Studying the Adaptive Changes of Transcript Abundance or Splice Variants upon Repeated Stimulation by KCl-induced Depolarization or by Dopamine

By

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Abstract

Objective: One of the key steps of the regulation of gene expression is alternative splicing of pre-mRNA. Previous studies have shown that alternative splicing is critical for adaptive changes of cells to repeated stimulation or stress. However, the underlying mechanisms of alternative splicing during these changes remain elusive. In this study we aim to identify the different effects of repeated versus single stimulation on the transcript abundance or alternative splicing of particular genes. **Method:** Effect of a single exposure or repeated dopamine (DA) or KCl treatment on the transcript abundance or alternative splicing of *FosB* in striatal cells was examined using RT-PCR. Moreover, to determine the changes of other candidate genes in this treatment scheme, RNA-Seq was carried out on the striatal cells treated with DA.

Results: While changes in splicing variants of *FosB* was evident after KCl or DA stimulation, there was no difference in changes observed after a single exposure or after repeated treatment with KCl or DA. RNA-Seq data showed that the splicing factor cluster of genes was the most significant group that changed in alternative splicing after repeated DA treatment compared with non-treated (NT) cells. The transcription factor cluster, was most significantly changed for splice variation after a single exposure to KCl or DA. A similar difference was seen between repeated exposure and single exposure condition. In addition, with respect to transcript abundance, there were unique signatures of gene clusters affected by either single exposure or repeated exposure treatment.

Conclusion: Alternative splicing of *FosB* does not change after repeated compared to first stimulation by KCl or DA. However, RNA-Seq reveals that repeated stimulation affects the expression, particularly alternative splicing of specific genes differently from that observed for a single stimulation.

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DEDICATION

I dedicate this work to my father, mother as well as my brothers.

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ABBREVIATIONS

ABCA1	ATP-binding cassette transporter A1
AD	alzheimer's disease
Ano1	anoctamin 1, calcium activated chloride channel
ApoA-I	apolipoprotein A1
AS	alternative splicing
BDNF	brain-derived neurotrophic factor
BK	big potassium channels
BSA	bovine serum albumin
C. elegans	Caenorhabditis elegans
Cacna1h	voltage-dependent, T type, alpha 1H subunit
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
cAMP	cyclic adenosine 3',5'-monophosphate
CaRF	calcium-response factor
Central nervous system	CNS
ChIP	chromatin immunoprecipitation
CREB	cAMP response element-binding protein
Cry	cryptochrome genes
DA	dopamine
DD	dark-dark
ddH2O	double-distilled water
dfmr1	*drosophila* fmr1
DG	dentate gyrus
DM1	myotonic dystrophy
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNMT	DNA methyltransferase
DS	down syndrome
DSPD	delayed sleep phase disorder
DTT	dithiothreitol
Dyrk1A	dual-specificity tyrosine-phosphorylated and regulated kinase 1A
EDS	excessive daytime sleepiness
EDTA	ethylenediamine tetraacetic acid
EGCG	green tea flavonol epigallocatechin-gallate
EPM	elevated plus maze
ERK	extracellular signal-regulated kinase
ESE	exonic splicing enhancer
ESSs	exonic splicing silencers
FBS	fetal bovine serum
FD	familial dysautonomia
FMR1	fragile X mental retardation gene
FMRP	fragile X protein
FosB	FBJ murine osteosarcoma viral oncogene homolog B
GPR126	G protein-coupled receptor 126

Gpr149	G protein-coupled receptor 149
H3K9me2	histone 3 lysine 9 dimethylation
HAT	KAT lysine acetyltransferase
HDAC	histone deacetylase
HnRNP	heterogeneous nuclear ribonucleoprotein
hnRNP LL	hnRNPL-Like
ICD	intracellular domain
Igfbp2	insulin-like growth factor binding protein 2
IGFBP-3	insulin-like growth factor binding protein 3
ISE	intronic splicing enhancer
ISSs	intronic splicing silencers
KH	K Homology
LD	light-dark
LTM	long-term memory
LTP	long term potentiation
MBNL1	muscleblind-like splicing regulator
miRNA	microRNA
MO	morpholino
MPH	methylphenidate
NAc	nucleus accumbens
NCAM	neural cell adhesion molecule
nM	nano molar
NMD	nonsense mediated mRNA decay
NRXN	neurexins
OFT	open field test
OPRM1	mu-opioid receptor gene
PAC1	pituitary adenylate cyclase-activating polypeptide type I receptor
PACAP	pituitary adenylate cyclase-activating peptide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	parkinson's disease
Per	period
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
Pp1c	protein phosphatase 1
Pre-mRNA	precursor messenger RNA
PSG	penicillin-streptomycin glutamine solution
PTB	polypyrimidine tract-binding protein
QTL	quantitative trait locus
RA	retinoic acid
RRF	retrorubral field
RRM	RNA recognition motif domain
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulfate

Sept7	septin7
shRNA	short hairpin RNA
SN	substantia nigra
snRNA	small nuclear RNA
snRNP	small nuclear ribonucleoprotein
SR	serine/arginine-rich
SR protein	serine/arginine-rich protein
Srpk2	serine/arginine-rich protein specific kinase 2
SRSF1	serine-arginine rich splice factor 1
Srsf4	serine/arginine-rich splicing factor 4
STAR	signal transduction and activation of RNA
STHdh	mouse striatal-derived cell line
STM	short-trem memory
STREX	stress axis-regulated exon
TCR	T-cell receptor
TPA	12-*O*-Tetradecanoylphorbol-13-acetate
TSA	trichostatin A
Ttc23	tetratricopeptide repeat domain 23
U2AF	U2 auxiliary factor
UTR	untranslated region
UV	ultraviolet
VEGF-A	vascular endothelial growth factor A
VEGFR2	VEGF receptor-2
WMT	water maze test
XBP1	X-box binding protein 1
µl	microliter
µm	micrometer
µM	micro molar

Chapter 1: Introduction

1.1. Adaptation

The environment of an organism changes; therefore, in order for the species to survive, they need to respond to the changes by achieving new steady state which may give them viability, new function and tolerance to the changes (1). Adaptation is usually useful in response to changing environmental conditions, and prepares an organism to adapt themselves to the changes leading to survival (1). However, sometimes, organisms also display different traits in response to the stimuli, which is harmful and called maladaptation, such as addiction (2). Adaptive changes can result from exposure to different stimuli, which through triggering signaling cascades can lead to downstream pathways and finally adaptation (3). There are a variety of adaptive events including addiction (4), learning and memory (5) and circadian period (6).

Addiction leads to a series of adaptive changes in the brain, which are influenced by genetics and environmental factors (4). Drugs of abuse can act as an external stimulus and modulate addictive behaviors through triggering various mechanisms within the brain cells. Changes in gene expression induced by the drugs can cause long-lasting changes in the brain's reward system (2).

Memory and learning is a physiological process which depends on the ability of the brain to store the retrieved information (7) It is classified into two forms, short (STM) and long term memory (LTM) (5). Enhancement of synaptic activity and signal communication between neurons is called long term potentiation (LTP), which leads to synaptic plasticity and plays an important role in the formation of LTM (8). Specific stimuli causes changes in gene expression and protein synthesis, leading to LTP formation (9).

Circadian rhythms are physiological phenomena, which roughly follow a 24-hour period and are present in many living organisms. It enables an organism to anticipate and adapt to environmental changes during the 24-hours and give appropriate responses to the changes. Production of proteins which regulate these physiological events are regulated through gene expression mechanisms, thereby leads to generation of appropriate proteins at the right time during the 24-hours (6).

1. 2. The regulation of gene expression in cell physiology and pathophysiology

During transcription, followed by translation, specific proteins are produced from coding genes which finally control different aspects of cell function. Protein synthesis is highly controlled by different regulatory mechanisms, allowing cells to generate appropriate proteins at different times in response to various stimuli. Chromatin modification including DNA methylation and histone modifications, transcription, post transcriptional processing (such as splicing and polyadenylation) are examples of processes involved in the regulation of gene expression (10, 11).

1. 2. 1. Transcription

During transcription, RNA polymerases synthesize mRNA using DNA as a template. Regulation of temporal activity of a gene, or level of RNA copies allows the cells to respond properly to the intra- or extra- cellular signals (12). Transcription factors are proteins which regulate transcription in response to different signals by binding to specific elements of DNA, generally the promoter or enhancer of target genes and thereby can promote or block the recruitment of transcriptional complex, leading to an increase or decrease of expression of the target gene. Activation and inhibition of transcription factors can affect transcription of their target genes (13-15).

1. 2. 1. 1. Transcription in physiology and pathophysiology

It has been known that the expression of many genes are changed in the brain after exposure to the drugs (12). For instance, FOSB, CREB, NF-κB and glucocorticoid receptors are the most prominent transcription factors that have been shown to be involved in the regulation of gene transcription in response to various drugs (16).

For the first time, during the years of 1990–1991, it was shown that after an acute exposure of cocaine or amphetamine, FOS and JUN family transcription factor, are expressed in the striatal region of the brain (17). ΔFOSB is a member of the FOS family, and is a truncated form of FOSB protein, which is produced through alternative splicing (17). ΔFOSB along with JUND, another transcription factors forms AP-1 complex which regulates transcription of target genes containing AP-1 responsive elements and finally leads to long term adaptive changes in the brain (12, 18-22). Mice lacking *FosB* were unable to develop sensitivity to the cocaine-induced locomotor activity, suggesting an essential role of FOSB in addiction (23). ΔFOSB protein is expressed rapidly in a region-dependent manner after exposure to many drugs as well as natural rewards and stress (17, 24). ΔFOSB functions both as an activator and repressor of transcription in the brain (24). One of the target genes for ΔFOSB is dynorphin, whose expression is inhibited by ΔFOSB and contributes to the enhancement of reward mechanisms after an ΔFOSB increase (25).

Overexpression of ΔFOSB in mice elevates locomotor activity in response to acute and repeated cocaine administration, increases reward responses to cocaine and morphine, and enhances anxiolytic responses to alcohol (25). Moreover, motivation and self-administration of low doses of cocaine were increased in mice overexpressing ΔFOSB (25). Another target for some drugs of abuse such as cocaine and morphine is CREB, which is a transcription factor performing an opposite role from ΔFOSB by inducing expression of dynorphin, resulting in the reduction of

the reward responses. Therefore, the opposite regulation of dynorphin by CREB and ΔFOSB could explain the reciprocal changes during early and late phases of withdrawal (25, 26).

ΔFOSB is a long lasting molecule and could be stable until a couple of weeks after withdrawal, but the drug-induced abnormalities persist for a longer time (25). Perhaps the gene expression changes by ΔFOSB, CREB and other transcription factors have long lasting effects in neuronal morphology and/or synapses. Another possibility is that, these transcription factors may lead to more lasting changes in the chromatin modification, such as changes in histone acetylation or deacetylation and in this way activate or repress expression of target genes, respectively. They also may be involved in other chromatin modification forms such as DNA or histone methylation (25). More studies are required to uncover the long lasting effects of these proteins on neuronal plasticity involved in addiction. Therefore, transcription regulation of specific genes are required for some adaptive events in physiology and pathophysiology.

1. 2. 2. Splicing and alternative splicing

In eukaryotes, exons contain coding sequences which carry the information for protein synthesis; however, these sequences in many genes are interrupted by non-coding sequences, so called introns. Before mRNA undergoes translation, pre-mRNA introns are removed and exons are joint together. This process is called splicing which generates mature mRNA, to produce protein through translation. Sometimes more than one mature mRNA is produced from a single gene during a process named alternative splicing (AS) (Fig. 1). AS is an essential biological event, and plays a critical role in the proteomic diversity in metazoans. Almost 95% of human genes undertake AS, often in response to changing cellular environments (27-32). AS regulates expression of a substantial set of genes that are not changed at the level of transcription (33).

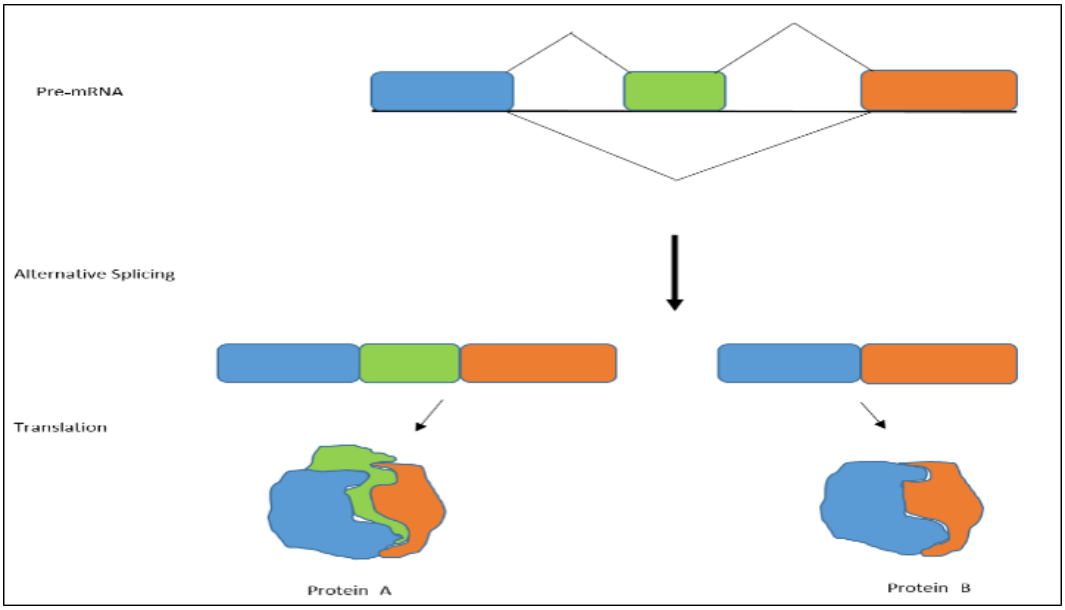


Figure 1. Schematic representation of AS mechanism, a common way of gene regulation that allows the generation of multiple mRNA and often protein isoforms from a single gene. Exons are exhibited as rectangles, and introns are shown as lines between exons.

1. 2. 2. 1. Mechanisms of pre-mRNA splicing

During AS an exon can be either excluded or included. The joining of different splice sites during this process leads to formation of a variety of mRNAs, which can encode different proteins (31, 34). There are different types of splicing such as canonical splicing (major spliceosome), noncanonical splicing (minor spliceosome) and self-splicing (35). Canonical splicing accounts for majority (99%) of splicing, while self-splicing accounts for rare introns which are involved in the formation of ribozyme (36). Spliceosome, is an RNA-protein complex of 5 small ribonucleoparticles (U1, 2, 4, 5, 6), which are called small nuclear ribonucleoproteins (snRNPs) (37, 38). In addition, some axillary proteins are involved in assembly of spliceosomes such as U2AF35 and U2AF65 (39). RNA component of snRNPs interact with the intron. Introns usually contain four conserved elements which are critical for a splicing event, including: 5´ splice site

(GU), 3´ splice site (AG), polypyrimidine tract and branch point. Polypyrimidine tract and branch point are located at the upstream of 3´splice sites (38). Assembly of spliceosome components happens in several steps in the following order: complex E, A, B and C. While complex E formation is ATP independent, the rest of process is ATP-dependent. At the last step of complex C, exons are joint together. In addition, production of spliced variants is regulated by *cis*-acting elements and *trans*-acting factors (40, 41).

1. 2. 2. 2. *Cis*-acting elements and *trans*-acting factors

Spliceosome needs more information than just the four elements of pre-mRNA for accurate slicing events. This information is provided by specific *cis*-acting elements, inside exons or introns (42, 43). *Cis*-acting elements are divided into two types, enhancer and silencer. Intronic and exonic splicing enhancers (ISEs and ESEs) increase exon inclusion, and exonic and intronic splicing silencers (ESSs and ISSs) inhibit splicing of mRNA (44, 45). Splicing enhancers or silencers are recognized by *trans*-acting proteins (45). *Trans*-acting factors also are either activators or repressors. Serine/arginine rich (SR) proteins are a family of proteins, which generally through binding within the enhancers, increase the splice site usage (46) by facilitating recruitment of other splicing factors such as U1 to the 5´ splice site (47), or U2AF to the 3′ splice site (48). Contrary to SR proteins, heterogeneous nuclear ribonucleoproteins (hnRNPs), mostly induce exon exclusion by binding to silencer elements (33) through their RNA recognition motif domain (RRM) or K Homology (KH) domains, leading to repressing the recruitment of splicing factors (49). Level of the inclusion of alternative exon is determined by the final decision as a result of balance between these regulatory factors (50). Defect in every step of splicing or AS, for example disruption of *cis*-acting elements, small nuclear RNA (snRNA) and their accessory factors, or *tans*-acting factors, can cause aberrant splicing. AS could be regulated by external signals such as stress,

neurotransmitters, depolarization and hormones (25, 30). Appropriate responses of cells to the various stimuli help cells to adapt themselves to the different conditions (51). Various stimuli control AS by changing regulatory proteins at the transcription and post-transcriptional level. However, the mechanisms involved in these processes are poorly understood (31, 34).

1. 2. 2. 3. Alternative splicing in physiology and pathophysiology

AS is necessary for some physiological events in the body such as adaptive immunity and learning and memory formation. It has been shown that memory and learning ability is affected by AS of specific genes. For example, reduced ratio of 4R-tau/3R-tau splice variants of tau gene (52), and loss of Nrxn1 and 3 AS4 splice variant (53), impair memory, while overexpression of XBP1s, a splice variant of XBP1 gene (54), enhances memory. Moreover, absence of some splicing factors such as Rbfox3 (55), MBNL1 (56) and hnRNP A/B (57) cause learning and memory impairment. Variety of internal and external stimuli, such as stress, neurotransmitters, depolarization and hormones can affect AS, in which a proper response can be given to the stimulation (25, 30). For example, some drugs of abuse affect splicing of DA receptors (58) and *FosB* (59), leading to production of proteins with different functions.

Membrane depolarization, which affects the excitability of neuronal cells, has been shown to change splicing of different genes. For example splicing of BK channels (60), Ca^{2+} channels (61) and NMDA receptor splicing (62) are affected by KCl-induced depolarization. However, it is still remains to be discovered how environmental factors induce splicing changes of different genes and what is the importance of the specific splice variants on adaptive changes.

1. 3. Adaptive responses controlled by alternative splicing of specific genes

Here we provide some evidences that show the essential role of specific splicing factors or splice variants of some genes in different types of adaptive behaviors.

1. 3. 1. Receptors

Dopamine receptor: DA is a neurotransmitter, which is released by a variety of rewards, such as drugs of abuse, and is involved in adaptive responses to the rewards (63). D2 receptor is a type of DA receptors, which has two splice variants: long variant (D2L), the postsynaptic receptor, and short variant (D2S), the autoreceptor (58, 64-66). D2 receptor alteration is associated with several neurological disorders including schizophrenia, posttraumatic stress disorder, movement disorders, Parkinson's disease (PD), Huntington's disease and drug addiction (64). Several investigations have shown the importance of D2 receptor variants in adaptive behaviors. Mice lacking D2L receptor, by deletion of exon 6, exhibited reduced locomotion and rearing behavior compared with wild type, which were examined by open field test (OFT). However, $D2L^{-/-}$ mice didn't show thigmotaxis, which is the tendency of mice to remain close to the walls of open-field, and is a behavior associated with increased fear. Thigmotaxis decreases gradually during the first minutes of exploration in the open-field (67).

Circadian pattern of mice lacking the D2L variant was similar to the wild types, however, spontaneous basal motor activity was decreased in their home cage during 24 hours.

Moreover, using ring test, D2L knockout mice showed longer spontaneous (or basal) immobility than wild type mice (64). D2L knockout mice exhibited less running activity examined by running wheels as well as reduced home cage activity than wild type mice (65).

These experiments show the essential role of D2L isoform in locomotion and rearing behavior, however, D2L rescue is needed to confirm the observations. Moreover, the importance of D2S isoform is not studied here. Whether D2L isoform is essential and enough for locomotor activity or D2S isoform is required as well, remains to be determined. Studying the locomotion behavior of mice that lack D2S, but not D2L, can help to reveal the importance of D2S isoform in

locomotor activity. Identifying the splicing factors and mechanisms by which they regulate splicing of D2 receptors, could help us to understand mechanisms that modulate locomotor activity.

Ethanol and sugar water consumption was examined in D2L deficient mice. Ethanol consumption in both sexes was increased in D2L knockout mice (65). Sugar water (sucrose solution) consumption in female knockout mice, but not males, was almost two times more than wild type animals (65), suggesting that rewarding response in knockout mice is different for female and male mice. More investigations are required to explain the different consumption of sugar water in male and female mice lacking D2L.

Interestingly it was found that knockout mice which lack D2L, overexpress D2S receptors, and had increased abundance of D2S receptors in comparison with wild type animals (64, 65). Therefore, to investigate whether the increased ethanol consumption is due to lack of D2L or overexpression of D2S, eticlopride, a D2 antagonist, was administrated before ethanol treatment. Mice lacking D2L in both sexes drank more ethanol at different concentrations of eticlopride, however, increasing eticlopride concentration reduced ethanol uptake in females but not males (65). Eticlopride treatment experiments suggest D2S overexpression could contribute to increase consumption of ethanol in the knockout mice.

ApoER2: Another evidence that showed the importance of AS in adaptive change was obtained by studying the apolipoprotein E receptor 2 (*ApoER2*). *ApoER2* is a member of the LDL receptor gene family, and is involved in LTP, learning, and memory. *ApoER2* signaling requires the 59 amino acids at the contextual (ICD), encoded by the alternatively spliced exon 19 (ex19), which interacts with cytosolic adaptors (68, 69). Learning and memory ability of mice was assessed using *ApoER2* knockout mice (*ApoER2* $2^{-/-}$), mice with exon 19 deletion (Δex19) and

mice overexpressing (ex19) (68). The learning ability was assessed using fear-conditioned learning task. In situation where the auditory component was absent, deletion of exon 19 caused contextual related learning impairment (68). Using water maze test (WMT), the importance of exon 19 in spatial learning of mice was examined. *ApoER2*$^{-/-}$ and *ApoER2* (*Δex19*) showed memory impairment compared to wild types and mice expressing exon 19, suggesting that exon 19 of *ApoER2* is required to maintain the normal spatial learning in mice.

