Universidade de Aveiro Departamento de Biologia
2010

Violeta Silva Loureiro Alves Ferreira

Expressão de miRNAs no cérebro de *Danio rerio* exposto a etanol

microRNA expression profile of *Danio rerio* brain exposed to ethanol

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Professor Manuel António da Silva Santos, Professor Associado do Departamento de Biologia da Universidade de Aveiro

o júri

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palavras-chave miRNA, alcolismo, regulação de genes, microarrays, *Danio rerio*

resumo

Os miRNAs são pequenas moléculas (~22 nt) de RNA não-codificante que regulam a expressão genética ao nível pós-transcripcional através da degradação ou inibição dos genes alvo. Os miRNAs são abundantes nos genomas eucariótas, onde desempenham funções importantes no controlo da proliferação e diferenciação celular, apoptose, neurogénese e plasticidade sináptica. Apesar de ser uma substância legal, o álcool é tóxico para os seres vivos e o seu consumo regular pode induzir dependência. O consumo excessivo de álcool aumenta o risco de doença hepática e de determinados cancros e tem efeitos deletérios ao nível do sistema imunitário, endócrino e nervoso. O cérebro, para além de ser um importante alvo dos metabolitos do álcool, é um dos órgãos com maior expressão de miRNAs. Através da análise de microarrays de miRNAs pretendeu-se entender de que modo a exposição crónica ao etanol (0.25% EtOH) e a exposição aguda a um pulso de etanol (0.25%; 0.5%, 1 e 1.5% EtOH) afectam a expressão dos miRNAs. A expressão de 32 miRNAs foi significativamente alterada pela exposição crónica ao etanol, destacando-se o aumento da expressão do miR-9, miR-23a, miR-30e, miR-133a, miR-181 e a diminuição na expressão do miR-16a, miR-145 e miR-181b. Genes envolvidos no ciclo celular, diferenciação, apoptose e adesão celular parecem ser os alvos preferenciais destes miRNAs. A exposição aguda também alterou a expressão de vários miRNAs, que variam consoante a concentração de etanol utilizada. Por exemplo, a expressão do miRNA pró-apoptótico miR-23a aumentou durante a exposição crónica ao etanol e diminuiu com o aumento da concentração do tóxico (1 e 1.5% EtOH) durante o teste agudo. Estes resultados sugerem que o consumo de álcool, mesmo num curto espaço de tempo ou concentração, afecta a expressão dos miRNAs. Possivelmente essas alterações têm implicações no ciclo celular e apoptose, podendo contribuir deste modo para um risco acrescido de desenvolver tumores.

keywords

miRNA, alcoholism, gene regulation, microarrays, *Danio rerio*

abstract

miRNAs are short (~22 nt) non-coding RNA molecules that control gene expression at post-transcription level by degrading or inhibiting particular target genes. miRNAs are abundant in vertebrate genomes where they play an important role on the control of cell proliferation and differentiation, apoptosis, neurogenesis and synaptic plasticity. Although legally commercialize, alcohol is toxic for living organisms and its consumption can induce dependence. The regular alcohol intake increases the risk of hepatic disease and certain cancers, having a deleterious effect on the immune, endocrine and nervous system. The brain is not only an important target for alcohol metabolites, but also a rich source of miRNAs. Using miRNA microarrays approach, we aimed to understand how chronic (0.25% EtOH) and acute (0.25%; 0.5%, 1 e 1.5% EtOH) ethanol exposure affects miRNA expression.

The results demonstrate that chronic ethanol intake deregulated the expression of 32 miRNAs. From those, miR-9, miR-23a, miR-30e, miR-133a, miR-181 were over-expressed and miR-16a, miR-145 and miR-181b were inhibited. The putative target genes for those miRNAs are implicated in cell cycle, differentiation, apoptosis and cell adhesion. The acute ethanol exposure also deregulated miRNA expression profile and each ethanol concentration shows a distinct miRNA expression profile. For example, the pro-apoptotic miRNA miR-23a is up-regulated in chronic ethanol exposure but is down-regulated in the higher ethanol concentrations (1 e 1.5% EtOH) of acute test. These results suggest that alcohol consumption, even consumed in a short-period or concentration, affects the expression of miRNAs. Possibly, these alterations have implications on cellular cycle and apoptosis and therefore they could contribute to a higher risk of developing tumours.

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Abbreviations

ADH	Alcohol dehydrogenase
Ago	Argonaute proteins
ALD	Alcohol liver disease
ALDH	Aldehyde dehydrogenase
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propriate
ATP	Adenosine triphosphate
BACE1	Beta-secretase 1
BDNF	Brain-derived neurotrophic factor
BK	Big potassium channels
Ca^{2+}	Calcium ion
CLOCK	Circadian Locomotor Output Cycles Kaput
CNS	Central Nervous System
CPP	Conditional place preference
Cy3	Cyanine 3
DoL	Degree of labelling
DNA	Deoxyribonucleic acid
D2	Dopamine receptor type 2
D1	Dopamine receptor type 1
EDTA	Ethylenediamine tetraacetic acid
EtOH	Ethanol
FAS	Fetal alcohol syndrome
GABA	Gamma-AminoButyric acid
GMP	Guanosine monophosphate
GR	Glucacorticoid receptor
GRIA2	Glutamete receptor ionotropic AMPA2
GRM5	Metabotropic glutamate receptor type 5
HER	Hydroxyethyl radical
HNE	4-hydroxy-2-nonenal
HSWS	High stringency wash solution
5-HT3	5-Hydroxytryptamine 3
IP	Inositol phosphate
Kb	Kilobases

KCl	Potassium chloride
LSWS	Low stringency wash solution
LTP	Long Term Potentiation
MAPK	Mitogen activate protein kinase
MDA	Malondialdehyde
mGlu	Metabotropic glutamate
mGlurR	Metabotropic glutamate receptor
MHB	Midbrain-hinbrain boundary
miRNA	microRNA
miR*	Star miRNA strand
mRNA	Messenger ribonucleic acid
NAc	*Nucleus accumbens*
NMDA	N-methyl-D-aspartic acid
NO	Nitric Oxide
nt	nucleotide
PDE	Phosphodiesterase
PI4K2B	Phosphotidylinositol 4-kinase type 2 beta
poly(A)	Polyadenylated tail
PTBP-I	Polypyrimidine Tract-Binding Protein I
P450	Cytochrome P450
$MgCl_2$	Magnesium chloride
NaAc	Sodium acetate
NPC	Neuronal progenitor cells
NSC	Neuronal stem cells
REST	Transcription repressor of neuronal genes
RGS4	Regulator of G-protein
RIN	RNA integrity number
RISC	RNA inducing silencing complex
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rotation per minute
rRNA	Ribosomal RNA
RT	Room temperature
R1	Replicate 1

R2	Replicate 2
siRNAs	Small interference RNAs
SYNJ1	Synaptojanin 1
TRBP	TAR-RNA binding protein
3´-URT	3´- untranslated region
UV	Ultra-violet
VTA	Ventral tegmental area
WHO	World Health Organization

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I. Introduction

1. Alcohol abuse

Alcohol abuse is a major concern of public health authorities. Although legally commercialized and generally consumed in social context, alcohol is a strong addictive substance that activates the reward circuits in similar ways to other recreational drugs such as cocaine and nicotine (Kalivas and O´Brien, 2008; Kily et al., 2008; Koob and Le Moal, 2008). Epidemiological studies reveal that pathologies associated with ethanol consumption are among the ten major risk factors of mortality in USA (Danaei et al., 2009), Canada and European countries (Her and Rehm, 1998; Hulrich and Hanke, 2002). The World Health Organization (WHO) statistics place Europe at the top of alcohol consumption per capita per year. Considering that ranking, Portugal occupies the 9th position, drinking 12.2 L of alcohol per capita, 2.7 L more than European average (Table 1) (World Health Organization, 2005).

Alcohol consumption has dose-dependent effects. Moderate drinking can be beneficial in healthy people (Holahane et al., 2010; Liang et al., 2010), however uncontrolled intake can result in severe health effects (Mayfield et al., 2002; Tateno et al., 2006; Markis et al., 2008; Schuckit, 2009). Considering heavy alcohol intake, two drinking profiles can be distinguished: chronic consumption, which includes every day drinkers and is related to the development of dependence (Epstein et al., 1995) and binge or heavy episodic drinkers (Crews et al., 2003; Ward et al., 2009). Both regimes have harmful effects on neurological function (Obernier et al., 2002a and Crews et al., 2004) and general metabolism (Ward et al., 2009). The chronic ethanol intake is associated with the development of various pathological conditions such as liver and pancreatic disorders (Lieber et al., 1979; Clemens and Mahan, 2010; O´Shea et al., 2010); cancer (Poschl and Seitz, 2004); fetal alcohol syndrome (FAS) (Kaminen-Ahola et al., 2010); cardiovascular disease (Spies et al., 2001) and central nervous system (CNS) injury (Jacobs and Miller, 2001; Herrera et al., 2003; Crews et al., 2004; Sullivan and Marsh, 2003). The binge consumption also induces cell damage, especially due to the increase of oxidative stress (Obernier et al., 2002b; Crews and Nixon, 2008). Alcohol deleterious effects also includes the risk of psychological disorders such as dementia (Järvenpää et al., 2005), depression (Brady, 2006) and aggressive or risky behaviour that lead to social problems such as violence, traffic accidents or unemployment (Kushner, 2005; Schuckit, 2009).

The severe ethanol effects are attributed to the toxic by-products that result from its metabolites (Das and Vasudevan, 2007). The ethanol breakdown occurs in the liver and is metabolised by alcohol dehydrogenase (ADH) and cytochrome P450 2E1 (P450) in a greater extent (Figure 1). ADH converts alcohol into acetaldehyde, a highly toxic compound that binds to proteins and generates adducts. The chemical reaction between cytochrome P450 2E1 and alcohol also produces acetaldehyde but the major reaction products are reactive oxigen species (ROS) (Zakhari, 2006). Oxygen radicals can interact with lipids, in a process called lipid peroxidation, resulting in the formation of high oxidative molecules such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) (Tuma et al., 2003). Lipids are essential for brain function, being the backbone of several membranes and also acting as second neurotransmitters in signal transduction pathways (Farooqui and Horrocks, 1998). On the other hands, oxidation of proteins results in the production of hydroxyethyl radical (HER) molecules that can also damage brain function (Tuma et al., 2003; Chattopadhyay and Paila, 2007).

Table 1: WHO statistics for alcohol consumption (2005). The European mean is 9.5L per year, per adult. The methodology to convert alcoholic drinks to pure alcohol made the correspondation: beer as 4-5%; wine as 11-16% and spirits as 40% of pure alcohol equivalent. Adult is considered a person over 15 years of age. (Adapted from WHO – Global Health Indicators: Risk Factors, 2005)

Rank	Country	Number of litres of pure alcohol consumed per adult, per year
1.º	Czech Republic	14.8
2.º	Ireland	13.4
3.º	France	13.2
4.º	Andorra	12.8
5.º	Austria	12.7
6.º	Croatia	12.5
7.º	Hungary	12.5
8.º	Lithuania	12.5
9.º	**Portugal**	**12.2**
10.º	Uganda	11.9
11.º	Republic of Korea	11.8
12.º	Germany	11.7
13.º	Luxembourg	11.7
14.º	Armenia	11.5
15.º	United Kingdom	11.5

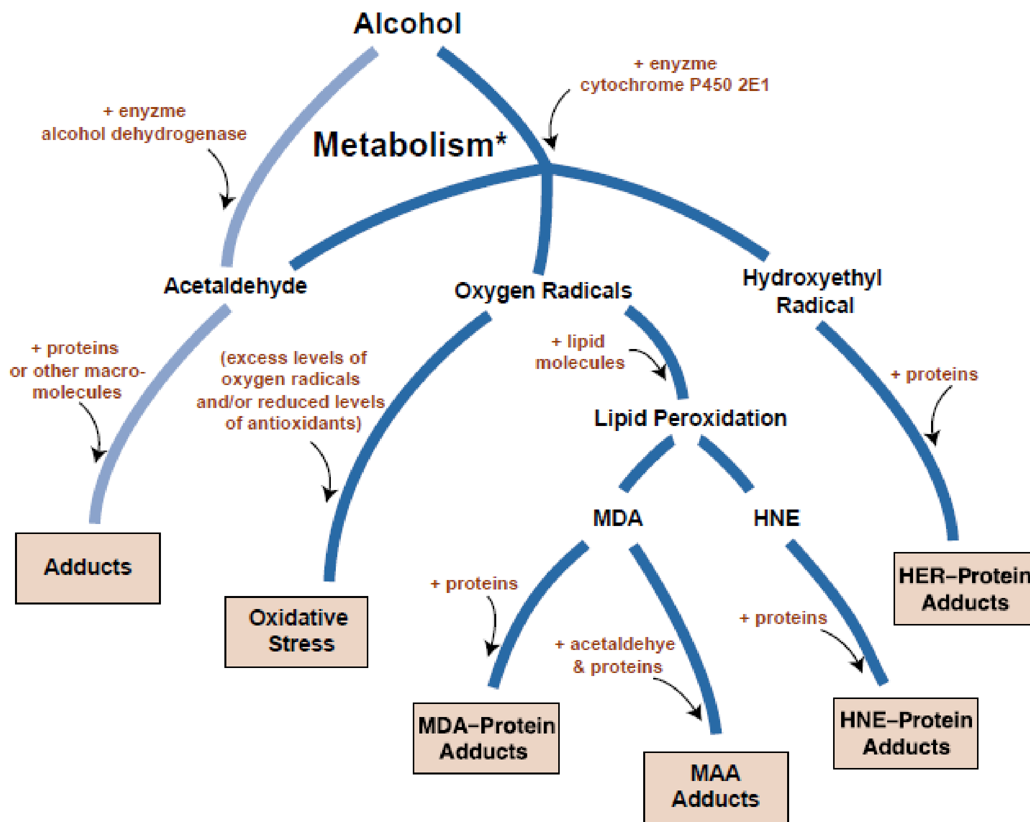


Figure 1: Toxic products resulting from ethanol breakdown (Tuma and Carol, 2003). The enzymes ADH and P450 are the major enzymes responsible for alcohol degradation. ADH converts ethanol to acetaldehyde that can react with proteins or other macromolecules to generate unstable molecules called adducts. Alcohol degradation by cytochrome P450 generates acetaldehyde and reactive oxygen species (ROS).

