STUDY OF ETHANOL METABOLISM IN MOUSE BRAIN

Master’s Thesis

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1. Background and Significance

1.1 Introduction

Alcohol is the oldest drug known to humans. It is consumed, and sometimes abused, all around the world. Though it is thought that moderate drinking can be beneficial to the health, higher amounts of alcohol affect almost all systems of the body in an undesirable manner. Alcohol produces a wide variety of effects on the body at different doses. It has abuse potential and its excessive usage causes physical and psychological dependence, which not only causes physiological damage to the individual but also represents social and economic burdens to the country. Although alcohol is regarded as a drug of abuse, it differs from other abused substances because an intoxicating dose corresponds to 1 or more moles of alcohol, corresponding to tissue concentrations of >10 millimolar. Other abused substances are effective at micromolar or lower concentrations. Alcohol has been studied extensively for many decades, and much is known concerning its toxicology, however, it is not known how alcohol causes the brain alterations that underlie its recreational use, and to what extent effects are due to alcohol itself, or to alcohol’s metabolites. It remains a big challenge to unravel the comprehensive mechanisms of action of alcohol and its effects on body. These studies are directed at improving our knowledge of such mechanisms in the expectation that this will aid the development of novel therapeutic measures for the treatment of alcohol abuse and dependence.
1.2 Epidemiology

Though alcohol consumption is decreasing in developed countries, about 2 billion people drink alcohol globally and 76.3 million people suffer from alcohol-related disorders (WHO 2004). Alcohol consumption can cause more than 60 types of diseases and injuries (WHO 2002). All over the world, alcohol is responsible for 3.2% of deaths (1.8 million) and 4% of disability adjusted life years (58.3 million). It is estimated that 20-30% of the esophageal and liver cancers, liver cirrhosis, motor vehicle fatalities, homicide and epilepsy are linked to alcohol consumption (WHO 2002). Besides these personal health problems, social and economical consequences of alcohol abuse are enormous. The economic impact of alcohol abuse was estimated to be $184.6 billion in 1998 which was about 25% more compared to $148 billion in 1992 (Harwood, Fountain et al. 1998; Harwood 2000). In the United States, the total numbers of motor vehicle fatalities were 37,261 in 1998; of which, 32% were associated with driving under the influence of alcohol (NHTSA 2009). There also seems to be a strong relationship between alcohol consumption and violence. In the United States, excessive alcohol drinking is involved in brutal crimes such as homicides (28-86% of offenders), robberies (7-72% of offenders), assaults (24-37% of offenders) and sexual offences (13-60% of offenders) (Roizen 1997; Brewer and Swahn 2005).

1.3 Effects of Alcohol on Health

1.3.1 Cardiovascular System

Chronic heavy alcohol consumption is associated with enlargement of the left ventricle and thickening of the left ventricular wall, and results in alcoholic cardiomyopathy (Lazarevic,
Heavy alcohol drinking can also cause hypertension, cardiac arrhythmia and heart failure (Djousse and Gaziano 2008). Chronic alcohol consumption of more than 36 g/day (about more than 3 drinks/day) increases the risk of atrial fibrillation significantly (Djousse, Levy et al. 2004). However, light to moderate alcohol consumption is linked with decreased risk of congestive heart failure and decreased mortality (Fuchs, Stampfer et al. 1995; Djousse and Gaziano 2008). Effects of moderate drinking on ischemic stroke remain controversial. Some studies have found alcohol drinking to be correlated with stroke while other studies including the Framingham study (Djousse, Ellison et al. 2002) did not find any relation between moderate drinking and risk of stroke. In fact they found beneficial effects of moderate drinking against stroke among sexagenarians. Effects of alcohol on heart function also depend on the type of alcoholic beverages (Djousse, Ellison et al. 2002). Wine was found to have protective effects in stroke whereas beer and spirits did not. One explanation for this would be effects of other constituents of wines.

1.3.2 Liver and Cancer

It is well established that chronic alcohol consumption can affect liver function. It causes alcoholic liver disease which starts from the development of steatosis, and if untreated, progressively develops into steatohepatitis, fibrosis, cirrhosis and finally hepatocarcinoma (Purohit, Gao et al. 2009). Chronic alcohol consumption is also a risk factor for the development of cancer of the alimentary tract including the oral cavity, esophagus, larynx, and pharynx (Poschl and Seitz 2004). Alcoholic cirrhosis is a precancerous condition which itself can develop into hepatocellular carcinoma. Alcohol metabolizing enzymes and alcohol metabolites (described under Metabolism of Alcohol & Its Consequences section) are crucial mediators in
the development of cancer (Stickel, Schuppan et al. 2002). Acetaldehyde, the initial metabolite of alcohol, is carcinogenic and mutagenic and can interfere with DNA repair mechanisms (Garro, Espina et al. 1986). Induction of Cytochrome P450 2E1 (CYP2E1) enzyme by alcohol can lead to the formation of reactive oxygen species, free radicals and lipid peroxides which can initiate carcinogenesis. In support of this mechanism, chlormethiazole decreased the alcohol associated morbidity by inhibiting CYP2E1 (Gouillon, Lucas et al. 2000). Other CYP2E1 inhibitors and inducers have also been found to modulate the effects of alcohol on liver. Additionally, high levels of alcohol consumption modestly increased the incidence of colorectal cancer (Cho, Smith-Warner et al. 2004). Alcohol usage may also be associated with breast cancer. Mild alcohol consumption was found to increase the risk of fatal breast cancer in postmenopausal women in one study (Feigelson, Calle et al. 2001) whereas alcohol, irrespective of amount, only marginally increased the incidence of breast cancer in another study (Kuper, Ye et al. 2000). Differences between these study findings illustrate the difficulties in controlling the risk factors other than alcohol consumption.

1.3.3 Nervous System

Long term alcohol use has a profound impact on brain structure and function. The alcohol-associated impairments include short-term memory loss, cognitive and motor function deficiencies, difficulty in coherent thinking, learning and though processing disabilities, and uncontrolled behaviour (Wolfgang 1997). The neuropsychological consequences of alcoholism are anxiety, major depression, hallucinations and delusions. Moreover, the suicide rate is very high among alcoholics. The Young female alcoholics are more prone to suicidal attempt than male alcoholics (Roy, Lamparski et al. 1990). Excessive chronic alcohol consumption can affect
emotional state and fMRI studies have indicated that impaired amygdala and hippocampal activities may be responsible for this (Marinkovic, Oscar-Berman et al. 2009). Since alcohol is addictive, chronic usage can result in physical and psychological dependence. This results in continued damage to the brain and other organs. Brain damage that makes loss of control over drinking more likely may increase alcohol consumption further increasing damage; a “vicious spiral” may thus ensue. Binge drinking during adolescence can significantly affect the cortical brain development (Crews, He et al. 2007). Wernicke-Korsakoff syndrome can result due to excessive long term alcohol usage. Alcohol can interfere with the absorption of thiamine and its utilization in cells which results in Wernicke’s encephalopathy developing into Korsakoff’s psychosis (Martin, Singleton et al. 2003).

1.3.4 Pancreas

There are considerable evidences to indicate that alcohol is converted in the pancreas to metabolites that may be involved in causing pancreatitis. Chronic excessive alcohol usage is a major risk factor for acute pancreatitis and its progression into chronic disease (Lankisch, Breuer et al. 2009). Alcoholics develop chronic pancreatitis independent of the severity of the initial pancreatic condition, and alcoholism is a major etiological factor in recurrent pancreatitis episodes (Pelli, Lappalainen-Lehto et al. 2008). Also, abstinence from alcohol impedes the recurrence of pancreatitis. Alcoholic pancreatitis can progress into pancreatic cancer. Diabetes is also strongly associated with chronic pancreatitis. The risk of diabetes mellitus increases with time among individuals with chronic pancreatitis. A survey in Japan showed that 29% of 418
patients with chronic pancreatitis developed diabetes mellitus within an eight year follow up period (Ito, Otsuki et al. 2007).