It has been shown that level of exon 19 inclusion in AD patients is decreased compared to non-AD participants, suggesting the possible role of lack of ICD in AD. To further examine the importance of exon 19 inclusion in cognitive ability in AD, AD transgenic mouse model which mimicked human AD, and AD transgenic mice expressing *ApoER2* exon 19 were produced (69). AD transgenic mice exhibited learning and memory impairment which was assessed by WMT. Rescue experiment through increasing exon 19 inclusion could improve memory and learning ability of AD transgenic mice (69).

These investigations suggest that lacking exon 19 of *ApoER2* is associated with learning and memory deficiency in AD mice. Moreover, rescue of *ApoER2* exon 19 inclusion**,** could help to improve the cognitive impairment in mouse model of AD which had reduction in exon 19 inclusion.

Knockout studies followed by expression of exon 19 confirmed the essential role of exon 19 of ApoE2 in spatial learning and memory in mice. However, while the possible involvement of exon 19 in cognitive ability in human is shown by comparing the level of exon 19 inclusion in AD patients compared to non-AD counterparts, due to limitation on human study, such as expressing exon 19 in AD patients, it is not still demonstrated whether exon 19 inclusion is essential for human learning and memory, and whether expression of exon 19 could improve cognitive impairments in

AD patients. However, these studies could reveal a new therapeutic approach for AD by controlling splicing of Apoer for increased inclusion of exon 19. Understanding the mechanisms involved in regulation of ApoE2 exon 19 in AD, could lead to new approach for cure of AD or even may be other relative adaptive disorders such as addiction.

Insulin/IGF receptor: More solid evidence to show the importance of AS in adaptive changes, is gained by studying behavioral response of *Caenorhabditis elegans* worm to salt concentration. This investigation indicates an essential role of splice variants in adaptive phenomena by studying the splicing of insulin/IGF receptor in *C. elegans.* Two splice variants of insulin/IGF receptor in *C. elegans* are DAF-2c and DAF-2a. These splice variants are generated during AS of exon 11.5, when its inclusion and exclusion produce DAF-2c and DAF-2a, respectively.

PTB-1 and Asd-1 are splicing factors which regulate the splicing of exon 11.5, where the presence of these splicing factors lead to inclusion of exon 11.5 and production of DAF-2c variant. Inclusion of this exon is reduced in mutated worms for these splicing factors. Importance of AS of this exon is reported by studying the behavioral response of *C. elegans* to NaCl concentration. Worms are able to change their preference for NaCl concentration. They prefer NaCl concentrations at which they were fed, and avoid the one they starved at. The failing in NaCl concentration avoidance after the starvation is called taste-avoidance learning, which is considered as a learning defect (70). *C. elegans* mutated for either *ptb-1* or *asd-1* showed taste-avoidance learning impairment, which was more severe in the *ptb-1; asd-1* double mutants (70). Neuronal expression of DAF-2c (exon 11.5+), but not DAF-2a (exon 11.5-), in the *daf-2* mutant *C. elegans* relieved the learning impairment. Moreover, learning defects of *ptb-1; asd-1* mutants are partially rescued by neuronal expression of DAF-2c but not of DAF-2a (70). Interestingly, overexpression

of either DAF-2a or DAF-2c in the wild-type *C. elegans* lead to learning defects, suggesting that proper expression levels or ratio of DAF-2a and DAF-2c is important for appropriate learning ability (70).

Collectively, these data exhibit the essential role of splicing factors and splice variants in adaptive responses to conditioning NaCl concentration in *C. elegans*. Performing rescue experiment has provided a solid evidence for the critical role of splice variants in learning ability of *C. elegans*. However, although in this report PTB-1 and ASD-1 are studied as splicing factors that control AS of insulin/IGF receptor during learning, the molecular mechanism underlying this process remains to be uncovered; and mechanism by which splicing of insulin/IGF receptor is regulated during learning process is required to be studied in more detail, such as recognizing RNA sequences which these regulatory factors interact to, how these RNA interaction are regulated during adaptive changes and what are the signaling pathway(s) which induce such changes during learning.

PAC1: PAC1 (pituitary adenylate cyclase-activating polypeptide type I receptor**)** is a membrane associated protein which is expressed in a tissue dependent manner, for example it is expressed in the brain. Activation of PAC1 increases level of cAMP, leading to recruitment of CREB to corticotropin-releasing hormone promoter.

Skipping and inclusion of pac1 exon 14 (hop cassette), which encodes 28 amino acids, leads to production of short and long isoforms, respectively (71). To study the importance of AS of Pac1 in stress response in zebrafish, pac1a-hop MO reagent was used, which leads to skipping of hop cassette exon (exon 14) in Pca1, thereby inhibits formation of long PAC1 isoform, while the short isoform remains intact (71). Stress-related anxiety-like behavior in zebrafish larvae is measured by light-dark (LD) preference test. Two-compartment LD measuring arena experiment showed

larvae spent most of the time in white, and exhibited aversion to the dark side of the box. Larvae injected by pac1a-hop MO, which block formation of the long isoform of PAC1, spent longer time on dark side in comparison with control (71), suggesting reduced anxiety behavior.

To confirm that LD preference experiment measures stress-related anxiety-like behavior, time spent in the dark was measured in both wild type and pac1a-hop MO-injected larvae treated with diazepam, which is an anxiolytic drug. Diazepam increased spending time in the dark side in the both groups, which confirms related anxiety-like behavior (71). To test for correlated likely behavioral phenotype, behavioral response of both groups to osmotic stress was assessed. Osmotic stress was induced by putting larvae to the artificial condition similar to 50% seawater. Dark avoidance of both wild type and pac1a-hop MO-injected larvae was measured during recovery osmotic stress. Dark avoidance behavior was attenuated rapidly in wild type group compared to pac1a-hop-injected larvae, suggesting that wild types recovered from osmotic shock rapidly and became less anxious compared to pac1a-hop-injected larvae (71).

These behavioral responses to stress suggest that PAC1-hop splice variant is essential for adaptive anxiety-like behavioral response. The rescue of long isoform could confirm the essential role of AS of pac1 in stress-induced responses. Moreover, whether the long isoform is enough for the behavior, or the presence of short isoform is required, has not been studied in this investigation. Splicing of pac1 is regulated by a2bp1, a RNA-binding protein, which is rapidly increased in response to foot shock stressor in mice (71). Therefore, it would be interesting to study the mechanism by which this factor can affect the splicing of pac1 during stress-induced responses.

1. 3. 2. Channels

Cav2.29: N-type calcium channels or Cav2.29 are voltage-gated calcium channels, and are encoded by the *Cacna1b* gene. Two exons of *Cacna1b* gene, e37a and e37b, which undergo

mutually exclusive AS, encode 32 amino acids in the C-terminus of protein. E37a and e37b are different in 14 amino acids (72). To explore the importance of e37a and e37b in morphine-induced analgesia, mice lacking either e37a or e37b were created. Analgesia defined as relief from pain, and induced by some groups of drugs such as morphine which interacts with opioid receptors (72). An exon substitution method was used to delete $Ca_V2.2$[e37a] splice variant. Using this technique, e37a was replaced with an e37b-encoding sequence in the *Cacna1b* gene producing *Cacna1b*$^{b*b/b*b}$ which lack e37a. *Cacna1b*$^{aa*/aa*}$ which only contained e37a was created by eliminating e37b and replacing with e37a-encoding sequence. *Cacna1b*$^{b*b/b*b}$ was only able to express e37b exon, while *Cacna1b*$^{aa*/aa*}$ expressed e37a (72). Plantar testing instruments was used to measure paw withdrawal threshold. After applying thermal stimuli, withdrawal latency of hind paw was measured in wild type, *Cacna1b*$^{b*b/b*b}$ and *Cacna1b*$^{aa*/aa*}$ mice. There was no significant difference in withdrawal latency between different groups; suggesting that expression of e37a or e37b is not required for noxious thermal stimuli response (72). Analgesic efficacy of morphine in different groups against thermal stimulus was measured. Morphine was significantly less effective in *Cacna1b*$^{b*b/b*b}$ compared to wild type, as *Cacna1b*$^{b*b/b*b}$ showed a lower latency withdrawal than wild types, which upon morphine administration had lengthened withdrawal latency. These results suggest that *Cacna1b*$^{b*b/b*b}$ animals have lower analgesic effect than wild type. However, there was no significant difference between *Cacna1b*$^{aa*/aa*}$ and wild type (72).

These results showed that e37a is necessary for maximum analgesic efficiency of morphine against thermal stimuli; however, the rescue experiment could provide more solid evidence. It would be interesting to study whether splicing of e37a is affected upon morphine treatment and/or thermal stimulation. Which splicing factors regulate AS of e37a and e37b? How the mechanisms which thereby splicing of e37a is regulated are affected by morphine exposure and/or thermal

stimuli? Whether repeated morphine stimulation and/or thermal stimulation induce adaptive AS of e37a, remains to be determined?

μ-opioid receptors: μ-opioid receptors provide binding sites for many drugs such as IBNtxA, are encoded by *Oprm1* gene. During AS of this gene, exon 1 which encodes full length 7 transmembrane domain is replaced with exon 11 resulted in truncated form of receptor (6TM variant) which lacks a transmembrane domain (73). To determine whether exon 11 is important in analgesic effect of some opioid drugs, double knockout mice for exon 1 and exon11 were created. Using thermal stimuli, no analgesic activity of μ opioids such as IBNtxA, morphine, and ketocyclazocine was found in double knockout mice. Truncated form of receptor (6TM) was rescued using lentivirus expressing the 6TM variant mMOR-1G. Rescue of 6TM variant recovered analgesic effect of IBNtxA in one week. Effectiveness of IBNtxA analgesic ability in the mice expressing 6TM variant was similar to wild types. Ketocyclazocine analgesia was also rescued using lentivirus expressing 6TM variant.

These experiments suggest that presence of exon 11 is required and sufficient for analgesic effects of these drugs. However, rescue of exon 1 alone has not been studied to see whether it can rescue the drug-induced analgesia (73). Rescue of exon 11 failed to restore morphine analgesia, which previously has been shown that its action is independent from exon 11 function (73), indicating exon 11 expression is not sufficient to maintain morphine analgesia. However, it is not studied whether exon 1 is essential and sufficient to preserve the morphine analgesia; or the expression of exon 11 along with exon 1 is also required for morphine induced analgesia.

It would be interesting to study how the splicing of Oprm1 gene is changed in exposure with different drugs, as well as thermal stimuli? And if there are any splicing changes by drugs, what are the mechanisms that different drugs might affect differently to induce Oprm1 gene splicing?

And which splicing factors are involved in regulating Oprm1 gene splicing? Various opioid drugs, such as morphine are used clinically as a pain killer. However, sometimes drug analgesic tolerance induces clinical limitation for using these drugs (74). Understanding downstream mechanisms thereby different splice variants of *Oprm1* gene could mediate analgesic effects of different opioid drugs, could be helpful for more effective clinical usage of these drugs by inhibiting pathways that lead to drug tolerance.

1. 3. 3. Transcription factors

FOSB: FOSB is a transcription factor encoded by *FosB* gene. During alternative splicing, intron sequence in exon 4 can be skipped, resulting in a highly stable truncated form, ΔFOSB, which accumulates after repeated exposure to stimuli that induce activation of dopaminergic neurons. Mutant mice which produce different expression levels of FOSB and ΔFOSB were created to study the effect of different *FosB* splice variants in locomotor activity and stress (75). Mutant mice with genotype $FosB^{d/d}$ expressed ΔFOSB but not FOSB; $FosB^{G/G}$ mice were *FosB*-null mice with no expression of FOSB and ΔFOSB; and $FosB^{+/d}$ mutants had a higher expression of ΔFOSB than FOSB. The expression of in $FosB^{+/d}$ mice mimics the conditions, where endogenous ΔFOSB accumulates during long periods of time (75). Several behavioral tasks, such as OFT, and elevated plus maze (EPM) tests were applied to examine the spontaneous locomotor activity of different mutants during 12 h night and 12 h day time in one week (75). Compared to wild-type and $FosB^{+/d}$ mice, locomotor activity of $FosB^{d/d}$ mutants increased by the third night, while locomotor activity of $FosB^{G/G}$ mice was lower than wild types. These results suggest that ΔFOSB enhanced locomotor activity during the night when mice are awake and active (75). Locomotor activity of $FosB^{+/d}$ mice which express higher level of ΔFOSB and lower level of FOSB, was less than $FosB^{d/d}$ mice expressing only ΔFOSB variant, indicating that FOSB might

reduce effect of ΔFOSB and consequently locomotor activity in *FosB*$^{+/d}$ mice. After one month, locomotor activity of all genotypes reduced; however, *FosB*$^{d/d}$ mice still showed higher activity than other groups. Also, *FosB*$^{+/d}$ mice had higher activity than wild types, indicating the effect of ΔFOSB in increasing locomotor activity in these mutants (75). Exploratory activity of different mutants, which represent anxiety-like behavior, was measured using OFT during day time. Exploratory activity of *FosB*$^{d/d}$ and *FosB*$^{G/G}$ mice was higher than wild types on the first day in the open field (75), suggesting that FOSB might increase anxiety-like behavior or inhibit curiosity, and after the second day, activity of *FosB*$^{+/d}$ and *FosB*$^{d/d}$ but not *FosB*$^{G/G}$ was higher than wild type mice, indicating less anxiety-like behavior (75). Analyzing data using another test of anxiety-like behavior, EPM, showed that compared to wild types, *FosB*$^{d/d}$ mice spent longer time in the open arms, indicating that ΔFOSB might reduce anxiety-like responses. Moreover, *FosB*$^{G/G}$ mice spent less time in the open arms compared to wild types. There was no significant difference between *FosB*$^{+/d}$ and wild type mice, suggesting that FOSB might normalize effects of ΔFOSB (75). Conventional forced swim test was applied to study the importance of *FosB* splice variants on inescapable stress. Repeated forced swim test was done during 4 consecutive days. Swimming time decreased over repeated swimming in the wild-type, *FosB*$^{d/d}$, and *FosB*$^{G/G}$ mice. However, there was no decreasing trend in swimming time in the *FosB*$^{+/d}$ mice. Moreover, upon antidepressant (paroxetine) injection before swimming test on the last day, swimming time increased in all type of genotypes, except *FosB*$^{G/G}$ mice (75).

To explore the effect of DA signaling pathway on locomotion of mice expressing different variants of *FosB*, mice were treated with methylphenidate (MPH) that increases DA level through inhibiting DA reuptake. *FosB*$^{d/d}$ and *FosB*$^{+/d}$ mice showed higher hyperactivity after MPH treatment at 4:00 PM, compared to wild type, which came back to wild type level after 4 hours.

However, during the dark phase, hyperlocomotion of *FosB*$^{+/d}$ mice reduced after MPH treatment, indicating that during the dark time, FOSB might prevent locomotor activity, which might be induced by ΔFOSB. Locomotor activity of *FosB*$^{G/G}$ mice after MPH treatment, was similar to wild type mice (75). While the effect of FOSB versus ΔFOSB is studied in *FosB*$^{d/d}$ and *FosB*$^{+/d}$ mice, there is no mutant which express only FOSB, and not ΔFOSB. Therefore, in this study, effect of FOSB alone and independently from ΔFOSB on the behavior of mice is lost. Moreover, while effect of ΔFOSB along with FOSB is studied in *FosB*$^{+/d}$ mice, rescue of FOSB in *FosB*$^{d/d}$ could provide stronger validation for the observed results.

To explore whether the hyperactivity of *FosB*$^{+/d}$ and *FosB*$^{d/d}$ mice in response to MPH is due to increased D1 receptor signaling sensitivity, locomotor activity of mice was examined after D1 receptor agonist (SKF81297 (SKF)) treatment. Upon SKF treatment no difference was found in different mutants and wild type mice. Therefore, D2 receptor antagonist (haloperidol) was applied, and subsequently locomotor activity of mice was measured. Upon haloperidol treatment, compared to the wild types, *FosB*$^{d/d}$ and *FosB*$^{G/G}$ mice exhibited higher and lower locomotor activity, respectively. It has been shown that haloperidol induces hypolocomotion (76). Therefore, higher locomotion in *FosB*$^{d/d}$ mice might be due to inhibitory effect of ΔFosB on D2 receptor signal (75).

To study whether ΔFOSB expression affects locomotor activity of mice, bi-transgenic animals with the ability of overexpressing ΔFOSB and tetracycline transactivator tTA were created (77). Mice were raised in doxycycline condition (dox mice), leading to inhibition of ΔFOSB expression; however, in the absence of the tetracycline derivative doxycycline, expressed tTA by the transgene induces ΔFOSB transcription through binding to tetracycline responsive promoter (77). Locomotor activity of mice was assessed using “locomotor activity chamber”. There was no significant difference between locomotor activity of ΔFOSB expressing mice and dox mice.

However, after first time treatment with cocaine, ΔFOSB expression enhanced locomotor activity of cocaine-treated mice than the other group. Moreover, after repeated cocaine treatment (up to 10 days), ΔFOSB expressing mice maintained higher activity than the dox mice (77).

Place conditioning test was used to examine the effect of ΔFOSB expression on rewarding response to cocaine. ΔFOSB expressing mice showed highest response to place conditioning in lower concentration of cocaine, suggesting high sensitivity of this group to rewarding effect of cocaine; while dox mice exhibited dose-dependent response to place conditioning, and their response to rewarding effects of cocaine increased in higher concentrations (77). Loss of function of ΔFOSB followed by rescue could provide stronger evidence on the effect of ΔFOSB on locomotor activity of mice in response to cocaine. Due to possible side effects of doxycycline treatment, wild type group should be included here. Endogenous effects of FOSB is ignored, to consider the possible effects of FOSB on behavioral response to cocaine, loss of function of FOSB and/or overexpression of FOSB is required. It would be interesting to study the effect of first versus repeated cocaine treatment on the splicing changes of *FosB*.

1. 3. 4. Kinases

B-Raf: B-Raf is a member of Raf kinases family which are activators of the mitogen-activated protein kinase kinase/extra-cellular signal-regulated kinase (ERK) module in regulated and deregulated proliferation. *B-raf* gene encodes several isoforms through alternative splicing. Both of spliced variants, exons 8b and 9b, are expressed in hippocampus. Conditional knockout mice for 8b or 9b exons were produced to evaluate the significance of B-raf splicing in learning and memory. Interestingly, using behavioral tasks, knockout mice for exon 9b exhibited behavioral impairment, such as defect in spatial and object recognition memory, but not in contextual fear

conditioning (78). However, the absence of exon 8b doesn't have any effect on hippocampal-dependent memory (78).

These experiments show the presence of exon 9b, but not 8b, is required for learning and memory associated with the hippocampus in the adult mice. The mechanism by which splicing of B-raf is regulated during development of learning and memory remains elusive. The recognition of splicing factor(s) which regulate AS of B-raf during memory formation could direct us to better understanding of this process (78).

1. 3. 5. Synapse related proteins

AchE: Cholinergic neurons play an important role in modulating activity of some parts of brain such as cerebral cortex, hippocampus and thalamus. ACh release is increased following pain or stress (79). AChE is an enzyme that hydrolyzes the neurotransmitter acetylcholine, thereby terminating synaptic transmission at cholinergic synapses. It has two splice variants: synaptic acetylcholinesterase (AChE-S) and Readthrough acetylcholinesterase (AChE-R). AChE-S is an abundant synaptic variant; AChE-R, however, is a stress-induced form containing a hydrophilic C-terminus, which is incapable of supporting membrane adherence. To investigate whether AS change of AChE is essential for stress-induced formation of fear memory, Nijholt et al., 2004, explored the relationship between AS change of the *AChE* gene and stress-induced consolidation of fear memory in mouse hippocampal neurons. Learning ability of mice were evaluated by fear conditioning task. Downregulated mice for AChE-R were produced using mEN101 as an antisense oligonucleotide. Overexpressed mice for AChE-R were generated by lateral ventricle microinjection of mARP, a synthetic peptide with the sequence of the mouse AChE-R C-terminus. Downregulation of AChE-R reduced learning ability of mice; while overexpression of AChE-R resulted in elevated learning performance on fear conditioning task (79).

To study the importance of AChE-R in LTP after acute stress, theta-burst-induced LTP was assessed in the hippocampal slices of stressed mice. LTP was enhanced in the stressed mice compared to the naïve mice. The stressed induced LTP was reduced in the stressed animals downregulated for AChE-R. High frequency tetanic stimulation increased LTP by 40% in mice overexpressing AChE-R compared to LTP of wild types (79).

This report suggests the essential role of an AChE-R splice variant in stress induced responses and LTP; however, rescue experiment is needed to confirm whether the observed loss of response is indeed due to loss of AchE-R. Moreover, the importance of the other splice variant, AChE-S, is not studied in this investigation. Downregulation/knockout studies for AChE-S can show whether or not absence of AChE-S is involved in stress responses as well as LTP formation. Splicing factor(s) that regulate splicing of AChE are not known here, and mechanism by which AChE splicing is controlled during facilitated responses remains unknown.

To study the importance of AChE splice variants in locomotion, learning and anxiety-related behavior, transgenic mice which overexpress AChE-S (TgS mice) and AChE-R (TgR) were tested by two exposures to the EPM, and compared to FVB/N control mice (79). Locomotor activity of male and female mice with different genotype (FVB/N control, TgS transgenic mice and TgR transgenic) was examined using automated activity, and the ambulatory activity was analyzed for different sex and genotypes (79). Female mice from the AChE-R group showed higher activity than the ones from the FVB/N control and TgS genotype. No significant differences were found among the males (79). Moreover, the females in the AChE-R group tended to run rapidly in circles in the middle of the arena rather than exploring the environment. There was no difference among the male mice (79). Ambulatory activity of TgS male and female mice was monitored for 10 min per day, and compared to that of in FVB/N mice (79). The male TgS mice exhibited higher activity

than the male FVB/N mice on days 1–6, suggesting absence of habituation to the novel environment. No difference was found among female TgS and female FVB/N mice, and females of both these genotypes exhibited significant habituation by the third day and every day thereafter (79).

