 2 and 4- hour time points animals were removed from the cone and placed back into their home cage after 1 hour of restraint. Similar to amphetamine treatment, *fosB* induction is greatest at one hour, whereas, Δ*fosB* levels continue to rise until the 2-hour time point. **B.** Rats were restrained for one hour once daily over a 9 day period. Like amphetamine the greatest induction as well as the largest change in *fosB* ratios occurred acutely.

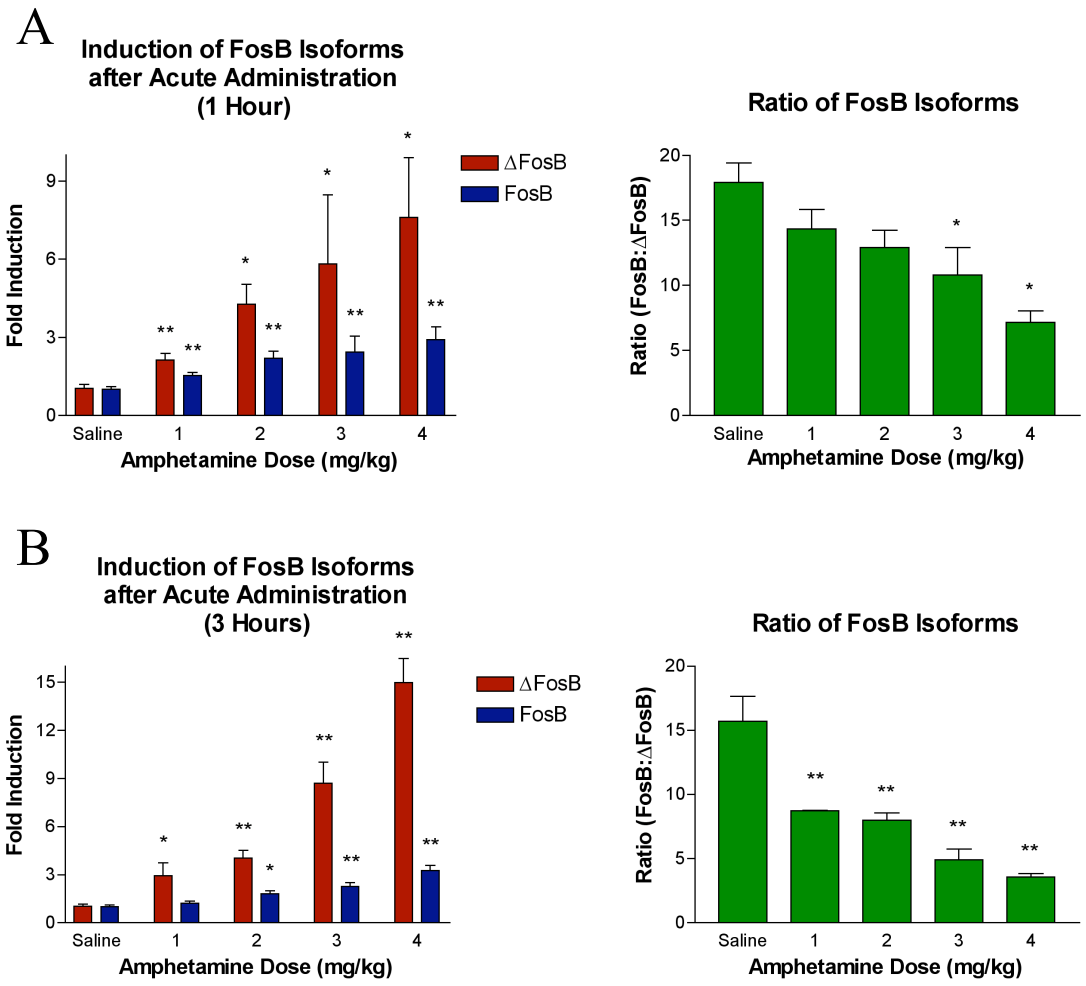


Figure 8. *In vivo* Dose Response. Animals were administered increasing amphetamine doses and killed 1 (**A**) or 3 (**B**) hours after the acute dose. *fosB* levels are induced to roughly equivalent levels regardless of dose; however, Δ*fosB* levels increase as the drug dose increases.

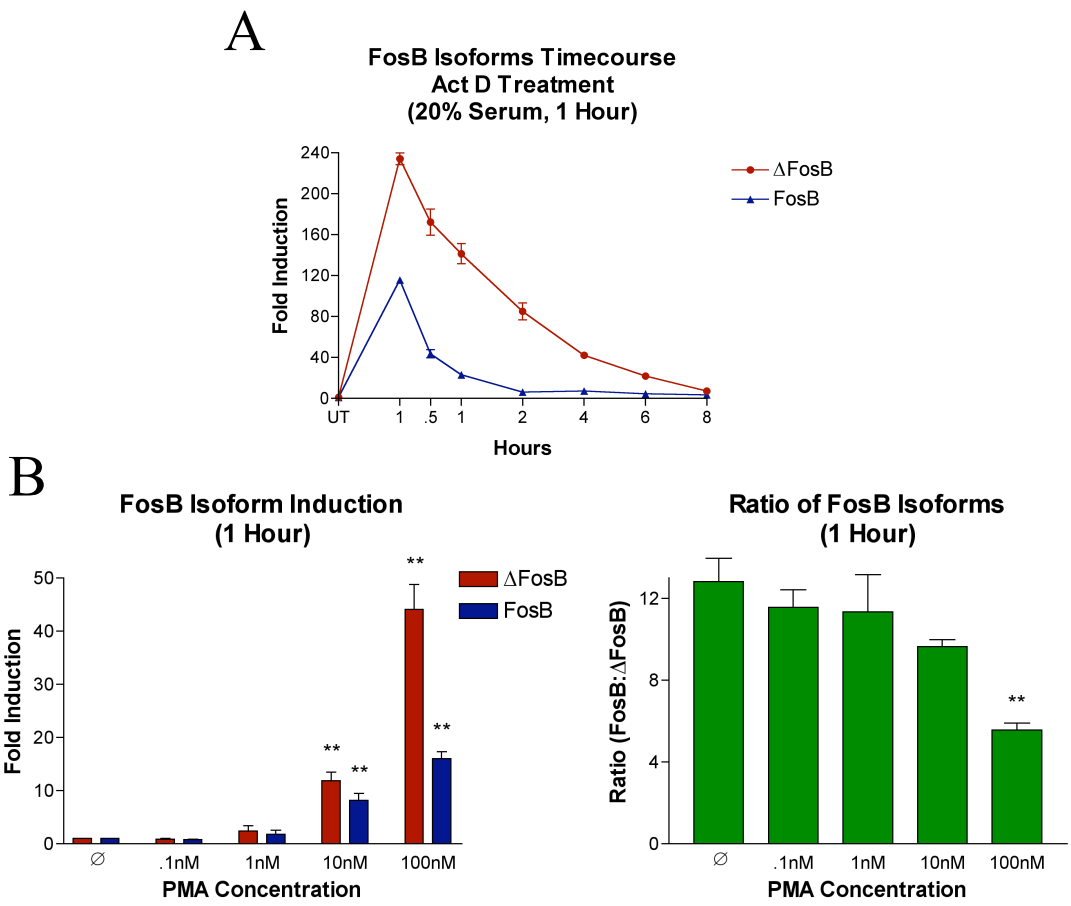


Figure 9. ***In vitro*** **Recapitulation.** **A.** Determination of *in vitro* half lives. PC12 cells were stimulated with 20% serum for 1 hour and then treated with Actinomycin D. As *in vivo* both *fosB* isoforms have similar half lives. **B.** Dose response curve with PMA. The shift in *fosB* ratios was most significant at the highest induction levels, which was generated at the peak dose of PMA.