2. miRNA overview

2.1. Biogenesis

miRNAs are short (~22 nt length) non-coding RNA molecules. They modulate gene-expression at post-transcriptional level by base-pairing to 3´-UTR of mRNA transcripts, inducing transcript repression or cleavage (Bartel, 2004). The first reference to a miRNA molecule dates from 1993, when Lee and colleagues identify a gene, *lin-4*, implicated in the development of *C. elegans* that was transcribed into a small RNA molecule instead of a protein (Lee et al., 1993). The miRNAs are present in a wide diversity of unicellular and multicellular organisms including animals, plants, algae and virus (Griffiths-Jones et al., 2008) and are highly phylogenetically conserved (Ambros, 2004). The number of miRNAs that can be found in certain species is directly proportional to the complexity of that organism, suggesting the importance of miRNAs in the course of evolution (Vreughenhil and Berezikov, 2009). It is estimated that approximately 30% of animal genes are regulated by miRNAs (Kapsimali et al., 2007). miRNA coding genes can either be located in genome coding regions, using their own transcription machinery to replicate, or lie within introns of coding and non-coding genes where there are generally transcribed together with the host genes (Lagos-Quintana et al., 2001). The miRNA genes are transcribed in the nucleus by polymerase II, generating a pri-miRNA molecule with ~1Kb length. This first transcript is similar to other mRNA transcrips, containing 5′ cap structure as well as 3′ poly(A) tail, but have the particularity to fold themselves generating hairpin structures (Lee et al., 2004). In the nucleus, pri-miRNA is recognized and cleaved by the complex constituted by the endonuclease Drosha and the protein DGCR8. The cleavage occurs near the base of the stem loop and generates a ~70 nt stem loop intermediate duplex with a 3´overhang called pre-miRNA (Lee et al., 2003). The pre-miRNA overhang is recognized by Ran-GTP and exportin-5 that enables the transport of the molecule into the cytoplasm. Once in the cytoplasm, the pre-miRNA characteristic hairpin is cleaved by the endonuclease Dicer and TRBP protein, producing a ~20 nucleotides length miRNA duplex. Depending on the duplex thermostability and 3 ´overhang identity, one of the two strands is selected to be biological active, while the other is target for degradation (Lee et al., 2002). The selected strand it is called mature miRNA, while the less stable strand is identified as passenger/star strand (miR*). The

mature miRNA is then incorporated in the RNA-induced silencing complex (RISC) that contains mostly Argonaute (Ago) proteins (Hammond et al., 2000). When the mature miRNA is assembled into the RISC, the complex is guided to the complementary mRNA transcript. Hybridization between miRNA:mRNA only occurs if there is perfect match between 2nd-8th nucleotides of the 5´end of the miRNA (seed sequence) and the 3´-UTR of the target mRNA (Lewis et al., 2003, Starck et al., 2003). The remaining miRNA sequence can bind to the 3'UTR of the mRNA with some mismatches. If perfect base-pairing is observed between miRNA:mRNA, a similar mechanism as the one observed for siRNAs takes place, with the mRNA being targeted for degradation, possibly in the P-bodies; if there are mismatches between the miRNA and mRNA the target translation is blocked at the initiation or elongation step (Bartel, 2004). If both miR and miR* strands are stable, miR* can also target mRNA instead of being degraded (Ghildiyal et al., 2010) (Figure 2).

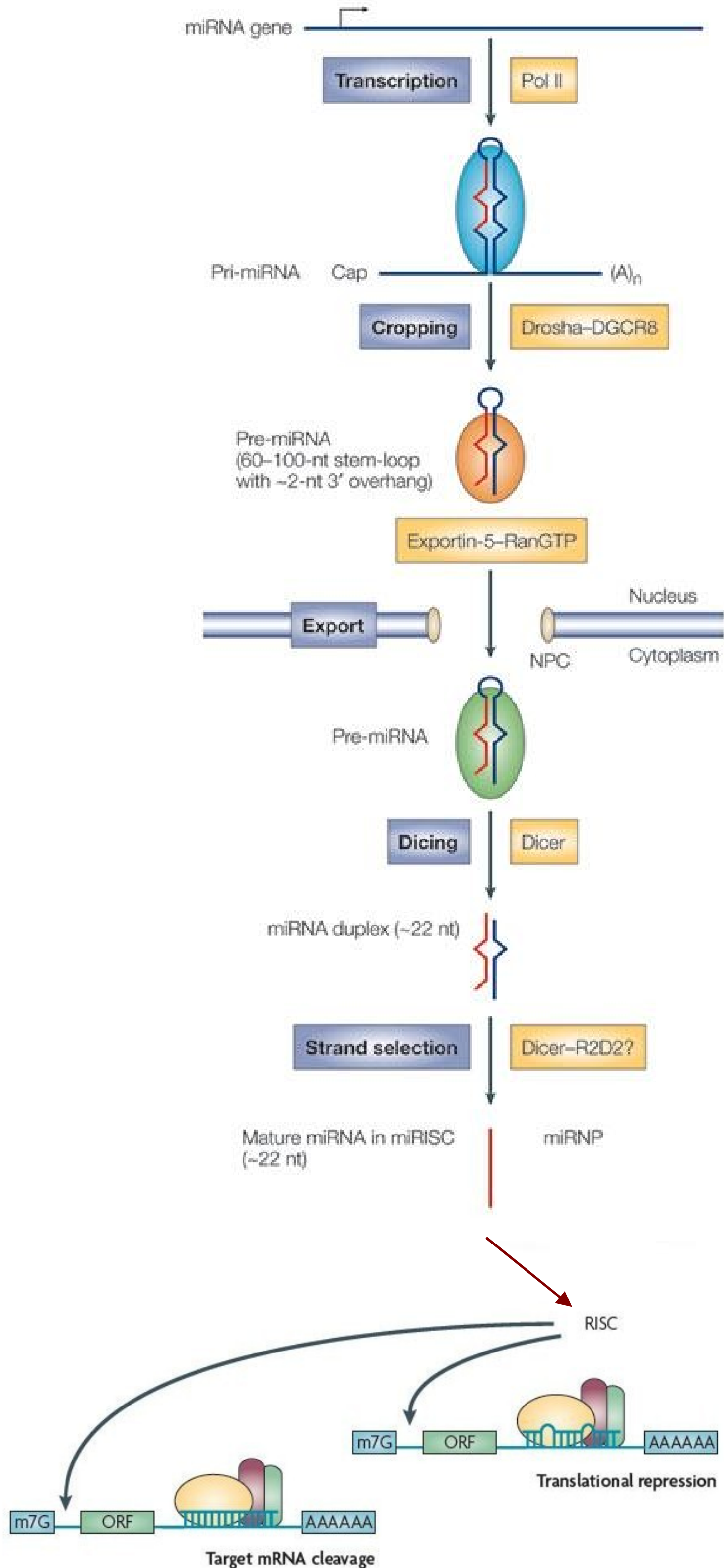


Figure 2: miRNA biogenesis and mechanism by which miRNAs regulates gene expression (adapted from Kim et al., 2005).

2.2. miRNAs in the central nervous system

miRNAs have been found to been expressed during brain development (Giraldez et al., 2005; Kloosterman et al., 2006) and in particular neuronal cell types (Karp et al., 2005), being implicated in cell proliferation (Johnnidis et al., 2008; Stadler et al., 2008) , neurogenesis (Zhao et al., 2009), synaptic plasticity (Schratt et al., 2006), dendritic morphology and learning (Li et al., 2001; Ashraf et al., 2006; Schratt et al., 2006; Luigli et al., 2008).

Most of the knowledge on miRNA function was achieve by experiments where Dicer was deleted (Yi and Piatigorsky, 2009), compromising miRNA biogenesis or by blocking the miRNA action by gain- or loss-function assays (Krützfeldt and Poy, 2006). Aberrant miRNA expression was found to be implicated in the loss of neuron phenotype (Conaco et al., 2006; Makeyev et al., 2007), development malformations (Giraldez et al., 2005), loss of homoeostasis and pathological stages (Kloosterman and Plasterk; Stark et al., 2008; Baudry et al., 2010). Together, those findings suggest that miRNAs play a central role in the regulation of brain function.

For example, miR-124 and miR-128 are ubiquitously expressed in the brain cells and have a central role on neural differentiation (Smirnova et al., 2005). The miR-124 was found to be involved in the preservation of neuronal phenotype by regulating the expression of neuronal differentiation-inducing factors, particularly the transcriptional repressor of neuronal genes (REST), the repressor of alternative splicing in non-neuronal cells (PTBP-I), glial cell generation gene (Sox9) and also glucacorticoid receptors (GR) (Vreugdenhi and Berezikov et al., 2009). Moreover, additional analysis showed that miR-124 expression in sufficient to revert the phenotype of non neuronal cells into neuronal cells (Kim et al., 2005). The miR-128 was also found to be fundamental to neuronal differentiation and its expression was enhanced when neuronal stem cells were incubated with retinoic acid, a neuronal inducing agent (Krichevsky et al., 2006). Other example of a brain-specific miRNA is miR-9. This miRNA is mainly express in cells undergoing differentiation and previous studies shown that the inhibition of this miRNA reduces the number of differentiated neurons (Krichevsky et al., 2006). The expression of this miRNA also defines the limits of midbrain-hindbrain boundary (MHB) in developing zebrafish (Leucht et al., 2008), an organization centre of neural tube formation. Other miRNAs display a cell specific expression, in zebrafish miR-222 is restricted to telencephalon, miR-

218a is expressed in motor neurons (kapsimali et al., 2007) while miR-26 and miR-29 are principally expressed in astrocytes (Smirnova et al., 2005).

Several authors have described the role of miRNA in synaptic plasticity (Christensen and Schratt, 2009). Short-term synaptic alteration implicates modifications in pre-existing proteins and stimulation of plasma membrane receptors (Kosik, 2006). On the other hand, long-lasting synaptic plasticity implicates protein synthesis as it is required for long-term memory formation (Frey et al., 1997). The protein synthesis occurs locally in the synaptic terminals and dendritic spines (Aakalu et al., 2001). The presence of Dicer and both pre-miRNA and mature miRNA on those locations, suggests the interaction between miRNAs and mRNA in mRNA translation control. It is possible that neuronal stimulation activates Dicer, which in turn allows the processing of pre-miRNA into mature miRNAs. The mature miRNA interacts with cognate mRNA regulating local mRNA translation (Ashraf et al., 2006; Luigli et al., 2008). The brain-specific miRNA, miR-134 is related to synaptic plasticity. This miRNA was found in synaptoneurosome and Lim domain containing protein kinase 1 (LimK1) was validates as one of its targets (Schratt, 2006). This gene modulates dendritic spine morphology by regulating actin filament organization. By repressing *LimK1*, miR-134 induced repression of LimK1 protein, inhibiting dendritic spine growth. However, the release of brain derived neurotrophic factor (BDNF) after synaptic stimulation reverted this phenotype, but the mechanism underlying this cascade of regulation between LimK1, miR-134 and BDNF is currently unknown (Christensen and Schratt, 2009).

Several studies demonstrated that miRNAs contribute to the onset of neurological diseases (Alzheimer, Parkinson, Huntingtin, and Schizophrenia) and affect cognitive function. Neuronal death is a common feature of neurodegenerative diseases such as Alzheimer, Parkinson and Huntington (reviewed by Hébert and Strooper, 2009). In Parkinson patients, the loss of dopaminergic neurons in the midbrain is related to the decrease in expression of miR-133b (Kim et al., 2007). A polymorphism in the pre-miR-30e is associated with risk of schizophrenia (Xu et al., 2010) and the cluster miR-29a/b-1 is responsible for regulating the BACE1 protein that, together with β-secretase, is responsible for cleaving amyloid percurssor plaques (APP) (Hébert et al., 2008).

Having in mind that aberrant cell death is a cause of several neurodegenerative diseases (Harper and Wilkie, 2003) it is important to relate deregulated apoptosis to the onset of these pathologies .The apoptosis, or programmed cell death, normally occurs throughout the life cycle, being implicated in development but also in the maintenance of adult tissues homoeostasis. The lack of apoptosis is related to cancer and some autoimmune diseases, while neurodegenerative diseases are associated with high apoptosis (Xu et al., 2004). Apoptosis pathways are highly conserved between zebrafish and mammals (Figure 3) (Eimon and Ashkenazi, 2009) and miRNAs regulate both pro-apoptotic and anti-apoptotic intermediates (Subramanian and Steer, 2010). Pro-apoptotic or tumor suppressor miRNAs include let 7a, miR-15a, miR-16 and miR-34a that are mainly under-expressed in cancer cells (Subramanian and Steer, 2010). The capacity of miR-15 and miR-16 to induce apoptosis is because they target the anti-apoptotic gene BCL2 (B-cell CLL/lymphoma 2) (Cimmino et al., 2005). Also, up-regulation of miR-23a-27a-24-2 cluster induces apoptosis in embryonic kidney cells (Chhabra et al., 2009) and the over- expression of miR-34a induces apoptosis in malignant peripheral nerve sheath tumour cell lines (Subramanian and Steer, 2010). The let-7a target caspase-3 effector (Tsang and Kwok, 2008). Considering anti-apoptotic miRNAs, the miR-17-5p and miR-20a morpholino injection induces apoptosis in lung cancer cells (Matsubara et al., 2007). Recently, miR-21, miR-130, miR-140 and miR-290 were associated with rat liver mitochondria and putative target genes of those miRNA seem to be related to apoptosis, cell proliferation and differentiation (Kren et al., 2009). Moreover, muscle specific miR-29, miR-1, miR-133 and miR-206, also seem to be implicated in the regulation of apoptosis. The miR-1 and miR-133 are implicated in various cardiovascular diseases and in the regulation of cardiomyocite proliferation (Tang et al., 2009). While miR-1 acts as a pro-apoptotic agent by targeting to HSP (Xu et al., 2007), miR-133 targets caspase 9, acting as an anti-apoptotic effectors (Xu et al., 2007).

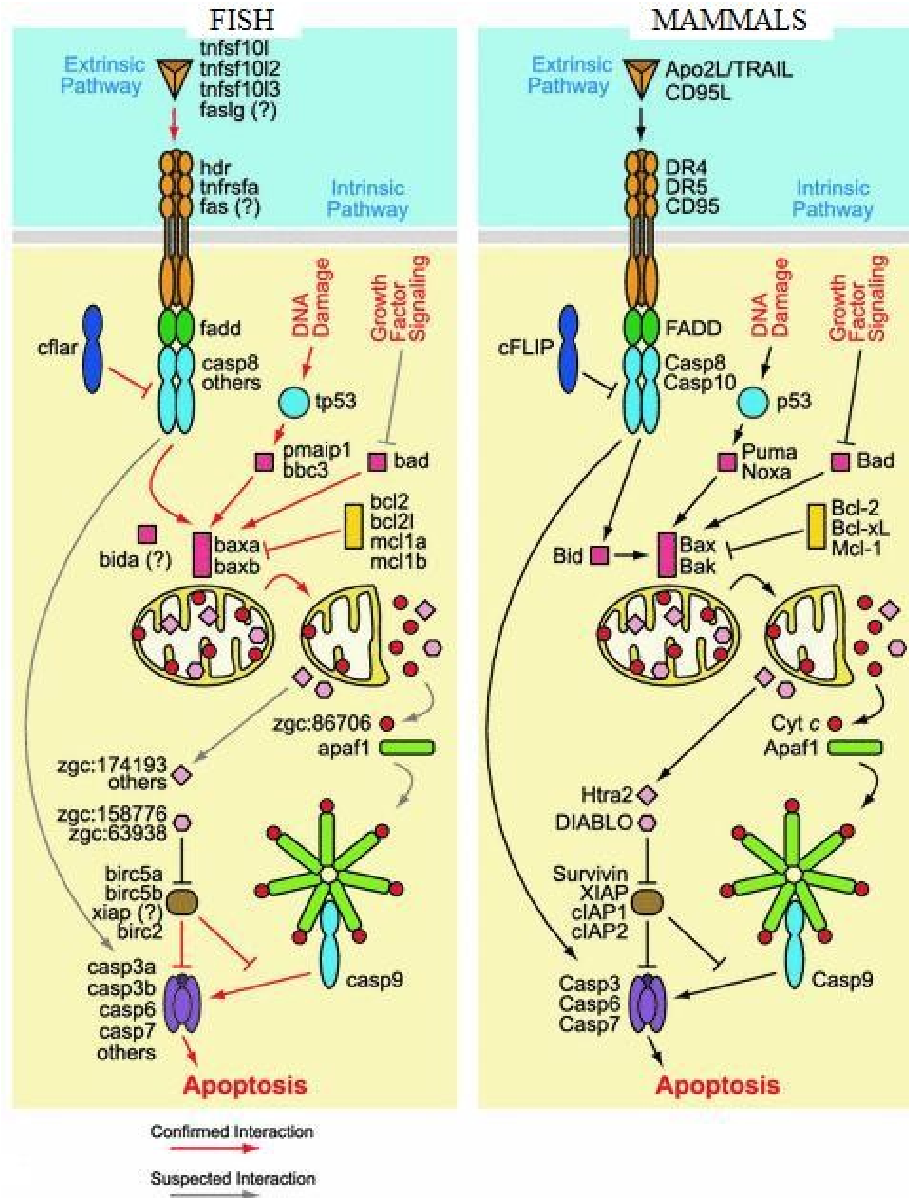


Figure 3: Comparison between apoptotic signalling pathways in fish and mammals. Several intermediates of apoptotic response are conserved or exist in and homologue form (Eimon and Ashkenazi, 2009).

