Surrogate Markers for Gammahydroxybutyrate (GHB) Exposure In Cell lines

By

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This thesis is the result of author's original research. It has been composed by the author and has not been previously submitted for the examination which has lead to the award of a degree.

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ABSTRACT

Gamma hydroxybutyric acid (GHB) is a metabolite of gamma-amino butyric acid (GABA) and considered as a natural neurotransmitter found in the brain in low concentrations. GHB has been used in general anaesthesia and is currently used to treat narcolepsy and alcoholism. The abuse of GHB, especially in date rape sexual assaults, has increased in recent years. GHB has a rapid rate of metabolism causing it to disappear quickly making criminal cases often difficult to prosecute. This study is aimed at extending the window of detection of GHB beyond 12 hours through finding robust surrogate markers of GHB exposure in cell lines. Two approaches were used: Agilent SurePrint G3 Human 8x60K arrays were used firstly to detect changes in gene expression in human monocytic leukaemia TH-P1 blood cells after 24hours GHB exposure and the two-dimensional gel electrophores (2-DGE) were used secondly to find proteomic changes in THP-1 and in two brain cell lines (SH-SY5Y and 1321N1 cells) after 24 h GHB exposure. The results show that 900 μM GHB induces an alteration in 2380 genes ($P<0.05$ and a fold change of >2) and the Gene Ontology (GO) enrichment analysis showed that the largest numbers of the altered genes were in the intracellular and membrane parts of the cell. In terms of GO molecular functions, the majority of altered genes code for proteins and nucleotide binding sites, and some of the altered genes encode steroid dehydrogenase enzymes. Furtheremore, the results of 2-DGE revealed that 900 μM GHB in THP-1 cells and 100 μM GHB in brain cells used were induced alteration in proteins after 24h exposure, some of altered proteins were glycolytic enzymes and chaperones. This information may be useful in forensic toxicology.

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LIST OF ABBREVIATIONS

%	percent
μg	micro gram
μl	micro litre
μM	micro molar
g	grams
AKR	Aldo-Keto Reductase
ALDH	Aldehyde dehydrogenase
ATP	Adenosin triphosphate
BBB	Blood brain barrier
BD	1,4- butanediol
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
C_{max}	Peak concentration
CNS	Central Nervous System
Da	dalton
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	dithiothreitol
EC	Entorhinal cortex
EDTA	ethylenediaminetetraacetic acid
FADD	Fas-associating protein with a novel death domain
FBS	Fetal Bovine Serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GABA	γ-aminobutyric acid
GBL	gamma-butyrolactone

GC/MS	Gas Chromatography/Mass Spectrometry
GH	Growth hormone
GHB	γ- hydroxybutyrate
h	Hour (s)
H2O2	hydrogen peroxide
IL	Interleukin
IPTG	isopropyl D-thiogalactoside
KDa	kilo Dalton
Kg	Kilogram
L	Liter (s)
LB	Lysogeny broth
M	Molar
MCT	Monocarboxylate transporter
MCTs	Monocarboxylate transporters
min	minute(s)
ml	milli litre(s)
mM	milli molar
mRNA	messenger RNA
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium Bromide
NAC	Nucleus accumbens
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	reduced form of Nicotinamide Adenine Dinucleotide Phosphate
NCS-382	6,7,8,9-tetrahydro-5-hydroxy-5H-benzocyclohept-6-ylideneacetic acid
nm	nanometres
nM	nanomolar

°C	degree Celsius
PBS	phosphate buffer saline
PCR	polymerase Chain Reaction
PPARγ	peroxisome proliferator-activated receptor
RNA	Ribonucleic acid
RT-PCR	quantitive Real Time Polymerase Chain Reaction
rpm	Revolutions Per Minute
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean
SH-SY5Y	human neuroblastoma cell line
SSA	succinic semialdehyde
SSR	succinic semialdehyde reductase
SSADH	succinic semialdehyde dehydrogenase
TCA cycle	Tricarboxylic acid cycle
TEMED	NNN-N′-tetramethylethylenediamine
T_{max}	Time to reach peak concentration (C_{max})
Tris	tris (hydroxymethyl) methylamine
UV	Ultraviolet
VTA	Ventral tegmental area

CHAPTER ONE

GENERAL INTRODUCTION

1 INTRODUCTION

1.1 Overview on Gamma hydroxybutyrate (GHB):

GHB is a four carbon fatty acid that was first synthesized as an anesthetic in 1960 and as a precursor of the neurotransmitter gamma aminobutyric acid (GABA), the main inhibitory neurotransmitter in the brain (Laborit, 1964). Furthermore, GHB was found to be present endogenously in mammalian brain and being present mainly in the hypothalamus and the basal ganglia (Bessman and Fishbein, 1963; Snead and Morley, 1981). However, the concentration of GHB in peripheral body tissues such as the kidneys and brown adipose tissue is more than 10 times the concentration in the brain (Nelson *et al*., 1981), indicating an additional role. GHB is structurally similar to GABA, having a hydroxyl group on the forth carbon instead of the amine group in GABA (Figure 1.1).

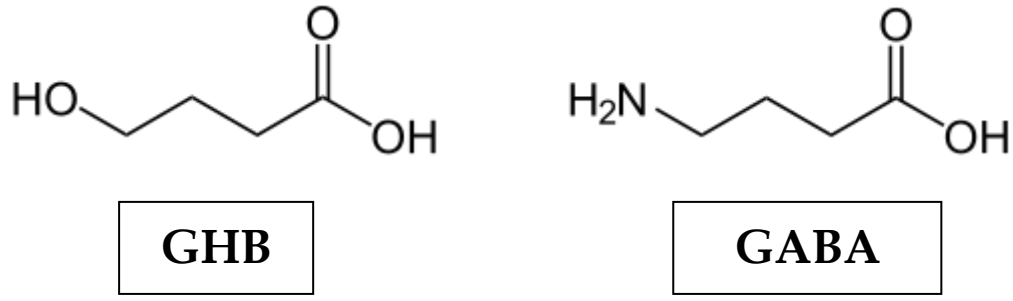


Figure 1.1: Chemical structures of Gamma hydroxybutyric acid (GHB) and its precursor gamma amino butyric acid (GABA).

GHB is also known as liquid ecstasy (Elliot and Burgers, 2005) and the sodium salt of GHB is distributed by Jazz Pharmaceuticals under the trade name Xyrem® (Figure 1.2).

Figure 1.2: The sodium salt of GHB is sold by *Jazz Pharmaceuticals* under the trade name Xyrem®.

GHB is an important compound in three respects: it is found to be present endogenously (Bessman and Fishbein, 1963; Nelson *et al*., 1981), it is prescribed for therapeutic purposes (Addolorato *et al*., 1999; Carter *et al*., 2009; Fuller and Hornfeldt, 2003) and it is an abused drug (Stillwell, 2002; Elsohly and Salamone, 1999; Rodgers *et al*., 2004); these three aspects are discussed below.

1.1.1 Endogenous GHB

GHB is a metabolite of gamma -amino butyric acid (GABA) and is found naturally in the human body. Its presence in the brain shows unequal distribution but it is important to note that relatively higher levels of GHB are found in kidney, heart and skeletal muscle (Nelson *et al*., 1981). The normal concentration of GHB in the human brain is two to three times higher in the basal ganglia than in the cerebral cortices (Okun *et al*., 2001). The cerebral concentrations of GHB are approximately 0.1% of the concentrations of GABA; it is suggested that GHB itself may serve as a neurotransmitter or neuromodulator (Maitre, 1997). Barbaccia *et al*., 2005 propose that the presence of GHB in mammals including humans, together with the unequal distribution in the brain and the fact that synthesis and release of GHB are regulated processes, indicates that GHB may act as neurotransmitter and/or neuromodulator. Although a range of effects on some neurotransmitter systems have been reported, the precise physiological function of GHB remains a subject of debate (Wong *et al*., 2004). Protective functions have been assumed, higher doses of GHB (more than 100 mg/kg) had a protective effect against oxidative stress on different tissues in vitro and in vivo e.g. heart, lung, liver, kidney, skeletal muscle, pancreatic b cells, gut, and brain (Mamelak, 2007). However, afterwards studies revealed that GHB and its precursors caused induction of oxidative stress in cerebral cortex of rats (Sgaravatti *et al*., 2007, 2009).