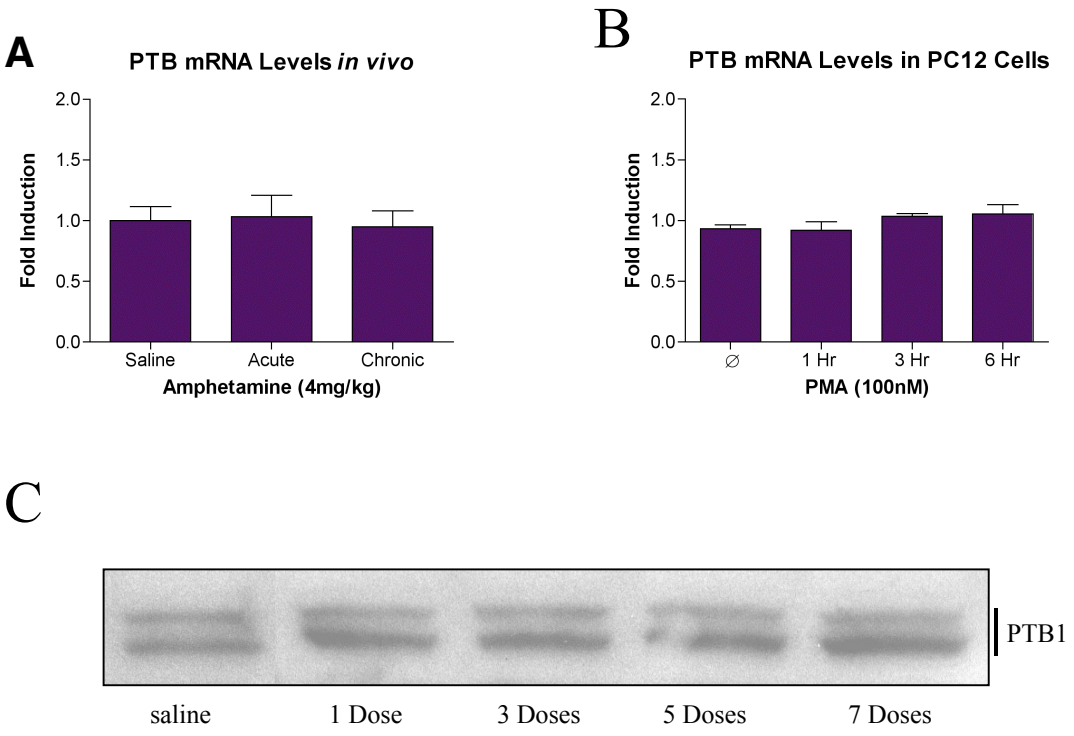


Figure 10. Accumulation of PTB1 Protein. *PTB1* mRNA levels were measured after *in vivo* amphetamine administration (**A**) and *in vitro* PMA treatment (**B**). **C.** Protein precipitated from the experimental samples described in Figure 5A (1 hour amphetamine administered rats) were immunoblotted with a PTB1 antibody. RNA and protein remain unchanged over the course of amphetamine or PMA treatment.

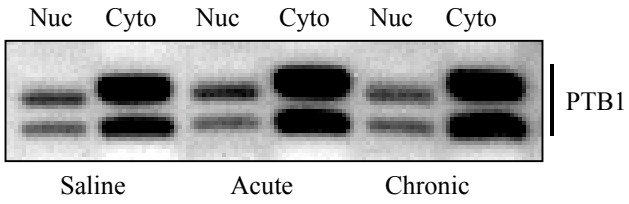


Figure 11. PTB1 Shuttling. Rats were administered saline or acute or chronic 4 mg/kg amphetamine and killed 1 hour after the last dose. The tissue was fractionated between nuclear (nuc) and cytoplasmic (cyto) fractions and immunoblotted with PTB1 antibody. Nuclear and cytoplasmic levels of PTB1 are unaltered over the course of amphetamine treatment.

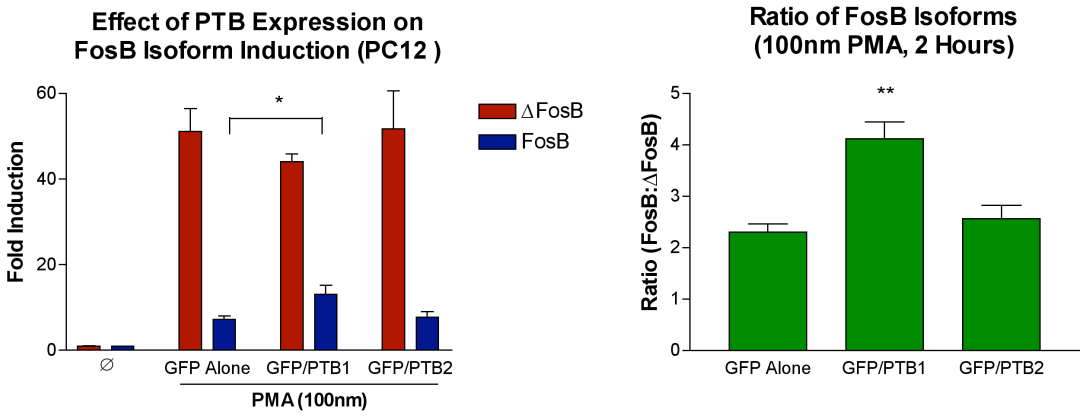


Figure 12. PTB Overexpression. PC12 cells were transfected with GFP or GFP plus Human PTB1 or PTB2 and simultaneously serum starved for 22 hours. Cells were then stimulated with PMA for 2 hours. Transfection efficiency was approximately 40%. *fosB* transcript is preferentially spliced relative to Δ*fosB* transcript in the presence of excess PTB1 protein. . Cells overexpressing PTB2 are indistinguishable in induction and ratio of *fosB* isoforms from control cells.

