“Dysregulation of a set of miRNAs by Ethanol leads to Alcoholism”

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Submitted by
SNEHA
(Enroll no. 003/12)
Under the Joint Supervision of

Prof. M. Y. Khan	**Dr. Anand Prakash**
Department of Biotechnology	Department of Biotechnology
B.B.A.U., Lucknow	B.B.A.U., Lucknow

At

Department of Biotechnology
School for Biosciences and Biotechnology
Babasaheb Bhimrao Ambedkar University
(A Central University), Vidya Vihar, Raebareli Road,
Lucknow - 226025, INDIA.

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Alcohol addiction is a chronic relapsing disorder and is characterized by repetitive alcohol drinking patterns leading to a loss of control over alcohol consumption (Koob, 2003). The alarming rate at which the use of alcohol is increasing the number of alcoholics it is imperant to decipher the molecular mechanisms underlying addiction. This will help to develop newer drug targets for rehabilitation and it will also shed some light on the molecular events and the pathways involved in memory formation as most of the pathways that are utilized for addiction overlap with that of memory formation. The neuroadaptational changes induced by exposure to alcohol and drugs of abuse may be related to dysregulation of signaling systems, gene transcription, and protein expression at the cellular level (Nestler, 2004; Koob et al., 1998; Pandey, 2004). The mesolimbic dopaminergic pathway has been shown to be a key mediator in the rewarding effects of alcohol. Molecular and cellular changes in the nucleus accumbens with acute and repeated alcohol exposure may underlie certain aspects in the development of alcohol addiction (Koob et al., 1998; Pandey, 2004).

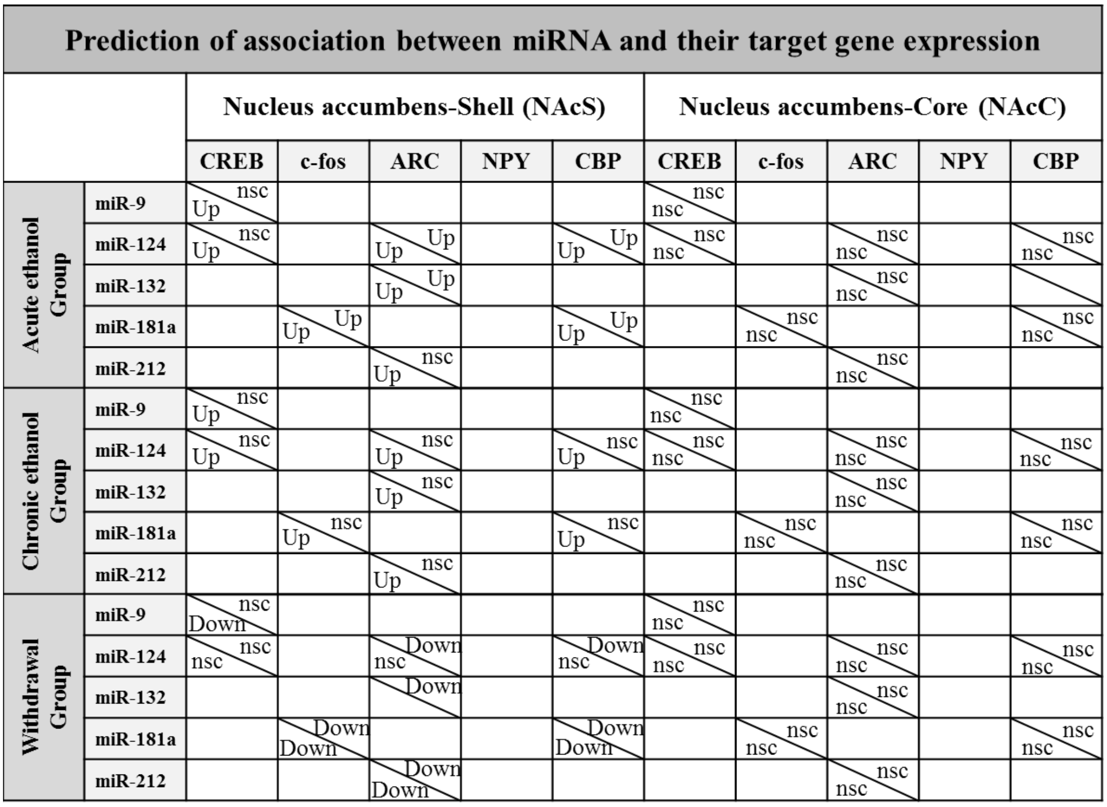
The dysphoric state induced during alcohol withdrawal is a robust factor in the maintenance of both alcohol drinking and the eventual development of alcohol addiction (Koob, 2003; Pandey, 2014). Amygdaloid brain regions, specifically the central nucleus of the amygdala (CeA) and medial nucleus of amygdala (MeA), appear to be associated with the dysphoric effects of alcohol withdrawal, particularly the promotion of anxiety-like behaviors (Koob,2003; Pandey, 2004, 2008, ; Gonzales et al., 2004). CREB plays a central role in the process of addiction (Nestler, 2001; Gonzales et al., 2004; Carlezon, 2005; Spanagel et al., 2009). Ethanol has a complex pharmacological profile and various signaling systems have been identified as modulators of CREB function that may serve as potential ethanol targets (Spanagel et al., 2009; Morrow et al., 2004; Harris et al., 2008). A great deal of research has focused on the role of CREB and its target genes, such as neuropeptide Y (NPY), brain-derived neurotrophic factor (BDNF), activity-regulated cytoskeleton-associated (Arc) protein, and corticotrophin-releasing factor (CRF) in the development of alcohol addiction (Heilig & Koob, 2007 ; Thorsell, 2008; Davis, 2008; Pandey et al, 2008). In addition, several studies have identified novel epigenetic mechanisms, such as histone modification-

