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NEUROCHEMICAL, NEUROINFLAMMATORY AND NEUROCOGNITIVE CHANGES INDUCED BY BINGE DRINKING IN THE ADOLESCENT FAMALE RATS

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To my Family.

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ABBREVIATION

AACs:	Axonal Associated Cells
Ach:	Acetylcholine
ADH:	Antidiuretic hormone
ALDH:	Aldehyde dehydrogenase
AMPA:	(2-amino-3-(3-hydroxy-5-methyl-isoxazol-4- yl)propanoic acid)
BAC:	Blood alcohol concentration
BCs:	Basket cells
BD:	Binge drinking
BrAC:	Breath alcohol concentration
CA1/2/3:	Cornu Ammonis areas
CFV:	Cresyl Fast Violet Staining
CNS:	Central nervous system.
COX-2:	Cyclooxygenase
DTI:	Diffusion tensor imaging
EAAC1:	Excitatory amino-acid carrier 1
EAAT2:	Excitatory amino acid transporter 2
EAAT4:	Excitatory amino acid transporter 4
ECT:	Electroconvulsive therapy
EEG:	Electrophysiological brain mapping,
eNOS:	Nitric oxide endothelial
EPSP:	Excitatory postsynaptic potential Physiology

EtOH:	Ethanol
GCs:	Glucocorticoids
GLAST:	Astrocytes glutamate transporter
GLT-1:	Glial glutamate transporter
HED:	Heavy episodic drinkers
IL-2/3/6/10:	Interleukin
INF-γ:	Interferon gamma
iNOS:	Nitric oxide synthase inducible
IPSP:	Inhibitory postsynaptic potential
LLP:	Long-lasting potentiation
LPS:	Lipopolysaccharide
LTP:	Long Term Potentiation
MEOS:	Microsomal ethanol oxidizing system
MRI:	Magnetic resonance imaging
N9:	Microglia cell line
NAc?:	N-acetyl-L-cysteine
NAD +:	Nicotinamide adenine dinucleotide
NADH:	Nicotinamide adenine dinucleotide dehydrogenase
NFκb:	Nuclear factor kappa B
NMDA:	N-methyl-D-aspartate
nNOS:	Nitric oxide synthase neuronal
NO:	Nitric oxide
NOS:	Nitric oxide synthase
NR2B:	NMDA receptor

OECD:	Economic Cooperation and Development statement Organisation
OPA:	o-phtaldialdehyde
PFC:	Prefrontal cortex
ST:	Septotemporal axis
STDP:	Spike-timing-dependent plasticity
TAU:	Taurine (2-aminoethanesulfonic acid)
TLR4:	Toll-like receptor 4
TNF-α:	Tumor necrosis factor-α
TRANS:	Orthogonal transverse axis
VTA:	Ventral tegmental region

I. INTRODUCTION

1. Alcohol

Ethanol is a clear flammable liquid that boils at 78.4 °C. It has a variety of uses; for example as an industrial solvent, car fuel, and raw material in the chemical industry. Both ethanol and methanol possess denaturing and inert rendering properties, which make them useful as anti-microbial agents in medicine, pharmacy, and industry. Alcohol is an organic compound where the hydroxyl functional group (-OH) is bound to a carbon atom. The carbon atom is saturated, having single bonds to three other atoms (Nic et al., 2006). The most commonly used alcohol is ethanol, C_2H_5OH.

1.1. Ethanol as an alcoholic beverage

Ethanol in alcoholic beverages has been consumed by humans since prehistoric times for a variety of hygienic, dietary, medicinal, religious, and recreational reasons. Fermentation was discovered in ancient times when a few grains of barley were left in the rain. Opportunistic microorganisms then fermented the starch-derived sugars into alcohols. In addition, fruits were fermented into wine and cabbage into Kimchi or sauerkraut. It is thought by some anthropologists that mankind settled down from nomadic wanderers into farmers and started to grow barley in order to make beer in 10,000 BC. There are many quotations in the bible, in both the Old and New Testament, which describe the adverse effects of excessive alcohol intake. For example, ‘Noah, who was a farmer,

was the first man to plant a vine yard. And he drank of the wine, and was drunken; and he was uncovered within his tent' (Genesis 9. 20-21), 'You are doomed! You get up early in the morning to start drinking and you spend long evenings getting drunk' (Isaiah 5.11), 'Let us conduct ourselves properly, as people who live in the light of day-no orgies or drunkenness' (Romans 13.13).

1.2. Ethanol use in Europe and USA

Ethanol is widely used throughout society, e.g. at parties, weddings and various celebrations, to induce relaxations, lessen anxiety and induce a sense of well-being in individuals. Early studies in the 1960s, suggested that the intake of red wine in moderate amounts, combined with a Mediterranean diet, might actually prolong life, although this was refuted in later studies. When alcohol intoxication occurs, a variety of acute adverse effects are observed, which include ataxia of gait, slurred speech, prolonged reaction times, poor memory consolidation, impaired emotional modulation and compromised judgment. Overall this will induce impaired judgment, blunted affect, poor insight, social withdrawal, reduced motivation and attention, and impulse-control deficits. In chronic abusers of alcohol, where excessive amounts of alcohol, 5-10 units/day have been consumed over a number of years, a variety of adverse effects may occur (Figure 1).

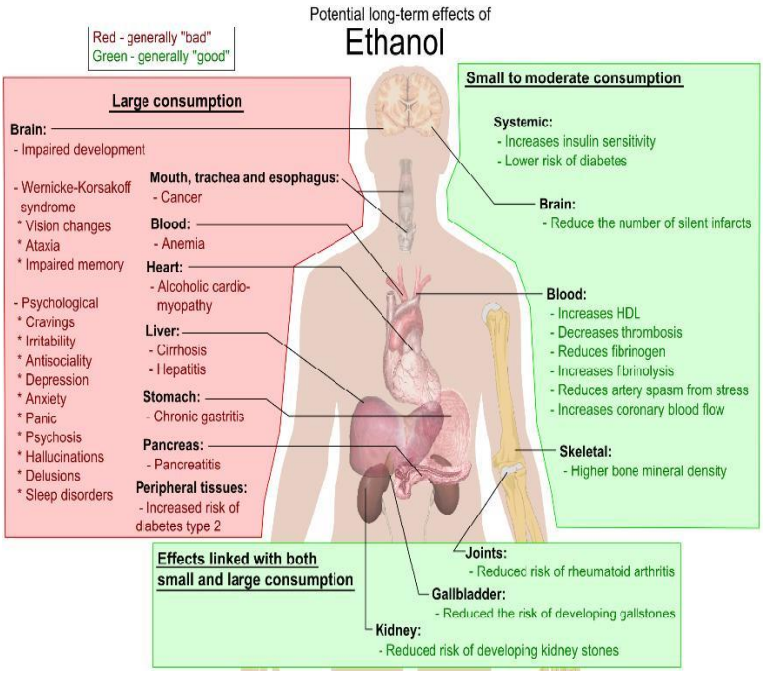


Figure 1: Adverse effects of long-term alcohol abuse.

1.3. Ethanol absorption, redistribution and elimination

- **Effects of nutrition**

The rate of absorption and re-distribution of alcohol after ingestion will be influenced by a variety of factors, which include nutrition. In the fasted state, ethanol will be absorbed principally in the duodenum and jejunum, due to its rapid transit through the empty stomach. In contrast, when food is present in the stomach, gastric emptying will be delayed and substantial amounts of ethanol will be absorbed from the stomach (70%) during 4-5 hours (Cortot et al., 1986). The composition of the food will also influence ethanol absorption; simultaneous administration of ethanol and liquid meals will retard ethanol absorption in the order of fat > carbohydrate > protein, (Sedman et al. 1976) while Welling et al. (1977) showed that solid high carbohydrate meals had the largest effects on

ethanol absorption, followed by high fat meals and then high protein meals. In addition, the rate of ethanol elimination was enhanced when a meal was eaten.

- **Effects of ADH isoenzymes**

The different isoenzymes of ADH will determine the rate of ethanol oxidation to acetaldehyde (Figure 2 and Table 1), which will determine the circulating concentration of ethanol in the blood as well as the CNS (Ammon et al., 1996).

Class	Allele	Enzyme	Km (mM)
I	ADH_2 [1]	β1β1	0.049
	ADH $_2$ [2]	β2β2	0.94
	ADH_2 [3]	β3β3	36
	ADH_3 [1]	γ1γ1	1
	ADH_3 [2]	γ2γ2	0.64
III	ADH5	χχ	no saturation
IV	ADH7	σσ	37

Table 1: Kinetic constants for ethanol oxidation by ADH in gastrointestinal tract.

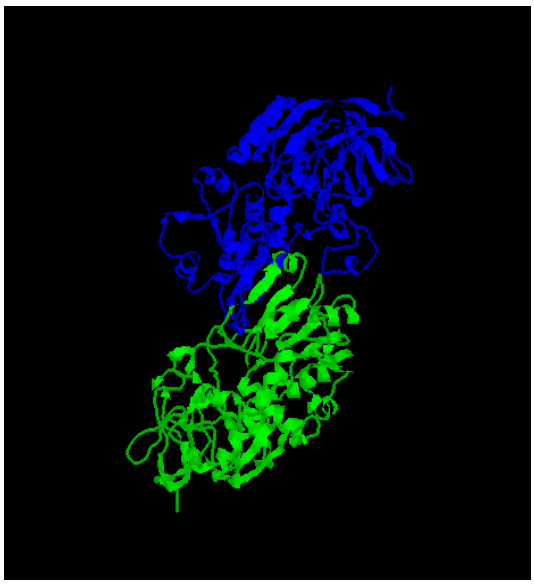


Figure 2: Protein structure of ADH together with its cofactor NAD +.

The genes ADH_2 and ADH_3 are the main enzymes involved in vivo in alcohol metabolism. The ADH_2 gene occurs in the form of three alleles: ADH_2^1, ADH_2^2 and ADH_2^3 which are responsible for β1, β2 and β3 subunit. ADH_2^1 and ADH_2^2 alleles are present in the white community while the ADH_2^3 allele occurs in approximately 25% of the Afro-American population. In vivo, in man, the β2 gene is highly active in the metabolism of alcohol. The ADH_3 gene has two alleles, ADH_3^1 and ADH_3^2, which code, for γ1 and γ2 respectively.

In the oxidation of ethanol to acetaldehyde by ADH, a hydrogen is transferred from the substrate to the co-factor nicotinamide adenine dinucleotide, NAD, producing NADH and acetaldehyde (Figure 3). NAD is also involved in the oxidation of acetaldehyde to acetate producing more NADH.

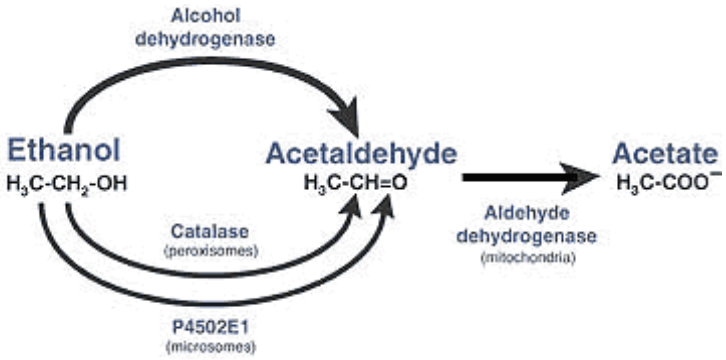


Figure 3: Ethanol metabolism via alcohol dehydrogenase and aldehyde dehydrogenase to acetate.

- **Factors affecting the distribution of ethanol in tissues**

Ethanol will distribute itself within the total body water, which will occur more rapidly in well vascularised organs (e.g., brain, liver, lung and kidneys) while poorly vascularised tissues, such as muscle, will show a slower uptake of ethanol. It is noteworthy that the concentrations of ethanol in the brain and arterial blood are

substantially higher than those in muscle and peripheral veins after ethanol ingestion. Therefore, breath alcohol concentration (BrAC) will more accurately assess the level of CNS exposure than venous blood during the early stages of the blood alcohol concentration (BAC) curve (Figure 4).

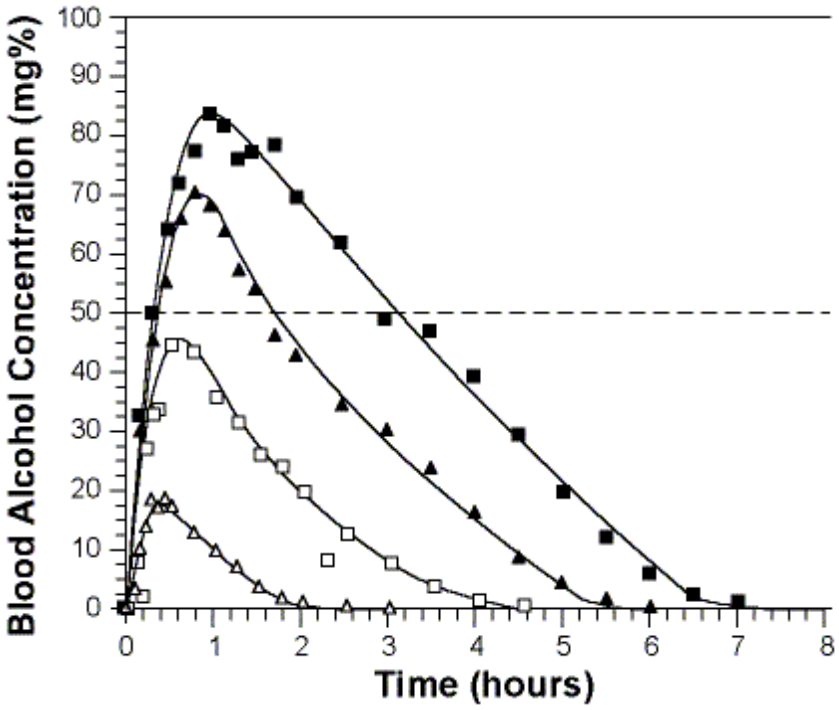


Figure 4. Blood alcohol concentration after the rapid consumption of different amounts of alcohol.

Various factors will contribute to the alcohol concentrations in the different tissues. These include the body water mass which can vary between 67 to 77% of the lean body mass, (this is altered during menstrual cycle, edema etc), variations of the fat content (women have higher body fat content than men), as well as age (lean body mass is generally decreased with age).

The elimination rates in moderate drinkers, (alcohol intake 1-3 units) are low (8 to 25 mg/dl/hr), while heavy drinkers (alcohol intake in excess of 5

units/day) show high elimination rates, (36 to 40 mg/dl/hr), this being caused by the induction of the P450 2E 1 cytochrome (MEOS) in the liver.

The blood alcohol level reached after 2 to 3 drinks will approximately depend on a number of factors. These include the time period over which the alcohol is consumed. If drinks are consumed at widely spaced intervals, and particularly if each drink is taken with a meal, it is probable that blood ethanol concentrations would peak at 2 to 5 mM (9 to 25 mg/%) after each drink. On the other hand, if three drinks were consumed within a relatively short time, such as within 1 hour or less, significantly higher peak blood ethanol concentrations would be evident. Such drinking is referred to as intermittent alcohol consumption or 'binge drinking'.

Ethanol is oxidised almost entirely in the liver by oxidative metabolism. In addition the P450 2E 1 cytochrome, MEOS, will be synthesised when high amounts of alcohol are consumed over a long period of time, 3-6 months, which has a high K_m for ethanol (10 mM). In contrast the capacity of brain to metabolize ethanol is somewhat limited. The ADH isoenzymes present in the brain, ADH_5 has an extremely high Km such that it will play no role in ethanol metabolism. Catalase which is present in the brain has a limited ability to metabolise ethanol, possibly because of it is rate-limited by the availability of the co-substrate hydrogen peroxide, while the inducible MEOS, if synthesised to any large extent, plays a minor role (Ward et al., 2011). Acetaldehyde is the major metabolite of ethanol metabolism and is extremely toxic such that its rapid elimination is essential to

prevent tissue toxicity. There are many ALDH isoenzymes but only 2, $ALDH_1$ and $ALDH_2$ play a role in the oxidation of acetaldehyde to acetate in vivo (Table 2).

- **ALDH1** Modulator of gene expression and cell differentiation.
- Biosynthesis of retinoic acid, (Km for retinal = 0.06umol/l)
- Oxidation of acetaldehyde (Km 10-30 uM).
- **ALDH2** Oxidation of acetaldehyde (Km 1-2 uM).
- (Point mutation of a single base, G - A causes a change in the amino acid glutamate at 487 to a lysine substitution. Enzyme inactivated).
- ALDH3 May play a role in the oxidation of toxic aldehydes.
- ALDH4
- ALDH5
- ALDH 6 Plays a role in the detoxification of aldehydes generated by alcohol metabolism and lipid peroxidation.
- ALDH7 Play a role in the detoxification of aldehydes generated by alcohol metabolism and lipid peroxidation.
- ALDH8
- ALDH9 High activity for oxidation of gamma-aminobutyraldehyde and other amino aldehydes
- ALDH10

Table 2: Shows the different ALDH isoenzymes present in humans, together with their Km for acetaldehyde metabolism.

$ALDH_2$ is present in the mitochondria in the liver and will play the major role in ethanol metabolism. Cytosolic $ALDH_1$ plays only a minor role, as its Km is relatively high for acetaldehyde, 10-39 μM. In many Oriental and Asian subjects a mutation of $ALDH_2$ occurs which reduces the ability of the enzyme to metabolize acetaldehyde. As a result of this mutation, flushing occurs in these individuals after alcohol ingestion caused by high circulating levels of acetaldehyde.

Excessive alcohol consumption will initiate a variety of detrimental effects on many tissues within the body which include the liver and the oesophagus. Over recent years particular attention has been directed at the vulnerability of the brain to excessive alcohol consumption.

1.4 Alcohol and the brain

Alcoholic brain damage is caused by chronic, long term and excessive consumption of alcohol. Not all alcohol abusers will show alcoholic brain damage, since environmental and genetic influences, as well as an individual's acquired and inherent modifying factors, will play important roles. Alcoholism will diminish life expectancy by approximately 4.2 years. Over the past 10 years the adverse effects of chronic alcohol abuse on brain structure and function have been characterized by advanced technologies such as magnetic resonance imaging, MRI, diffusion tensor imaging DTI, positron emission tomography and electrophysiological brain mapping, EEG, which evaluate brain structure and function.

Binge drinking (BD) has received considerable attention over the past 5 years, primarily since there is accelerated alcohol-induced brain damage over a much shorter time period < 2-3 years. Binge drinking is defined as an intake of at least 5 units over a 2 hours period, followed by a period of abstinence. The alcohol consumption over this time period is often 50% of the weekly recommended intake and elevates blood alcohol levels to 0.8 g/L or above (NIAAA, 2004). Adolescents appear to be the group that indulges in this type of drinking pattern. Changes in cognitive function have been identified in some individual, although the exact mechanisms involved await clarification. The neuro-toxicity associated with binge drinking may also be related to the periods of abstinence between the few days of excessive alcohol consumption, when alcohol withdrawal occurs, which is associated with the release of a variety of ROS and RNS species together with excessive amounts of the excitatory neurotransmitter glutamate. Such patterns of alcohol abuse may increase the individual's vulnerability to develop alcohol

induced brain damage and alcoholism in later life, if such excessive alcohol intake continues.

Particular attention has been directed to this form of ethanol consumption in adolescents because during this period neurogenesis occurs in particular regions of the brain, e.g. hippocampus, which may be adversely affected by such patterns of ethanol intake (Taffe et al., 2010). This may result in cognitive impairment in vulnerable individuals (Brumback et al., 2007; Witt, 2010). Adolescence is acknowledged to be a time when certain characteristic behaviours occur which include high social interaction and play behaviour, high levels of risk-taking, high activity and exploration, impulsivity, and novelty and sensation seeking (Ernst et al., 2009; Spear, 2000).

Heavy alcohol consumption in the young college adults is a public health concern, (Dawson et al., 2004), since this represents a public health concern, due to reduced judgment and increased risky behaviours induced by BD. Crucial neurogenesis may be interrupted by repeated binge alcohol consumption, (Luna, 2009; Spear, 2009) yet the processes underlying the toxicity during this developmental period are still not fully understood.

It is clear that such excessive alcohol intakes occur among college students; 44% report BD every 2 weeks and 19% report more than 3 BD episodes per week (O'Malley et al., 1998; Wechsler et al., 1995). Since adolescents have low sedative responses to alcohol, this will allow a greater consumption of alcohol, and subsequently higher blood alcohol levels (Silveri and Spear, 1998).

Italy is at the lower positions for alcohol consumption in a list produced by the OECD (Economic Cooperation and Development statement Organisation) but it

is in the first place for the earliest exposure to ethanol: at twelve and half years by comparison to the European average of 14.6 years. An International study has reported that alcohol will be the third leading cause of disability, morbidity and mortality in the forthcoming years. Alcohol consumption in Italy has recently been studied by a survey carried out by Eurispes between 2009 and 2010 among the 18 years aged population. According to data released by the Presidency of the Ministers Council-Department of Drug Policy, 70% of Italians declare to drink alcohol.

Of these:

•55.7% drink occasionally;

•11% drink often;

•4.1% daily.

29% of respondents however, never drinks, especially women (56.9% *vs* 43.1% of males), especially among those over 65 (26.9%), followed by 45-64 years age classes (23.7%) and 35-44 years age (22.8%).

The mechanisms underlying brain damage induced by BD are poorly understood. Since such alcohol abuse in adolescents coincides with changes in the structural development of the brain, i.e. neurogenesis, ethanol may adversely affect this process which could lead to defects in intelligence (Shaw et al., 2006) and behavioural control of executive functions (Ernst et al., 2009). This may be more prevalent in adolescent females (Scaife and Duka 2009). Adverse effects may include blackouts, impairment of functional brain activity, particularly in brain regions more responsible for learning and memory (Zeigler et al. 2005).

Furthermore, BD may be associated with an increased risk of dementia (Gupta and Warner, 2009).

In initial studies the hippocampus has been the focus to investigate the underlying processes involved in the adverse effect of BD (Stephens and Duka, 2008), since this brain region plays a crucial role in learning and memory, it appears to be the region most damaged from BD.

2. Hippocampus, structure and function

The hippocampus (Figure 5) is an area of the central nervous system (CNS) that together with other important regions constitutes the limbic system.

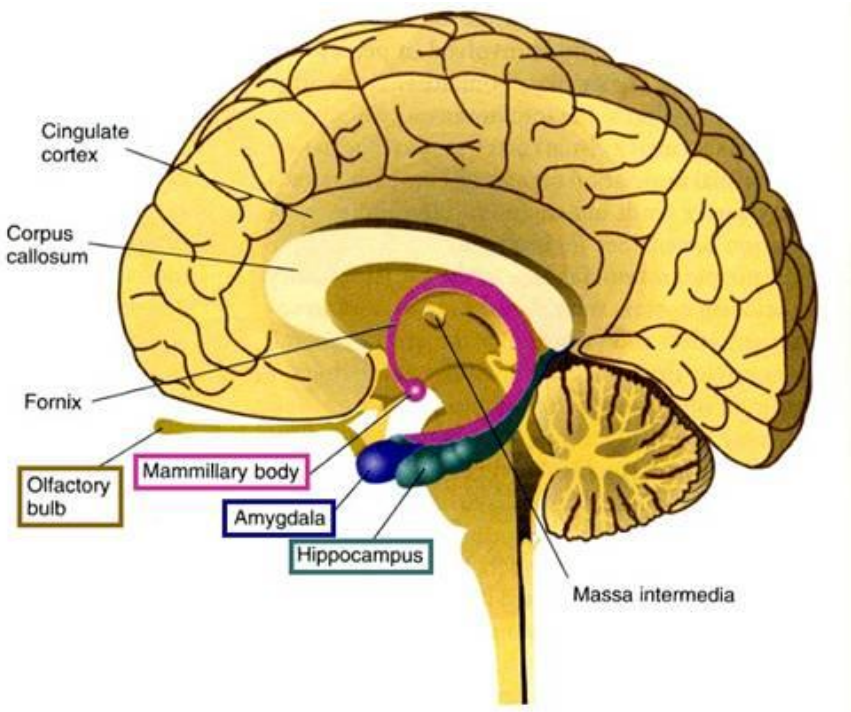


Figure 5: *Lateral view of some anatomical structures of the human brain.*

Overall it is difficult to give an overview of the functional significance of the limbic system. It is an important link between olfactory sensations, emotional, instinctive and vegetative activity. In this system, the hippocampus merits particular attention because of the memory processes, the complexity of events due to hippocampal neurons. At macroscopic level the hippocampus has a characteristic structure easily identifiable: it has an elongated shape that extends to form a C which makes it similar to a sea horse, from which the name itself, hippocampus, derives from the Greek word *hippo* meaning horse, and *kampos*: meaning monster of the sea. Another reason for interest in this region of the brain

dates back to the early 50s when it was assigned a major role in memory and learning processes.