To examine the learning ability, mice were placed in the Y maze to explore and find food. Vanilla or rum was considered a correct choice and other essence was the wrong choice. Two learning criteria were used: first criterion was the discrimination criterion, defined as a day that 8 correct responses in 10 consecutive trials were completed, and the second criterion was learning criterion, that is the day that criterion 1 was attained for 3 consecutive days (79). The day after mice reached the second criterion, intra/Extra Dimensional Shift task which is a test of rule acquisition and reversal was used. The TgS mice were slower to attain the discrimination criterion in the first odor discrimination compared to the TgR and the FVB/N control mice, but once they attained the criterion, they were not slower to attain rule learning (79). On the first discrimination, but not on the reversal learning, TgS males attained the rule learning criterion slower than the FVB/N males. No difference was found between TgS females and FVB/N females (79).

To test the effects of genotype for the exploration index and avoidance index the EPM was used (79). Less exploratory behavior by the TgS mice was seen on the second exposure. There was no effect of sex. On the second exposure, but not on the first exposure, TgS mice showed lower exploration compared with the FVB/N and TgR groups (79). TgS mice had less avoidance on the first exposure compared to the FVB/N and TgR mice; however, no differences among the genotypes on the second exposure was seen (79).

This investigation showed that overexpression of AChE splice variants affects locomotion, discrimination learning and anxiety behavior of transgenic mice which overexpress either AChE-

S (TgS mice) or AChE-R (TgR). However, no loss of function study followed by rescue experiment is done to confirm the role of AChE splice variants in these behaviors. The downstream mechanisms which affect behavior by each of these splice variants is not studied. Moreover, the regulation of AChE splicing by splicing factors is not investigated.

NRXN: The three neurexins genes (NRXN1/2/3) encode synaptic adhesion molecules. AS of segments 1 to 6 (AS1-6) produce thousands of spliced variants. Alternatively spliced segment four (AS4) is encoded by exon 21 (53). To examine the importance of exon 21 inclusion in STM, conditional knockout mice for *Nrxn3 AS4* splice isoform were created, and then exon 21 was conditionally removed using $Pvalb^{cre}$ mice. To eliminate the possibility of overlapping or redundant function of other Nrxn family member, *Nrxn1,2,3* genes, $Nrxn1/3^{ex21\Delta PV}$ double mutants were created by combining $Nrxn3^{ex21\Delta PV}$ single mutant with $Nrxn1^{ex21flox}$ knock-out mice (53).

Novel object recognition task was used to evaluate the STM of mutants. $Nrxn1/3^{ex21\Delta PV}$ mutant showed the similar preference for exploring novel and familiar objects. Wild type mice spent more time to explore novel objects than familiar objects, which suggest**s** short memory deficiency in $Nrxn1/3^{ex21\Delta PV}$ mutant mice. No anxiety behavior and locomotor deficiency was seen in $Nrxn1/3^{ex21\Delta PV}$ mutant compared to the wild types (53). This study showed loss of exon 21-containing *Nrxn1/3* transcripts led to short term memory deficiency in mice.

The STM ability is studied in double knock out mice ($Nrxn1/3^{ex21\Delta PV}$) compared to wild type, and no comparison between $Nrxn1/3^{ex21\Delta PV}$ and $Nrxn1^{ex21flox}$ knock-out mice is done. The redundant function of *Nrxn*2 is not eliminated. The splicing factors and upstream mechanisms in which they regulate AS of AS4 is not studied. It would be interesting to investigate the downstream mechanisms in which splicing of AS4 could affect memory.

Suv39h1 is a histone methyltransferase for H3K9me3, and maintains repressive histone markers by binding to H3K9me3. Doxycycline (dox) induces knockdown of *Suv39h1* through expression of TurboRFP/shRNAmir (80). KCl stimulation induces *Nrxn1* SS4 inclusion, which was abolished in *Suv39h1* knockdown by dox as well as *Suv39h1*$^{+/-}$ mice (80). To examine the importance of Suv39h in memory, Tet-on system was used to knock down *Suv39h1* in hippocampus of mice. Contextual fear conditioning test was applied to assess the fear memory of *Suv39h* knockdown mice (80). Retrieval of recent memory was not changed in *Suv39h1* knockdowns, indicating encoding of memory might not be affected by lack of Suv39h1. Compared to wild types, *Suv39h1* knockdown mice showed reduced freezing time in the fear conditioning test, which suggest Suv39h1 is required for preventing fear memory (80).

To see whether SS4 inclusion is important for memory preservation, mice were injected with LV-CMVp-Nrxn1B SS4^{+}. Knockdown of *Suv39h1* using dox didn't affect the contextual fear conditioning memory of mice expressing SS4. Indicating lack of *Suv39h1* could affect memory by reducing SS4 inclusion (80). Inclusion of *Nrxn1* SS4 is essential for memory, and lack of *Suv39h1* impairs memory by decreasing SS4 inclusion which is rescued by SS4 expression.

This study shows the importance of a methyl transferase on regulation of splicing of *Nrxn1*, which leads to controlling memory, suggesting that epigenetic modification can control adaptive changes by regulating AS of specific genes. However, the mechanisms in which this modification can regulate AS of *Nrxn1* is not studied. For example, which splicing factors are involved in regulation of AS of *Nrxn1*, and how epigenetic modification by Suv39h1 could affect their function. Moreover, the mechanisms in which *Nrxn1* splice variants could affect memory are not studied.

1. 3. 6. Splicing factors

SAM68: KH-domain RNA-binding protein SAM68 (Src-associated in mitosis 68 kDa protein) is an RNA binding protein which regulates alternative splicing. To explore the importance of Sam68 in motor activity and spatial learning and memory, Sam68 knockout mice were created (81). These parameters were assessed in KO mice in comparison with wild type mice by behavioral tests. The Sam68-null mice display normal learning and memory in the WMT, which is used to evaluate hippocampal-dependent spatial learning and memory. Motor coordination, which is assessed by beam balance and rotorod is abnormal in the Sam68-null mice compared with the wild-type mice (81). These experiments showed that lack of Sam68 leads to motor coordination defects in mice. While KO mice have provided interesting observation of the importance of splicing factors in adaptive changes, rescue experiment could confirm these results. Considering alternate roles for splicing factors, it remains unknown whether the motor coordination impairment in SAM68 KO mice is due to change in AS of target genes of SAM68 or not. Rescue for different splice variants of SAM68 target genes could address this issue.

RBFOX: The RBFOX family, which are encoded by three genes *(Rbfox1*, *Rbfox2*, and *Rbfox3)*, regulate AS. To study the role of Rbfox3 in spatial learning and memory as well as anxiety behavior in mice, *Rbfox3*$^{-/-}$ mice were tested using WMT (55). Knockout mice exhibited moderate spatial learning impairment, however, after day 6 there was no alteration between *Rbfox3*$^{-/-}$and wild types, suggesting that in the mice lacking Rbfox3 there is a delay in spatial learning (55).

To test the role of Rbfox3 in hippocampal dentate gyrus related anxiety behaviors, novelty-suppressed feeding test was used. Food-deprived wild type and knockout mice were placed in the familiar environment where they were presented with food. No latency in feeding

was observed between the groups in the familiar environment. However, in the new environment, Rbfox3 knockouts fed earlier than wild type mice, indicating reduced anxiety behavior. To further examine the anxiety behavior, some other behavioral tests including marble-burying test, elevated plus-maze test and locomotion task were applied. These results obtained by the four tasks also suggest that *Rbfox3*$^{-/-}$ mice show reduced anxiety (55).

A rotarod test, gait analysis and grip force test were used to investigate whether absence of Rbfox3 could cause motor coordination deficiency. These experiments showed that there was no difference between performance of knockout and wild type mice, suggesting that Rbfox3 deletion do not affect motor coordination of mice (82).

A sensory nervous system's response to certain harmful or potentially harmful stimuli is called nociception, which triggers different physiological and behavioral responses. To examine the nociceptive behavior and sensorimotor gating in mice lacking Rbfox3, hot/cold plate test and prepulse inhibition test, were used. Prepulse inhibition is a neurological occurrence in which a weaker pre-stimulus stops response to the subsequent stronger stimuli (82). Rbfox3 knockout mice showed cold hyperalgesia, while there was no difference in prepulse inhibition test (82). To investigate the recognition memory, the novel object recognition test was used. Rbfox3 knockout mice showed impaired cognitive abilities and learning when compared with wild type mice (82).

Overall, these investigations demonstrated the importance of Rbfox3 in hippocampal related behaviors in male mice, and suggest that Rbfox3 knockout mice display cold hyperalgesia, delay in spatial learning and memory, impaired cognitive ability as well as reduced anxiety related behavior compared to wild type mice. However, no rescue was done to confirm the role of Rbfox3 in observed behavioral changes in Rbfox3 knockout mice.

Splicing factors often have additional different functional roles in the cells. To understand the mechanisms in which Rbfox3 regulates behavior, it is essential to determine the functional role of Rbfox3 in this process. It is still not discovered how Rbfox3 might regulate behavioral responses to environmental stimuli: What are the pathways leading to activation of Rbfox3 by environmental stimuli which influence behaviors? What are the essential genes involved in behavior regulation, and if their splicing is controlled by Rbfox3? And through what mechanisms Rbfox3 can regulate splicing of these genes? In these investigations, effect of Rbfox3 deletion in hippocampal related behavior is studied; however, the importance of this splicing factor in regulation of behavioral responses in other region of brain remains elusive.

GEMIN2: Studying the importance of splicing factors in regulating circadian rhythm could help us understand the role of splicing in controlling circadian clock. The circadian rhythm maintains a constant period over different temperatures resulted in seasonal fluctuations. GEMIN2 is a spliceosomal small nuclear ribonucleoprotein assembly factor which modulate the assembly of U1–U5 snRNPs and is conserved from yeast to humans (83).

To investigate whether the circadian network in plants is regulated by spliceosome assembly, Arabidopsis thaliana mutants with defect in GEMIN2 gene were used (83). When plants were kept at 10 °C for 4 weeks, the survival rate of GEMIN2 mutants decreased compared to wild types. Moreover, the effects of temperature on circadian period were strongly enhanced in GEMIN2 mutants resulting in disruption of temperature compensation. *Arabidopsis* plants with GEMIN2 mutation showed mild growth and developmental alterations such as shorter petioles and smaller leaves at 22 °C under long-day conditions (16 h light:8 h darkness). Rescue of GEMIN2, however, improved these phenotypes to the wild types. Flowering time is a clock-dependent phenotype in *Arabidopsi*, and mutants showed an early flowering phenotype. Moreover, mutants exhibited a

short-period phenotype for the circadian rhythms of leaf movement (83). In this study, the essential role of GEMIN2 for appropriate acclimation of Arabidopsis thaliana to seasonal temperature changes was shown, and the rescue experiments confirmed its effect.

U2af26: To investigate the significance of splicing factors in controlling circadian clock, the importance of *U2af26* on circadian biology in mice was examined using knockout mice for U2AF26 (84). U2-auxiliary-factor 26 (U2AF26) is one of the U2 auxiliary factor (U2AF) subunit, which undergoes AS (84). Under 12 hr LD conditions mice lacked U2AF26 showed wheel-running behavior similar to wild types, and no strong alteration upon dark-dark (DD) was observed (84). To test the importance of U2AF26 in experimental jet lag, phase advance experiment was done. The required time to adapt to the new activity onset was measured after animals were phase-advanced by 4 hr. The activity onset in U2AF26- deficient animals was changed by over 3 hr on the first day already, while wild types advanced their activity onset by 1 hr per day. This experiment showed U2AF26 deficient mice adapted faster to jet lag (84). However, more studies are required to show this splicing factors regulate circadian clock through controlling alternative splicing. In addition, it is interesting to investigate the importance of their target genes in regulation of circadian period in absence or presence of these splicing factors. It is said that U2AF26 itself undergoes alternative splicing; however, the effect of different spliced variants on circadian behavior is not tested.

MBNL1: Muscleblind-Like Splicing Regulator (MBNL1) regulates splicing of their target genes in a tissue specific manner (85). One of these target genes is *DMPK*. CTG repeat expansion in the 3′UTR of *DMPK* gene leads to Myotonic dystrophy (DM1). Expanded CUG repeat leads to misregulation of splicing by MBNL1. DM1 patients have behavioral disorders such as mental retardation and spatial memory impairment, anxiety related behavior and depression. These patient

also show lack of attention, cognitive impairment and hyperactivity. Apathy, which is lack of interest and enthusiasm, is another problem of these patients. (86).

To study the importance of MBNL1 and DMPK in anxiety-related behavior, mice lacking either MBNL1 or DMPK were created. Anxiety-related behaviors (thigmotaxis) and general activity were measured by the OFT. When mice were placed in the open- field, they tended to remain close to the walls and incline to explore the peripheral zone, this tendency to remain close the walls is called thigmotaxis which is an index of anxiety in mice. There was no difference between thigmotaxis, ambulation or average velocity between *Dmpk*$^{-/-}$ mutant and wild types mice. However, thigmotaxis increased, and ambulation and average velocity decreased in *Mbnl1*$^{-/-}$ mice. These results suggest that *Mbnl1*$^{-/-}$ mice have decreased general activity (86). The WMT was used to study the visuo-spatial learning and memory. The *Dmpk*$^{-/-}$ showed normal acquisition (latency to find the platform), swim speeds and thigmotaxis. While *Mbnl1*$^{-/-}$ mice exhibited normal acquisition, the swim speed was decreased; and levels of thigmotaxis were increased in these mutants. The *Mbnl1*$^{-/-}$ but not *Dmpk*$^{-/-}$ mice spent more time "floating" as training proceeded, indicative of decreased motivation (86). To directly test apathy in *Mbnl1*$^{-/-}$ mice to study whether these mutants have motivational deficits, a sucrose consumption test, a classic anhedonia test, was chosen. The *Mbnl1*$^{-/-}$ mice lacked interest in sucrose, indicating decreased motivation (86). To test the importance of *Mbnl2 on* spatial memory, adult *Mbnl2* knockout and wild-type mice were examined by WMT, which indicated that mice lacked *Mbnl2* have spatial memory deficiency (56).

Hypersomnia, or excessive daytime sleepiness (EDS), and associated perturbations in rapid eye movement (REM) sleep patterns are several phenotypes of myotonic dystrophy. *Mbnl2* knockout mice exhibited related REM sleep changes over 24 hours, such as increased REM sleep

episode numbers. However, these changes were more profound during the active (dark) period. REM sleep episodes were two times more in *Mbnl2* knockouts than that in wild types. The large portion of REM sleep episodes had short latencies from the proceeding wake episodes in *Mbnl2* knockouts when compared to wild type mice. Moreover, after sleep deprivation, *Mbnl2* knockout mice showed profound REM sleep rebound. In contrast no change was seen in wake and REM sleep parameters in Mbnl2 deficient mice (56). These studies show the importance of Mbnl1 in anxiety, motivation and spatial learning and memory, and the role of Mbnl2 in regulation of sleeping in mice. Rescue experiment could confirm these results.

It has been suggested that Mbnl2 may have a compensatory role when the expression of Mbnl1 is compromised (56); therefore, double knockout for Mbnl1 and Mbnl2 could help us to understand the role of these factors in regulating observed phenotypes. These results indicate that Mbnl2 knockout mice are useful to study DM1-associated splicing alternations which may be related to phenotypes of DM1 disease. Identifying the target genes of Mbnl1 and Mbnl2, and their expression or functional changes in Dm1 patients could help us to understand the molecular mechanisms which are related to this disease, and could give new therapeutic approaches. It would be interesting to study the mechanisms which thereby Mbnl1 and Mbnl2 could regulate their target genes and to indicate how these mechanisms are altered in DM1 patients. Since it has been shown that Mbnl1 is important in inducing motivation, the importance of these factors in other adaptive events such as addiction could be studied.

hnRNP A/B: hnRNPs are a large group of RNA-binding proteins. Among them, the hnRNP A/B paralogs including A1/A1^{B}, A2/B1, B2, and A3, are the most abundant (57). To explore the importance of hnRNP A/B in learning and memory in mice, WMT was used. HnRNP A/B knockdown mice were created by injections of shRNA lentivirus against hnRNP A1 or A2/B1 to

the entorhinal cortex (57). WMT results suggested that memory and learning capabilities are reduced in knockdown mice for hnRNP A1, hnRNP A2 and both A1+A2. This study showed that absence of hnRNP A1, A2 and A1+A2 resulted in reduced memory and learning ability in mice. Double knockdown of A1 and A2 could prevent any possible compensatory effect of A1/A2 reduction by the other one. However, rescue experiment was not done. The downstream mechanisms by which these splicing factors affect learning and memory formation were not studied. Moreover, the target genes of A1 and A2 were not determined. The upstream mechanisms induced by environmental condition leading to activation of hnRNP A/B and thereby memory formation were not studied as well. It would be interesting to investigate the target gene/genes of hnRNP A/B which play a critical role in memory formation, and upon their deletion the memory is lost.

SRSF1: Vascular endothelial growth factor A (VEGF-A) is a member of VEGFs family which are secreted polypeptides, and has an important role in vascular development. VEGF-A AS is induced by serine-arginine rich splice factor 1 (SRSF1). Serine-arginine rich protein kinase 1, SRPK1 controls phosphorylation of SRSF1. AS of VEGF-A produces two isoforms which differ by their C' terminals, VEGF-A_{xxx}a (pro-angiogenic) and VEGF-A_{xxx}b (anti-angiogenic). Phosphorylation of SRSF1 splicing factor induces production of VEGF-A_{xxx}a. Both VEGF-A isoforms could activate VEGF receptor-2 (VEGFR2) (87). Neuropathic pain resulted from maladaptive plasticity in the nociceptive neuronal system, which leads to alterations in sensory neuronal excitability. Noxious stimuli (hyperalgesia) and innocuous stimuli (allodynia) could lead to neuropathic pain (87).

To explore the importance of SRSF1 in neuropathic pain in rodents, SRPK1 inhibitor (SRPIN340) was used to inhibit SRPK1 in the spinal cord of rats (87). Nociceptive behavioral

testing was used to test the effect of SRPK1 antagonist application on the neuropathic pain. Mechanical and cold allodynia were induced by performing the partial saphenous nerve ligation injury (PSNI) model of neuropathic pain. The mechanical withdrawal threshold is defined as a maximum pressure applied that cause paw withdrawal. Using PSNI, mechanical withdrawal thresholds of the ipsilateral hind paw is decreased, however, this reduction is blocked by SRPIN340 application. Moreover, compared to control group, SRPIN340 induced a reduction in tactile and cooling allodynia, which refers to central pain sensitization (87).

Hargreaves method was used to determine responses to thermal stimulation. Which showed that the SRPK1 inhibition increased the tolerance of animals to injury, both ipsilateral and contralateral (87). Interestingly, SRPK1 inhibition enhanced level of VEGF-A_{xxx}b, and reduced VEGF-A_{xxx}a isoform, while there was no change in the total level of VEGF-A. These results raise the question whether VEGF splice variants can have a different role in neuropathic pain (87). VEGF antagonists (PTK787) was administrated in mice and rats, and subsequently pain behaviors were measured. Mechanical withdrawal thresholds and heat-induced nociceptive withdrawal time were increased in PTK787 treated animals compared with control vehicle treated groups (87), indicating increased tolerance to pain. To study the effect of VEGF-A_{xxx}a in neuropathic pain, VEGF-A_{165}a was injected to mice. Mechanical thresholds and time taken for withdrawal from heat were enhanced upon VEGF-A_{165}a injection, indicating VEGF-A_{165}a resulted in pain. However, VEGF-A_{165}b administration enhanced mechanical withdrawal thresholds as well as heat withdrawal latencies, suggesting central anti-nociceptive effect of VEGF-A_{165}b (87). Rats that received neutralizing antibody against VEGF-A_{xxx}b, showed reduced mechanical withdrawal thresholds and heat withdrawal latencies similar to VEGF-A_{165}a treated groups, which suggest that neuropathic pain could be induced by loss of endogenous VEGF-A_{xxx}b from the spinal cord (87).

Since SRPK1 inhibition enhance the level of VEGF-A_{165}b, by increasing the proportion of VEGF-A_{165}b, the effect of SRPK1 inhibition is mimicked. Therefore, two days after start point of neuropathic pain in rats, exogenous VEGF-A_{165}b protein was administrated in spinal cord. Mechanical thresholds, cold allodynia and withdrawal latencies both ipsilaterally and contralaterally were reversed to control group (87). This study shows that production of VEGF-A_{xxx}a which is controlled by the SRSF1 splicing factor, induces pain in rodents, while the VEGF-A_{xxx}b isoform has an anti-nociceptive effect. These findings are proved by different experiments, such as using SRPK1 inhibitor which inactivates SRSF1 splicing factor, administration of endogenous VEGF-A_{165}b and applying neutralizing antibody against VEGF-A_{xxx}b. Applying neutralizing antibody against VEGF-A_{xxx}b rescued VEGF-A_{165}a effect, which could confirm the different roles of VEGF-A_{xxx}a and VEGF-A_{xxx}b isoforms in neuropathic pain.

These experiments suggested a possible link between pain and the VEGF variants and SRPK1. However, more functional coupling evidences between these factors are needed before a conclusion can be reached. For example, using inhibitors could have unknown effects on the various mechanisms and pathways in the cell which subsequently affect the observed results; therefore, knockout experiment might give more specific results. The mechanisms in which different splice variants could affect pain was not studied. Moreover, the mechanisms SRSF1 splicing factor can regulate VEGF splicing were not studied.

