Serotonergic activity and hypothalamic-pituitary-adrenal axis response in alcohol administered and subsequently withdrawn rats

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Abstract: Present study aims to depict the role of serotonergic pathways in discrete brain areas (hypothalamus, amygdala, and hippocampus) and their interaction with hypothalamic pituitary adrenal (HPA) axis in alcohol dependence and subsequent withdrawal syndrome in rats. Albino Wistar rats were fed a liquid diet containing alcohol for 4 weeks. Matched control rats were fed isocaloric amounts of the alcohol-free liquid diet, in which the alcohol contribution was substituted with maltose-dextrin. Brain regional tryptophan, 5-hydroxytryptamine (5-HT), 5-Hydroxyindoleacetic acid (5-HIAA) concentrations were determined using high performance liquid chromatography with flourimetric detector. Serum corticosterone was determined spectrofluorimetrically. Data analysis showed that there was significant increase in tryptophan (hippocampus), 5-HT (hippocampus and amygdala) and 5-HT turnover in all the three regions examined when alcohol administered rats were compared with matched controls. In contrast withdrawal from alcohol decreased brain tryptophan, 5-HT and its turnover. It is concluded that the prolong alcohol use boost functions of serotonergic neuronal pathways, in particular, hypothalamus that regulate HPA-axis function and develop tolerance and adaptation. In addition, withdrawal from alcohol exacerbates serotonergic functions that results in failure to suppress corticosterone levels and hence induce low mood states and other signs and symptoms of alcohol withdrawal syndrome.

Keywords: Alcohol, serotonin, corticosterone, tryptophan, 5-HT.

INTRODUCTION

Alcohol addiction is common worldwide, and its uncontrollable consumption is associated with health problems. Being an addictive drug, alcohol alters brain functions by interacting with neurotransmitter systems that mediate the reinforcing effects of alcohol. Serotonin dysfunction seems to cause depression, anxiety and low mood states. Preclinical studies in animal models of alcohol dependence and withdrawal have revealed that even a single dose of alcohol could bring anxiety, emotional reactivity and aggression. In association with this framework serotonin dysfunction and depression in alcoholics are less well established.

Preclinical and clinical studies have proven that acute alcohol consumption exerts biphasic effect possibly by the initial increase in serotonin levels followed by the reduction in its concentration across the synapse (LeMarquand, 1994a, b). Further depletion in serotonergic functioning would no longer inhibit behavior and provoke anxiety (Spoont, 1992). Alcohol intake increases tryptophan/large neutral amino acids (LNAAs) ratio there by increasing TRP availability to the brain. This mechanism also increases the availability of free tryptophan to the liver (LeMarquand, 1994a, b) and hence hepatic tryptophan 2, 3 dioxygenase (TDO) enzyme activity that allow the degradation of TRP along kynurenine pathway. This may result in decrease in serum tryptophan and hence its uptake into the brain that explains biphasic effect on brain serotonin concentration (Badawy and Evans, 1976). Chronic alcohol consumption, however, seems to increase brain 5-HT via decreasing hepatic kynurenine enzyme activity (LeMarquand *et al*., 1994b) and increasing substrate availability to the brain. In contrast, withdrawal from chronic alcohol consumption increased hepatic TDO enzyme activity that decreases TRP uptake to the brain and consequently decreased 5-HT concentration and turnover (Badawy *et al.* 1980a, b). Clinical data suggest that low serotonin turnover rate is responsible for the aggressive behavior in alcoholics (Virkunnen *et al*., 1994). Quinolinic acid is known to activate *N*-methyl-D-aspartate (NMDA) receptors during alcohol dependence and therefore alters brain responses to emotional states after alcohol is abstained that possibly generate alcohol withdrawal seizures and the effects can be reduced by NMDA receptor antagonists. Atrophy of brain regions and impairment in cognitive functions has been widely observed in alcohol dependence and withdrawal syndrome. Alcohol stimulates certain receptors such as 5-HT3 receptors on gamma-aminobutyric acid (GABA) neurons that increases the (GABA)-ergic activity, thus mimic the inhibitory response of the neurons that receive signals from these neurons. Amygdala is viewed to control impulsive aggression against rewarding and aversive stimuli. Further hypothalamus also contains dense serotonergic fibers that influence rewarding responses to drug of abuse by releasing several neuropeptides.