2.1 Structural organization of the rat hippocampus.

The hippocampus (Figure 6) appears as an elongated structure.

Its main axis extends to form a C from the rostral region of the nucleus of the septum, above and behind the diencephalon, until it reaches, ventrally and caudally, the temporal lobe. The main axis of the hippocampus is known as the septotemporale axis, while the axis orthogonal as the transverse axis. The hippocampal formation comprises four regions: the dentate gyrus, the hippocampus proper, the complex subiculare (divided into *subiculum*, *presubiculum* and *parasubiculum)* and the entorhinal cortex than in rodents is divided into medial and lateral.

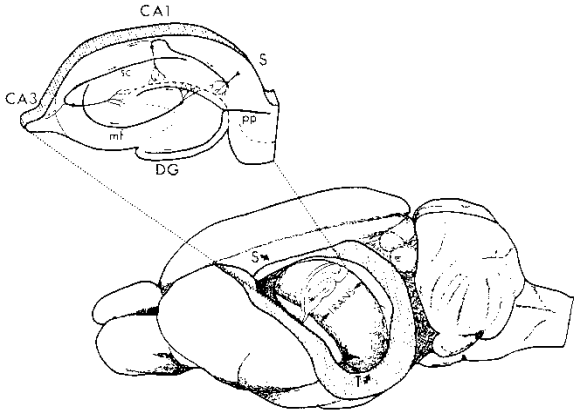


Figure 6: Lateral representation of the rat brain showing the localization of the hippocampal formation. The hippocampus appears as a curved structure which extends from the region of the rostral nucleus of the septum until it reaches caudally the temporal cortex. The main axis is called septotemporal axis (indicated with ST), and the axis is orthogonal to said transverse axis (TRANS) (Amaral and Witter, 1989).

Dentate gyrus, hippocampus and *subiculum* are constituted by layers of a few cells and by some acellular layers located above and below it, while the

entorhinal cortex is formed from 6 cell layers. The dentate gyrus is made up of three layers: the molecular acellular layer, the granular layer (the main) and the polymorphic cells layer (also called *hilus*). Even the hippocampus has a main layer called pyramidal cells layer that has been divided into three regions: CA1, CA2, CA3 (Lorente de No', 1934) depending on the aspect of the size and morphology of pyramidal neurons. Above and below it a number of other layers are distributed (*stratum radiatum*, *stratum Oriens*, *stratum lucidum*, *stratum caudatum*, alveus, *lacunoso-molecular layer*). In addition, it used the old term, CA4 region to indicate the polymorphic layer of the dentate gyrus. The areas CA3 and CA2 correspond to the lower area and the area CA1 to the top.

2.2 Main neurons

The principal neurons of the hippocampal region are the granule cells of the dentate gyrus and pyramidal neurons (Figure 7).

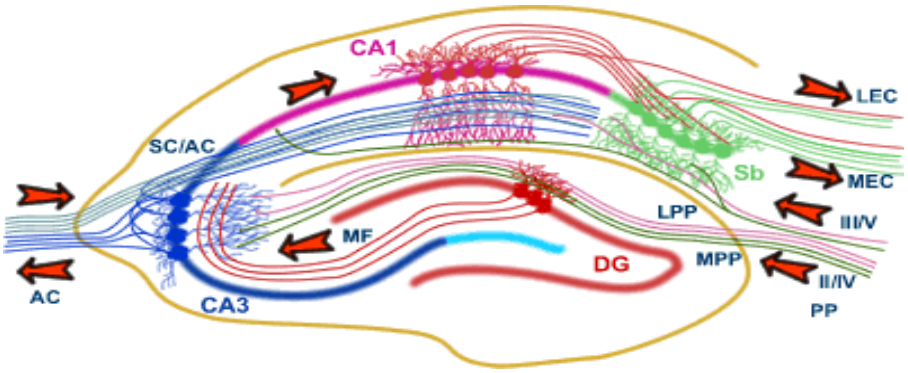


Figure 7: The hippocampal Network.

The dentate gyrus is a region uniquely situated to control the effects of incoming cortical inputs on the hippocampus. The perforant path, formed by cells

of layer II in the entorhinal cortex, constitutes the main input to the dentate gyrus (Steward, 1976; Varga et al., 2010). The principal cells of the dentate gyrus are the granule cells that releases the neurotransmitter glutamate (Spruston and McBain, 2006)and are about 1 million in rats and five million in non-human primates (Claiborne et al., 1986;Seress, 1988) and the mossy cells (Amaral, 1978). In the dentate gyrus, granule cells have small and spherical cell bodies (100 μm diameter) and are grouped in clusters of 4-6 cells. The dendrites of granule cells extend perpendicular above to the molecular layer that receives synaptic connections from different origins. The granular cells are considered unipolar neurons because the dendrites emerge from the apical portion of the cell field only. The axons of granule cells are called mossy fibres due to the appearance of their synaptic terminals. They originate from the basal portion of the cell body together with some neurons of the polymorphic cells layer before meeting as a beam that comes out of this layer and enters into the *stratum lucidum* of the CA3. The polymorphic neurons, as the name suggests, have various morphological characteristics but all of these have the characteristic of projecting only towards the dentate gyrus and receiving from the other cells of the polymorphic stratum: the mossy and basket cells that are interneurons (see paragraph: interneurons) (Ribak and Seress, 1983; Ribak et al., 1985, Scharfman et al., 1990a). The hippocampus pyramidal cell bodies of neurons are sorted into groups of 3-6 cells. These neurons have elaborate dendritic trees which extend perpendicularly to the cell layer in both directions and for this reason they are considered multipolar neurons. The apical dendrites, longer than the basal one, extend from the apex of the cell body of pyramidal cells in the central zone towards the dentate gyrus of the

hippocampus. The apical dendrites of pyramidal cells of the CA3 extend through three layers: the *stratum lucidum*, the *stratum radiatum* and the layer *lacunoso-molecular*, and in each of these layers they receive different types of synaptic contacts. The basal dendrites extend from the basal region of the pyramidal cells layer and reach the *stratum Oriens*. These dendrites are covered with spikes and on it ending many synapses of excitatory nature. Some of the biggest thorns in the CNS are those located on the dendrites of pyramidal cells of the CA3 and do synapses with mossy fibres. The rest of the dendritic tree of the CA3 pyramidal cells and all the tree of the CA1 pyramidal cell dendritic spines are similar to those of cortical excitatory and in then they make an asymmetric synapses

The axons of pyramidal cells of the CA3 region are the way of the Schaffer collaterals that goes to have synapses on interneurons and pyramidal neurons of CA1 region. Instead, the axons of pyramidal cells of the CA1 region project to the *subiculum* and the entorhinal cortex.

Although significant excitatory input arrives at the dentate through the perforant path, few granule cells (GCs) will fire to pass along this input to the CA3 region (Fricke and Prince, 1984; Mody et al., 1992a, b; Scharfman, 1992; Staley et al., 1992; Williamson et al., 1993; Coulter, 1999; Nusser and Mody, 2002; Pathak et al., 2007). In the three pyramidal regions of the hippocampus, the cells present, in addition to a different morphology, differentiation at the level of connections. The pyramidal cells in CA3 receive inputs from mossy fibres of the dentate gyrus, which, however, do not send an input to the pyramidal cells in CA1. The area CA2 has been a matter of debate. As originally defined by Lorente de No (1934) it was a limited area of cells located between CA3 and CA1 with the cell bodies as large as

those of the area CA3 without contracting synapses with mossy fibres, as well as with cells of the area CA1. In fact it was also noted that there is a limited area in CA2 that has both features of the CA1 and CA3 regions and has even functional diversity from the rest of the region. This relatively narrow region of the CA2 localized distally, on which end the projections of mossy fibres, is formed by large and dark color pyramidal cells as those of the CA3 region (Lorente de No', 1934; Tamamaki et al., 1988). Immunohistochemical studies have shown that the CA2 area has a different immunoreactivity. Furthermore, the presence of acetylcholinesterase and proteins that bind Ca_2^+, was always found in the CA2 region, but not in the adjacent CA3 and CA1 areas (Bainbridge and Miller, 1982). This aspect is interesting since the Ca_2^+ binding proteins appear to play a protective role in the case of ischemia or cell death of cytotoxic nature; in fact the area CA2 appeared to be more resistant to cell death of epileptic nature than CA3 and CA1, and it is often referred to as the "field strength" (Corsellis and Bruton, 1983).

- **Interneurons**

The Interneurons in the hippocampus are mostly spineless, they branch locally and they contain GABA. In the dentate gyrus and hippocampus proper there are at least five different types of interneurons including: the basket cells, the axonal associated cells (AACs) and ganglion cells. The term "basket cells" (BCs) comes from the basket-like appearance of their preterminal axonal segments around the soma of target neurons.

The basket cells make synapses with the soma of neurons and each can form multiple contacts to constitute a sort of basket around the cell body of the neuron. These cells are divided into three distinct subclasses: large, small and nest basket cells (Marin-Padilla, 1969; Wang et al., 2002). The majority of BCs belong to the category of fast-spiking cells (Kawaguchi and Kubota, 1993, 1997; Zaitsev et al., 2005). BCs are mutually interconnected by chemical synapses as well as by electrical synapses (gap junctions) (Hestrin and Galaretta, 2005).

The cells associated axon make synapses initially with the segment of the axon with thus exerting a strong control on the potential action. Finally the ganglion cells form synaptic contacts on the dendrites of neurons. In the dentate gyrus, pyramidal basket cells (Ribak and Seress, 1983) are located between the layer of granule cells and the layer of polymorphic. Each axon can get out of a high number of granule cells. The terminations are GABAergic, thus form inhibitory synapses primarily with the cell bodies, but also with the dendritic tree (Kosaka et al., 1984). The same GABAergic neurons of the polymorphic layer are innervated by other GABAergic terminations (Misgeld and Frotscher, 1986). The AACs cells are present in the molecular layer and the make synapsis on the axonal initial segment of granule cells (Kosaka et al., 1984; Freund and Buzsaki, 1996b). In this way it receives an inhibitory input (Kosaka, 1983; Soriano and Frostscher, 1989). Other interneurons are localized at the level of the molecular layer and the polymorphic layer and have axons that remain locally (Freund and Buzsaki, 1996b) In the polymorphic layer there is always a class of cells called mossy neurons (Amaral, 1978), which have excitatory nature and which project only to the molecular layer of the dentate gyrus (Blacksyad, 1956; Laurberg and Sorensen,

1981). These neurons are therefore an exception to the terms traditionally used to define interneurons. They do not project locally, but for long-distance and on both sides in the septum of the hippocampal dentate gyrus. Since this projection originates both from the ipsolateral and contralateral side, it has been called associative-commissural ipsilateral projection, and it seems that takes its origin as a collateral fibre of the mossy cell axons of the *hilus* (Laurberg and Sorensen, 1981). The dentate hilus is located subjacent to the granule cell layer and extends to the border of the dendritic layer of CA3 that is interposed between the upper (suprapyramidal) and lower (infrapyramidal) blades of the dentate gyrus. The principal and most numerous cell types in the hilus is the mossy cell. These neurons are characterized by their densely spiny dendrites and several thorny excrescences on both the cell body and proximal dendritic shafts and their dendrites are mostly confined to the hilus (Amaral, 1978) Most of the terminations of this region form an excitatory synapse on dendritic spines of granule cells present in the molecular layer (Laatsch and Cowan, 1966; Kishi et al., 1980). In the hilus and granule cell layers of the dentate gyrus, the microcircuits involving GABAergic interneurons and GCs have been explored (Buzsaki, 1984; Seress and Ribak, 1984; Sloviter, 1991; Freund and Buzsaki, 1996; Penttonen et al., 1997; Kraushaar and Jonas, 2000; Alle et al., 2001). However, although careful descriptions of individual molecular layer interneurons exist (Seress and Ribak, 1983; Soriano and Frotscher, 1989; Halasy and Somogyi, 1993; Han et al., 1993; Freund and Buzsaki, 1996; Chittajallu et al., 2007; Capogna and Pearce, 2011), the precise identities of interneurons performing feed-forward roles in this layer have yet to be delineated. Because of the anatomical proximity of molecular layer

interneurons to incoming perforant path input, feed-forward interneurons in the molecular layer would be expected to contribute to the sparse firing observed in dentate GCs. This role may be crucial in proper circuit function. Indeed, feed-forward inhibition has recently been shown to have dramatic computational effects on network dynamics (Ferrante et al., 2009). Since the mossy cells were immunoreactive for the glutamate (Soriano and Frotscher, 1993) it is easy to tell that this is the neurotransmitter released at the level of the molecular layer. The mossy cells' membranes are densely innervated by granule cells in the stratum polymorphic and ranging in synapses or with the dendrites of granule cells in the molecular layer, or with the dendrites of basket interneurons. The granular cells innervate the mossy cells in the same septotemporal level in which the cell bodies are located, while the mossy cells project to a more distant level. It seems that the mossy cells transmit an output signal from a septotemporal level towards the granular cells located in distant levels of the dentate gyrus. The associative fibres make a synapsis with the shaft of the dendritic cells to the GABAergic basket (Frotscher and Zimmer, 1983; Seress and Ribak, 1984); in this way the associative commissural projection can be either excitatory or inhibitory. Hippocampal interneurons have cell bodies located in the pyramidal layer or close to it; associated axonal cells make a synapsis with the initial segment of pyramidal neurons, the basket cells make a synapsis with the soma of pyramidal neurons, the bistratificated cells form synaptic contacts on apical dendrites and basal pyramidal neurons. Although there is a clear overlap between the regions of dendrites projection of all three classes, these are directed to the *stratum radiatum* and the *stratum Oriens*, and can thus receive excitatory inputs from Schaffer collateral,

through hippocampal commissural projections (see section 2.4: synaptic connections of the CA3 region) and pyramidal neurons (Buhl et al., 1996; Halasy et al., 1996). GABAergic interneurons are present in the *stratum radiatum* and stratum lacunosum-moleculare where they receive excitatory inputs, respectively, from the Schaffer collateral and perforant path, and make synapses on the pyramidal dendrites of the various regions. There is also a mutual inhibitory connection between these inhibitory interneurons, whose function would be to synchronize the various pulses producing oscillations at various frequencies including theta (5 Hz) and gamma (40 Hz) (Jefferys and Whittington, 1996). Among the various interneurons, whose properties and connections are less well known, there are presumably excitatory interneurons in the *stratum lucidum* (Spruston et al., 1997) that receive inputs from mossy fibres and reach the CA3 pyramidal neurons (Soriano and Frotscher, 1993 Kobuyashi, 2012). These interneurons can be divided into interneurons with or without spines. It has been suggested that the first ones are glutamatergic interneurons, and therefore responsible for the excitation of pyramidal neurons, whereas the second ones would be responsible for a GABAergic inhibition of the same pyramidal neurons. The function of these interneurons is as yet unknown.

2.3 Neural Circuits

The hippocampal formation has four areas connected by a single large one-way excitatory connection referred to by Andersen and colleagues (1971) with the term "*trisynaptic loop*" (Figure 8).

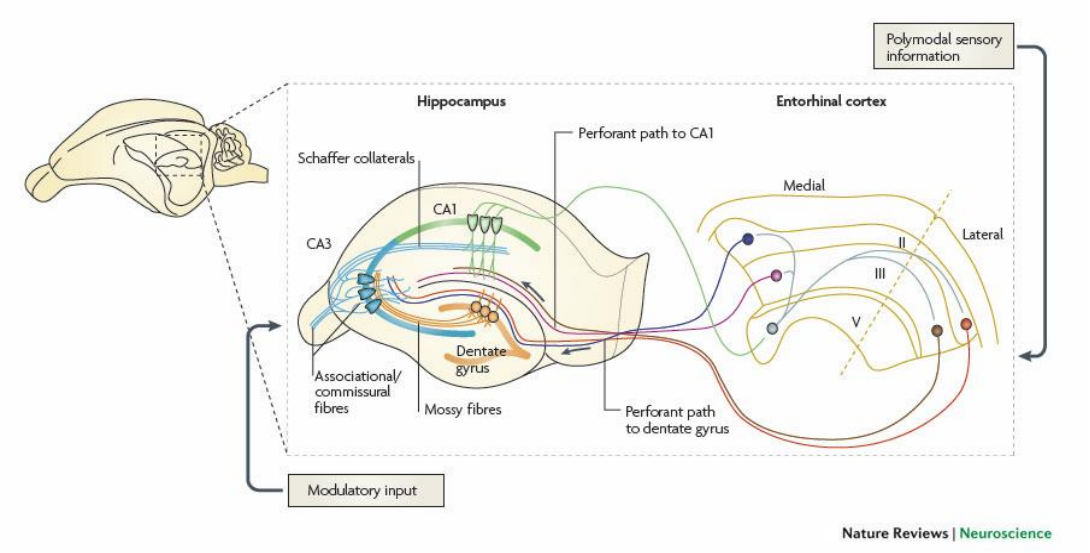


Figure 8: Traditional representation of the hippocampus as a trisynaptic loop (modified by Guilherme et al., 2008).

The neurons localized in the entorhinal cortex II layer give origin to a pathway, called perforant pathway, which crosses the region of the subiculum penetrating it and ending at the level of the dentate gyrus and CA3 area.

The portion of this pathway that reaches the dentate gyrus is in turn constituted by two components: the medial and lateral cells. The first one contributes with its axons to create a projection that reaches a narrower area in the medial portion of the layer stratum lacunosum-moleculare, close to the CA3, the second one, however, reaches the third outermost, or a portion of the layer-stratum lacunosum-moleculare located on the border between the CA1 area and the *subiculum*. The perforant path ends in the molecular layer of the dentate gyrus, where asymmetric synapses, strictly confined to the 2/3 of the surface, are formed. They are at least 85% of the total synaptic population (Nafstad, 1967). They reach most dendritic spines of the granular cells of the dentate gyrus and to a lesser extent the interneurons of the pyramidal basket cells (Zipp et al., 1989). The majority of inputs to the dentate gyrus comes from the entorhinal cortex and

especially from the II layer (Steward and Scoville, 1976; Schwartz and Coleman, 1981; Ruth et al., 1982). A minor component comes also from the lower layers (IV-VI) (Kohler, 1985). From the dentate gyrus stems a number of myelinated axons called mossy fibres. From each of them depart about 7 thinner collaterals, crossing first the polymorphic layer and then the area CA3 (Claiborne et al., 1986). Within the polymorphic layer there are about 160 small varicosities (0.5-2 μm) that form synaptic contacts branching locally (Claiborne et al., 1986). This layer is also connected to other levels of the dentate gyrus through associative connections. At the end of each side, in general, there is a single larger varicosity (3-5 μm): it is irregular and reaches the dendrites of the pyramidal cells of the CA3 region.

The mossy fibres tend to form bundles when extended across the *stratum lucidum* (Claiborne et al., 1986). The presynaptic expansions form a single synaptic complex with the *thorny excrescence*. These large spinous processes are surrounded by a single expansion of the mossy fibre which can form up to 37 synaptic contacts with a single dendrite of CA3 pyramidal cells (Chicurel and Harris, 1992). The granular cells are in a privileged condition to influence the activity of hippocampal pyramidal cells, even if the mossy fibres make contact with a relatively small number of them: each pyramidal cell, receives about 50 synaptic contacts from granule cells. The mossy fibres remain approximately at the same septotemporal level of their cells of origin (Gaarskjaer, 1978a, b; Swanson et al., 1978; Claiborne et al., 1986). Near the border area between CA3 and CA2 they change direction and extend for 1 mm or more through the temporal area of the hippocampus; the functional significance of this component is not jet known. Finally a part of the mossy fibres goes to make synapses on pyramidal neurons of a

narrow area of the CA2 region. CA3 pyramidal cells give rise to the Schaffer collateral projection. Their neurons have synapses in CA1, on the pyramidal neurons and interneurons (see section 2.4: synaptic connections of the CA3 region), the latter giving rise to connections both direct towards the subiculum and to the lower layers of the entorhinal cortex. Finally, also in the subiculum there is a direct connection to the entorhinal cortex. So the information that flows from the entorhinal cortex runs along the entire hippocampal circuit ending in the cortex, from which it originated. Probably, this transversal pathway plays an essential role in information related to long-term memory.

- **Septal-ippocampal pathway**

Most of the direct inputs to the dentate gyrus originate mainly from the medial septum nuclei and nucleus of the diagonal band of Broca (Mesulam et al., 1983). These projections are called septal-hippocampal pathway (Pepeu, 1983) and consist of cholinergic and GABAergic neurons. The cholinergic neurons projecting towards the pyramidal cells of CA3, CA1 and CA2 areas, to the granular cells of the dentate gyrus have the nature of inhibitory interneurons (Lewis et al., 1967; Lynch et al., 1978), while the GABAergic neurons project mainly towards the inhibitory interneurons of the above areas (Babb et al., 1988; Freund and Antal, 1988). The other cells of the septum, which project to the dentate gyrus and seem to have a preference for GABAergic cells (Freund and Antal, 1988), contain the glutamic acid decarboxylase (GAD) and are presumably considered as GABAergic (Kohler et al., 1984). In the CA3 area, the septal-hippocampal projection ends mostly in the *stratum Oriens* and, to a lesser extent, in the *stratum radiatum*

(Nyakas et al., 1987; Gaykema et al., 1990). The GABAergic component of the septal projection sends its terminals on the CA3 GABAergic interneurons, as in the dentate gyrus (Freund and Antal, 1988; Gulyas et al., 1990). So we know that the area of the medial septum contains at least two types of neuronal populations: a cholinergic one, 30%, and the other 70% GABAergic (Kohler et al., 1984).

2.4 Synaptic connections in the hippocampus proper

- **CA3 region**

From the area CA3, pyramidal cells send a whole series of collateral axons directed to the entire hippocampal region, including CA3, to the contralateral hippocampus, at subcortical level, and also to the lateral nucleus of the septum. CA3 and CA2 neurons also contribute with a small number of collaterals to innervate the polymorphic layer of the dentate gyrus. All CA3 and CA2 pyramidal cells give rise to projections diverging towards the different hippocampal portions (Ishizuka et al., 1990). Projections from CA3 and CA2 are typically known by the name of associative connections, those from CA3 to CA1 are called, as already mentioned, Schaffer collateral pathway. The CA3 projections, that branch locally reaching the CA1 region, are highly organized and spatially ordered (Ishizuka et al., 1990). All portions of the CA3 and CA2 neurons project to CA1, but the distribution of their terminations in CA1 depends on the location of their neuronal cell bodies. The topographical organization of the Schaffer collateral pathway determines a network where it is likely that certain cells in CA3 establish contact with as many cells of CA1. Thus, cells of CA3 localized near the dentate gyrus tend to project

above the levels of the septum of CA1. CA3 cells close to CA1 are projected primarily to the temporal levels of CA1. Pyramidal cells localized at the proximal side of CA3 give rise to cells that end superficially in the *stratum radiatum*, while those located more distally terminate deeper in the *stratum radiatum* and the *stratum Oriens*. Finally, pyramidal cells near the dentate gyrus project also to the more distal portions of CA1, near the subiculum, while the projection of CA3, which originates from cells localized distally, terminates in a portion of CA1 which is close to CA2.

It should be noted that each pyramidal neuron of CA3 makes contact with several cells of CA1; for example, it has been estimated that a single CA1 neuron is innervated by more than 5,000 ipsilateral CA3 pyramidal cells (Amaral et al., 1991). The projections from CA3 to CA1 terminate with asymmetric ace-spiky synapses at the level of basal and apical dendrites of pyramidal cells. Size and shape of the spines and presynaptic profiles in this region are variable, and this has to be related to the physiological effects of synapses in CA1. Even the associative projections of CA3 that branch locally are tightly organized and also end in the *caudatum* and *Oriens* layers. It was also demonstrated that in the rat the pyramidal cells of CA3 give rise to commissural projections towards the regions of the contralateral hippocampus, CA3, CA1 and CA2. The commissural projections follow the same topographical organization and generally terminate in homologous regions of both sides (Swanson et al., 1978).