1.1.2 GHB as a therapeutic drug and dietary supplement

GHB was synthesized in 1960 to produce an analogue for the omnipresent inhibitory neurotransmitter GABA that can cross the blood-brain barrier. It

has been used as an anaesthetic drug for minor surgical procedures, but now the use of GHB as an anaesthetic is decreasing, although it is still permitted in Germany for intravenous anaesthesia (Bessmann and Fishbein, 1963; Caputo *et al*., 2009).

The US Food and Drug Administration approved a pharmaceutical form of GHB, sodium oxybate (Xyrem), in 2002 for a limited use in the treatment of narcolepsy (Fuller and Hornfeldt, 2003). Narcolepsy is considered as a sleep disorder that is characterised by fragmented night time sleep and daytime drowsiness, and can also include cataplexy which is characterised as loss of muscle tone with intact consciousness. Studies in narcolepsy illustrate that GHB has been valuable in treating daytime cataplexy which is weak or paralysed muscles and helped in treating disjointed sleep/wake cycles of these patients. GHB night doses were selected to reduce the number of night-time awakenings and daytime cataplexy; also it can develop a good sleep pattern in narcoleptic patients (Carter *et al*., 2009).

In addition, GHB is under study for insomnia associated with schizophrenia and there are some preclinical studies for the usefulness of GHB for these sleep disorders (Balla *et al*., 2009; Kantrowitz *et al*., 2009).

GHB was also found to reduce alcohol withdrawal signs and symptoms such as tremor, sweating, nausea, depression, and anxiety (Gallimberti *et al*., 1989). It also helped patients to keep abstinent (Addolorato *et al*., 1996). Reports have shown the effectiveness of GHB in promoting abstention in alcoholics leading to the approval of GHB in Italy and Austria for the treatment of alcoholism under the trade name Alcover (Beghè and Carpanini, 2000). A new study verified that GHB was more valuable than diazepam in

treating alcohol-withdrawal syndrome. GHB reduced anxiety, agitation and current depression more rapidly than diazepam and as efficiently as clomethiazole. GHB was used at the dose of 50-100 mg/kg in three or four daily doses, and no severe side effects were registered (Caputo *et al*., 2009).

A systematic review of the role of GHB in treating alcohol withdrawal syndrome and the prevention of relapse were published by the Cochrane Library and the study concluded that GHB is effective to treat alcohol withdrawal syndrome and to prevent relapse at a dose of 50 mg/kg/day. However, as the dose increased to 100 mg/kg/day, the most common side effects such as dizziness and vertigo were reported (Leone *et al*., 2010).

In the USA, GHB was manufactured during the late 1980s and marketed in the 1990s as a dietary supplement (Chin *et al*., 1992; Okun *et al*., 2001 and Wong *et al*., 2004). Throughout the 1980s GHB was consumed by body builders to increase the release of growth hormone (GH), and it has been sold as an anabolic supplement over the counter drug (Van Cauter *et al*., 1997; Miotto *et al*., 2001; Donjacour *et al*., 2011). The mechanism behind the increase in GH release by GHB is not fully explained. It was hypothesized that GH is released during slow-wave sleep induced by GHB, especially in the first two hours following sleep onset (Van Cauter *et al*., 1997). Another study proposed that GH release is mediated by cholinergic receptors because of the complete suppression of GH release in normal control and parkinsonian patients that were pre-treated with a cholinergic antagonist, pirenzepine before GHB administration (Volpi *et al*., 2000).

1.1.3 GHB as a drug of abuse

The abuse or misuse of GHB in different ways has lead to GHB becoming a notorious drug that is banned in many countries.

Throughout the late of 1990s GHB became popular as "club drug" due to its widespread use among individuals while attending nightclubs (Wong *et al*., 2004). GHB known as a club drug or recreational drug, because of a euphoric feeling, relaxation, and socialization that can be produced by low doses of GHB (Rodgers *et al*., 2004; Andresen *et al*., 2010). Illegal preparations of GHB were produced under street names such as, liquid X, Liquid Ecstasy, growth hormone booster and Salty water (Nicholson and Balster, 2001). The abuse of GHB and its precursors resulted in a number of overdoses and death related cases (Ingels *et al*., 2000; Knudsen *et al*., 2010; Zvosec *et al*., 2011).

Abuse of GHB has lead to an increase in overdoses and toxicological effects, characterized by dizziness, respiratory depression, vomiting, and unconsciousness, as well as coma and death. However, the mechanism of action contributing to the observed toxicological effects of GHB is controversial, and appears to vary based on the dose administered and the behavioural or physiological endpoint that is measured (Carter *et al*., 2009; Mamelak, 2009).

In spite of being banned by Food and Drug Administration on November 8, 1990, this substance is still used. More than 40 cases of GHB poisoning have been reported in California with further cases in other states. The prognosis for GHB intoxication is not bad; there were few reports of long-term adverse effects or deaths, nor any proof for physiological addiction (Chin *et al*, 1992).

The more serious consequences of GHB misuse are related to the loss of consciousness of the individual.

GHB has an extremely steep dose-response correlation. GHB doses of more than 36.8 mg/kg can lead to serious consequences such as bradycardia, decreased respiration, unconsciousness and even death. These consequences are more serious when consumed with other central nervous system depressants such as ethanol. The USA Drug Enforcement Administration classified GHB as a Schedule I controlled substance because of these dangerous effects (Bravo *et al*., 2004).

GHB is also abused significantly as a "date-rape" drug, in drug-facilitated sexual assault due to its sedative and hypnotic effects (Schwartz *et al*., 2000; Halkitis *et al*., 2007). GHB is a colourless and odourless compound, so it is easily spiked in to drinks. It is a rapidly absorbed drug; it reaches a peak plasma level in 20-45 minutes (Brenneisen *et al*., 2004). GHB exhibits its sedative effects in a short period of time and it is metabolized and eliminated in 6 to 8 hours from the blood circulation and in 12 hours from the urine (Palatini *et al*., 1993). There are many reports involving GHB in criminal cases (Stillwell, 2002; Varela *et al*., 2004; Anderson *et al*., 2006).

1.2 Neuropharmacology of GHB

As illustrated in Figure 1.3., the primary precursor of GHB in the brain is GABA, which is transformed into succinic semialdehyde (SSA) through a GABA-transaminase and then transformed into GHB by a specific succinic semialdehyde reductase (SSR). GHB can also be retransformed into SSA by a GHB dehydrogenase, and then SSA can be transformed back to GABA. SSA

can also be converted to succinic semialdehyde dehydrogenase (SSADH) into succinic acid and then metabolized by the Krebs cycle in mitochondria (Caputo *et al*., 2009). This means that exposure to GHB may also lead to changes in GABA levels, making it difficult to separate out the specific effects of GHB.

