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Consequences of Glutamatergic and GABAergic Neurotransmitter Changes Aggravating the Complications of Ethanol Withdrawal Can Be Challenged by Administration of Extract of *Zingiber officinale*

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Abstract: Although the basic metabolic and homeostatic mechanisms governing functional aspects of GABA-mediated neurotransmission under ethanol withdrawal has long been worked out decades ago, elucidating the cellular and molecular targets for alcohol's important pharmacological actions has always turned out to be challenging. Among other things, it has become evident that the withdrawal from chronic ethanol consumptionhasa profound adverse impacton the GABAergic function and promotes neuronal hyperexcitability. Contemporary techniques such as NMR spectroscopy as well as cloning of enzymes, transporters and receptors may provide fine information allowing a much more sophisticated and detailed knowledge about the area under discussion. This may in future lead to development of new therapeutic strategies by which neurological disorders involving disturbances of GABAergic activity may be treated.

Key words: Ethanol Withdrawal · Neuronal Hyperexcitability · GABA-Mediated Neurotransmission · Ginger · Therapeutic Strategies

INTRODUCTION

Prolonged administration of ethanol to animals and humans can cause adaptive cellular changes resulting in a requirement for the presence of ethanol for normal function, i.e., physical dependence [1]. Discontinuation of ethanol administration reveals this adaptive increase in neuronal excitability, resulting in the development of withdrawal signs such as anxiety and seizure susceptibility. With more prolonged and heavier alcohol consumption, 'complicated alcohol withdrawal syndrome' sets in which include the presence of any of the three i.e. psychotic symptoms, confusion and seizures [2]. Ethanol has potent effects on both excitatory and inhibitory neurotransmitter systems. Chronic ethanol exposure leads to an up-regulation of NMDA receptors [3] and excitotoxicity occurs following excessive excitation of neurons through glutamate receptors. On a neurochemical level alcohol dependence syndrome is characterized by neuroadaptation of a number of different types of receptor, the most important being that of the glutamate and GABA systems. NMDA receptors play a key role in tolerance development [4,5] and the pharmacological activity of the NMDA receptor may be influenced by the history of alcohol consumption is clearly demonstrated [6]. Numerous studies have suggested that ethanol dependence and withdrawal result in part from adaptive changes in GABAergic transmission [7,8], although a number of cellular changes are observed following chronic ethanol treatment [9]. Excessive levels of extracellular glutamate in the brain are known to be excitotoxic and lead to neuronal death [10,11]. It is quite logical to assume that rapid removal of the glutamate released into the synapses may prevent the unwarranted excitation of glutamate receptors. Moreover, GABA is known to act as an inhibitory neurotransmitter in the brain and the enzyme glutamate decarboxylase (GAD) plays an important role in the balance of glutamate/GABA

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concentrations in the brain [12,13]. About 30% of brain neurons contain glutamic acid decarboxylase (GAD), which synthesizes GABA from glutamic acid, the GABA inhibitory effects play an important role in the protection of the brain neurons [12,14]. Drugs enhancing the tissue content of GABA act as anticonvulsants [15], the present study was designated to determine the possible influence of ginger extract administration on glutamate, GAD and GABA levels and their interrelations in selected brain regions of alcoholic and withdrawal rat.

MATERIAL AND METHODS

Drugs and Chemicals: Standards for GABA, glutamate and GAD and BSA were purchased from Sigma Chemicals (St. Louis, MO, USA). All other chemicals are of analytical grade unless otherwise mentioned.

Collection of Plant Material: Aqueous ginger extract was prepared from locally available ginger roots. Ginger rhizomes were purchased fresh from the local markets and were authenticated by the staff, Department of Botany, Sri Venkateswara University, Tirupati, India.

Extract Preparation: Whole rhizome of ginger was thoroughly washed, sliced, grated and grind to fine paste. A weighed quantity (30g) of the paste was subjected to continuous hot extraction in soxhlet apparatus using double distilled water. The extract was evaporated under reduced pressure using rotary evaporator and then lyophilized until all the solvent has been removed to give an extract sample and stored at 4°c for further studies.