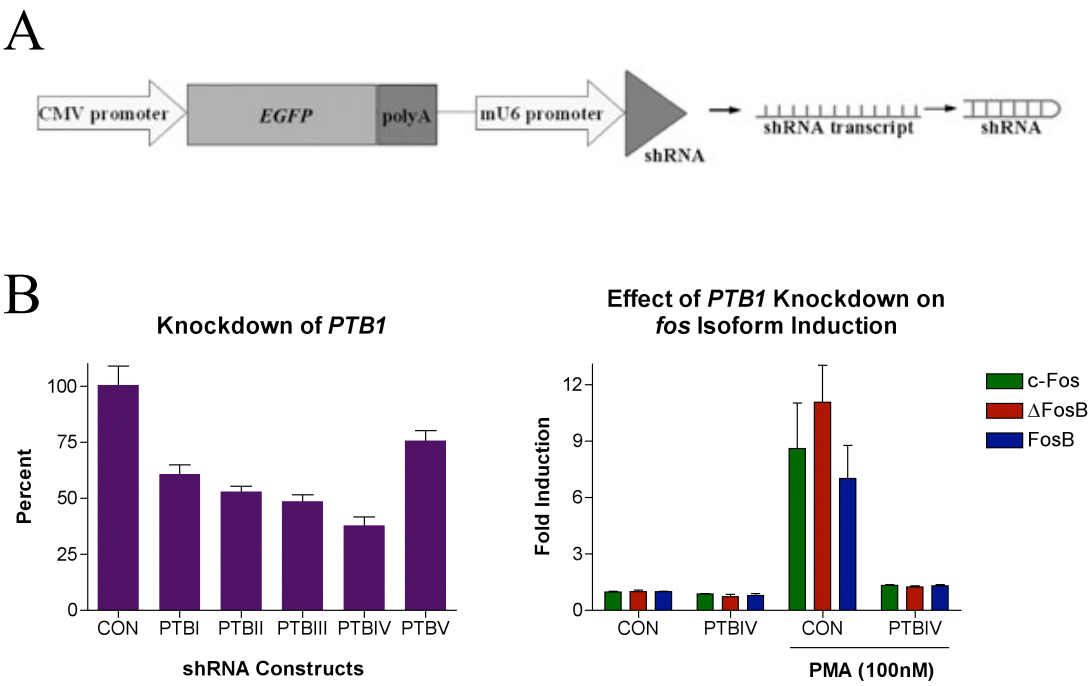


Figure 13. ***PTB1*** **Knockdown. A.** AAV vector backbone expressing both EGFP and shRNAs. **B.** shPTBIV (having a knockdown efficiency of ~70%) was transfected into PC12 cells. EGFP positive cells were selected by FACS and replated. 72 hours after the initial transfection cells were treated with PMA for 1 hour. shPTBIV cells were unable to induce any of the *fos* isoforms.

CHAPTER FOUR
Discussion

Neuronal vs. Non-neuronal cell lines

The mechanism of alternative splicing described here is specific to neuronal or neuronal-like cell lines. PC12 cells were selected because they are one of few immortal cell lines that are neuronal-like; however, they grow poorly and aggregate and are difficult to transfect. PC12 is a clonal cell line derived from a transplantable rat adrenal pheochromocytoma, which responds reversibly to nerve growth factor (NGF)[40]. Though both isoforms are induced in a variety of cell lines, only PC12 cells have a similar basal level ratio of *fosB* to Δ*fosB* found in the brain (Figure 4B). In HEK293, HeLa and NIH3T3 cells, the ratios of the isoforms are almost one to one. Interestingly, "fresh" or undifferentiated PC12 (round cells depicted in Figure 2) have a *fosB* to Δ*fosB* ratio of approximately 6:1; however, after several rounds of passage, the cells begin to differentiate, extend processes (irregular shaped cells depicted in Figure 2) and the relative ratio of the *fosB* isoforms begins to resemble the *in vivo* ratio. Unfortunately, the use of fully differentiated PC12 cells is not an option because these cells stop dividing and become extremely difficult to transfect. Towards the end of these studies Neuroscreen-1 cells were purchased from Cellomics (Pittsburgh, PA). Neuroscreen-1 cells are a subclone of PC12 cells that are marketed to have robust growth, less tendency to aggregate and are to be more "neuron-like" in their reactivity to stimuli than PC12 cells. The Neuroscreen-1 cell line most closely replicated striatal tissue with respect to relative ratio of *fosB* to Δ*fosB* transcript. Future studies should focus on this cell line because of its relative ease to maintain and transfect.

This variation between relative ratios of the fosB isoforms may be explained by the differing functions of the ΔFosB molecule in non-neuronal cells. For example, ΔFosB is implicated in mechanical stress-induced intracellular signaling pathways that activate the osteogenic program in osteoblasts[28]. In addition, fosB gene transcription is differentially regulated depending on the cell type. Lazo et al.[41] demonstrated that the Serum Response Element (SRE) found in the fosB promoter was necessary and sufficient to induce gene transcription in PC12 cells. Mutations to the Cyclic AMP Response Element (CRE) and the AP-1 site had little to no effect on induction of the reporter. This finding has been further substantiated by the generation of a conditional serum response factor (SRF) knockout[42]. SRF, which induces transcription by binding SREs, was identified as a candidate regulator of activity dependent IEG expression by early studies of transcriptional regulation[43]. Mutant mice with SRF ablation specific to hippocampal neurons were subjected to electroconvulsive shock (ECS), a procedure that induces robust activity of IEGs in the dentate gyrus[44]. In contrast to wild-type litter mates, the SRF mutant mice showed little to no induction of IEGs (including c-Fos and FosB) in SRF deficient cells. This study suggests SRF (and consequently SRE) are crucial to IEG expression in neuronal cells. Conversely, Inoue et al.[45] showed the CRE site was necessary and sufficient to induce fosB gene transcription in osteoblasts and mutations in the SRE had no effect.

Finally, modulation (overexpression or knockdown) of PTB1 protein in HEK293 cells has no effect on fosB isoform ratios (see Appendix). The presence of neuronal PTB (found only in neuronal cells) may explain the variation seen in neuronal vs. non-neuronal cells. The Black Laboratory has shown neuronal PTB, like PTB1, can similarly repress

alternative splicing in *in vitro* splicing assays[38]. The concept that varying levels and types of heterogeneous nuclear ribonucleoproteins (hnRNPs) in different tissues lineages can alter the splicing of the same transcript has been well documented in the splicing field[46, 47]. For example, the ubiquitously expressed c-src (proto-oncogene rous sarcoma) has a neuron specific N1 exon, which is only expressed in neuronal cells[48]. It is further shown that this regulation of the N1 exon is mediated by PTB1. Grabowski has compared the dynamic and specific nature of hnRNP binding to chromatin. He suggests that neuronal cells might contain lower levels of PTB or altered distribution of isoforms. Or perhaps there "might be activities in neuronal cells that disrupt splicing repressor complexes after their assembly with pre-mRNA, analogous to the disruption of nucleosomes at promoter sites during transcriptional activation[49]." Additionally, similar to PTB2, other neural specific hnRNPs have been identified which regulate the generation of neural specific transcripts such as KSRP (KH-type splicing regulatory protein)[50], Hu (Human embryonic lethal abnormal vision) protein[51] and Nova[52].