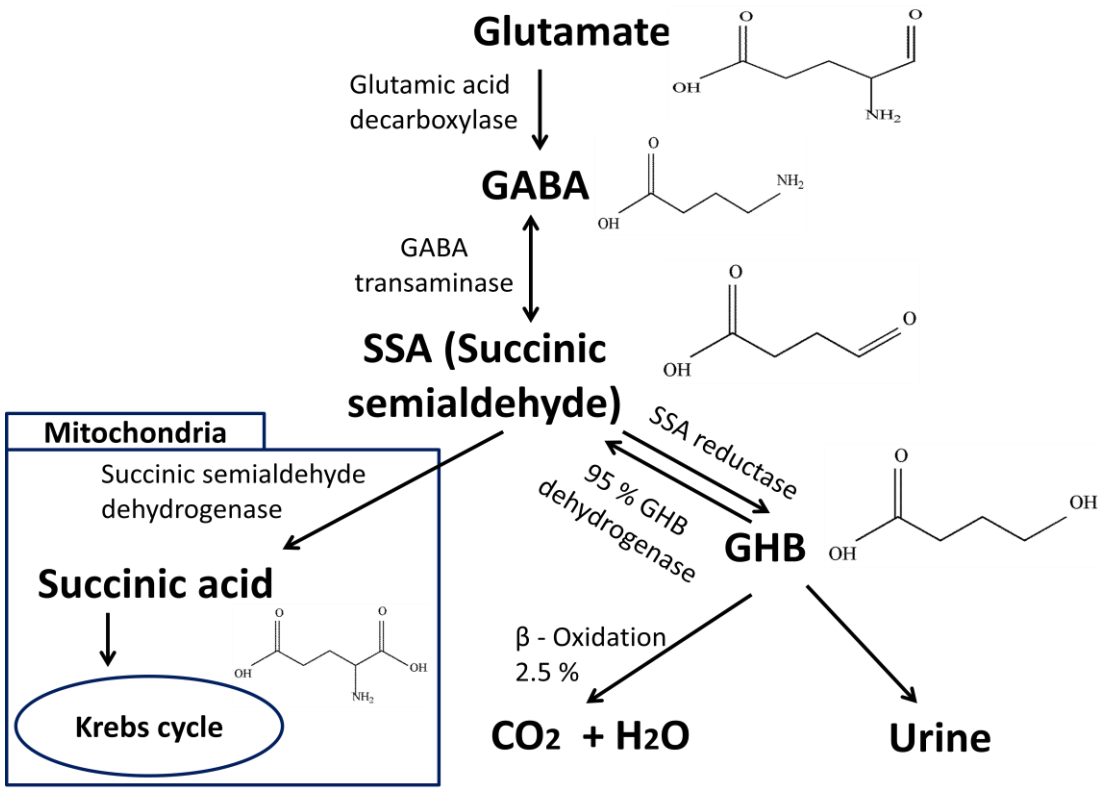


Figure 1.3. GHB metabolism in the central nervous system. GHB has a very rapid metabolism and it is cleared within 12 hours, a problem for detection after this time, adapted from (Caputo *et al*., 2009; van Amsterdam *et al*., 2012).

1.2.1 GHB role as a neurotransmitter or a neuromodulator

It has been shown that GHB is a naturally occurring substance in the brain of mammals and its role was suggested as a neurotransmitter (Bessmann and Fishbein, 1963). The physiological effects of GHB on sleep and sleep disorders are assumed to occur via specific GHB receptors, while the pharmacological and toxicological effects of GHB are thought to occur through interactions with the GABAB receptors (Maitre, 1997; Carai *et al*., 2001; Mamelak, 2009).

1.2.2 GHB binding to receptors

In the brain, GHB binds to both specific high-affinity GHB receptors and to $GABA_B$ receptors, making the understanding of its neuropharmacology quite complex (Høg *et al*., 2008). There are several probable pharmacological mechanisms for GHB; it can metabolize to GABA, which binds to $GABA_A$ and $GABA_B$ receptors and also, GHB can bind to $GABA_B$ and/ or GHB receptors (Carter *et al*, 2009), as illustrated in Figure 1.5.

GHB effects differ from the effects of GABA, even though GHB is closely related to GABA and can activate $GABA_B$ receptors in elevated doses (Molnar *et al*., 2009). GHB is known to be metabolized to GABA and to bind to $GABA_B$ and GHB receptors without showing considerable binding to sites on $GABA_A$ receptors (Carter *et al*., 2009).

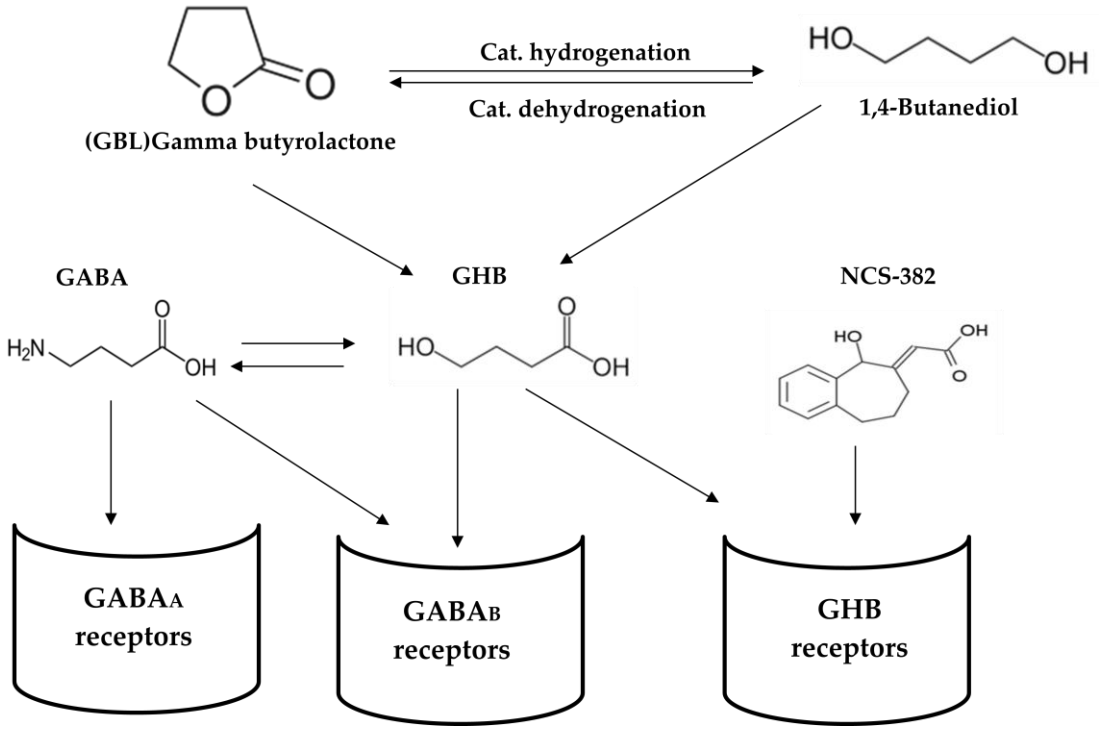


Figure 1.4: Possible pharmacological mechanisms of GHB, GHB is metabolized to GABA, which binds to $GABA_A$ and $GABA_B$ receptors, GHB binds to GHB and $GABA_B$ receptors, NCS-382 binds to GHB receptors. Structures of 1,4-butanediol (1,4-BD) and gamma-butyrolactone (GBL) (GHB prodrugs), GABA, GHB and NCS-382 are shown (Carter *et al*., 2009).

1.2.2.1 GHB receptors

GHB binds to specific receptors with high affinity at low concentrations and at endogenous levels. In rat and human brain, an isoform of GHB receptor has a K_d of 30-580 nM (Benavides *et al*., 1982; Maitre, 1997). GHB receptors are found mainly in the presynaptic neurons of cortex and hippocampus (Kemmel *et al*., 2006) and not in glia (Maitre, 1997). Not only GHB an endogenous substrate for GHB receptors but also succinate, a tricarboxylic acid cycle metabolite can bind to the same site (Molnar *et al*., 2009).

GHB receptors are thought to be linked to a G-protein and binding lowers adenylyl cyclase activity which leads to a reduction in cAMP (Snead, 2000), but some other studies disagree with this (Castelli *et al*., 2003; Odagaki and Yamauchi, 2004). Nanomolar concentrations of GHB were able to activate cAMP response while micromolar levels activate calcium gating (Coune *et al*., 2010). A study carried out *in vitro* on a neuronal cell line (NCB-20 cells) revealed that binding of GHB to the receptor stimulated the entering of Ca2+ via T-type calcium channels when the membrane is at resting potential. This leads to hyperpolarisation of the cell membrane by activating potassium channels (Kemmel *et al*., 2003).