2.3. miRNAs and ethanol

Cells have the ability to adapt to changing environments. As a neurotoxic substance, ethanol elicits several cell responses that act together to minimized ethanol toxic effect. In the liver, alcohol is metabolized by ADH and ALDH into less toxic intermediates and in central nervous system alcohol induces changes in neuronal function. The central

nervous system is constituted by a complex neuron network and communication is established through synapses of those neurons (Pietrzykowski, 2010; Peng and Yin, 2009). Changes in neuronal plasticity induced by ethanol include alteration of neurotransmitter basal levels, deregulation of endocrine system and alteration in both signal transduction pathways and gene expression (Ward et al., 2009) as it will be later discuss. These neuro-adaptations are associated with several aspects of drug addiction such as seeking-behaviour; withdrawal; tolerance and relapse (Kalivas and O´Brian, 2008). Alcohol administration has been shown to alter protein translation without changing mRNA levels (Dodd and Lewohl, 1998) suggesting the implication of miRNA in post-translational control of gene expression.

Recent findings identify some miRNAs that are triggered by ethanol and that can modulate some of the ethanol addiction symptoms. In fetal mouse brains, microarray analysis shown that ethanol teratogenic effects were related to enhance expression of miR-10a, miR-10b, miR-9, miR-145, miR-30a-3p and miR-152 and reduced expression of miR-200a, miR-496, miR-296, miR-30e-5p, miR-339, miR-29c and miR-154 (Wang et al., 2009). Several authors also demonstrated the importance of miR-9 in ethanol response. Pietrzykowski and colleagues (2008) reported that an increase on miR-9 expression after 15 minutes of ethanol exposure. In this case, the amount of alcohol used was similar to an episode of social drinking. However, exposure to a higher ethanol doses (similar to alcoholics level) inhibited the miR-9 expression (Adachi et al., 1991; Sathyan et al, 2007), suggesting the dose dependent effects of this drug. The miR-9 is implicated on the regulation of BK channels that contributes to neuron excitability, modulation of action potentials and neurotransmitter release (Shipson et al., 1999; Hu et al., 2001 and Martin et al., 2004). Different isoforms of BK channels have different sensibility to ethanol and the most sensible isoforms are targeted by miR-9. Cosnequently, loosing sensibility to ethanol could explain why alcoholics increase the alcohol intake to achieve the drug reward effects (Kalant, 1998). Other miR-9 targets are also implicated in the molecular response to ethanol intake, for example, mutations on the dopamine receptor 2 (DRD2) was associated with higher risk of ethanol abuse (Volkow et al, 2006), the GABA(A) receptor contributes to the development of dependence (Sander et al., 2009) and the SYNJ1 regulates synaptic vesicles recycling, which is important for synaptic plasticity (Nemoto et al., 2001). miR-9

also target CLOCK genes important to the regulation of circadian cycles (reviewed by Pietrzykowski, 2010).

Ethanol effects also seem to affect miRNAs involved in the neurogenesis, by counter-balance the ratio between undifferentiated neuronal stem cells (NSCs) and progenitor cells (NPCs) responsible for the generation of most adult neurons (Bayer et al., 1993). The miR-21, miR-335 and miR-9 are implicated in control of NSCs/NPCs proportion (Miranda et al., 2010). In normal conditions, the anti-apoptotic miR-21 and the pro-apoptotic miR-335 counter-balance cell proliferation and cell survival (Figure 4). Under ethanol influence, expression of miR-21 and miR-335 is repressed (Xu et al., 2009). Theoretically, block of anti-apoptotic miR-21 should result in apoptosis but, the block of pro-apoptotic miR-335 not only increases cell proliferation but also repress apoptosis mediated by miR-21 (Sathyan et al., 2007). The resulting phenotype is an increase on cell proliferation, inducing differentiation of NSC without cell death. The miR-9 was also implicated in NSC fate by targeting to *Foxg1* in cajal-retzius cell (Shibata et al., 2008) and in the differentiation of embryonic stem cell (Krichevsly et al., 2006). The simultaneously expression of miR-21, miR-135 and miR-335 also targets Jagged-1, a notch ligand and the RNA binding protein ELAVL2/HuB implicated in neuronal identity (Sathyan et al., 2007).

Large screening of liver miRNAs found that ethanol modifies the expression of ~2% of analyzed miRNAs. Deregulated miRNAs were related to lipid homeostasis and inflammatory pathways (Dolganiuc et al., 2009). Alcohol liver disease (ALD) is common in heavy drinkers (Burbige et al., 1984) and it is hypothesis that gut leakiness and endotoxins invasion facilitate ALD onset (Keshavarzian et al., 2009). Integrity of intestine gut is assured by adhesion molecules. Ethanol increased the miR-212 level which is related to disruption of thigh junction protein ZO-1 (Tang et al., 2008) and thus loss of cell adhesion. Those are some examples of how ethanol regulates miRNAs and the phenotypic effects of this deregulation. Having in mind that each miRNA can regulate hundreds of genes and that a single gene can be targeted by different miRNAs, it is important to experimental validate the predicted miRNA targets in order to achieve a higher knowledge of miRNA regulation and function.

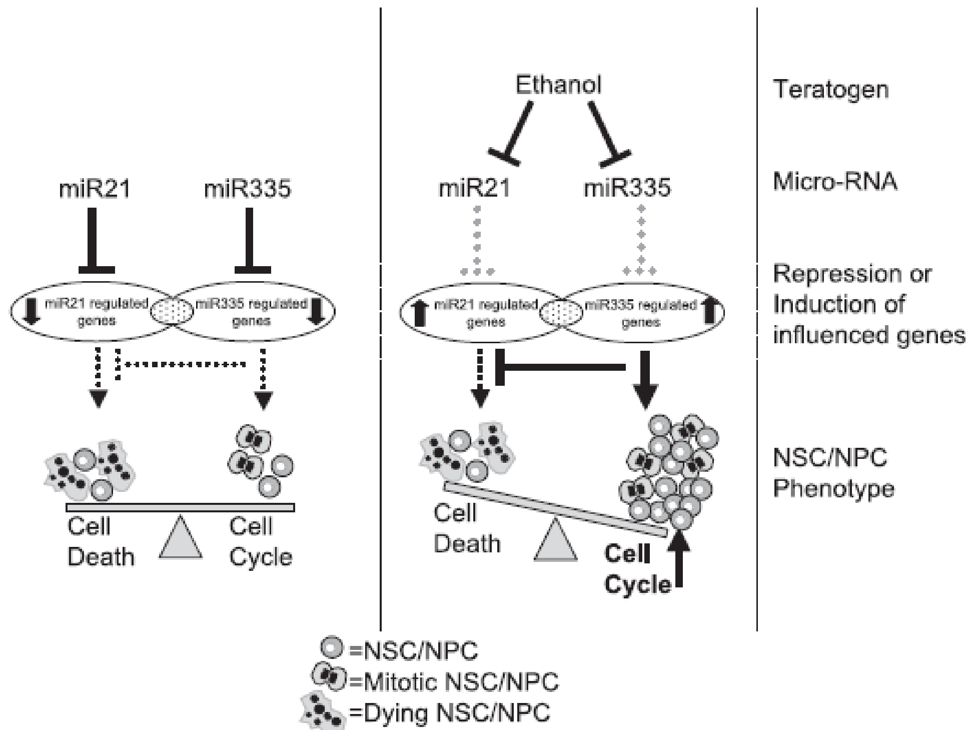


Figure 4: Regulation of neural fate by miR-21 and miR-335 under normal physiological conditions and ethanol effect. Ethanol induces differentiation of neural stem cells without affecting cell viability. NSC: neural stem cell; NPC: neural progenitor cell (Miranda et al., 2010)

3. Neurobiology of addiction

The chronic alcohol administration induces adaptive changes manifested as tolerance and addiction (Damodaran, 2006) that reflect the neurotoxic effect of this substance. The development of tolerance in manifested as lost of sensitivity to a certain drug doses (Torregrossa and Kalivas, 2008) and reflects the profound neurological modifications induced by drug intake (Torregrossa and Kalivas, 2008), while addiction resumes the vulnerability to relapse after long periods of drug abstinence. However, tolerance can be triggered by a single episodes of drug intake (Morato and Khanna, 1996). The mechanisms inherent to tolerance development are similar to those implicated in learning and memory acquisition. The effect of ethanol on brain “communicators” – the neurotransmitters glutamate, dopamine, opiates, serotonin and Gamma-aminobutyric acid (GABA) will be discussed below (Koob and Le Moal, 2001).

Glutamate

Glutamate is the major neurotransmitter implicated in learning and memory acquisition (Riedel et al., 2003). It is released in synaptic gaps, where it targets the glutamate receptors N-methyl-D-aspartic acid (NMDA), α-amino-3-hydroxyl-5-methyl-4-isoxazole (AMPA) and metabotropic glutamate (mGlu) (Ward et al., 2009). The binding of this neurotransmitter to the NDMA receptors activate the Long Term Potentiation (LTP) mechanism, which is a synaptic mechanism that allows for memory formation. Briefly, the activation of NMDA receptors increases the calcium ion (Ca^{2+}) influx, leading to the activation of calcium dependent proteins. These calcium proteins enhance the production of nitric oxide (NO) by activation of nitric oxide synthase (NOS). NO acts as a retrograde messenger by diffusing through the membrane to the pre-synaptic terminal and the repetitive stimulation of pre-synaptic cells results in increased synaptic strength, leading to the increase of LTP and thus memory formation (Bliss and Collingridge, 1993) (Figure 5). However, excessive glutamate release is also a major cause of neuronal cell death (Stelmashook et al., 2010). Generation of NO leads to mitochondrial depolarization and activation of Na^{+} influx, leading to a unsustainable increase in ATP demand, microtubule depolymerization, mitochondrial damage and dendritic beading (Greenwood et al., 2007). Furthermore, the calcium influx also stimulates phospholipase (PLA) to generate ROS (Murphy et al., 1989; Choi, 1992) (Figure 6).

Several studies have described an increase on glutamate release in *nucleus accumbens* (NAc) in animals submitted to binge drinking (Szumlinski et al., 2007). A decrease in the sensitivity of NMDA receptors occurs during ethanol detoxification (Kumari and Anji, 2005) despite of the apparent increase in the number of receptors. Other studies show no differences in the density of NMDA receptors after consumption of high amounts of ethanol (Crews et al., 1996). A study of Khanna and colleagues (1993) demonstrated that rapid tolerance to ethanol can be blocked using NOS antagonists.

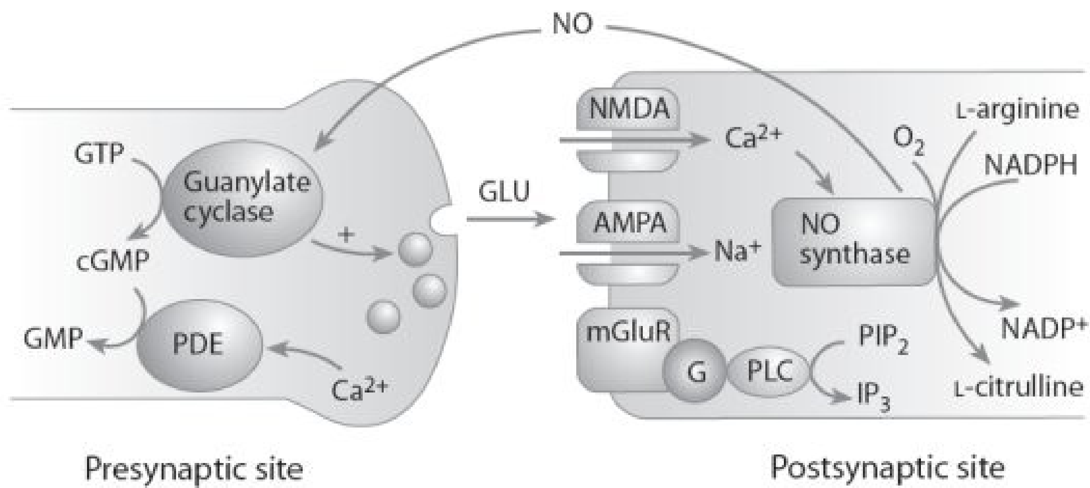


Figure 5: LTP mechanism mediated by retrograde nitric oxide in the hippocampus glutamate synapse. The excitatory neurotransmitter glutamate activates AMPA and mGluR leading to the recruitment of NMDA receptor activity. Ca^{2+} enters into the post-synaptic site via NMDA receptor channels, activating NOS production. The NO diffuses into the pre-synaptic neuron where it is absorbed by a group of NO-sensitive guanylate kinase enzyme which in turn triggers the production of cyclic GMP, increasing neurotransmitter release. mGluR: metabotropic glutamate receptors; IP: inositol phosphate; IP_3: inositol triphosphate; PDE: phosphodiesterase; NO: nitric oxide; NOS: nitric oxide synthase (Adapted from Holscher, 1997).