Animals: The study involved young (3 months old; 200-220g) male albino rats of wistar strain purchased from Sri Venkateswara Traders Pvt. Limited, Bangalore, maintained in the animal house of the department in polypropylene cages. The animals were allowed to habituate to the animal facilities for at least for two weeks adaptation period upon arrival and were maintained under standard conditions of humidity (50± 9% relative humidity), room temperature (25-28ºC) and 12 h light/ dark cycle (6:00 A.M. to 6:00 P.M.). A standard rodent diet (M/s Hindustan Lever Ltd., Mumbai) and water were provided *ad libitum*. All experimental procedures were approved by the CPCSEA on Animal Care with CPCSEA No. 438 / 01/a / CPCSEA / IAEC / SVU / KSR-1 (dt: 11.09.2008).

Treatment Protocol: Animals were maintained six per group and grouping involved 3 batches, first batch includes control group given normal saline. Second batch involves two groups of rats given 20% Alcohol(p.o.) and another group is set to receive alcohol along with ginger extract (200 mg/kg body weight)while third batch is also treated the same way as the second batch except that they are allowed for withdrawal. All the treatments are carried out for 42 days excluding withdrawal groups that were allowed for 3 days of withdrawal from the drug after the last dose.

Tissue Collection: After the period of last dose of ethanol treatment, the animals were sacrificed exactly after 72 hrs by cervical dislocation. The whole brain was removed, washed with ice-cold saline, dry freezed in liquid nitrogen and immediately transferred to the ice chamber at-80°c. Cerebrum, hippocampus, pons medulla and cerebellum were separated as described by Nayak and Chatterjee [16].

Biochemical Procedures

Estimation of Brain Glutamate Levels: The brain glutamate content was measured according to Bernt and Bergmeyer [17].

Estimation of Glutamate Decarboxylase Activity: The activity of GAD was measured in the supernatant of the brain regions spectrophotometrically as described by Cozzani [18].

Estimation of GABA Content: The brain gamma-aminobutyric acid (GABA) content was estimated according to the method of Lowe [19].

Data Analysis: Statistical analyses were carried out by one-way ANOVA with multiple comparisons measurements were used to assess the statistical significance of effects using the statistical package for social sciences (SPSS) software 12.0. Scheffe's *post hoc* analyses were used when appropriate. Results are presented as mean ± SD, with P values of <0.05 considered significant.

RESULTS

Effect of Aqueous Ginger Extract (AGE) on amino acid neurotransmitter levels in rats under withdrawal stress induced due to chronic ethanol administration.

Table 1: Regional glutamate contents (imoles / g wet tissue) of brain

	Groups of animals					Statistical calculation (F)
Brain regions	Control	Alcohol	EtoH+Ginger	EW	EW+Ginger	ANOVA Single factor
CC	11.344±0.686	12.403±0.371*	11.702± 0.504	15.363±0.417*	11.933 ±1.167	32.778†
CB	11.755±0.666	12.870±0.101*	11.947±1.313	14.708±1.522*	11.424±1.715*	7.079†
HC	11.455±1.094	12.194±0.316*	11.844±1.209*	15.149±0.800*	12.306±0.292*	18.591†
PM	10.624±0.563	12.377±0.145*	10.551±0.746	12.501±0.213*	10.101±0.335*	35.864†

Data is mean of six observations ± SD. * indicates significant difference ($p< 0.05$) in comparison to respective control. † indicates calculated F is higher than the critical F ($\alpha = 0.05$)by ANOVA (Scheffe's test).

Table 2: Regional GAD activity (imoles of GABA produced / hr/ mg wet tissue) of brain

	Groups of animals					Statistical calculation (F)
Brain regions	Control	Alcohol	EtoH+Ginger	EW	EW+Ginger	ANOVA Single factor
CC	24.834±0.550	31.523±3.547*	30.998±8.444	18.633±3.004*	20.234±7.278	7.260†
CB	22.925±2.081	23.709±5.894	22.035±2.887	12.584±3.166*	14.450±1.497*	13.644†
HC	29.868±1.691	39.692±3.775*	38.969±3.506*	32.847±4.924*	12.106±6.858*	37.258†
PM	38.827±0.789	40.749±1.162*	40.762±6.105*	21.800±3.536*	35.509±4.160*	27.578†

Data is mean of six observations ± SD. *indicates significant difference ($p< 0.05$) in comparison to respective control. † indicates calculated F is higher than the critical F ($\alpha = 0.05$) by ANOVA (Scheffe's test).