Molecular Tolerance

With respect to drug addiction, tolerance is generally considered a behavioral term. It refers to the decreasing effect of a drug after repeated administration at the same dose[2]. Perhaps, the effects seen in Figure 5, can be considered molecular tolerance. Initially, at an acute dose all the *fos* isoforms are strongly induced; however, levels of the transcripts return to near baseline levels after chronic dosing. This correlation between the molecular response and the behavioral phenotype may be a useful tool to identify molecules or pathways that

affect both phenomena. The *fosB* promoter contains three cis acting elements that may mediate this "molecular desensitization": a SRE, a CRE and an AP-1 site. The Polakis Laboratory has suggested that activation of the Wnt pathway interferes with serum response element-driven transcription of immediate early genes[53]. In addition, after long- term opiate treatment, cAMP pathways are up-regulated in distinct areas of the brain[54, 55]. A consequence of the upregulation of the cAMP pathway is the activation of Cyclic AMP Response Element Binding Protein (CREB)[56]. CREB binds to CRE sites as a dimer and activates transcription when serine 133 is phosporylated[2]. Recently, Brami-Cherrier et al.[57] showed that in mitogen- and stress-activated protein kinase (MSK1) knock-out mice CREB serine 133 phosphorylation in response to cocaine (10 mg/kg) was blocked, and induction of c-Fos was prevented, whereas the induction of other IEGs such as Egr-1 (early growth response-1) was unaltered. Consequently, MSK1 could modulate IEG expression by regulating CREB transcriptional activity by phosporylation of serine 133. Finally, ΔFosB accumulates within a subset of neurons after chronic administration of drugs of abuse and has been shown to be a functional transcription factor by heterodimerizing with JunD and binding to AP-1 sites[17]. Therefore, ΔFosB could bind its own promoter and regulate its own expression. To identify which putative mechanisms control the induction of the *fosB* gene, a 2 kb promoter fragment of the *fosB* gene, which includes 5' untranslated sequence, the transcription start site and upstream regulatory sequences, has been cloned upstream of a luciferase reporter. Overexpression of the above transcription factors coupled with mutations could partially elucidate the mechanism of "molecular tolerance."

Epigenetics may also be a factor in transcription of the *fosB* gene. It has been shown that transcription factors regulate target genes by recruiting to the gene promoters a class of enzymes that modify the core histones and the other chromatin components by regulating their acetylation, phoshporylation, methylation, etc[58, 59]. For example, the acetylation of histones H3 and H4, as well as phosphoacetylation of H3, at gene promoters is linked to increased gene activity, while deacetylation is linked with suppression and silencing of gene activity. Using chromatin immunoprecipitations, Kumar et al.[60] investigated the effects of acute and chronic cocaine administration on acetylation of the histones H3 and H4. They found that acute induction of *fosB* involves H4 acetylation, whereas chronic induction of the gene involves H3 acetylation. These findings are consistent with acute and chronic seizures effects on the *c-fos* promoter. Tsankova et al.[61] demonstrated H4 acetylation is induced after acute ECS, whereas chronic ECS is associated with H3 acetylation. Understanding the molecular switch in the *fosB* promoter from H4 acetylation acutely to H3 acetylation chronically may provide critical insight on the transcriptional regulation of the *fosB* gene as well as the phenomena of molecular tolerance.

Δ*fosB Regulation*

The initial hypothesis that Δ*fosB* would preferentially accumulate relative to *fosB* after chronic administration of amphetamine, and thereby contribute to the selective accumulation of ΔFosB during chronic treatment, was disproved by this study. Figure 5 illustrates that the relative ratio of *fosB* to Δ*fosB* is basically unchanged after chronic dosing when compared to saline controls. In addition, Figure 6 demonstrates that *in vivo* Δ*fosB*

mRNA does not accumulate after acute induction. By 12 hours, *fosB* as well as Δ*fosB* have returned to control levels. This is further substantiated by the *in vitro* determination of each transcripts half-life. Figure 9A shows that the Δ*fosB* transcript has only a slightly longer half-life then the *fosB* transcript.

Expectedly, cocaine administration does not differ greatly from amphetamine because both drugs have a similar mode of action (unpublished data). Since it has been shown that ΔFosB protein can be induced by a variety of stimuli outside of drugs of abuse, a restraint stress paradigm was employed to determine if the phenomena illustrated in Figure 5 is a general mechanism. Figure 7 demonstrates that although restraint stress does not generate *fosB* transcripts to induction levels of amphetamine treatment, both isoforms are again induced acutely and diminish over the course of the experiment. Additionally, Figure 7A corroborates data from Figure 6, as both restraint stress and amphetamine administration show that *fosB* transcript levels reach maximal induction at 1 hour whereas Δ*fosB* reaches its peak at about 3 hours.

Surprisingly, the only instance when Δ*fosB* was preferentially spliced relative to *fosB* was during acute administration of drug or restraint stress. Due to the tolerance phenomena described earlier, it is during the acute doses that there is the greatest overall induction of both transcripts. This indicates that the production of Δ*fosB* is directly proportional to the transcription of the *fosB* gene. The dose response experiment displayed in Figure 8 proved this to be the case. Δ*fosB* mRNA increases as the dose increases. In contrast, *fosB* peaks at approximate a 2.5-3 fold increase. As indicated by Figures 5A, 6A and 7, *fosB* stimulation is limited to a 3 fold induction and the remaining transcript is presumably converted to Δ*fosB*.

The *in vivo* dose response was recapitulated *in vitro* by treating PC12 cells with 10 fold increases of PMA (from 0.1nM to 100nM). As dose increases, the ratio of *fosB* to Δ*fosB* shifts further from the baseline. Interestingly, PC12 cells differ from striatal tissue, in that *fosB* levels are not limited or capped. Figure 9A shows that *fosB* levels can be induced as high as 80 fold under conditions of 20% serum stimulation. Obviously, the stimulus described her is not physiologically relevant. It is likely there is not enough dopamine in the brain to elicit such a response nor enough dopamine receptors on the cell surface to respond. Neurons would likely undergo excitotoxicity if the levels of dopamine began to approach stimulus levels described *in vitro*. It would be interesting to test if PC12 cells could react as robustly once they go undergo differentiation. Or conversely, striatal neurons could be disassociated and then treated for their responsiveness to stimuli.

Alternative Splicing

The sequencing of several metazoan genomes has revealed that overall complexity cannot be fully explained by the number of genes[62]. Alternative splicing is one mechanism that contributes to protein diversity in metazoan organisms. It is estimated that 60% of genes undergo alternative splicing in the human genome[63]. Moreover, many transcripts have several splicing patterns and some have thousands, opening the possibility of generating multiple proteins from one gene transcript[64].