- **CA2 region**

As in the CA3 region, CA2 cells give rise to projections towards CA1 (Ishizuka et al., 1990). It should be noted that a greater number of collateral projections of CA2, as compared to CA3, are distributed in the polymorphic layer of the dentate gyrus.

- **CA1 region**

In contrast to what it was observed in the CA3 region, the pyramidal cells of CA1 do not have collateral connections with local ramifications (Amaral et al., 1991; Tamamaki et al., 1987), but only a few associative connections. The axons of neurons in the CA1 stratum extend towards the *alveus* or the stratum *Oriens,* through the subiculum, and, occasionally, some collateral connections enter in the stratum Oriens and in the layer of pyramidal cells. Possibly, these collateral projections terminate on the basal dendrites of other cells of CA1 (Deuchars and Thomson, 1996). What is clear, however, is that CA1 completely lacks the massive associative network present in CA3. Two projections depart from CA1: the first one, topographically organized directed towards the subiculum (Amaral et al., 1991), the second one, directed to the deeper layers of the entorhinal cortex. The axons of pyramidal cells descend towards the stratum Oriens and towards the alveus then bend sharply towards the subiculum (Finch et al., 1983; Tamamaki et al., 1988; Amaral et al., 1991). Subsequently, they fall in the pyramidal layer of the subiculum, where they branch profusely and bear the deepest molecular layer.

- **Projections to other regions of the CNS**

Swanson and Cowan (1975) have shown that most of the connections that reach the basal forebrain and the diencephalon, originate from the subiculum. The CA3 receives NAergic inputs from the *locus coeruleus*. NAergic fibres are more densely distributed in the *stratum lucidum* and in the superficial portion of the stratum lacunosum-moleculare layer. A plexus thinner than noradrenergic axons is distributed through all other layers of CA3.

Serotonergic fibres are spread in the area CA3 and terminate on interneurons (Freund et al., 1990) whose axons innervate the distal dendrites of pyramidal cells. In this region, it was also demonstrated the presence of dopaminergic fibres (Swanson et al., 1987). CA1 receives, as already reported, a projection from the septum (Nyakas et al., 1987) and, as well as CA3, receives NAergic and serotoninergic fibres. The distal portion of CA1 receives a substantial input from the amygdala (Krettek and Price, 1997b) that appears to be restricted to one third of CA1. Herkenham (1978) has demonstrated the presence of a non specific projection from a central region of the thalamus to different regions of the hippocampus. The CA2 region seems to receive a particularly abundant innervations from mammillary bodies (Haglund et al., 1984). The dentate gyrus is also a target of projections from the hypothalamus through a single innervation that rises from mammillary bodies (Wyss et al., 1979a, b; Dent et al., 1983; Haglund et al., 1984).

An input of noradrenergic nature arrives at the dentate gyrus from the *locus coeruleus* (Pikel et al., 1974; Swanson and Hartman, 1975) and reaches the polymorphic layer where also a projection originating in the core of the

serotonergic raphe terminates. Freund and colleagues (1990) have demonstrated that these serotonergic fibres preferentially reach a class of interneurons of the dentate gyrus, which innervate the distal dendrites of granule cells (Halasy et al., 1991). Finally, the dentate gyrus receives a light and diffuse dopaminergic projection from cells localized in the ventral segmentum (Swanson, 1982).

2.5 Hippocampus and Memory

Memory is the ability of an organism to acquire and retain new information and to utilize that information during behaviour in an environment. Memory compresses time. This means that long bygone events can be remembered now and also in the future, and that future events can be simulated and anticipated in the present, so that an organism can remember and behave more appropriately in subsequent situations similar to the initial learning experience (Tulving, 1995a). Memory and learning are closely-related concepts; on one hand learning requires some information-storing facilities and retention mechanisms like a memory, on the other hand a memory always entails learning. The hippocampal neurogenesis and the cholinergic hippocampal activity play a major role in learning/memory processes and in the state of attention and arousal (Decker and McGaugh, 1991; Matsuzaki et al., 2004). Electrophysiological recordings and molecular imaging studies in animals, as well as MRI imaging studies in humans provided correlative evidence that episodic learning and memory involve hippocampal activity (Vazdarjanova et al. 2004, Guzowski et al. 2001, Gabrieli et al. 1997). In addition, recent data have shown that the main hippocampal neurons are associated to structural plasticity, and suggested that remodelling of hippocampal circuits might

underlies an important aspect of episodic learning and memory (Muller et al. 2002, De Paola et al. 2003 and Galimberti et al. 2006). It has been proposed that changes in neurogenesis in the hippocampus may be involved in some of the alterations of cognitive function.

In 1972, Endel Tulving identified a theoretically far-reaching dissociation of long-term memory: the distinction between episodic and semantic memory. Episodic memory is thought to be the memory system responsible for storing personally-based memories and experienced events. The remembering of such information is accompanied by the conscious retrieval of the temporal (subjective time on a bi-directional time axis, (when)) and spatial (space/location, (where)) setting of those events and experiences. Existent models of memory have demonstrated that episodic memory can be differentiated functionally (Yonelinas, 2002) and neurologically (Eichenbaum et al., 2007) from other forms of explicit memory. Recent evidence indicates that episodic recollection of ideas, and not a generalized sense of familiarity for them, is preferentially involved in reading comprehension because it supports the ability to integrate ideas from the text during retrieval (Mirandola et al., 2011).

Semantic memory describes our general knowledge of the world and is retrieved without knowing when and where it was acquired. That is, no temporal or spatial contextual setting is remembered concurrently with a fact. Semantic memory contains the meaning of words and all other vocabulary, grammatical and arithmetical factual knowledge, and is therefore a repository of facts and concepts (Murre et al., 2001; Simons et al., 2002; Graham et al., 2000; Markowitsch, 1995; Tulving, 1995b).

Clinical observations made in humans and numerous studies conducted on animals damaging have reinforced the belief that the hippocampus has a central role in many forms of learning and memory and the potential contribution of adult neurogenesis to these processes at the system level has been a central question in the field (Squire et al., 1993; Teng and Squire, 1999; Winocur, 1990; Kim et al., 1995; Nadel and Moscovitch, 1997; Song et al., 2012;, et al., 2012).

While the hippocampal system has been shown to be involved in several types of cognitive tasks such as working memory (Olton and Papas, 1979; Nadel et al., 2000), associational memory (Sutherland and Rudy, 1989), representational memory (Eichenbaum et al., 1994), and trace conditioning (Thompson and Kim, 1996), it is also critical for the formation and use of spatial cognitive maps (O'Keefe and Nadel, 1978; Nadel, 1991). For example, lesions of the hippocampal system impair the performance in spatial learning tasks (O'Keefe et al., 1975; O'Keefe and Conway, 1980) and in previously learned spatial tasks (Jarrard, 1983). A decline in neurogenesis may underlie cognitive impairments associated with aging and disorders such as Alzheimer Disease (Clelland et al., 2009; Lazarov et al., 2010). In contrast, the discovery of a *de novo* production of neurons in the adult DG has introduced the possibility of a new form of plasticity that could sustain memory processes. A growing body of evidence supports the view that promotion of adult hippocampal neurogenesis improves pattern separation and spatial memory (Sahay et al., 2001; Stone et al., 2011).

Olsen et al. (2012), reviewed recent research showing that the hippocampus is also involved in short-delay recognition and perception. They concluded that the hippocampus rapidly and continuously forms associations between disparate

environmental inputs, including comparing current perceptual input with internal representations.

The hippocampus is therefore part of a large extended network involving the recognition and learning of material available within working memory. This network incorporates multiple brain regions, including the prefrontal cortex (Buchanan et al., 2004). These functional characteristics have been attributed to the neocortex as a result of various kinds of studies, both pharmacological and electrophysiological. Injuries caused in certain areas *in vivo* of the rodent's brain led to a lower efficiency in the execution of behavioural tests: for example, it has been observed that CA3 lesions are able to destroy the spatial memory (Handleman and Olton, 1981; Morris et al., 1982b). Cholinergic and glutamatergic systems in the hippocampus play a crucial role in cognitive processes (Staubli et al., 1994; Everit and Robbins, 1997; Philippu and Prast, 2001). The septo-hippocampal pathway is activated in the process of memory formation, learning new information and retrieval of old memories. This pathway originates from neurons located in the medial septum nucleus and the diagonal band of Broca (Olton et al., 1979; Gray and McNaughton, 1983; Walker and Olton, 1984; Nicoll, 1985). Hippocampal lesions and the block of cholinergic inputs largely affect exploratory behaviour and habituation to a new environment (Poucet, 1989; Ukai et al., 1994). Memory is not fully determined during the first phase of learning (Hebb, 1949; McGaugh, 1966), but rather remains in a labile form for a certain period of time (minutes or hours) during which it is susceptible to positive or negative modifications before being consolidated. It is therefore correct to say that the storage of memories is a time-dependent process (McGaugh, 1966). Nearly

contemporaneous with the discovery of place cells, the synaptic responses in the hippocampus were found to display plasticity with several features advantageous for memory storage (Bliss and Lomo, 1973). Studies on some forms of human amnesia have shown that some individuals exhibited a selective retrograde amnesia after ECT or brain damage. In these patients, short-term memory had been lost because the information could not be established. The phenomenon is called *sharp wave* and it occurs during a state of drowsiness and sleep whilst in long-wave it is probably the result of a synchronous activity of a small group of CA3 neurons recurrent connections. This phenomenon can be put in relation to the formation of memory processes (Buzsaki, 1989). Stimulation with a high-frequency train of action potentials was shown to produce a prolonged strengthening of synaptic transmission in all three of the major hippocampal pathways (Lüscher and Malenka, 2012). Sutherland and McNaughton (2000) have shown that neuronal activity in the hippocampus and neocortex recorded during certain episodes of behavior is spontaneously re-expressed in some particular states such as in slow-wave sleep. This reactivation of memory traces, in a phase in which the brain is not receiving new stimuli from the outside, appears to be a necessary step in the selection of those synaptic impulses that determine the storage of new experiences towards long-term memory. Through experimental studies on the acquisition of information in the rat, it was observed that the habit of a type of exploratory behaviour can be taken as a reference for a mnemonic acquisition process, even if it is a complex paradigm that involves different mechanisms. These include the answer to the new state of arousal, emotional and environmental conditions, and factors that cause stress. Vice versa, when the

environment becomes familiar, trigger mechanisms and processes of memory recall of previously learned tasks are triggered (Gerhardt et al., 1993, 1994; Tomaz et al., 1990). Thiel and others (1998) using the vertical microdialysis technique in ventral hippocampus of rats have observed an increase in the extracellular levels of ACh positively correlated with the behavioural activity when the animal was placed in an open field for the first time. Re-exposure to the same environment, 24 hours later, resulted in the same activation of the cholinergic system observed on the previous exposure, although the exploratory behaviour was decreased, thus suggesting the onset of habituation. These data have suggested that ACh is released in the hippocampus, not only following novelty and the associated exploratory behaviour, but also during habituation.

2.5.1. Long Term Potentiation (LTP)

The hippocampus had a considerable importance in the study of long-term potentiation (LTP), as an example of synaptic plasticity (Anwyl, 1989 Bliss and Collingridge, 1993), which is believed to represent an important mechanism of learning and memory (Bliss and Lomo 1973; Gruart et al. 2006 and Whitlock et al. 2006). This significant and sustained potentiation of synaptic transmission takes place, for example, following a short and intense pre-synaptic stimulation (100 Hz), for one second, of the Schaffer collateral pathway forming synapses with the CA1 pyramidal cells. In 1973, Bliss and Lomo discovered that, as stimulations were repeated, the synaptic response was greatly increased compared to the first one. A physiological event lasting a few milliseconds was able to cause a change at synaptic level prolonged in time. This phenomenon is termed LTP.

It is known that glial cells actively take part into the immune response in the CNS and might represent a third element of the synapse in addition to the pre- and the postsynaptic neurons, influencing synaptic transmission and in particular synaptic plasticity processes (Bains and Oliet, 2007; Di Filippo et al., 2008a). What was really fascinating was the discovery that this synaptic memory phenomenon recalled closely the learning process and it is assumed to be the underlying mechanism of the memorization and learning process. As mentioned above, the LTP is induced by experience and is durable. It has been demonstrated that not only direct central nervous system inflammation but also systemic inflammatory triggers have the potential to influence synaptic function and memory (Vereker et al., 2000a, 2000b; Cibelli et al., 2010). It has been proposed that inflammation in peripheral tissues results in the synthesis of pro-inflammatory cytokines, which communicate through different ways with CNS synapses to induce a spectrum of behavioural/cognitive changes known as “sickness behaviour” (Cibelli et al., 2010; Dantzer et al., 2008; Konsman et al., 2002; Perry, 2004).

The event of LTP may involve both, the pre-synaptic fibres that release the chemical neurotransmitter and the post-synaptic terminal sensitive to it. When the pre-synaptic terminal is invaded by an electrical impulse, due to transmission of the action potential, the Ca^{2+} ion, present in high concentrations in the synaptic cleft, enters through the voltage-activated channels localized on the pre-synaptic cell membrane. This ion influx triggers a cascade of events leading to the fusion of vesicles with the plasmatic membrane and, consequently, the release of an excitatory neurotransmitter like glutamate. In a few microseconds the released neurotransmitter interacts with the surface of the post-synaptic fibres, where the

AMPA receptor-channels are located, determining the rapid transfer of information (EPSP) to the receiving neuron. In addition to these receptors, as mentioned earlier, there are also NMDA receptors, which play an important role in the slow signalling process: they are able to control the Ca^{2+} ions entry at post-synaptic level, where they reach very high concentrations such as to activate a series of Ca^{2+}-dependent enzymes. Other forms of activity-dependent hippocampal plasticity have been found, including, EPSP-spike (E-S) potentiation, spike-timing-dependent plasticity STDP, depotentiation and de-depression (Dan and Poo 2004; Staubli and Lynch, 1990). Recent study of Malenka and Bear (2004) said that just the opening of NMDA channels and the consequent influx of Ca^{2+} in the post-synaptic fibres is the key event for the induction of LTP processes. There is a mechanism that allows the synapse to maintain the information for a long time. There are two possible theories of interpretation for this phenomenon: a pre-synaptic and a post-synaptic theory. According to the first theory, the pre-synaptic terminal releases more neurotransmitter, whereas according to the post-synaptic theory the amount of neurotransmitter released remains constant, while it is the post-synaptic terminal that becomes more sensitive to the neurotransmitter. It has been discovered that the maintaining mechanism resides in part, if not entirely, at the pre-synaptic level. This is a significant simplification of the problem due to the enormous diversity between the two compartments from both a structural and a biochemical point of view. Pre-synaptic changes can occur because they require a retrograde messenger, something that moves in the opposite direction relative to the pre-and post-synaptic neurotransmitter flow. The process of LTP initiation is post-synaptic, it requires the activation of NMDA receptors localized on the post-

synaptic fibre, while the synaptic modification, capable of supporting a lasting LTP occurs at pre-synaptic level. In view of the well-known anatomical separation of the two compartments, the intervention of a molecule capable of diffusing from the post-synaptic portion, from which it is released, rapidly interacting with the pre-synaptic portion, is required. In the past, arachidonic acid had been considered as a potential candidate for this role. Strong evidence has been gathered in favour of gaseous substances, such as nitric oxide (NO) and monoxide carbon (CO), which obviously can diffuse more easily between the two compartments. The enzymes that produce, respectively, NO synthase and emossigenase, are present in the hippocampal post-synaptic neurons. *In vivo* studies have shown that drugs inhibiting NO synthase are able to cross the blood brain barrier and produce a dose-dependent decrease of learning in experimental animals. A high-frequency hippocampal stimulation via the perforant path induces EPSP in hippocampal neurons that may last for months, triggering the LTP phenomenon (Bliss and Lomo, 1973; West et al. 2002). It is well known that the hippocampal NMDA receptors are involved in the LTP induction and antagonists of these receptors, not only block the formation of such a process, but at the same time deplete, in some behavioural tests, the spatial memory, and some forms of olfactory memory (Morris, 1989; Staubli, et al., 1989; Giurfa and Sandoz, 2012). GABA agonists administration abolished the IPSP induced by GABAergic interneurons located in CA3, CA2 and in the dentate gyrus, it is therefore likely that LTP is blocked by an increase of GABAergic tone in the medial septum. It has been shown that in rats, after LTP induction, there is an increase in endogenous glutamate and GABA release, 60 minutes after tetanus (Ghijsen et al., 1992). The mechanism of this

effect is not known, it seems likely that GABA acts on pre-synaptic $GABA_B$ receptors controlling the release of GABA from GABAergic neurons or of other neurotransmitters. It has been seen that applications of taurine are able to induce an LLP (long-lasting potentiation) on excitatory synaptic potentials hippocampus independently of the action on $GABA_A$ and NMDA receptors for glutamate (Galarreta et al., 1996). This LLP has been explained either by an increase in synaptic efficacy with increased axonal excitability and also seems to be linked with an intracellular accumulation of taurine and seems to be dependent on an increase in intracellular calcium levels. Data from the literature shows that the induction of LTP tetanus involves several families of Ca^{2+}-dependent kinase, which in turn convert the initial synaptic potentiation in a LLP phenomenon (Larkman and Jack, 1995). A subsequent study formulated the hypothesis that some of the mechanisms involved in LTP are also required for the induction of LLP (Del Olmo et al., 2000). In conclusion, the most fascinating aspect of LTP is that it can be studied experimentally at various levels, from a physiological to a molecular study up to the behavioural studies in *in vivo* animals. The study of this form of synaptic memory is linked to the hope of obtaining detailed knowledge on the biological mechanisms at the basis of the coding of the information by lasting synaptic matrices and the structural modifications to load them.

2.6. Interactions of alcohol with neurotransmitter receptors

Alcohol will interact with a range of neurotransmitter receptors present on neurons, as well as glial cells, in specific brain regions, which may induce an imbalance between the different neurotransmitters. DA neurons in the ventral tegmental region (VTA) (Figure 9) will be directly stimulated by an acute ethanol

dose to release dopamine in the NAc. (which is associated with the reinforcing and rewarding action of ethanol) as well as in the prefrontal cortex, PFC.

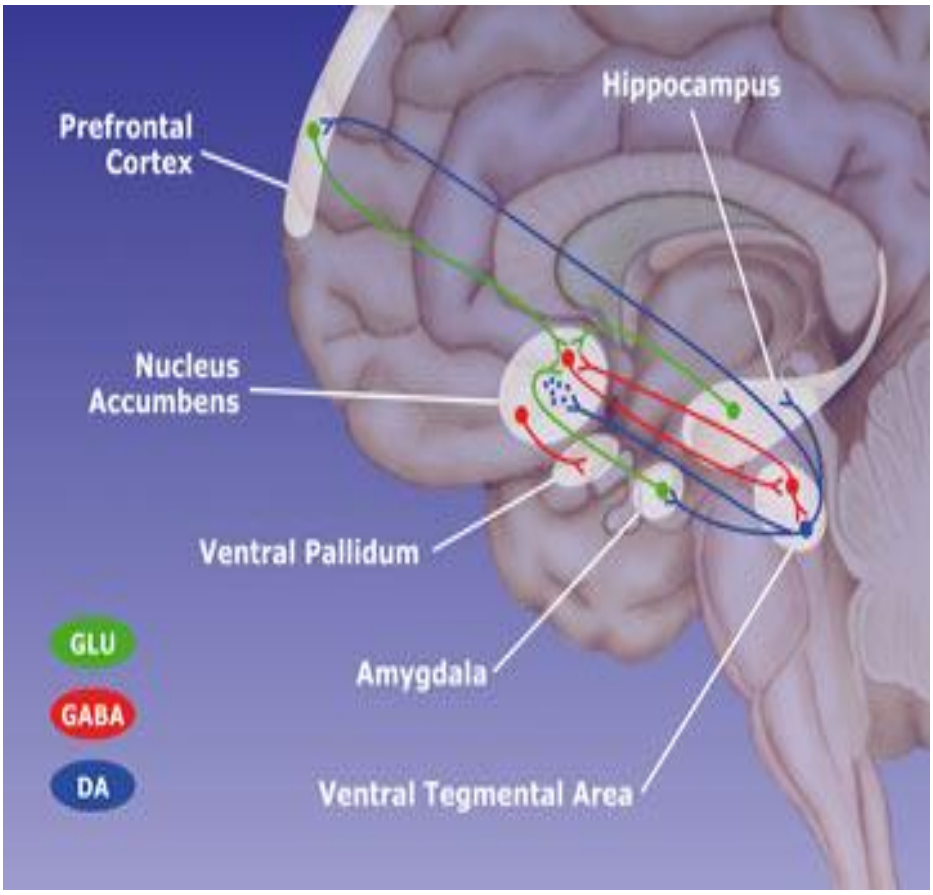


Figure 9: View of neurotransmitter systems affected by alcohol intake.

In addition, in the substantia nigra the neuronal bodies of the nigrostriatal neurons, which synthetise dopamine and release it at their terminals in the striatum, may be targeted by long term alcohol abuse (Figure 9). Increasing alcohol use will induce a decrease of dopamine release, which means less pleasure associated with alcohol, so that more alcohol needs to be drunk to obtain the increased dopamine release associated to pleasure. The glutamatergic input to VTA will preferentially target DA neurons that project back to the PFC and GABAergic neurons that project to the NAc. Glutamatergic inputs will activate the AMPA and NMDA receptors on DA neurons, to induce their firing. There are major

GABAergic feedback projections from the NAc and the ventral pallidum to the VTA. With increasing alcohol intake, the effect of ethanol on each of these neurotransmitter receptors will be modulated, depending on their different subunit composition, (i.e. the presence of different subunits in NMDA and GABAA receptors). Increasing alcohol abuse will result in the down-regulation of both $GABA_A$ and NMDA receptors function.

2.6.1. GABA receptors

The most important inhibitory neurotransmitter in the hippocampus is GABA (gamma-aminobutyric acid) (Roberts et al., 1976). Although glycine is a major inhibitory neurotransmitter in the spinal cord and in some regions of the CNS (e.g. olfactory bulb), this amino acid plays a negligible neurotransmitter role in the hippocampus. More detailed studies on the activity of GABA in the cerebellum, cortex, striatum and hippocampus have shown that this neurotransmitter is mainly localized in short interneurons. Furthermore, it was estimated that GABA acts as a transmitter in approximately 30% of all synapses in the CNS.

In the CNS of vertebrates, there are two types of GABA receptors: $GABA_A$ and $GABA_B$, according to their pharmacological differences (Bowery, 1989), the molecular structure and the different signal transduction mechanisms (Bowery et al., 1987). The $GABA_A$ receptors are ion channel-receptors, permeable to Cl, while $GABA_B$ receptors are coupled to inhibitory G proteins. The distinction between the two types of receptor is based, respectively, on the affinity of $GABA_A$ for the selective agonist (muscimol) and for the competitive antagonist (bicuculline), and on the $GABA_B$ affinity for the selective agonist baclofen. Given that activation of

$GABA_B$ by baclofen decreases the permeability to Ca_2^+, this receptor seems to be linked, via G protein, to a Ca_2^+ ion channel. Since the potential of Nerst relative to chloride ions is negative and close to the values of the rest of the membrane, the opening of ionotropic $GABA_A$ receptors in adult hippocampal neurons results in membrane hyperpolarization and may thus be responsible for a decrease in the excitatory post-synaptic response. The activation of $GABA_B$ receptors leads to the opening of K^+ channels at both, pre-and post-synaptic level (Dutar and Nicoll, 1988a, b; Thalmann, 1988).