induced chromatin remodeling and DNA methylation, in the process of alcohol-related neuroadaptation (Shukla et al, 2008; Pandey et al., 2008).

A well-known means of post-transcriptional regulation of gene expression is the inhibition of translation via microRNA (miRNA). MiRNAs are expressed at high levels in the brain (Fiore et al., 2008; Bartel, 2009), and the involvement of miRNA in numerous aspects of normal and abnormal brain function has been reported (Fiore et al., 2008; Bushati & Cohen, 2008). In addition to classically defined epigenetic mechanisms, microRNAs (miRNAs) can also convey epigenetic-like characteristics through post-transcriptional regulation of gene expression (Saetrom et al., 2007). MiRNAs can rapidly regulate gene expression by targeting certain mRNAs for degradation or through specific inhibition of mRNA translation. How the environmental changes or drugs of abuse bring about changes in the expression pattern of miRNAs is not well known. So such studies may shed light on the regulatory mechanisms controlling gene expression of miRNAs and might reveal newer targets which can be used for novel drug development for overcoming different brain disorders and addiction etc. The selected set of microRNAs (miR-9, -124, -132, 181a and -212) has been found active in many types of neurological processes such as, synaptogenesis, synaptic plasticity and neurotoxicity related to drug abuse and most drugs of abuse use the same neural circuitry involved in the addiction which might be directly or indirectly regulating the ethanol responsive transcription factor CREB and its target genes. The present proposal is envisaged to closely look at this mechanism.

The hypothesis of the work was to understand the mechanism during chronic exposure to ethanol which may lead to dysregulation of a subset of miRNAs, normally resisting the changes in the gene expression on acute exposure of ethanol, resulting in loss of this homeostatic control through miRNAs and allowing the modulation of circuitries responsible for alcohol addiction. The animal model of alcoholism developed by treatment with chronic ethanol and withdrawal group by the sudden cessation of ethanol for 24 hrs after prolonged ethanol exposure and in acute exposure group single dose of ethanol injected (1 gm/kg dose; ethanol was diluted to 0.2 gm/ml in n-saline and was injected as 5µl/gm of body weight) and subjected to anxiety measurement paradigms i.e. elevated plus maze (EPM) and light dark box (LDB). Nucleus accumbens (shell and core) and amygdala (basolateral amygdala, central amygdala and medial amygdala) regions were analyzed due to their vulnerability during exposure of ethanol. The changes occur in these regions during acute and chronic ethanol exposure assessed by molecular studies, using TaqMan assay based qRT-PCR, DAB-immunohistochemistry and chromatin-immunoprecipitation-qPCR (ChIP-qPCR).