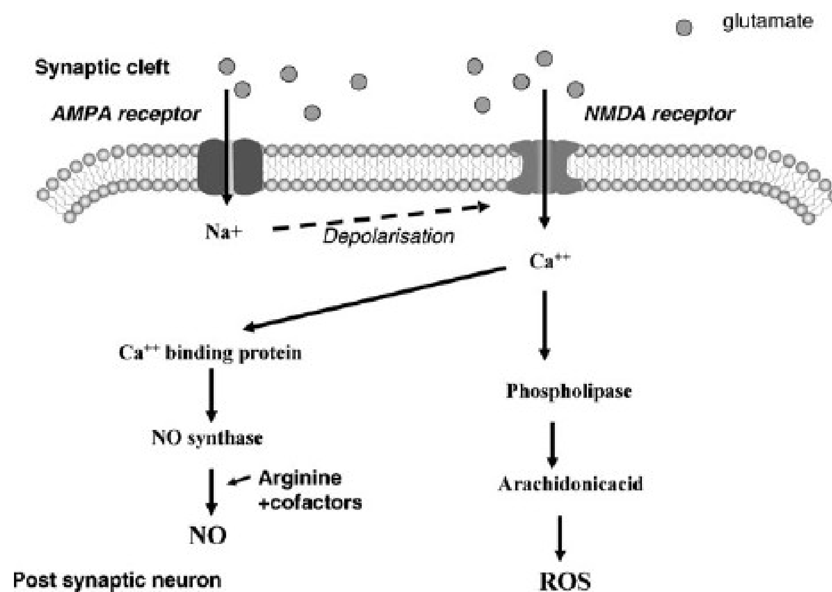


Figure 6: Cytotoxic effect of excessive glutamate release (Ward et al., 2009). The glutamate released on the synaptic cleft stimulates AMPA and NMDA receptors inducing Na^+ and Ca^{2+} influx respectively. The calcium will bind to calcium binding proteins that activate NO synthase to produce NO and also activate PLA to produce ROS.

Dopamine

Dopamine is an excitatory molecule that plays an important role on the motivational and reinforcement mechanisms related to behaviour, by interacting with the mesolimbic system. The mesolimbic system includes the brain amygdala, hippocampus, *nucleus accumbens* (NAc), *ventral striatum*, ventral diencephalon (including basal forebrain, ventral tegmentum, and hypothalamus), as well as cortical areas (Oscar-Berman and Bowirrat, 2005). Alcohol increases dopamine release in the mesolimbic system, particularly on the NAc and also increases the firing rate of dopaminergic frontal cortex neurons (Heidbreder and De Wittem, 1993; Weiss et al., 2003 and Gatto et al., 1994).

Defects in dopaminergic circuits, including the death of dopaminergic neurons, are related to the development of drug addiction and a variety of neurodegenerative diseases such as schizophrenia, attention-deficit, hyperactivity disorder and Parkinson disease (Silitoe et al., 2008).

Stimulation of dopamine release in the NAc and ventral tegmental area (VTA) were observed in rats submitted to self-administration of ethanol (Weiss et al., 1993 and Gatto et al., 1994). In chronic alcohol abuse, large amount of alcohol are need to achieve the false sensation of pleasure induced by ethanol intake. Moreover, during withdrawal, dopamine levels decrease dramatically which may cause disphoria and depression (Wang et al., 2009).

Serotonin

Serotonin is an excitatory neurotransmitter responsible for several aspects of behaviour: mood; sleep; pain; appetite (Carlson et al., 1998). A pulse of alcohol increases the serotonin release in the CNS, but repeated alcohol consumption leads to decrease in extracellular serotonin concentrations in the NAc (Szumlinski et al., 2007). Considering serotonin receptors, the $5\text{-}HT_3$ receptor is described as a strong ethanol target and the presence of ethanol deregulates this receptor, probably through the interaction with the receptor proteins (Lovinger, 1999; Rood et al., 2007).

Opiates

The opioid system modulates pain, mood reinforcement and response to stress (Buttner, 2010). Alcohol administration enhances the opioid activity and the result is the

increase in endogenous opiates (Herz, 1997). In occasional alcohol consumption events, opiates can modulate the dopamine release, contributing for the activation of reinforcement pathways (Herz, 1997). However, in chronic ethanol users, the levels of endorphins (opiate molecules) are low (Wand et al., 1993) which can explain some of the physical dependence symptoms.

4. Zebrafish: a model organism for studying alcohol addiction

Zebrafish (*Danio rerio*) is a freshwater teleost fish that emerged in the last twenty years as a promissory model organism for biological research (Grunwald and Eisen, 2002). The fish small size, short life cycle (larvae hatch in 5 days and reach sexual maturity is 3 - 4 month), high fertility rate (each female can produce c.a. 200 eggs per day), embryo transparency that allows the monitorization of embryo development, together with the genome similarity to other vertebrates, are some of the characteristics that make this fish so appreciated by scientific community (Lawrence, 2007) (Figure 7). At the beginning, zebrafish was mainly used by embryologist (Kimmel et al., 1995) and geneticist in forward genetic studies (Streisinger et al., 1981). The extensive mutagenesis allow large screen of mutations in genes implicated in development (Driever et al., 1996). Nowadays reverse genetic tools, like antisense morpholino oligonucleotide injection and *in situ* hybridization are also very useful to gain/loss function studies. Moreover, methods for large-scale screening as microarray analysis increased the researchers interest for this organism and justify why zebrafish is widely used in cancer research (Feitsma et al., 2008), in vivo drug discovery (Zon and Peterson, 2005), development (Driever, 1996; Thisse and Zon, 2005), neurobiology (Elicerci et al., 2011), toxicology (Adrian et al., 2005) and aquaculture (Dahm and Geisler, 2006).

Zebrafish shares considerable similarity with mammals in both genetic background (Liu et al., 2004) and organ structures (Guo, 2009) and demonstrates higher capacity to throughput high screenings when compared to rodents (Ninkovic et al., 2005). A set of behavioural tests have also been established to allow the study of complex behaviours such as addiction, anxiety, learning capacity and memory (Ninkovic et al., 2005; Gerlai et al., 2006). Zebrafish show positive response to alcohol by exhibiting conditioned place preference and alteration of genetic function (Killy et al., 2008). These results contributed

to validate the use of zebrafish as a model to study addiction and the implicated alterations on neuronal function. Still attention should be paid when using adult fish to study addiction because they are sensible to handling and because the breeding procedures are more complex than in larvae (Ninkovic et al., 2005).

Figure 7: External morphology of adult zebrafish (*Danio rerio*)
http://www.focusonnature.be/keywords/zebrafish (17-12-2010)

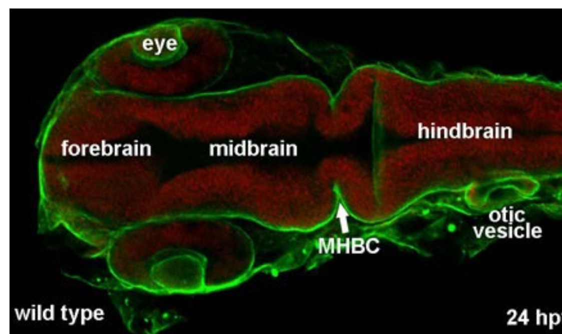


Figure 8: Zebrafish larvae brain. MHBC: midbrain-hindbrain barrier
http://web.wi.mit.edu/sive/pub/Ongoing%20Projects2.htm (17-12-2010)

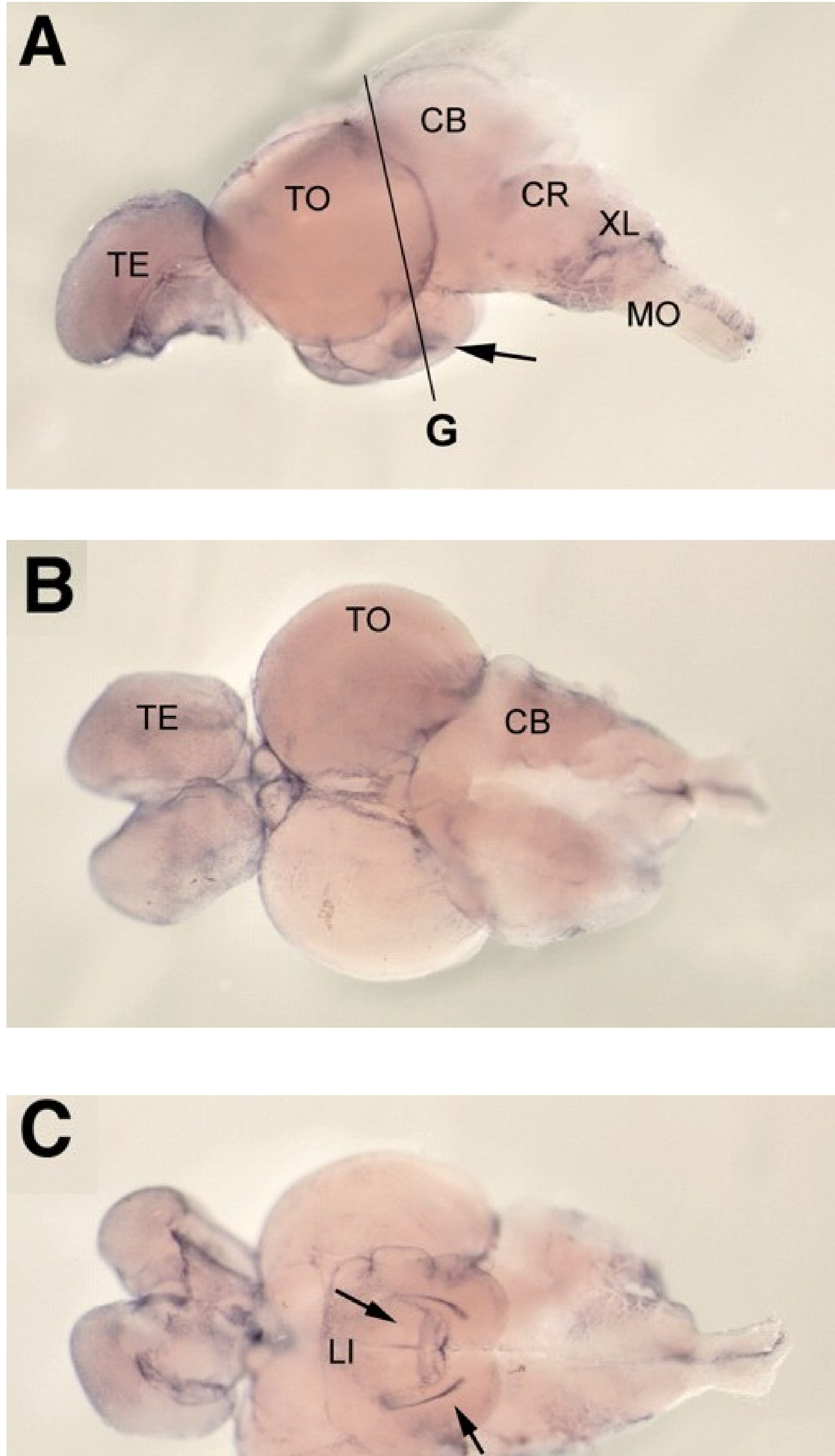


Figure 9: Sections on adult zebrafish brain. **A)** Lateral view: TE) Telencephalon; TO) tectum opticum; CB) corpus cerebelli; CR) crista cerebellaris; (XL) lobus vagi; MO) medulla oblongata; Arrow) Hypothalamos. **B)** Dorsal view. **C)** Ventral view (Adapted from Winkler et al., 2003).

5. Microarrays: a useful platform for miRNA expression profiling.

Large screening studies require high throughput technologies. miRNA microarrays based on hybridization between sample miRNAs and complementary nucleotide sequences (probes) are normally used for miRNA profiling. The hybridization principle is used in several molecular biology techniques, namely in Southern and Northern blotting. However while blotting techniques only analyse few fragments, microarrays analyse thousands of molecules simultaneously (Figure 10) (Southern, 2001).

Microarray assay is semi-quantitative methodology, because hybridization signal is just indicative of the amount of molecules present in our sample and there is no quantification of the number of molecules that have been expressed. In other words, the measured intensities of each array correspond to relative expression values.

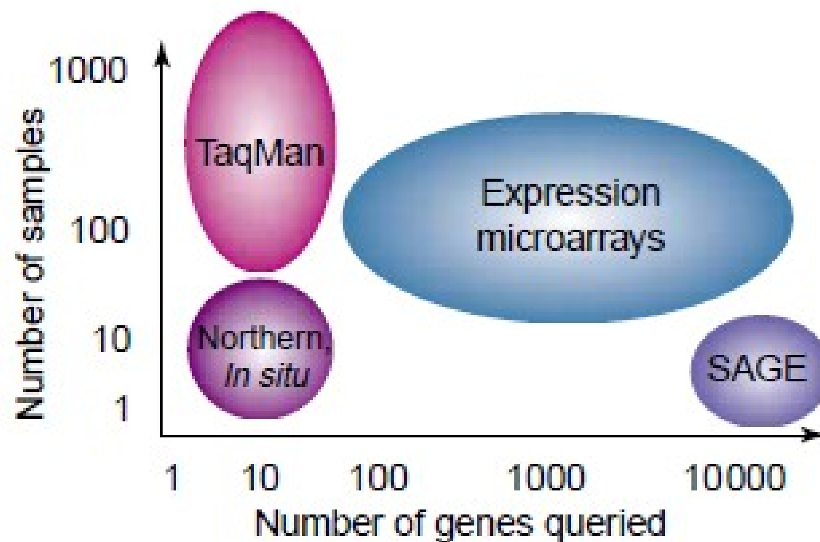


Figure 10: Comparison on molecular techniques available for the study of RNA expression profiles. The graphic highlights the high throughput capacity of microarrays in gene expression studies. A large number of molecules are detected in a considerable huge number of samples (adapted from Mirnics et al., 2001).

6. Aims

miRNAs are critical regulators of cell physiology, particularly on cell proliferation, differentiation and plasticity. Ethanol, by affecting miRNA expression will in turn destabilize those fundamental cell processes. By comparing the expression values of basal treatment (no alcohol) with chronic and short-acute ethanol exposure we aimed to verify if alcohol affects miRNAs expression, explore the impact of different patterns of ethanol exposure on zebrafish miRNA expression profile and give insights on the possible effects of deregulated miRNAs on brain function.

II. Material and methods

1. Zootechnical conditions and experimental animals

Wild-type (AB strain) zebrafish were maintained at 28°C ± 0.5ºC in osmosis water (SGwaters) on a 14:10h (light:dark) photoperiod in a ZebTech (Tecniplast) closed flow-through system . The system includes a set of 3.5 litre tanks and a combination of biological, mechanical and carbon filters. System water sterilization is assured by UV light. One year old adult Zebrafish (*Danio rerio*) were used for the ethanol exposure assays. The same number of males and females were used in the assays, because females are typically heavier than males.

2. Experimental design - ethanol exposure test

Males and females were kept separately and placed in 2.5 L transparent tanks (Tecniplast), where they stayed for acclimatizing. Fishes were kept in tanks with continuous air flow. Absolute ethanol (PanReac) was used for the preparation of ethanol dilutions. All solutions where prepared in system water. Solutions were replaced daily at the same time (14h:00m ± 15 minutes) and fishes were fed twice a day (10h:00m and 17h:00m) with commercial fry food ZM400 (ZM).

To perform the ethanol exposure assay, six groups of twelve fishes (50% females; 50% males) were exposed to a range of ethanol (EtOH) concentrations (v/v) after accommodation.