For a long time it was thought that the GABAergic inhibitory synapses were mainly located on the cell bodies of pyramidal neurons (Andersen et al., 1964a). More recently it has been observed that these synapses are not only present on the cell bodies but also in the dendritic shaft. In particular, there appears to be a different localization of the two types of $GABA_A$ receptors because the responses of $GABA_B$ receptors are dendritic, while those of $GABA_A$ are distributed along the neuron. It is unclear whether $GABA_A$ and $GABA_B$ coexist on the same synapse and it has been proposed that the final answers is that they are mediated by different interneurons. The GABAergic interneurons located in areas CA3, CA1 and dentate gyrus, respectively, generate an inhibitory postsynaptic potential (IPSP) on the dendrites and neuronal cell bodies and on hippocampal granule cells (Wigstrom and Gustafsson, 1983, Gustafsson and Wigstrom, 1988). Administration of GABAergic antagonists abolished the IPSP and facilitated the establishment of long-term potentiation (LTP) (see section 2.5.1) (Del Cerro et al., 1991).

The $GABA_A$ receptor complex belongs to the super family of ligand-gated ion channel receptors (Figure 10).

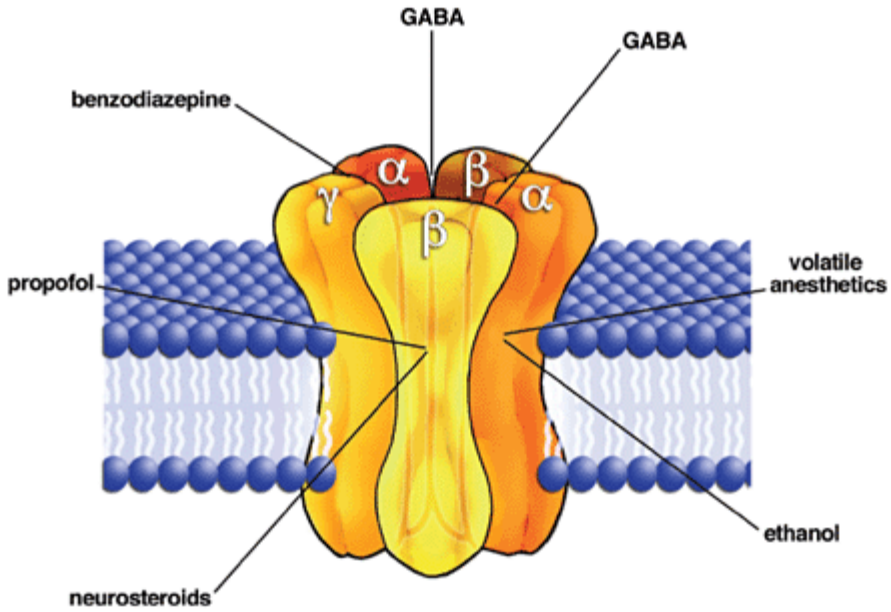


Figure 10: GABA receptor.

When $GABA_A$ receptor is stimulated by GABA, there is an increase in chloride ion permeability, causing hyperpolarization of the neuronal membrane (Pritchett et al., 1989). With increasing alcohol abuse there will be a generalized depression of neural activity, which will induce an anxiolytic effect, together with cognitive, psychological and behavioural changes. Both chronic and acute ethanol ingestion will affect GABA receptors (Faingold et al. 1998; Grobin et al. 1998; Harris 1999; Ueno et al. 2001). Ethanol may enhance GABA-stimulated chloride fluxes at low to moderate doses of ethanol. However, ethanol does not stimulate $GABA_A$ receptor-mediated chloride flux in a similar way in different brain regions (Celentano et al., 1988; Aguayo, 1990; Reynolds and Prasad, 1991; Osmanovic and

Shefner, 1990; Mihic et al., 1992) because of the heterogeneity of the subunit composition of the $GABA_A$ receptors complexes (Givens and Breese, 1990). $GABA_B$ receptors may also play an important role in modulating the interaction between ethanol and $GABA_A$, particularly in the hippocampus, where ethanol induces a blockade of the presynaptic $GABA_B$ receptors (Wan et al., 1996). The $GABA_B$ receptor is a G protein-coupled metabotropic receptor that regulates the activity of K^+ and Ca^{2+} channels, which in turn modulate adenylyl cyclase activity. $GABA_B$ receptors are mainly located presynaptically and their activation reduces GABA release (Misgeld et al., 1995).

2.6.2. Glutamate and NMDA receptors

Glutamate and aspartate are the main transmitters and ubiquitous mediate fast excitatory synaptic responses in the CNS. Glutamate is the most important excitatory neurotransmitter in the hippocampus (Storm-Mathisen, 1977; Roberts et al., 1981). It is released from the perforant path, mossy fibres, the Schaffer collateral and a whole series of excitatory interneurons.

Glutamate plays an important metabolic role, being implicated both in the carbohydrates and in the nitrogen metabolism. It is a constituent of many proteins and has a key role in the synthesis of important cofactors such as folic acid and glutathione. Finally, it is the precursor of the synthesis of GABA. Since the blood-brain barrier is almost impermeable to glutamate, this must be synthesized in the nervous tissue. A source of glutamate and aspartate is represented by intermediates of the Krebs cycle in particular alpha-ketoglutarate through an aminotransferase. The most important way for the neosynthesis of glutamate as a

neurotransmitter is the deamination of glutamine by glutaminase, an enzyme found in excitatory neurons. The intermediates of the reactions which lead to glutamate and glutamate it-self are present in all cells, therefore are not useful for the identification of neurons that contain excitatory amino acid neurotransmitters. Glutamate acts on two types of receptors: ionotropic and metabotropic receptors, characterised not only in terms of their structure, but also according to which mechanism of signal transduction they use. In the case of the NMDA receptor, the receptor site is coupled, directly or not, to an ion channel the opening of which allows the passage of ions through the membrane with consequent variation of the action potential; in the case of AMPA and KA receptors (non NMDA receptors), the receptor site is coupled, via G protein, to a system of second intracellular messengers.

The ionotropic receptors are further divided, according to their pharmacological and electrophysiological responses to exogenous agonists, in three main subtypes (Morrison et al., 1996).

At the level of the nervous system, aspartate, and above all glutamate, also cover important physiological functions, such as the dendritic and axonal development and consolidation, synaptogenesis, synaptic plasticity (Collingridge and Singer, 1990) as well as learning and memory (McDonald and Johnston, 1990). In addition, alteration of glutamatergic neurotransmitters are associated with hypoxia and ischemia (Choi, 1992) as well as chronic conditions, such as dementia, Alzheimer's, Huntington's and Parkinson's disease (Monaghan et al., 1989). In the hippocampus, most neuronal networks use the excitatory neurotransmitter glutamate (Davies and Collingridge, 1989); this brain area containing a high

density of glutamatergic receptors (Monaghan et al., 1983). Various combinations of AMPA, kainate and NMDA receptors are present on the whole hippocampal excitatory network; receptors of the NMDA type, for example, are located at the synaptic level of mossy fibres (Monaghan et al., 1983). It has also been suggested that some synapses of the Schaffer collateral contain only receptors of the NMDA type (Isaac et al., 1995; Liao et al., 1995). Metabotropic receptors are present both at pre- and post-synaptic sites, where they coexist with ionotropic receptors modulating the pre-synaptic release of neurotransmitters (Schoepp and Conn, 1993).

Alcohol has been shown to block the binding of glutamate to the NMDA receptor, (Figure 11), and reversibly reduce sodium transport together with an up-regulation of the density of voltage-dependent calcium channels.

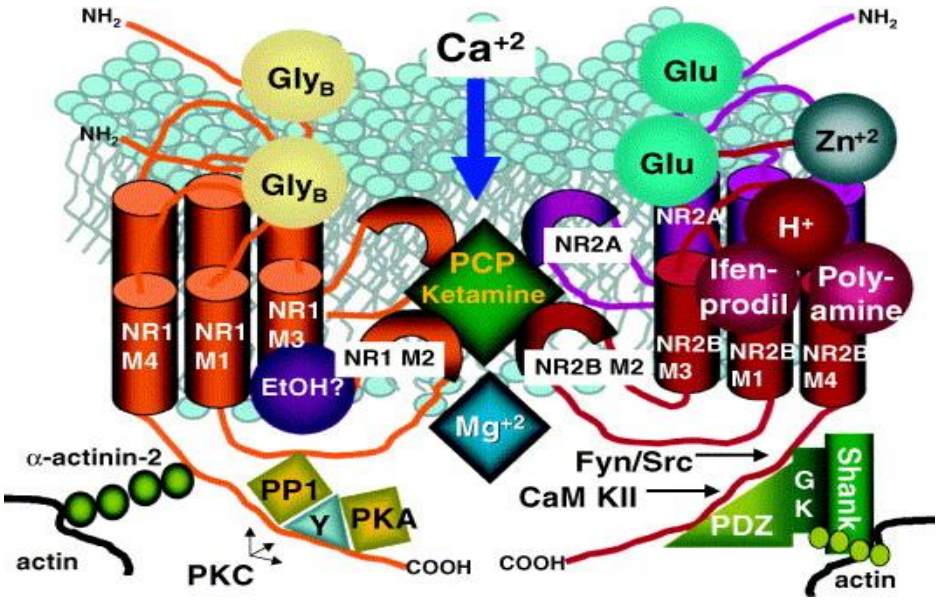


Figure 11: NMDA receptor.

The NMDA receptor has been considered to be the most sensitive to the effects of ethanol (Hoffman et al., 1989; Lovinger et al., 1989). Low concentrations (5 to 10 mM) of ethanol inhibit the function of this receptor. It is a cation channel permeable to Ca^{2+}, as well as to Na^{+} and K^{+}, and various pharmacologically distinct sites on the receptor protein also modulate the ion flux through this channel. These sites include (1) the glutamate and NMDA recognition site, (2) a strychnine-insensitive glycine binding site, (3) a site within the channel that binds phencyclidine-like compounds, (4) a voltage-dependent Mg^{2+} binding site, and (5) a modulatory site that binds Zn^{2+} (Mori H and Mishina M, 1995). NMDA receptors in different anatomical areas of the brain differ in their sensitivity to ethanol, (Tabakoff and Hoffman, 1996) which has also been related to the subunit composition of the receptors. Ethanol has been reported to affect the function of "non-NMDA" ionotropic glutamate receptors, inhibiting these receptors too (Dildy-Mayfield and Harris, 1994).

2.7. Neurotransmitters

There are many other neurotransmitters which are believed to act on metabotropic receptors through G proteins, these include: norepinephrine, dopamine, serotonin, and neuropeptides. Because of their indirect action through G proteins it is difficult to classify their nature as excitatory or inhibitory, because their action depends on the state of the neuron, and thus we refer to them as neuromodulators (Kaczmarek and Levitan, 1987). Another neurotransmitter present in the hippocampus, is serotonin, which interacts with 5-HT3 ionotropic

receptors (Jackson and Yakel, 1995). These receptors are not channel-selective for cations and their opening produces a depolarization of the membrane.

2.7.1 Neuromodulators

At the pre- and post-synaptic hippocampal level there are inhibitory neuromodulators. They are epinephrine, serotonin, dopamine and neuropeptides. In addition there is taurine, which is considered a neuromodulator, although its role in the CNS has not been defined precisely. It is involved in many important physiological functions which include calcium homeostasis, and osmolarity.

2.7.2. Taurine

Taurine (TAU) (2-aminoethanesulfonic acid) is a nonessential sulfonated amino acid that contains a sulfonic group instead of a carboxylic one. It is an β amino acid, and it is a structural analogue of GABA. TAU was isolated for the first time in 1813 in the beef bile (Bos Taurus). In the liver taurine is conjugated with cholic acid to form the corresponding taurocholic acid with an emulsifier function and with the capability of facilitating the absorption of triglycerides in the intestine.

This inhibitory amino acid is abundant in mammalian tissues (millimoles), second only to glutamate (Jacobsen and Smith, 1968; Saransaari and Oja 2000), while it is absent or present only in trace amounts in plants and in bacteria (Huxtable, 1992). High amounts are present in muscle, particularly in the heart. The concentration of taurine in the developing hippocampus is markedly greater than in the adult hippocampus (Oja and Saransaari 2009). Most taurine synthesis

occurs in the liver (Tappaz et al., 1992), the brain being able to synthesize a limited amount of TAU. The major route for the synthesis in the liver as well as the CNS is through the sulfhydryl group of cysteine (Figure 12).

TAU is first oxidized to cysteine sulfinic acid by the enzyme cysteine dioxygenase. Cysteine sulfinic acid, in turn, is decarboxylated by sulfinoalanine decarboxylase to form hypotaurine. It is unclear whether hypotaurine is then spontaneously or enzymatically oxidized to yield TAU.

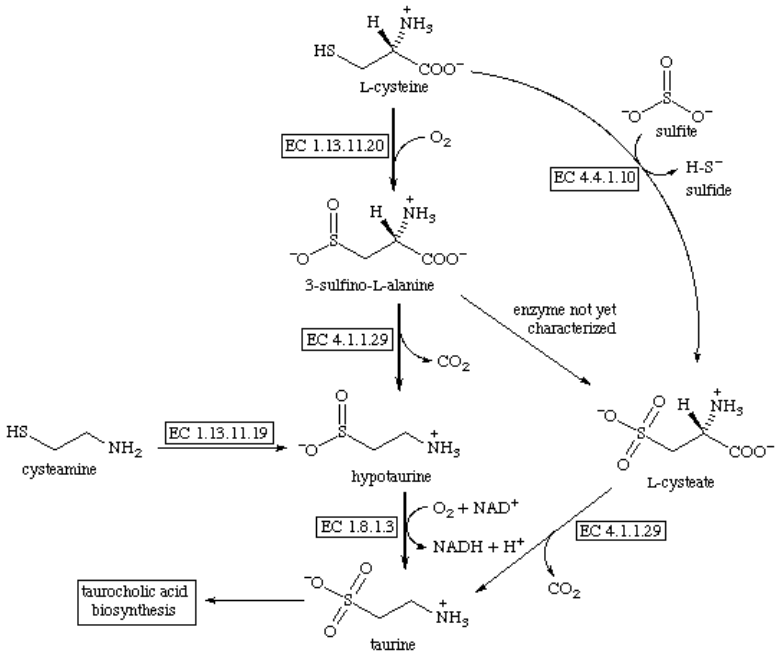


Figure 12: Biosynthetic pathways of taurine.

The uptake of TAU across cellular membranes is an energy dependent process, temperature sensitive and Na^+ dependent. Such transporter mediates the uptake of other amino acids like GABA and glycine, at the BBB, the presynaptic terminals and surrounding glial cells. Two types of transporter for TAU have been described: a saturable low-and high-affinity, and a non-saturable form. The

constants for the saturable high affinity transporter are of the same order of magnitude as those of GABA and glycine transport systems. These high affinity transporters have been described in many brain regions such as cortex, hippocampus and striatum, using brain slices of rat (Oja and Kontro, 1983).

TAU produces a neuroinhibitory effect interacting as an agonist with $GABA_A$ receptors (Okamoto and Sakai, 1981) and it has therefore been proposed as an inhibitory neurotransmitter (Taber et al., 1986), although, as yet, there is no overall consensus of the existence of a membrane taurine receptor. Taurine is widely distributed in the nervous tissue and it is present in high concentrations (mM) with marked differences in various brain regions (Kontro and Oja, 1983). Studies on the cellular and intracellular distribution by immunocytochemical techniques have revealed its presence in neurons as well as in glial cells (Ida et al., 1987). The function of TAU in the CNS remains unclear. Evidence of a possible role of neurotransmitter is suggested by the presence of an uptake system of high affinity for TAU in various preparations of the CNS (Borg et al., 1976; Oja et al., 1976; Lombardini 1977) and by the demonstration in microdialysis experiments of a DNQX- and TTX-dependent KA-evoked release from striatonigral terminals (Bianchi et al., 1996) in a fashion similar to GABA, the main neurotransmitter of this pathway. However, more than 30 years of work by several groups of researchers have not clarified the physiological and pharmacological actions of TAU. Nevertheless, TAU appears to play an important role in several essential biological processes such as development of the central nervous system and the retina, reproduction, osmoregulation and neuromodulation (Albrecht and Schousboe, 2005). In several processes involving brain development and

neurotransmission has been shown to exhert neuroprotective actions in neural tissues (Saransaari and Oja., 2000; Della Corte et al., 2002) and immunomodulatory actions (Schuller-Levis and Park, 2003; Huxtable, 1992, 2000; Schuller-Levis et al., 1990; Sturman, 1993). However, the role of TAU in hippocampal neurogenesis during brain development is still not clear (Mattu et al., 2012). TAU is suggested to have an important role in brain development: as its levels are 3–4 times higher in developing and neonatal brain than in adult brain (Agrawal et al., 1968) and in children are about 4-fold greater than those in adults (Schuller-Levis and Park, 2003).

TAU is essential for the mammalian development. Studies *in vivo* in various species have shown that low levels of TAU are associated with various pathological lesions, especially if the deficiency occurs during development (Sturman, 1993). It has been widely demonstrated that taurine is involved in many important physiological functions. It is a trophic factor in the CNS development (Sturman et al., 1985,1993), it is responsible for maintaining the membrane structural integrity (Pasantes-Morales and Cruz, 1985; Moran et al., 1988). It has a regulatory role in the binding and transport of calcium (Lazarewicz et al., 1985; Lombardini, 1985), it is a neuromodulator (Kuriyama, 1980) and it is neuroprotective against neurotoxicity induced by excitatory aminoacids (Tang et al., 1986; Pan et al., 2010).

Taurine can effectively prevent glutamate-induced neuronal injury in cultured neurons. This amino acid has been shown to protect against H_2O_2-induced cell injury in PC12 cell cultures by reducing H_2O_2-induced ER stress. It is generally believed that taurine's neuroprotective functions are due to its role in reducing

intracellular free Ca^{2+} concentration, $[Ca^{2+}]_i$, and its anti-oxidative stress capacity (Chen et al., 2001; Schaffer et al., 2003). Wu and Prentice (2010) have shown that taurine can shift the ratio of the anti-apoptotic protein, Bcl-2 and the pro-apoptotic protein, Bax, in favour of cell survival (Leon et al., 2009). In addition, it has been demonstrated that glutamate-induced activation of calpain is inhibited by taurine, resulting in a decrease of the formation of Bcl-2 hetero-dimers and Bax with the subsequent inhibition of the release of cytochrome C and the apoptosis cascade (Leon et al., 2009).

- **Taurine in hippocampus**

The activation of excitatory hippocampal network is modulated by GABA as the main inhibitory transmitter released by the interneurons. TAU, a structural analogue of GABA, found in high concentrations in the hippocampus where it plays an inhibitory role on CA1 pyramidal cells, acts as an agonist of $GABA_A$ receptors, by increasing the conductance of chloride ions and determining a membrane hyperpolarization (Lombardini, 1976; Kontro et al., 1980; Palkovits et al., 1986; Taber et al., 1986).

TAU immunoreactivity in rat hippocampus has been localized in interneurons, in pyramidal neurons and in the dentate gyrus granular cells (Clements et al., 1989; Magnusson et al., 1989). In addition, its synthetic enzyme, cysteine sulfinic decarboxylase, has been identified in the pyramidal interneurons of the basket cells (Taber et al., 1986).

An involvement of TAU in the LLP (long-lasting potentiation) has been observed, which in turn seems to be induced by the LTP (Del Olmo et al., 2000).

In vivo microdialysis studies on adult animals have shown that TAU is released from the hippocampus following stimulation of AMPA, kainate and NMDA receptors (Lehmann et al., 1986; Menendez et al., 1989). This may indicate an interaction with the glutamatergic system. This interaction is probably important for the protective role that TAU can play against epilepsy, hypoxia, ischemia and excitotoxicity (Oja and Kontro, 1983; Schurr et al., 1987; French et al., 1986). For example, it has been observed that the extracellular levels of glutamate and TAU increase in the hippocampus of rats during ischemia (Benveniste et al., 1984; Korf et al., 1988; Lekieffre et al., 1992).

In the hippocampus, cell-damaging conditions increase the release of excitatory amino acid neurotransmitters, and of TAU (Saransaari and Oja 1997a, 1998). Furthermore, the activation of ionotropic glutamate receptors enhances TAU release (Saransaari P and Oja, 1997b). Metabotropic glutamate receptors also participate in the regulation of TAU release in both developing and adult hippocampus (Saransaari and Oja, 1999).

Despite the considerable amount of data on the subject a specific role of TAU in the hippocampus has not yet been identified.

- **Taurine and its analogues**

A taurine based analogue, acamprosate, calcium acetyl-homotaurine, was developed for clinical use as an anti-craving drug in detoxified alcohol abusers which is structurally related to GABA and taurine (Figure 13).

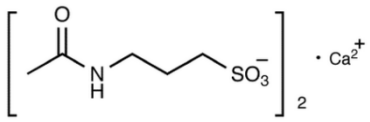


Figure 13: 3-Acetamidopropane-1-sulfonic, also known as N-acetyl homotaurine

More recently another TAU analogue ethane-β-sultam (β-sultam) (Figure 14) has been developed by chemists at Huddersfield University, UK, which has the advantage of being able to cross cellular membranes before being hydyolysed to TAU.

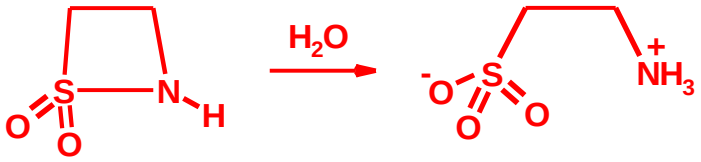


Figure 14: N-ethane β-sultam hydrolyses slowly to taurine.

In such a way it is possible to override the cellular control of TAU uptake which is under normal circumstances carefully controlled by the TAU transporter. It is therefore proposed that administration of β-sultam may elevate intracellular TAU levels, which will have various beneficial actions including suppression of inflammatory stimuli via stabilization of NfκB (Ward et al., 2011). This transcription factor plays a major role in orchestrating the inflammatory response by cells, particularly phagocytic cells such as macrophages and microglia.

3 Microglia

Inflammation is an innate non-specific defence mechanism which helps to defend the organism against damage from physical, chemical and biological agents. Neuroinflammation is a process which occurs in the brain, which is mediated by glial cells, particularly microglia and results in the release of inflammatory substances which includes cytokines. Approximately 5-10% of the brain cells are present as microglial cells. Microglia are the immune cells of the central nervous system (CNS), which act as a surveillance system to protect the brain from damage by engulfing dead cells and debris (Allen and Barres, 2009), it is functionally similar to macrophages (Vallejo et al., 2010) and play important roles under physiological and pathophysiological conditions (Tremblay et al., 2011).

Under normal physiological conditions, microglia is involved in immune surveillance and host defence against infectious agents (Perry et al., 1993). Microglia has also been implicated in synaptic remodelling during the development of the nervous system, when they are proposed to remove inappropriate synaptic connections through the process of phagocytosis (Allen and Barres, 2009).

Microglia readily becomes activated in response to a variety of CNS diseases including ischemia, inflammation, injury or immunological challenges. Once activated, the microglia exhibits a distinct neuronal plasticity, consisting of morphological changes in the number of cells, the expression of surface receptors and in the production of growth factors and cytokines. (Streit, 2000).

The microglial cells morphology in the quiescent shape is defined by a cell body and oval long and thin branched prolongations, which project radially to the

body. In the active form, however, such cells take on a form amoeboid and all the typical characteristics of macrophages at the functional level (Figure 15) (Davis et al., 1994; Streit, 2000).

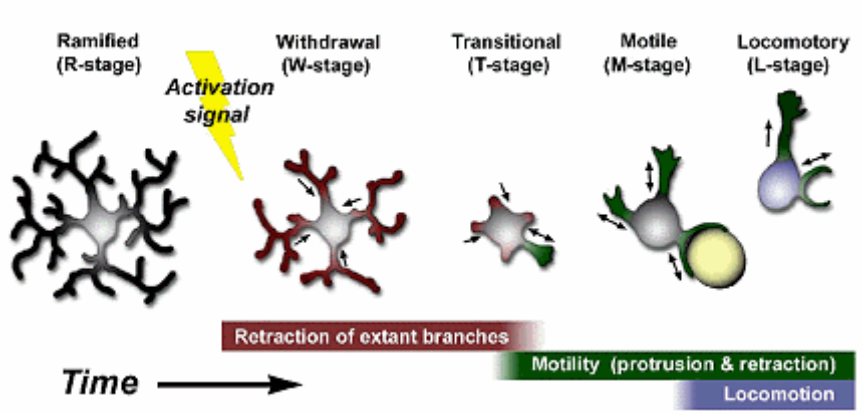


Figure 15: Microglial activation sequence.