1.3.5 Endocrine System

Alcohol interferes with optimal endocrine functions which can have metabolic, developmental and functional consequences. Alcoholism can interfere with secretion of growth hormone. Chronic drinking in men can cause testicular atrophy, impaired spermatogenesis, decreased testosterone level, decreased libido and erectile dysfunction (Van Thiel, Gavaler et al. 1980; Maneesh, Dutta et al. 2006). In women, alcohol can disturb the menstrual cycle, the fertility rate, and sexual function. Rates of abortions, stillbirths, and hysterectomy are also linked to increased drinking (Wilsnack, Klassen et al. 1984). As noted earlier, alcoholic women are prone to develop breast cancer; increased secretion of estrogen, due to alcoholism, may be responsible for that.

1.3.6 Immune System

Chronic alcohol abuse depresses the immune system by significantly decreasing cytotoxic activity and the count of natural killer cells (Blank, Pfister et al. 1993). Decreased immunity may be responsible for the increased risk for cancer and microbial infections in alcoholics (Rehm, Room et al. 2003). Effects of alcohol on the immune system are dose dependent. Low to moderate doses of alcohol decrease the immune responses whereas its heavy usage can cause excessive inflammatory responses (Goral, Karavitis et al. 2008). It is evidenced that alcohol can affect antigen recognition capabilities of immune cells by modulating expression of TLRs (Toll-like Receptors) on these cells (Nishiyama, Ikejima et al.)
2002; Zuo, Gong et al. 2003). Since TLRs initiate inflammatory reactions through intracellular signaling pathways including activation of mitogen activated protein kinases (MAPKs) and NFκB, modulation of TLRs may result into abnormal inflammatory reactions (Kawai and Akira 2006).

1.3.7 Fetal Alcohol Syndrome

Alcohol drinking during pregnancy affects the growth and development of the fetus, and babies born to alcohol-dependent mothers may suffer from fetal alcohol syndrome (FAS). FAS is mainly characterized by mental and physical growth retardation and craniofacial abnormalities (Chaudhuri 2000). Alcohol easily crosses the placental barrier and can cause defects in the growth and development of the fetus.

1.4 Metabolism of Alcohol & Its Consequences

Although alcohol is metabolized primarily by liver, it is also metabolized by extraheptic tissues including the brain, pancreas and some gastrointestinal organs. Both oxidative and non-oxidative pathways are involved in alcohol metabolism. The enzymes that play roles in oxidative metabolism are alcohol dehydrogenase (ADH), cytochrome P450 2E1 and catalase (Zakhari 2006). ADH plays a major role in metabolizing alcohol in liver. CYP2E1, present in microsomes, also functions significantly in liver. CYP2E1 is thought to be important for alcohol metabolism in the brain because brain has low ADH activity (Zakhari 2006). Catalase which is found in cell peroxisomes forms a minor pathway for alcohol metabolism. Oxidative pathways of alcohol metabolism are summarized in Figure 1.
Figure 1 Oxidative pathways of alcohol metabolism. (ADH – Alcohol dehydrogenase, ALDH – Acetaldehyde dehydrogenase, NAD⁺ - Nicotinamide adenine dinucleotide, NADH – Nicotinamide adenine dinucleotide reduced, CYP2E1 – Cytochrome P450 2E1)


The ADH family comprises a wide variety of isozymes present in cytosol. ADH oxidizes alcohol into acetaldehyde, a toxic and highly reactive chemical entity. There are some evidences that acetaldehyde is responsible for the effects of alcohol to some extent (Deitrich, Zimatkin et al. 2006). Acetaldehyde can react and form adducts with protein, enzymes, nucleic acids or other biological building blocks to impair their functions. Acetaldehyde is known to be capable of reacting in vivo with the aminergic neurotransmitters to form tetrahydroisoquinolines (TIQs) which may be responsible for some of the CNS effects of alcohol. Similarly, it can form tetrahydro-β-carboline derivatives, which can affect brain functions, by condensing with serotonin. Acetaldehyde can also indirectly affect the elimination of various other aldehydes (eg. metabolites of aminergic neurotransmitters) by competitively inhibiting
their metabolism. However, the notion that acetaldehyde has significant roles in mediating the effects of alcohol on the brain are disputed due to following factors. Normally, acetaldehyde is quickly metabolized by liver such that the blood level of acetaldehyde remains extremely low, whereas it has been believed that a high concentration of acetaldehyde in blood is required for effective penetration of the blood-brain barrier (Sippel 1974; Tabakoff, Anderson et al. 1976; Westcott, Weiner et al. 1980). In addition, alcohol is intoxicating to animals even after blockage of ADH by administration of pyrazole and thus inhibition of formation of acetaldehyde. This suggests that acetaldehyde has little to do with the most noticeable of alcohol’s CNS effects (sedation, loss of motor control). It is possible, however, that some effects of alcohol in humans, for example, anxiolysis, loss of social inhibition, euphoria, “hangover”, are associated with acetaldehyde or compounds formed from acetaldehyde.

Acetaldehyde is further oxidized into acetate in mitochondria by acetaldehyde dehydrogenase (ALDH). Acetate produced from alcohol in liver appears to be mainly exported into the blood (Zakhari 2006). Eventually, it is oxidized to carbon dioxide via acetylcoA and the Krebs cycle, in extra-hepatic tissues. Acetate can enter the brain, and has behavioral effects when administered to laboratory animals (Carmichael, Israel et al. 1991). Normally, blood-borne acetate makes only a very small contribution to energy metabolism in the brain, because its concentration is very low (Hertz and Dienel 2002). However, after heavy alcohol consumption it is possible that acetate becomes a significant source of energy, reducing consumption of glucose (Volkow, Wang et al. 2006). Regional glucose metabolism in the brain is tightly regulated and linked to demand for energy and to regional blood flow. It also involves substrate cycling between glia and neurons. Since acetate is metabolized predominantly in glia,
one can speculate that cerebral consumption of acetate derived from alcohol might dysregulate cerebral metabolism and contribute towards the effects of alcohol on the brain.

In summary, the effects of alcohol could arise from three distinct general mechanisms: (1) effects of alcohol itself on neurotransmitter receptors, transports and enzymes; (2) effects of compounds formed from its reactive metabolite acetaldehyde, such as tetrahydroisoquinolines, that might bind to receptors, transporters and enzymes, and (3) interference with cerebral glucose metabolism as a result of acetate consumption.

1.5 Difference between Metabolism of [1-14C]ethanol & [2-14C]ethanol

The two carbons of acetate that enter the Krebs cycle are converted to CO2 at different rates, because their residence times in cycle intermediates (and in compounds in equilibrium with the intermediates) are different. Most of the radioactivity of [1-14C]ethanol is removed in first two turns of the Krebs cycle whereas radioactivity from [2-14C]ethanol is more slowly released over a greater number of turns. Furthermore, Most of the radioactivity in tissue is probably associated with compounds in equilibrium with the Krebs cycle intermediates, such as aspartate and glutamate, transamination products of oxaloacetate and α-ketoglutarate respectively. Over time, some radioactivity from labeled acetate will be incorporated into tissue constituents such as proteins (eg. from aspartate and glutamate) and complex lipids (from long chain fatty acids via acetylCoA). However, we have assumed that oxidation to CO2 is the major process in the brain and that we could detect differences in distribution between [1-14C]ethanol and [2-14C]ethanol.
The metabolism of [1-14C]ethanol in the Krebs cycle is shown in Figure 2. [1-14C]acetate, formed from [1-14C]ethanol, reacts with nonradioactive oxaloacetate to form citrate (2-hydroxypropane-1,2,3-tricarboxylate). Due to the achirality of citrate, 50% of the citrate molecules will have a radioactive carboxylate group bonded to 1C of propane backbone of citrate and 50% of the citrate molecules will have radioactive carboxylate group bonded to 3C of propane backbone of citrate. Citrate isomerizes to isocitrate. Isocitrate is then metabolized into α-ketoglutarate with the loss of one carbon in the form of carbon dioxide. This carbon comes from nonradioactive oxaloacetate. During conversion of α-ketoglutarate to succinyl CoA, the carboxylate group attached to the ketone group of α-ketoglutarate will be lost as CO2. It means 50% of the radioactivity will be lost at this step. Succinyl CoA looses coenzyme A to form succinate. Due to the symmetry of succinate, the remaining 50% of the radioactivity will be distributed between two carboxylate groups of succinate. Succinyl CoA will be successively converted to fumarate, malate and finally to oxaloacetate. In oxaloacetate, both the carboxylate groups will be radioactive at the end of the cycle. Thus, 50% of radioactivity will be lost in this first turn of the cycle and both the carbons of [1-14C]acetate become the part of the carbon backbone of oxaloacetate. In the second cycle, during the formation of citrate from oxaloacetate and due to the achirality of citrate molecule, the radioactive carboxylate group, attached to the ketone group of oxaloacetate, will be positioned at 2C of the propane backbone of citrate and the radioactivity of the other carboxylate group of oxaloacetate will be shared by the carboxylate groups positioned at 1C and 3C of the propane backbone of citrate. The radioactive carboxylate groups at 2C and 3C position of citrate will be removed as carbon dioxide during the formation of α-ketoglutarate and succinyl-CoA respectively. As a result, the
37.5% of the radioactivity will be lost during the second turn of the cycle. In this manner, 99% of the radioactivity from [1-\(^{14}\)C]ethanol is lost in initial four turns of the Krebs cycle.