To study the effects of chronic exposure to a continuous ethanol concentration, a control group (6 females + 6 males) was maintained in tanks containing system water with 0% EtOH, while the remaining group where kept on a tank with 0.25% EtOH for 15 days. After 15 days of chronic exposure, fishes were dissected and brains were removed, fast frozen with liquid nitrogen and stored at -80ºC until RNA extraction.

To evaluate the effects of an acute exposure to higher ethanol concentrations, the pre-adapted fishes were placed on distinct ethanol concentrations. First, all the 5 groups of twelve fishes (6 females + 6 males) were placed in 0.25% EtOH for 15 days and on the last day each group was placed on a different concentration, described as follows: 0% System water; 0.25% EtOH, 0.5% EtOH; 1% EtOH and 1.5% EtOH. The concentrations were chosen based on previous data regarding zebrafish behavioural tests (Gerlai et al., 2000;

Duglos et al., 2003) and alterations on gene expression (Kily et al., 2008) and neurotransmitters levels (Chatterjee and Gerlai, 2009). After 1h of exposure to these new concentrations, fishes were dissected and brains were removed, fast frozen with liquid nitrogen and stored at -80ºC until RNA extraction. All the exposure assays were performed in duplicate.

3. RNA extraction

3.1. RNA isolation

Total RNA extraction was performed using TRIzol reagent (Invitrogen), a combined solution of phenol and guanidine isothiocyanate, according to the manufacturer protocol. This method is a modification of the RNA isolation protocol developed by Chomczynski and Sacchi (1987) that allows for fast and easy isolation of high quality nucleic acids. The presence of TRIzol during homogenization avoids RNA degradation by RNases, since guanidine isothiocyanate is a chaotropic agent and induces loss of three dimensional structures of macromolecules helping cell lysis and preventing ribonuclease activity. One millilitre of TRIzol reagent was added to brain tissues and homogenized using a Precellys 24 rotator (Bertin) and zincronium oxide spherical beads. Samples were subjected to three cycles of 10 seconds at 5000 rpm, followed by 5 min incubation at RT to complete tissue dissociation. Samples were always kept on ice between homogenization cycles.

For RNA extraction, 200 µl of chloroform (Merck) was added to the samples, mixed and centrifuged at 12000*g* for 15 minutes at 4ºC. After centrifugation, total RNA remained in the upper aqueous phase while most of DNA and proteins remained either in the interphase or in the lower organic phase. Supernatants were collected into new eppendorf tubes and 500 µl of isopropanol (Sigma) was added for RNA precipitation. Samples were incubated for 10 min at RT with isopropanol and then centrifuged at 12000*g* for 10 min at 4ºC. After centrifugation, the supernatant was discarded and the pellet was washed with 1 ml of ice-cold 75% ethanol (Merck) and centrifuged at 7500*g* for 5 min at 4ºC. Ethanol was discharged and the pellet, often invisible, was allowed to dry for approximately 2 min on a speed vaccum dryer (ThermoFisher) in medium temperature. The dry pellet was ressuspended in 20 µl of RNase-free water to achieve a final

concentration of ≈ 10 µg/µl. RNA concentration was measured using a NanoDrop-1000 spectrophotometer (NanoDrop).

3.2. RNA purification

In order to remove possible DNA contamination, total RNA was digested with DNase I - amplification grade (Invitrogen)followed by phenol extraction. For DNA digestion, 1 µl of DNase I (10000 units/mg) and 2 µl of 10X DNase I Reaction Buffer (200 mM Tris-HCl (pH 8.4), 20 mM $MgCl_2$, 500 mM KCl) were added to 17 µl of sample. Samples were incubated at RT for 40 min to allow digestion. After incubation, 1 µl of EDTA was added and samples were incubated for 10 min at 65ºC to inactivate the enzyme.

Phenolic extraction (1:1) was performed after adjusting sample volumes to 300 µl with RNase-free water. 300 µL of phenol:chloroform:isoamyl alcohol (Sigma) was added to the RNA samples. Tubes were shaken for 15 sec and then centrifuged at 12000*g* for 15 min at 4ºC. Supernatants were collected into new eppendorf tubes and 300 µl of chloroform was added. Tubes were again shaken and centrifuged in the same conditions. RNA kept in the aqueous phase was collected into new eppendorf tubes and precipitated overnight at -80ºC with 1 ml of absolute ethanol and 20 µl of sodium acetate 3M, pH 5.2. After precipitation, RNA samples were centrifuged at 12000*g* for 15 min at 4ºC. Supernatants were discarded and the RNA pellet was washed with ice-cold 75% ethanol, re-centrifuged and dried as described before using “RNA isolation step”, after isopropanol addition. The RNA pellet was reconstituted in 20 uL RNase-free water and the RNA concentration was determined using the NanoDrop-1000 spectrophotometer (NanoDrop).

4. miRNA microarrays

4.1. RNA quality assessment

RNA integrity was assessed using the Agilent RNA 6000 nano kit and the 2100 Bioanalyzer (Agilent). Samples were submitted to capillary electrophoresis and RNA fractions were detected either as gel bands or as intensity peaks histograms. For this analysis, 1 µl of RNA was diluted in RNase-free water to achieve the final concentration of 25 - 500 ng/µl RNA. The RNA samples were denaturated for 3 min at 85ºC and 1 µl of RNA was loaded into the chip. Only samples with RNA Integrity Number (RIN) above 7.0 were considered for microarray analysis.

4.2. miRNA microarray production

The miRNA microarrays used were designed and manufactured in the National DNA-Microarray Facility located in the Department of Biology of the University of Aveiro. The microarray chip (miRNAChip_MS_V1) contained a total of 1164 probes spotted in quadruplicate onto Nexterion E slides (SCHOTT) using a MicroGrid II compact spotter (Digilab Genomic Solutions). Printed slides were further processed according to manufacture's recommendations. NCode™ Multi-Species miRNA probe set V2 from Invitrogen was used to miRNAChip_MS_V1 production. This probe set contains 1140 optimized probes targeting all known mature miRNAs deposited in mirBase 9.0 for human, mouse, rat, Drosophila, *C. elegans*, and zebrafish and also mismatch controls for monitoring hybridization specificity and the NCode™ Positive control for labelling quality control and easier spot finding. In addition, we have also included in the probe set 24 novel dre-miRNAs identified by parallel DNA pyrosequencing (Soares et al., 2009).

4.3. miRNA labelling and hybridization

Total RNA was labelled with the fluorophore Cy3 using the ULS™ (Universal Linkage System) microRNA labelling kit (Kreatech) according to the manufacturer´s protocol. The ULS is a nonradioactive technology for labelling and detection of nucleic acids. This method is based on the binding of a detectable molecule, a complex that

includes a fluorophore and a platinum (II), which binds on N7 position of guanine residue of nucleic acids.

Two micrograms of total RNA (Vt=17 µl) was incubated in 2 µl of labelling solution 10X plus 2 µl of Cy3-ULS for 15 min at 85ºC. In oder to remove non-reacted dye molecules, samples were filtered thought KREApure columns previously prepared by centrifugation for 1 min at 16000*g*. Eluted solution contains the labelled RNA, including miRNAs. Dye incorporation was monitored by UV-visible spectroscopy and the efficiency of labelling (DoL) was determined using formula 1. DoL levels should be between 1 – 3.6% (Kreatech application notes).

Formula 1: DoL (%) =

$$\frac{340 * [Cy3] \times 100\%}{[Nucleic\ acid] \times 1000}$$

[Cy3]: pmol/µl

[Nucleic acid]: ng/µl

Labelled RNA (20 µl) was incubated for 3 min at 85ºC with 83 µl of preheated 3X hybridization buffer plus 84.5 µl of RNase-free water. The mixture was then incubated on ice for 1 min and spin-down to collect the sample in the bottom. Afterwards, 62.5 µl of KREAblock 4X solution was added to the previous mixture followed by 1 min incubation on the dark at RT. Samples were hybridized at 42ºC for 16h.

After hybridization, microarray slides were disassembled with a low stringency wash solution (LSWS) followed by a high stringency wash solution (HSWS). Microarray slides were immersed in LSWS to remove the gasket slides and then washed during 30 sec in stirred LSWS solution. Slides were then quickly placed on HSWS and washed twice in stirred solution for 30 sec. Slides were dried with compressed nitrogen and immediately scanned using the Agilent G2565AA microarray scanner (Agilent).

5. Data analysis

The miRNA microarray images were analyzed using QuantArray v3.0 software (PerkinElmer). The pixel intensity of spots was processed, background subtracted and quantified. The saturated or incorrectly printed spots were excluded from the analysis (Figure 11). The median and standard deviations of each probe were calculated considering the fluorescence intensities of the quadruplicated spots. The median and standard deviations were first normalized using BRB-ArrayTools v3.4.0 software (http://linus.nci.nih.gov/BRB-ArrayTools.html) and then combined with the NCode internal control value. Normalized data were filtered to sort Zebrafish miRNAs and used to compare the miRNA expression patterns in different conditions by ratio calculation ($Intensity_{treatment}/Intensity_{control}$). Differentially expressed miRNAs were determined using the MeV - MultiExperimental Viewer software (TM4 microarray software suite) by comparing group means using pair Student´s T-test (p=0.05) and Bonferroni correction.

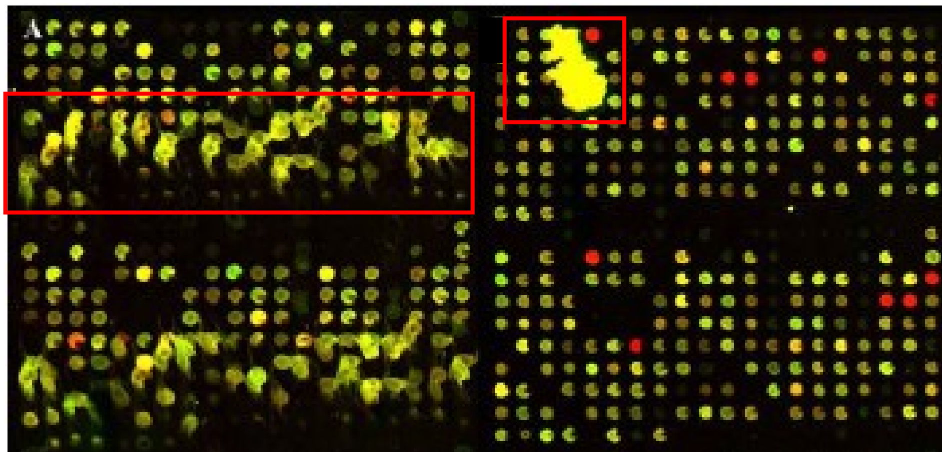


Figure 11: Example of spots excluded from the analysis (red squares)

6. Target Prediction

It is the ability to target multiple genes that allows the relatively small population of different miRNAs to exert their central role in regulating cell function and homeostasis. Several databases and algorithms are available for miRNA target prediction. Microcosm, miRanda, and EIMMO are databases for prediction of miRNA targets in Zebrafish. To understand the biological significance of deregulated miRNAs, putative targets of up-regulated miRNAs (miR-9, miR-23a, miR-30e, miR-133a, miR-181a, miR-736, miR-737)

and down-regulated miRNAs (miR-16a, miR-181b, miR-145) were identified using the databases mentioned above. Only putative targets that were common to at least two prediction algorithms for the same miRNA were considered. DAVID Bioinformatic Resources 6.7 tool was used for gene functional classification and pathways (Huang et al., 2009).

III. Results

1. RNA integrity

Before performing the microarray assay, samples were submitted to gel electrophoresis to assess RNA integrity. Most of the RNA is ribosomal 18S and 28S rRNA. Therefore, it is assumed that analysis of these rRNAs summarize the stability of total RNA samples. Gel electrophoresis (Figure 12) allows one to distinguish the 18S (2 Kd) and 28S (4 Kd) and identify good quality samples.

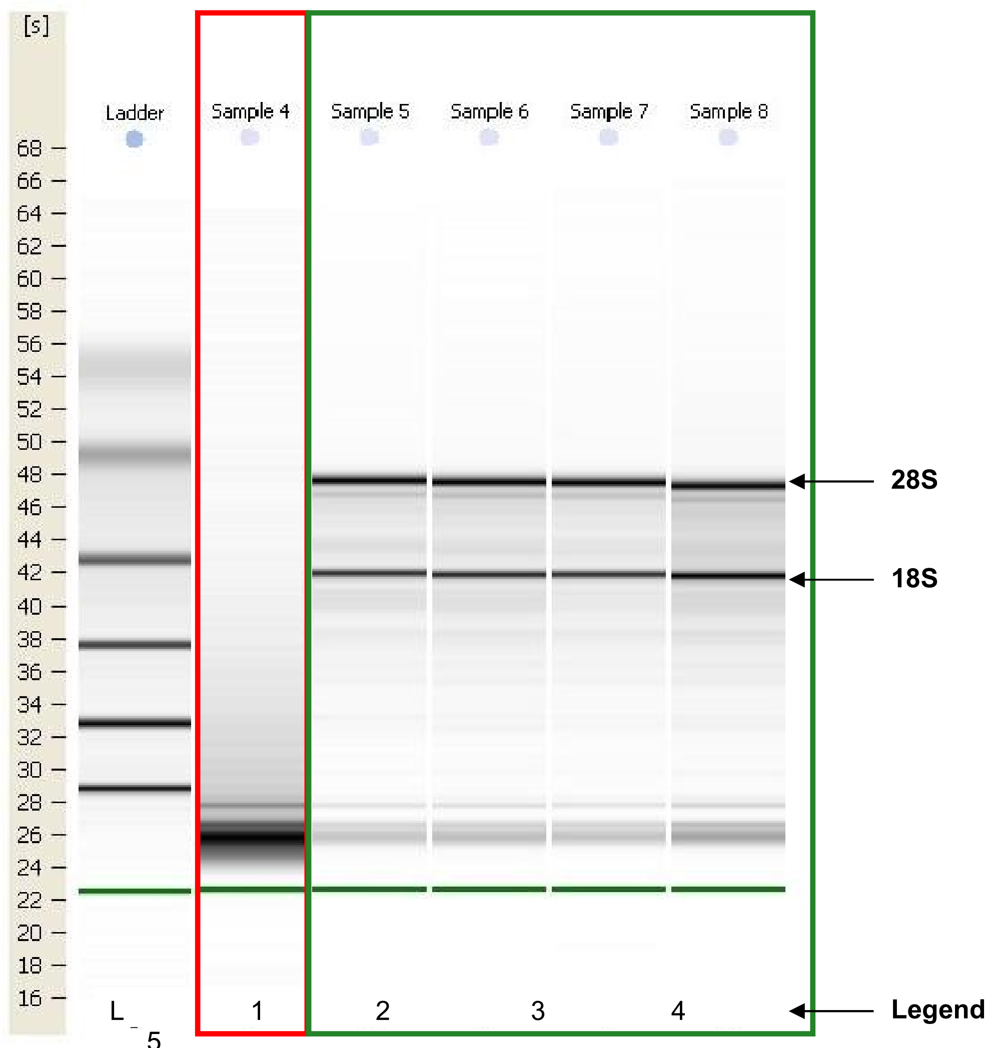


Figure 12: RNA fractionation using gel electrophoresis. The two dark bands (black arrows) represent 28S rRNA (~4000 nt) and 18S rRNA (~2000 nt). The first column – L – is load with the molecular marker and the remaining (1 to 5) are loaded with RNA samples. Sample-1 shows bad quality RNA (red square) and samples 2-4 contains good RNA (green square).