The amoeboid phenotype is characterized by a big cell body and by the change in the number and morphology of the extensions, compared to quiescent form, which are decreased in number, being shorter and not branched.

Following an ischemic event, microglial cells change their morphology and begin to proliferate, produce neurotoxins (Dirnagl et al., 1999), migrating from their seats of rest towards the damaged area (Bruss-Keller, 1999; Stoll et al., 1998; Marks et al., 2001). Activated microglia release a variety of neurotoxic factors, such as IL-10 and TNFα as well as reactive oxygen and nitrogen species. These are the central mechanisms for microglia-mediated neurotoxicity (Block et al., 2007).

4 Nitric oxide, NO

NO plays vital roles in various physiological and pathological processes in many organ systems, including the brain (Ritt et al., 2011; Souza et al., 2010; Sato et al., 2011). NO is enzymatically formed from arginine by the enzyme NO synthase (NOS). It was discovered in 1992. Nitric oxide (NO) is a short-lived, endogenously produced gas that has a signalling function in the immune, cardiovascular, and nervous systems (Bredt and Snyder, 1992; Esplugues, 2002; Holscher, 1997; Prast and Philippu, 2001). NO is produced by nitric oxide synthases (NOSs), which catalyze NO formation from L-arginine, O_2, and NADPH and can react with superoxide to generate the toxic molecule, peroxynitrite (Kavya et al., 2006) (Figure 16).

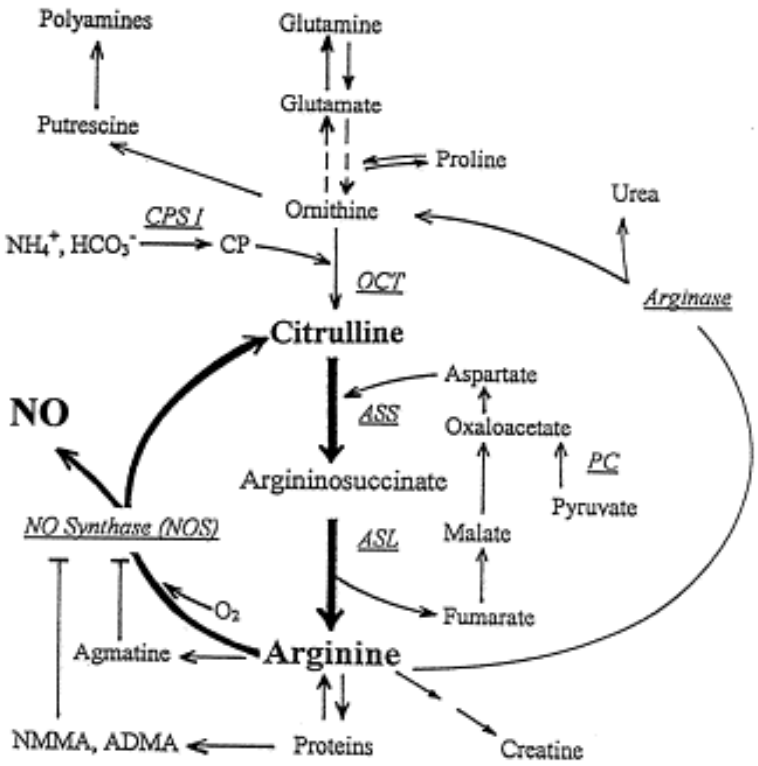


Figure 16: NO synthesis in mammalian cells (Wiesinger, 2001).

Constitutively expressed NOS, neuronal NOS, nNOS, endothelial NOS, eNOS, and mitochondrial NOS, mtNOS, continuously produce NO in beneficial amounts. The brain levels of NO are determined by the expression and/or activity of NO synthase which is present in three isoforms: two constitutively expressed: neuronal (nNOS or Type I) and endothelial (eNOS or Type III) and one inducible (iNOS or Type II).

Both nNOS and eNOS are constitutively regulated by calmodulin, a protein sensitive to Ca^{2+} (Bredt and Snyder, 1990; Bredt, 1999). iNOS shows Ca^{2+}-independent activities (Ferreira et al., 2010; Wang et al., 2010). During neuronal damage, the isoforms nNOS and eNOS can be induced and synthesized de novo (Wu et al., 1994; Chen and Aston-Jones, 1994).

All three isoforms require coenzymes, such as the Heme complex tetrahydrobiopterin, to carry out their catalytic function.

Endothelial NOS (eNOS) is bound to membranes in blood vessels and regulates vascular function through NO; mitochondrial NOS, via NO, helps regulate mitochondrial oxygen consumption and transmembrane potential (Giulivi 2003; 2007). Neuronal NOS (nNOS) is present in both neurons and endothelial cells and is immediately stimulated upon insult to the CNS, potentially producing damaging concentrations of NO. In pathological situations, a second phase of NO production is triggered by the transcriptional activation and production of the inducible form of NOS (iNOS) (Conti et al., 2007). iNOS is known to produce large amounts of NO over prolonged periods of time. The inducible form of NOS as well as eNOS is present in activated astroglia and microglia, (Barna et al., 1996; Gabbott and Bacon, 1996; Iwase et al., 2000).

Inducible NOS is not normally present in the healthy brain and is present only in excessive amounts in pathological conditions (Conti et al., 2007), when it may be found in many cell types (Knott et al., 2000). Normally glial cells will release small regulated amounts of NO which will play a role in several functions in the CNS including the induction and maintenance of synaptic plasticity, neurotransmitter release, and removal of pathogens (Dawson et al., 1992; Chen et al., 2004) as well as learning and memory (Prast and Philippu, 2001). Many studies demonstrated that in several brain areas including the cortex, cerebellum, and hippocampus, NO is involved in the induction of long-term potentiation (LTP) and long-term depression (LTD) which are considered cellular models of learning and memory (Calabresi et al., 1999; Haley et al., 1996; Haul et al., 1999; Hawkins et al., 1998; Lev-Ram et al., 1997; Lu et al., 1999; O'dell et al., 1994; Wang et al., 2005; Zhuo et al., 1994; Zhuo, et al., 1998). However, the role of different isoforms of NO in the learning process is still not clear and the data are inconsistent.

NO synthesis may be beneficial or destructive based on the levels of NO, from where it is produced, by the oxidative stress entity and the type of neurodegenerative process (Figure 17). NO acts as a neurotransmitter by mediating the release of neurotransmitters such as acetylcholine in the nucleus accumbens through the stimulation of glutamatergic neurons in many brain areas. Basal concentrations of NO may also reduce the release of GABA while high levels will increase the release of this inhibitory transmitter (Ohkuma et al., 1998). NO increases the release of noradrenaline and glutamate in the hippocampus (Lonart et al., 1992).

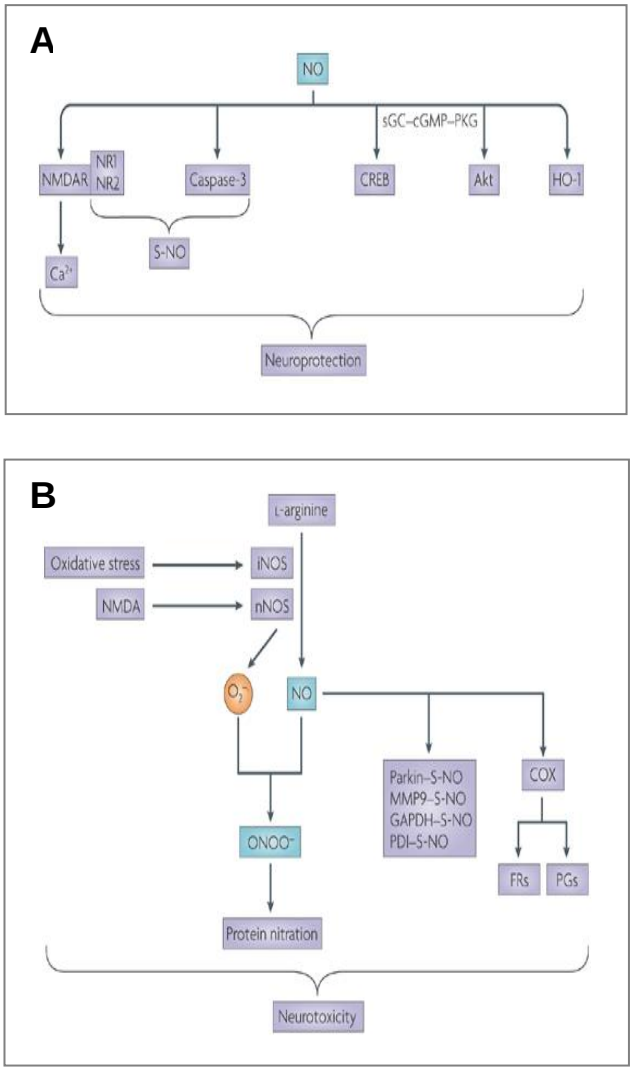


Figure 17: Beneficial and toxic effects of NO synthesis.

5 Cytokines

Cytokines are small cell-signaling protein molecules that are secreted by numerous cells, including microglia and astrocytes, in picomolar concentrations. During neuroinflammation their concentrations can increase up to a 1000 fold.

Tumour necrosis factor-α (TNF-α) is one of the main cytokines in the inflammatory process. TNF-α exists in soluble and in transmembrane form, after cutting by the enzyme TACE (TNF-α converting enzyme). The balance between transmembrane and soluble form depends on the state of cell activation and is crucial to its activity. TNF-α soluble receptors acts on trasnmembrane receptors (TNFR1 and TNFR2) that activate different signalling pathways, which involves the transcription factor NFκB, which positively regulates the transcription of several pro-inflammatory genes.

In the nervous system TNF-α has many important functions which include the regulation of blood brain barrier permeability, as well as glutamatergic transmission and synaptic plasticity (McCoy and Tansey, 2008). Over expression of TNF-α has many neurotoxic effects. It has been shown to be increased in the serum of patients with Alzheimer's Disease, Parkinson's disease and multiple sclerosis (Fillit et al. 1991) as well as during cerebral ischemia. It has been shown that treatment with drugs that inhibit TNFR1 receptor will lead to a reduction of ischemic damage. However it would seem clear that treatment with anti-TNF-α, will not discriminate the activity on the various receptors, and may lead to a reduced hippocampal neurogenesis level. These observations suggest a different role of TNF-α receptors (Figure 18) (McCoy and Tansey, 2008).

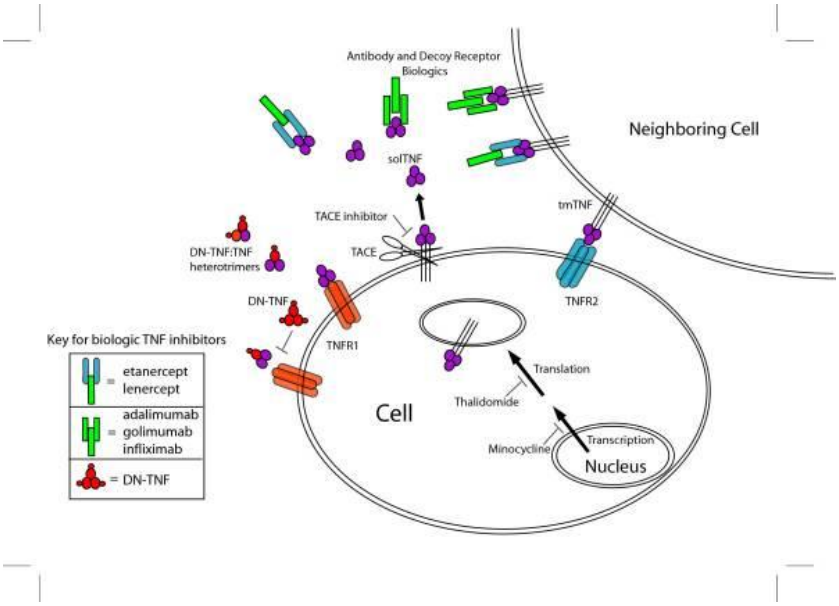


Figure 18: Schematic representation of TNF inhibitors and their mode of action.

IL-6 is also a pro-inflammatory cytokine produced by macrophages, microglia, astrocytes, T lymphocytes, fibroblasts, endothelial cells and keratinocytes. IL-6 will induce fever and will also coordinate immune response. IL-6 is able to activate B cells inducing them to synthesize antibodies. Unlike IL-1, IL-6 also possesses anti-inflammatory functions, in particular inhibits TNF-α synthesis and induces the synthesis of soluble receptors for IL-1 and TNF-α, which decrease the proportion of cytokines available. High serum levels of IL-6 were measured in patients with acute ischemia, and in animal models, IL-6 is induced following ischemic insult in the CNS, in particular in the peripheral region of the ischemic zone. It has a dual role: contributing to both brain damage as well as repair mechanisms. This is due to the binding of IL-6 with the gp 130 receptor. Such observations suggest a protective role of IL-6 in cerebral ischemia (Clark et al. 2000).

IL-10 is a potent anti-inflammatory cytokine, produced primarily by monocytes-macrophages, microglia and, although in lesser amounts, by lymphocytes. It is capable of inhibiting the expression of pro-inflammatory cytokines, such as TNF-α, INF-γ, IL-2 and IL-3. In the brain plays an important role in the control of the neuro-inflammatory state. It is up-regulated after ischemia, produced by glia, and exerts neuroprotective action.

Knock out IL-10 animals, which were subjected to focal ischemia, show an infarcted area wider, and other studies report that the administration of IL-10 or it's over expression causes a reduction of infarct volume and inflammation (Planas et al., 2006; De Bilbao et al., 2009).

II. AIM OF THESIS

1. To develop an appropriate animal model of binge drinking.
2. To investigate changes which occur in hippocampal neurotransmitters after a binge drinking regimen.
3. To investigate whether inflammation occurs in the periphery and the brain after a binge drinking regimen.
4. To show whether such inflammation is associated with neuronal loss.
5. To associate such changes with cognitive function, with respect to memory.

III. MATERIALS AND METHODS

1. Materials

The following commercial products were used: 17 amino acids stock solution (Pierce, Rockford, Illinois, USA), GABA, 2-aminoethanesulfonic acid (taurine), o-phtaldialdehyde (OPA), 2-mercaprtoethanol, 3, 3'-diaminobenzidine Tetrahydrochloride hydrate (DAB), Glacial acetic acid, Chloral hydrate and Formaldehyde (Merck, Darmstadt, Germany), Methanol (BDH, England), Xylene, Ethanol, Hydrogen chloride, Hydrogen peroxide, Gelatin from bovine skin (Panreac, Barcelona, Spain), triton X100 (Probus Genève), Glycine, Tris-HCl (USB). Vectastain ABC peroxidase kit, OX-6 antibody (Serotec, Oxford, UK), Biotinilate horse antimouse secondary antibody (Vector Laboratory, Peterborough, UK).

Lypopolyshaccaride, LPS, interferon gamma (Sigma).

N9 microglial cell line donated by Dr. Paola Ricciardi Castagnoli (CNR Cellular and Molecular Pharmacology Centre, Milan, Italy), IL-6 and TNFα ELISA kit (R&D System, Abingdon, UK).

Ethane β-sultan was supplied by Professor Mike Page.

Multiple well plates (Corning Inc, NY, USA), Dulbecco media.

All tissue culture media and chemicals were purchased from Sigma (Belgium) unless stated otherwise.

2 Methods

2.1 Synthesis of ethane β-sultam

Taurine sulfonyl chloride (30.4 g, 169 mmol) was added to finely ground sodium carbonate (35.9 g, 339 mmol) in ethyl acetate (950 ml and stirred at ambient temperature for 48 hours. The reaction mixture was filtered through celite and the solvent removed by reduced pressure rotary evaporation at 30.8C, giving a fine white powder (15.9 g, 89%).

Melting points were determined on a Gallenkamp melting point apparatus, and were 50–51 8C (lit. 51–52.8C (Page MI, 2004). 400 MHz 1H and 67 MHz 13C NMR spectra were determined on a Bruker Advance 400 MHz spectrometer, while for the 500 MHz 1H and 100MHz 13C NMR spectra a Bruker AMX 500 spectrometer was utilized. The results were; 1HNMR: d (CDCl3) 3.39 (2H, dt, J 4 and 7, CH_2N); 4.32 (2H, dt, J 2 and 7, CH_2SO_2); 5.53 (1H, bs, NH). 13CNMR: d (CDCl3)60.6, 26.8. Infra-red measurements were determined on a Gallenkamp melting point apparatus and were: nmax (cm 1): 3307, 3048, 3022, 2991, 2918, 1416, 1336, 1299, 1249, 1212, 1171, 1156, 1107, 966, 803, 760, 668, and 615. GC–MS were determined on a Varian GC–MS with a Finnigan MAT ion trap detector. For mass spectrometry a Fisons Quatro VG quadrupole mass spectrometer was utilsed; m/z (GC–MS) (M+H):108, 77, 54, 42. The pKa of ethane b-sultam was determinedbytitration using the reversible chromophoric change at 230 nm, while hydrolysis of ethane β-sultam to the β-amino acid taurine as a function of pH was followed at 300 nm.

2.2 Animals housing

The experimental procedure were performed using adolescent female Wistar rats (Harlan-Nossan, Udine, Italy) at puberty (6 weeks of age) with average body weights around 125-135g. They were housed under controlled humidity and temperature (≈ 23 ± 1 °C) with a dark/light cycle consisting in 12 light and 12 dark hours and with free supply of food and water within a polypropylene cage. All animals were treated to Italian Guidelines for Animal Care (D.L. 116/92) and European Communities Council Directives (86/609/ECC) whit all efforts to minimize animal sufferings and the number of animal necessary to collect reliable scientific data.

2.3 Treatment & Binge Drinking Regimen

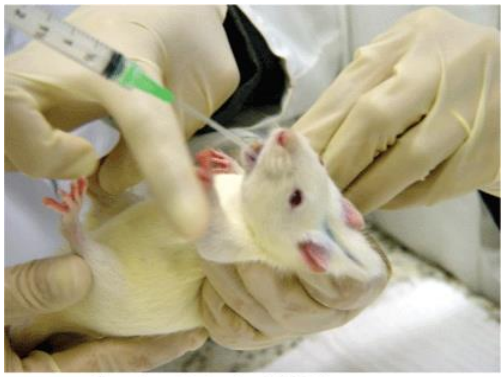


Figure 1 - Administration of DMBA by gavage.

Animals were administered 1g/kg or 2g/kg ethanol +/- β-sultam by gavage. In the Binge Drinking (BD) regimen rats received 1g/kg or 2g/kg of ethanol (20%) by gavage, 3 times per day with 3 hours intervals on 2 consecutive days, followed by 5 days of abstinence. This was repeated for a total of three weeks. At the same time points the control rats were administered water alone as the BD ethanol rats.

β-sultam was freshly prepared before each administration, (2.86 mg/ml) and also administered by gavage at a dose of 0.028 g/kg. The group of rats, which were to be given β-sultam, received the compound alone each morning starting from one

week before the BD regimen, at 09.00 h. During the subsequent 3 weeks of BD, β-sultam was administered 1 hour before the BD regimen.

2.4 Surgery and Microdialysis procedure

At the end of the third week the rats were prepared for microdialysis by insertion and cementing of the cannula (concentric design, CMA Microdialysis AB, Stockholm, Sweden) (Figure 19).

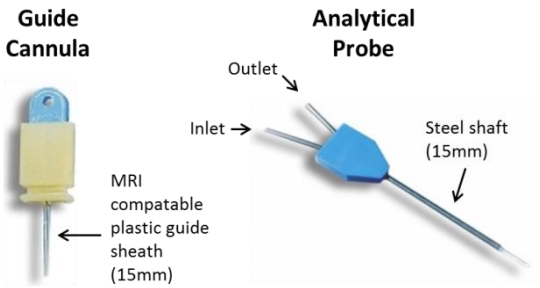


Figure 19: Guide Cannula and Analytical Probe used.

Rats were anaesthetised with chloral hydrate (400 mg/kg i.p.) and were mounted securely on a sterotaxic frame (Stellar, Stoelting Co., Wood Dale, IL, USA) (Figure 20).

The scalp was then cut and separated to expose the cranium. The skull was levelled by ensuing that the lambda and bregma points were at the same horizontal co-ordinates and then the stereotaxic frame was use to locate the point of the right ventral hippocampus by using the bregma as a "calibration point" and a transformation from that position of AP -4.8, L- 5.2, V- 4.0 (Figure 21).

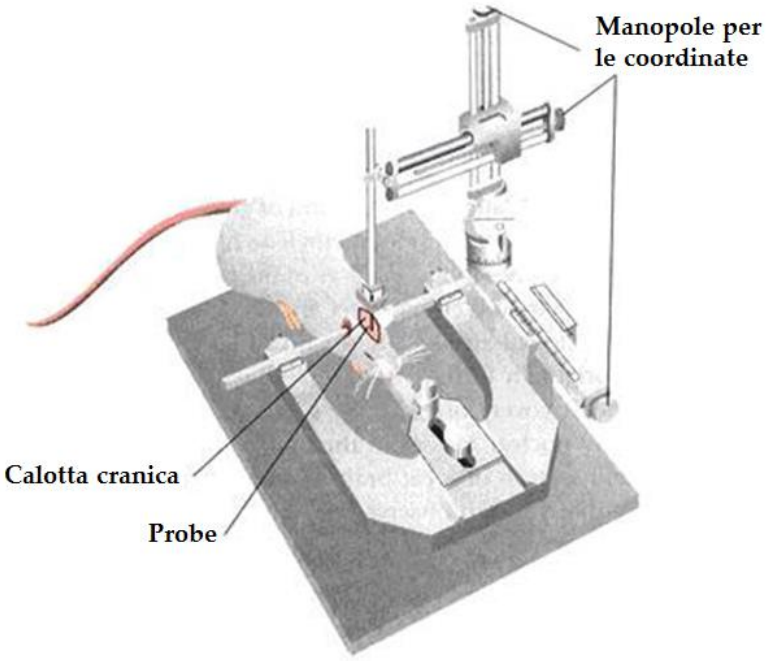


Figure 20: Stereotaxic and right location of the animal.

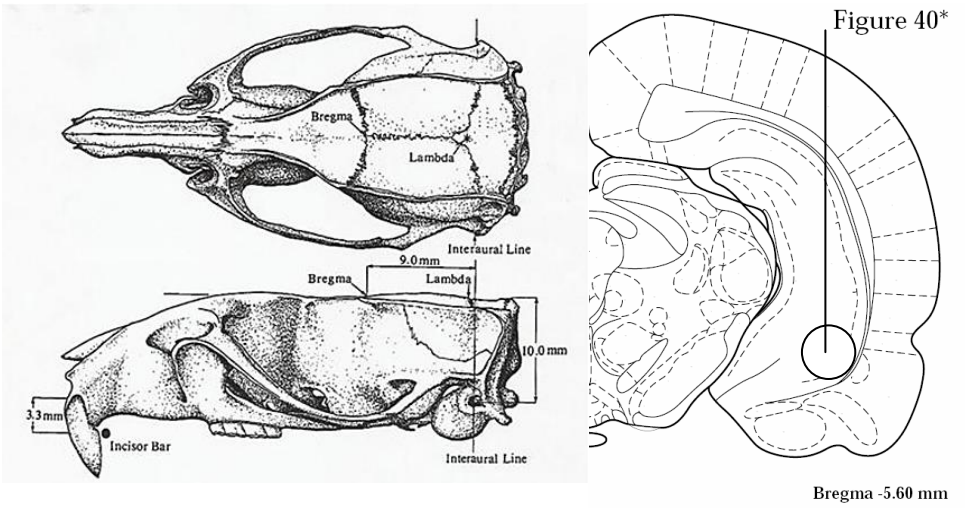


Figure 21: The lambda and bregma points (left) and the position of the probe in the brain (right).