Figure 2 Metabolism of [1-\(^{14}\)C]ethanol in the Krebs cycle. The radioactive \(^{14}\)C is indicated as \(^{14}\)C in the first turn of the Krebs cycle and as \(^{13}\)C in the second turn of the Krebs cycle. It means the radioactive carbon of oxaloacetate (\(^{13}\)C) at the end of the first turn of the cycle is shown as \(^{13}\)C in citrate during the second turn of the cycle.

In contrast, as shown in Figure 3, “condensation” of [2-\(^{14}\)C]acetylCoA with oxaloacetate results into citrate with radioactive carbons at 1\(^{st}\) (50% of citrate molecules) and 3\(^{rd}\) (50% citrate molecules) carbon positions of propane backbone due to the achirality of citrate molecule. Therefore, the radioactive carbon will not be released as CO\(_2\) during the formation of \(\alpha\)-ketoglutarate and succinyl CoA. Since succinate, formed from succinyl CoA, is a symmetrical compound, radioactivity will be distributed over all four carbons of succinate in the same
proportions and will remain distributed in oxaloacetate at the end of the first turn of the cycle.

So, in contrast to the metabolism of [1-\(^{14}\)C]ethanol, there is no loss of radioactivity from [2-\(^{14}\)C]ethanol in the first turn of the cycle. During the second turn, the label is again distributed among the carbon atoms of citrate, 1\(^{st}\) and 3\(^{rd}\) carbon of the propane backbone of citrate and carboxylate groups attached to these carbons, because of the symmetry of this molecule. Therefore, total 37.5% of the radioactivity will be lost during the formation of \(\alpha\)-ketoglutarate and succinyl-CoA. Interestingly, the remaining radioactivity will be again redistributed over all

Figure 3 Metabolism of [2-\(^{14}\)C]ethanol in the Krebs cycle. The radioactive \(^{14}\)C is indicated as \(^{\ast}\)C in the first turn of the Krebs cycle and as \(^{\ast}\)C in the second turn of the Krebs cycle. It means the radioactive carbon of oxaloacetate (\(^{\ast}\)C) at the end of the first turn of the cycle is shown as \(^{\ast}\)C in citrate during the second turn of the cycle.
four carbons of succinate. In the same way, the radioactivity will be lost in fractions in the subsequent turns of the cycle. In conclusion, most of the radioactivity from [1-\textsuperscript{14}C]ethanol is lost in first two turns of the cycle whereas slightly more than half of the radioactivity from [2-\textsuperscript{14}C]ethanol will be lost in the same time. Therefore, the radioactivity will remain for a longer time in the form of metabolites after treatment with [2-\textsuperscript{14}C]ethanol compared to [1-\textsuperscript{14}C]ethanol treatment.
2. Specific Aims

2.1 Rationale

The metabolism of ethanol in brain has been extensively studied; however, the specific sites of action are still not understood in detail. It is very important to know about the distribution and metabolism of alcohol in brain, which can explain toxicological, pharmacokinetic and pharmacodynamic aspects of alcohol. As described in Section 1.4, ethanol is initially metabolized into acetaldehyde which further oxidized into acetate. Acetate finally enters Krebs cycle and is eventually metabolized into carbon dioxide (CO₂). Since several intermediates of Krebs cycle can be converted into amino acids, carbons of ethanol can eventually be distributed into a wide variety of chemical entities. Anatomical, physiological and biochemical factors can affect this distribution. So, to examine this feature of alcohol metabolism, ethanol radiolabeled at the two different carbon positions ([1⁻¹⁴C]ethanol and [2⁻¹⁴C]ethanol) can be utilized. In addition, manipulating the metabolic steps of alcohol metabolism by enzyme blockers should also provide useful information about the natural metabolic pathways. Disulfiram, an aldehyde dehydrogenase blocker, is useful in treating alcoholics. The effects of disulfiram on radioactivity distribution patterns in brain may give insight into the pathways associated with alcohol metabolism.
2.2 Objectives

1) To compare distribution patterns of radioactivity in different brains regions using autoradiography, 30 min following intravenous administration of either [1-\(^{14}\)C]ethanol or [2-\(^{14}\)C]ethanol to mice.

2) To compare the effects of pretreatment of mice with disulfiram, acetaldehyde dehydrogenase inhibitor, on radioactivity distribution patterns in brain regions 30 min after intravenous administration of [1-\(^{14}\)C]ethanol.

3) To measure volatile and non-volatile radioactivity in brain homogenates 2 and 5 minutes after intravenous administration of a tracer dose of [1-\(^{14}\)C]ethanol to mice pretreated with disulfiram and to control mice.

4) To measure volatile and non-volatile radioactivity in brain homogenates 2 and 5 minutes after intravenous administration of a tracer dose of [1-\(^{14}\)C]ethanol to mice pretreated with disulfiram as well as pharmacological dose of ethanol and to control mice (only treated with pharmacological dose of ethanol).

2.3 Hypotheses

1) The radioactivity distribution patterns would be broadly similar but distinct in detail, because \(^{14}\)C from [2-\(^{14}\)C]ethanol remains in intermediary metabolites longer than \(^{14}\)C from [1-\(^{14}\)C]ethanol, and it was considered likely that brain regions would differ from one another in flux through metabolite pools.
2) The radioactivity distribution patterns would be different. This expectation was based on the work by a previous MS student (Shachi Bhatt). She found different patterns with [1-\(^{14}\text{C}\)]acetaldehyde than with [1-\(^{14}\text{C}\)]ethanol.

3) In control mice, there would be less volatile \(^{14}\text{C}\) after 5 minutes of [1-\(^{14}\text{C}\)]ethanol treatment than after 2 minutes, but more non-volatile \(^{14}\text{C}\) at 5 minutes than 2 minutes. Disulfiram would markedly decrease the reduction in volatile \(^{14}\text{C}\) between 2 and 5 minutes, and also reduce the increase in non-volatile \(^{14}\text{C}\) between 2 and 5 minutes.