Newly established derivatives of phenylacetic acid, including the nonsteroidal anti-inflammatory drug (NSAID) diclofenac, bind to definite GHB binding sites with affinity similar to GHB, and the presence of these separate GHB binding sites is also supported by the production of selective GHB ligands such as NCS-382 (Høg *et al*., 2008).

1.2.2.2 $GABA_B$ receptors

Some of the behavioural effects of GHB are not reversed by NCS-382, the GHB receptor antagonist, and are blocked by $GABA_B$ receptor antagonists. NCS-382 does not affect $GABA_A$ or the $GABA_B$ receptors and this indicated that some of the effects of GHB are independent of GHB receptors (Ticku and Mehta, 2008).

Relatively high levels of GHB can activate $GABA_B$ receptors, indicating that GHB is a weak agonist of $GABA_B$ receptors (Lingenhoehl *et al*., 1999).
$GABA_B$ receptors are G-protein coupled receptors that are negatively joined to adenyl cyclase (Kaupmann *et al*., 1997; Odagaki *et al*., 2000). In the

beginning, it was identified in the peripheral nervous system (Bowery and Hudson, 1979), although, it has been established to be present in the CNS (Andrade *et al*., 1986, Becher *et al*., 2001). It consists of two subunits: $GABA_{B1}$ and $GABA_{B2}$ (Kaupmann *et al*., 1998; Jones *et al*., 1998). On the contrary to GHB receptor, activation of $GABA_B$ receptors inhibits calcium influx in presynaptic neurons, but it reduces cAMP levels similar to GHB receptor and also causes postsynaptic neuronal hyperpolarization (Sakaba and Neher, 2003).

Additional mechanisms of action for GHB have been suggested including the involvement of $GABA_C$ receptors and $GABA_B$ mediated increase in synthesis of $GABA_A$ modulating neuroactive steroids (Carter *et al*., 2009; Mamelak, 2009).

$GABA_B$ receptors are present on GABAergic and non-GABAergic neurons and can modulate neuronal (GABAergic) signalling pre- and post-synaptically by decreasing neurotransmitter release (Bonanno and Raiteri, 1993). Activation of $GABA_B$ receptors can increase the production of some neuroactive steroids that positively modulate $GABA_A$ receptors (Barbaccia *et al*., 2002). Animal studies revealed that GHB causes absence-like seizures and this type of seizures is aggravated by $GABA_A$ agonists, activation of $GABA_B$ receptors by GHB can explain the effects of GHB on $GABA_A$ receptor, (Snead *et al*., 1992).

The changing of nucleus accumbens (NAC) dopamine levels is the key action of many drugs of abuse, (Di Chiara and Imperato, 1988) but most of them also act on other transmitter systems. It was revealed that GHB enhances the activity of ventral tegmental area (VTA) dopaminergic neurons by the inhibition of VTA GABAergic neurons and enhances the release of dopamine

into NAC (Cruz *et al*., 2004; Pistis *et al*., 2005). The other major target is the glutamatergic system: improved glutamate transmission is necessary to express drug-seeking, restoration or sensitization (Famous *et al*., 2008; Kalivas *et al*., 2009), also many drugs are stimulating their own receptor in the NAC (Nestler, 2005).

It was shown that GHB in small doses has anxiolytic effects in rats. These anxiolytic effects are not opposed by the GHB receptor antagonist, NCS-382, nor by naloxone, which is the opioid receptor antagonist. However, the anxiolytic effects of GHB are reversed by flumazenil, which is a benzodiazepine receptor antagonist indicating GHB interaction with $GABA_A$ receptors that mediate the anxiolytic effects of benzodiazepines (Schmidt-Mutter *et al*., 1998).

Conversion of GHB to GABA which activates $GABA_A$ receptors causes the use of GHB exogenously to reduce the symptoms of alcohol withdrawal syndrome in humans (Caputo *et al*., 2009). The main significant clinical effect of exogenous GHB is a central nervous system depression by binding to the $GABA_B$ receptor. Studies show that GHB activates the $GABA_B$ receptor either directly or after conversion into GABA (Ticku and Mehta, 2008). Regular administration of GHB causes down regulation of GABA receptors. This leads to drug dependence as a means to maintain homeostasis (Constantinides and Vincent, 2009). A number of animal studies indicate that the addictive properties of GHB may be mediated through the $GABA_B$ receptor (van Noorden *et al*., 2009).

Numerous previous studies have also proposed that $GABA_B$ receptors are chiefly essential for various behavioural effects of GHB, including hypolocomotion, catalepsy, ataxia, loss of righting, decreased operant

responding, and discriminative stimulus effects. The typical $GABA_B$ receptor agonist baclofen also produces all of these effects of GHB. $GABA_B$ receptors have a significant role on the GHB effects; however, the effects of GHB are like but not identical to the effects of the baclofen, which is a typical $GABA_B$ receptor agonist (Koek *et al.*, 2009).

1.2.3 GHB interaction with other neuromodulators

Glutamate

Glutamate is the main excitatory neurotransmitter in the brain. Synaptic hyperactivity leads to excessive release of glutamate. A microdialysis study in rat to find the effect of GHB on extracellular glutamate levels in the hippocampus revealed that a locally perfused GHB into rat hippocampus results in a biphasic concentration-dependent effect on extracellular glutamate, specifically, the nanomolar GHB concentrations were found to increase whereas the millimolar concentrations decreases glutamate levels (Ferraro *et al*. 2001). Moreover, the influence on hippocampal glutamate levels might be important to distinguish between the agonistic or antagonistic features of GHB receptor ligands; it was revealed that the stimulant effect, the excretion of glutamate, was mediated by GHB receptors whereas the inhibitory effect, inhibition of glutamate excretion, was mediated by $GABA_B$ receptors (Castelli *et al*., 2003).

Dopamine

Roth *et al*. (1980) find out that GHB administration to rats causes an inhibition of striatal dopaminergic neuronal firing and possibly reduced release. This inhibition of activity and release is accompanied by augmented dopamine biosynthesis and reduced catabolism (Roth and Suhr, 1970; Morgenroth *et al*., 1976; Nowycky and Roth, 1979; Roth *et al*., 1980).

Furthermore, it was revealed that GHB expresses dual action on the dopaminergic system, where low doses of GHB stimulate dopamine release, while high doses inhibit dopamine release in the mesolimbic dopamine system (Cruz *et al*., 2004; Hechler *et al*., 1991). Most of the abused drugs as well as ethanol were found to increase dopamine release in the mesolimbic dopamine system (Pierce and Kumaresan, 2006).

Acetylcholine

Previous study in rats indicates that GHB administration results in reduction of extracellular acetylcholine levels in the rat hippocampus in a dose-dependent manner and this effect was mimicked by a $GABA_B$ agonist baclofen and blocked by a $GABA_B$ receptor antagonist signifying that this effect is mediated by the $GABA_B$ receptor (Nava *et al*., 2001). Another study proposed physostigmine, an acetyl cholinesterase inhibitor for the treatment of GHB toxicity (Yates and Viera, 2000).

Additional study carried out on normal men and parkinsonian patients in order to find whether muscarinic cholinergic receptors mediates the GHB dependent growth hormone (GH) secretion using the anticholinergic agent pirenzepine pre-treatment and they found that pirenzepine completely repressed the GHB-induced GH release in both groups, leading them to conclude that the GH response to GHB in normal men is mediated by cholinergic mechanisms (Volpi *et al*., 2000).

Serotonin

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter present in the central nervous system and also in the periphery. Serotonin has extended physiological functions in the brain including modulation of sleep, emotion

mood, memory and learning (Lei, 2012). The entorhinal cortex (EC) is assumed as the gate of the hippocampus to control the inflow and outflow of information, so, EC is important for many physiological functions like memory, learning and emotion, also important for numerous pathological disorders such as schizophrenia, Alzheimer's disease and temporal lobe epilepsy. Serotonin cause hyperpolarization of entorhinal neurons and inhibits the excitatory synaptic transmission and also, facilitates GABA release (Lei, 2012), so it may mediate the pharmacological effects of GHB. Moreover, GHB interacts with the serotonergic system, making the build-up of tryptophan and increasing serotonin turnover in rat brain (Gobaille *et al*., 2002).