1. 3. 7. Other proteins

Per: The *period* (*per*) gene, which has two splice variants, is important for controlling the circadian clock in *Drosophila.* These two types are different from each other by the presence of an alternative intron in the 3′ untranslated region. Type A encodes prototypical 1,218-amino-acid PER protein and retain intron in the 3′UTR; type B lacks this intron and is 96 amino acid-shorter

than type A (88). To determine the importance of these splice variants in regulation of circadian clock and behavior in drosophila, transgenic flies, which expresses modified *per* genes (per^{A}, $per^{B'}$ and per^{G} genes) were generated. The per^{A} transgene, which produced only type A transcript, was generated by mutagenizing both the 5′ and 3′ splice junctions of the alternative intron in the 3′UTR. Type B′ transcripts was generated by $per^{B'}$ transgene, which was made by removing the alternative intron in the 3′UTR. per^{G} transgene, produced both transcript types A and B′, was constructed of an unmodified *per* genomic DNA fragment (88). per^{G} transgenic flies which express both splice variants mimic wild types, and were considered as control. Male flies were kept in 12-h light/12-h dark cycles for 72 h and then in constant darkness for 7 days. The circadian period was analyzed by collected locomotor activity data during constant darkness (88). All genotypes could rescue locomotor activity. Wild type flies have the period of 24.0 hours. While per^{A} showed average circadian periods of 25.4 hours, which was 1.0 hour longer than per^{G} with the average circadian period of 24.4 hours. $per^{B'}$ flies had average circadian periods of 24.7 hours, which was closer to per^{G} as a control group compared to per^{A} flies (88).

These experiments showed that all transgenic flies with different splice variants of per gene could preserve locomotor activity, while the period of different genotypes are different to some extent.

Synchronizing by daily changes in environmental modalities such as light and temperature is an important adaptive feature of circadian clocks, therefore, circadian clocks enable organism to organize their physiology and behavior. To investigate response of circadian clock to cold temperatures *Drosophila melanogaster* was used as a model (89). The locomotor activity rhythm as a function of temperature was measured. Wild type flies were maintained in 18°C, 25°C, or 29°C and kept in 12 hours light followed by 12 hours dark and afterwards exposed in constant dark

condition for 6-10 days. At the different temperatures, endogenous period length of the activity rhythm remained constant. There were two peaks for the activity of flies during LD at the standard temperature of 25°C, one morning peak, and another evening peak (89). Following a midday, the evening activity of wild type flies gradually increases which anticipates the light to dark transition (89). To examine the role of three different genotypes on activity at different temperatures, the locomotor activity of different transgenic flies was measured at two temperatures (18°C and 29°C) and photoperiods (12:12 LD and 6:18 LD) (89). At 25°C and 29°C (higher temperature), flies showed more nocturnal as the photoperiod is shortened; however, at 18°C (colder temperatures), flies even in short photoperiods (only 6–8 hr in length) had their evening activity during daytime hours. There was a similar distribution of activity in wild type and per^{G} flies. However, under 6:18 LD, per^{A} and $per^{B'}$ flies exhibited delay in the peak of evening activity, and displayed lack of anticipatory rises in evening activity. Moreover, under the shorter photoperiod, per^{A} and $per^{B'}$ showed delay in activity rhythms. Under both photoperiods, the activity peak of per^{A} flies was delayed compared to per^{G} flies; moreover, per^{A} flies exhibited lengthened free-running period compared to per^{G} flies (89).

This experiment suggests the importance of *per* splice variants in activity of drosophila in colder temperature (89). These study showed the importance of *per* splice variants in regulation of circadian clock. While all transgenes could rescue locomotor activity, the difference in behavioral rescue suggests that splicing of 3′UTR alternative intron affects function of transcripts to rescue locomotor activity. While the role of *per* splicing in circadian clock is shown, the molecular mechanism of per splicing by environmental factors such as light and temperature remains

to be studied.

FMRP: Fragile X syndrome is a form of heritable intellectual disability, which is caused by mutation in the fragile X mental retardation gene (*FMR1)*. The fragile X protein (FMRP) is an RNA binding protein, located in the neuronal nucleoplasm and within nuclear pores. Human FMRP has at least 20 isoforms, produced by alternative splicing. FMRP isoforms can have alterations in domains such as second KH domain, a nuclear export sequence, a major phosphorylation site, and C-terminal domain. While FMR proteins are highly conserved in their N-terminal RNA binding regions, they show more diversity in their C-terminal domain. Most AS of this protein occurs in the regions that encodes C-terminal domain, enriched in glutamine and asparagine (Q/N) residues (90). Similar to human *FMR1, Drosophila fmr1 (dfmr1)* pre-mRNA undergoes alternative splicing, resulting in isoforms with alterations in the second KH domain and the C-terminal peptide (90).

To assess the importance of isoforms of *D. melanogaster* in short and LTM formation, various mutants were produced: larvae homozygous for a null allele of *dfmr1,* larvae expressing a rescue fragment introduced by *P* element transformation in *dfmr1* null background, where the mutant transgene is the only source of dFMR1 protein expressing dFMR1 with a deletion of the Q/N domain, and wild type rescue expressing a wild type *dfmr1* allele in *dfmr1* null background. To produce an allele ($dfmr1^{L}$) that is unable to generate the Q/N- isoform, a derivative of the *dfmr1* genomic rescue fragment was produced: a *dfmr1* cDNA clone was substituted with *dfmr1* genomic DNA where AS that produces the Q/N- isoform occurs, resulting in eliminating Q/N- synthesis. These mutants were the only source of dFMR1 protein (90).

The conditioned courtship suppression paradigm is a method to test learning ability in various kinds of mutants. Using conditioned courtship, the importance of the dFMR1 Q/N isoform on immediate recall and STM was assessed. During training session, flies were paired with

unreceptive females, and then either faced with receptive females immediately (immediate recall), or after one hour isolation to test their STM. A comparison of naïve and trained flies showed that similar to wild type rescue, flies with ΔQ/N alleles of *dfmr,* but not null mutants, exhibited reduced courtship at the immediate recall stage, indicating that they retained memory immediately after training. However, contrary to wild type, no difference was observed between the naïve CI of ΔQ/N and null flies and the CI measured one hour after training, indicating that the memory index declined in ΔQ/N mutants and nulls flies. Flies lack dFMR1 Q/N-isoform (*dfmr1*^{L}) and wild type flies have a similar naïve courtship level as well as one hour STM of courtship training. However, flies expressing either the *dfmr1*^{L} or *ΔQ/N* allele exhibited a deficit in 4-day LTM of courtship training, indicating that dFMR1 Q/N- isoform is essential for LTM formation (90).

These experiments, especially wild type rescue, demonstrate the essential role of isoforms containing Q/N domain in short and LTM formation in Drosophila. It would be interesting to investigate the role of other domains with/without presence of Q/N domain. Identifying splicing factors that regulate FMR1 mRNA splicing during memory formation can help us better understand the mechanisms that modulate memory formation. It is proposed that human FMRP autoregulates *FMR1* pre-mRNA splicing by binding to a G-quartet-forming segment of its transcript (90); however, further studies are required to reveal whether this splicing autoregulation is important during memory formation.

1. 4. Depolarization

The concentration of K^+ and Na^+ is higher inside and outside of the cell, respectively. Stimuli that changes the distribution of these ions across the cell membrane, could lead to membrane depolarization. Depolarization is required for the function of cells, including neuronal cells. The movement of depolarization to the end of axon of neuronal cells is called action potential (91)

1. 4. 1. Role of depolarization in physiology and pathophysiology

A variety of inhibitory or excitatory stimuli, such as chemical, physical and electrical stimuli are able to affect the membrane potential. Excitatory and inhibitory stimuli induce depolarization or hyperpolarization, respectively. Changing the concentration of K^+ in culture media can induce depolarization. Glutamate is a neurotransmitter, which physiologically is able to induce depolarization. Depolarization of neurons can lead to release of different neurotransmitters and changes the intracellular concentration of Ca^{2+} (92), which can regulate different physiological events, such as synaptic plasticity and learning and memory formation (93, 94). It has been shown that both amoeboid locomotion and pinocytosis of *Amoeba proteus* are regulated by membrane depolarization, in which upon environmental conditions that induce depolarization, amoeboid locomotion is stopped and pinocytosis is increased (95).

1. 5. Dopamine

DA is a neurotransmitter and belongs to the catecholamine group. Catecholamine is a monoamine which is made by amino acid tyrosine. Tyrosine hydroxylase coverts tyrosine to dopa, which later is changed to DA by dopa decarboxylase. DA could be hydrolyzed to norepinephrine by DA beta hydroxylase (96). When DA is made, it is stored in the vesicles at terminus of dopaminergic neurons. Calcium influx induced by action potential stimulates the fusion of storage vesicles and release DA in the synaptic space (96, 97).

DA binds to the receptors. DA receptors are G protein coupled receptors which perform their role through second messenger cAMP. DA receptors are classified into two groups: D1-like including D1 and D5, and D2-like consist of D2, D3 and D4. D1-like receptors which coupled to G_s could have an excitatory effect by activating adenylyl cyclase which results in increasing the level of cAMP, while D2-like receptors are inhibitory receptors which coupled to G_i/G_o and inhibit

adenylyl cyclase, resulting in inhibition of cAMP production (98, 99). The affinity of D2 receptors are higher than D1 (100); therefore, D2 receptors are tonically activated by even low level of DA (101), leading to opening of potassium channels which results in less excitability of the neurons. Activity of D2 receptors are important for the motor activity. Upon deletion of D2 receptors, but not D1 receptor, mice exhibit parkinsonian symptoms (102-104). D1 receptors which are activated by higher level of DA are involved in LTP formation and reinforcement (101). DA is reuptaken by the autoreceptors on the presynaptic neuron, and then be re-stored in the vesicles of be degraded by monoamine oxidase (105).

Basal ganglia, which is located at the base of forebrain is associated with regulating various body functions, such as motor activity, learning, habits, cognition and emotion. Striatum is one of the main and largest component of basal ganglia which is divided into two parts, dorsal and ventral. Dorsal striatum itself is composed of caudate nucleus and putamen, and ventral striatum, NAc and olfactory tubercle (97). Dopaminergic neurons send inputs to the striatum. Most of the striatal cells are GABAergic neurons which send the outputs to the others parts of basal ganglia (97). DA reaches to basal ganglia mainly from the substantia nigra pars compacta (SNc), ventral tegmental area (VTA), and the retrorubral field (RRF) (106). Striatal sells receive DA trough DA receptors and thereby DA triggers the downstream pathways (98, 99).

1. 5. 1. Role of dopamine in physiology and pathophysiology

DA has been known to modulate various aspects of adaptive behavior, such as learning and memory and addiction, in response to different adaptive stimuli. However, there are a lot to be learned about the mechanisms by which DA regulates these behavioral activities. Moreover, the association of different aspects of behavior with each other and the similarity and differences of DA action of these aspects remained to be discovered.

1. 5. 1. 1. Learning and memory

The importance of DA in learning and memory has been examined in different investigations. For example, T-maze task showed that DA depletion in the striatum of rats impairs the learning behavior, which was rescued with L-dopa injection (107). In another study, mice lacked DA exhibited memory and learning ability deficiency in WMT, which was partially rescued using L-Dopa injection into the striatum (108). Moreover, in DA-depleted mice, which are suffering from PD, LTP impairment as well as learning ability deficiency was observed which was examined by food pellet reaching task (109). These experiments suggest an essential role of DA in learning ability.

1. 5. 1. 2. Motivation

Studies on the DA deficient mice have shown that lack of DA leads to less food consumption and animals were suffering from hunger because of less motivation to feeding. However, L-Dopa could rescue these behaviors (110). Moreover, rewarding responses of rats to amphetamine and cocaine was lost upon administration of DA antagonists (111-115). DA-deficient mice showed less locomotor responses to morphine as well as reduced analgesic effects of morphine, which were recovered by L-Dopa administration (116). Using D1 and D2 receptors antagonist suggest that both of these two types' antagonists reduce preference of mice to sucrose consumption (117).

Moreover, sugar preference of *Drosophila* flies which lacked DA was abolished, which was rescued by L-DOPA (118).

1. 5. 1. 3. Locomotor activity

Importance of DA on the locomotor activity is investigated by several studies. For example, it has been shown that mutant drosophila flies lacking tyrosine hydroxylase in the nervous system, which is required for DA synthesis, have locomotor deficiency and are less active than wild types.

Moreover, these mutants, had a longer sleep as well as impaired aversive olfactory learning. Rescue with L-DOPA restored all of these behaviors to wild type level (119). DA deficient mice were created by inhibiting tyrosine hydroxylase (TH) production. A few weeks after birth, these mutants showed hyperactivity and stopped food consumption. L-DOPA injection rescued activity and feeding. These experiments suggest that DA is essential for locomotor activity (120).

Taken together, these studies have demonstrated that splicing of specific genes is required for some adaptive events. Therefore, we set on to address the underlying molecular basis by trying to test splicing changes of genes in striatal cells after repeated compared to single stimulation.

Chapter 2: Statement of Hypothesis

Prior studies have revealed that: AS of specific genes and splicing factors can be regulated by adaptive stimuli; and, some adaptive responses disappear upon loss of specific splice variants or splicing factors (75, 81, 90). However, no study has addressed whether and how AS of specific genes is changed after repeated versus single exposure to adaptive stimuli has been performed. Neuronal activity and DA have been shown to be essential for some adaptive events; therefore, here we ask the question: Does repeated stimulation change the transcript abundance of splicing factors or alternative splicing of specific gene(s) differently from that induced from single stimulation?

In this project, I completed studies that tested the hypothesis that repeated stimulation with KCl-induced depolarization or dopamine causes adaptive and/or sensitized changes of the abundance of transcript for splicing factors and the pattern of alternative splicing of certain genes in neurons.

Chapter 3: Materials and Method

3. 1. Materials

3. 1. 1. Cell line

Mammalian cell line	Source
Mouse striatal-derived cell line (STHdh(Q111/111))	Coriell Institute

3. 1. 2. Materials for cell culture

Material	Manufacturer
Cell culture plate	BD FALCON
Disposable serological pipet	FALCON
Fetal bovine serum (FBS)	Invitrogen
Gibco® Dulbecco's modified eagle medium (D-MEM) powder	Invitrogen
Gibco® Penicillin-Streptomycin, 100X solution	Invitrogen
L-Glutamine, 200mM solution	Invitrogen
Polypropylene centrifuge tubes	BD FALCON
Polypropylene tubes with snap cap	VWR
T175 tissue culture flask	FALCON
Trypsin, 2.5%	Cellgro

3. 1. 3. Chemicals and reagents

Name	Manufacturer
ß-Mercaptoethanol	Sigma-Aldrich
Agarose	American Bioanalytical
Glycerol	Fisher
Bromophenol blue	Fisher

Calcium chloride	Fisher
Dimethyl sulfoxide (DMSO)	Fisher
Ethanol	Fisher
Ethidium bromide (EtBr)	Sigma-Aldrich
Ethylenediamine tetraacetic acid (EDTA)	Fisher
Gibco® D-glucose	Sigma-Aldrich
Glycine	Fisher
Isopropanol	Fisher
Magnesium chloride	Fisher
Methanol	Fisher
$MgCl_2$ 25mM	Promega
Phosphate buffered saline (PBS) tablet	MP Biomedicals
Polyoxyethylene-20-sorbitan monolaurate (Tween 20)	Fisher
Potassium chloride (KCl)	Fisher
Sodium chloride (NaCl)	Fisher
Sodium dodecyl sulfate (SDS)	Fisher
Sodium fluoride (NaF)	Sigma-Aldrich
Sodium orthovanadate (Na3VO4)	Fisher
Tris base	Fisher (J.T. baker)
Triton X-100	Fisher
Hydrogen chloride	Fisher
Dopamine hydrochloride, 98.5%	Alfa Aesar

3. 1. 4. Enzymes and buffers

Name	**Manufacturer**
Source 5x first strand buffer	Invitrogen
Benzonase® nuclease	Sigma-Aldrich
dNTPs	Invitrogen
Dithioreitol (DTT), 1M	Invitrogen
M-MLV reverse transcriptase	Invitrogen

Protease inhibitor cocktail	Sigma-Aldrich
SUPERase• In™ RNase inhibitor	Invitrogen
Taq DNA polymerase	Xie lab
DNase I recombinant	Roche

3. 1. 5. Kit

Name	Source
Gel extraction kit (250)	QIAquick
GenElute mammalian total RNA miniprep kit	Sigma-Aldrich
RNeasy® plus mini kit	QIAGEN

3. 1. 6. Others

Product	Source
Acrodisc® syringe filters (0.45 μm)	PALL
Syringe filters cellulose acetate (0.2 μm)	NALGENE

3. 2. Recipe for the buffers and solutions

NP-40 Buffer (Nonidet-P40)

Component	Concentration
NaCl	150 mM
Tris· HCl (pH 7.5)	10 mM
EDTA·2Na	1 mM
Igepal	0.65%

5 × TBE Buffer

Component	Concentration
Tris	445 mM (54g)
Boric acid	27.5g
EDTA (pH: 8.0)	10 mM
ddH2O	Fill up with to 1 L

6 × SDS Loading dye (50 ml)

Component	Concentration/Volume
Tris· HCl (pH 6.8)	300 mM (15 ml)
Glycerol	50% (25 ml)
ß-Mercaptoethanol	6% (3 ml)
SDS	12% (6 g)
Bromophenol blue	0.12% (6 mg)

RT Mix I

Component	Concentration/Volume
5 × First strand buffer	400 µl
dNTP (100 mM)	2.5 µl each (2.5 × 4 µl)
DTT (0.1 M)	200 µl
ddH_2O	90 µl

10 X PCR buffer (40ml)

Component	Concentration/Volume
Tris.HCL PH: 8.3	200mM (8.0 ml)
$MgCl_2$	15mM (6.0 ml)
KCl	250mM (2.5 ml)
Tween-20	0.5% (667 µl)
BSA	1mg/ml (800 µl)
ddH2O	22.5ml

PCR Mix 1

Component	Concentration/Volume
10XPCR buffer	25ml
dNTP (100mM)	50ul ×4

$MgCl_2$ (100mM)	125ul
ddH_2O	18.814ml

3. 3. Methods

3. 3. 1. Cell culture

Striatum is important in some adaptive activities such as motor activity, learning and cognition (97). Therefore, we used E14 striatal precursor cell line, from a knockin transgenic mouse containing homozygous Huntingtin (HTT) loci with a humanized Exon 1 containing 7 polyglutamine repeats (STHdhQ7/Q7), which were bought from Coriell Institute (https://www.coriell.org/) (Fig. 2). Cells were immortalized by retrovirus infection transducing the tsA58/U19 large T antigen (121). Since large T antigen is only expressed at 33°C (122), cells are temperature-sensitive and die at the temperature higher than 33°C (121). Striatal cells were seeded in the 100 mm cell culture dish (DMEM media, plus 10% fetal bovine serum, 1% penicillin-streptomycin glutamine solution) at the 33°C as recommended by Coriell Institute and described before (121). Cells were passaged when they were almost 70-80% confluent.

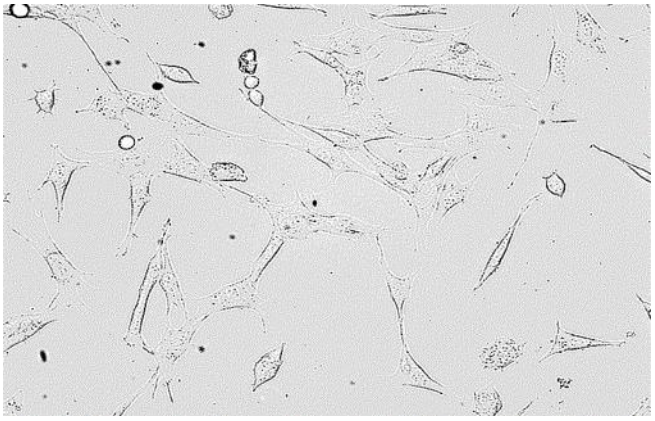


Figure 2. Phase contrast micrograph (40 ×) of wild-type mouse striatal-derived cell line (STHdh (Q111/111)).

3. 3. 2. Treatment procedure

3. 3. 2. 1. KCl treatment

The cells were divided into three groups: First group was treated just once with KCl (50mM for 3 hours). In the second group, the treatment was repeated up to 8 times with 21 hours of interval between each treatment. In each treatment, cells were treated for 3 hours with KCl, and then media was replaced with fresh media. Third group was non-treated negative control. At the end of treatment period, RNA was extracted (0.5, 1, 3, 6, 12 and 21 hours) after adding KCl (Fig. 3).

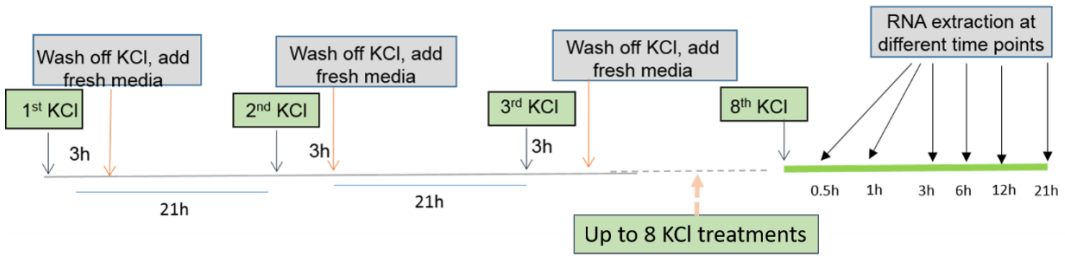


Figure 3. Experimental design. Striatal cells were treated with KCl followed by wash-off and then addition of fresh media. Treatment was repeated for 8 times with 21 hours interval between each treatment. At the end of treatment period cytoplasmic RNA was extracted at different time points.

3. 3. 2. 2. DA treatment

The cells were divided into three groups: first DA treated group, which was treated just once with DA, repeated DA treated group, which was treated with DA over 7 times with the 24 hours of interval between each treatment, and the third group was not treated, considered as a negative control. For DA treatment, DA was added to the cells without replacing DA containing media with fresh media, and after the 24 hours interval, fresh DA was added to the media. RNA extraction was done 9 hours after the last treatment (Fig. 4).