4) In control mice, percentage changes in volatile and non-volatile radioactivity would be smaller than that for the tracer dose. Percentage changes in non volatile \(^{14}\text{C}\) due to disulfiram treatment would be larger.
3. Materials and Methods

3.1 Materials and Equipment

All the materials and the equipments used for this project are mentioned here as follows—

[1-\(^{14}\text{C}\)]ethanol; [2-\(^{14}\text{C}\)]ethanol; 200 proof ethanol (USP/NF); Solvable™, aqueous based tissue
solubilizer by Perkin Elmer Life & Analytical Sciences, Boston, MA; Tetraethylthiuram disulfide
(Disulfiram) by Sigma-Aldrich, St. Louis, MO; Tween® 20 by Sigma-Aldrich, St. Louis, MO; Ultima
Gold™ XR, high flash-point liquid scintillation counter cocktail by PerkinElmer, Waltham, MA;
NVSL Manual Advance Vibroslice, Vibrating Microtome (Vibratome) by World Precision
Instruments; Cyclone® Plus Storage Phosphor Imager by PerkinElmer; LS6500 Multi-Purpose
Scintillation Counter by Beckman Coulter; Super Resolution (SR) Storage Phosphor Screen by
PerkinElmer.

3.2 Animals

Male Swiss Webster mice (Charles River laboratories, Cambridge, MA) were used for all
in vivo studies. Mice were maintained at the animal facility of DLAM (Division of Laboratory
Animal Medicine) on 12 hour alternative light and dark period with access to food and water ad
libitum. Mice were treated in compliance with NIH guidelines for the use of laboratory animals
and a protocol approved by IACUC (Institutional Animal Care and Use Committee).
3.3 Method

3.3.1 Mouse brain sectioning by using vibratome

The mouse was injected 40 μCi of [1-¹⁴C]ethanol intravenously. Before injecting, 20 μl of [1-¹⁴C]ethanol was pipetted out into liquid scintillation vial and scintillation fluid was added to it. The radioactivity of this sample solution was counted in liquid scintillation counter and CPM (counts per minute) data was noted. After 30 minutes of injection, the mouse was euthanized by cervical dislocation method and the brain was taken out. The cerebellum and brain stem were cut off and the forebrain was affixed in upright position on the stage of vibratome for performing coronal sectioning. The cerebellum was weighed and solubilized by using tissue solubilizer. The vibratome stage was kept in tissue bath and the later was filled with 0.9% saline until the forebrain completely submerged. The vibratome frequency was adjusted maximum because high frequency with slow cutting speed gives high quality sections of soft tissue (Prusky and McKenna 1999). The cutting head with razor blade was lowered till it reached an apex of the forebrain and the coronal sections of 300 μm were taken by cutting head attached with razor blade. The brain sections were collected on glass slides and kept overnight on slightly warm plate for drying. The following day, the dried sections were exposed to the storage phosphor screen along with standard for 24 hours. The scintillation fluid was added to the dissolved cerebellum sample and radioactivity was counted by liquid scintillation counter. After 24 hours, the autoradiograph of the exposed plate was obtained at 600 DPI (dots per inches) resolution by storage phosphor imager.
The same procedure mentioned here was followed for mice injected with [2-14C]ethanol. 10 µCi of [2-14C]ethanol was used.

**3.3.2 Pharmacological study of disulfiram by using vibratome brain sectioning method**

2% disulfiram suspension was prepared in 0.9% saline using Tween 20 as a suspending agent. The mixture was sonicated briefly to get an even suspension of disulfiram. A total of 9 mice were included in this study, of which, six were injected 100 mg/kg of disulfiram and three were injected 200 mg/kg of disulfiram. The disulfiram suspension was injected intraperitoneally immediately after sonication. Mice behaviour was observed for at least half an hour after disulfiram injection. After about 10 minutes of injection, they looked uncomfortable. Most conspicuous behaviour observed was that they stopped moving and grooming and became quiet. They were quite shivering and they partially closed their eyes. After one hour of disulfiram injection, mice were injected [1-14C]ethanol intravenously. Before injecting [1-14C]ethanol, 20 µl of it was pipetted out into liquid scintillation vial and scintillation fluid was added to it. The radioactivity of this tracer sample solution was counted in liquid scintillation counter and CPM data was noted. For 200 mg/kg disulfiram dose, mouse was initially treated with 100 mg/kg disulfiram and the remaining 100 mg/kg disulfiram was injected after 30 minutes. After 30 minutes of [1-14C]ethanol treatment, mice were euthanized; cerebellums were weighed, brains were taken out and sliced coronally with 300 µm slice thickness as described earlier.

**3.3.3 Mouse brain microdissection study using trace dose of ethanol**

This study consisted of 20 mice (n=20), of which, half of the mice were treated with only [1-14C]ethanol and the remaining half were treated with 100 mg/kg intraperitoneal dose of
disulfiram before treating them with [1-\(^{14}\text{C}\)] ethanol. Each mouse was injected with a tracer
dose of [1-\(^{14}\text{C}\)] ethanol intravenously. The duration between disulfiram and [1-\(^{14}\text{C}\)] ethanol
treatment was 30 minutes. The radioactivity of 20 µl of [1-\(^{14}\text{C}\)] ethanol was measured by liquid
scintillation counter before injecting into mice. The total radioactivity and radioactivity in the
nonvolatile forms were measured in hippocampus, striatum, cerebellum and total brain after 2
minutes (n=5) and 5 minutes (n=5) of [1-\(^{14}\text{C}\)] ethanol injection.

During microdissection of the brain, the precautions were taken to minimize the loss of
volatile radioactivity by keeping the plate, on which the brain microdissection was performed,
ice cold. After microdissecting the brain, each region was weighed and 2 ml of 0.9% saline was
added to the vial. Each brain region was homogenized and 0.2 ml of the homogenate was taken
out into two scintillation vials. The liquid scintillation fluid was added to one of the vials and the
total amount of radioactivity was measured. To measure the nonvolatile forms of radioactivity,
0.2 ml of alcohol was added into the other vial and kept it in the fume hood until it became
completely dry. The dried samples were dissolved in liquid scintillation fluid and the
radioactivity was measured.

3.3.4 Mouse brain microdissection study using pharmacological dose of ethanol

This study also consisted of 20 mice (n=20), of which, half of the mice were treated with
the pharmacological dose (1 g/kg) of nonradioactive 200 proof ethanol followed by the tracer
dose of [1-\(^{14}\text{C}\)] ethanol and the remaining half were treated with 100 mg/kg intraperitoneal
dose of disulfiram followed by 1 g/kg dose of nonradioactive ethanol before treating them with
the tracer dose of [1-\(^{14}\text{C}\)] ethanol. The pharmacological dose and the trace dose of ethanol were
given to mice by intraperitoneal and intravenous route of administration respectively. The
duration between nonradioactive ethanol and disulfiram treatments was 20 minute; whereas the duration between ethanol and [1-14C]ethanol treatments was 10 minute. The radioactivity of 20 µl of [1-14C]ethanol was measured by liquid scintillation counter before injecting into mice. The behavior of mice was observed after injecting disulfiram and 1 g/kg dose of ethanol. Within 5-10 minute of disulfiram treatment, the activity of mice subsided. Their normal behavior such as grooming and roaming became diminished. Intermittently, they sprawled at the corner of the cage without much movement. They looked quite uncomfortable. After treating disulfiram pretreated mice with 1 g/kg of ethanol, most of them became very calm without any movement. After 2 minutes (n=5) and 5 minutes (n=5) of [1-14C]ethanol injection, the mice were euthanized. The total radioactivity and radioactivity in the nonvolatile forms were measured in hippocampus, striatum, cerebellum and total brain by performing microdissection of the brain as described in Section 3.3.3. The same precautions were taken to limit the loss of volatile radioactivity.
4. Results and Discussion

4.1 Comparison of distribution of radioactivity in mouse brain after injecting [1-\(^{14}\)C]ethanol and [2-\(^{14}\)C]ethanol

The distribution of radiotracer (\(^{14}\)C) in mice brain slices, after injecting mice with [1-\(^{14}\)C]ethanol and [2-\(^{14}\)C]ethanol, has been shown in Figure 4 and Figure 5 respectively.