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The HPA axis comprises of three hormones: (1) corticotropin-releasing hormone (CRH), which is produced in a brain region called the hypothalamus; (2) adrenocorticotropic hormone (ACTH), which is released from the pituitary gland (3) glucocorticoid hormones, which are secreted from the adrenal glands. Activation of neurons in the brain in response to stress, results in the production and release of CRH from certain cells in the hypothalamus. Through specific blood vessels in the brain, CRH is transported from the hypothalamus to the pituitary gland, where it induces the production and secretion of ACTH into the body's general circulation. Through the blood, ACTH reaches the adrenal glands and initiates the production and release of glucocorticoid hormones. (The major glucocorticoid in humans is cortisol, whereas the major glucocorticoid in rodents is corticosterone.) These glucocorticoids induce and regulate the body's varied physiological responses to stress. The activity of the HPA axis is regulated by a negative feedback mechanism in which glucocorticoids released into circulation act on hypothalamus and/or pituitary gland to suppress further release of CRH and/or ACTH. The body's neurochemical and hormonal responses to stress are tightly interconnected.

Serotonergic neurons from raphe nuclei project to hippocampus and para ventricular nucleus (PVN) of the hypothalamus exist components of HPA-axis and serotonin system (Lopez *et al.,* 1991). And, serotonergic neurons that project to other brain areas such as amygdala are thought to modulate functions of PVN (Tork, 1990). It has been reported that corticotrophin releasing hormone (CRH) is released from the parvocellular zone of the PVN of the hypothalamus (Feldmen and Weidenfeld, 1991) and catecholamines have been found to have excitatory role on CRH secretion (Feldmen and Weidenfeld, 1991, Negro-Vilar *et al.,* 1987). Pharmacological agents can also lead to stimulate adrenocorticotropin hormone (ACTH) and corticosterone release (Fuller, 1992). Behavioral manifestation during alcohol dependence and withdrawal is one of the challenging aspects of neuroendocrine system. Numerous studies on animals and humans have shown that acute exposure to alcohol has been shown to induce corticosterone levels via releasing corticotrophin releasing factor (CRF). Whereas prolong alcohol consumption engenders a transient hyperactivity to the HPA-axis response (Rivier *et al.,* 1984; Redei *et al.,* 1988). Both the humans and animal studies have declared the development of behavioral phenomenon known as anxiety and relapse behavior in response to adaptive changes in the heightened HPA -axis response. And, that transient rise in CRF release has been recorded to peak between 10-12 h after onset of withdrawal (Merlo Pich *et al.,* 1994). Concomitant with these findings several preclinical and clinical studies have documented significant rise in plasma ACTH levels following acute alcohol withdrawal (Ellis, 1965; Merry and Marks, 1972). And administration of alcohol during withdrawal tends to normalize the elevated corticosterone levels (Merry and Marks, 1972). It has been reported that the levels of corticosterone remained elevated for as long as after the last alcohol intake (Willenbring *et al.,* 1984). Much less attention has been paid to inter- relationship of corticosterone with serotonin neurotransmission in brain areas, in particular, hypothalamus, amygdala and hippocampus. The involvement of these brain regions in alcohol dependence and withdrawal is still under investigation. Present study aims to depict the role of serotonergic pathways in discrete brain regions (hypothalamus, amygdala, and hippocampus) and their interaction with HPA axis system during alcohol dependence and subsequent withdrawal syndrome in rats.

METHODS

Chemicals and reagents

Tryptophan, Serotonin (5-HT),5-hydroxyindoleacetic acid (5-HIAA) and corticosterone were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ethanol, Methanol (HPLC grade).