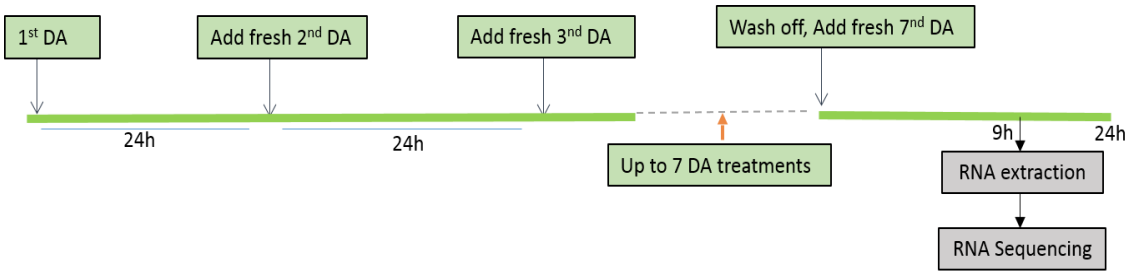


Figure 4. Experimental design. Striatal cells were treated with DA once or up to 7 times with 24 hours interval between each treatment. At the end of treatment period cytoplasmic RNA was extracted at different time points.

3. 3. 3. RNA preparation

The striatal-derived cells are adherent. At the end of treatment period, cells were scraped using rubber scraper. After washing with ice-cold PBS containing 1mM EDTA, the total RNA was extracted using RNeasy® Plus Mini Kit (QIAGEN) according to its protocol.

The whole-transcriptome RNA-Seq sequencing was carried out for non-treated, 1st and 7th DA treatment groups. Differential levels of total transcripts were determined using edgeR, and alternative splicing, alternative transcription start site and polyadenylation were analyzed using DEXSeq and rMATS.

3. 3. 4. RNA sequencing (RNA-Seq)

The efficiency of DA treatment on gene expression and splicing was confirmed by RT-PCR, which showed the effect of DA on expression and splicing of *FosB*. Approximately 2 µg of the total RNA extracted from different treatment groups (non- treated, 1st DA treated and 7th DA treated), each in triplicate, were sent to the Génome Québec Innovation Centre at McGill University (Montréal, Québec, Canada) for RNA sequencing (Illumina Hi-Seq 4000 PE 100

paired-end 100bp sequencing). Reads were mapped to the mus musculus genomic database (Mus-musculus GRCm38/mm10) allowing maximal two mismatches.

The initial library size was ~380 million of reads, of which ~250 million reads survived quality control measurement and an average of 92.1 % were uniquely properly mapped, and used for subsequent analysis (i.e. AS and transcript abundance).

3. 3. 5. Bioinformatics analyses of RNA-Seq read data

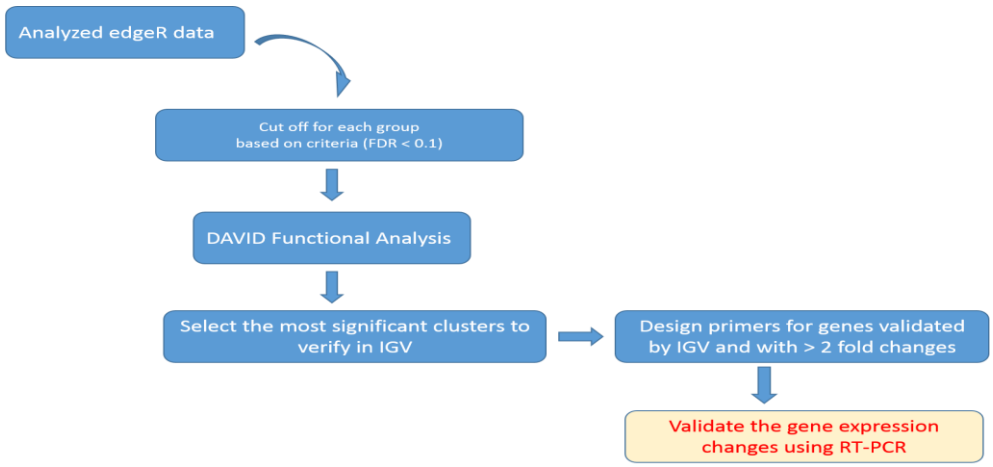
Using edgeR (differential expression analysis of digital gene expression data in R), which is a Bioconductor software package for analyzing gene expression and uses an exact test analogous to the Fisher's exact test, differential expression of counted data in different samples were examined (123, 124) (Fig. 5. A).

To estimate the accuracy of level of expression we used logCPM (logarithm of counts per million reads) method. logCPM can be converted to RPKM (Reads per kilo base per million mapped reads) using the formula: RPKM=2^(logCPM-log2(genelength)). However, we used logCPM instead of RPKM, because logCPM is a more accurate descriptive measure for filtering out genes with low counts which have a similar chance in expression estimation (125, 126).

DEXSeq (differential exon usage using RNA-Seq data) and rMATS (replicate Multivariate Analysis of Transcript Splicing) method were applied to calculate for differential exon usage in replicate RNA-Seq data. The R Bioconductor package DEXSeq is a statistical method which uses ANODEV (Analysis of Deviance) for detecting differential exon usage from RNA-Seq data (127), and rMATS is a tool to test differential AS events corresponding to all different types of AS patterns in comparative replicate RNA-Seq experiments by calculating the P-value and false discovery rate using likelihood ratio test (128, 129). Difference at adjusted p values < 0.05 were considered significant.

Integrative Genomics Viewer (IGV) was used to visualize the sequencing data (developed at the Broad Institute of MIT and Harvard, available for download from http://www.broadinstitute.org/igv) (130). The significantly changed genes were classified according to their function by DAVID functional analysis (developed at the U.S. National Institute of Allergy and Infectious Diseases, available on the Internet at http://david.abcc.ncifcrf.gov) (131) (Fig. 5. B).

A)



B)

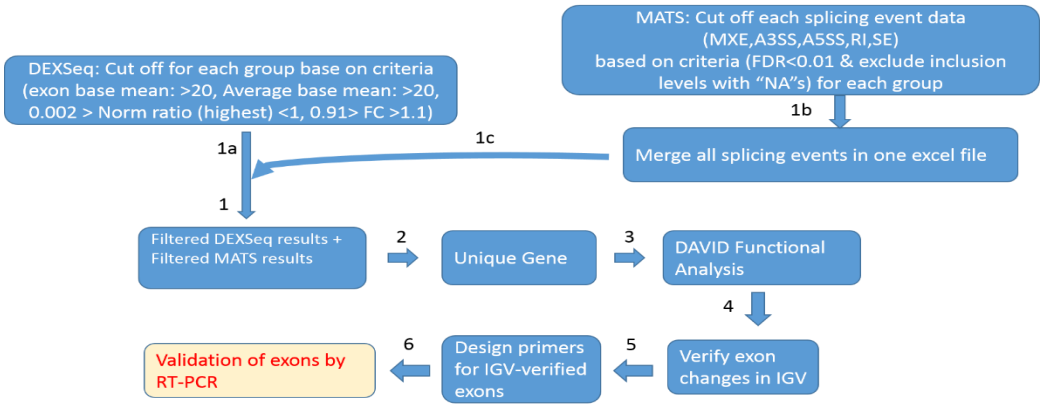


Figure 5. Analysis of genes from edgeR, DEXSeq and rMATS output of RNAseq reads. A) Analysis of genes from edgeR output of RNAseq reads. B) Analysis of unique genes from DEXSeq and rMATS output of RNAseq reads.

3. 3. 6. Reverse transcription-polymerase chain reaction (RT-PCR)

Reverse transcription reaction was made using M-MLV Reverse Transcriptase (Life Technologies), SUPERase In™ RNase inhibitor (Ambion) and Oligo (dT)18 primers. About 200 ng of total RNA was used in 10 μL reverse transcription reaction.

PCR was done in 12.5 μL PCR reaction containing 1 μL RT sample for 30 cycles. PCR products were visualized with a digital camera under ultraviolet (UV) after running on 2.5 % agarose gels containing ethidium bromide (EtBr). The bands were then quantified using ImageJ software (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/ij/).

3. 3. 7. Primers for RT-PCR validation

Primer	Sequence
Actb	F: TGGGACGATATGGAGAAGATTTG R: CCATCACAATGCCAGTGGTAC
Gapdh	F: CTTCATTGACCTCAACTACATGGTT R: GCTCCTGGAAGATGGTGATG
FosB	F: CTGCAGCTAAGTGCAGGAAC R: GTGAGGACAAACGAGGAAGTG
Ddx18	F: GGACACTTCATTTGCTTCCTTG R: CTGGTAGGAGAGAGAATCAG
Spg7	F: GCCGACATCGCCAACATCTG R: GTGAAGAGGTACTGGTCCCGAG
Map3k4	F: CTGCTGAAATCCACAGGGAG R: GTCAGGAGCAGGAAGGCATC
Mtor	F: GGCTTTGTGGACCCTGGGAC R: GGAACTGCACACACTTGAG
Stk38	F: GACAGTATTAGCCATAGACTCC R: GGAGCAATGTAGTCAGGAG
Cdk8	F: GCAGAACTACTAACGTCAGAACC R: GTGGAATGCTTTACTATCGGG
Camk2d	F: GGGATGGACTTTCACAGATTC R: GAGTTTGCCAACAGTAACGGAAG
Vwa8	F: CACAGTCCTTGGCAGCCTC R: GTAAGTGTTCGCCAAGGAC
Kif5	F: CCAGAAGGCATGGGAATTATTC R: CATAGGGAACACGGTTTTTG
Gabrg1	F: CCGACACTTTCTTCAGGAAC

	R: GATCAGCTACTTCCACAGAGG
Ints2	F: CAGAGTTCTTGGTGCCTGAGTC R: CGACGAGGGGAATCACTGTGTTC
Sf1	F: GGTGGACAGGATGGTTCGGCAAAG R: CCTATTGAGCAACGAGTCAG
Ccn2	F: CTTCAGCAGACTCAAGAACTGG R: GAATCCTCTTCAACCTACTAG
Nr1h2	F: GCCTTGCTTATCGCCATCAAC R1: GGAGGAGAGGTGAAGAGTCC R2: GCTTCTTGTCCTGGAGTCGCAATG
Srebf1	F: CAGCGGTTTTGAACGACATC R: GGTGGCTGCTGAGTGTTTCC
Tle3	F: GTCAATGCCGGGCAAACCTC R: CACAGCACATACCACCTCTCC
Zfp276	F: CCTGAGCGAAGATGAGAGTG R: CTGGTGGACGAGAAGGTGC
Samd1	F1: GAGTGGATTCTGGACACCATC F2: GAGTGCTAAGGAGAGGGTC R: CACCGGAGATGGAGAATC
Cdc34	F1: GCTGCTGCTGGAACTGAAGG F2: CAGAGCGAGTGGAGCCTTC R: GGAATACGGGTAGTCGATGG
Tulp3	F: GGAAAGCAAGCCTCAGGTTCTC R: CTCATCTTCCTCCTTGTCAGGC
Mga	F: GATGCCCTGGATTCAGTGAGG R: GCTGCACACCTGAACTTGCTCAG
Comt	F: GAGCATTGTTCCAGGTTTCC R: GTCTGAGTCGTTGCTGCCATTG
Gemin5	F1: GGCATCTTTGGCTTCGCTG F2: CTCATCACCCTGGACAATACAACC R: GTCCTGGGCTCTGTGAAG
Pus1	F1: CTTCGTGCACGTGGTCAGCTTC F2: CCAAGACATCTGGAAGACAG R: GAGTAGGCCATGAGCAGCAC
Spata24	F1: GAGAAGTTACAGTTTGCTCTGG R1: CTTACGCTTCTGATCCAGCAC R2: GACTTGCCTGTATCCTCAGC
Gm1673	F: CTGCTTGTTCGCGGTTCCTTG R: GCTGCATGAATTCAAATGCGTG
Il33	F: CCAGGTGCTACTACGCTACTATGAG R: GATGTCTGTGTCTTTGATGGG
Per2	F: CATACACAGAGAGAGGAGCAGGG R: CAGACCCTGTGCTCTCAGAAG
Dok7	F: GAAGCACTGGAAGCCCTG R: GGTCCTTCTTGTGATGTGCCG
Tbc1d30	F: GAGGATGCTGTTGCGAGTGC

	R: CATCCGCTCCATCATCATACTG
Tgfb2	F: GAGTTCAGGGTCTTCCGCTTGC R: CCTCCGCTCTGGTTTTCAC
Abca1	F: CAAGATGCTGACTGGAGACACC R: GCTCTGTGATGGCGTCAAACTG
Grem1	F: CTCTCCTTCGTCTTCCTCC R: GACTCAAGCACCTCCTCTCCAG
Rbm38	F: GACCCTAACCCTATCATC R: CAAGGTGGGGTGTAGTTGCTG
Sf3b4	F: CTCCTGGCTCTTTTCCACCTC R: GATGAGGCATTCCAGGAGGTG
Hnrnpf	F: GTGTTTCCTACAGAAGGC R: CAATCCATCTCGGTTCTGTGTG
Ddx5	F: GCTCCTATTCTGATTGCTACC R: GGTCGCTCACTTGCTTTATG
Ept1	F: CATAGTGACTGCGGTTGTGG R: GGCTTCATAGACGGACTTG
Spp1	F: GTGACTGATTCTGGCAGC R: CAAGGAGATTCTGCTTCTG

3. 3. 8. Image data analysis

Image J software was used to quantify TIFF images (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/ij/) (132).

3. 3. 9. Statistical analysis

Two-tailed unpaired Student's t-test was done to analyze significance of experiments repeated three times. P values < 0.05 was considered statistically significant.

CHAPTER 4: Results

4. 1. Effect of KCl-induced depolarization and dopamine on *FosB* splicing

To examine the effect of KCl-induced depolarization on the splicing of *FosB*, striatal cells were treated with KCl (50 mM) for different times. After cytoplasmic RNA extraction and RT-PCR, the expression of *FosB* and *ΔFosB* were analyzed. KCl-induced depolarization promoted *ΔFosB* expression strongly after 3, 6, and 9 hours at the concentration of 50 mM (Fig. 6). Cells treated with KCl for 0.5, 1 and 2 hours were extracted at the same time with NT group for 2 hours.

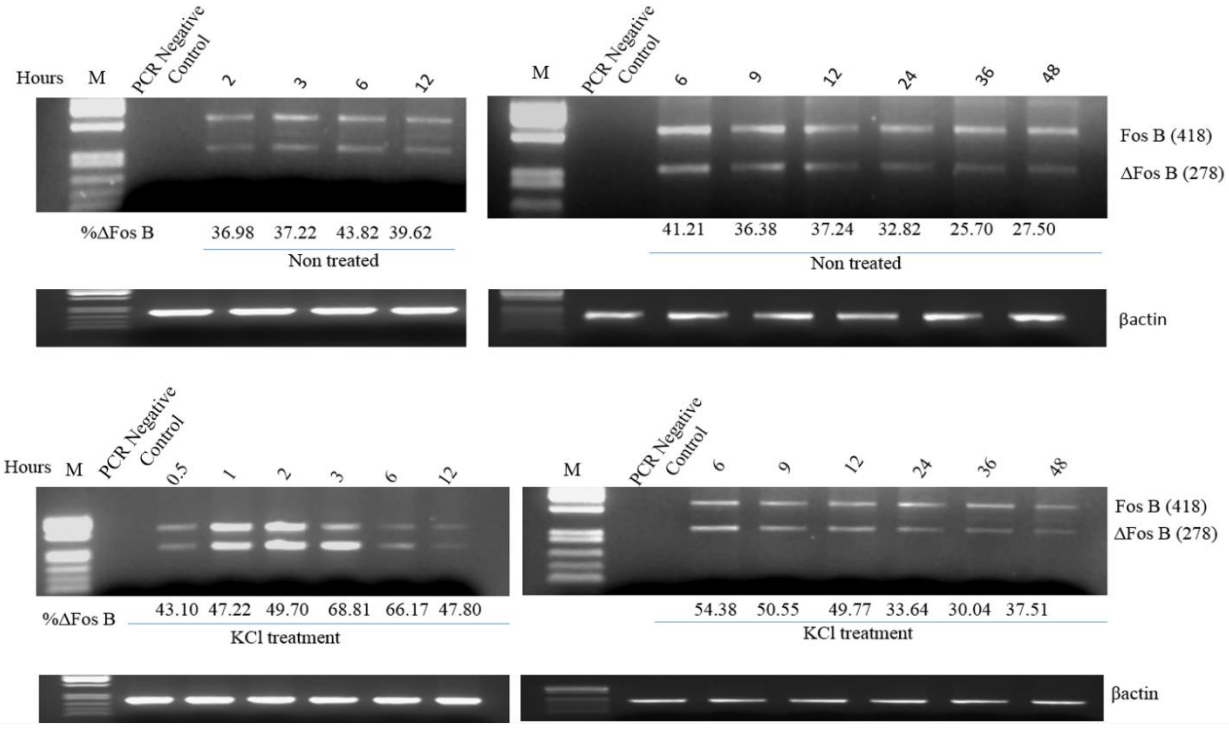


Figure 6. Splicing change of *FosB* in striatal cells after treatment with KCl (50mM) at different times (n = 2). KCl induced *FosB* splicing after 3, 6 and 9 hours. M: Marker.

Many rewards and drugs of abuse induce secretion of DA as well as the expression level of ΔFosB protein in the brain (25, 133-135), which consequently regulates transcription of many

different genes involved in neuronal plasticity (12, 25, 136). Therefore, we examined the effect of DA on *ΔFosB* expression as well.

To determine whether or not DA induces splicing of *FosB*, striatal cells were seeded in 6 well plate, and each well was treated with different concentration of DA, ranging from 1nM to 500uM, for 3 hours. Then cytoplasmic RNA was extracted, and splicing of *FosB* was studied by RT-PCR. DA strongly induced splicing of *FosB* at all concentrations, while the expression of *ΔFosB* reached to its highest level at the concentration of 250 nM (Fig. 7). Therefore, DA could induce *ΔFosB* in a dose dependent manner.

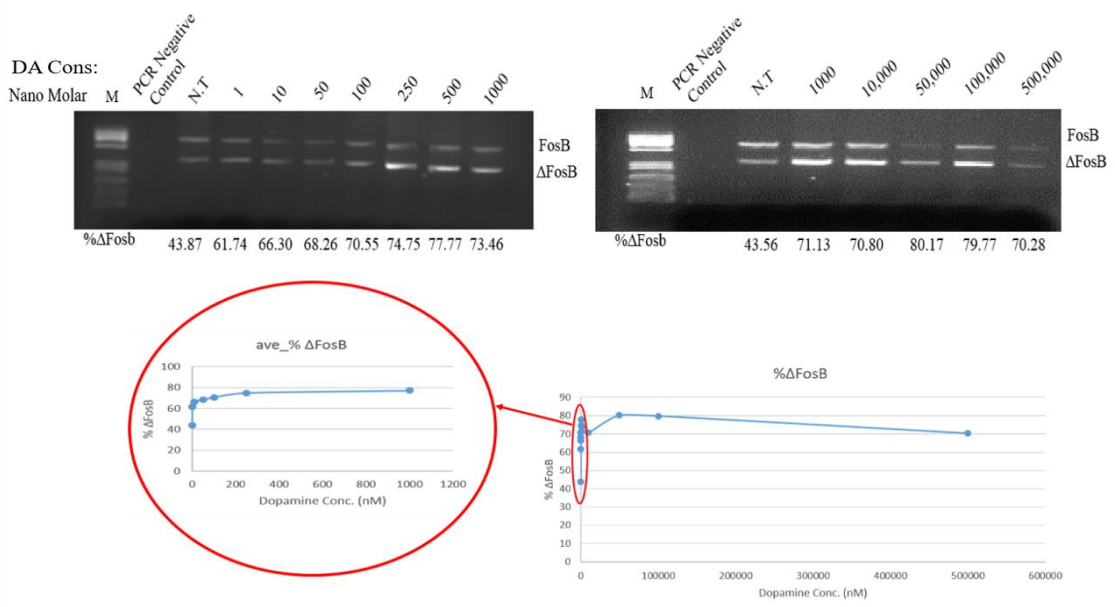


Figure 7. DA treatment at different concentration for 3 hours induces *FosB* splicing (n = 2). M: Marker.

We also examined the pathways involved in regulation of *FosB* splicing, using nifedipine which is a L-type Ca^{2+} channel blocker, and trichostatin A (TSA), a deacetylase inhibitor. Also, effect of 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) as a protein kinase C (PKC) stimulator was studied on the induction of *FosB*, with or without KCl treatment. Cells were seeded in 6 well plate,

pre-treated with, either nifedipine (20uM for 30minutes) (137), TSA (1uM for 2 hours) (138), TPA (120ng/ml for 10 min) (139). KCl (50mM for 3 hours) was added after the end of pre-treatment. Vehicle-DMSO treated group, KCl treatment only and non-treated groups were controls.

Figure 8 shows that TSA alone induced ΔFosB almost at the same level as KCl, while, co-treatment of TSA and KCl increased ΔFosB level higher than KCl or TSA treatment alone, indicating that KCl and TSA might induce *FosB* splicing independently through effecting different pathways, which enhance *ΔFosB* expression compared to KCl or TSA treatment alone.

Stimulation of PKC pathway by TPA induced splicing of *FosB* dramatically, co-treatment with KCl, however, didn't enhance TPA effect, suggesting that KCl might enhance splicing of *FosB* through the same mechanisms as TPA (Fig. 8).

Nifedipine induced *FosB* splicing, however, KCl+nifedipine treatment didn't enhance the effect of nifedipine treatment (Fig. 8).