Opioids

The precise interactions between GHB and the opioid system are not fully understood; it was shown that dopamine release in the striatum is probably accompanied by the release of endogenous opioids (Hechler *et al*., 1991). A Microdialysis study revealed that GHB enhances the release of opioid-like substances in the striatum, and also, GHB-induced dopamine accumulation and release were blocked by Naloxone. Furthermore, the specific GHB receptor antagonist was totally blocked the dopamine response and the opioid-like substances release. This information indicates that GHB induces dopamine release through particular receptors that could modulate the activity of opioid interneurons (Hechler *et al*., 1991). Another study revealed that naloxone and naltrexone which are the specific opiate antagonists reduced or eliminated the effects produced by gamma-butyrolactone which is the GHB prodrug in a dose-dependent manner (Snead and Bearden, 1980).

1.3 Pharmacodynamics of GHB

1.3.1 GHB transport

Exogenous GHB passes the blood brain barrier (BBB) by a carrier-mediated mechanism, mainly by the monocarboxylate transporter (MCT) family (Bhattacharya and Boje, 2004) which are proton-linked transporters that play a vital role in cellular metabolism (Lam *et al*., 2010). This information is very important in GHB therapeutics, drug interactions and overdoses and it is suggested that the use of GHB transport inhibitors in overdose cases may help in decrease brain GHB concentrations (Bhattacharya and Boje, 2004). Moreover, MCTs are expressed in many tissues in the body includes the kidney, liver, intestine and brain (Morris and Felmlee, 2008). Coadministration of monocarboxylate transporter (MCT) substrates like L-lactate or pyruvate with GHB increased its renal and total clearance and decreased its plasma concentrations in the rat (Morris and Wang, 2005). This is because of the competition of these substrates and GHB over the transporter.

1.3.2 GHB prodrugs

The GHB analogues gamma-butyrolactone (GBL) and 1,4- butanediol (BD) became popular drugs, as a consequence of the Food and Drug Administration ban on nonprescription GHB in 1990. These are rapidly metabolized to GHB by the enzymes serum lactonase and alcohol dehydrogenase, respectively. The clinical effects including drowsiness, euphoria and sensual feeling produced by GBL and BD are equivalent to the effects of exogenously administered GHB (van Noorden *et al*., 2009). In the brain of adult rat, GHB levels are about 0.4 pM in the frontal cortex, 1.2 PM in the hippocampus, 1.8 PM in the striatum to 4.6 KM in the substantial nigra

(Maitre, 1997). GBL and BD are present naturally in rat brain at concentrations of about 1/10 of concentrations of GHB. These compounds may be of significance to the synthesis of GHB under limiting conditions (Lyon *et al*., 2007).

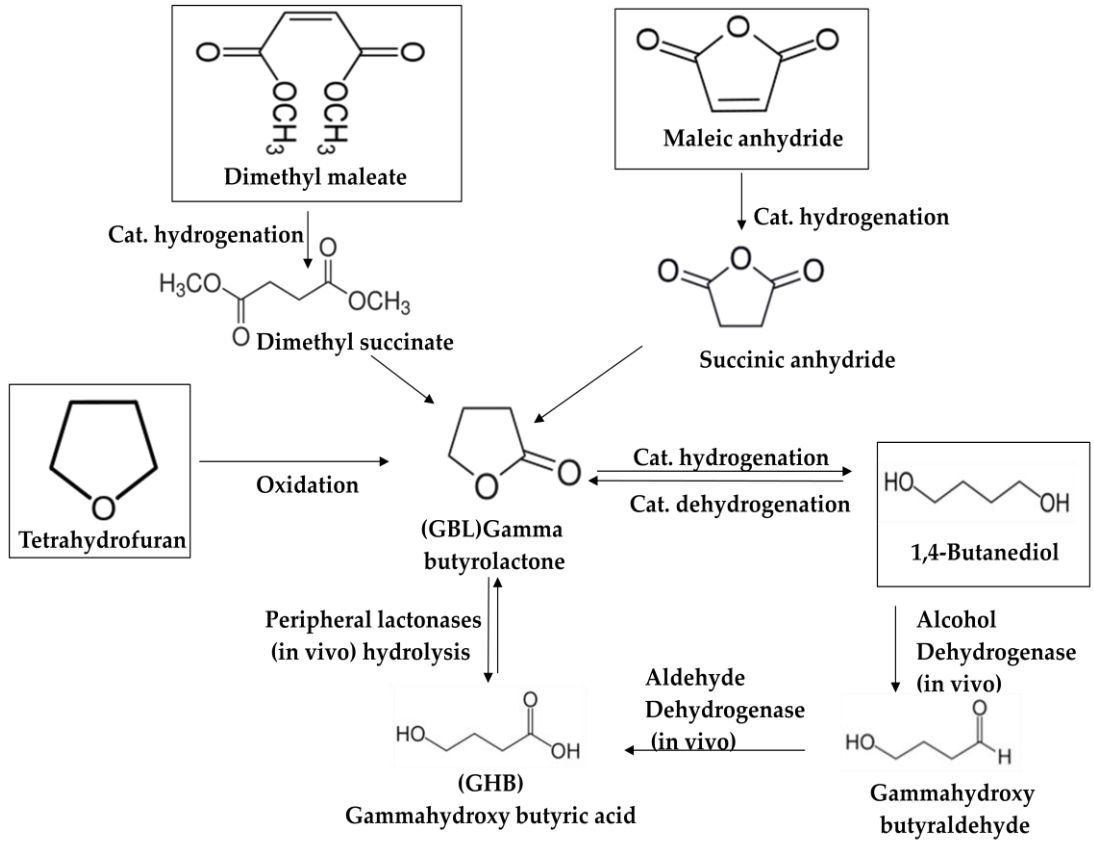


Figure 1.5: Structures and synthesis pathways of synthetic precursors of gamma-butyrolactone (GBL) (Marclay *et al*., 2010).

Usage of GBL has increased in the recent years mostly as a chemical intermediate to produce polymers and also a biodegradable degreaser or paint remover. The major portion of GBL is presently being chemically

produced through the dehydrogenation of BD which is produced from the reaction of acetylene with formaldehyde; this reaction is known as the Reppe process. More recent manufacturing ways of GBL is based on the two-stage hydrogenation of cheaper raw materials such as dimethyl maleate or maleic anhydride. Also tetrahydrofurane can be used as a precursor to manufacture GBL after a single oxidation step (Marclay *et al*., 2010), the synthesis pathways of GHB precursors is illustrated in (Figure 1.5).

1.4 Pharmacokinetics of GHB

1.4.1 Absorption and distribution of GHB

GHB is usually administrated orally in the form of capsules, solution, or as powder dissolved easily in water, and it is quickly absorbed from the gastrointestinal tract.

GHB absorption is rapid and the highest clinical effect is achieved following 30- 60 minutes of oral administration. Unlike GABA, GHB easily crosses the blood–brain barrier, and the plasma half-life of GHB is 20–30 min (van Noorden *et al*., 2009).

Oral bioavailability was measured in rat to be dose-dependent, ranging from 62% to 94% (Lettieri and Fung, 1979) and the absorption shown to be capacity-limited, meaning that plasma peak concentration (C_{max}) is dose-independent (Ferrara *et al*., 1992).

A pharmacokinetic human study on GHB estimate drug concentration in plasma, urine, and oral fluid specimens after a single 25-mg/kg body weight oral dose of GHB analysed the specimens using gas chromatography-mass spectrometry (GC-MS). Plasma peaks of GHB occurred in 20 to 45 min after

drug administration. GHB could be detected up to 360 min in oral fluid however, the peak concentration was found in the 10-min samples. GHB estimation in urine samples showed that less than 5% of GHB excreted unchanged in the urine with a maximum concentration after about 60 min. Therefore, GHB cannot be detected after 12hours following administration and the urine samples can be used in forensic investigations only when the sample is collected almost immediately after consumption (Brenneisen *et al*., 2004). These values were same in alcohol-dependent patients (Ferrara *et al*., 1992).