Animals and Treatment

All animal procedures described below were conducted in strict accordance with the national research council for the care and use of laboratory animals (1996). Ethical approval was obtained from institutional animal ethics committee, University of Karachi. All efforts were made to minimize the number of animals and any pain/distress they might incur. Locally bred male Adult Albino Wistar rats, weighing 200-250g at the start of experiment, were housed in a quiet, temperature and humidity-controlled room in which 12h dark: 12h light cycles at 22±3^{o}C was maintained. The rats were housed 6 animals per cage and were given an alcohol-free liquid diet *ad libitum*, for three days before introducing alcohol into the diet. Alcohol was then added into the liquid diet in the proportion of 5% or 8% (v/v). Matched control rats for each group were fed isocaloric amounts of the alcohol-free liquid diet, in which the alcohol contribution was substituted with maltose-dextrin. All rats were housed in grid –bottomed cages to prevent coprophagia. Matched control rats, being limited in their food intake were noted to ingest their total daily food ration more rapidly than their corresponding alcohol-fed counter parts, resulting in altered feeding pattern and hence periods of starvation. Additionally in all experiments, a control group of rats maintained *ad libitum* on solid lab chow was included. Treatment of rats was continued for 28 days. Fresh alcohol and (as well as control) liquid diets were replaced daily between 16:00 and 17:00 and all rats had free access to drinking water (Bano *et al*., 1996).

Assessment of alcohol withdrawal syndrome

For assessment of the effects of alcohol withdrawal, the alcohol-containing liquid diet was substituted with

drinking water for 7hrs, as by this time there were demonstrable signs of withdrawal including the ability to induce seizures audiogenically and other behavioral signs including agitation, tremors, stereotyped behaviors and wet dog shakes were monitored (Hunter *et al.*, 1975). Matched controls for the alcohol withdrawal groups were also starved for 7hrs to counter any likely effects of the liquid diet itself in relation to the interpretation of the effects of alcohol withdrawal. It should be noted that during the 7hrs alcohol-withdrawal period, rats do not consume any diet and, accordingly, it was neither necessary nor desirable to provide the control liquid diet during the withdrawal period. Rats were killed between 10:00 and 12:00 hour. Audiogenic seizures were examined at the 7hrs time point, because this is the time interval after withdrawal of alcohol-containing liquid diets at which seizure intensity as well as the TRP metabolic changes are both maximal, as reported (Oretti *et al*., 1996). Rats used for the biochemical studies were different from those used for assessment of the withdrawal seizures. Rats were killed by decapitation and brains were rapidly removed and frozen in liquid nitrogen and were stored at -70^{o}C until analysis. Serum was also collected and stored until analysis,

Measurement of blood alcohol concentration

Blood alcohol concentration was determined by an alcohol dehydrogenase (EC 1.1.1.1) based enzymatic procedure (Badawy and Aliya, 1984) in rats given chronic treatment of ethanol liquid diet. The blood ethanol concentration was determined in alcohol administered rats, 233±40 mg/dl are means ± SEM of six rats.

Brain indoleamine analysis

Brain neurochemical analysis was done by using HPLC-FL. After decapitation brains were rapidly removed and the brain regions (hippocampus, amygdala and hypothalamus) were dissected. After weighing the respective regions, the tissues were homogenized and deproteinised in volumes of 0.1M perchloric acid (1g in 4ml 0.1M perchloric acid). The brains were sonicated at 0-4^{0}C at a medium setting for two 15-sec periods. After adding 0.5ml of 4M perchloric acid and mixing the samples were spun at 10,000g for 10 min and a portion of supernatant was taken and stored at -70^{o}C for analysis. The analytical measurements were performed by high performance liquid chromatography with fluorescent detector. A reverse phase chromatography was used in all analysis of TRP, 5-HT and its metabolite 5-hydroxyindolacetic acid 5-HIAA.The ratio of 5-HIAA/5-HT was used as an index of 5-HT turnover. For mobile phase 0.01 M sodium acetate was made and pH 4.5 was adjusted with glacial acetic acid and finally the volume was made up to 1L with deionized water. After filtering mobile phase, 15% methanol was added. and was passed through the ODS separation column (25cm in length 4.6 mm in diameter) at a constant flow rate (2ml/min) with an operating pressure of 2000-3000 *psi*, using a 200 series pump. Fluorescence detection was performed on Shimadzu VT 03 detector at an operating potential of 0.8V. The flourimetric detector was used with a 254nm excitation and 360nm excitation (Anderson *et al.*, 1981).

Corticosterone estimation

Serum corticosterone was determined spectro-fluorimetrically by Glick *et al*. (1964).