![Figure 4](image1.png)  ![Figure 5](image2.png)

**Figure 4** Autoradiograph of brain slices of mice injected with [1-\(^{14}\)C]ethanol. Results from three mice have been shown.
The amount of radioactivity in a particular brain region was analyzed by OptiQuant™ software. The brain regions considered in this study were cortex, hippocampus, striatum, thalamus, hypothalamus and diagonal band. For each mouse, portion of each concerned brain region was marked in different brain sections and radioactivity data were obtained in DLU/mm² (DLU – Digital Light Unit). The background DLU/mm² value was deducted from DLU/mm² values.
for all the regions and their averages were calculated. The average DLU/mm^2 radioactivity of specific region was considered for the further analysis. Amount of radioactivity accumulated in different brain regions was calculated in terms of percent injected dose per milligram of the tissue. For that, mean DLU/mm^2 values were first converted to CPM/mm^2. Relationship between DLU and CPM was studied by scrapping some brain sections out, dissolving them in tissue solubilizer and measuring the radioactivity counts by liquid scintillation counter. The images of the same whole sections, those had been scrapped out, were studied to get the gross DLU data. The ratios of gross DLU to CPM were obtained and the average ratio was used to convert data from DLU/mm^2 to CPM/mm^2. By considering 300 µm thickness of brain sections, radioactivity per mg of tissue (i.e., CPM/mg) was calculated. Finally, CPM/mg values were converted to % injected dose per mg of brain region by dividing these values by the total radioactivity of the injected ethanol. The % injected dose per mg of tissue after treating mice with [1-^14C]ethanol and [2-^14C]ethanol were compared as shown in Figure 6. The radioactivity in all of the brain regions were found significantly higher after injecting mice with [2-^14C]ethanol compared to mice treated with [1-^14C]ethanol (p-value <0.01). This result proves that radioactivity in the form of [1-^14C]ethanol is lost faster than in the form of [2-^14C] ethanol. There was also difference in radiotracer distribution pattern between [1-^14C]ethanol treated and [2-^14C]ethanol treated mice. For [1-^14C]ethanol treated mice, maximum radioactivity was measured in hypothalamus followed by hippocampus and thalamus. Whereas, for [2-^14C]ethanol treated mice, maximum radioactivity was found in thalamus followed by hippocampus and hypothalamus.
Figure 6 Comparison of radioactivity distribution in different brain regions after injecting $[1^{-14}C]$ethanol and $[2^{-14}C]$ethanol. Radioactivity is shown in terms of % injected dose (% ID) per mg of the tissue.

After 30 minutes of radiolabeled ethanol treatment, the lowest radioactivity was observed consistently in striatum. The data was standardized with respect to the radioactivity in the striatum to account for the variability in radioactivity accumulation in some regions and to study the relative radioactivity distribution. For that, the radioactivity data of all the regions were first normalized with that of standard and the standardized radioactivity was finally normalized with the average standardized radioactivity of striatum. These radioactivity ratios with striatum for all mice injected with $[1^{-14}C]$ethanol are shown in Table 1.
Brain Slice Regions | Average DLU/mm² of particular brain region (after normalized with standard)/ Average DLU/mm² of striatum (after normalized with standard)
---|---
| Mouse 1 | Mouse 2 | Mouse 3 | Average | σ |
**Striatum** | 1.00 | 1.00 | 1.00 | 1.00 | 0.00 |
**Cortex** | 1.40 | 1.46 | 1.65 | 1.50 | 0.13 |
**Hippocampus** | 1.73 | 1.67 | 1.98 | 1.79 | 0.16 |
**Thalamus** | 1.74 | 1.56 | 1.61 | 1.64 | 0.09 |
**Hypothalamus** | 2.01 | 1.54 | 1.92 | 1.82 | 0.25 |
**Diagonal Band** | 1.51 | 1.34 | 1.73 | 1.53 | 0.19 |

Table 1 Normalized radioactivity in specific brain region after injecting mice with [1-¹⁴C]ethanol. The radioactivity data of particular region has been standardized with that of striatum. (σ = standard deviation)

The brain slices, obtained from mice injected with [2-¹⁴C]ethanol, were also analyzed in the same way. The ratios of average standardized radioactivity in particular region to that of striatum for all mice treated with [2-¹⁴C]ethanol has been shown in Table 2.

| Brain Slice Regions | Average DLU/mm² of particular brain region (after normalized with standard)/ Average DLU/mm² of striatum (after normalized with standard)
---|---
| Mouse 1 | Mouse 2 | Mouse 3 | Mouse 4 | Average | σ |
**Striatum** | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.00 |
**Cortex** | 1.44 | 1.35 | 1.26 | 1.33 | 1.35 | 0.07 |
**Hippocampus** | 1.64 | 1.44 | 1.33 | 1.29 | 1.43 | 0.16 |
**Thalamus** | 1.60 | 1.61 | 1.53 | 1.59 | 1.58 | 0.04 |
**Hypothalamus** | 1.43 | 1.41 | 1.35 | 1.43 | 1.41 | 0.04 |
**Diagonal Band** | 1.40 | 1.34 | 1.43 | 1.29 | 1.37 | 0.06 |

Table 2 Normalized radioactivity in specific brain region after injecting mice with [2-¹⁴C]ethanol. The radioactivity data of particular region has been standardized with that of striatum. (σ = standard deviation)

The normalized data for [1-¹⁴C]ethanol and [2-¹⁴C]ethanol is plotted in Figure 7. It is observed that radioactivity is significantly higher in all the regions compared to striatum after
injecting [1-^{14}C]ethanol and [2-^{14}C]ethanol with low variability in data. Comparative relative distribution of radiotracer was found significantly higher in hippocampus and hypothalamus in mice treated with [1-^{14}C]ethanol compared to [2-^{14}C]ethanol treated mice.

![Comparison of Distribution of Radiotracer after Treatment with [1-^{14}C]ethanol & [2-^{14}C]ethanol](image)

**Figure 7** Comparison of normalized radioactivity distribution in different brain regions after injecting [1-^{14}C]ethanol and [2-^{14}C]ethanol.

These results clearly show the difference between the metabolism of [1-^{14}C]ethanol and [2-^{14}C]ethanol. Since ^{14}C distribution patterns in brain differ between [1-^{14}C]ethanol and [2-^{14}C]ethanol treated mice, the flux of ^{14}C, from either [1-^{14}C]ethanol or [2-^{14}C]ethanol, through the Krebs cycle might be different significantly. The biochemical activities and requirements of different brain regions differ from each other. For example, it is possible that some of the regions require higher amount of glutamate, an excitatory neurotransmitter and a
transamination product of $\alpha$-ketoglutarate, and therefore Krebs cycle activities may differ between such regions and may show different radioactivity distribution patterns.

4.2 Validating the method for analyzing the distribution and concentration of radiotracer in mice brain slices

All the brain sections obtained after injecting [1-$^{14}$C]ethanol and [2-$^{14}$C]ethanol were exposed additionally for 7 days and for 51 days to study the changes in the radioactivity with respect to time. During the entire exposure time, the sections were kept at -80 °C to prevent the decay of radioactivity. After the exposure time, autoradiography was conducted. The comparison of images obtained at different time exposure is shown in Figure 8 for [1-$^{14}$C]ethanol and in Figure 9 for [2-$^{14}$C]ethanol.

![Figure 8 Autoradiograph of mouse brain slices after injecting [1-$^{14}$C]Ethanol. Two mice brain slices are shown as Sample 1 and Sample 2. Panel (A, D, G, J) - Slices exposed for 1 day. Panel (B, E, H, K) - Slices exposed for 7 days. Panel (C, F, I, L) - Slices exposed for 51 days. Panel I and L indicate overexposure of the phosphor imager plate.](image-url)
Figure 9 Autoradiograph of mouse brain slices after injecting [2-\textsuperscript{14}C]Ethanol. Two mice brain slices are shown as Sample 1 and Sample 2. Panel (A, D, G, J) - Slices exposed for 1 day. Panel (B, E, H, K) - Slices exposed for 7 days. Panel (C, F, I, L) - Slices exposed for 51 days. Panel I and L indicate overexposure of the phosphor imager plate.