Food significantly alters the absorption of GHB by decreasing the plasma peak concentration (C_{max}) and extending the time required to reach it (t_{max}), so, decrease the bioavailability of the GHB (Borgen *et al*., 2003).
GHB has a quick distribution into the tissue following a two compartment model with a fast distribution phase and a small apparent volume of distribution (Van-Sassenbroeck *et al*., 2001). It is easily moves through the blood-brain barrier (van Noorden *et al*., 2009). GHB does not bind to plasma proteins at any dose (Morris and Wang, 2005).

1.4.2 Metabolism and elimination of GHB

GHB undergoes first pass metabolism in the liver and is subjected to different enzymatic reactions. As mentioned previously GHB is converted to succinic semialdehyde (SSA) then to succinate which enters the tricarboxylic acid cycle (TCA cycle) and turns in to carbon dioxide and water (Doherty *et al*., 1975). Radioactive studies in rat established that about 0.5 to 2% of GHB is converted to GABA in particular regions of the brain, about 3hours after administration. Radioactive glutamate and glycine were also detected;

succinic semialdehyde (SSA) was the intermediate in these two reactions (Gobaille *et al*., 1999). It has been also confirmed the β-oxidation metabolism of GHB to acetyl-CoA, which also enters the TCA cycle (Brown *et al*., 1987).

Clearance of GHB is 14.0 ml min $^{-1}$ kg $^{-1}$ at a dose of 12.5 mg/kg and its half-life is 20 minutes. GHB metabolism is very fast and leads it to enter the citric acid cycle as succinate which is converted to CO_2 + H_2O (Doherty *et al*., 1975). GHB is almost totally oxidized to carbon dioxide and approximately 2-5% was eliminated in the urine (Gallaway *et al*., 1997).

Elimination half-life corresponds to the time needed for the concentration of drug in the plasma, or the drug amount in the body, to reduce by half or 50 %, the t½ of GHB is 0.3 -1.0 h, and the volume of distribution is 0.4 L/kg. Bioavailability of GHB after oral ingestion is quite low, after five half-lives or about 97 % of a drug is eliminated from the body (Jones *et al*., 2009). A small percentage of GHB is recovered unchanged in the urine (less than 5 %) one hour after consumption (Brenneisen *et al*., 2004).

GHB is quickly cleared from the blood stream, having final plasma elimination half life of 20- 30 min (Borgen *et al*., 2003, Brenneisen *et al*., 2004). The rate of elimination of GHB was established to be dose independent at high doses (zero order) (Van-Sassenbroeck *et al*., 2001).

1.5 GHB Concentrations

1.5.1 Endogenous GHB concentrations

GHB exists in the neuronal and peripheral tissues. GHB concentrations were measured in rat to be 1.85 μM in brain, 1.59 μM in liver, 10.8 μM in heart, 1.12 μM in lung, 21.6 μM in kidney, 11.3 μM in muscle, 0.42 μM in white

adipose tissue and 0.55 μM in blood (Nelson *et al*., 1981). Also, GHB concentrations were measured in brain regions of rats killed by microwave irradiation to be 2.42 μM in hypothalamus, 2.04 μM in cerebellum, 1.87 μM in cerebral cortex, 1.86 μM in stratum, 1.41 μM in hippocampus, and 1.73 μM in white matter, this increase in GHB levels particularly in the hypothalamus was observed in the decapitated animals and this information is indication for a post-mortem formation of GHB (Eli and Cattabeni, 1983).

Previous human studies measured an endogenous concentration of GHB to distinguish the endogenous GHB levels from the concentrations after GHB exposure, one of these studies revealed 9.3 – 11.98 μM in blood samples and 10.6 – 45.6 μM in urine samples (Elian, 2002). Other study revealed 31.7 – 39.7 μM and 79.3 μM for blood and urine respectively (Elliott, 2003). Another human study suggested the urinary concentration as 47.6 μM (Andresen, *et al*., 2010). Higher levels of GHB and its precursor GBL were found in the urine of pregnant women (Raknes, 2010).

GHB concentrations in saliva were measure to be ranged from 1.2 – 26.4 μM (De Paoli *et al*., 2011). Furthermore, GHB levels were measured in hair to be ranged from 0.3-12 ng/mg (Goulle *et al*., 2003; Kintz *et al*., 2003).

1.5.2 Post-mortem endogenous GHB concentrations

Studies were curried out to measure the post-mortem endogenous GHB concentrations revealed that relatively high concentrations of endogenous GHB were found in body fluids after death, in contrast to the low levels of endogenous GHB concentrations found in living humans, some of these studies measured endogenous GHB concentrations in whole blood (Fieler *et al*., 1998; Sakurada *et al*., 2002; Kintz *et al*., 2004; Moriya and Hashimoto,

2005). A study screened 71 autopsy cases less than 3 days after death and reported elevated levels of GHB in cardiac blood rather than femoral blood (Kintz *et al*., 2004). Other study found that the amount of GHB in femoral blood were higher than cardiac blood and aortic blood in 25 cases measured less than 2 days after death (Moriya and Hashimoto, 2005).

A study in mouse liver revealed that the GHB concentration in liver were increased over the time designated that GHB might be produced due to post mortem breakdown (Sakurada *et al*., 2002).

Other studies were carried out to measure endogenous GHB concentrations revealed that no GHB or low concentrations of GHB were found in the post mortem urine samples (Fieler *et al*., 1998; Sakurada *et al*., 2002; Moriya and Hashimoto, 2005; Berankova *et al*., 2006). One study stated that a significantly higher endogenous GHB levels were found in urine from hanging and drowning cases (Elliot, 2001).

Significant GHB concentrations were detected in vitreous humour and bile samples post mortem (Kintz *et al*., 2004).

1.6 GHB detection samples and techniques used

1.6.1 Samples

The narrow window of GHB detection causes difficulties in prosecution of drug facilitated sexual assault cases. GHB can be detected in blood and saliva for 5 h only (Kintz *et al*., 2001) and can be detected in urine for 12 h only after GHB exposure (Brenneisen *et al*., 2004). Therefore, it is very important that the samples should be taken quickly after GHB exposure to be

analysed. However, GHB can be detected in hair samples after one month of single exposure to GHB (Kintz *et al*., 2003).

1.6.2 Techniques used to detect GHB

The preferred method for the qualitative and quantitative measurement of GHB concentration was the gas chromatography (Elliott, 2003; Moriya and Hashimoto, 2005; Lyon *et al*., 2007). However, there were some studies using liquid chromatography/ mass spectrometry (LC/MS) and (LC/MS/MS) methods for GHB detection analysis (Borgen *et al*., 2004; Kaufmann and Alt, 2007; Stout *et al*., 2010).

Furthermore, the enzymatic techniques were also used for GHB detection, since the enzymatic techniques a rapid screening method for the drugs in samples. One of the enzymatic techniques that were used for GHB detection is based on the conversion of GHB to SSA using GHB dehydrogenase enzyme which reduces NAD to NADH causes an increase in absorbance at 340 nm (Sciotti *et al*., 2010).

1.7 Changes in gene expression in GHB treated cells

1.7.1 Changes in mRNA

Although it has been more than 40 years since GHB was synthesised (Laborit, 1964; Caputo *et al*., 2009) relatively little is known about its mechanisms of action. There has been a great deal of discussion about which receptors are involved in mediating GHB activity but little is known about the changes in gene expression that GHB may induce.

GHB is known to have broad pharmacological activity, through binding to either the specific high- affinity GHB receptors or GABA receptors, interacting with the dopaminergic system, affecting serotonin levels, interacting with neurosteroids, interacting with the cholinergic system decreasing acetylcholine levels, as well as increasing the secretion of growth hormone, as illustrated in Figure 1.6. (Drasbek *et al.*, 2006). It is therefore possible that exposure to GHB can be observed beyond 12 hours by measuring GHB-dependent changes in gene expression.