STATISTICAL ANALYSIS

The results were expressed as mean ± SEM. Data was analysed using student's t test. A Significant difference was determined when $P<0.05$.

RESULTS

Table 1-3 shows the effects of alcohol administration and subsequent withdrawal on serotonergic changes in the brain regions. When compared with chow controls, matched controls showed insignificant changes in the brain TRP concentration in all the three regions with insignificant change in 5-HT in hypothalamus and hippocampus while amygdala showed significant increase t=3.9 ($P<0.01$). And, insignificant difference was observed in 5-HIAA in hypothalamus while both amygdala t=2.45 ($P<0.05$) and hippocampus t=2.3 ($P<0.05$) showed significant increase and decrease respectively ($P<0.05$). Further, significant decrease ($P<0.01$) was found in 5-HT turnover in hypothalamus t= 3.5 and hippocampus. t=4 ($P<0.01$).

Rats consuming alcohol (alcohol dependent rats) showed significant increase in TRP concentration in hippocampus region only t=3.0 ($P<0.05$) when compared with matched control liquid diet group (table1) while increase in 5HT in amygdala t=2.29 ($P<0.05$) and hippocampus t=3.1 ($P<0.05$) region. Also, significant increase ($P<0.01$) in 5-HIAA was found in all the three brain regions hypothalamus t=3.86, amygdala t=3.9, and in hippocampus t=3.9. Significant rise in 5-HT turnover in all the three brain areas hypothalamus t=8 ($P<0.001$), amygdala t= 2.5 ($P<0.05$), hippocampus t=3 ($P<0.05$).

AW (7h) group of rats showed significant decrease in brain TRP levels in hypothalamus t=2.59 $P<0.05$ and in amygdala t=3.19 ($P<0.01$). Significant reduction ($P<0.001$) was found in 5-HT in hypothalamus t=5.75and amygdala t=5.86 ($P<0.001$) and in hippocampus t=3.6 ($P<0.01$). Similarly significant reduction in 5-HIAA was shown in hypothalamus t=7.36 ($P<0.001$) and amygdala t=6.24 ($P<0.001$) and in hippocampus t=3.47 ($P<0.01$) was found with the significant reduction in 5-HT turnover in hypothalamus t=8 ($P<0.001$), amygdala t=8 ($P<0.001$) and in hippocampus t=4 ($P<0.01$).

Table 1: Effect of alcohol administration and subsequent withdrawal on brain regional (hypothalamus) trp metabolism

	HYPOTHALAMUS			
	TRP (ng/g)	5-HT (ng/g)	5-HIAA (ng/g)	5-HIAA/ 5-HT
Chow control	1476±132.8	1180.5±106.5	618.5±38.7	0.52±0.02
Matched control	1441.5±102.4	1427.3±100.2	644.8±34.1	0.45±0.01*
	NS	NS	NS	P<0.05
AW (0hr)	1622±159	1541.8±112.2	882±51.0**	0.53±0.01***
	NS	NS	P<0.01	P<0.001
AW (7hrs)	1112±75.0*	825.6±54.7***	315±29***	0.37±0.01***
	P<0.05	P<0.001	P<0.001	P<0.001

Table 2: Effect of alcohol administration and subsequent withdrawal on brain regional (amygdala) trp metabolism

	AMYGDALA			
	TRP (ng/g)	5-HT (ng/g)	5-HIAA (ng/g)	5-HIAA/ 5-HT
Chow control	1304±159.6	974.5±24.6	501±31.1	0.50±0.02
Matched control	1281±107.4	1278±73.7**	625.8±40.1*	0.48±0.01
	NS	*P*<0.01	*P*<0.05	NS
AW (0hr)	1530.3±112	1591.5±115.0*	841.5±37.6**	0.53±0.02*
	NS	*P*<0.05	*P*<0.01	*P*<0.05
AW (7hrs)	914.6±40.8**	768.5±46.06***	315.1±29.6***	0.40±0.01***
	P<0.01	*P*<0.001	*P*<0.001	*P*<0.001

Table 3: Effect of alcohol administration and subsequent withdrawal on brain regional (hippocampus) trp metabolism