Table 3: Regional GABA contents (imoles /gm wet tissue) of brain

	Groups of animals					Statistical calculation (F)
Brain regions	Control	Alcohol	EtoH+Ginger	EW	EW+Ginger	ANOVA Single factor
CC	2.757 ±0.314	3.107±0.502*	2.855±0.105*	1.041±0.651*	2.648±0.697*	15.951†
CB	3.297±0.110	3.563±0.582*	3.239±0.056*	1.478±0.445*	3.155±0.556*	24.224†
HC	3.153±0.399	3.363±0.854*	3.260±0.391*	0.993±0.46*	3.478±0.813*	17.013†
PM	2.635±0.205	3.758±0.233*	2.945±0.411*	1.599±0.554*	2.037±0.347*	29.981†

Data is mean of six observations ± SD. * indicates significant difference ($p< 0.05$) in comparison to respective control. † indicates calculated F is higher than the critical F ($\alpha = 0.05$) by ANOVA (Scheffe's test).

Glutamate (Glu): In alcohol and withdrawal models, Glutamate levels significantly ($p<0.01$) increased in different brain regions of withdrawal and found to be much greater than chronic ethanol treated group were observed. AGE at the doses of 200 mg/kg bw, treated animals showed a significant ($p<0.05$) decrease in Glu levels in brain regions of rats (Table 1).

Glutamate Decarboxylase (GAD): In alcohol and withdrawal models, GAD levels significantly ($p<0.05$) decreased in decreased in the brain regions of withdrawal animals compared to control group. Ginger extract at the doses of 200mg/kg bw, treated animals showed a significantly ($p<0.05$) increased in GAD levels in forebrain of rats (Table 2).

Gamma Amino Butyric Acid (GABA): In alcohol and withdrawal models, GABA levels significantly ($p<0.05$) decreased in forebrain of withdrawal control animals were observed. Aqueous Ginger extract at the dose of 200mg/kg bw, treated animals showed a significantly ($p<0.05$) increased in GABA levels in brain of rats (Table 3).

DISCUSSION

Alcohol withdrawal seizures, defined as adult-onset seizures occur in 5-15% of people with alcohol dependence, 7-48 h (can be upto 72h) after cessation of drinking [20]. Such seizures are the fourth most common cause of status epilepticus [21]. Repeated episodes of

withdrawal symptoms, as may occur during repeated detoxifications, can be detrimental to the CNS, a process known as 'kindling of withdrawal symptoms'. Proposed mechanisms underlying these harmful kindling effects include repeated bouts of excitatory-induced neuronal cell death, along with repeated episodes of lability of the hypothalamus-pituitary-adrenal (HPA) axis. With chronic alcohol misuse, neuroadaptation by NMDA and GABA receptors in the brain i.e. up-regulation of NMDA receptors and down-regulation of GABA receptors leads to tolerance while drinking and to relative hyperactivity of NMDA receptors and hypoactivity in the GABA system at times of lowered blood alcohol levels. Up-regulation occurs in NMDA receptors secondary to the long-term, direct inhibition of these receptors by alcohol, while direct excitation of the GABA receptors due to long-term alcohol consumption leads to their down-regulation in alcohol dependence. On acute withdrawal of alcohol this results in excitatory effects via, respectively, an increased calcium flux and a reduced chloride shift in CNS neurons [22].

GABA is formed *in vivo* via a metabolic pathway called the GABA shunt. The initial step in this pathway utilizes α-ketoglutarate formed from glucose metabolism via the Krebs cycle. α-Ketoglutarate is then transaminated by α-oxoglutarate transaminase (GABA-T) to form glutamate, the immediate precursor of GABA. Finally, glutamate is decarboxylated to form GABA by the enzyme(s) glutamic acid decarboxylase (GAD). In addition to the GABA binding site, the $GABA_A$ receptor complex appears to have distinct allosteric binding sites for benzodiazepines, barbiturates, methanol etc [23]. The results indicate that the activities of the enzyme are significantly lower in the brain of withdrawal rats as compare to that of normal and ethanol groups. Lower activity of GAD may alter the balance of glutamate and GABA concentration in favor of glutamate accumulations in the synaptic parts of the studied brain regions.