For a typical gene, the pattern of splicing can be altered in numerous ways. Exons that are always spliced or included in the final mRNA are termed constitutive exons. Alternatively, cassette exons are regulated in that their inclusion in the final mRNA is

variable. In certain instances, several cassette exons can be mutually exclusive as their inclusion is inhibitory to another exon[65]. Exons can also be altered in length by the position of their 5' or 3' splice site. Further, 5'-terminal exons can be regulated by the inclusion of alternative promoters[66]. Similarly, the 3'-terminal exons can be substituted by combining alternative splicing with alternative polyadenylation sites[67]. Finally, as in the case of the *fosB* gene, a small subset of genes are alternatively spliced to contain small introns in the open reading frame of the mRNA by a process termed intron retention[68].

Cis acting elements

The removal of an intron from a pre-mRNA and subsequent joining of the exons flanking the removed intron is controlled by sequences found at the intron/exon boundary known as splice sites. The 5' end of the intron, known as the 5' splice site, is defined by the AG/GURAG consensus sequence ("R" is a purine nucleotide and "/ "denotes the exon-intron boundary). The 3' end, termed the 3' splice site, is characterized by the YAG/R motif ("Y" is a pyrimidine nucleotide). The pyrimidine tract, a stretch of 15-25 nucleotides enriched with pyrimidine residues, immediately precedes this motif. Further upstream of the acceptor site, typically 18-40 nucleotides, is the branch point sequence (BPS). The BPS is characterized by a highly degenerate sequence that contains an adenine residue and roughly follows the consensus YNCURAY[69].

Spliceosome

The splicing of an intron is directed by a complex of five small nuclear

ribonucleoproteins (snRNPs) U1, U2, U4, U5, U6 and a number of accessory proteins, which assemble in a stepwise manner on the pre-mRNA and are collectively known as the spliceosome (see Figure 14). The Early (E) complex contains the U1 snRNP bound to the 5' splice site (the GU dinucleotide). Each element of the 3' splice site is bound by a specific protein, the branch point (A) by SF1, the polypyrimidine tract (PY) by U2AF 65, and the AG dinucleotide by U2AF 35. The A complex forms when U2 engages the branch point via RNA/RNA base-pairing. This complex is joined by the U4/5/6 Tri-snRNP to form the B complex. The B complex is then extensively rearranged to form the catalytic C complex. During this rearrangement the interactions of the U1 and U4 snRNPs are lost and the U6 snRNP is brought into contact with the 5' splice site[69]. After the formation of the C complex is formed the intron is catalytically removed by 2 transesterifcation steps. The first step begins with the 2' hydroxyl of the branch point A attacking the phosphate at the 5' splice site. This reaction results in cleavage of the 5' exon, ligation of the 5' end of the intron to the branch point A, and formation of a lariat intermediate. The second step involves the attack of the 3' end of the intron by the 3' hydroxyl group of the free exon. This reaction results in ligation of the two exons and release of the intron lariat[30].

Intron Retention

Intron retention is by far the least common of the alternative splicing mechanisms mentioned above. Galante et al.[70] predicted less than 15% of human genes retain introns in the final mRNA.; however, the majority of intron retention events described in this paper occur in untranslated regions or are not conserved between humans and mice. Using a new

computational algorithm, Ohler et al.[31] recently showed that Galante et al. overestimated the frequency of intron retention. Ohler et al. predicted approximately 25 human genes have an evolutionarily conserved retained intron in the open reading frame of the final mRNA. Both papers did, however, agree that retained introns are significantly smaller than introns that are constitutively excluded.

Many factors that affect intron retention events are the same as those affecting other alternative splicing mechanisms. For example, in female flies an intron is retained in the final mRNA of the *Drosophila msl2* (*male specific lethal 2*) transcript[71]. Sxl (Sex lethal), a well characterized RNA binding protein (RBP)[72], binds the 5' and 3' splice site of the expressed intron and is required for intron retention. It is likely that Sxl protein prevents recognition of the intron as well as shuttling of the transcript into the cytoplasm.

The P element transcript in *Drosophila* is another well-characterized system of intron retention[73]. The P element is a transposon whose expression is limited to the germ line. In germ cells all the P element introns are excised and the expressed protein is a fully functional transposase enzyme. In somatic cells, the third intron is retained and this prevents translation of the P element mRNA, consequently blocking somatic transposition. The intron retention event is mediated by P element Somatic Inhibitor (PSI) and hrp48 (the *Drosophila* homolog of hnRNP A and B, discussed below), which bind the 5' splice site and thus prevent the action of the spliceosome component snRNP U1. In addition to splicing repression, both PSI and hrp48 have been implicated in other processes including transcription, RNA localization and RNA degradation[74, 75]. The above examples illustrate that alternative splicing and in particular intron retention are mediated by both the presence of cis acting elements as well as

multifunctional RBPs that prevent recognition of the retained intron by the spliceosome or its components.

RNA binding proteins (RBPs)

There are two major classes of RBPs that participate in pre-mRNA splicing: serine/arginine-rich (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs). SR proteins have RNA binding domains (the RRM domain) at the N-terminus and an arginine/serine rich RS domain at the C-terminus that participates in protein-protein interactions[76]. SR proteins function predominantly in the splicing of constitutive exons through exon definition. Many constitutive exons have SR binding sites marking the exon for recognition by the spliceosome. Through the C-terminal domain, SR proteins recruit spliceosome factors to the pre-mRNA, and may assist in E complex assembly by mediating interactions between U2AF 35 at the 3' splice site and U1 snRNP at the 5' splice site[77].

In contrast to SR proteins, which recruit and promote spliceosomal activity, hnRNPs affect pre-mRNA splicing by negatively regulating alternative splicing. HnRNP proteins have either an RRM (RNA Recognition Motif), or a KH motif (K homology RNA binding domain) binding domain. They are most often present in the nucleus, although some shuttle between the nucleus and cytoplasm[78]. During transcription hnRNPs coat the pre-mRNA and it is thought that each transcript has an individual pattern of hnRNP binding, depending on the mRNA sequence and the cell type. This unique combination of hnRNP proteins bound to the pre-mRNA facilitates the formation of the H complex, which directly precedes generation of the E complex[77]. HnRNP function is not restricted to alternative splicing.

These RBPs also contribute to mRNA transcription, mRNA stabilization and transport to the cytoplasm, polyadenylation and translation[79].

PTB Regulation

The hnRNP I or PTB1 protein is an attractive candidate to regulate the production of *fosB* transcript for several reasons. First, PTB1 protein was first described as a negative regulator of the U2AF splicing complex by competitively binding polypyrimidine tracts to which the splicing complex bound[35]. Next, the intron flanked by exons IVa and IVb of the *fosB* gene contains a consensus binding site for the PTB1 protein[80]. Finally, the Potashkin group has shown in preliminary studies that the PTB1 protein will bind *in vitro* transcribed *fosB* transcript. When the consensus binding site is mutated, PTB1 protein will no longer bind the transcript[81].