	HIPPOCAMPUS			
	TRP (ng/g)	5-HT (ng/g)	5-HIAA (ng/g)	5-HIAA/ 5-HT
Chow control	1494.6±133.2	1655±105.8	818.3±49.7	0.49±0.008
Matched control	1720.8±101.2	1401±109.3	637.5±58.4*	0.45±0.01**
	NS	NS	*P*<0.05	*P*<0.01
AW (0h)	2176±113.0*	1875±106.6**	910.5±36.2**	0.48±0.01*
	P<0.05	*P*<0.01	*P*<0.01	*P*<0.05
AW (0-7h)	1676±111	961.5±48.6**	409.6±29.7**	0.418±0.01**
	NS	*P*<0.01	*P*<0.01	*P*<0.01

Experimental details are given in material and methods section. All values are means ± for each group of six rats. Statistical analysis was done using students *t*-test The values obtained in matched control rats were compared (with those in solid chow control group, whereas alcohol withdrawal (AW) 0 & 7 h were compared with those in matched control group. The significance of difference is indicated as follows **P*< 0.05, ***P*<0.01, ****P*<0.001

Table 4 shows the effects of alcohol administration and subsequent withdrawal on serum corticosterone concentration in rats. No significant difference was found in corticosterone between two controls groups (chow and matched). However, chronic alcohol administered rats when compared with matched control showed significant decrease in corticosterone levels t=2.83 (*P*<0.05). In contrast, significant increase in corticosterone t=8.5 (*P*<0.01) levels were seen in alcohol withdrawal group when compared with matched control group.

DISCUSSION

Present data shows that chronic alcohol administration increases brain TRP concentrations only in hippocampus with non-significant effects on hypothalamus and amygdala. The increased serotonin concentration in amygdala and hippocampus with the increase in 5-HT turnover rate in all the three brain regions may therefore explain the increase in tryptophan availability to the brain for serotonin synthesis by alcohol intake. It has been reported that acute alcohol intake increases the release of catecholamines, which via lipolysis increases free TRP uptake to the tissues (brain and liver). This mechanism exerts biphasic effect possibly by the initial rise in serotonin levels followed by the reduction in its concentration across the synapse (LeMarquand, 1994a, b). However, acute alcohol intake appeared to increase hepatic TDO enzyme activity (Bano *et al*., 1996). It was reported that acute alcohol exposure facilitates 5-HT functions by increasing brain TRP concentration via altering free TRP to LNAAs ratio, which is independent to the activation of hepatic TDO enzyme activity (Le Marquand 1994 a, b). Chronic alcohol consumption,

however, seems to increase brain 5-HT by increasing substrate availability from the periphery via decreasing hepatic kynurenine enzyme activity (LeMarquand *et al*., 1994b). Inhibition in TDO enzyme activity was also seen when ethanol was given in drinking water (Badawy and Evan, 1972 1973). It is revealed from the present data that chronic alcohol facilitates serotonergic neurotransmission by increasing tryptophan availability to the brain and hence 5-HT synthesis, release and metabolism (Badawy, 2005). Thus, the observed increase in 5-HT turnover in all the three brain areas (hypothalamus, amygdala and hippocampus) provides evidence for the involvement of these brain areas in facilitating alcohol-induced serotonergic neurotransmission. Since the decreased central 5-HT function has been implicated in aggression and violent behavior, these patterns may explain that the alcohol induces serotoninergic neurotransmission that provide neurochemical adaptation and imbalance in the brain neurochemistry when alcohol exposure ceases. Thus facilitation of 5- HT neurotransmission by alcohol accounts for the enhancement of mood (Artigas, 1995; Barr *et al*., 1994). In addition presence of alcohol for long duration can increase the rise in number of receptor proteins on the surface of target neurons that bring adaptation and tolerance to alcohol intake. These changes are consistent with the development of aggressive behavior in our present findings when alcohol was withdrawn from animals. Serotonin neurons from the raphe nuclei project to limbic brain areas are synapse onto CRH neurons in the PVN of the hypothalamus that stimulate the release of CRH from that region and consequently increased the release of ACTH from pituitary and corticosterone from the adrenals (Fuller, 1981, 1992). Further 5-HT neurotransmission imply the control of HPA- axis on CRH release in the hypothalamus (Pazos and Palacios, 1987). Alcohol, itself is a powerful inducer of HPA axis and its acute exposure increases HPA-axis, while its chronic administration has been demonstrated to develop tolerance to the HPA-axis stimulating effects. Preclinical studies by Spencer and McEwen (1990) have demonstrated that this HPA axis activity is associated with decrease in its response on the last day of the treatment than on the first day when alcohol was administered repeatedly (Spencer and McEwen, (1990). And the decreased CRF release was found when acute alcohol was administered in chronically-alcohol treated subjects that suggest the development of tolerance to the CRF stimulating effects (Redie *et al*., 1998; River *et al*., 1984). Therefore the release of corticosterone by alcohol is suggested to be multifactorial that is directed by alcohol or serotonin-induced CRF release. Further, mineralocorticoid receptors interaction with CRH provides mainstay in establishment of adaptation phenomenon in the brain against aversive stimulants (Gesing *et al*., 2001). It can be suggested that the endocrinological responses and serotonin are the two consistent components of the limbic system that are responsible for adaptation to behavioral patterns that leads to aggression and anxiety.