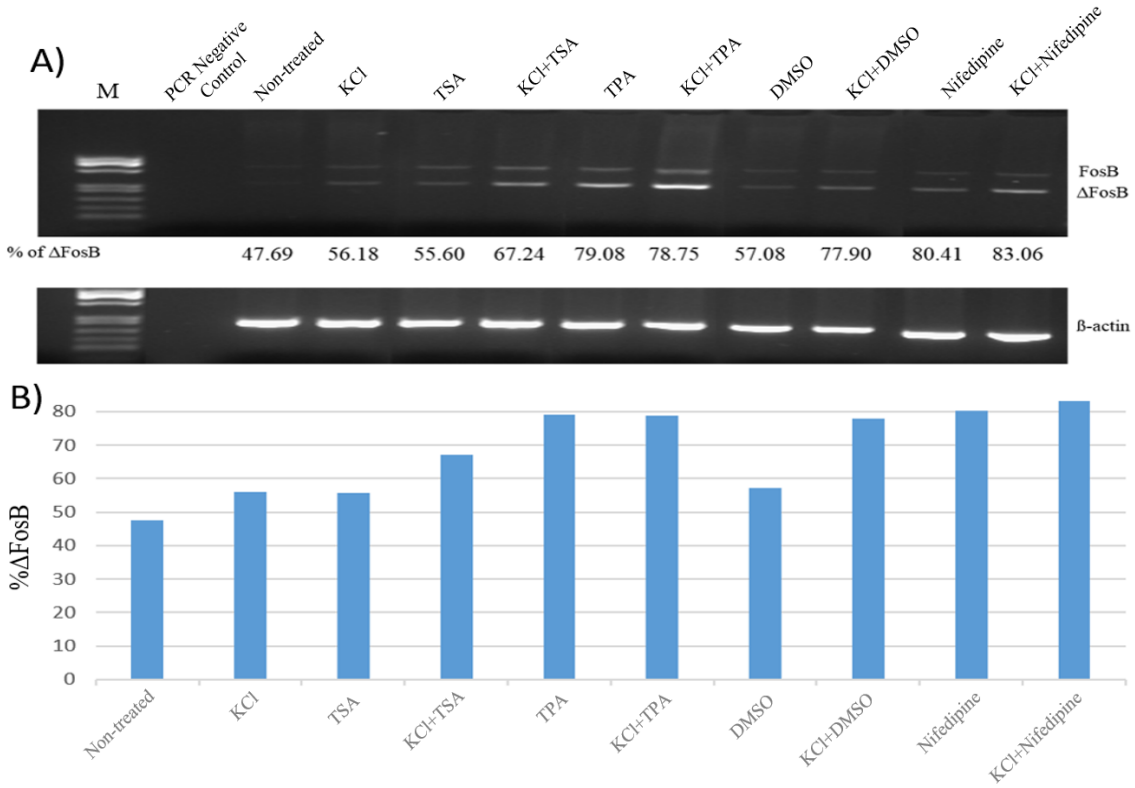


Figure 8. A) Effect of inhibition or stimulation of different pathways on KCl-induced splicing of *FosB* in the striatal cells (n = 1). B) The diagram shows the percentage of *ΔFosB* mRNA level after different treatments. M: Marker.

4. 2. The splicing changes of *FosB* after repeated KCl-induced depolarization or DA treatment, compared to a single treatment

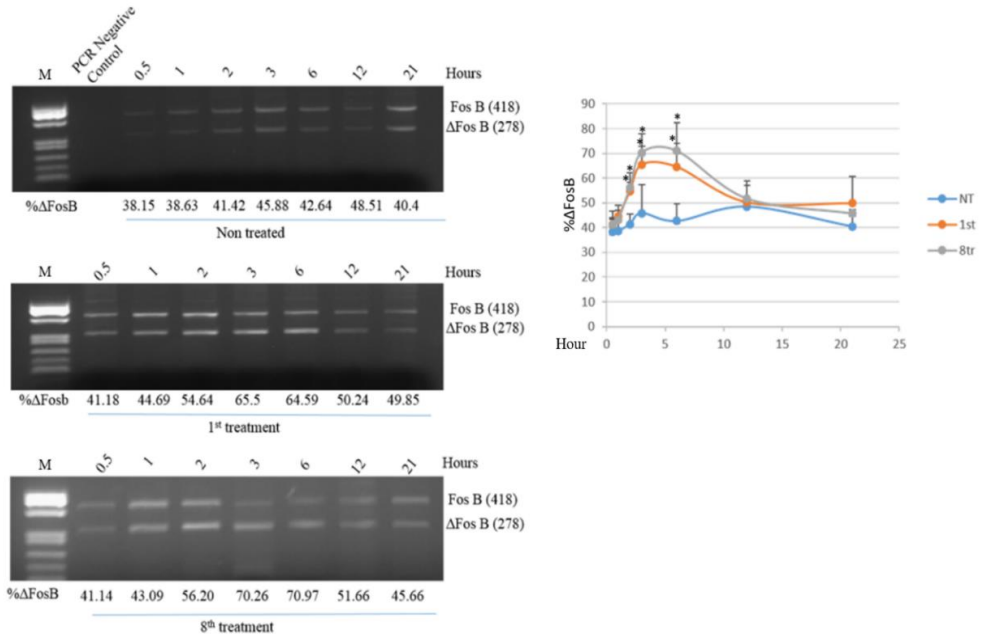
Many adaptive changes are developed after repeated, but not just after single exposure to adaptive stimuli. For example, addiction occurs after several exposures to the drug. However, the molecular mechanisms for differential effect of single and repeated stimulation on gene expression remains to be understood.

It has been shown that first time treatment of GH3 cells with KCl, which induces membrane depolarization, leads to skipping of STREX (*Stress-Axis* Regulated Exon) exon of *Slo1* gene which

encodes BK channels, through phosphorylation of hnRNP L (140). As shown in Figure 6, first time KCl (50mM) treatment induces *FosB* splicing. Since first time KCl-induced depolarization regulates splicing of important genes involved in adaptive responses, such as *Slo1* and *FosB*, we assumed that repeated KCl treatment might have a different effect than the single treatment on the splicing of target genes, leading to induction of adaptive or sensitized splicing, which might be involved in development of adaptive responses in organisms.

Therefore, we focused on the effect of first and repeated KCl treatment on *FosB* splicing in striatal cells. To this purpose, cells were treated with KCl (50mM) for 1, 8 or 10 times. 8 times treatment experiment was done in three independent experiments. However, no splicing pattern change in *FosB* was found after 8 or 10 times, compared to the single KCl treatment (Fig. 9), although the total transcript and *ΔFosB* level increased briefly.

A)



B)

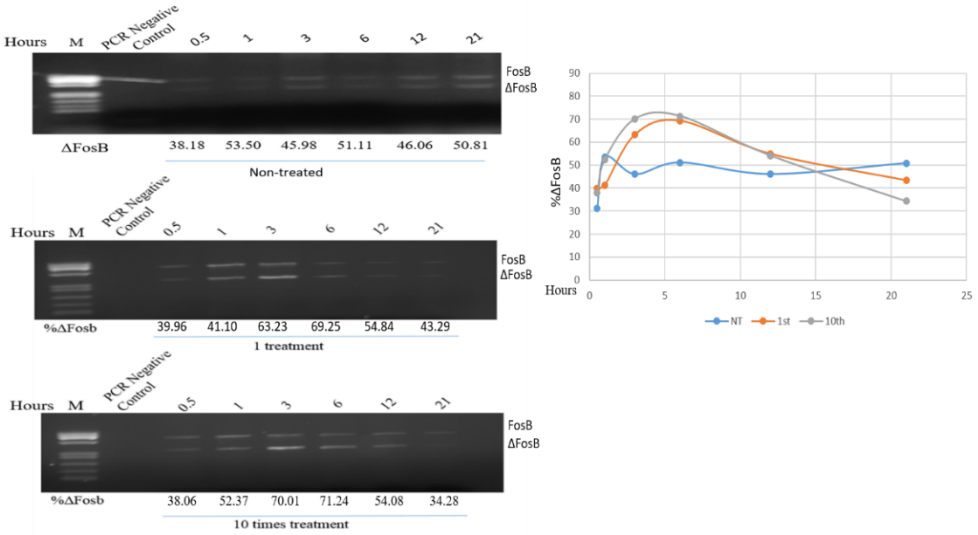


Figure 9. Comparison of single and repeated KCl treatment (50 mM) on splicing of *FosB* in striatal cells. Cell were treated with KCl for either 8 (Mean ± S.D., n=3, *: $p<0.05$) (A), or 10 times (n=2) (B). No significant change in the level of *ΔFosB* was observed after 10th treatment compared to 1st treatment. Line graphs show the percentage of *ΔFosB* mRNA level after KCl treatments in different groups. M: Marker.

Induced membrane depolarization using lower concentration of KCl (25mM) didn't show any dramatic difference on the splicing pattern of *FosB* in striatal cells. Therefore, striatal cells were treated with KCl (25mM) once or up to 8 times. Subsequently splicing change of *FosB* was analyzed. No change in *FosB* splicing was observed after single and repeated treatment compared to non-treated cells (Fig. 10), suggesting that even repeated treatment with lower concentration of KCl doesn't affect the pattern of *FosB* splicing. However, this experiment was only done once with single samples at each time point.

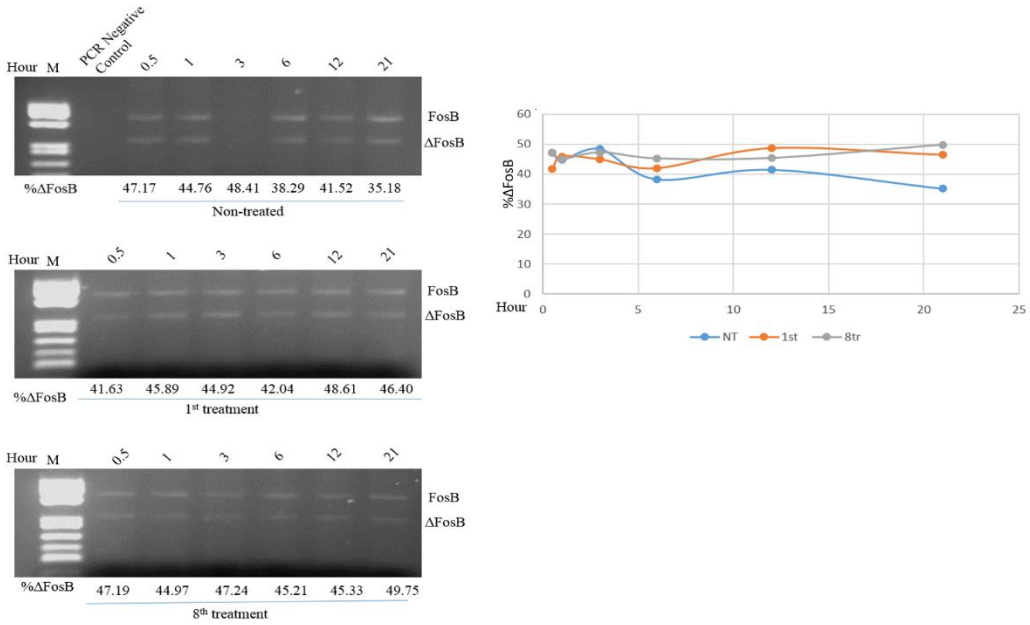


Figure 10. Comparison of single and repeated treatment (8 times) with KCl at lower concentration (25mM). No significant change in the level of *ΔFosB* was observed between 1st treatment, 8th treatments and non-treated cells (n = 1).

Tonic level of DA is released ranging from 5 to 20 nM depending on the different region of the brain in rats and mice (141-147). However, upon stimulation of DA neurons, DA could be released as high as 1 μM (148-150). Variety of drugs of abuse increase DA level in the synaptic space of different regions of brain such as striatum (135, 151). In several *in vivo* investigations that

studied the effect of various drugs of abuse on behaviors of rodents, animals were treated with the drug for up to 7 consecutive days to show adaptive behaviors (17, 135, 152). Therefore, here we treated striatal cells with 10 nM DA (3 hours) for up to 7 times with a 21 hour interval between each treatment. Treatment was done every day at the specific time to mimic the *in vivo* condition where animal seeks drugs at the same time as they administrated drugs before. Cells were treated for 1st or up to 7 times, and then splicing of *FosB* was examined after different times (0.5, 1, 3, 6, 12, and 21 hours) after the last treatment. Expression of *FosB* were induced rapidly, and level of both *FosB* and *ΔFosB* were increased after half an hour exposure to DA, and returned to basal level after almost 12 hours. The percentage of *ΔFosB* mRNA, however, increased after 3 hours, and returned to control level almost after 12 hours (Fig. 11).

By comparison between the first time treatment and 7th treatment, no obvious difference at the level *ΔFosB* was observed (Fig. 11), suggesting that repeated DA had the similar effect as single DA treatment on the splicing of *FosB* in the cultured striatal cells.

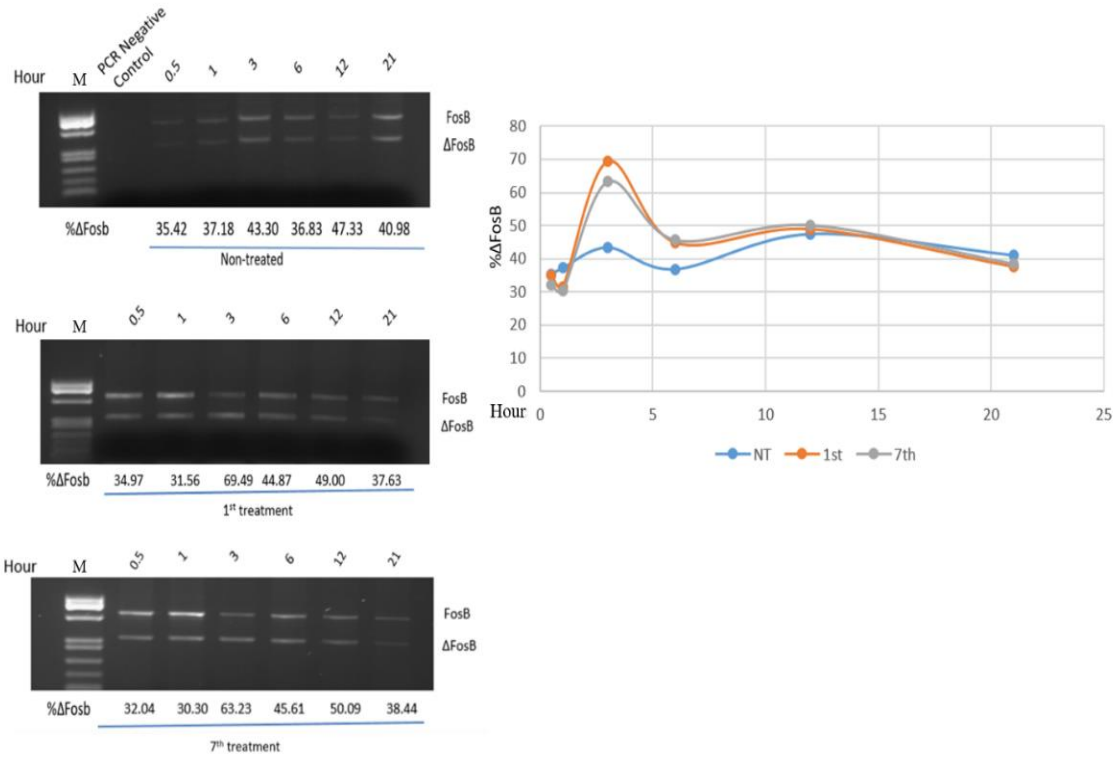


Figure 11. Repeated (7 times) DA treatment (10nM) induces %ΔFosB expression at the same level as single treatment in cultured striatal cells (n=2). M: Marker.

We also examined a lower concentration of DA (1nM) on splicing of *FosB* after repeated (7 times) compared to single treatment, to see whether or not repeated treatment with lower concentration can affect splicing changes of *FosB* differently from the single one. No significant difference was observed after repeated compared to the first time treatment (Fig. 12).

In summary, repeated KCl or DA treatment changed the transcript abundance (%) of *FosB* and *ΔFosB* variants compared to non-treated cells, but they show no dramatic difference compared to 1st treatment.

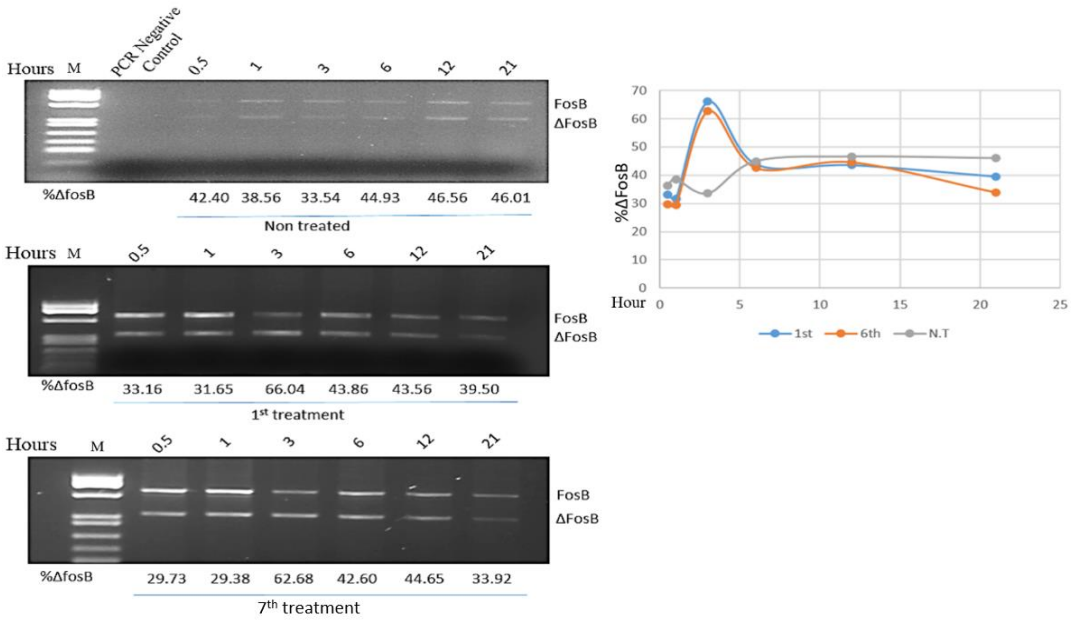


Figure 12. Effect of repeated (7 times) lower concentration of DA (1nM) treatment on splicing of *FosB* (n=2). M: Marker.

4. 3. Splicing changes of genes in DEXSeq and rMATS analysis

Striatal cells treated with 10 nM DA for 1 or 7 times were sent for RNA-Seq sequencing. By DEXSeq and rMATS, exons with the most significant splicing changes were identified. DAVID Functional Analysis of the genes that undergo AS showed different clusters with the highest enrichment score as it is mentioned in Table 1.

Table 1. Splicing changes of genes by DEXSeq and rMATS analysis

Treatment groups	Most Significant Cluster of Genes	Example
NT vs 1st	Transcription regulation cluster (4.00E-07)	Hnrnpdl, Srsf10, Hmga1
NT vs 7th	mRNA splicing cluster (7.20E-07)	Hnrnph1, Srsf4, Srpk2
1st vs 7th	Transcription regulation cluster (1.40E-05)	CaRF, Per3, Crem

Interestingly, after repeated DA treatment compared to non-treated group, mRNA splicing (7.20E-07) was among the clusters with the most significant enrichment score. Transcription regulation cluster of the genes showed the most predominant splicing changes after 1st treatment compared to non-treated (4.00E-07) as well as after repeated treatment compared to 1st treatment (1.40E-05), suggesting the splicing changes of genes involved in transcription might affect their function and consequently expression of the genes important in adaptive responses.

In the NT vs 1st group there was 101 genes in Transcription regulation cluster; however, the number of genes in the same cluster in NT vs 7th group was much lower, 34 genes. *Son* gene was the only common gene in the transcription regulation cluster between these two treatment groups. 95 genes belong to the mRNA splicing cluster in the NT vs 7th group. There were 23 common genes between mRNA splicing cluster in NT vs 7th and transcription regulation cluster in the 1st vs NT group. The only common gene between mRNA splicing cluster and transcription regulation cluster in the NT vs 7th was *Zfp638*.

Feng et al., in 2014 (135) studied the effect of repeated cocaine treatment on gene expression in mice NAc. To this purpose, the animals treated either with saline or cocaine for once or up to or 7 consecutive days, and 24 h after the last treatment, NAc samples were used for RNA-Seq analysis (135). These experiments showed that promoter usage and AS alteration induced by repeated cocaine were more prevalent than gene expression changes compared to saline treatment. Moreover, these genes were involved in a variety of cell function such as synapse, neuron projection, ion binding, chromatin remodeling complex and nucleotide binding.

Septin7 (Sept7), nuclear antigen Sp100 (Sp100) and tetratricopeptide repeat domain 23 (Ttc23) are example of the genes which underwent splicing changes after repeated treatment

compared to saline treated animals recognized by Feng et al., 2014 Study (135). They also performed RNA-Seq in NAc of mice after single cocaine treatment; therefore, using DEXSeq, we analyzed the RNA-Seq data obtained by Feng et al., 2014 study to compare the splicing changes after repeated and single cocaine treatment. DEXSeq analysis showed that almost 800 different genes which underwent AS changes after 7^{th} cocaine treatment compared to 1^{st}.

Since cocaine, similar to many drugs of abuse, increases DA level in striatum (153), we examined whether splicing of these genes is also changed after repeated DA treatment. Therefore, we used IGV to check the splicing changes determined by DEXSeq. RT-PCR was performed for some of the genes which showed the most splicing changes as follows: *Ddx18, Spg7, Map3k4, Mtor, Stk38, Cdk8, Camk2d, Vwa8, Kif5* and *Gabrg1*. However, no change was observed after repeated versus single DA treatment. This difference could be due to the different systems by Feng et al., 2014 and us. Although cocaine increases DA level, splicing changes observed after the repeated treatment could be due to pathophysiological effect of cocaine on different region of the brain and different mechanisms which are triggered by cocaine finally leading to differential splicing regulation after repeated versus single treatment. For example, Feng et al., 2014 showed the epigenetic changes after repeated cocaine administration compared to saline treated mice, which was associated with gene expression and spicing changes after repeated treatment (153). Moreover, the splicing changes after repeated versus single cocaine exposure not validated by RT-PCR in the Feng et al., 2014 study.

In our study, heterogeneous nuclear ribonucleoprotein H1 (Hnrnph1), a splicing factor, was identified after 7vs NT in the mRNA splicing factor cluster. Using Quantitative trait locus (QTL) mapping and positional cloning in mice, *Hnrnph1* was identified to be important for response to methamphetamine, a drug of abuse, which stimulate DA associated pathways (154). Behavioral

test on mice downregulated for Hnrnph1 showed that they methamphetamine-induced locomotor activity was decreased, suggesting the critical role of Hnrnph1 in neuronal plasticity and drug addiction (154).

Another example in mRNA splicing factor cluster is serine/arginine-rich (SR) splicing factor 4 (Srsf4), a splicing factors which is involved in the AD through dysregulation of tau gene, leading to disturbing the appropriate ratio of 3R /4R tau isoforms (155).