If PTB1 protein does regulate the generation of the *fosB* isoform then its levels must stay constant or decrease. Otherwise, as illustrated in Figure 5, if PTB1 protein increased, *fosB* transcript levels would continue to rise at the 5 and 7 dose time points. Figure 10 shows that PTB1 levels (protein and mRNA) do not change over the course of amphetamine or PMA treatment. Additionally, it is well documented that chronic administration of several types of drugs of abuse increase PKA levels as well as activity in many cell types, including *in vivo* in striatum[14, 37]. PKA has also been shown to phosphorylate PTB1 protein, which results in the translocation of the protein from the nucleus into the cytoplasm[36]. After fractionating amphetamine treated tissue, protein fractions were immunoblotted with an anti-PTB antibody. PTB1 protein levels remain constant and do not seem to shuttle between the

nucleus and cytoplasm (Figure 11). Previous microarray data has shown that, along with PKA, many other kinases as well as phosphatases are upregulated after chronic drug administration[82]. These may negatively modulate the PKA phosphorylation of the PTB1 protein and thereby prevent the shuttling phenomenon described *in vitro*. This regulation could be better investigated with the use of phospho-specific PTB antibodies; however, no such antibodies currently exist.

Overexpression of PTB1 protein in PC12 cells further confirms the role of PTB1 protein in *fosB* splicing. Figure12 demonstrates that *fosB* transcript is preferentially spliced relative to Δ*fosB* transcript in the presence of excess PTB1 protein. A common criticism of overexpression studies in the splicing field is that hnRNPs, which negatively regulate splicing, may act as partial dominant negatives by interacting with the spliceosome and thus causing an overall depression in splicing. To resolve this issue, PTB2 and hnRNP A1 were also overexpressed in PC12 cells. Both proteins act as negative regulators of splicing and are the 2 closest homologues in sequence identity to PTB1[39]. PTB1 and PTB2 are so similar in structure that it is predicted that PTB2 arose from a gene duplication event of PTB1[52]. Cells overexpressing either PTB2 or hnRNP A1 are indistinguishable in induction and ratio of *fosB* isoforms from control cells. This suggests the effect of PTB1 on *fosB* regulation is specific and not due to a general depression in splicing.

If PTB1 protein overexpression results in a specific increase of the *fosB* transcript, then knockdown of PTB1 protein via RNAi should yield a decrease in the *fosB* transcript relative to Δ*fosB* transcript. The use of the method described by Hommel et al.[34] to generate shRNAs for RNAi knockdown experiments has several advantages over transfecting siRNA

oligos. First, as shown in Figure 13A, the shRNA is driven by a constitutively active mU6 promoter, thereby, generating many copies of the shRNA throughout the course of the experiment for the RNA-induced silencing complex (RISC) to utilize. In addition to encoding a shRNA, the vector expresses EGFP for the detection of cells that have been transfected. Finally, the plasmid is an AAV construct that can be expressed as a virus to produce temporal and spatial knockdowns *in vivo*.

Five shRNA constructs were transfected into PC12 cells and evaluated for knockdown efficiency of *PTB1* transcript (Figure 13B). shPTBIV (having a knockdown efficiency over 70%) was further evaluated for its effect on *fos* isoform expression. In comparison to cells expressing a control shRNA (CON), the shPTBIV expressing cells are unable to induce either *fosB* isoform. The *c-fos* transcript, which has no putative consensus sequences for the PTB1 binding, is also depressed in the experimental cells. This finding is not unexpected because several distinct functions of the PTB1 protein have been reported since its original description as an inhibitor of alternative splicing. Functioning as an RBP, PTB1 also has roles in 3'-end processing, internal initiation of translation, stabilization and RNA localization[83]. Similarly, Wickens et al.[84] has illustrated that Pumilio–Fem-3-binding factor (Puf) proteins from *S. cerevisiae* belong to a structurally related family of cytoplasmic RBPs implicated in developmental processes in various eukaryotes. Using DNA microarrays to identify the specific mRNAs that interact with the five *S. cerevisiae* Puf proteins, the Brown laboratory found that Puf family proteins bound a large set (approximately 700 transcripts) of distinct and functionally related mRNAs. These results suggest a system for large-scale coordinated control of cytoplasmic mRNAs and provide insights into the

"physiological logic of the gene expression program" in *S. cerevisiae* [85]. Though no such analysis has been conducted for PTB1, it is likely due to its diverse set of putative functions that PTB1 bind hundreds of transcripts in the cell. The Keene laboratory has also demonstrated that many higher eukaryotic RBPs associate with multiple mRNAs both *in vitro* and *in vivo*. This multi-targeted binding property of RBPs has led to a model of regulated gene expression in eukaryotes termed the post-transcriptional operon[86]. Post-transcriptional operons are postulated to mediate complex functions in the cell; therefore, disruption of the RBPs that modulate specific post-transcriptional operons could cripple cellular function. Accordingly, knockdown of PTB1 may alter the function of a post-transcriptional operon controlling IEG expression.

PTB1 RNA Immunoprecipitation

Similar to chromatin immunoprecipitations employed by Kumar et al.[60] to measure levels of post-translationally modified histones at various promoter regions, an RNA immunoprecipitation was attempted to identify transcripts bound to PTB1 *in vivo*. As mentioned earlier, the demonstration by the Potashkin laboratory that PTB1 bound the intronic mRNA sequence of *fosB* transcript was performed strictly *in vitro*. This study tried to recapitulate those finding *in vivo* using a method described by Niranjanakumari et al.[87]. PC12 cells were crosslinked with formaldehyde and lysed by sonication. A monoclonal mouse PTB1 antibody purchased from Zymed (San Francisco, CA) was used to immunoprecipitate PTB1 from the lysed extracts. Unfortunately, this antibody did not effectively pull down PTB1 protein using several conditions listed in published protocols.

The polyclonal rabbit PTB1 antibody (employed in the immunoblots in Figures 10C and 11) was not used because of limited quantity and because the antibody recognizes both PTB1 and PTB2 isoforms as well as other related hnRNPs[88]. There have been several reports of successful RNA immunoprecipitations in the literature, including a method in which formaldehyde is replaced by UV light to crosslink RNA to protein[89, 90]. In these examples, however, immunoprecipitated RBPs were endogenously abundant or exogenously overexpressed and the antibodies used for immunoprecipitation were commercially available and well characterized.

Cyclooxygenase (COX)-1

Cyclooxygenase (COX) enzymes are involved in *de novo* prostaglandin biosynthesis and thus their inhibition by nonsteroidal anti-inflammatory drugs relieve pain and inflammation. Very recently, Qin et al.[91] identified a splicing variant of COX-1, arising via the retention of intron 1. Strikingly similar to the generation of Δ*fosB* transcript, *COX-1$_b$* arises via retention of the entire 94 base pair intron, leading to a shift in the reading frame and consequent early termination. Additionally, intron 1 contains a canonical PTB1 binding site in its polypyrimidine tract. Due to its parallel structure, *COX-1$_b$* and other genes like it, which retain introns may be regulated by the mechanism elucidated by this work.