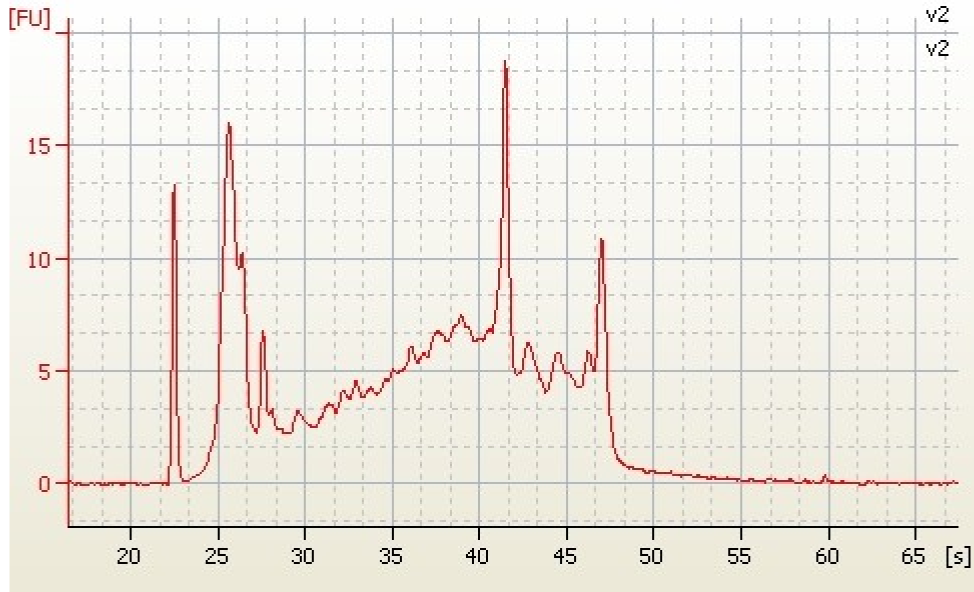


Figure 13: Histogram of fluorescence intensity of low quality RNA sample. The emission peaks (~2000 and ~4000 nt) represent the rRNA subunits. The ratio between rRNA 28S:18S is lower than 1, as a consequence of rRNA 28S degradation into small fragments.

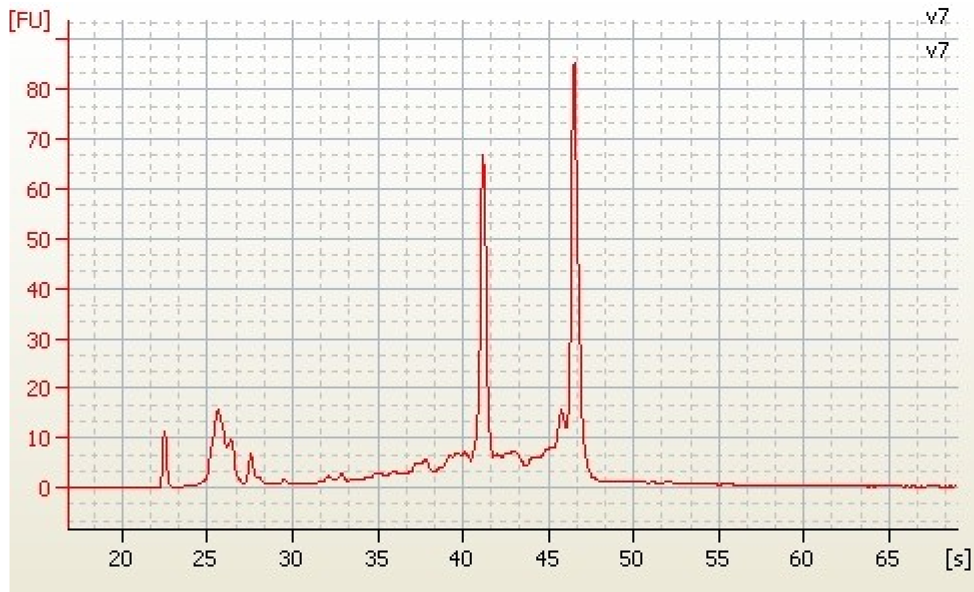


Figure 14: Histogram of fluorescence intensity of a high quality RNA sample. The two clear peaks of emission represent the rRNAs . The ratio between rRNA 28S:18S should be ~2 however, >1 ratio <2 are acceptable for RNA isolation from living tissues.

2. Effect of chronic ethanol exposure in miRNA profile

To test if chronic exposure to low ethanol concentrations affects miRNA expression, we performed miRNA microarray analysis of zebrafish brains isolated from fishes that were not exposed to ethanol or that were chronically exposed to 0.25% ethanol during a 15 day period. Hybridization intensity of miRNA from basal (0% EtOH) (Control group) were compared to the data obtained from the chronic ethanol group (0.25% EtOH).

After RNA hybridization and data normalization, hierarchical clustering (Pearson correlation, average linkage) of hybridization intensities was performed to assess the similarity between samples and among replicates (Figure 15). The replicates from chronic exposure cluster together, indicating similarity within replicates.

The differences in miRNAs expression profile between chronic and basal group was assessed using a paired t-test ($p<0.05$). Significant differences in the expression of thirty two miRNAs were found (Figure 7 e 8). More specifically, twenty-nine miRNAs showed expression enhance, while three exhibited decrease in the expression (see table in Annex A). The up-regulated miRNAs: miR-202, miR-181a, miR-27c, miR-373 and miR-30e duplicated their expression in chronic ethanol exposed fishes when compared to basal, while the remaining up and down-regulated miRNAs increased or decreased their expression in, at least, 1.5 times comparing to basal.

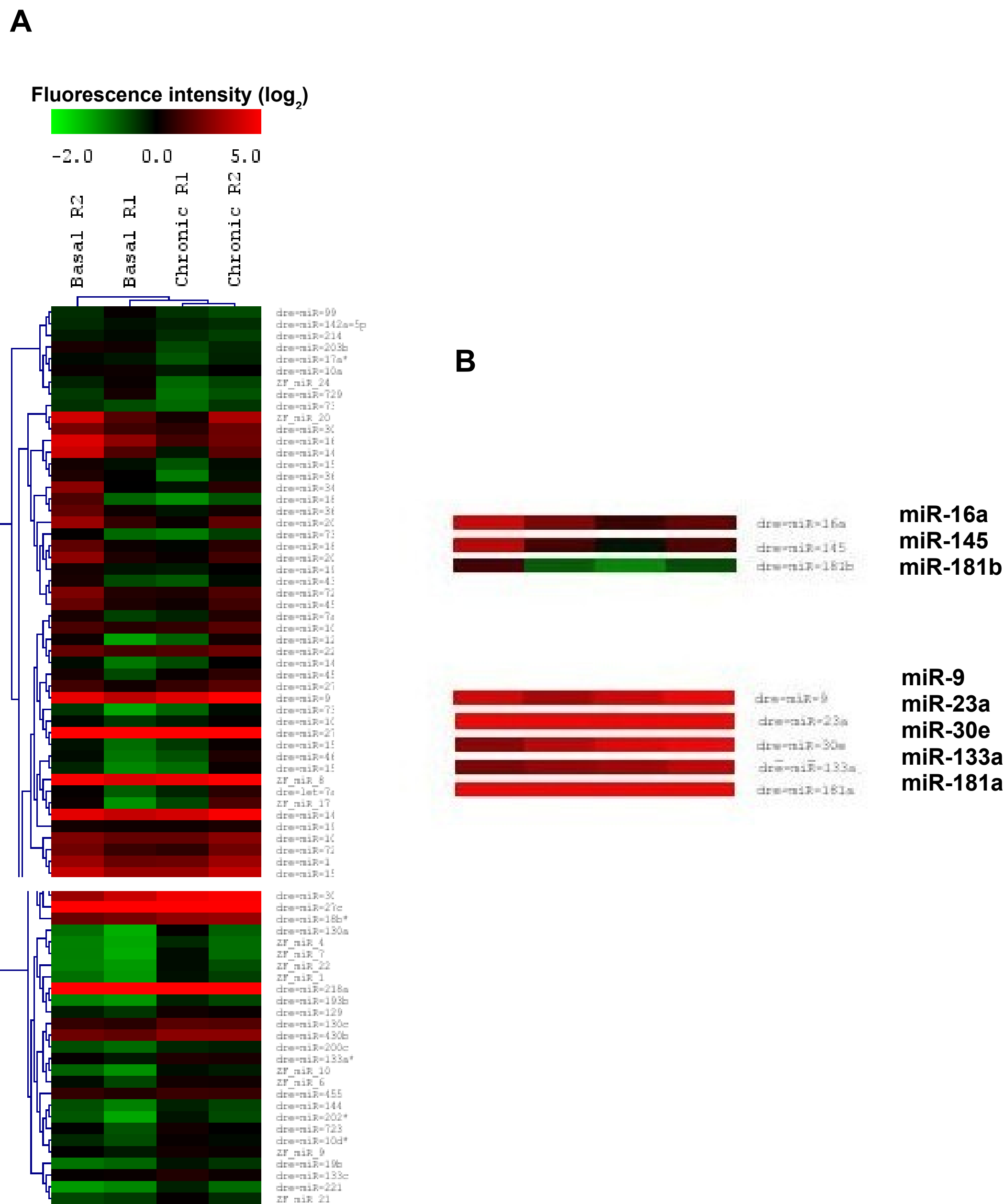


Figure 15: A) Hierarchical cluster (Pearson correlation, average linkage) of chronic alcohol and basal samples. The tree was generated by comparing the miRNA intensity values (log_2 transformed) of basal (0% EtOH) and chronic (0.25% EtOH) groups. The two replicates of chronic group (chronic R1 and chronic R2) cluster together, however the intensities of the first basal replicate (Basal R1) are more similar to the chronic group than with the other basal replicate. Colours in the map indicates normalized intensity values: green (intensity value below 0); red (intensity value above 1) and black (intensity value 0). **B)** Zoom-in with some of the significant deregulated miRNAs.

Figure 16: Relative expression ratios of significant deregulated miRNAs **A)** Relative expression ratios of significant up-regulated miRNAs after 15 days of chronic ethanol exposure (0.25+0% EtOH) ($p<0.05$). The expression ratios were calculated by dividing the mean intensity of chronic by basal values. The twenty eight up-regulated miRNA (red bars) have expression ratios above 1.5. **B)** Relative expression ratios of selected miRNAs that were used for functional analysis.

Figure 16 A)

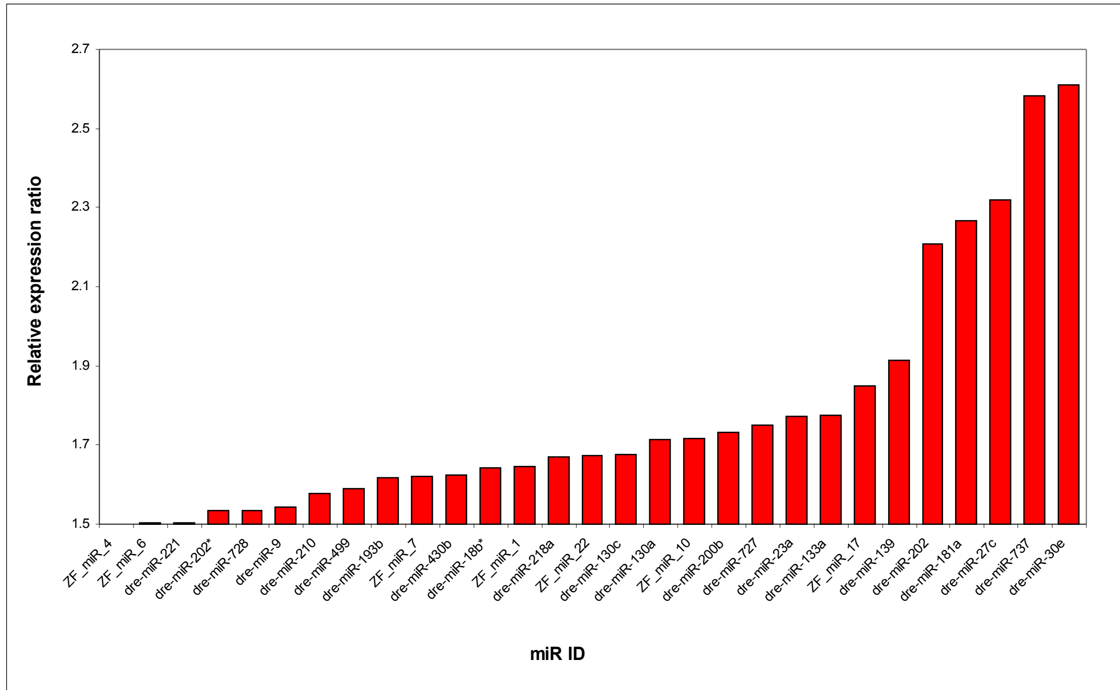


Figure 16 B)