The cannula was implanted vertically by drilling a hole through the hardening mixture (Kerr tab 2000, KERR UK Ltd., Peterborough, UK) and fixed to the skull

with self-curing acrylic (Kerr Italia, Salerno, Italy) and the skin was sutured. The rats were then allowed to recover for 3 days, after which time they received a one day regimen of their treatment followed by microdialysis the next day. The inner cannula was then removed and replaced with a dialysis probe (2 mm exposed surface, CMA 12, CMA Microdialysis AB, Stockholm, Sweden). The brain was then perfused (CMA/100, CMA Microdialysis AB, Stockholm, Sweden) with artificial cerebrospinal fluid (aCSF) consisting of, 1.2 mM NaCl, 3 mM KCl, 140 mM $CaCl_2$, 1 mM $MgCl_2$, 0.27 mM NaH_2PO_4, 7.2 mM glucose and 1.2 mM Na_2HPO_4 (pH 7.4) at a rate of 2µl/min (Ward et al., 2009) via polyethylene tubing (i.d. 0.38mm) connected to a 1mL syringe mounted on a micro-infusion pump (CMA/100, CMA/Microdialysis AB, Stockholm, Sweden). After a period of stabilisation of 1.30 hours, the perfusion fractions were collected every 30 minutes. During the day of microdialysis rats were treated with their relevant doses of either ethanol or water +/- β-sultam . The rats were then continuously sampled every 30 minutes for 5 hours (0 to 300 min). The following day, the alveolar macrophages were isolated from each animal, which was then killed by decapitation and the brain removed.

The dialysate samples were either analysed immediately or frozen before analysis. The HPLC system (High-performance liquid chromatography) used for the amino acid detection consisted in 2 LC-6A pumps, a SIL $10AD_{VP}$ refrigerated autoinjector, and a RF-551 fluorescence detector (kex= 340nm and kem= 455 nm, for the amino acid OPA-derivatives) with SCL $10AD_{VP}$ control system (Shimadzu, Milano). Column effluent was analysed using the software Class VP 7.2 (Shimadzu, Milano, Italia). The microdialysis samples were derivatised with OPA-reagent (Figure 22)

for pre-column derivatisation, which consisted of mercaptoethanol, o-phthalaldehyde (OPA) and $NaHCO_3$ (pH 9.5).

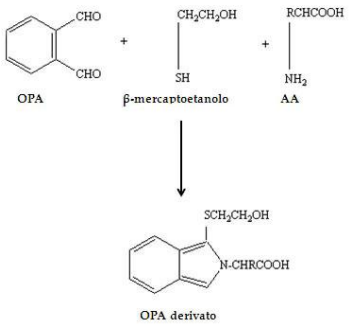


Figure 22: Reaction of OPA derivatization.

These derivatives were separated with 5µm reverse-phase Nucleosil C18 column (250 x 4.6 mm; Machery-Nagel, Duren, Germany), maintained at room temperature, using a mobile phase consisting of methanol and potassium acetate (0.1 M, pH adjusted to 5.48 with glacial acetic acid) at a flow rate of 1.0 mL/min, in a three linear step gradient (from 25% to 90% methanol) (as described in Ward et al., 2009).

2.5 Alveolar Macrophage Isolation

Alveolar macrophages were isolated from rats 24 hours after the microdialysis. Rats were anaesthetised with chloral hydrate (4%) and then a small incision was made in the trachea, allowing a small tube to be inserted into the lungs. A phosphate buffer solution (1.36M NaCl; 0.0268M KCl; 0.0113M KH_2PO_4; 0.0912M NaH_2PO_4) pH 7.25, approximately 50 ml, was used to lavage the alveolar macrophages from the lungs, which was then centrifuged at 1,500 rpm for 10

minutes. Cells at densities of $1x10^5$ and $2x10^5$ were pipetted into wells (Corning Inc. USA) containing culture medium Dulbecco media, 10% foetal calf serum, 100 µg/ml streptomycin and 100 µg/ml penicillin. The alveolar macrophages were left for 24 hours to adhere to the wells. The supernatant was then removed and the cells resuspended in culture medium and stimulated with 1 µg/ml LPS for 24 hours. The supernatants were removed and stored at –20 ^oC prior to further analysis.

2.6 Nitrite analysis

The level of nitrite in the supernatants was evaluated by combining 100 µl aliquots with an equal volume of Greiss reagent (2.5% phosphoric acid, 1% sulphanilamide and 0.1% naphthalene diamine dihydrochloride). The mixture was incubated for 10 minutes at room temperature prior to their optical density being measured at 540 nm. Standards of sodium nitrate were prepared in the range from 1-50 µM.

2.7 IL-6 and TNFα Quantification

IL-6 and ELISA kit (R&D Systems, Abingdon, UK) were used for the quantitative measurement of this cytokine in the supernatants.

2.8 Taurine analysis in Plasma

The plasma level of taurine was evaluated using 100 µL of plasma and 40 µL of 2% TCA. (After a 3000 r.p.m. centrifugation for 15 minutes, the supernatant was collected and analysed by HPLC.

2.9 Preparation of immortalised N9 glial cells

The N9 microglial cell line, donated by Dr. Paola Ricciardi Castagnoli (CNR Cellular and Molecular Pharmacology Centre, Milan, Italy), was originally derived from embryonic day 13 mouse microglial cultures (Corradin et al., 1993). The responses from these cells are very similar to those from primary rat microglia (Kingham and Pocock, 2000; Taylor et al., 2005). N9 cells were maintained in DMEM supplemented with 5% foetal bovine serum, 50 mM β-mercaptoethanol, 50 U/l penicillin and 50 mg/ml streptomycin at 37.8 °C in 5% CO_2. The immortalised glial cells, N9 were grown to confluence and cells recovered for 10 minutes after centrifugation at 1200 rpm. Cell viability was measured by trypan blue uptake extrusion (>98%). The glial cells were then plated at densities of $1x10^5$ or $2x10^5$ cells, in Dubecco media supplemented with 10% foetal calf serum, containing 100 mg/ml penicillin and 100 mg/ml streptomycin.

2.10 Brain Preparation for *ex vivo* studies

Brains were removed from each rat at the completion of the pulmonary lavage. They were initially preserved in 4% formaledehyde in buffer solution for a minimum of 2 days, then cryopreserved in 30% sucrose solution for a further 7 days and then frozen in isopentane at -80 ^oC prior to analysis.

The frozen brains were mounted on the cryostat (Bright Instruments, UK) and 10 to 30 microns coronal sections cut through the hippocampus and transferred onto slides (2 per slide). After cutting slides were kept at -80 ^oC to avoid degradation and only defrosted when required for staining.

2.11 Cresyl Fast Violet Staining (CFV)

Cresyl fast violet staining is used to stain with nissel substance, nuclei and neurons. In this experiment the stain was used to a) identify the exact position of the probe and b) perform cell counts in cortical regions and in the hippocampus. Every 7th frozen sections, they were rehydrated through changes of ethanol (100% to 70%), then stained (3 min) with filtered cresyl fast solution (0.1% cresyl violet, 5% acetic acid). Excess stain on these slides was removed by rinsing with distilled H_2O and differentiated (95% ethanol, 5% acetic acid) to reduce the intensity of the stain and make the background clear. The slides were then dehydrated in increasing ethanol concentrations (70% to 100%) and cleared in xylene before mounting in DPX (VWR, UK).

2.12 Immunohistochemisty

- **OX-6 staining – MHC-II**

Glia cells up-regulate MHC-II antigen when they have been activated. MHC-II can be detected by the use of OX-6 antibodies (Serotec Ltd., Oxford, UK). Every 7th slide was stained for presence of MHC-II by a well-tested and optimised protocol (Ward et al., 2009). Slides were first rehydrated in changes of ethanol, circled with a pap pen (Daido Sangyo Co. Ltd., Tokyo, Japan) and then left in phosphate buffer saline (PBS: 16g NaCl, 2.3g NA_2PO_4, 0.4g KH_2PO_4, in 400ml adjusted to pH 7.4). Endogenous peroxide activity was blocked by use of 0.3% H_2O_2 in 100% in PBS (40 minutes). Slides were washed and incubated (1 hour) first in 5% normal horse serum (Vector Laboratories, UK) with PBS/Triton X (PBS, 0.1% Triton X-100; Sigma-Aldrich, UK) then in the same solution with the OX-6

antibody in a 1:500 dilution (refrigerated for 20 hours). The following day, slides were washed in PBS and then incubated (2 hours) anti-mouse IgG (secondary antibody) (1:200) in PBS/TX. 30 minutes before this time had elapsed, the ABC mix (2 drops A, 2 drops B, in 5 ml PBS/TX; Vectorstain Elite Kit, Vector Laboratories, UK) was made and kept in the dark. The slides were washed from the secondary antibody and the ABC mix was applied and slides covered for 1 hour. ABC mix was then washed off with PBS and then the chromogen, 3,3'-Diaminobenzidine (DAB: 5 ml H_2O, 2 drops buffer, 4 drops DAB, 2 drops H_2O_2; Vector Laboratories, UK) was added and left from 5 to 15 minutes until the brain sections had turned pale brown.

Slides were dehydrated in increasing alcohol concentration, cleared in xylene and cover-slipped with DPX mountant (VWR, Lutterworth, UK).

- **iNOS**

A similar protocol to that used for OX-6 was utilised with the double staining: OX-6/iNOS. After the DAB staining we washed very well in H_2O (5 minutes) and then in PBS (5-10 minutes). They were then blocked again with 5% normal horse serum and the iNOS first antibody (1:300) was added and left over night at 4 °C.

The following day the same protocol described for the OX-6 immunohistochemistry was applied.

2.13 Stereological Quantification

CFV stained slides (4-8 slides per brain in 3 to 4 animals for each treatment group) were utilised to count cells of the whole hippocampus. Hippocampal regions were examined in the same position in both hemispheres: within the "fork" of the hippocampus, encompassing the polymorphic layer of the dentate gyrus and CA3 neurons (Figure 23).

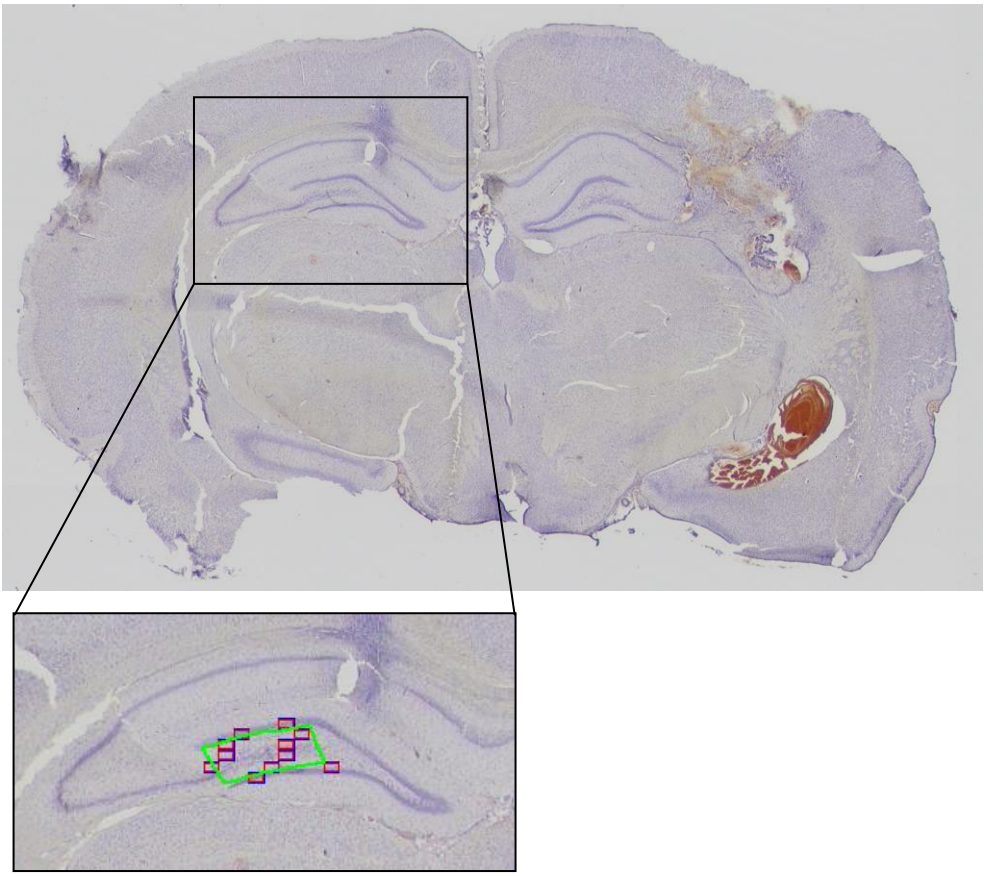


Figure 23: Cells counts were made on the CFV stained slides from the fork within the hippocampus encompassing the polymorphic layer of the dentate gyrus and CA3 neurons. Counts were made using computer-based stereological software linked to a JVC SLR camera mounted on a Nikon Eclipse E800 microscope. All types of cells were counted except for thoses which made up capillaries and blood vessels. The cell numbers were calculated per 10 µm.

Two cortical regions were counted in the left hemisphere; the right hemisphere was avoided due to probe related damage. The first region was defined by a rectangle (average size of 1100 μm x 1200 μm) from the midline and 200- 300 μm from the corpus callosum.

This region included retrosplenial agranular cotex (RSA), motor cortices (M1, M2), visual motor cortex (V2MM), somatosensory cortex (S1TR) and parietal association cortex (PtA). The second cortical area counted was a rectangle (average size of 1000 μm x 1100 μm) perpendicular to the midline and the dorsal 3rd ventricle or pineal recess and 200-300 μm from the corpus callosum. This region included the somatosensory cortex (S1BF, D2) and auditory cortex (AuD, Au1) (Paxinos & Watson, 1998). Counts were made using computer based stereological software (MAG, UK), attached a JVC SLR camera mounted on a Nikon Eclipse E800 microscope (Nikon, Japan). Cells were counted by selecting the area of interest (AOI), then the software computed counting frames using a uniform systematic random. The number of counting frames was dependant on the size of the area of interest and also the sampling fraction. The cortical areas were sampled at a fraction of 1/8 and hippocampal areas were set to a fraction of 1/4, mainly due to the relative side of the AOI. The system based software moved between these randomised frames for counting the individual cells. Cells that were within the box and touching the green line were selected, but cells touching the red line were not selected (Figure 24).

In addition all types of cells were counted except for cells which made up capillaries and blood vessels. The cell numbers were calculated per 10 μm as half

of the brains were cut at 30 µm and the rest at 20 µm, this allows a comparison to be made across all the brains.

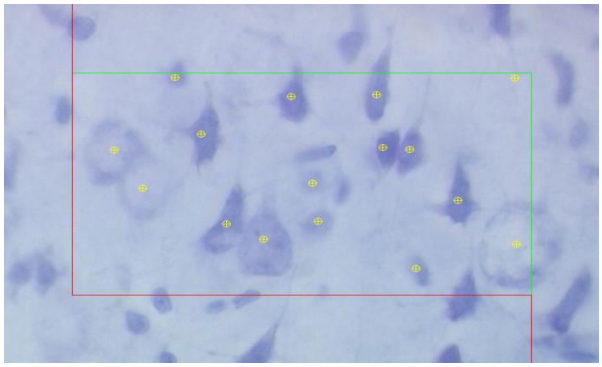


Figure 24: Cell counts were made on the CFV stained slides from the fork within the hippocampus encompassing the polymorph layer of the dentate gyrus and CA3 neurons.

2.14 Behavioural test

- **Morris Water Maze test**

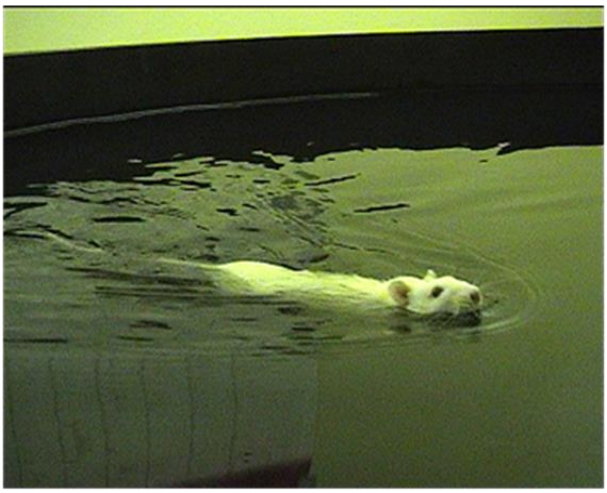


Figure 25: a swimming rat

The Morris water maze is one of the most widely used tasks in behavioural neuroscience for studying the psychological processes and neural mechanisms of spatial learning and memory. The basic task is very simple.

Animals, usually rats or mice, are placed in a large circular pool of water and required to escape from water onto a hidden platform whose location can normally be identified only by the use of a specific cognitive task, a spatial memory (Figure 25).

There are no local cues indicating where the platform is located. It was developed by Richard Morris at the University of St Andrews in Scotland and first described in two publications in the early 1980s (Morris et al., 1982b). Place navigation in the water maze is now often used as a general assay of cognitive function (Brandeis et al., 1989); for example for testing the impact of various disturbances of the nervous system in animal models of stroke (Nunn et al., 1994), aging (Gallagher and Rapp, 1997), neurodegenerative disease or the potential impact of novel therapeutic drugs (D'Hooge and De Deyn, 2001). The task has been used by computational neuroscientists and roboticists who are interested in navigation (Krichmar et al., 2005).

Various drugs can be applied to test animals before, during, or after maze training, which can reveal information about both mental and physical abilities. For example rats treated with the NMDA receptor blocker perform poorly in the Morris water maze, suggesting that NMDA receptors play a role in learning (Davis et al., 1992), as well as LTP and possibly spatial learning.

In this study the Morris Water Maze task was used to test learning and memory. A pool, of 160 cm diameter, filled with 30-35 cm water at 20-22 °C was used in this experiment. A sidewall above the waterline prevented the rats from being distracted by laboratory activity and from climbing out from the pool. The water in the maze was tinted black with a nontoxic child-paint so that the escape platform was not visible.

A video camera was placed above the centre of the pool to capture images of the swimming animals. The pool was located in a laboratory room with distinctive distal cues (music, light and coloured shapes) which would aid orientation.

Spatial reference memory: reference memory used a protocol in which the platform is in a fixed location relative to the room cues. The animals were placed into the water, facing the sidewalls of the pool, at a different starting position for each trial (n=4), performed each day (n=4). They quickly learnt to swim to the correct location, i.e. the hidden platform, with decreasing escape latency and more direct swim paths, from day 1 to day 4. When animals found the hidden platform, the time was recorded and a further 20 seconds elapsed before the rats were removed from the tank. During this time they could identify the position of the hidden platform so that on subsequent testing, they were expected to swim more quickly to the platform. The time taken to reach the platform is referred to as the latency and it was recorded in seconds. The experimenter was blinded as to the treatment groups of rats undergoing the water maze tests.

- **Probe Trial**

After the training was completed, the experimenter conducted a probe trial in which the escape platform was removed from the pool and the animals allowed to swim for 30 seconds.

Probe trial was used to test memory. Rats were given 30 seconds in the pool without the platform in place. During the probe trial the time spent into 2 of the 4 quadrants of the pool was measured: dial entry (where the animals were initially placed) and target dial (the position of the platform).

2.15 Statistical Analysis

Results are presented as mean ± standard error of the mean (S.E.M.). Data were analyzed by one or two-way analysis of variance (ANOVA), as appropriate, followed by the *post-hoc* Fisher protected LSD test for group comparisons (GB-Stat 5.3 for Windows, Dynamic Microsystems, MD, USA). Criteria for statistical significance were set at $p < 0.05$.

IV. Results

Animal body weight

The effect of the BD regimen on body weight was examined on the last week of treatment. The body weight of each animal was measured every morning, before the administration of β-sultam, in order to assess differences in their mean body weight during the last week of the BD regimen.

Figure 26 shows that the mean body weight of the animals receiving 1g/kg EtOH was significantly lower as compared to controls. However, pre-treatment with β-sultam prevented such decrease (Two way ANOVA: EtOH treatment $F_{2,103}$ =5.18, p=0.0072; β-sultam pre-treatment $F_{1,103}$=0.689, p=0.0249. Fisher protected LSD *post hoc* comparisons: 1g/kg bw EtOH *vs* control, p< 0.01, and 1g/kg EtOH +β-sultam *vs* 1g/kg EtOH, p< 0.05).

No significant changes were evident in the rats administrated 2g/kg EtOH +/- β-sultam.

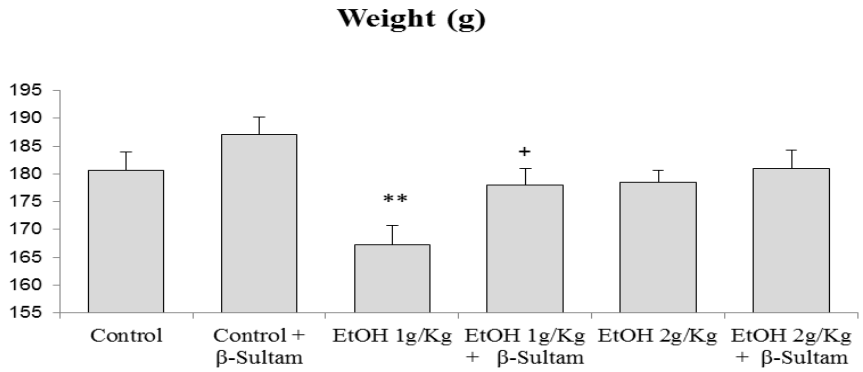


*Figure 26: Animals growth performance. Values are expressed as mean ± SEM. Number of observations were 10-12 for each treatment group. Post hoc Fisher LSD 1g/kg EtOH vs control, **p< 0,01 and 1g/kg EtOH β-sultam vs 1g/kg EtOH, +p< 0,05.*

Macrophages

Release of pro-inflammatory markers from alveolar macrophages

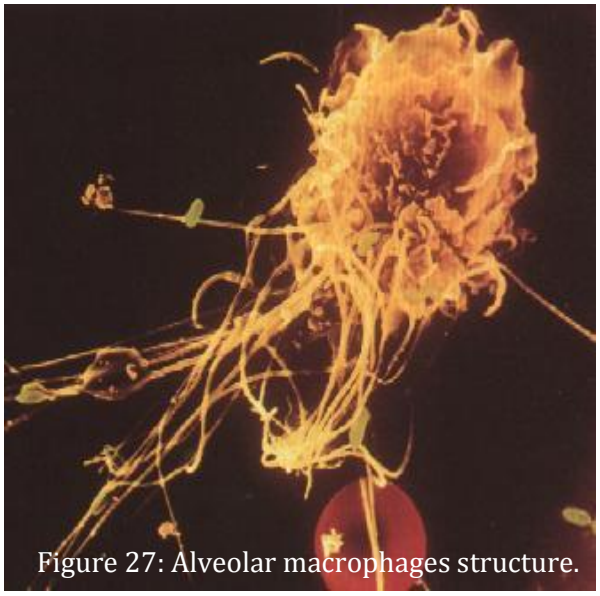
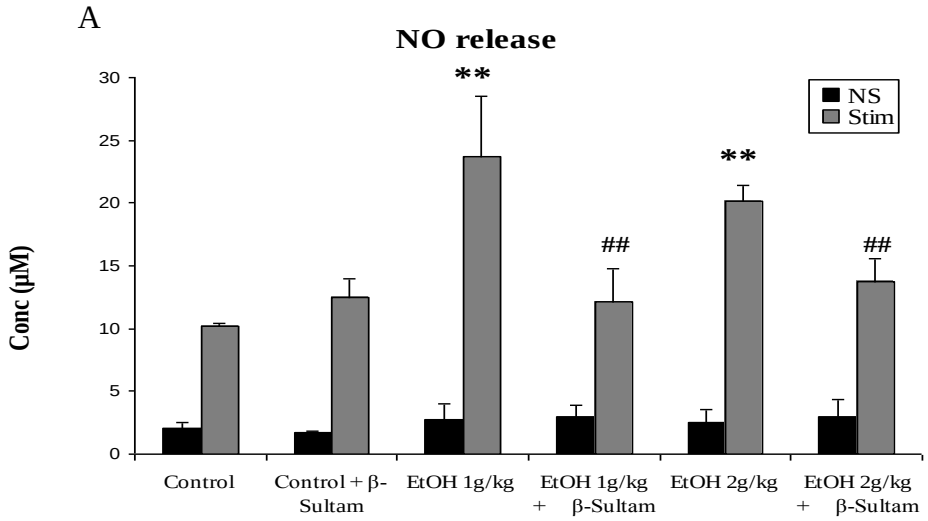


Figure 27: Alveolar macrophages structure.