To verify the results obtained from autoradiography, the radioactivity in entire brain section was calculated by liquid scintillation counter. The two brain sections of mice treated with [1-\textsuperscript{14}C]ethanol and two from mice treated with [2-\textsuperscript{14}C]ethanol were scrapped off carefully with razor blade and collected in separate liquid scintillation vial. The scraps were dissolved by tissue solubilizer. The scintillation fluid was added to the vials and the radioactivity was counted in CPM. The images of the sections, which had been scrapped off, were analyzed by OptiQuant™ software. The whole brain section region was marked and radioactivity was obtained in gross DLU. The radioactivity data in both CPM and gross DLU for these four sections
is shown in Table 3. The ratios of gross DLU to CPM were measured and their average was considered.

<table>
<thead>
<tr>
<th>Brain Slice</th>
<th>CPM</th>
<th>Average Gross DLU After Different Exposure Time</th>
<th>(Gross DLU)/CPM</th>
<th>Average [(Gross DLU)/CPM]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 Day</td>
<td>7 Days</td>
<td>51 Days</td>
</tr>
<tr>
<td>Mouse 1</td>
<td></td>
<td>[1-\textsuperscript{14}C] Ethanol</td>
<td>10527</td>
<td>52193600</td>
</tr>
<tr>
<td>Mouse 2</td>
<td></td>
<td>[2-\textsuperscript{14}C] Ethanol</td>
<td>12428</td>
<td>63540520</td>
</tr>
<tr>
<td>Mouse 1</td>
<td></td>
<td>[2-\textsuperscript{14}C] Ethanol</td>
<td>7824</td>
<td>37648634</td>
</tr>
<tr>
<td>Mouse 2</td>
<td></td>
<td>[2-\textsuperscript{14}C] Ethanol</td>
<td>4513</td>
<td>25970552</td>
</tr>
</tbody>
</table>

Table 3 Radioactivity data of four brain slices (two slices from [1-\textsuperscript{14}C]ethanol injected mice and two from [2-\textsuperscript{14}C]ethanol injected mice) obtained from liquid scintillation counter and from autoradiography at different exposure time period.

To study the changes in radioactivity pattern over various time points, the average ratio of gross DLU to CPM was plotted against the exposure time. The plot is shown in Figure 10. It is observed from the graph that radioactivity increases linearly with respect to the exposure time. This result substantiates the method, used for comparison study of radiotracer distribution and concentration after injecting [1-\textsuperscript{14}C]ethanol and [2-\textsuperscript{14}C]ethanol, of counting the amount of radioactivity in brain regions as a percentage of injected radioactive doses of alcohol per mg of tissue.
Figure 10 Changes in the radioactivity of brain sections of mice, injected either [1-\textsuperscript{14}C]ethanol or [2-\textsuperscript{14}C]ethanol, after different exposure time period.

4.3 Effects of pretreatment of disulfiram on \textsuperscript{14}C distribution pattern obtained after injecting [1-\textsuperscript{14}C]ethanol

The distribution pattern of radiotracer after injecting [1-\textsuperscript{14}C]ethanol in mice, pretreated with 100 mg/kg disulfiram and 200 mg/kg disulfiram, is shown in Figure 11 and Figure 12 respectively.
Figure 11 Autoradiograph of brain slices of mice, pretreated with 100 mg/kg of disulfiram, injected with [1-\textsuperscript{14}C]ethanol. Results from three mice have been shown.

Figure 12 Autoradiograph of brain slices of mice, pretreated with 200 mg/kg of disulfiram, injected with [1-\textsuperscript{14}C]ethanol. Results from three mice have been shown.

The amount of radioactivity in a specific brain region was analyzed by OptiQuant™ software as described earlier. The brain regions considered in this study were cortex, hippocampus, striatum, thalamus, hypothalamus and diagonal band. Amount of radioactivity in brain regions were calculated in terms of percentage of injected dose per milligram of the brain region. For this analysis, mean DLU/mm\textsuperscript{2} data for regions obtained from Autoradiograph study were first converted CPM/mm\textsuperscript{2} by considering the relationship between DLU/mm\textsuperscript{2} and CPM.
obtained from liquid scintillation counting of the scrapped brain regions. By considering 300 µm thickness of brain sections, radioactivity per mg of tissue was calculated. Finally, CPM values were converted to % injected dose per mg of brain region by dividing the data by radioactivity of the injected ethanol. The % injected dose per mg of the tissue after treating mice with 100 mg/kg disulfiram followed by [1-14C]ethanol and mice with 200 mg/kg disulfiram followed by [1-14C]ethanol is shown in Figure 13. The data for the mice treated with [1-14C]ethanol is also shown in Figure 13. A significantly higher amount of radioactivity was found in hippocampus of the mice treated with 100 mg/kg of disulfiram followed by [1-14C]ethanol compared to the mice treated only with [1-14C]ethanol. However, pretreatment of mice with 200 mg/kg of disulfiram, before injecting [1-14C]ethanol, did not alter the results significantly that obtained from only [1-14C]ethanol treated mice. There was no difference in the concentration of radioactivity in any of the brain regions of mice treated with either 100 mg/kg or 200 mg/kg dose of disulfiram followed by [1-14C]ethanol.
Figure 13 Comparison of radioactivity distribution in different brain regions after injecting young and aged mice, pretreated with 100 mg/kg disulfiram, with [1-\textsuperscript{14}C]ethanol and young mice, pretreated with 200 mg/kg disulfiram, with [1-\textsuperscript{14}C]ethanol. Radioactivity is shown in terms of % injected dose (% ID) per mg of the tissue. (DS - Disulfiram)

After 30 minutes of radiolabeled ethanol treatment, the lowest radioactivity was consistently observed in striatum. To account for the variability of radioactivity data in different brain regions, the data was standardized to the radioactivity in the striatum. For that, the radioactivity data of all the regions were first normalized with that of standard and the standardized radioactivity was finally normalized with the average standardized radioactivity of striatum. These radioactivity ratios with striatum for all mice treated with 100 mg/kg disulfiram followed by [1-\textsuperscript{14}C]ethanol are shown in Table 4.
<table>
<thead>
<tr>
<th>Brain Slice Regions</th>
<th>Average DLU/mm² of particular brain region (after normalized with standard) / Average DLU/mm² of striatum (after normalized with standard)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mouse 1</td>
</tr>
<tr>
<td>Striatum</td>
<td>1.00</td>
</tr>
<tr>
<td>Cortex</td>
<td>1.44</td>
</tr>
<tr>
<td>Hippocampus</td>
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</tr>
<tr>
<td>Thalamus</td>
<td>1.67</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>1.55</td>
</tr>
<tr>
<td>Diagonal Band</td>
<td>1.46</td>
</tr>
</tbody>
</table>

Table 4 Normalized radioactivity in specific brain region after injecting mice, pretreated with 100 mg/kg of disulfiram, with [1-14C]ethanol. The radioactivity data of particular region has been standardized with that of striatum. (σ = standard deviation)

The brain slices were analyzed in the same way for mice treated with 200 mg/kg of disulfiram. The ratios of average standardized radioactivity in particular region to that of striatum for all mice treated with 200mg/kg of disulfiram followed by [1-14C]ethanol has been shown in Table 5.