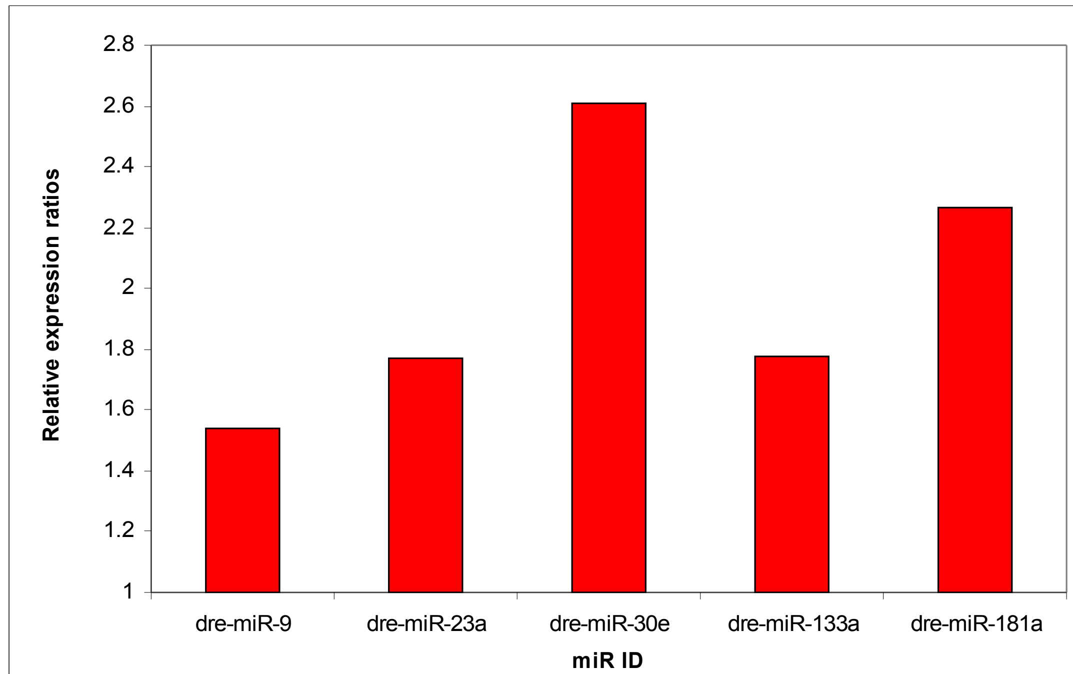
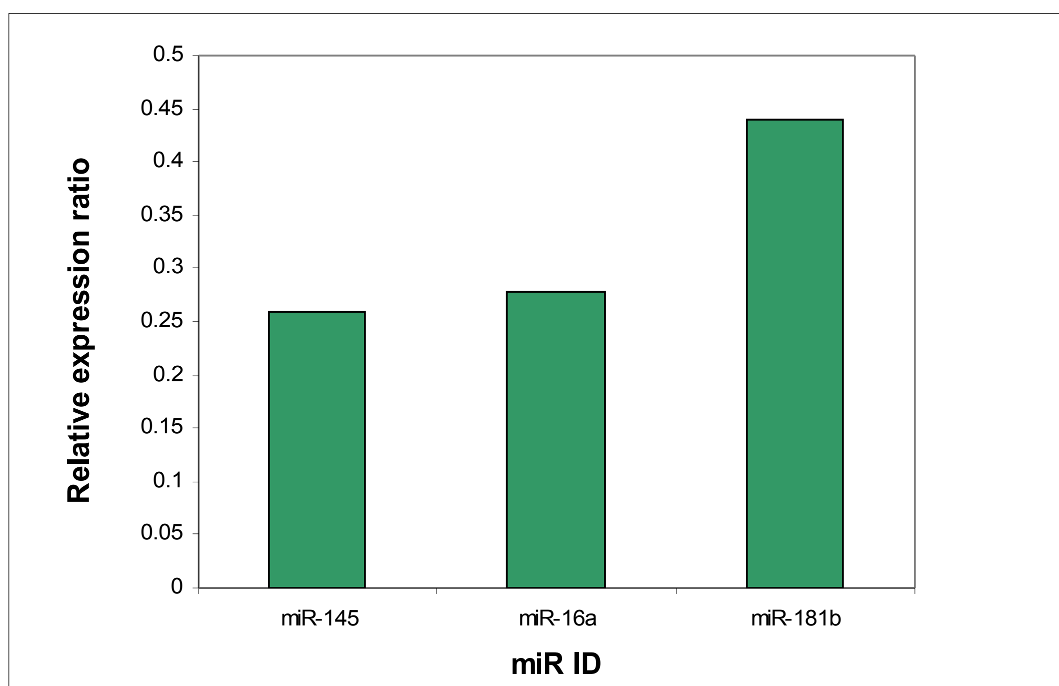


Figure 17: Relative expression ratios of significant down-regulated miRNAs after 15 days of chronic ethanol exposure (0.25+0% EtOH) ($p<0.05$). The expression ratios were calculated by

dividing the mean intensity of chronic by basal value. The three down-regulated miRNA (miR-145, miR-16a, miR-181b) have expression ratios below 0.5.



To understand the biological consequences of deregulated miRNAs, the putative target for some deregulated miRNAs (miR-9, miR-23a, miR-30e, miR-133a, miR-181a, miR-181b, miR-145, miR-16a) were predicted using bioinformatic tools. The functional analysis shown that most of these miRNAs may target genes implicated in the regulation of cell cycle and cell viability, neurogenesis and in other cell functions like cellular adhesion, vesicular trafficking and lipid biosynthesis (Table 2). Most miRNAs, both under or over expressed, were somehow related to the regulation of MAPK and Notch signalling pathways. The MAPK phosphorylation cascade establishes the cellular communication between extracellular stimuli into the nucleus, where it controls gene expression of several transcriptional factors that regulate cell proliferation, differentiation, inflammation and apoptosis. The Notch pathway also controls gene expression, however in a more strictly context as activation of this pathway requires cell-cell interaction. In adult cells, notch modulates regeneration by inducing differentiation of neural stem cells, oligodendrogenesis, apoptosis and angiogenesis. Therefore, alcohol induces strong alterations on cell physiology by modulating transcription activity in the cell nucleus.

Table 2: Functional classification of the putative target genes (DAVID) of deregulated miRNAs and their role in biological processes and signalling pathways. DR: Down-regulated | UP: Up-regulated

miRNA	Predicted target gene	miR status	presumable phenotype
Mitogen activated protein kinase pathway (MAPK)			
miR-9	*dusp6*	UP	stimulates proliferation
miR-23a	*fgfr4*	UP	inhibitis proliferation
miR-30e	*bdnf*	UP	inhibit synaptic function
miR-16a	*bad*	DR	increases apoptosis
Notch signaling pathway			
miR-30e	*dll4 & jag2*	UP	inhibits notch
miR-9	*her6*	UP	decrease regulatory genes
miR-133a	*rbpjb*	UP	decrease regulatory genes
mir-181a	*dll4*	UP	inhibits notch
miR-16a	*dla*	DR	stimulates notch
miR-145	*dll4*	DR	stimulates notch
Hedgehog signaling pathway			
miR-145	*zic2b*	DR	negatively modulates hg
Endocytic and Autophagy pathways			
miR-16a	*pik3c3*	DR	neural homeostasis / vesicular trafcking
Cardiac muscle contraction			
mir-181b	*atp1b1a*	DR	facilitates Na2+ reuptake
mir-133a	*aldh1a(a)*	UP	aldh1a modulates retinoic acid
Lipid biosynthesis			
miR-133a	*elovl6*	UP	early diabetes
Celular adhesion			
miR-133a	*nadl1.2*	UP	no relashionship fould
miR-181a	*itga5*	UP	invasiveness in tumor cells
miR-145	*ptk2.1*	DR	invasiveness in tumor cells

3. Effect of acute ethanol exposure in miRNA expression of pre-adapted fishes

A pulse of a different ethanol concentration was given to the chronic exposed fishes after the 15th day of exposure. Each group received an extra dose of ethanol, increasing the ethanol concentration to 0.25%, 0.5%, 1% and 1.5%. After 1h of exposure to the acute pulse, zebrafish brains were extracted and RNA was prepared for microarray analysis.

The effect of the short on miRNAs expression of pre-adapted fishes was accessed by comparing the miRNA profile of chronic exposure concentration (0.25+0%) to the 0.25+0.25%, 0.25+0.5%, 0.25+1% and 0.25+1.5% EtOH groups.

The analysis of hierarchical clustering (Pearson correlation, average linkage) reveals no clear cluster among the different groups (Figure 18). This result could be indicative that one hour of acute exposure is not sufficient to alter the overall expression of miRNAs. However, differences were detected in the higher ethanol group (1.5%). The 0.25+1.5% EtOH R2 group belongs to the same monophyletic group (red square) of chronic, 0.25+0.5% and 0.25+1% R1 conditions, but it has the most different profile comparing to the rest of the groups. The other 0.25+1.5% replicate R1 has also a very different profile, which make it belong to a different branch of the tree.

Besides the samples variability, significant differences were found between the expressions of several miRNAs but the population of deregulated miRNAs was not sustained throughout all the concentrations tested (Table 3). The expression of miR-23a, miR-736 and miR-737 were deregulated in some of the acute concentrations tested (Figure 19). While miR-737 expression did not vary much with the ethanol increase, miR-23a was the only deregulated miRNA found in higher ethanol pulses. miR-23a down-regulation was more pronounced in the highest ethanol concentration. On the other hand, increase in ethanol concentration induced miR-736 expression.

Target prediction analysis shown that miR-737 may be implicated in transcription machinery, as *gabpa* were retrieved as putative targets and miR-23a putative targets *fgfr4* are mostly implicated in cell proliferation. No targets were detected for miR-736.

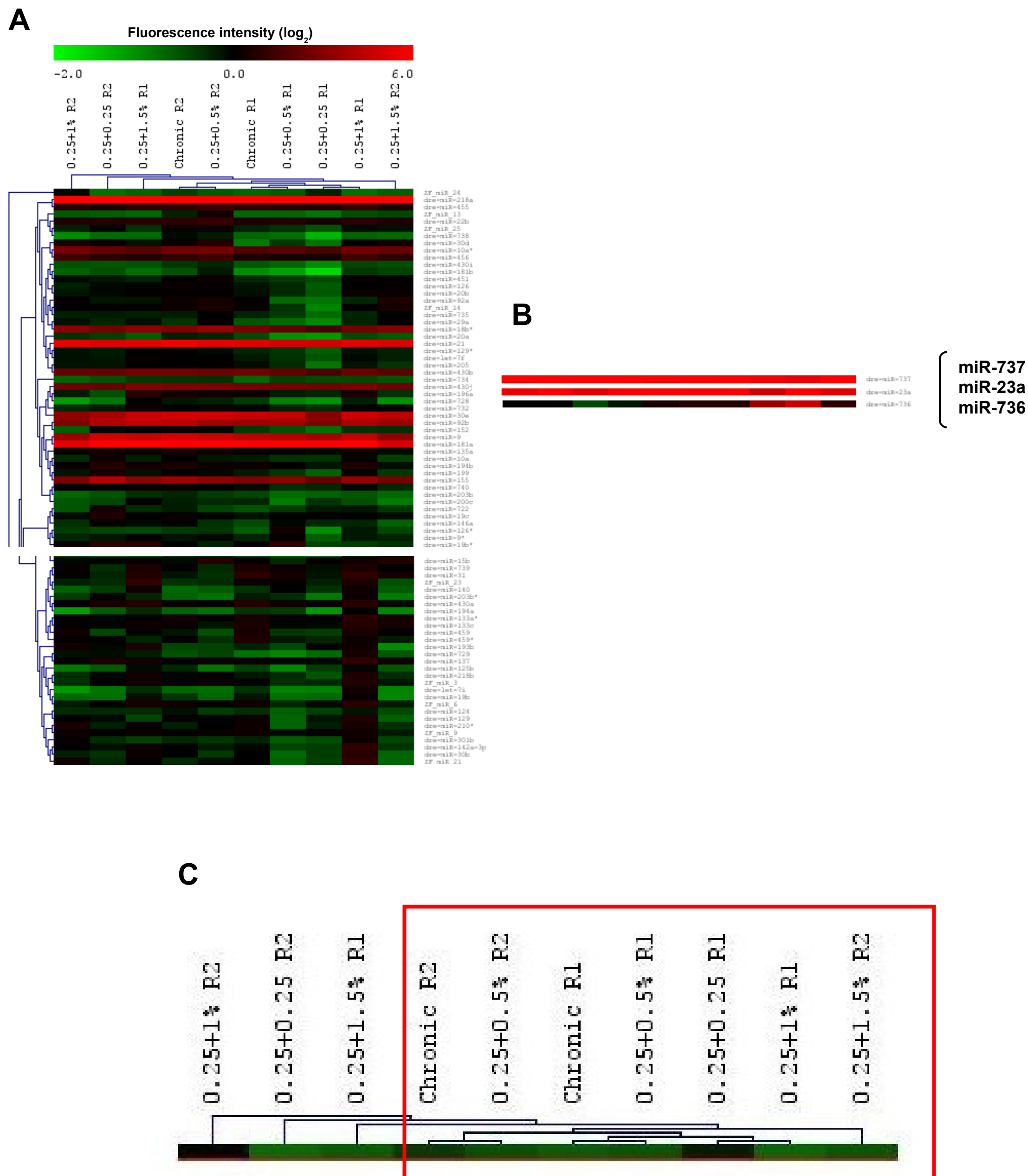


Figure 18: A) Hierarchical cluster (Pearson correlation, average linkage) of chronic alcohol samples and acute ethanol pulse samples. The tree was generated by comparing the miRNA intensity values (log_2 transformed) of chronic (0.25+0% EtOH) with acute ethanol exposure groups (0.25+0.25%, 0.25+0.5%, 0.25+1% and 0.25+1.5% EtOH). Colours in the map indicate normalized intensity values: green (intensity value below 0); red (intensity value above 1) and black (intensity value 0). **B)** Zoom-in showing some of the deregulated miRNAs. **C)** Zoom of the tree cluster. The cluster shows no clustering among replicates, demonstrating the high variability of the measurement. The red square indicates the closely related samples that belong to the same monophyletic group.

Table 3: General overview of miRNAs deregulated one hour after the ethanol pulse. All comparisons were performed between the chronic ethanol group (0.25+0% EtOH) and the groups pre-conditioned in chronic concentration that were exposed to higher ethanol concentrations, at the end of the assay.

Effects of ethanol pulse on miRNA profile (after chronic exposure to 0.25+0% EtOH)

	Chronic vrs. 0.25+0.25%		Chronic vrs. 0.25+0.5%		Chronic vrs. 0.25+1%		Chronic vrs. 0.25+1.5%		
miR ID	miR ID	Expression ratio	miR ID	Expression ratio	miR ID	Expression ratio	miR ID	Expression ratio	
	miR-736	2.770	miR-128	1.881	miR-736	4.241	miR-27a	2.172	
	miR-454b	2.068	miR-737	0.477	miR-729	1.902	miR-146b	2.005	
	miR-204	1.820			let-7c	1.670	miR-135b	1.665	
	miR-206	1.800			miR-193b	1.646	miR-1	1.563	
	miR-30a	1.632			miR-365	1.569	miR-192	0.494	
	miR-192	0.412			miR-30d	1.519	let-7b	0.467	
	miR-30e	0.406			miR-23a	0.498	miR-23a	0.246	
	miR-737	0.403			miR-737	0.191			

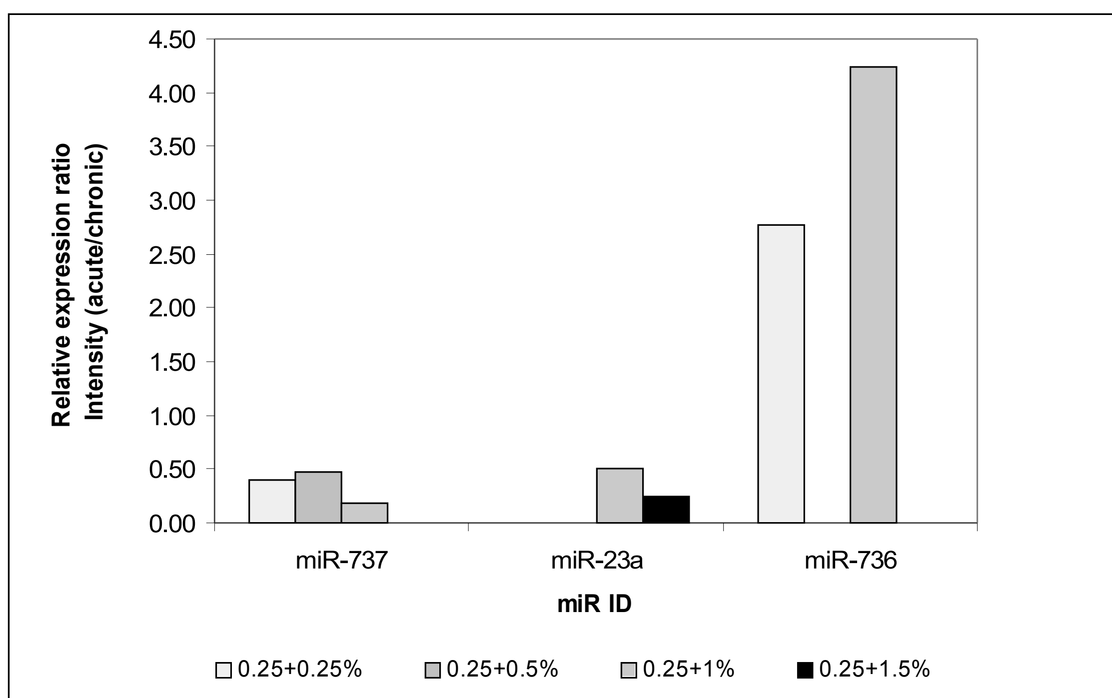


Figure 19: Variation in the expression of deregulated miR-737, miR-23a and miR-736 after acute ethanol exposure of pre-adapted fish. The expression ratios were calculated by dividing the mean intensity of the acute condition by the chronic value. Expression of down-regulated miR-737 remains stable at low ethanol concentrations, decreasing at 1% ethanol. The miR-23a is only deregulated at higher ethanol concentrations and it is negatively affected by increasing ethanol concentration. On the other hand, enhanced miR-736 expression is observed at 0.25+0.25% and 0.25+1% of ethanol, with increase miR expression at higher ethanol concentration.