SR protein specific kinase 2 (Srpk2), another example of the gene in the splicing regulator cluster, is a kinase which by phosphorylation of SR splicing factors regulates their function. However, Srpk2 expression is elevated in AD, diminution of Srpk2 in AD model of mice improved cognitive behavior, synaptic plasticity and LTP (156).

Calcium-response factor (CaRF) is an example gene in the transcription regulation cluster in the 7^{th} vs 1^{st} treatment group. CaRF is a transcription factor, which has been shown to regulate expression of *Grin3a*, a subunit of NMDA receptors. Depletion of CaRF reduces *Grin3a* expression (156). CaRF activity is dependent on the intracellular Ca^{2+} level. Upon depolarization and Ca^{2+} elevation both CaRF and Grin3a are downregulated (157, 158). On the other hand, lack of CaRF similar to depolarization, increases the expression of *Bdnf* and *Arc* which are important in synaptic formation and experience associated plasticity in brain (158).

Another gene in this category, Period circadian clock 3 (Per3), which is involved in regulating circadian clock. It has been shown that a length polymorphism is the human *Per3* gene is associated delayed sleep phase disorder (DSPD) (159), which is dysregulated circadian rhythms and affect the sleep timing (160), diurnal preference (159), and the circadian period is longer than 24 hours (161).

In summary, for the genes identified with splicing change by DEXSeq and MATS, mRNA splicing cluster has the highest enrichment score in NT vs 7th, and transcription regulation cluster is the most significant in the NT vs 1st and 1st vs 7th groups, and some genes in these clusters are involved in adaptive responses.

4. 4. Single and repeated treatments target gene expression at the transcript abundance of different functional clusters

The RNA-Seq data were also analyzed by edgeR tool to identify the transcript abundance changes after 7 treatment compared to first treatment. Analyzing edgeR results showed that single and repeated DA stimulation regulates different clusters of genes (Table 2).

Table 2: Single and repeated treatments target gene expression of different functional clusters

Treatment groups	Number of Regulated genes (P<0.05, FDR<0.1)	Fold Change >2	Most Significant Cluster of Genes	Example
NT vs 1st	785	43	Glycoproteins (P<3.6E-10)	Cacna1h, Gpr149
NT vs 7th	1795	108	Extracellular matrix (P<1.2E-7)	Gpr126, Igfbp3
1st vs 7th	295	24	Secreted (P<3.6E-5)	Igfbp2, Ror1

After 1st DA treatment compared to non-treated group, glycoproteins cluster (P<3.6E-10) was the significant cluster of genes. Glycoproteins cluster of genes are involved in a variety of cell functions such as regulation of development, cell-cell interaction, transport molecules, enzyme, hormones and receptors which are important in hormone response and drug action (162).

G protein-coupled receptor 149 (Gpr149) Calcium channel, Voltage-dependent, T type, alpha 1H subunit (Cacna1h) and Anoctamin 1, calcium activated chloride channel (Ano1) are some examples of the genes belonging to the glycoproteins cluster. Calcium channels have been shown

to be involved in a variety of adaptive changes; such as regulation of neuronal plasticity (163), and are involved in drug addiction and LTP. T-type calcium channels are low voltage-dependent and are activated during depolarization leading to influx of Ca^{2+} into the cell membrane. They are involved in repetitive neuronal firing (164) and synaptic plasticity (165). L type Ca^{2+} channels are inhibited by DA through PKA pathway (164, 166). Repeated activation of these channels leads to high Ca^{2+} level in the mitochondria and damage dopaminergic neurons in substantia nigra (SN). Loss of dopaminergic neurons results in reduced DA release into the striatum, which is involved in PD. High activity of these channels increase D2 receptor expression as well (167). These channels are also involved in pain and epilepsy (168). Ano1 is a voltage sensitive channel (169), which is identified by edgeR analysis. Ano1 are involved in regulation of ionic plasticity through reducing GABAergic activity cerebellum (170).

After 7th treatment compared to non-treated the most significant cluster was extracellular matrix (P<1.2E-7), including G protein-coupled receptor 126 and Insulin-like growth factor binding protein 3 genes. Extra cellular matrix is a group of molecules released by cells to outside of membrane (171). G protein-coupled receptor 126 (GPR126) is a member of adhesion GPCR family (172), which is important for schwann cells development (173).

Insulin-like growth factor binding protein 3 (IGFBP-3) binds to the insulin-like growth factors IGF-1 and IGF-2. It is important for preserving neuronal structure and signaling. It has been shown that lack of IGFBP-3 cause hyperactivity, spatial memory impairment. Moreover, the level of DA and serotonin neurotransmitters are reduced in IGFBP-3 knockout mice (174).

Secreted cluster (P<3.6E-5) showed the most significant changes in the 1st vs 7th group. Insulin-like growth factor binding protein 2 (Igfbp2) and receptor tyrosine kinase-like orphan receptor 1(Ror1) are examples of the genes in this cluster.

It has been shown that Igfbp2 is involved in AD, and high level of Igfbp2 leads to poor memory performance (175). Ror1 is important in synaptogenesis and synapse creation in hippocampus through regulation of Wnt-5a signaling pathway (176).

There were some common genes between these clusters among different treatment groups. The number of genes in glycoprotein and extracellular matrix clusters was 22 and 50 genes, respectively, which among these number of genes 14 genes were in common. In secreted cluster there was 13 genes of which 4 of these genes were in common between this cluster and extracellular matrix cluster. There was no gene in common between glycoproteins and extracellular matrix clusters. All of these common genes between the most significant clusters had a similar gene expression direction. However, the expression level of some of these genes was different among different group.

Analyzing RNA-Seq data by Feng et al., 2014 showed that expression of 55 genes were regulated differently after repeated compared to single cocaine administration in NAc of mice (153), which are consistent with our RNA-Seq results that repeated stimulation regulates gene expression differently from single stimulation.

c-Fos as well as *zif/268* mRNA are induced upon DA treatment in cultured primary striatal neurons of rat through activation of D1 receptors (177). Moreover, acute cocaine evaluates *c-Fos* mRNA while upon repeated treatment its expression is decreased (178). However, our RNA-Seq result didn't show expression change of neither *c-Fos* nor *zif/268* in any group. Our RNA-Seq analysis showed higher expression of *FosB* both after 1st and 7th treatment compared to non-treated, but not after 7th compared to 1st treatment, which is consistent with RT-PCR results (Fig. 11).

It has been shown that prolonged ethanol taking increases the level of D2 receptor mRNA but not D1 in rat Nac (179). Single cocaine taking increases the level of D1 mRNA, while after

cocaine withdrawal, the level of D1 mRNA decreased (180). However, neither acute nor chronic cocaine change D2 expression in the rat striatum (181). We didn't see any change in the D1 and D2 expression in any treatment group. While proenkephalin (Penk) mRNA was increased after acute but not repeated cocaine administration. Acute cocaine increases prodynorphin (*Pdyn*) mRNA while during withdrawal it is reduced (180). In another study repeated cocaine exposure induce dynorphin expression (178). While according to our RNA-Seq data, there was no change in *Pdyn* expression in any treatment group; the level of *Penk* mRNA increased after first and repeated DA treatment compared to non-treated group, but not after repeated compared to first treatment.

In summary, single and repeated dopamine stimulation regulate different clusters of genes. Some genes in these clusters are involved in adaptive responses.

4. 5. Expression changes of RNA splicing factor genes

In contrast to DEXSeq and rMATS analysis where mRNA splicing cluster was among the ones with high enrichment score; for the genes identified with gene expression changes by edgeR, this cluster was not the most significant in NT vs 7^{th}.

Figure 13 shows the expression changes of splicing factors in Nt vs 1^{st} and NT vs 7^{th} treatment groups, indicating that while more splicing factors are changed after repeated treatment, their expression change is less than two fold (FC<2); suggesting that other changes of splicing factors might be the key step to induce adaptive splicing.

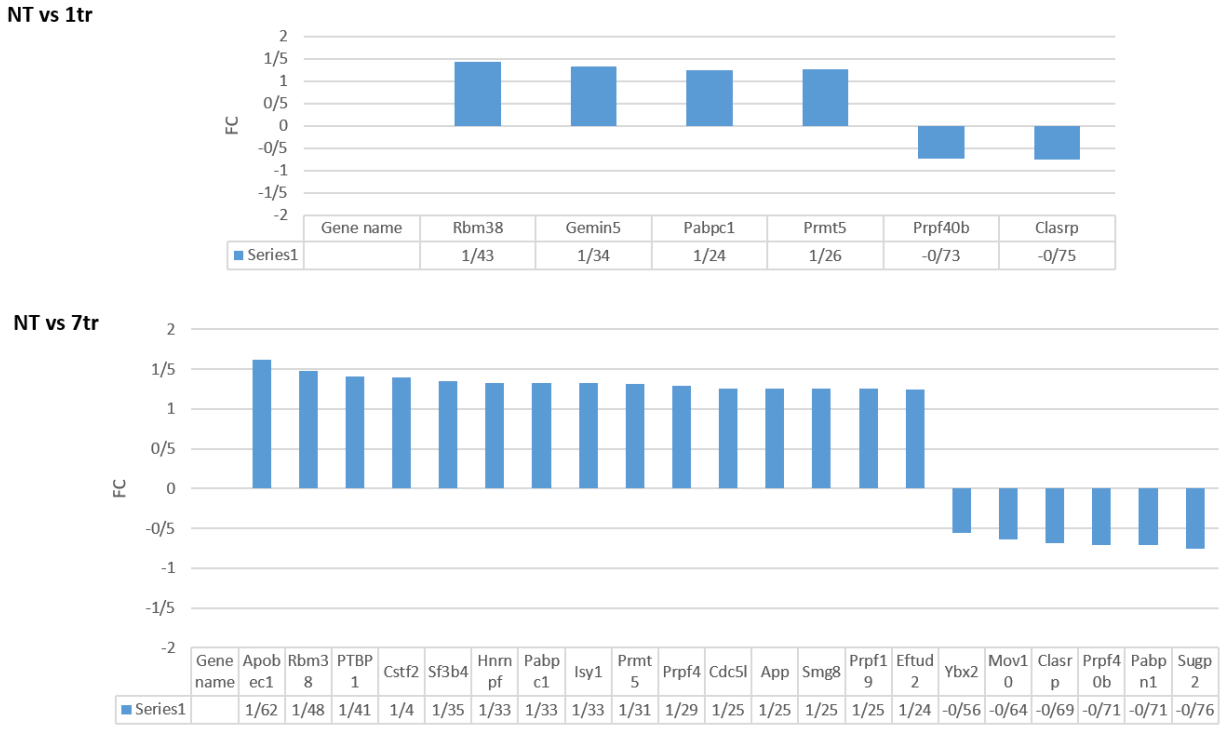


Figure 13. Expression changes of RNA splicing factor genes.

Splicing factors such as Rbm38 and Pabpc1 were in common between these two different treatment groups. There was no splicing factor cluster identified by DAVID Functional Analysis in the 1st vs 7th treatment group.

As it is indicated in Figure 13, the expression changes of splicing factors in both NT vs 1st and NT vs 7th treatment is not very big; however, there might be different mechanisms that could affect the splicing factors function other than transcript abundance (140, 182). For example, as the DEXSeq and rMATS analysis showed (Table 1), the splicing factor cluster had the most significant changes of splicing after 7 treatment compared to non-treated cells. Splicing changes of splicing factors might affect their function. Moreover, different post-translational modification mechanisms could be involved in regulation of splicing factor function. For instance, it has been shown that repeated cocaine exposure as well as depolarization induce A2BP1 nuclear localization with no change in total protein or mRNA level (182, 183). A2BP1 is a splicing factor which is

important for some adaptive changes, such as neuronal excitability (184), neuronal adaptations to stress (71) and rewarding response to cocaine (183). Nuclear migration of A2BP1 might facilitate stimuli-induced AS (183). Our RNA-Seq data also didn't show any expression change in A2BP1 mRNA level. In addition Liu et al., in 2012 showed that membrane depolarization induces phosphorylation of hnRNP L by CamkIV, while it doesn't affect the mRNA and protein level (140). Depolarization-induced phosphorylation of hnRNP L could regulate AS of the target genes. For example, phosphorylation of hnRNP L inhibits binding of U2AF65/35 a splicing regulator to the RNA element which finally leads to skipping of STREX exon in *Slo1* gene, leading to generation of BK channels with altered function skipping of STREX (140).

In summary, the transcript abundance of more splicing factors are changed after repeated treatment compare to 1st treatment. However, their expression change is not very dramatic.

4. 6. Patterns of expression changes among all pairs

Ten genes were in common between all the groups, which are shown in Figure 14. Three different patterns for the expression changes of these genes were identified. Expression could increase after the first treatment compare to non-treated and after repeated treatment increase more, or the gene is downregulated after single treatment and the repeated treatment enhances downregulation even more. *Abca1* and *Tbc1d30* expression changed in a special pattern after first and repeated treatment. Initial DA treatment reduced *Abca1* expression while increased *Tbc1d30* mRNA level; however, after repeated treatment they were expressed in opposite direction. This suggests that certain genes could be regulated differently after repeated versus single DA stimulation, which might be functionally important in adaptive responses.

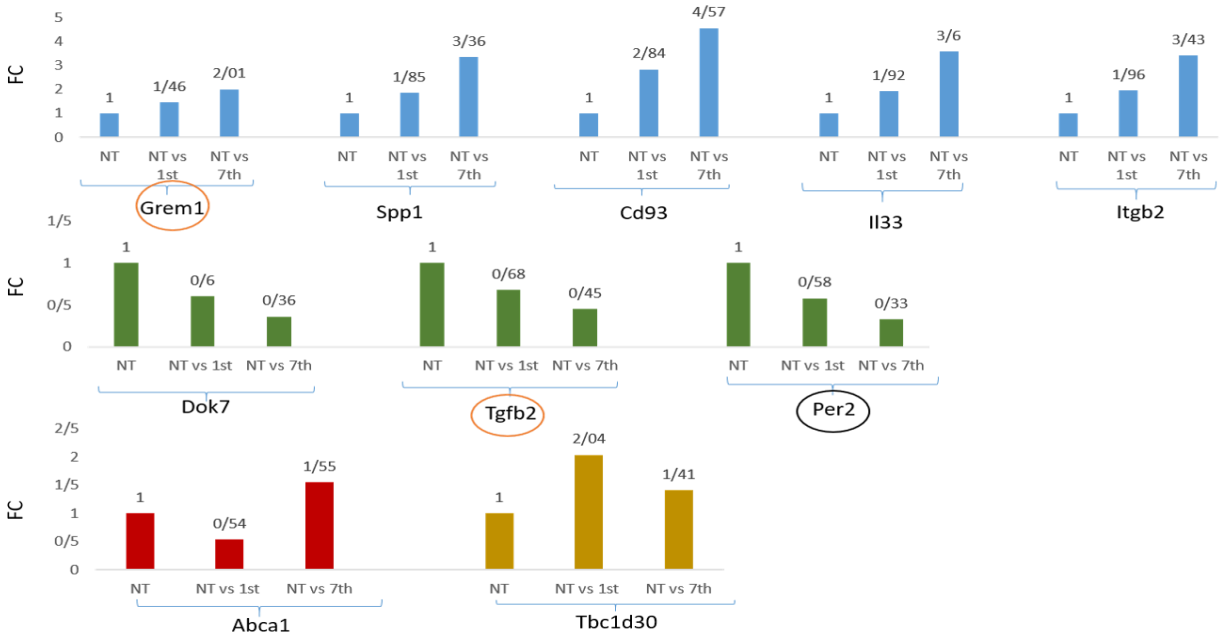


Figure 14. Patterns of expression changes among all pairs. n = 3. P<0.05.

Moreover, Figure 14 shows some interesting genes, such as *Per2*, which are already known to play important roles in some adaptive changes. Per2 is one of the key regulators of circadian clock (185). DA is important for regulation of circadian clock; for example, circadian clock is disrupted in PD (186) in which DA neurons are degraded and DA level is decreased in striatum (187).

It has been shown that Per2 regulates DA release, and its expression itself is regulated by extracellular level of DA in striatum. Expression of *Per2* is induced using DA agonists in *Xenopus laevis* retina (185). Moreover, DA depletion as well as blocking of D2 receptors reduced Per2 expression which is rescued by activation of D2 but not D1 receptors in the absence of DA in the striatum (188). Moreover, D2 agonists enhance *Per1* expression during the night and reduce it at daytime (189), suggesting that D2 activation modulates rhythm of *Per1* and *Per2* expression. DA

release in the rat striatum reaches to its highest level and night time, 6 hours ahead of Per2 peak which is during the day (188).

Per2 regulates monoamine oxidase A (MAOA) expression, an enzyme that inhibits DA activity. Rhythmic expression of MAOA is absent in *Per2* mutant mice leading to increase in DA level (190).

Increase of DA level induces D1 receptors expression, while reduces D2 receptors function (191, 192) , on the other hand, inhibiting D2 receptors reduces *Per2* expression (188). We observed that first time DA treatment reduce *Per2* expression, which is consistent with previous studies, however, repeated DA treatment reduced *Per2* expression more, which could be due to increased internalization or dimerization and as a result reduction of function of D2 receptors.

Gremlin1 (*Grem1*) is another gene whose expression was increased by DA treatment, and is a neuroprotective factor which protects neuronal cells from degeneration MAP kinase signaling pathway (193).

Tfdb2 has been shown to protect cultured dopaminergic neuronal cells from death and is an important factor for protection and survival of dopaminergic neurons (194).

Osteopontin (Spp1) is a glycoprotein which facilitates macrophage recruitment (195). The expression of osteopontin is increased in PD and is involved in neuronal degeneration process (196). Moreover, the increased level of Spp1 is also seen in AD which is associated with reduced cognitive ability (197).

Expression of Interleukin-33 (IL-33), and alamin cytokine, is increased in the brain in AD patients (198). ST2 is the IL-33 receptor. In AD IL-33/ST2 signaling deficiency is observed. Stimulation of IL-33/ST2 pathway and IL-33 injection improved memory impairment of AD model of mice (199).

ATP-binding cassette transporter A1 (ABCA1) regulates production of high density lipoproteins by transferring cholesterol to the lipid-free apolipoprotein A-I (ApoA-I) (200), which its expression showed an opposite direction after 7th treatment compared to 1st in our RNA-Seq results. It has been shown that lack of ABCA1 is associated with spatial memory and cognitive impairment in mice (201). Moreover, risk of AD might be influenced by polymorphisms of *ABCA1* gene (202).

In summary, transcript abundance of specific genes could be regulated differently after repeated versus single dopamine stimulation, which might be functionally important in adaptive responses.

4. 7. Visualizing RNA-Seq data using IGV and validation using RT-PCR

DEXSeq and rMATS results for all pairs were analyzed, and the genes which passed the filtering criteria were visualized using IGV (Fig. 15). After visualizing IGV, primers for some of the genes with the most significant splicing changes were designed, and RT-PCR was carried out to confirm the splicing changes. However, no splicing change was validated and confirmed. Figure 15 shows the example of IGV images visualizing RNA-Seq reads mapped for Srebf1 and Zfp961, two transcription factors which suggested splicing changes after 7th compared to 1st treatment.

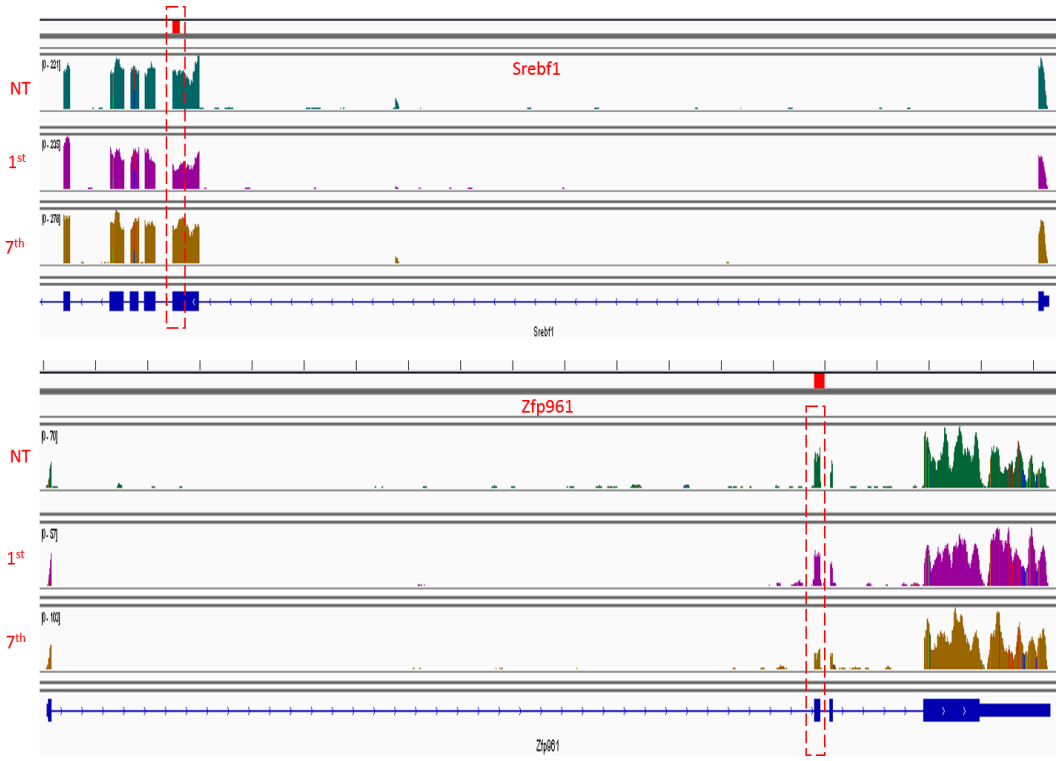


Figure 15. IGV images visualizing RNA-Seq reads mapped on selected genes after DEXSeq and rMATS analysis. Each row shows one sample from a specific treatment group. Treatment groups are separated by black tick line. The peaks show the reads which are represented by colorful area. Read scale is given in the bracket on the left. The tick blue, and the thin blue lines represent exons and introns, respectively, on the reference gene at the bottom of the view. The dotted box shows the target sequence underwent splicing changes in different groups. The splicing changes between different groups was determined by comparing the target sequence expression with flanking exons.