Alveolar macrophages (Figure 27) were isolated from rats 24 hours after the microdialysis experiment. Figure 28 shows the LPS-induced NO (Figure 28 A) and IL-6 (Figure 28 B) release in the extracellular culture media of the alveolar macrophages of each animal group, before and after stimulation with LPS (1 μg/ml for 24 hours), apart from TNFα (Figure 28 C), for which only the LPS-induced release was evaluated. A similar behaviour of the release of each of these pro-inflammatory markers was evident. Following *in vitro* stimulation with LPS a statistically significant increase in the release of both, NO (Two way ANOVA EtOH $F_{2,27}$= 7,223, p=0,0031; β-sultam $F_{1,27}$= 14,74, p= 0,0007; Fisher protected LSD *post hoc* comparisons: 1g and 2g/kg EtOH *vs* control, p<0.01 and 1g and 2g/kg EtOH + β-sultam *vs* respectively 1g and 2g/kg EtOH), and IL-6 (Two way ANOVA: EtOH $F_{2,10}$= 60,73, p<0,0001; β-sultam $F_{1,10}$= 68,20, p= 0.0037; Fisher protected LSD *post hoc* comparisons: 1g and 2g/kg EtOH *vs* control, p<0.01; 1g and 2gr/kg EtOH + β-sultam, respectively, *vs* 1g and 2gr/kg EtOH, p< 0.01) was observed in the binge drinking rats administered either 1g/kg or 2g/kg EtOH, as compared to the control group administered water alone (Figure 28 A, B). IL-6 graph shows also a statistically significant increase before LPS stimulation (Two way ANOVA: EtOH $F_{2,11}$= 4,31, p= 0,041; β-sultam $F_{1,11}$= 7,42, p= 0,0124. Fisher protected LSD *post hoc*

comparisons: 1g/kg EtOH *vs* control, p< 0.05, and 2g/kg EtOH *vs* control, p< 0.01; 1g/kg EtOH + β-sultam *vs* 1gr/kg EtOH, p< 0.05, and 2gr/kg EtOH + β-sultam *vs* 2gr/kg EtOH, p< 0.01). Pre-treatment with β-sultam prevented the BD-induced increase of IL-6 release, which went almost down to control values.

A similar pattern of response was shown by the TNFα release which was increased after stimulation with LPS. Pre-administration with β-sultam prevented the increase of TNFα induced by both doses of ethanol (Figure 28C) (Two way ANOVA: EtOH $F_{2,16}$= 0,743 p= 0,492; β-sultam $F_{1,16}$= 15,18 p= 0,0002. Fisher protected LSD *post hoc* comparisons: 1g and 2g/kg EtOH *vs* control, **p< 0.01, 1g and 2g/kg EtOH + β-sultam *vs* respectively, 1g and 2g/kg EtOH, $^{\#\#}$p<0,01).



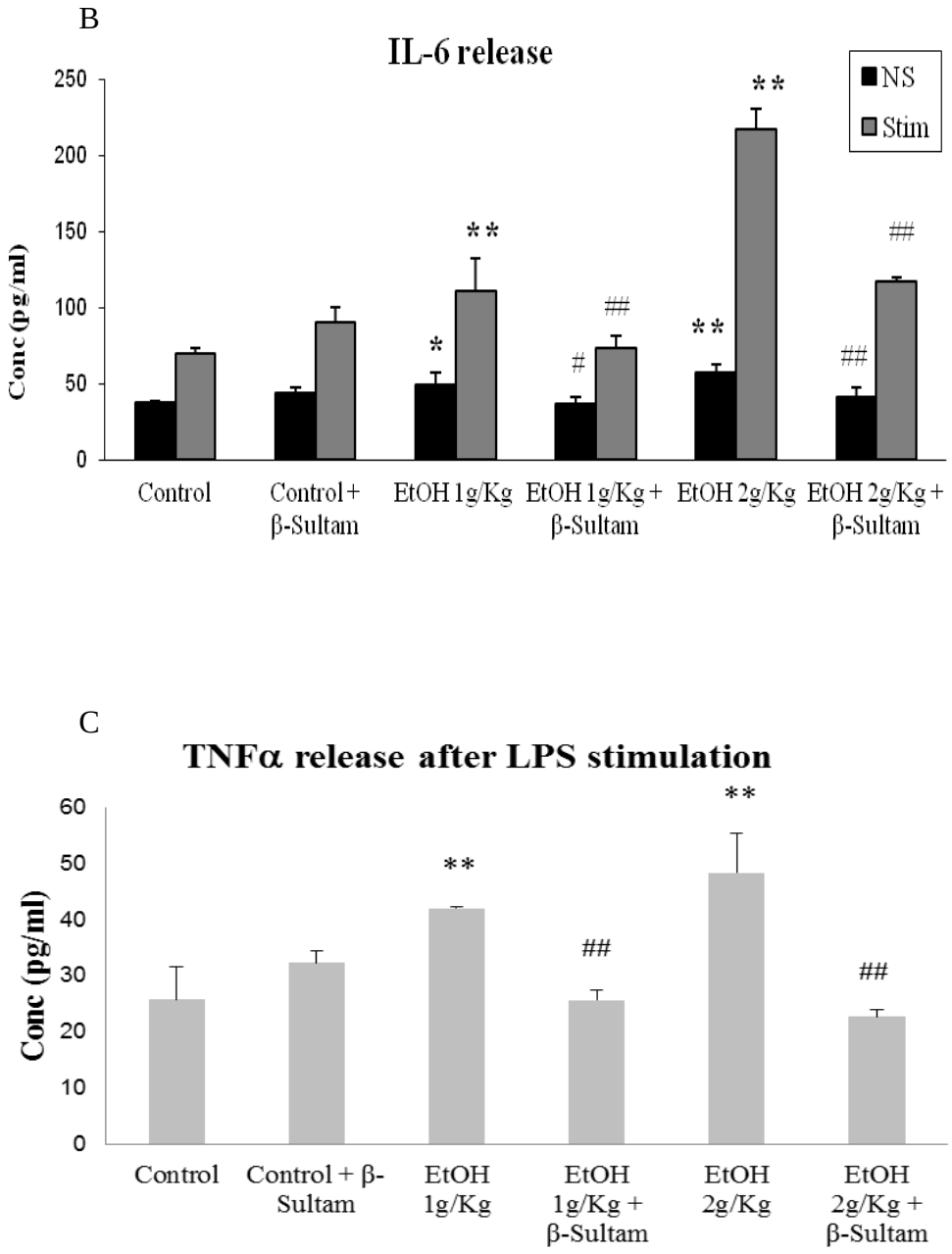


Figure 28: Release of inflammatory markers from isolated alveolar macrophages. Values are expressed as mean ± SEM. Number of observations were 6-8 for each treatment group. Post hoc Fisher LSD ***A)*** *NO, **p< 0.01 vs control and 1g and 2g/kg EtOH + β-sultam vs respectively, 1g and 2g/kg EtOH ##p< 0,01;* ***B)*** *IL-6, before LPS stimulation, 1g/kg EtOH vs control, *p< 0.05 and 2g/kg EtOH vs control **p< 0.01; 1g/kg EtOH + β-sultam vs 1gr/kg EtOH, p< 0.05, and 2gr/kg EtOH + β-sultam vs 2gr/kg EtOH, p< 0.01. After LPS Stimulation **p< 0.01 vs control; 1g and 2g/kg EtOH + β-sultam vs respectively, 1g and 2g/kg EtOH. ##p< 0.01.* ***C)****, 1g and 2 g EtOH vs control **p< 0.01, 1g and 2g/kg EtOH + β-sultam vs respectively, 1g and 2g/kg EtOH ##p< 0,01.*

In vitro stimulation of N9 cells

NO release

In preliminary experiments N9 microglial cell line was stimulated *in vitro* for 24 hours with increasing doses of ethanol (20 mM, 50 mM, and 200 mM) before and after LPS stimulation. Figure 29 shows a tendency of the non stimulated NO release to increase with increasing doses of ethanol. Following LPS stimulation, NO release appeared to behave in an opposite way, decreasing with increasing ethanol concentrations. However, more experiments are needed to draw any conclusion.

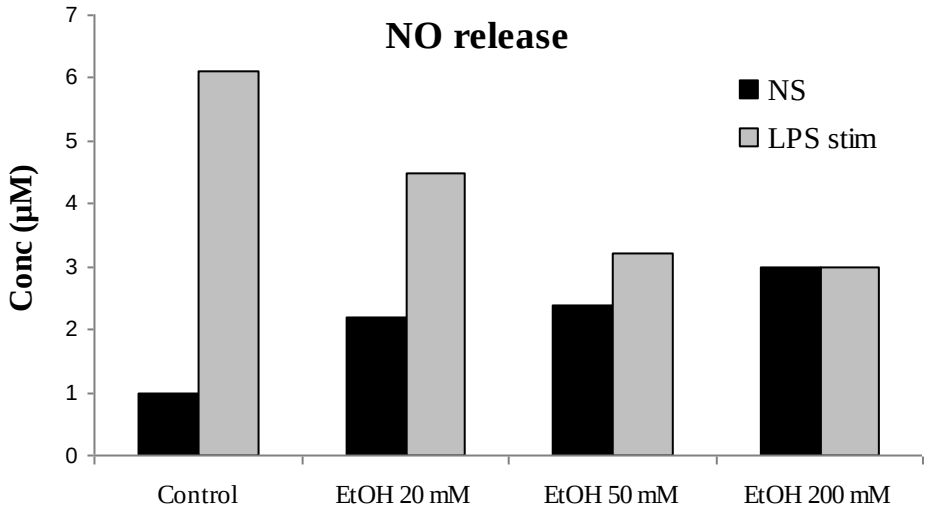


Figure 29: Preliminary data (n=2) of NO release by N9 microglial cells before and after LPS stimulation.

Taurine release

As shown in figure 30, taurine concentration, expressed as (µM)/100.000 N9 cells, seemed to decrease after incubation with 50 µM or 100 µM ethanol for 24 hours, as

compared to control. Furthermore, LPS stimulation of these cells seemed to cause a further decrease of taurine concentration.

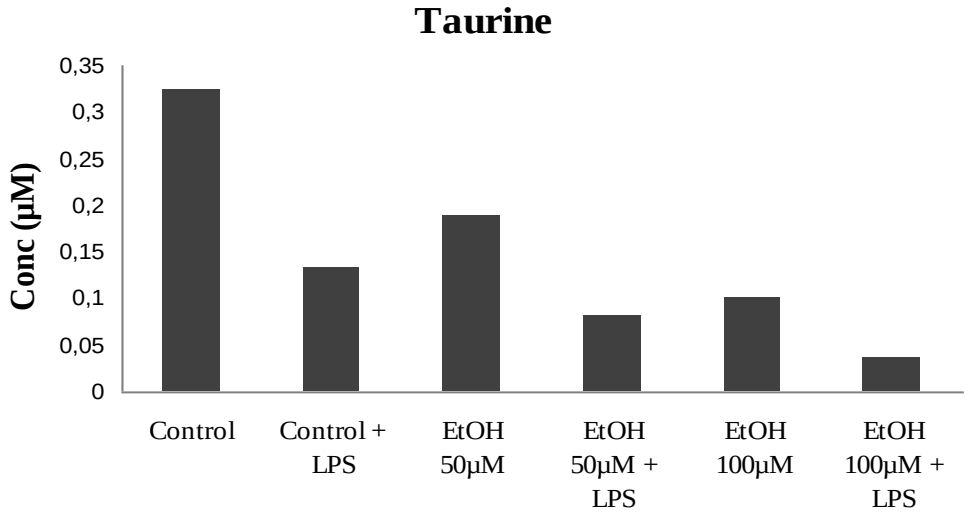


Figure 30: Preliminary data (n=2) of turbine concentration in N9 cells under ethanol and LPS stimulation.

Plasma Taurine Concentration.

As shown in Figure 31, only the administration of 2g/kg EtOH induced a statistically significant decrease in plasma taurine concentration as compared to controls. Following β-sultam pre-treatment the decrease in plasma taurine induced by 2g/kg EtOH was significantly reduced (Two way ANOVA: EtOH $F_{2,23}$=13,56, p= 0,0001; β-sultam $F_{1,23}$= 2,09, p= 0,1622; Fisher protected LSD *post hoc* comparisons*:* 2g/kg EtOH *vs* control, $^{**}p< 0.01$, 2g/kg β-sultam *vs* 2g/kg EtOH, $^{\#\#}p< 0.01$.). Animals treated with 1g/kg EtOH + β-sultam did not show any significant change in plasma taurine concentration (Figure 31).

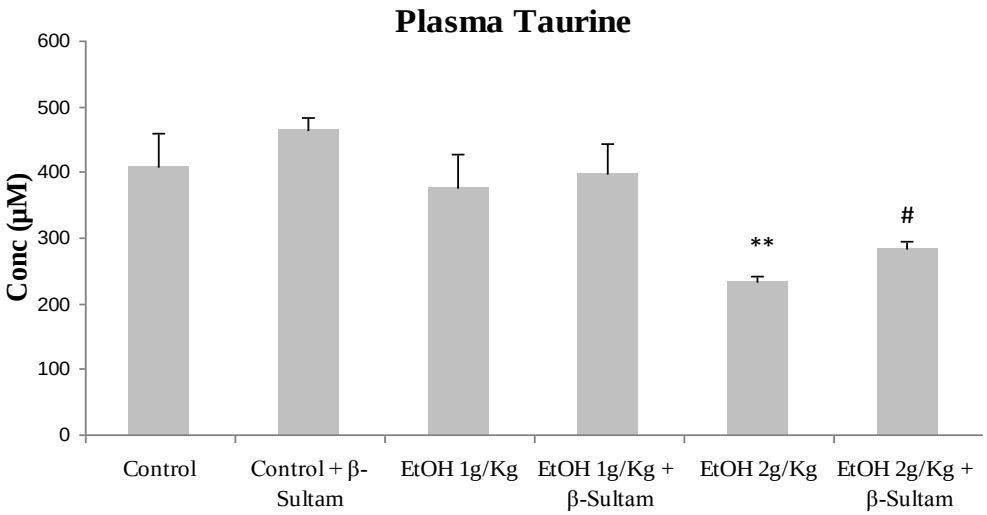


Figure 31: Plasma taurine concentration in a binge drinking regimen.
Values are expressed as mean ± SEM. (n= 5-6) Post hoc Fisher LSD.$^{**}p< 0.01$ vs control, $^{\#}p< 0,05$ vs 2g/kg EtOH.

Microdialysis experiments.

At the end of the third week the rats were prepared for microdialysis where the animals were implanted with a guide cannule.

The microdyalisad was collected every 30 minutes to measure amino acids glutamate and taurine and then measured by HPLC coupled to fluorimetric detection as previously described (Ward et al., 2009).

Taurine

The basal extracellular levels of hippocampal taurine did not show any significant change after the BD regimen. Taurine maintained its basal levels, even after a further ethanol challenge, over the 5 hours period of the microdialysis experiment (Table 3).

Glutamate

The basal levels of glutamate released in the ventral hippocampus region of BD rats, following 1 and 2g/kg EtOH +/- β-sultam are shown in table 3. Basal glutamate extracellular concentrations were significantly increased after 2g/kg EtOH. Pre-treatment with β-sultam prevent the glutamate increase only in animals treated with 2g/kg (Two way ANOVA: EtOH $F_{2,113}$= 1,077 p= 0,344; β-sultam $F1_{,113}$= 1,22, p= 0,0328. Fisher protected LSD *post hoc* comparisons*:* 2g/kg EtOH *vs* control, $^{*}p< 0.05$, 2g/kg EtOH + β-sultam *vs* 2g/kg EtOH, $^{\#\#}p< 0.01$).

The further challenge with the last BD dose of ethanol did not induce any significant change in the release of glutamate.

	H_2O	**H_2O + β-sultam**	**1g/kg EtOH**	**1 g/kg EtOH +β-sultam**	**2g/kg EtOH**	**2 g/kg EtOH +β-sultam**
Taurine						
Basal	1536.35 ±95.32	1441.20 ±129.31	1535.10 ±112.01	1619.76 ±112.47	1588.32 ±113.71	1627.07 ±65.79
EtOH	1694.09 ±419.37	1354.78 ±99.56	1141.80 ±375.19	1653.77 ±421.17	1483.49 ±567,42	1609.10 ±90.83
Glutamate						
Basal	2469.85 ±127.85	2199.43 ±25.60	2353.38 ±55.43	2267.55 ±71.63	2916.81* ±279.02	2228.30## ±31.16
EtOH	2468.17± 90.87	2425.63 ±158.42	2222.88 ±33.85	2477.30 ±125.77	2588.11 ±201.41	2174.71 ±19.79

Table 3: Glutamate and taurine extracellular levels monitored in the ventral hippocampus at the end of BD regimen, under basal conditions and following the last dose of ethanol. Concentration values (nM) are expressed as mean ± SEM. Number of observations were 8–9 for each treatment group. Post hoc Fisher LSD $^{*}p< 0.05$ vs control; $^{\#\#}p< 0.01$ vs 2g/kg EtOH.

Hippocampal immunohistochemistry

Brain microglial activation induced by binge drinking +/- β-sultam

Sections from all areas of the brain were examined for activated microglia by the presence of immunopositive OX-6 cells.

The monoclonal OX-6 antibody, used for the immunohistochemistry studies, showed activated microglial cells to be present with enlarged somas and thickened processes in the hippocampal region (Figure 36) of the BD-treated animals. Activated microglia were not present in any brain region of control animals.

In the hippocampus, as clearly shown in figure 37 b, both BD groups, but in particular the 2g/kg administered rats, showed a significantly elevated activation of microglia by comparison with controls. Pre-treatment with β -sultam was able to decrease the microglial activation in both treatment groups. (Two way ANOVA: EtOH $F_{2,16}$= 29,332 p< 0.0001; β -sultam $F_{1,13}$= 2,567 p= 0,128. Fisher protected LSD *post hoc* comparisons*:* 1 and 2 g/kg EtOH *vs* control, **p< 0,01; 2g/kg EtOH + β-sultam *vs* 2g/kg EtOH, $^{\#}$p< 0,05).

There was no evidence of microglial activation in any other brain regions in the BD-treated rats (Figure 37A).

Double staining: iNOS and OX 6

Double immunohistochemical staining with OX-6 and iNOS was used to understand if iNOS expression was present in the activated microglial cells. Indeed, iNOS activation was expressed all around the activated microglial cell, especially in those animals treated with 2g/kg EtOH (Figure 37B).

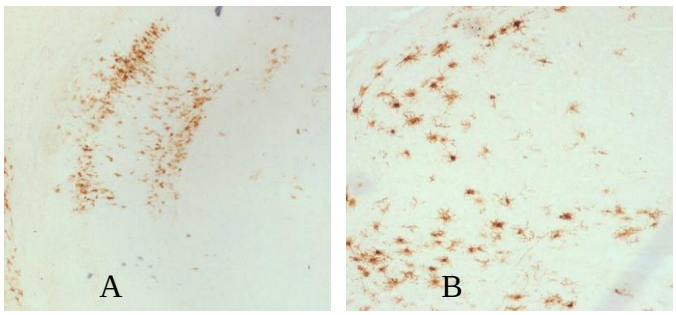


Figure 36: Activation of microglia by OX-6 immunohistochemistry in the hippocampal region. A) Magnification 4X; B) Magnification 20X.

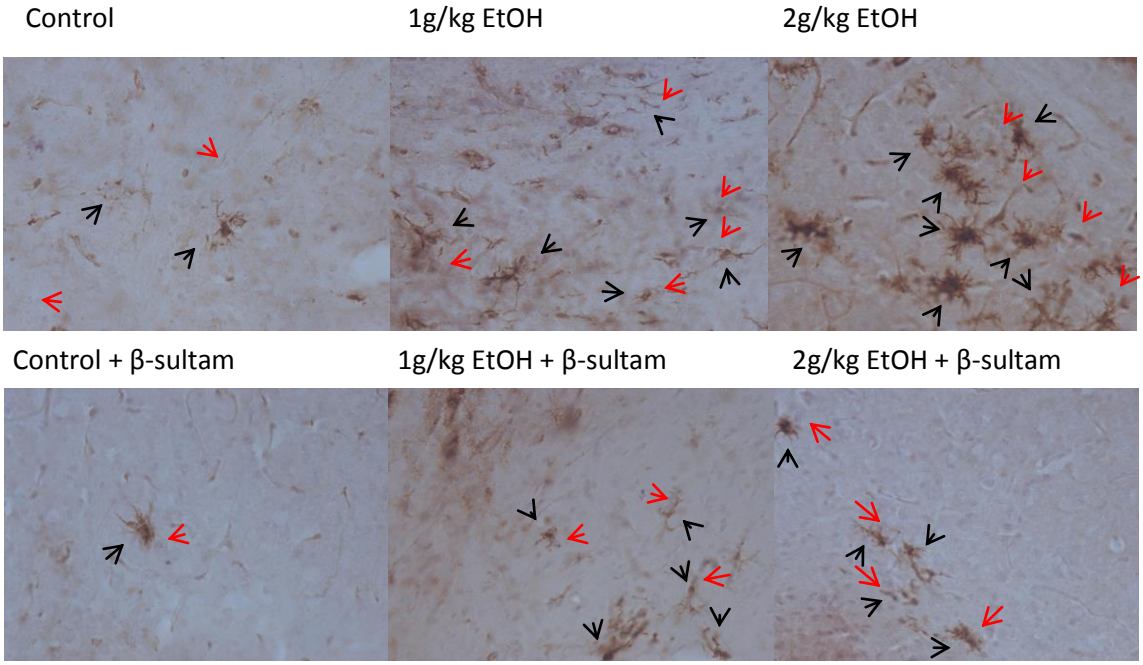


Figure 37b : OX 6 (black arrows) and iNOS (red arrows) double immunostaining in all the treatment groups. (40X).

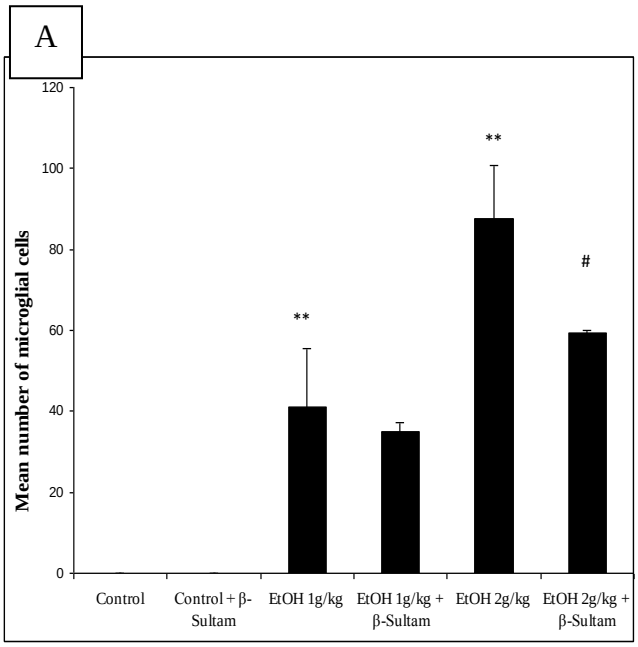


Figure 37A: Microglial activation after a BD regimen. Data are expressed as mean ± SEM of number of OX-6 stained cells per 10 µm. Number of observations were 4-5 for each treatment.

*In control sections, if there were less than 10 activated microglia after the counting procedure, the slide was ignored. 1 and 2 g/kg EtOH vs control, **p< 0,01; 2g/kg EtOH + β–sultam vs 2g/kg EtOH, #p< 0,05.*

Hippocampal neuronal loss after binge drinking +/- β-sultam

In rats which received doses of ethanol (1g/kg or 2g/kg) significant losses of neurons were evident in those areas where the activated microglia had been observed. Pre-treatment with β-sultam prevented the neuronal loss in BD rats which had been administered 1g/kg ethanol (Two way ANOVA: EtOH $F_{2,58}$= 7,661 p= 0,0011 Two way ANOVA: β-sultam $F_{1,58}$= 0,624 p= 0,432; Fisher protected LSD *post hoc* comparisons*:* 1 and 2g/kg EtOH *vs* control,$^{**}p< 0.01$, 1g/kg + β-sultam *vs* 1g/kg EtOH, $^{‡}p< 0.01$). However, no protection against neuronal loss was evident in the rats treated with 2g/kg + β-sultam (Figure 38).