IV. Discussion & Conclusion

Alcohol is an addictive substance that induces neuroadaptation (Moonat et al., 2010). The ethanol effects start with the alteration of cell signalling systems (neurotransmitters, hormones, signalling cascades (Ward et al., 2009)) and activation of transcription factors that modulate gene expression (Mayfield et al., 2002), culminating in proteome alteration (Damodaran et al., 2006). The miRNAs play a central role in the modulation of gene expression and central nervous system homeostasis. Therefore, the impact of alcohol on miRNA expression may result in phenotypes that are related to the deleterious effect of this drug (Pietrzykowsky et al., 2010). Following this hypothesis, we have tested the effect of different ethanol exposure patterns on zebrafish brain miRNA profile. Several authors validated this fish as a suitable model to study addiction (Ninkovik and Bally-Cuif, 2006) and molecular responses (Jonah and Eisen, 2002). In zebrafish, ethanol not only modifies the behavioural response (Gerlai et al., 2000) but also affects gene expression and elicits conditional place preference (CPP) in presence of adverse stimuli, which is indicative of dependence development (Kily et al., 2008).

Our results show that chronic alcohol exposure induces expression of twenty-nine miRNAs and decreases the expression of three. In particular, expression of miR-9, miR-23a, miR-30e, miR-133a, miR-181a increases more than 1.5 fold while miR-16a, miR-145 and miR-181b decreases over 1.5 times. The response of pre-adapted fishes (to the chronic concentration) to a higher ethanol doses show distinct expression patterns depending on the concentration used.

The effects of chronic ethanol exposure on miRNAs were assessed by continuous exposure of adult zebrafish to 0.25% (v/v) ethanol. This concentration was chosen based on the work of Gerlai and collaborators (2000), where 0.25% was the lowest ethanol concentration that induced alterations in zebrafish behaviour. Although we did not measure the ethanol concentration in water tanks, all solutions were replaced daily to avoid fluctuations in ethanol concentration due to evaporation. Our results demonstrate that chronic exposure to 0.25% ethanol significantly deregulates 32 of the 243 analysed miRNAs (~13% deregulated) (See table in the Annex A). We have selected some of the deregulated miRNAs to focus on the biological function analysis. The chosen miRNAs were expressed in zebrafish brain or are related to myogenseis and visual functions (Kapsimali et al., 2007; Leucht et al., 2008; Laterza et al, 2009). The exclusion of some up-regulated miRNAs is mainly due to lack of annotation in databases (in the case of ZF

newly described miRNAs) and lack of conservation in humans tissues (miR-27c and miR-728). However, literature shows that some of these excluded miRNAs (miR-221, miR-139 and miR-193b) are related to liver cancer (Pineau et al., 2010; Wang et al., 2010; Xu et al., 2010); miR-218a is expressed in motor neurons (Kapsimali et al., 2007); miR-200b is implicated in cell migration (Chan et al., 2010); miR-499 is over-expressed during heart failure and miR-210 is indicative of hypoxia conditions (Huang and Giaccia, 2010). The remaining miRNAs (miR-202, miR-727, miR-130c, miR-18b* and miR-430) are poorly described in the literature but it will be interesting to investigate their role in organisms and validate putative targets genes.

To understand how miR-9, miR-23a, miR-30e, miR-133a, miR-181a, miR-181b, miR-145 and miR-16a affect cell function we have performed an *in silico* analysis to predict putative target genes. The prediction associates deregulated miRNAs to cell-cycle regulation, apoptosis, vesicular trafficking, lipid metabolism, adhesion and modulation of transcript factors activity, however we did not validate the predicted target genes experimentally and references associating those miRNA to the respective cognate gene are also missing. In fact, besides the substantial growth in miRNA research, the lack of validated targets genes is an important weakness of the field (Khun et al., 2008; Thomas and Lieberman, 2010).

Considering the brain specific miR-9, it is described that this molecule is implicated in neurogenesis (Kapsimali et al., 2007, Leucht et al., 2008) and in the control of ethanol tolerance by targeting to ethanol sensible BK isoforms (Pietrzykowsky et al., 2008). Our array results are in concordance with other authors that describe miR-9 enhancement after ethanol exposure in mouse fetal brains (Wang et al., 2009) and in striatal neurons (Pietrzykowsky et al., 2008). Nevertheless, Sathyan et al. (2007) described the opposite effect in cultured cortical neurons. Probably these differences are due to the model choice, because the brain is a highly regionalize organ and different regions have different cell populations with specific functions, but can also be influenced by the type of ethanol exposure. Our data also suggest the cytoplasmatic dual specific phosphatase 6 gene (*dusp6*) as a possible target for miR-9. This phosphatase negatively controls the activity of signal-regulated kinase (MAPK/ERK), a MAP kinase that activates transcription factors that regulates cell cycle and that is also described to be affected by ethanol (Miranda et al., 2010). Thus, when over-expressed, miR-9 probably increases proliferation by inhibiting

dusp6. Nass and colleagues (2009) also observed increase of miR-9 in early-phase brain tumours (Nass et al., 2009). Increase of miR-9 can also be related to tolerance development, because this microRNA targets to alcohol sensitive form of BK channels inhibiting the channels activity (neuron excitability, control of neurotransmitters and action potentials). This effect can explain why, in addicted individuals, it is necessary to increase the ethanol doses to fire the release of certain neurotransmitters and activate the reward response.

The miR-23a is other brain specific miRNA that was up-regulated in zebrafish brains after ethanol exposure. From the analysis, up-regulation of this miRNA may inhibit fibroblast growth factor 4 expression, which may result in reduced cell proliferation. This miRNA is also described to induce apoptosis when associated with miR-23a approximately 27a approximately 24-2 cluster (Chhabra et al., 2009). Together, these results suggest that up-regulation of miR-23a repress cell proliferation. Regarding miR-9 and miR-23a, their over-expression result in opposite responses. These results can be related to ethanol deleterious effect in different brain areas responsible for coordination, learning and stimuli reception.

The miR-30e was found to be up-regulated after chronic ethanol exposure. One of the possible targets for this miRNA is the brain derived neurotrophic factor (BDNF). Down regulation of BDNF after chronic ethanol exposure was previously observed in rats (Jung et al., 2010) and it is likely that reduction of BDNF is involved in neurodegeneration and in the development of neurological alcohol-related disorders (reviewed by Moonat et al., 2010). So, the chronic ethanol consumption also elicit a certain risk to develop psychological diseases.

We found that after chronic ethanol exposure miR-181a was up-regulated while miR-181b was down-regulated. Both miR-181a and miR-181b act as tumour suppressor factors in human glioma (Shi et al., 2008). Increase in miR-181a expression was also observed after chronic cocaine administration (Chandrasekar and Dreyew; 2009). This miRNA modulates synaptic plasticity by negatively controlling *RGS4* (regulator of G-protein), *GRM5* (glutamate receptor), *GRIA2* (glutamate receptor, ionotropic, *AMPA2*), *PI4K2B* (phosphatidylinositol 4-kinase type 2 beta) genes (Pietrzykowski, 2010). These genes are related to neurotransmitter receptors and signalling transduction. It was also described that miR-181a also regulates immune function by modulating B-cell

differentiation and target T-cell (Okada, 2010). We found that miR-181a may be implicated in notch signalling by targeting Delta-like 4 ligand (*dll4*). Considering that *dll4* targets immune cells, contributing to their differentiation (Schaller et al., 2007; Mukherjee et al., 2009), it is possible that alcohol acts as an immune suppressor agent.

Considering the down-regulated miRNAs, apoptosis is modulated by miR-181b which represses the pro-apoptotic factor BCL-2 (Zhu et al., 2010). In our case, low miR-181b levels were observed suggesting that ethanol may have a positive effect on apoptosis. Down-regulation of miR-145 under chronic ethanol conditions seems to be implicated in Notch and Hedgehog signalling pathways. The Hedgehog signalling is required for the maintenance of progenitor cell populations in some areas in adult brain (Machold et al., 2003) and also for the oligodendrocyte differentiations (Lu et al., 2000), while the Notch pathway is important in cell regeneration (Schwanbeck, 2010). Modulation of proliferative capability of multipotent cells by miR-145 was also observed by Cordes and colleagues (2009) and Chivukula (2009). Moreover, miR-145 knockout results in alterations in smooth muscle cells and in the vascular system (Elia et al., 2009) and induces the expression of pro-apoptotic c-Myc (Sachdeva et al, 2009). The loss of cell adhesion can relate to the capability of miR-145 to induce target junctional adhesion molecule A and fascin (Goote et al., 2010). The under expression of miR-16a, inhibits MAPK pathways by activating the phosphorilation of *bad*, a proapoptotic factor that leads to apoptosis through caspase activation (Panka et al, 2006). Together our findings suggest that, even at low concentrations, continuous alcohol intake has negative effects on CNS. Ethanol may increase neurodegeneration by decreasing cell proliferation (at least in some brain regions) and slows cell regeneration. These processes may be regulated by miRNAs. Regarding apoptosis, we have conflicting results, having miRNAs that may either be related to apoptosis induction (miR-16a) or repression (miR-9). As the brain is a complex organ containing regions dedicated to specific functions, this pattern of pro/anti apoptosis may be related to neuronal differences. To examine which areas are more affected by apoptosis one should do *in situ* hybridizations with probes against miR-16a, miR-145 and miR-181b targeting different brain sections. Additionally, chronic alcohol exposure induced the expression of miR-9 and miR-181a that are also related to addiction to other drugs, such as cocaine (Chandrasekar and Dreyew; 2009). So, these two miRNAs can be useful for diagnosing drug addiction and may be used as potential therapeutic targets to treat addiction.

The effects of acute pulses of ethanol on miRNA expression were assessed by exposing pre conditioned zebrafish (0.25% ethanol) to different ethanol concentrations (0.25%; 0.5%; 1%; 1.5%). The data shows high variability between replicates and between

samples. This may suggest that one hour of high ethanol exposure is not enough to induce strong mirnome destabilization and highlights the necessity to increase the number of replicates in future analysis. The duration of acute pulse exposure was defined based on a time-course assay where highest brain level of dopamine and serotonin was achieved one hour after ethanol exposure (Chatterjee and Gerlai, 2009). The acute-pulse concentrations were chosen based on previous behavioural studies (Gerlai et al., 2006) that show some alteration on the extent of behavioural response in fishes pre-exposure to chronic 0.25% ethanol and then exposure to acute ethanol concentration. Despite the variability observed, deregulated miRNAs could be identified. Distinct miRNA expression profiles were found in different acute pulses. This complicated the biological functional analysis and indicates that each concentration may affect cells in specific ways. miR-737, miR-736 and miR-23a were deregulated at least in two different concentrations. While miR-737 was down-regulated and had low expression at 1% EtOH, miR-736 was up-regulated in 0.25% and 1%, but one cannot exclude the technical concerns mentioned above. The miR-23a expression was reduced in 1 and 1.5% of ethanol after acute exposure, conversely to what was observed in chronic ethanol addicted fishes. Additionally, it was observed that increasing ethanol concentration resulted in lower miR-23a expression.

Conclusions

Our data suggest the involvement of new miRNAs in ethanol response and identifies putative genes targeted for those miRNAs. Deregulated miRNAs are related to physiological responses that have already been described as being involved in ethanol addiction namely, regulation of MAPK pathway and loss of brain progenitor multipotent cells (Miranda et al., 2010). We show that low concentration chronic-alcohol exposure is enough to alter miRNA expression profile and suggest that some of the deregulated miRNAs (miR-9, miR-30e, miR-181a, miR-181b) may be potential regulators of neuro-apoptosis. Considering the decrease of undifferentiated multipotent cells and the increase of neuroapotosis, long-lasting effects of ethanol in brain function may be related to loss of regenerative capacity. Finally, the microarray technique used here is suitable for screening ethanol effects on miRNAs deregulation, although high number of replicates should be considered to minimize variability between data sets. In this study, the high number of

fishes that were scarified per replicate (72 fishes) limited our assays.. Nevertheless, future work should screen the effects of acute ethanol exposure on miRNAs deregulation and should also compare that data with our chronic group.

Prespectives

Future work includes the increase on the number of replicates per assay and the increase on the exposure frequency to acute pulse ethanol doses. Moreover, it will be useful to validate some of the putative target genes of deregulated miRNA by RT-qPCR and localize the brain areas where specific miRNAs are being expressed by *in situ* hybridization. It will be also interested to explore different patterns of ethanol exposure because, as we observed from our data, different concentrations and exposure conditions affects differently miRNAs expression and have serious implication in cell homeostasis.

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Annex A

List of deregulated miRNAs after chronic ethanol exposure

Deregulated miRNAs	
up-regulated	**down-regulated**
miR-9	miR-16a
miR-18b*	miR-181b
miR-23a	miR-145
miR-27c	
miR-30e	
miR-107b	
miR-126b	
miR-130a	
miR-130a	
miR-130c	
miR-133a	
miR-139	
miR-181a	
miR-193b	
miR-196c	
miR-196d	
miR-200b	
miR-202*	
miR-202	
miR-210	
miR-218a	
miR-221	
miR-222b	
miR-429b	
miR-430b	
miR-455b	
miR-499	

miR-727	
miR-728	
miR-737	
miR-2185	
miR-2186	
miR-429b	
miR-1388	
miR-1788	
miR-2187	
miR-2188	
miR-2189	
miR-2190	
miR-2191	
miR-2192	
miR-2193	
miR-2194	
miR-2195	
miR-2196	
miR-2197	
miR-2198	