General Conclusion

This is the first study to characterize the mechanisms underlying the generation of *fosB* and Δ*fosB* mRNA. Our data support a clear scheme (shown in Figure 15). According to this scheme, the splicing of *fosB* RNA is regulated by the quantity of unspliced transcript available to the splicing machinery. That is, above a certain threshold of full-length *fosB*, the remaining primary transcript is alternatively spliced into Δ*fosB*. This splicing phenomenon is likely regulated by the Polypyrimidine Tract Binding (PTB1) protein. Under basal conditions PTB1 protein binds the majority of the *fosB* pre-mRNA, thereby inhibiting the generation of the Δ*fosB* transcript. Only when PTB1 protein is saturated with transcript does the ratio of *fosB* to Δ*fosB* decrease significantly, because the unbound pre-mRNA is spliced into Δ*fosB*.

Originally, it was hypothesized that chronic stimulation of a cell might be associated with the increased splicing of *fosB* transcripts to Δ*fosB*. This would then contribute to the selective accumulation of ΔFosB protein seen under chronic stimulation conditions. However, contrary to the predicted hypothesis, the selective accumulation of ΔFosB protein with chronic drug exposure does not involve its preferential generation by splicing. Using the cell culture system generated by this study, Carle et al.[12] has demonstrated that endogenously expressed ΔFosB protein has a half life greater than 48 hours whereas, FosB protein has a half life of 1-2 hours. This discrepancy in half lives is explained by the absence of conserved C-terminal degrons, which prevent poly-ubiquitination and consequent proteasomal degradation of ΔFosB protein. Therefore, it is likely that post-translational modifications are the primary reason for the accumulation of ΔFosB protein after chronic administration of drugs of abuse. In addition, Ulery et al.[92] has shown that ΔFosB is phosphorylated on its N-terminus by CK2 and perhaps other protein kinases and that this

phosphorylation further stabilizes ΔFosB, but not FosB. Together, these data support a scheme that can explain the shifting pattern of FosB and ΔFosB protein expression shown here (Figure 3). As FosB and ΔFosB are expressed, both isoforms are phosphorylated, which explains their increasing apparent M_r on SDS gel electrophoresis. At the same time, the native and phosphorylated isoforms of FosB are subjected to rapid degradation by the proteosome and hence do not accumulate. In contrast, ΔFosB is spared proteasomal degradation, which enables phosphorylated, stabilized isoforms to persist in the cell and accumulate.

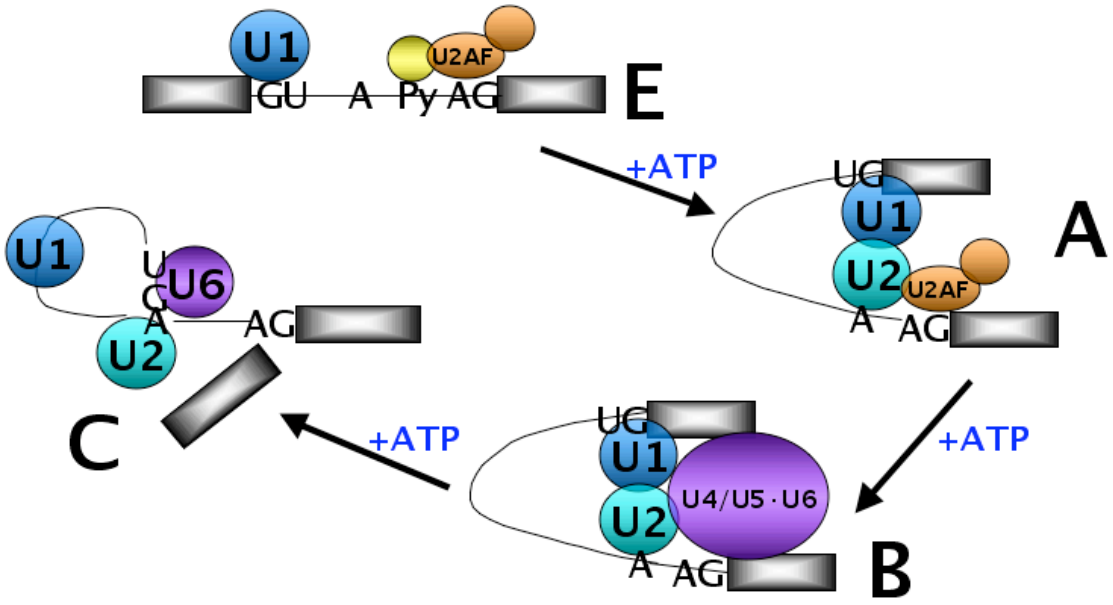


Figure 14. Spliceosome Formation. The spliceosome assembles on pre-mRNA in a stepwise manner. E complex formation consists of U1 snRNP bound to the 5' splice site, SF1 bound to the branch point sequence, U2AF 65 bound to the pyrimidine tract, and U2AF 35 bound to the 3' splice site. A complex formation consists of U2 snRNP bound to the branch point sequence, and release of SF1. B complex consists of tri-snRNP (U4/U5/U6 snRNP) addition, and release of U2AF 65 and U2AF 35. This complex rearranges to form the catalytic C complex. Figure adapted from Black, 2003[30].