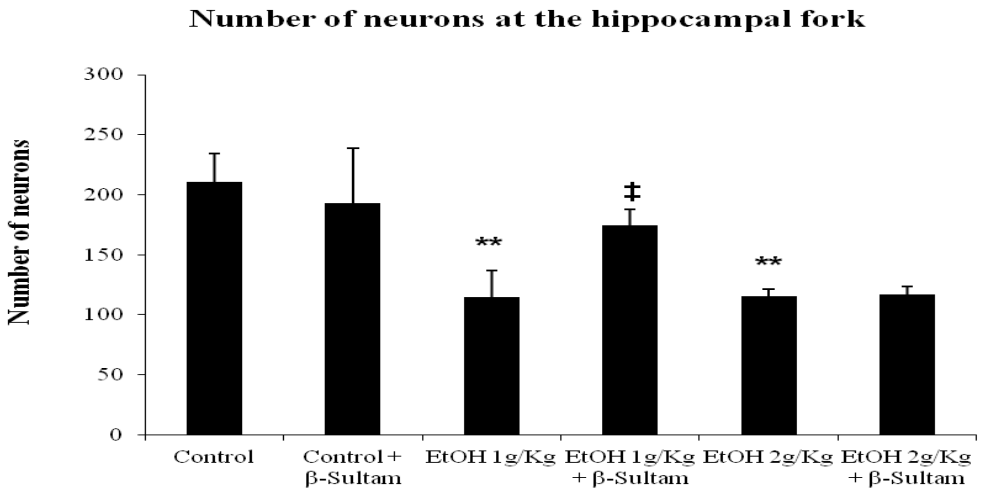


Figure 38: Neuronal loss after BD regimen.
Values are expressed as mean ± SEM. (n= 6-10). Post hoc Fisher LSD $^{**}p< 0.01$ vs control, $^{‡}p< 0.01$ vs 1g/kg EtOH.

Morris Water Maze

The Morris water maze (A) and the probe trial (B) were used to test, respectively, the spatial learning and memory after a BD regimen in rats.

A)

The Morris water maze test measured, from different access points, the escape latency, i.e. the time (to a maximum of 60 seconds) taken by each rat to escape from water onto a hidden platform, with the extra maze cues to examine acquisition of spatial reference memory. The Platform was always placed in the same quadrant (Figure 39).

At the end of all trials, (4x4 days), the controls rapidly found the hidden platform at a time significantly lower than 1g or 2g/kg EtOH-treated animals. The time spent by 1g/kg BD animals to find the platform was significantly decreased by pre-treatment with β-sultam as compared to those not undergoing pre-treatment (Two way ANOVA: EtOH $F_{2,29}$=3,394 p= 0,0474; β-sultam $F_{1,29}$= 0,933 p= 0,342; Fisher protected LSD *post hoc* comparisons*:* 1g and 2g/kg EtOH vs control, $^{*}p< 0.05$, 1g/kg EtOH + β-sultam vs 1g/kg EtOH, $^{\$}p< 0.05$).

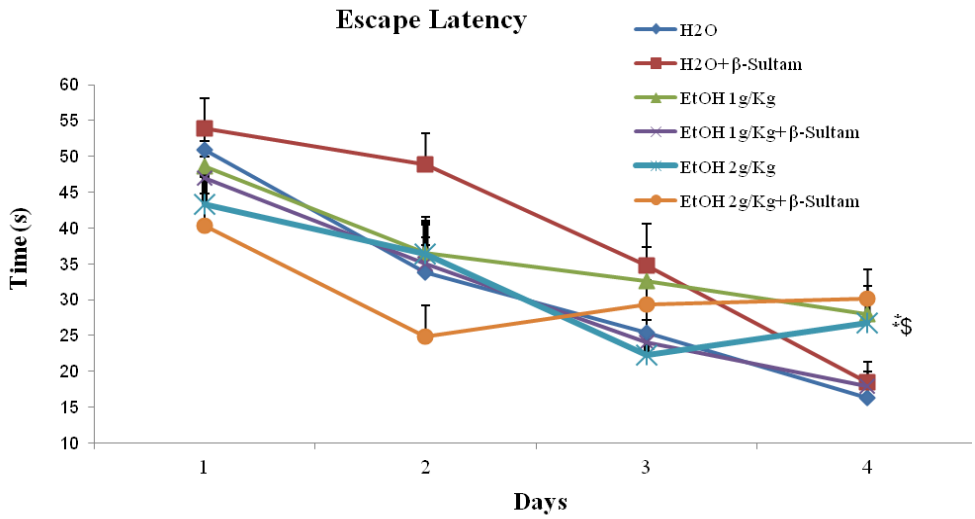


Figure 39: Escape Latency.
*Values are expressed as mean ± SEM, (n= 6). Post hoc Fisher LSD 1g/kg EtOH and 2g/kg EtOH vs control * p< 0.05; 1g/kg EtOH vs 1g/kg EtOH + β-sultam, $p< 0.05.*

B)

The probe trial was performed on the last day of the escape latency trials, to evaluate memory. Five hours after the last trial, the hidden platform was removed, and the animals were placed in the maze and the time spent in the target dial (the quadrant where the platform was) was measured.

A different behaviour of the various treatment groups appeared evident to the operator. The BD-treated rats were less anxious when placed in the water, starting immediately to quickly swim as compared to controls, which did not start swimming for a few seconds while squealing.

Figure 40 shows a tendency of ethanol BD animals to spend more time in the target dial as compared to controls, which swam quickly back to the entrance quadrant, waiting for the operator to remove them from the pool.

Similarly, in both treatment groups, pre-treatment with the prodrug β-sultam showed a tendency to decrease the time spent in the target dial looking for the platform. However, these effects were in no case statistically significant.

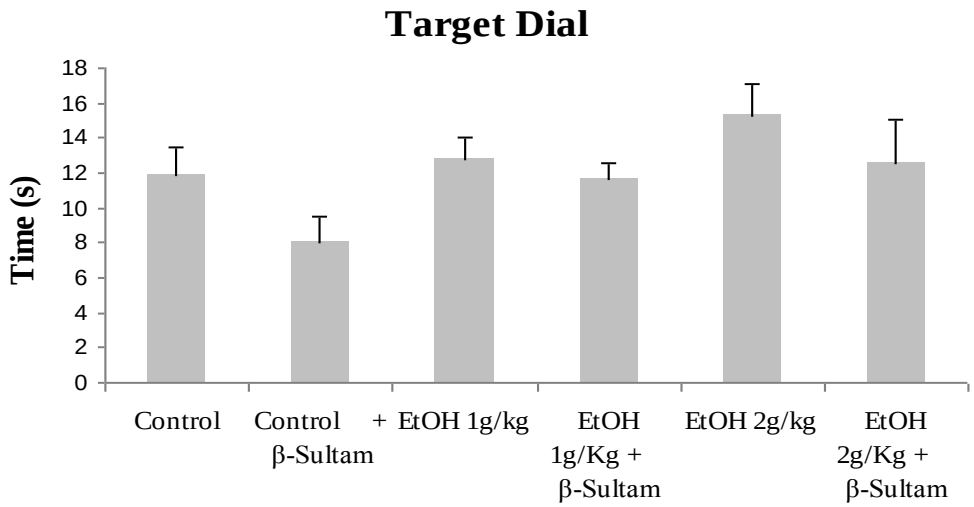


Figure 40: Probe trial performance.
Values are expressed as mean ± SEM, (n= 6).

V. DISCUSSION

Binge drinking (BD), heavy episodic drinkers (HED) is a topic of rising media and research interest throughout Europe since 1995. Over 80 million Europeans, aged 15 plus (over one-fifth of the adult European population), reported such a pattern of alcohol consumption in a survey in 2006 (Farke and Anderson, 2007). BD has been researched mainly in Northern America, Northern European and Anglo-Saxon countries (''dry countries''), showing a more sporadic alcohol intake than Mediterranean countries (''wet countries''), where consumption is more frequent and regular. This ''dry / wet country'' dichotomy seems to be diminishing in recent years, with a trend towards uniformity among countries (Bloomfield et al., 2003).

Neurogenesis, which occurs mainly in the hippocampus and in the olfactory bulb during adolescence, is adversely altered by ethanol although the various neurochemical pathways involved remain undefined. Therefore, the aim of this study was to identify factors that might be involved and then test specific possible therapeutic agents that might ameliorate such toxicity.

One of the major problems in trying to collate and compare the results in experimental 'binge drinking' animals, has been the wide variety of methods used to induce the binge type drinking regimen, in addition to the age, type of species and route of the ethanol administration. Kapasova and Szumlinski (2008) used repeated injection regimen, 8 x 2g/kg every other day to alcohol preferring (C57BL/6J, B6) and alcohol non-preferring (DBA2/J, D2). Szumlinski et al., (2007), restricted fluid intake for 12 hours before experimentation, after which time the mice were presented with a 50 ml sipper tube containing a 5% alcohol solution for

varying lengthened time periods every 3rd day for 18 days. Smith et al., (2008) utilised rats which drank a solution of 10% ethanol / 5% sucrose for 90 minutes, 5 days / week and then pulse labelled the brain tissue to estimate turnovers of various neurotransmitters including glutamate. Penland et al (2001) gave high doses of ethanol, 5% with 50% Vanilla Ensure, through an intragastric catheter every 8h for 4 days. A weight loss was evident after the conclusion of this treatment, and caused 10% mortality and a further 26% mortality during the withdrawal stage, suggesting that this model was more indicative of chronic abuse than a binge drinking model. In the binge drinking model of Pascual et al., (2009) rats received ethanol, 3g/kg, (eight injections) on two consecutive days followed by of two days of abstinence for two weeks.

Many of these models exhibited behavioural changes at the end of the BD regimen including ethanol preference, e.g. withdrawal symptoms after ethanol cessation and a greater propensity to consume ethanol. Indeed, preference is not considered to occur in binge drinking individuals. In the BD rats utilised for these present studies, no incidence of ethanol preference was observed, i.e. no increased motility after ethanol withdrawal or ethanol preference during the first 48h ethanol withdrawal (Ward et al., 2009).

The binge drinking regimen was well tolerated by the rats in these present studies, only marginal changes in weight occurred in these animal during the last week of the ethanol treatment period at the lower dose of 1g/kg EtOH, but not at the higher one of 2g/kg EtOH.

The alveolar macrophages isolated from these binge drinking rats showed an IL-6 activation before and prior to ex vivo stimulation with LPS, thereby indicating that

this form of ethanol drinking evokes an inflammatory response. This is in complete contrast to the situation in macrophages isolated from chronically alcoholised rats where a reduction in stimulation is evident (Zhang et al., 1998). This reflects the neuromodulation that occurs with chronic alcohol abuse. Furthermore, a stimulatory effect was also evident in the hippocampus, where activated microglia was a common finding in all of the rats administered 1 and 2 g/kg ethanol, intermittently.

Microglia is classified into three types. The resting ramified microglia continually monitor their microenvironment through pinocytosis and interaction with neurons (Nimmerjahn et al., 2005) When encountering infectious, neurodegenerative or other insulting stimulations, microglia are activated and transform into amoeboid phagocytic cells (Zhang et al., 2001). In addition to the different morphological types, microglia comprises a family of cells with diverse functional phenotypes, some of which are beneficial while others are harmful for neurons in CNS (Butovsky et al., 2006). Recent studies have shown that, as a type of tissue-resident macrophages, microglia is activated in a polarized manner (Michelucci et al., 2009).

In some preliminary *in vitro* experiments, microglial activation was examined together with the release of taurine, exposing N9 microglial cells to different concentration of ethanol pre- e post- LPS stimulation. Preliminary observations (n= 2) indicated a tendency of ethanol to increase NO concentration while decreasing taurine concentration in these cells with exposure to increasing ethanol concentrations, while the co-stimulation with LPS seemed to decrease both NO and taurine as the ethanol concentration increased. These very preliminary data

stimulates further experiments to investigate the association of a decrease in taurine concentration with increased concentrations of EtOH and whether in this model pre-treatment with β-sultam can lead to an increase in taurine concentration.

Binge drinking clearly altered neurotransmitter homeostasis in the hippocampus. After only three weeks of the BD regimen, the basal hippocampal glutamate level had increased approximately 2 fold, by comparison to controls, confirming previous findings (Ward et al., 2009).

A number of factors could contribute to the ethanol-induced elevated hippocampal glutamate levels. These include changes in the sensitivities of various glutamate receptors. Chronic ethanol administration will alter the sensitivities of NMDA receptors, which will show neuro-adaptation, such that the numbers of these NMDA receptors increase but show a diminished sensitivity. Therefore, when chronic ethanol intake ceases, such inhibition is removed, which results in an increase in glutamate release. It remains questionable as to whether 2 days of binge drinking followed by 5 non-alcohol days would induce such changes in the number and sensitivities of NMDA receptors. Clearly further studies are warranted in this area.

The cystine/glutamate exchanger Xc, also plays an important role in the brain, uptaking cysteine in order to synthesise reduced glutathione, and extruding glutamate from the cell, as a by-product of this synthesis. Alcohol, either administered acutely or chronically will alter the redox of the cell, leading to the generation of a variety of different reactive oxygen species, ROS. This in turn would

produce oxidised glutathione, thereby requiring the cell to synthesis additional amounts of reduced glutathione.

Glial cells may also be implicated in glutamate release into the hippocampus. Upon activation microglia will enhance glutamate release (Ward et al., 2011) together with cytokines which could contribute to the increased basal hippocampal glutamate levels. In addition, ethanol-induced changes in glutamate transporter on microglial, EAAT2, astrocytes, GLAST and GLT-1 as well as neurons, EAAC1 and EAAT4, may also be involved.

Such increases in glutamate could adversely affect neuronal cognitive functions. Significant frontal neurodegeneration (Crews et al., 2000a) as well as diminished neurogenesis (Crews et al., 2006), would indicate that the adolescent brain is uniquely sensitive to ethanol neurotoxicity (Crews et al., 2007).

Pascual et al. (2009) showed an increase in baseline glutamate levels after repeated ethanol administration in male adolescent but not adult rats, approximately 2 fold, both of these groups showing ethanol preference. In 2008, Kapasova and Szumlinski showed elevated NAC basal glutamate levels in alcohol preferring mice (B6) but not alcohol avoiding mice (D2). Although Smith et al (2008) showed no change in NAC glutamate turnover in their binge drinking model, this was attributed to the fact that there may be changes occurring either post-synoptically or through subtle modulations which did not affect overall synthesis. Kapasova and Szumlinski (2008) binge drinking model showed a trend, which was not significant, of an increase in basal NAC glutamate content. In the scheduled high alcohol consumption mice model no changes in basal glutamate levels were discernible (Szumlinski et al., 2007). Biochemical pathways within the

NAC, which have been implicated in such changes, include decreases in the phosphorylation of NR2B in adolescent but not in adults rats (Pascual et al., 2009) and increases mGluR5-Homer2-P13K pathway in binge drinking scheduled high alcohol consumption mice (Cozzoli et al., 2009). Such changes have been implicated in the impulsive behaviour and lack of control over drinking (Weitlauf and Woodward, 2008) and regulation of the propensity to binge drink (Cozzoli et al., 2009), respectively. Lastly in the model of Saellstroem Baum et al. (2006) rats showed ethanol withdrawal symptoms and a lower basal glutamate extracellular content. This would be comparable to that found in chronically alcoholised rats (Lallemand et al., 2006) probably induced by the inhibition of glutamic acid decarboxylase (Dahchour and De Witte, 1999).

Adolescent brain maturation has been reported to involve intermittent increases in cholinergic, dopaminergic and serotoninergic inputs into frontal cortex, as well as the cortical width, which are notably present during adolescence and then decline to stable levels with adulthood (Giedd, 2004; Giedd et al., 2008; Gould et al., 1991; Kalsbeek et al., 1988; Kostovic, 1990; Rosenberg and Lewis, 1994; Spear, 2000). However, in these present studies all other neuro-transmitters and neuromodulators monitored in the hippocampus showed comparable results to that of the control animals in each of the experiments. In other studies of this binge drinking model (Ward et al., 2009b); significant increases in extracellular glutamate content were also evident in the nucleus accumbens (NAc). The NAc extracellular glutamate is an important determinant of ethanol intake (Kapasova and Szumlinski, 2008), which may be mediated via upregulation of mGlu5-

Homer2-P13K signalling during BD (Cozzoli et al., 2009). However no other changes in neurotransmitter release were evident in the NAc.

The activation of the microglia in the hippocampus was associated with a decrease in neuronal counts. The loss of neuronal cells in the hippocampus after various regimens of ethanol have been previously reported.(e.g. Crews et al., 2004; Crews and Nixon, 2009) although the ethanol concentrations used in such studies were higher, 4-9 g/kg. The dentate gyrus region is particularly vulnerable since it contains neural progenitor cells which will proliferate throughout life, but particularly during adolescence, to form neurons, astrocytes and oligodendrocytes. High doses of ethanol were shown to decrease the survival of these neural progenitor cells (Nixon and Crews, 2002). In another study of marque monkeys, where a binge type regimen was administered for varying time periods, there were significantly decreased numbers of actively dividing type 1, 2a, and 2b cell types without significantly altering the early neuronal type 3 cells. Such results the authors concluded were caused by alcohol interfering with the division and migration of hippocampal preneuronal progenitors (Taffe et al., 2010). This was paralleled by an increase in neural degeneration which was mediated by nonapoptotic pathways. Such changes may underlie the deficits in hippocampus-associated cognitive tasks which were evident in our current studies.

Such losses of neurons could be associated with increased inflammation in this brain region. In these present studies, activation of NOS was identified after immunohistochemical staining in the hippocampal region. Previous studies have also identified markers of inflammation; pro-inflammatory cytokines and monocyte chemo-attractant protein 1, cyclo-oxygenase 2 as well as inducible nitric

oxide synthase (iNOS) in the neocortex, hippocampus and cerebellum of a binge drinking animal model (Pascual et al., 2007) and upregulation of COX2 and iNOS levels with increases in cell death in neocortex, hippocampus and cerebellum (Valles et al., 2004). A reduction in the ethanol induced inhibition of hippocampal pyramidal neural activity (Tokunaga et al., 2006) leading to brain damage has also been reported.

The facilitatory effect of NO in several types of behavioural learning has been reported in various animal models (Paul and Ekambaram, 2011; Susswein et al., 2004) such as olfactory learning (Kendrick et al., 1997; Okere and Kaba, 2000; Samama and Boehm, 1999), cerebellum-dependent motor learning (Yanagihara and Kondo, 1996), acquisition of active and passive avoidance (Antonov and Ha et al., 2007; Bernabeu et al., 1995; Huang and Lee, 1995; Pitsikas et al., 2005), and hippocampus-dependent spatial learning, the latter one accepted as an animal model of human episodic memory (Zhang et al., 1998). In these present studies the cognitive ability of the binge drinking rats was investigated by the Morris Water maze, and was shown to be impaired to a limited degree.

The increase in NO production has also been implicated in the pathophysiology of neurodegenerative disorders, such as Parkinson's disease and heart attack (Dawson et al., 1998). In neuropathological conditions, peroxynitrite may be formed, from the association between superoxide and NO, which is very neurotoxic (Dawson et al., 1998; Deckel, 2001; Wink et al., 1999).

Such inflammatory changes may contribute to cognitive alterations. Neurocognitive impairments have been characterized in adult alcoholism, particularly in the domains of spatial skills, learning and memory, and executive

functioning (Chanraud et al., 2010; Grant, 1987). Neuroimaging studies have identified structural, chemical, and functional abnormalities that parallel cognitive decrements (Pfefferbaum et al., 2000, 2001a, b, 2009; Sullivan and Pfefferbaum, 2005). Consistent with neuropsychological findings of working memory and spatial functioning, functional magnetic resonance imaging (fMRI) has revealed aberrant activation patterns during spatial working memory (SWM) tasks, particularly in frontal regions (Pfefferbaum et al., 2001a). While the majority of the literature suggests that females are more susceptible to alcohol related brain damage than males (Hommer et al., 1996, 2001; Jacobson, 1986), other research suggests males may be more vulnerable (Pfefferbaum et al., 2001b).

Pharmaceutical agents which may prevent the neurotoxicity of binge drinking have been reported. Recent reports provided evidence that microglia are able to adopt activation states that can promote neuroprotection, neurogenesis, and repair of damaged neuronal tissues (Colton, 2009). Certain compounds can inhibit the activation of microglia. Inhibition of microglial activation and subsequent inflammatory process in numerous CNS diseases is regarded as an important therapeutic target (Zhang et al., 2010; Hensley, 2010; Chakraborty et al., 2010). Studies with peripheral macrophages suggest that Transforming Growth Factor-b1 (TGFb1) might be a potent cytokine to control and regulate microglia activation states (Gordon and Martinez, 2010).

The anti-oxidant buytlated hydroxytoluene reversed binge induced brain damage, possibly via NFκb inhibition, and blocked ethanol inhibition of neurogenesis in several brain regions after the administration of very high doses of ethanol (8-12 g/kg/day) 3/day for 4 days with no abstinence period (Crews et al., 2006).

Administration of indomethacin, a COX-2 inhibitor to adolescent rats exposed to ethanol 3g/kg for 2 consecutive days at 48 h intervals abolished both COX-2 and iNOS expression, as well as cell death and behavioural deficits (Pascual et al., 2007). Knockdown of TLR4 by small interfering RNA abolishes the activation of NFκB and protein kinase, and production of inflammatory mediators by glia in the cerebral cortex of mice chronically receiving ethanol 10% for 5 months (Alfonso-Loeches et al., 2010). Furthermore mice lacking TLR4 receptors are protected against ethanol-induced inflammation, (Pascual et al., 2011) emphasising the importance of these receptors in inflammation However another approach, as used in this present study was to prevent the activation of transcription factors which mediate inflammation, i.e. NFκB. Taurine a sulphonated amino acid will prevent NFκB activation by stabilising IκBα and preventing its phosphorylation (Ward et al., 2009). Since taurine uptake by cells is limited by the taurine transporter, TauT, (Della Corte et al., 2002) a taurine prodrug, ethane-β sultam, was utilised which slowly hydrolyses intracellularly to the parent compound taurine. The prodrug, ethane- β-sultam diminished the activation of the innate immune system in both the alveolar macrophages in the periphery, in neuronal loss and in the glial cells in the hippocampus.

Evidence from animal studies suggests that adolescence may be a period of heightened sensitivity to the adverse cognitive squeal of alcohol exposure, perhaps because neuromaturation continues throughout this stage of life (Giedd et al., 1999, Giedd et al., 1996, Jernigan and Gamst, 2005; Lenroot and Giedd, 2006, Paus, 2001 and Sowell et al., 2004). It is commonly reported that adolescent rats are more sensitive to the cognitive impairing effects of alcohol compared to adults

(Markwiese et al., 1998). Acheson et al. (2001) reported that ethanol in adolescent rats inhibited acquisition of spatial memory but not in adult rats. The results from the Morris Water Maze indicate that BD adolescent rats exhibit deficits in spatial acquisition compared to controls. In the probe trial, ethanol didn't affected BD performance and probably the memory. The current work demonstrates that control animals spent less time in the target dial, where the platform was, than BD rats. This result could be due to an increase in thigmotaxis, (Novier et al., 2012). When mice are introduced into an open-field, they are inclined to explore mainly the peripheral zone of this open-field. This tendency to remain closes the walls, called thigmotaxis, decreases gradually during the first minutes of exploration (Simon et al., 1994). Thigmotaxis might be related to anxiety, therefore future research should address whether control rats show greater anxiety to swim as compared to the ethanol-treated rats. This suggests that in the hidden platform task other factors such as motoric impairment may be responsible for the poor performance shown by the rats in this test.

Overall these studies have identified some of the damaging effects of binge drinking. After a very short period of a binge drinking regimen, such as 3 weeks, a specific area of the brain, the hippocampus, showed evidence of neuronal loss and neuroinflammation which may contribute to such toxicity. Clearly these results are of significance for the numerous adolescents who partake in binge drinking. If a similar degree of toxicity is induced in their brains, it is likely that cognitive dysfunction may occur in susceptible subjects. Education of adolescents is therefore vital to inform them of the possible damage caused by such binge

drinking behaviour, as well as the possibility of a greater vulnerability to develop chronic alcohol abuse.

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