Table 4: Effect of alcohol administration and subsequent withdrawal on serum corticosterone

Groups	Corticosterone (µg/dl)
Chow Control	58.3±2.8
Matched Control	52.6±3.07
	NS
AW (0 hr)	41.4±2.5*
	(P<0.05)
AW (7 hrs)	87±2.64**
	(P<0.01)

Experimental details are given in material and methods section. All values are means ± for each group of six rats. The values obtained in matched control rats were compared with those in solid chow control group, whereas those obtained in the group of rats withdrawn from alcohol (AW) for 0 & 7h were compared with those in matched control group. Statistical analysis was done using Student's t-test. The significance of difference is indicated as follows *P<0.05, **P<0.01.

Present data also shows depletion in brain tryptophan, 5-HT and 5HIAA and in its turnover when AW rats were compared with matched controls. An inverse relationship exists between TDO enzyme activity and brain serotonin levels has been documented under many circumstances (Badawy and Morgan 1991; Oretti *et al*., 1996). The importance of TDO in regulation of serotonin synthesis is evident from the observation that majority of patients suffering from depression have elevated cortisol levels (Curzon, 1988). And, low serotonin turnover is associated with negative mood states and related to aggressive behavior, feeling of insecure and threat that leads to the development of anxiety (Virkunnin *et al*., 1994). The serotonergic changes in the brain areas examined in the present findings during AW were consistent with the development of behavioral aggression and anxiety following alcohol withdrawal. Our data shows that subsequent withdrawal from alcohol (7hrs) increased corticosterone levels when compared with matched controls, consistent with the previous reports (Bano *et al*., 1996). It can be suggested that the increased corticosterone levels during abstinence may result into failure of negative feedback response from mineralocorticoid and glucocorticoid receptors in the hippocampus. And, decreased CRF release was shown by the acute alcohol administration in chronically treated alcoholic subjects that were shown to develop tolerance in CRF-stimulating effects (Rivier *et al*., 1984; Redei *et al*., 1988). Decreased 5-HT turnover rate has been associated with reduced response to excessive alcohol consumption (Wrase *et al*., 2006). Moderate reductions in alcohol consumption were found in alcoholics treated with SSRIs (Naranjo *et al*., 1994). It can be considered that low serotonin turnover may also reduce the sensitivity of

glucocorticoid receptors to provide negative feedback control on corticosterone release. And, the elevated HPA-axis contributes to emotional reactivity during alcohol abstinence that also leads to stimulate aggression and anxiety.

It can be concluded that the neuroendocrine changes in the limbic brain areas play crucial role in alcohol-induced alteration in 5-HT neurotransmission. These changes may contribute to sedative and relaxing effects of alcohol that bring adaptation to neurochemical and behavioral changes. And therefore alcohol withdrawal consequently leads to aggression, anxiety and atrophy of brain areas, impairment in cognitive functions and other problems. A better understanding of the neurobiological impact of alcohol consumption will facilitate the development of novel intervention strategies that target both the prevention and treatment of alcohol dependence.

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