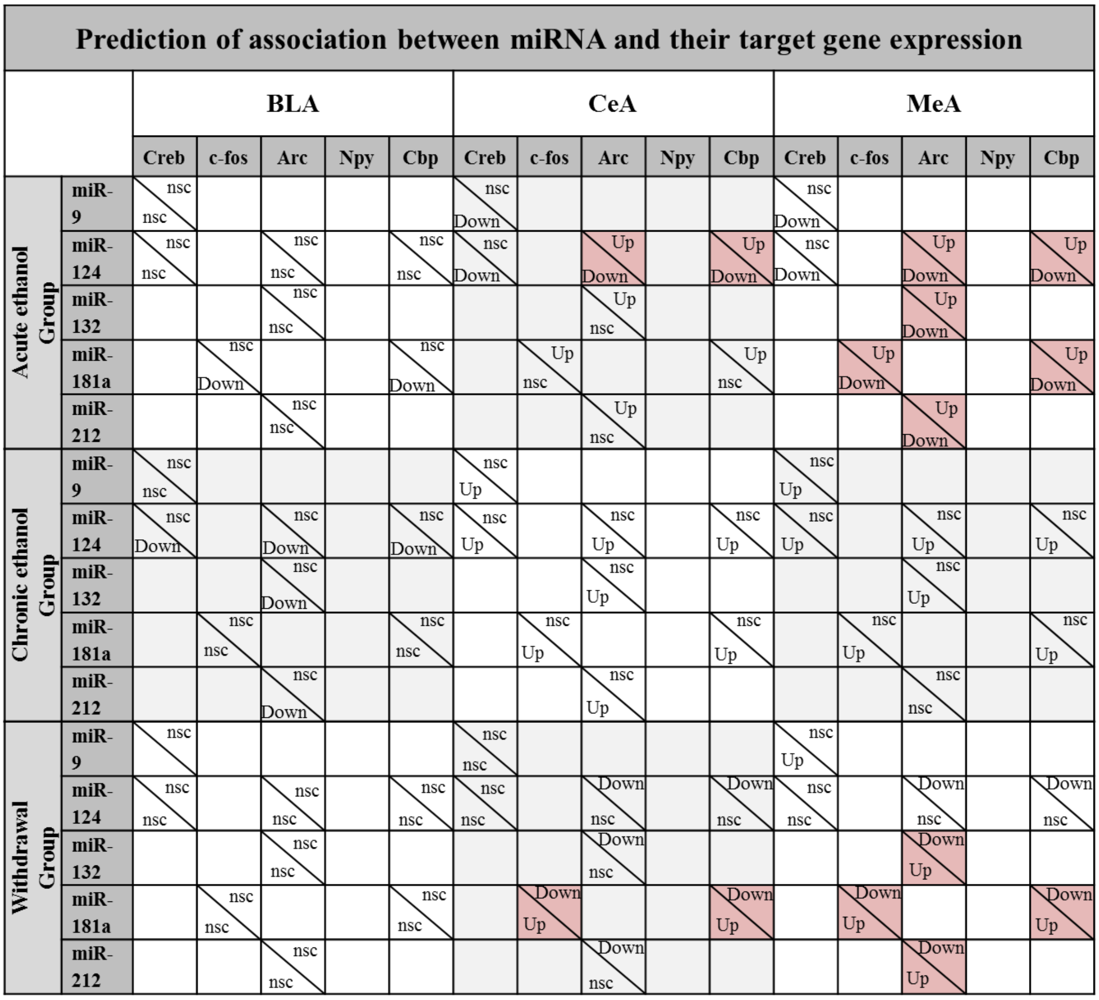
The indexing of the change in expression of selected microRNAs and their predicted target genes expression, differ very prominently, specifically in nucleus accumbens-shell (NAcS), central amygdala (CeA) and medial amygdala (MeA) regions compared to the core of nucleus accumbens (NACc) and basolateral amygdala (BLA), during acute ethanol and chronic ethanol exposure. Further, to find the changes occur during acute ethanol and chronic ethanol exposure in miRNA activity due to the CREB mediated control on the microRNA promoter suggests, though the changes observed during acute and chronic ethanol treatment in NAcS, CeA, and MeA, were statistically significant, yet the fold change was extremely less than 1.2 fold, which suggests that there is no direct role of CREB on the promoters of selected microRNAs. Remarkably, in NAcS region, chronic ethanol exposure had a significant increase in miR-132 promoter expression due to increased occupancy CREB rather than insignificant change during acute exposure to ethanol. We have utilized two different criteria's for the interpretation of microRNA and mRNA expression in this series which include statistical analysis and threshold fold change must be more than 1.20 (>1.20). Though the fold change is slightly more (1.27) than the threshold (i.e.1.20) and also not highly significant ($p<0.01$), so the result demands further investigation.