<table>
<thead>
<tr>
<th>Brain Slice Regions</th>
<th>Average DLU/mm² of particular brain region (after normalized with standard) / Average DLU/mm³ of striatum (after normalized with standard)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mouse 1</td>
</tr>
<tr>
<td>Striatum</td>
<td>1.00</td>
</tr>
<tr>
<td>Cortex</td>
<td>1.60</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>1.75</td>
</tr>
<tr>
<td>Thalamus</td>
<td>1.44</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>1.92</td>
</tr>
<tr>
<td>Diagonal Band</td>
<td>1.50</td>
</tr>
</tbody>
</table>

Table 5 Normalized radioactivity in specific brain region after injecting mice, pretreated with 200 mg/kg of disulfiram, with [1-14C]ethanol. The radioactivity data of particular region has been standardized with that of striatum. (σ = standard deviation)
The normalized data for mice treated with 100 mg/kg disulfiram followed by [1-\(^{14}\)C]ethanol and 200 mg/kg disulfiram followed by [1-\(^{14}\)C]ethanol is plotted in Figure 14. The data for the mice treated with [1-\(^{14}\)C]ethanol and the mice treated with [1-\(^{14}\)C]acetaldehyde is also compared. It is observed that radioactivity is significantly higher in all of the regions compared to striatum after injecting [1-\(^{14}\)C]ethanol with low variability in data. Comparative relative distribution of radiotracer was found significantly higher in cortex, thalamus and hypothalamus of mice treated with [1-\(^{14}\)C]acetaldehyde compared to mice treated with [1-\(^{14}\)C]ethanol and mice treated with different doses of disulfiram followed by [1-\(^{14}\)C]ethanol. Surprisingly, there was no significant difference in results between the mice treated with either higher or lower doses of disulfiram followed by [1-\(^{14}\)C]ethanol and the mice treated with only [1-\(^{14}\)C]ethanol. In addition, the mice treated with 200 mg/kg of disulfiram followed by [1-\(^{14}\)C]ethanol did not seem to have any significant difference in results compared to the mice treated with 100 mg/kg of disulfiram followed by [1-\(^{14}\)C]ethanol. The results of both absolute (Figure 13) as well as comparative (Figure 14) radioactivity measurements are very unexpected. Disulfiram did not seem to block aldehyde dehydrogenase.

Earlier studies found different \(^{14}\)C distribution patterns between mice treated with [1-\(^{14}\)C]ethanol and [1-\(^{14}\)C]acetaldehyde. It was speculated that the distinct distribution patterns from [1-\(^{14}\)C]acetaldehyde may be due to acetaldehyde adducts. However, disulfiram pretreated mice did not show different \(^{14}\)C distribution patterns, following treatment with [1-\(^{14}\)C]ethanol, compared to control mice. Disulfiram treated mice behaved in different manner compared to control mice and this indicates effectiveness of disulfiram. However, is might be possible that disulfiram blocked acetaldehyde dehydrogenase peripherally effectively but failed to do so
centrally. It is speculative that acetaldehyde concentration in brain tissues was not high enough to form acetaldehyde adducts which may give patterns similar to that obtained from [1-\(^{14}\)C]acetaldehyde treatment. Most of the acetaldehyde might have been metabolized to acetate and the other nonvolatile metabolites to give distribution patterns similar to that obtained from [1-\(^{14}\)C]ethanol treated mice. Whereas, in [1-\(^{14}\)C]acetaldehyde treated mice, significant amount of acetaldehyde, despite being readily metabolized to acetate peripherally, might be present in brain to be metabolized by different pathways. However, all of these studies used tracer dose of radiolabeled acetaldehyde and alcohol. So it would be important to study the effects of pharmacological dose.

![Comparison of Distribution of Radiotracer](image)

**Figure 14** Comparison of normalized radioactivity distribution in different brain regions after injecting [1-\(^{14}\)C]ethanol in mice pretreated with either 100 mg/kg disulfiram or 200 mg/kg disulfiram.
4.4 Mice brain microdissection studies

4.4.1 Effects of disulfiram on the amount of radioactivity in mice brain homogenates after treating mice with tracer dose of [1-\textsuperscript{14}C]ethanol

Total as well as nonvolatile radioactivities in the whole brain homogenate after 2 and 5 minutes of [1-\textsuperscript{14}C]ethanol treatment and the similar data from the mice pretreated with disulfiram have been compared in Figure 15. The average total and nonvolatile radioactivities in brain homogenate from mice treated only with tracer dose of [1-\textsuperscript{14}C]ethanol at 2 minutes were 0.002961 and 0.000206 \% IR (injected radioactivity) per mg of the brain respectively and at 5 minutes were 0.0022 and 0.000632 \% IR per mg of the brain respectively. From this data, volatile radioactivity at 2 and 5 minutes were calculated as 0.002755 and 0.001569 \% IR per mg of the brain respectively. This shows the decrease of 25.68\% and 43.05\% in total and volatile radioactivities respectively and the increase of 206.56\% in nonvolatile radioactivity between 2 and 5 minutes of [1-\textsuperscript{14}C]ethanol treatment. The average total, nonvolatile and volatile radioactivities in brain homogenate from mice treated with disulfiram followed by tracer dose
Figure 15 Comparison of total (Upper Panel) and nonvolatile (Lower Panel) radioactivity in whole brain after 2 and 5 minutes of [1\(^{14}\)C]ethanol treatment. [DS – Disulfiram; (+DS) – mice treated with disulfiram, (-DS) – mice not treated with disulfiram]

of [1\(^{14}\)C]ethanol at 2 minutes were 0.003075, 0.000223, and 0.002853 % IR per mg of the brain respectively and at 5 minutes were 0.002426, 0.000488 and 0.001938 % IR per mg of the brain respectively. It means total and volatile radioactivities decreased by 21.09% and 32.05% respectively and nonvolatile radioactivity increased by 118.95% during this time interval. It is concluded from these results that disulfiram reduced the percentage decrease in volatile and
percentage increase in nonvolatile radioactivity in brain between 2 and 5 minutes by 25.56% and 42.42% respectively.

Total amount of radioactivity in hippocampus, striatum, cerebellum and whole brain after 2 and 5 minutes of intravenous injection of [1-^{14}C]ethanol have been shown in Figure 16 and Figure 17 respectively. The similar data for mice treated with 100 mg/kg of disulfiram followed by tracer dose of [1-^{14}C]ethanol have also been mentioned in these figures. Disulfiram pretreatment increased total radioactivity in hippocampus significantly ($p<0.05$). However, effects of disulfiram was not evident in striatum, cerebellum as well as in whole brain at 2 minutes. Total amount of radioactivity was found significantly higher in cerebellum and in whole brain after 5 minutes of [1-^{14}C]ethanol treatment; whereas disulfiram did not increase total radioactivity significantly in hippocampus and striatum.

![Figure 16 Total radioactivity in different brain regions and in whole brain after 2 minutes of [1-^{14}C]ethanol treatment.](image-url)
Radioactivities in the form of nonvolatile metabolites in hippocampus, striatum, cerebellum and whole brain after 2 and 5 minutes of [1-\textsuperscript{14}C]ethanol treatment, along with corresponding results from the mice pretreated with 100 mg/kg of disulfiram followed by [1-\textsuperscript{14}C]ethanol, have been shown in Figure 18 and Figure 19 respectively. There was no significant difference found in nonvolatile radioactivity in any of the concerned brain regions and in the whole brain between these two groups of mice. Duration of [1-\textsuperscript{14}C]ethanol treatment did not affect these findings. The nonvolatile radioactivity, after 2 minutes of [1-\textsuperscript{14}C]ethanol treatment, was found significantly higher in hippocampus compared to that in the whole brain (p<0.05); both in disulfiram treated and not treated mice groups. Whereas, after 5 minutes of [1-\textsuperscript{14}C]ethanol injection without disulfiram pretreatment, nonvolatile radioactivity was significantly higher in hippocampus compared to that in striatum and cerebellum (p<0.05), but not in the whole brain. In group of mice pretreated with disulfiram, hippocampus had significantly higher amount of nonvolatile radioactivity than in whole brain (p<0.05) after 5
minutes of [1-\textsuperscript{14}C]ethanol injection. The brain slicing studies also found higher amount of radioactivity in hippocampus. These results indicate that alcohol may significantly affect the functions of hippocampus by disrupting the metabolic homeostasis.

\textbf{Figure 18} Radioactivity in the forms of nonvolatile metabolites in different brain regions and in whole brain after 2 minutes of [1-\textsuperscript{14}C]ethanol treatment.