Most significant gene expression changes analyzed by edgeR were also visualized by IGV. RT-PCR was done for the expression changes of the genes among all pairs, and 6 out of 8 were validated. Figure 16 shows the validated RT-PCR result for *Tbc1d30*, *Per2* and *Dok7* genes after normalization with *Gapdh*.

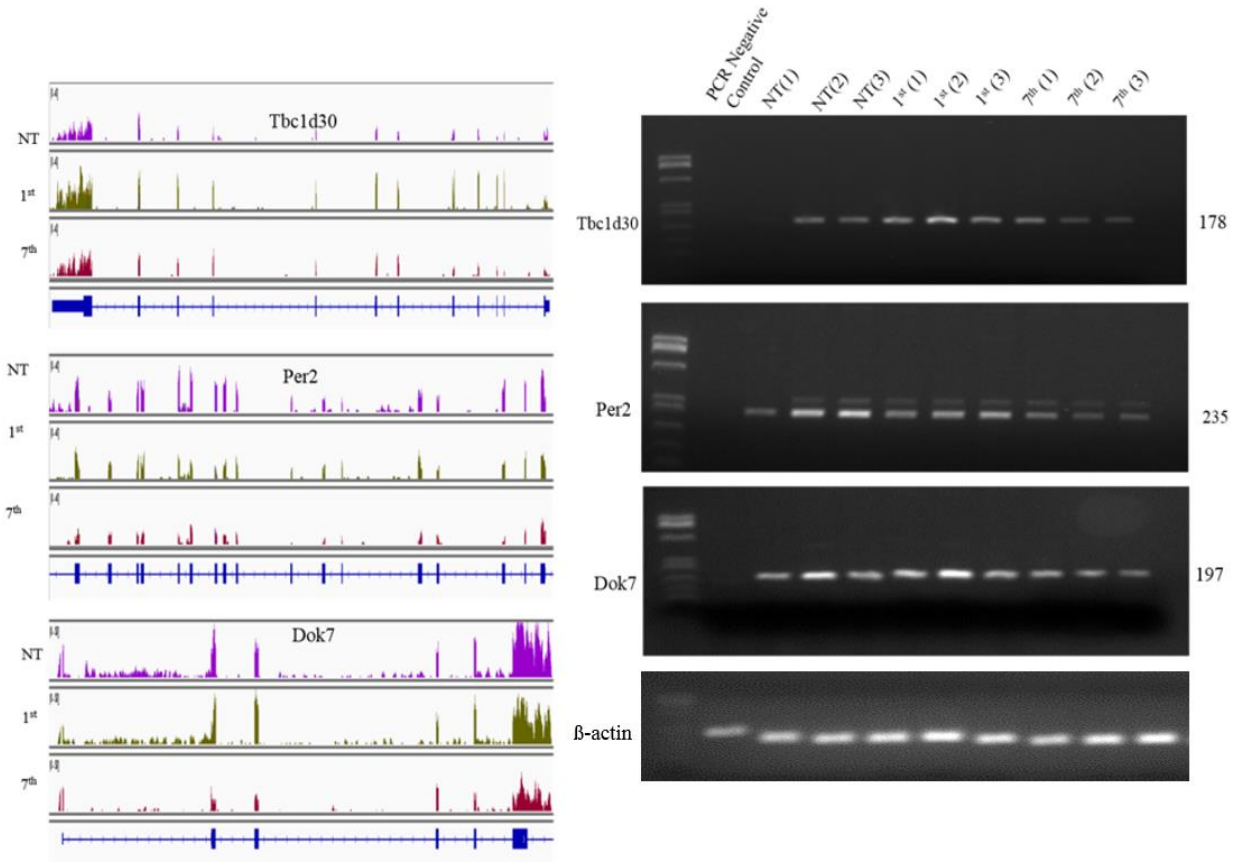


Figure 16. A) IGV images view of edgeR analysis for some genes among different treatment groups. Each row shows one sample from a specific treatment group. The peaks show the reads which are represented by colorful area. The higher peaks are the more reads. Read scale is given in the bracket on the left. The tick blue, and the thin blue lines represent exons and introns, respectively, on the reference gene at the bottom of the view. Comparing the length of the peaks represent the expression level of the gene among different groups. B) Validation of RNA-Seq data by RT-PCR (n = 3). The expression of different genes changes after repeated and 1st treatment which was determined by RNA-Seq data and validated by RT-PCR.

CHAPTER 5: Discussion

5. 1. Dopamine and KCl-induced depolarization promote *FosB* splicing

Our experiments show that 50 mM KCl treatment induces *ΔFosB* expression after 3 hours. KCl-induced depolarization has been shown to stimulate both L-type calcium channels and NMDA receptors to increase Ca^{2+} influx, which thereby leads to activation of CREB transcription factor by phosphorylation. Depolarization-induced CREB phosphorylation depends on the both L-type Ca^{2+} channels and NMDA receptors in striatal cells. Depolarization removes Mg^{2+} block from NMDA with the aid of AMPA receptors, leading to activation of NMDA receptors (203). NMDA activation by glutamate which is an excitatory neurotransmitter (204), amplifies depolarization, results in stimulation of L-type Ca^{2+} channels, which leads to Ca^{2+} entries, and finally through CaMKII phosphorylates CREB. Phosphorylated CREB regulates transcription of immediate early genes such as *FosB*, and increases the level of ΔFosB. This is a calcium-dependent pathway which is not dependent on cAMP as a second messenger (203).

We showed that the level of ΔFosB was increased by DA treatment at different concentration (1nM-500µM). Stimulation with natural rewards or drugs of abuse increases the level of DA in the synaptic cleft. Binding of DA to D1-like receptors which are G-protein coupled receptors in the NAc, activates adenylate cyclase (AC) leading to production of cAMP. Activation of protein kinase A (PKA) by cAMP, induces phosphorylation of CREB which finally leads to production of ΔFosB (12, 18-22).

Activation of D2-like receptors, however, leads to inhibition of AC and subsequently cAMP production and ΔFosB expression. Upon increase of DA level at the synapses, D1-like receptors are upregulated (191), while the D2-like receptors are decreased (192), leading to activation of D1-like receptors-dependent pathways (192).

It has been previously shown that amphetamine, a drug of abuse, induce ΔFosB expression in dose dependent manner (17) which is consistent with our observation that higher concentration of DA induce ΔFosB more than lower amount.

Our experiments exhibited increase of the level of ΔFosB after TPA treatment. Increase of ΔFosB after TPA treatment suggest that activation of PKC pathway is involved in induction of ΔFosB. The effect of TPA on induction of FosB has been previously studied (17, 205). It has been shown that TPA induce both FosB and ΔFosB mRNA, and increases the ratio of ΔFosB /FosB after 2 hours in PC12 *in vitro* (17). PKC activators also increases transcription of *c-fos* in nucleus pulposus (NP) cells. PKC activation increases transcription of *c-Fos* through activating of MAPK pathway, in which inhibition of MAPK in presence of TPA suppresses induction of *c-fos* (206). Phosphorylated PKC increases intracellular Ca^{2+} concentration (207), which might finally through calcium dependent pathway affects expression of *FosB*. Moreover, phosphorylation of PKC induces acetylation of histone (H3) at the promotor of some specific genes such as *Fos*, *Nrxn*, *Creb* and *BDNF*, which induce their expression (207). However, the exact role of PKC pathway in induction of *FosB* and *ΔFosB* has remained elusive.

Amphetamine and cocaine induce H3 acetylation at the promotor of *FosB* gene in NAc (207, 208), leading to activation of *FosB* transcription (208). HDAC enzymes have an opposite functions as KATs, and remove acetyl group from histones leading to alteration of gene expression by affecting transcription factors access to DNA(209). TSA is an HDAC inhibitor, which through increasing acetylation of histones (210) promotes gene expression. Probably histone acetylation at the promotor of *FosB* is increased by TSA leading to higher expression of *FosB* gene.

KCl affect expression of *FosB* through L-type calcium channels, independently form cAMP pathway. Ca^{2+} influx is required for activation of CREB which finally regulates transcription of

FosB. Nifedipine blocks Ca^{2+} channels, leading to inhibition of Ca^{2+} release from Ca^{2+} resources inside the cell to the cytoplasm (211). The inhibitory effect of nitrendipine, a blocker of L-type Ca^{2+}-channel on activation of *c-Fos* on PC-12 cells has been studied (212).

According to these studies, our expectation was that nifedipine treatment should inhibit *FosB* expression. However, our observation was contradictory to the expected results. This experiment was done once. To confirm this observation, this experiment must be done in three times. Moreover, the cell types and experimental condition might affect the observed results.

For pathway, striatal cells were treated with the stimulator and inhibitor drugs just one time. It would be interesting to see whether repeated treatment affect any of these mechanisms, and thereby change expression/splicing of *FosB* differently from first treatment.

5. 2. Repeated dopamine as well as KCl-induced depolarization induce *ΔFosB* expression at the same level as single treatment in cultured striatal cells

We studied the effect of DA and KCl-induced depolarization on the gene expression and splicing changes of *FosB* in cultural striatal cells after single and repeated treatment. However, no splicing change was observed after repeated compared to single treatment. DA as well as KCl-induced depolarization increase ΔFosB transcript abundance in striatal cells after either first or repeated treatment. However, there was no difference between first and repeated treatment.

In 2007, Alibhai et al., investigated the role of AS of *FosB* in accumulation of ΔFosB protein after chronic stimulation on rats treated either with amphetamine for 1 to 7 days or exposed to restraint stress for 1 to 9 days. Using qPCR the level of FosB and ΔFosB was measured (17). After the initial amphetamine or stress exposure, both FosB and ΔFosB transcripts was increased after 1 hour, however, after repeated stimulation their mRNA level decreased (17).

Ratio of FosB to ΔFosB in non-treated animals is 16:1, first stimulation reduced this ratio to 8:1. Upon repeated treatment the ratio gradually increased, suggesting desensitization of FosB and ΔFosB mRNA expression after chronic exposure to amphetamine. However, the ratio and the expression of FosB and ΔFosB mRNA after chronic exposure to amphetamine or stress was still significantly different compared to non-treated group. Examination of FosB and ΔFosB mRNA level during 12 hours after single and repeated treatment of rats with amphetamine showed that FosB mRNA reached to the highest level after 1 hour, while ΔFosB mRNA reached the maximum level after 3 hours treatment with amphetamine, however, both FosB and ΔFosB mRNA level returned to the normal after 12 hours, suggesting that accumulation of ΔFosB protein is not due to sustained ΔFosB mRNA expression (17). Actinomycin D treatment which inhibits transcription, showed that FosB and ΔFosB mRNA stability is almost similar and they are degraded at around the same rate. Level of FosB and ΔFosB mRNA returned to normal 24 hours after both initial and chronic amphetamine and stress exposure (17). These results suggest adaptive splicing of *FosB* gene after repeated stimulation.

The importance of this study relating to our study is due to the effect of amphetamine on DA release, as well as similarity between the mechanisms of action of amphetamine, stress and DA on DA receptors.

Amphetamine increases DA level by inhibiting the reuptake of DA into the presynaptic neuron by blocking DA transporters (DAT). Also it increases DA release from the storage vesicles into the synaptic space (192, 213-216).

The molecular mechanisms of action of stress are not well known. However, it has been shown that rats exposed to stress express lower level of D2 receptors (217, 218), while there is no change in D1 receptor expression (217). Amphetamine, on the other hand, increases expression of

D1 but not D2 receptors in NAc (219-221). Amphetamine affects D2 receptors function by increasing dimerization (222) and internalization (223) of D2 receptors, resulting in decreased apparent D2 receptors (220) and as a result reduced their function (224). Increased D1, and reduced D2 functions is important to mediate the rewarding responses to drug taking (192, 224).

Elevation of DA release activates D1 receptors leading to initiation of DA signaling pathway through PKA which results in expression of immediate early genes such as *FosB* (22). Inhibitory effect of D2 receptors on the AC resulted in reduction of cAMP production. *FosB* is expressed upon activation of PKA by cAMP (22), therefore, reduction of either D2 expression or function by stress or amphetamine, respectively, might lead to increase of cAMP level and activation of PKA which finally results in transcription of *FosB*.

In our study, we directly treated striatal cells with DA for either one or seven times which mimics the effect of amphetamine and stress on induction of DA. According to these finding and considering the expression of FosB and ΔFosB upon activation of DA signaling, our assumption was to observe sensitization of ΔFosB expression after repeated DA treatment compared to single treatment. However, there was no change in the expression of *FosB* and its splice variant ΔFosB after seven DA treatment compare to first treatment. The different observation from Alibhai et al., 2007 (17) could be due to several points. First of all, Alibhai et al., studied the effect of chronic amphetamine and stress on ΔFosB expression in male Sprague-Dawley rats. ΔFosB mRNA induction might be directly related to the stimulus administrated to the experimental model; therefore, applying different stimuli could have different effect on ΔFosB expression. Therefore, difference between our results and Alibhai study could be because of different physiological mechanisms induced by amphetamine and stress which could not be mimicked in *in vitro* study. Moreover, in Figure 7, we showed that the strength of ΔFosB expression is dependent on the DA

concentration; hence, the amount of released DA as a result of amphetamine or stress might affect the induction of ΔFosB differently from DA concentration we used in our experimental system.

Amphetamine and stress could affect D1 and D2 receptors differently and affect expression of *FosB*. Effect of these stimuli on D1 and D2 function could be involved in the observed results. However, *in vitro* study we cannot mimic these pathophysiological functions on the expression of FosB. Moreover, *FosB* is also induced independently form DA pathway through inducing calcium-dependent pathway by glutamate which is released from cortex and reaches to striatum and induce *FosB* expression by stimulation of NMDA ligand-gated ion channels (203). Amphetamine and stress induce glutamate secretion in striatum (225-227), which consequently leads to induction of *FosB*. In addition, other neurotransmitters could be involved indirectly in the regulation of *FosB* expression. For example, stress and amphetamine induce glucocorticoids secretion which subsequently enhance DA (228-230), and glutamate release (231-233). Increased level of DA and glutamate by glucocorticoids could induce *FosB* expression.

By *in vitro* study we ignored the effect of other neurotransmitters such as glutamate on the expression of *FosB*, which might be important in adaptive responses to simulation. Moreover, the networking between different neurotransmitters such as glutamate with DA function is missed, while in rat different neurotransmitters are present which could affect each other function, and play a role in adaptive splicing of *FosB*. In addition to glucocorticoids and glutamate, there might be more complex relations between different neurotransmitters on the induction of *FosB* which are unknown.

The NAc plays the most important role in the reward system. However, the NAc is associated with different regions of the brain especially with ventral tegmental area (VTA) which synthesized DA, and through axons direct it to the NAc. Prefrontal cortex, locus coeruleus, amygdala and

hippocampus are others regions of the brain which are important in rewards system. Wiring and interaction between different parts of the brain involve in rewarding system allow development of adaptive changes. *In vivo* study provides a model system to study adaptation in real physiological condition; however, since we used an *in vitro* model, the role of interaction between different brain regions in adaptive splicing is absent.

Striatal cells grow and divide fast in the culture, and they were passaged three times during the 7 days of treatment period; therefore, in the culture, cell division might affect induction of adaptive changes. Neuronal cells don't divide in the brain; therefore, in every treatment the same cells are exposed to the stimuli in the brain. Since striatal cells are adherent cells, trypsin was used to detach them. Trypsinization of cells might affect promoting of adaptive changes during the repeated stimulation.

In the study done by Alibhai et al., 2007 (17), the splicing changes of *FosB* was studied particularly in the NAc of male rats. NAc is one part of ventral striatum; olfactory tubercle is also located in the ventral striatum. Dorsal striatum composed of caudate nucleus and the putamen, which along with ventral striatum form the whole striatum (234). In our study the extracted cells from the whole striatum were used.

Moreover, the striatal cells were derived from mouse; while the Alibhai et al. (17), studied rats' brain cells. These factors might also be involved in the different results obtained by us and Alibhai et al. The striatal cells we used were infected with tsA58/U19 large T antigen. It has been shown that infection with large T antigen can affect epigenetic modification. For example large T antigen induce DNMT3b expression which results in increase in DNA methylation (235). Moreover, histone methylation is induced by large T antigen, due to its interaction with the histone

acetyltransferases (236). Chromatin modification might influence the effect of DA on the regulation of gene expression of striatal cells and affect the results.

5. 3. Genome wide analysis of RNA-Seq data after repeated compared to single DA treatment

To further examine the effect of repeated stimulation in comparison with single one on the gene expression and splicing of other genes, RNA-Seq was applied on DA (10 nM) treated striatal cells. Spicing changes of genes were analyzed using DEXSeq and rMATS. Splicing of splicing factors enriched the most between NT vs 7^{th} group. Between the NT vs 1^{st} and 1^{st} vs 7^{th} groups, the highest enriched are transcription regulation clusters. According to RNA-Seq analysis, splicing factor's splicing seems underwent prominent changes upon repeated stimulation. We speculate that splicing changes of splicing factors affect their function, and consequently control AS of their target genes, leading to adaptive splicing changes, which is consistent with our hypothesis. Since the functional product of gene expression is protein, importance of AS is depending on its impact on the proteins production. The splicing changes observed were very subtle (FC<2), however, the small changes of splicing might or might not affect the function of the protein product depending on which sequences of amino acids are changed as a result of splicing. To assess the importance of AS splicing changes, the level and function of produced protein should be studied. Moreover, the role of AS changes in adaptive responses to the repeated stimulation could be investigated *in vivo*.

Gene expression was analyzed using edgeR. Expression of different cluster of genes are affected differently after repeated compare to first treatment. The expression of several splicing factors changed in 1^{st} and 7^{th} treatment groups compared with NT (FC<1.5), but few are common between NT vs 1^{st} and NT vs 7^{th} groups, suggesting repeated treatment affects splicing factors

differently from single one. However, upon repeated treatment, in contrast to DEXSeq and rMATS result, splicing factor cluster was not among the most significant cluster of genes with gene expression change. In this case, other mechanisms such as epigenetic and post translational modification could affect the function of splicing factors. In addition, edgeR analysis showed that the expression of some genes involved in adaptation (e.g. circadian period) changed in different ways after repeated and first treatment compared to NT.

All of the adaptive changes happened after 2 passages of the neurons; suggesting that the changes could pass on to the next generations of cells. Many transcripts, including the ones which changed at the transcript abundance or underwent AS changes, might not be translated due to mechanisms such as NMD (235); therefore, it is important to study the effect of repeated and single stimulation at the level of translation, and analyze the impact of transcript abundance changes or AS changes of specific genes on the proteins.

However, RT-PCR failed to confirm the splicing changes after repeated compared to first DA treatment. The small splicing changes (FC<2) might be the reason that the change was not observed by RT-PCR. We should consider that the genes of interest may not be an appropriate target in these experiments; therefore, we should expand our work on new gene candidates. The primer designing and possible flaws in RT-PCR experiments.

Moreover, considering that different cells might response differently to stimulation, the cell type used for experiments should be considered while interpreting results. The number of repeated treatment and the concentration of the drug, the type of drug, are all important factors which may affect our experiments.

We should consider that there could be impact of different unknown mechanisms induced by DA which could affect our results. Moreover, there might be other possible mechanism which may

be important in adaptation, such as epigenetic modifications, post-translational modification etc., moreover, indirect effect of drugs through physiological responses on AS cannot be assessed by using *in vitro* settings. Therefore, it should be further addressed in animal models.

5. 4. Biological repeats of the RNA-Seq data

The current RNA-Seq data were obtained from a triplicate experiment. The triplicate experiment showed consistent changes in the transcript abundance of specific genes after repeated compared to single treatment. RT-PCR could confirm the transcript abundance changes of some of these genes, for example *Per2*, *Tbc1d30*, and *Dok7* genes upon repeated treatment compared to 1st treatment. However, in the independent tests, the transcript abundance changes in the different treatment groups were not consistent with the RT-PCR results. The reason could be experimental conditions or inherent to cell line division issues, but remains to be further investigated.

CHAPTER 6: Conclusion and Significance

6. 1. Conclusion

FosB does not change adaptively at the level of splicing in striatal cell line treated with KCl-induced depolarization or DA, but RNA-Seq data suggest that, although there was not significant change at the transcript abundance of splicing factor cluster of genes, there is a global change in their alternative splicing after repeated treatment with DA, which indicates there might be splicing changes of other genes which needs to be identified in the future.

Repeated and single stimulation of striatal cells induced transcript abundance changes of different cluster of genes differently. The transcript abundance of specific genes involved in adaptation (e.g. circadian period) changed in different ways, which may be functionally important in adaptive responses. However, these changes need to be validated in independent biological repeats.

6. 2. Significance

Understanding adaptive splicing in adaptation will provide us better approaches to design novel strategies to treat maladaptive events such as addiction. Addressing these mechanisms in the brain will also help decipher other adaptive events such as memory. The results of this study provide preliminary observations suggesting that adaptive splicing likely exists.

CHAPTER 7: Future direction

How repeated stimulation changes gene expression and AS of certain genes differently from single one? By answering this question, we would propose the potential mechanisms which are important in importance of repeated versus single stimulation on alternative splicing, which will be important step toward understanding of adaptive changes. Identifying certain genes which show sensitized or adaptive changes to repeated stimulation, can lead to further examination of possible mechanisms involved in adaptive responses of certain genes to repeated stimulation. For example, studying importance of epigenetic modification, such as DNA and histone modification, or post translational modification in adaptive changes after repeated compared to single stimulation.

Moreover, to unveil the effect of repeated versus single stimulation on the gene expression and splicing changes of certain genes in real physiological condition, this study could be expanded *in vivo*. By confirmation of splicing changes of certain genes, further investigation could be done to identify possible splicing factors and mechanisms which they thereby change AS. Knockout experiment followed by rescue could be useful to confirm the importance of target genes on adaptive responses.

In addition to striatum, it would be interesting to investigate the effects of repeated versus first treatment on other part of the brain. There are variety of adaptive stimuli which could be used in studying the effect of repeated treatment on adaptive changes.

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