Prediction of association between miRNA and their target gene expression

		Nucleus accumbens-Shell (NAcS)					Nucleus accumbens-Core (NAcC)				
		CREB	c-fos	ARC	NPY	CBP	CREB	c-fos	ARC	NPY	CBP
Acute ethanol Group	miR-9	nsc Up					nsc nsc				
	miR-124	nsc Up		Up Up		Up Up	nsc nsc		nsc nsc		nsc nsc
	miR-132			Up Up					nsc nsc		
	miR-181a		Up Up			Up Up		nsc nsc			nsc nsc
	miR-212			nsc Up					nsc nsc		
Chronic ethanol Group	miR-9	nsc Up					nsc nsc				
	miR-124	nsc Up		nsc Up		nsc Up	nsc nsc		nsc nsc		nsc nsc
	miR-132			nsc Up					nsc nsc		
	miR-181a		nsc Up			nsc Up		nsc nsc			nsc nsc
	miR-212			nsc Up					nsc nsc		
Withdrawal Group	miR-9	nsc Down					nsc nsc				
	miR-124	nsc nsc		Down nsc		Down nsc	nsc nsc		nsc nsc		nsc nsc
	miR-132			Down					nsc nsc		
	miR-181a		Down Down			Down Down		nsc nsc			nsc nsc
	miR-212			Down Down					nsc nsc		

Table 1.: Summary of the overall change in microRNAs expression and CREB and its target genes expression in Nucleus accumbens. (nsc= nonsignificant change; Up= upregulation; Down= downregulation).



Prediction of association between miRNA and their target gene expression																
		BLA					**CeA**					**MeA**				
		Creb	**c-fos**	**Arc**	**Npy**	**Cbp**	**Creb**	**c-fos**	**Arc**	**Npy**	**Cbp**	**Creb**	**c-fos**	**Arc**	**Npy**	**Cbp**
Acute ethanol Group	**miR-9**	nsc nsc					nsc Down					nsc Down				
	miR-124	nsc nsc		nsc nsc		nsc nsc	nsc Down		Up Down		Up Down	nsc Down		Up Down		Up Down
	miR-132			nsc nsc					Up nsc					Up Down		
	miR-181a		nsc Down			nsc Down		Up nsc			Up nsc		Up Down			Up Down
	miR-212			nsc nsc					Up nsc					Up Down		
Chronic ethanol Group	**miR-9**	nsc nsc					nsc Up					nsc Up				
	miR-124	nsc Down		nsc Down		nsc Down	nsc Up		nsc Up		nsc Up	nsc Up		nsc Up		nsc Up
	miR-132			nsc Down					nsc Up					nsc Up		
	miR-181a		nsc nsc			nsc nsc		nsc Up			nsc Up		nsc Up			nsc Up
	miR-212			nsc Down					nsc Up					nsc nsc		
Withdrawal Group	**miR-9**	nsc					nsc nsc					nsc Up				
	miR-124	nsc nsc		nsc nsc		nsc nsc	nsc nsc		Down nsc		Down nsc	nsc nsc		Down nsc		Down nsc
	miR-132			nsc nsc					Down nsc					Down Up		
	miR-181a		nsc nsc			nsc nsc		Down Up			Down Up		Down Up			Down Up
	miR-212			nsc nsc					Down nsc					Down Up		

Table 2. : Summary of the overall change in microRNAs expression and CREB and its target genes expression in Amygdala regions. (nsc= nonsignificant change; Up= upregulation; Down= downregulation).

To the best of our knowledge this is the first study which shows the changes in the activity of microRNAs which may help to maintain the homeostasis during the ethanol exposure get affected during the chronic ethanol exposure and withdrawal after protracted ethanol consumption may be responsible for the development of addiction Thus this study may provide a glimpse in darker sky of addiction field and may open new vistas for better prognosis and drug development of alcoholism and other drugs addiction.

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