\textbf{Figure 19} Radioactivity in the forms of nonvolatile metabolites in different brain regions and in whole brain after 5 minutes of [1-\textsuperscript{14}C]ethanol treatment.
4.4.2 Effects of disulfiram on the amount of radioactivity in mice brain homogenates after treating mice with pharmacological dose of ethanol followed by tracer dose of [1-^{14}\text{C}]ethanol

As described in Section 4.4.2, disulfiram did not increase the level of total radioactivity in all the brain regions studied after 2 or 5 minutes of [1-^{14}\text{C}]ethanol treatment. It increased total radioactivity level in the whole brain after 5 minutes of [1-^{14}\text{C}]ethanol injection but not after 2 minutes of [1-^{14}\text{C}]ethanol; whereas, in hippocampus, it increased the total radioactivity after 2 minutes of [1-^{14}\text{C}]ethanol treatment but not after 5 minutes of [1-^{14}\text{C}]ethanol treatment. Moreover, it also did not significantly decrease the nonvolatile radioactivity in brain regions. The reason behind these results could be the tracer dose of [1-^{14}\text{C}]ethanol. Though acetaldehyde dehydrogenase was blocked by disulfiram, [1-^{14}\text{C}]ethanol concentration could be too little to have any effect on its metabolism. Therefore, it was decided to inject mice with therapeutic dose of nonradioactive ethanol before injecting with tracer dose of [1-^{14}\text{C}]ethanol.

Total as well as nonvolatile radioactivities in the whole brain homogenate, at different time periods, from control mice treated with 1 g/kg of ethanol followed by [1-^{14}\text{C}]ethanol and the similar data from the mice pretreated with disulfiram followed by 1g/kg of ethanol and [1-^{14}\text{C}]ethanol have been compared in Figure 20. The average total, nonvolatile and volatile radioactivities in brain homogenate from control mice at 2 minutes were 0.003545, 0.000057, and 0.003488 % IR per mg of the brain respectively and at 5 minutes were 0.003123, 0.000122 and 0.003001 % IR per mg of the brain respectively. This shows the decrease of 11.91% and 13.97% in total and volatile radioactivities respectively and the increase of 113.51% in nonvolatile radioactivity between 2 and 5 minutes of [1-^{14}\text{C}]ethanol treatment. The average
total, nonvolatile and volatile radioactivities in brain homogenate from disulfiram pretreated mice at 2 minutes were 0.003710, 0.000072, and 0.003639 % IR per mg of the brain respectively and at 5 minutes were 0.003241, 0.000098 and 0.003143 % IR per mg of the brain respectively. Thus, total and volatile radioactivities decreased by 12.64% and 13.62% respectively whereas nonvolatile radioactivity increased by 36.94% during this time interval. In conclusion, these results indicate that disulfiram only marginally (2.48%) reduced the percentage decrease in volatile radioactivity compared to significant reduction in mice, not treated with pharmacological dose of ethanol (Refer Section 4.4.1). On the other hand, disulfiram sharply (67.46%) reduced the percentage increase in nonvolatile radioactivity in brain between 2 and 5 minutes.
Figure 20 Comparison of total (Upper Panel) and nonvolatile (Lower Panel) radioactivity in whole brain after 2 and 5 minutes of [1-14C]ethanol treatment. [DS – Disulfiram; (+DS) – mice treated with disulfiram, (-DS) – mice not treated with disulfiram]

In Figure 21 and Figure 22, total amount of radioactivity in the brain regions have been shown for 2 and 5 minutes of [1-14C]ethanol treatment respectively. The similar data for the mice treated with disulfiram is also plotted. Surprisingly, even after increasing the concentration of substrate of aldehyde dehydrogenase, acetaldehyde, by treating mice with
pharmacological dose of ethanol, disulfiram did not increase the total as well as volatile forms of radioactivity in any of the brain regions after 2 or 5 minutes of [1-\(^{14}\)C]ethanol treatment.

**Figure 21** Total radioactivity in different brain regions and in whole brain after 2 minutes of [1-\(^{14}\)C]ethanol treatment.

**Figure 22** Total radioactivity in different brain regions and in whole brain after 5 minutes of [1-\(^{14}\)C]ethanol treatment.
The amount of nonvolatile radioactivity after 2 or 5 minutes of $[1^{-14}C]$ethanol treatment, along with the similar data from mice treated with disulfiram, has been shown in Figure 23 and Figure 24 respectively. After 2 minutes of $[1^{-14}C]$ethanol dose, disulfiram did not affect the level of nonvolatile radioactivity. However, after 5 minutes, it significantly decreased the nonvolatile radioactivity in striatum and in the whole brain. The absence of prominent effects of disulfiram may be due to insufficient inhibition of aldehyde dehydrogenase.

![Nonvolatile Radioactivity (After 2 min)](image)

**Figure 23** Radioactivity in the forms of nonvolatile metabolites in different brain regions and in whole brain after 2 minutes of $[1^{-14}C]$ethanol treatment.
As with the brain slicing studies, the microdissection studies also showed unexpected results. Disulfiram did not seem to affect acetaldehyde dehydrogenase function prominently and consistently despite producing behavioral effects in mice. Although disulfiram is lipophilic drug, these results raise the questions about its central effects. Disulfiram is also believed to cause its effects through its metabolites. It is possible that its metabolites do not attain sufficient CNS concentration. On the other hand, since disulfiram also blocks activities of other enzymes, it is possible that behavioral effects of disulfiram might have resulted from the blockage of dopamine-β-hydroxylase, which blocks the formation of norepinephrine and thus subsides excitation. The future studies are required to measure the concentration of enzymes, disulfiram and its metabolites in blood as well as in tissue, both peripherally and centrally, to explain this conundrum.
5. Conclusion

The $^{14}$C distribution patterns across mice brain regions were different for $[1-^{14}\text{C}]$ethanol and $[2-^{14}\text{C}]$ethanol. Quantitative autoradiographic analysis showed that hypothalamus and hippocampus contained the highest fractions of total injected radioactivity from $[1-^{14}\text{C}]$ethanol; whereas maximum radioactivity from $[2-^{14}\text{C}]$ethanol was found in thalamus. This shows that there are differences in Krebs cycle activity occurring in different brain regions. It was previously shown that radioactivity distribution patterns of $[1-^{14}\text{C}]$acetaldehyde are different from that of $[1-^{14}\text{C}]$ethanol (S. Bhatt, MS thesis 2009). However, distribution patterns did not change in brain slices when mice were treated with disulfiram, either 100 mg/kg or 200 mg/kg, followed by $[1-^{14}\text{C}]$ethanol. The hippocampus of mice pretreated with 100 mg/kg of disulfiram had significantly higher amount of total injected radioactivity compared to control mice, but the other regions did not show markedly different results. Comparative radioactivity concentration was found a little higher in disulfiram pretreated mice than in $[1-^{14}\text{C}]$ethanol treated mice. This data indicates that although disulfiram induced behavioral changes, it does not appear to be very effective in inhibiting aldehyde dehydrogenase in the brain in vivo. This result was later substantiated by measuring the total, volatile and nonvolatile radioactivity in mouse brain homogenate. The total radioactivity increased significantly, after 5 minutes of $[1-^{14}\text{C}]$ethanol treatment, in the whole brain homogenate of mice treated with disulfiram followed by $[1-^{14}\text{C}]$ethanol compared to mice treated with $[1-^{14}\text{C}]$ethanol alone, however, this was not true after 2 minutes of treatment. It is possible that disulfiram, or its active metabolite S-methyl-$N,N$-diethyldithiocarbamate (MeDDC), does not achieve a sufficiently high concentration in brain to inhibit aldehyde dehydrogenase.
6. Future directions

To address the question that whether disulfiram blocks aldehyde dehydrogenase effectively or not, it would be interesting to measure the concentration of aldehyde dehydrogenase in homogenates of different brain regions. Radiochemical analysis of the brain homogenate might clarify the differences in radioactivity distribution in different brain regions obtained from treatment with \([1^{14}\text{C}]\)ethanol and \([1^{14}\text{C}]\)acetaldehyde. It might be also useful to study the pharmacokinetics of disulfiram in detail, because S-Methyl-\(N,N\)-diethyldithiocarbamate (MeDDC), immediate metabolite of disulfiram, is active and can be responsible for the effects of disulfiram.
Bibliography