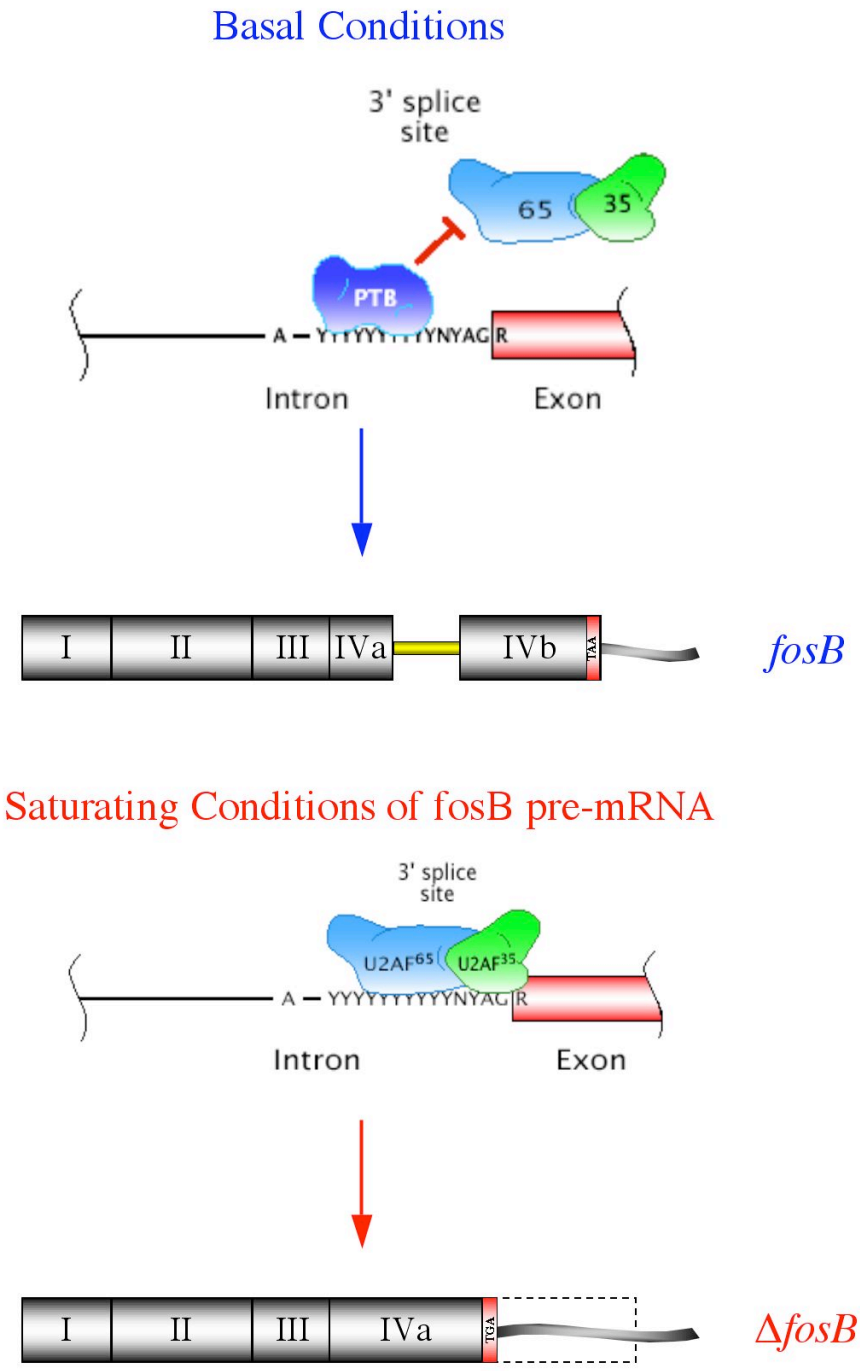
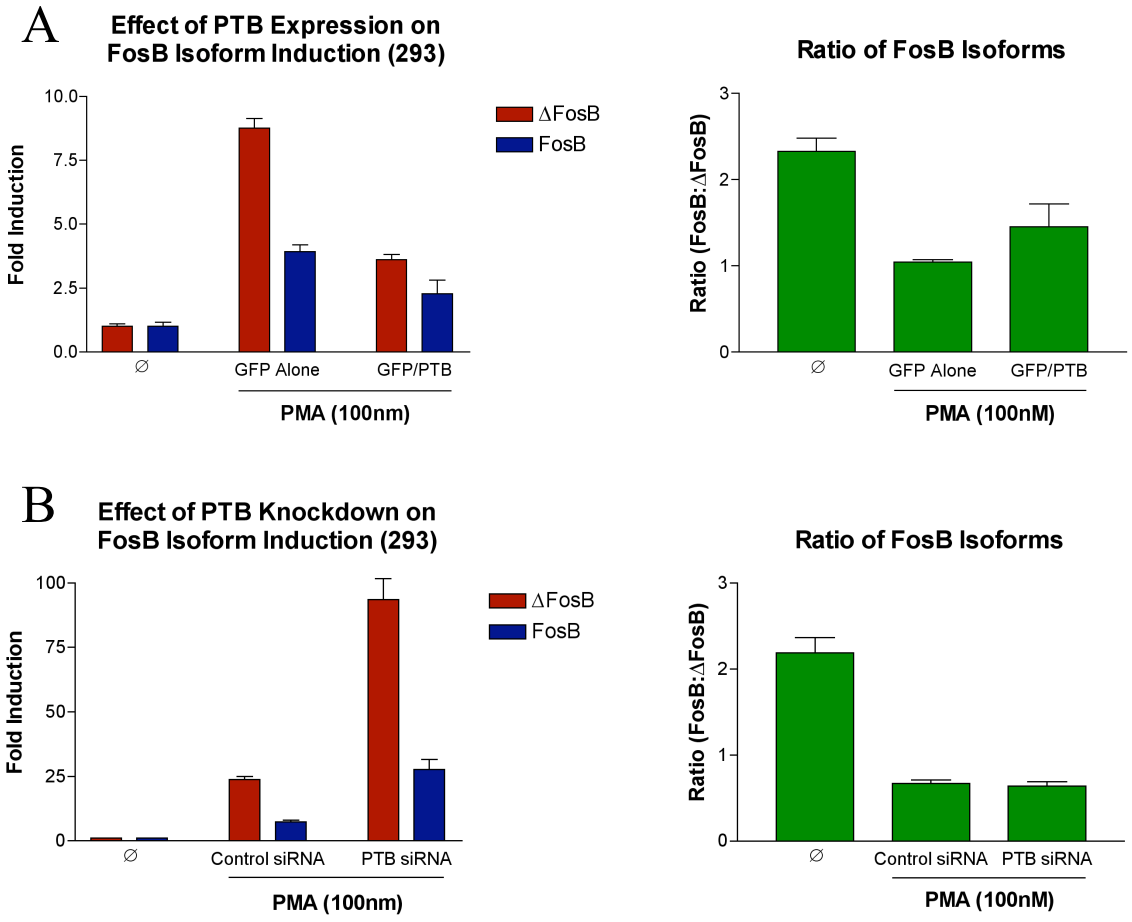


Figure 15. Model of *fosB* RNA Splicing. . Under basal conditions PTB1 protein binds the majority of the *fosB* pre-mRNA, thereby inhibiting the generation of the Δ*fosB* transcript. Only when PTB1 protein is saturated with transcript does the ratio of *fosB* to Δ*fosB* decrease significantly, because the unbound pre-mRNA is spliced into Δ*fosB*.

APPENDIX A
PTB Modulation in HEK293 Cells



PTB1 modulation in HEK293 cells. A. Overexpression of PTB results in an overall reduction of both transcripts but has no effect on the ratio of isoforms. **B.** Knockdown of PTB results in an overall induction of both transcripts but has no effect on the ratio of isoforms. Note the basal ratio of *fosB* to Δ*fosB* mRNA is ~ 2:1. These results suggest that *fosB* RNA splicing is differentially regulated in neuronal versus non-neuronal cell lineages.

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VITAE

Imran, son of Almas and Nizam Alibhai, was born on December 5, 1975 in London, England. In 1979, his family immigrated to Dallas, Texas, where he was introduced to America's (actually God's) Team: The Dallas Cowboys. After graduating from RL Turner High School in 1994, Imran attended Duke University. It was here he picked up two more unhealthy obsessions: golf and Duke Basketball. After graduating with a BS in Biology and a minor in Chemistry in 1998, he returned home to Dallas. In the intervening two years before beginning graduate school in 2000, Imran honed his skills in synthesis by working in an organic chemistry laboratory at UT Southwestern. He began his work in the Nestler Laboratory in the summer of 2003. After completing his degree, Imran will move to Seattle, Washington to try his hand in the world of venture capital and bio-equity. Due to limited practice time, his dreams of playing either professional football or golf will probably never be realized.

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