The changes in the levels or activity of the synthesizing enzyme of GABA, glutamic acid decarboxylase (GAD), is altered in the EW animals or with chronic ethanol treatment under the effect of ginger and long-term treatment with ginger extract has restoration of altered GABA and glutamate levels due to chronic ethanol administration and also withdrawal in brain regions selected under study exploring neuroprotective and neuroexcitotoxic preventive properties of ginger. It seems that the significant activation of the enzyme in withdrawal rat brain with ginger treatment, may also be due to the interaction of prydoxal-5-phosphate with the enzyme at the molecular levels and might be resulted from either an increased GAD apoenzyme level in alcoholic animals, or lower affinity of the enzyme for pyridoxal 5'-phosphate. The latter is likely to be related to the posttranslational modifications of the proteins as consequences of ethanol dependence although, provided data is not adequate to suggest this. Or unknown mechanisms operating at stages regulating subtype heterogeneity and receptor dynamics through trafficking, regulation of stability and plasticity might also be responsible for the prevention of generation of cytotoxic levels of glutamate and reactive oxygen species, allowing the fine tuning of these chemical messengers in neural networks.

GABA is a major inhibitory neurotransmitter of CNS and increase in its level in brain has variety of CNS dependent effects including anticonvulsant effect [24]. Inactivation of GABA as a neurotransmitter occurs exclusively by diffusion in the synaptic cleft and active transport into presynaptic nerve endings and astroglial cells ensheathing the synapse [25]. It is therefore clear that inhibition of such transport could lead to increased GABA levels in the synapse and thus to enhanced efficacy of inhibitory neurotransmission [26]. *In vivo* studies demonstrate that EW induced rats showed increased levels of Glu and have developed EW induced hyperexcitability and that this hyperexcitability may be attributable, in part, to a reduction in new GABA synthesis in the cerebral regions. Scientific studies also suggest that glutamate transporters in general and EAAC1 specifically, have a role in synthesis and release of new neurotransmitter GABA in the hippocampus of normal naive rats [27].

Since GABA catabolic enzyme GABA-T, like GAD, is pyridoxalphosphate requiring enzyme [28], developing agents that specifically inhibit GABA-T leaving GAD activity intact, it might be possible to develop an anti-convulsant drug like Gamma-vinyl GABA that was subsequently developed into a clinically active drug (vigabatrin) used to treat certain types of epilepsy [29], since GABA is a substrate for GABA-T with no affinity for GAD.

Glutamate transport is the major mechanism controlling extracellular glutamate levels, preventing excitotoxicity and averting neural damage associated with many neurodegenerative diseases [30-37]. Glutamate transporters are localized to the membranes of synaptic terminals and astroglial processes that ensheath synaptic complexes [38-43]. Astroglial glutamate transporters are responsible for at least 80% of the high-affinity glutamate transport and the majority of synaptic inactivation. Increased expression

of glu transporters either due to transcriptional regulation or by translation mechanisms as part of ginger protection cannot be overlooked and also needs to be considered.

The prospects for identifying novel therapeutic agents acting on glutamatergic transmission that are effective in the conditions described above are now exceptionally good. NMDA and AMPA receptor antagonists protect against acute brain damage and delayed behavioral deficits. Such compounds are undergoing testing in humans, but therapeutic efficacy has yet to be established. Other clinical conditions that may respond to drugs acting on glutamatergic transmission include epilepsy, amnesia, anxiety, hyperalgesia and psychosis. In this way, it should be possible to identify powerful novel agents with highly selective actions in terms of function and the target brain region or cell type.

In any event such goals depend upon a better knowledge of brain physiology, which in itself needs the development of new approaches, technologies and ideas involving indepth studies exploring alterations in $GABA_A$ receptors and receptor subunits, their distribution, GAD mRNA expression, synaptic regulation and neurotransmitter transport.

In all, our findings suggest that regulations of adaptations to chronic ethanol by ginger treatment expressed in the cerebral regions appear to be mediated by factors regulated by distinct cellular and molecular mechanisms that remain yet to be elucidated. This field of study can prove to be best alternative to antipsychotic drugs and presents fine hope in drug withdrawal treatments.

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