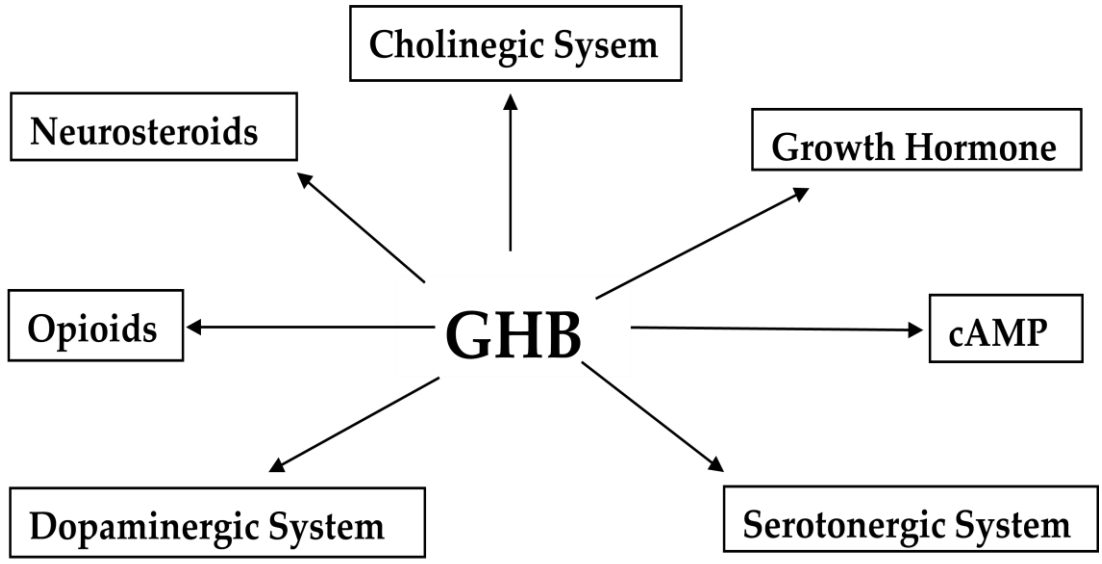


Figure 1.6: GHB interacts with multiple neuromodulatory systems in the brain. Though GHB has low or no affinity for receptors other than $GABA_B$ and GHB receptors, it will cause change in a variety of systems in the brain.

A number of previous studies have investigated specific genes expressed in response to treatment with GHB. The majority of these studies have focused

on changes in brain tissue or cells. One of these studies showed an increase in expression of the dopamine receptors D1 and D2 mRNA after a single 500 mg/kg i. p. dose of GHB given to rats and observed in different rat brain structures rich in GHB receptors (Schmidt-Mutter *et al*., 1999). However, another study in mice revealed that no significant change was found in the expression of dopamine receptor genes in the mouse brain after 4 hours of GHB administration of a single 500 mg/kg i. p. dose (Schnackenberg *et al*., 2010).

Another report on specific gene expression evaluated the effects of GHB on the expression of six sleep-associated genes: egr-3, fra-2, grp78, grp94, ngfi-b, and nr4a3 in the cortex of rat brain. This identified only fra-2 (FOS-like antigen 2) as significantly altered in response to GHB (Wisor *et al*., 2006). However, Schnackenberg *et al*., 2010, found no significant change in fra-2 or any of these sleep-associated genes in mouse brain using the same time points after GHB administration. These differences might be related to the variation in protocols or to the sensitivity of methods used, (QPCR, in situ hybridization and microarray technology) or could be related to the selected filters used to determine the differentially expressed genes (Schnackenberg *et al*., 2010).

A genome wide study was carried out in rat but this was limited to only a single time point 2 h after administration of a 1 g/kg dose of GHB to evaluate gene expression changes in the hippocampus and prefrontal cortex. 282 genes were differentially expressed in the hippocampus and 98 were differentially expressed in the prefrontal cortex. In terms of GO functional categories, the majority of these genes were involved in metabolic processing, biological regulation, binding, catalytic activity, developmental

processes, transcription regulation or gene expression, cell death, stress response, signal transduction, and immune system processes (Kemmel *et al*., 2010).

An additional study used microarray technology to investigate the changes in gene expression of the whole mouse genome in three brain regions at three time points 1, 2 and 4 hours after GHB administration (Schnackenberg *et al*., 2010).

This study reported that GHB induces gene expression changes in many genes in the hippocampus, cortex and striatum and the number of changed genes was increases during a 4-h time course. Many of these genes are involved in neurological disease, apoptosis, and oxidative stress (Schnackenberg *et al*., 2010).

In other tissues that may lack specific GHB receptors, specific and global changes in gene expression have also been investigated. A genome wide study was carried out using blood samples from mouse following a single 1 g/kg dose of GHB to identify possible biomarkers of GHB abuse or toxicity. The study identified nine genes that were considerably up regulated (PEA-15, Epiregulin, Chrm3, Drd2, Gal, Nola1, Ntsr2, Csf2rb1 and Hdc) and three genes that were suppressed (Extl1, Mrpl17 and Slc7a5) in blood after GHB administration (Larson *et al*., 2007). Again, Schnackenberg *et al*., 2010, found no significant change in any of these genes in the hippocampus, cortex and striatum of mouse brain. It is worth noting that the last time point in the Schnackenberg study was 4 hours, while the starting time point in the Larson study was 24 hours.

1.7.2 Changes in protein expression

A previous proteomic study was carried out on rats to analyse long-term changes in hippocampus protein expression following GHB exposure. Rats were treated once a day for 10 days with GHB followed by a washout period of 8 weeks, then protein extraction and gel analysis of hippocampal samples showed that there is a significant down regulation of two proteins glutathione-S-transferase (GST) Yb4 protein by 3 fold and PEA-15 by 1.9 fold and a significant up regulation of Neuronal pentraxin-1 (NP1) by 2.2 folds after GHB exposure (Van Nieuwenhuijzen *et al*., 2010).

The study of Sgaravatti *et al*., (2009) revealed that frequent administration of GHB and its precursors (for example gamma-butyrolactone (GBL)) may increase oxidative stress in the rat brain. The GST comprises a family of detoxification enzymes that protect against oxidative stress (Hayes *et al*., 2005); their inhibition in GHB-treated rats might help to explain the increase in oxidative stress after GHB treatment.

Also using a proteomic approach, Ryu *et al*. (2007) investigated changes in protein expression in adult mouse thalamus at very short periods, three different time points (5, 10, and 30 min) after an intraperitoneal dose of 50 mg/kg of the GHB precursor gamma-butyrolactone (GBL). The analysis revealed a transient dysregulation of 16 proteins during loss of consciousness only. Their expression level was returned back to normal after 30 min when the mice awakened from absence seizure, signifying that these proteomic changes are reversible. These transiently altered proteins had been formerly concerned in neurological disorders such as Alzheimer's disease, epilepsy and schizophrenia. The study also revealed that collapsin response mediator protein 2 (CRMP2), collapsin response mediator protein 4 (CRMP4) and the

mouse stress induced phosphoprotein 1(mSTI1) were differentially expressed in GBL-treated mice compared to the saline controls. CRMP2 and CRMP4 proteins are phosphoproteins known to mediate signals involved in axonal outgrowth and guidance in the nervous system (Goshima *et al*., 1995; Arimura *et al*., 2000). mSTI1 protein is known to reduce neuronal apoptosis and to take part in neuroprotection (Zanata *et al*., 2002).

1.8 Aim of the study

This study is aimed at extending the window of detection of GHB beyond 12 hours through finding robust surrogate markers of GHB administration.

GHB abuse and especially in date rape sexual assaults has increased in recent years. Current detection methods rely on sampling blood and urine but the rapid rate of metabolism of GHB means that GHB can disappear and the criminal cases are often difficult to prosecute. However, GHB is known to have profound pharmacological effects, through binding to either the GHB receptor or GABA receptors. It is therefore possible that exposure to GHB can be monitored beyond 12 hours by measuring the GHB dependent changes in gene expression.

1.9 Study Plan and Experimental Approach

In this study, the effect of different doses of GHB exposure on the expression of epiregulin, PEA-15 and other genes in human astrocytoma 1321N1 cells, human neuroblastoma SH-SY5Y cells and human monocytic leukaemia THP-1 cells will be evaluated using a combination of quantitative RT-PCR and Western blots. It is also planned that a full expression profile will be obtained using microarray analysis and 2D-gel electrophoresis to find novel

biomarkers for GHB exposure in cell lines, particularly the blood cell line (THP-1). Furthermore, to elucidate the mechanism by which GHB exerts these changes using another GHB receptor ligand (NCS-382).

1.9.1 Choice of human cell lines:

1.9.1.1 Human astrocytoma (1321N1) cell line

The central nervous system consists of a number of cell populations, mostly neurons and macroglial cells. The main macroglial cell types are astrocytes and oligodendrocytes. Many functions including cellular support, ion homeostasis, neurotransmitters uptake, CNS immune system contribution and neuromodulation are attributed to astroglia (Ridet *et al*., 1997).

Astrocytes are recently considered as cells that propagate Ca^{2+} over extended distance in response to stimulation, and, like neurons, release transmitters (called gliotransmitters) in a Ca^{2+}-dependent manner to adjust a host of significant brain functions. Astrocytes are also stated to play an important role in modulating neuronal excitatory synaptic activity by Ca^{2+}-dependent release of the gliotransmitters glutamate and ATP (Fiacco *et al*., 2009).

Recent studies found that the main effect of GHB in the nucleus accumbens (NAC) was the activation of subpopulation of astrocytes. It induced Ca^{2+} transients in a subpopulation of astrocytes and this effect is dose-dependent. GHB did not act on $GABA_B$ receptor or GHB receptor in the NAC, but induced Ca^{2+} transients in a subpopulation of astrocytes. The activation of astrocytes by GHB proposes their association in GHB-mediated physiological effects (Molnar *et al*., 2009).

1.9.1.2 Human neuroblastoma (SH-SY5Y) cell line

The SH-SY5Y cell line is a three times cloned neuroblastoma cell, originated from the neuroblastoma line SK-N-SH which is originally established from a bone marrow biopsy of a neuroblastoma patient and neuroblastoma line SK-N-MC. SH-SY5Y cells are described initially in 1978 and became available for a range of studies subsequently (Biedler *et al*., 1973 and 1978).

SH-SY5Y cells are frequently used as a neuronal cell model due to their sympathetic feature and low resting membrane potential, these cells are used in many studies relating to tumour cell growth and neuronal cell biology (Tosetti *et al*., 1998).

Recent studies in SH-SY5Y cell lines illustrate the efficiency of conversion of 1, 4-butanediol to GHB when the main GHB synthesis pathway from GABA is closed, once the concentration of GHB is lowered, the cell respond to compensate GHB, this explains the presence of specific threshold for GHB in the cell, once the GHB concentration declines below the threshold, the cell responds in a substitute way to make GHB (Lyon *et al*., 2007).

Neuronal cells are considered as a good model to expresses most of the functional properties of a GHB system. The neurohybridoma NCB-20 cell line was used to investigate the effect on cytosolic free Ca^{2+} level and cAMP production by GHB receptor(s) inspired with micromolar concentrations of their natural ligand or the structural analogues of GHB (Coune *et al*., 2010).

1.9.1.3 Human monocytic leukaemia (THP-1) cell line

THP-1 is single and round suspension cells (Tsuchiya *et al*., 1980). After exposure to phorbol-12-myristate-13-acetate (PMA), all the THP-1 cells start to adhere to culture plates accompanied by differentiation into macrophage-like cells with clear morphological changes (Tsuchiya *et al*., 1982).

These cells are well-known model in toxicology, immunology and atherosclerosis study, as regard monocyte and macrophage role and biology. Phorbol esters, for example PMA, are frequently used to obtain the differentiation of THP-1 monocytes into macrophage-like cells which mimic native monocyte-derived macrophages in several respects (Maess *et al*., 2010).

THP-1 cells have some advantages over human primary monocytes /macrophages; the main advantage is their homogeneous genetic setting which helps minimise the degree of variability in the cell phenotype, this feature is especially important when studying the biological function of chemicals with elevated variability (Rogers *et al*., 2003; Cousins *et al*., 2003). Second advantage is the transfection efficiency which is a problem in human primary monocytes and macrophages (Burke *et al*., 2002). Third advantage is that THP-1 cells can be stored for an indefinite period in liquid nitrogen and then after a suitable procedure, this cell line can be recovered without clear injurious effect on monocyte and macrophage features and cell viability, while the availability of primary human monocytes and macrophages is limited (Altieri and Edgington, 1988).

A review study carried out on THP-1 cells revealed that THP-1 cells mimic the *in situ* change of macrophages in the adipose tissue of the overweight

subjects and in atherosclerotic lesions, in addition to the resemblance of these cells to the primary monocytes and macrophages isolated from normal donors or donors with disease, for example diabetes mellitus (Qin, 2012).

Monocytes/macrophages are important in the progression of disease, at the blood brain barrier (BBB) junction, macrophages, fibroblasts and endothelial cells are the main sources of matrix metalloproteinases (MMPs). Therefore, macrophages may act as amplifiers of the pathogenic cascade, especially by activation of fibroblasts by molecules such as interleukin (IL)-1 and tumour necrosis factor alpha (TNF-α) (Engelhardt, 2006).

It was revealed that the THP-1 cells do not have a functional membrane monocarboxylate transporter (MCT) and that 3-bromopyruvate can enter these cells without MCT (Verhoeven and van Griensven, 2012). MCT4 is predominantly expressed in cells with a high glycolytic rate such as white blood cells, where it is involved in the removal of lactic acid produced from glycolysis (Juel and Halestrap, 1999; Manning Fox *et al*., 2000).

CHAPTER TWO

MATERIALS AND METHODS

2 Materials and Methods

2.1 Materials

2.1.1 Human cell lines

Human astrocytoma 1321N1 cells at passage 40 (Clark *et al*., 1975) and human neuroblastoma SH-SY5Y cells at passage 15 (Biedler *et al*., 1973; Biedler *et al*., 1978), were provided by Dr. Elizabeth Ellis, University of Strathclyde. Human monocytic leukemia THP-1 cells (Tsuchiya *et al*., 1980) were obtained from the European Collection of Cell cultures, UK.

2.1.2 Cell culture plastic wares and equipments

Tissue culture flasks (Easy Flask 75 cm^2 and 25 cm^2), also the 6-well, 12-well and 24-well plates were obtained from Fisher Scientific (UK). 96-well plates were obtained from Greiner Bio- one (UK) and cell Scrapers were obtained from BD bioscience (UK).

2.1.3 Chemicals and reagents

Eagle's minimal essential medium (EMEM), Ham's F-12, Dulbecco's modified eagle's medium (DMEM), RPMI 1640, Hank's Balanced Salt Solution (HBSS), phosphate-buffered saline (PBS: 137 mM NaCl, 10mM sodium phosphate, 2.7 mM KCl, pH 7.4), Trypsin - EDTA Solution (1X), sodium chloride, sodium citrate, Penicillin- Streptomycin (10,000 units penicillin; 10mg streptomycin/ml 0.9 % NaCl) Solution, Sodium Pyruvate (11.0 mg/ml sodium pyruvate in tissue culture water), L-glutamate, Non - Essential (100 x) Solution, GHB, NCS-382, Gel red, calcium chloride, rubidium chloride, magnesium chloride, MOPS, tryptone, yeast extract, bacteriological agar, glucose, sodium acetate, sodium dodecyl sulfate (SDS), Urea, Triton X, 2- Mercaptoethanol, dithiothreitol (DTT), bromophenol blue,

tetramethyl ethylenediamine, iodoacetamide, Coomassie brilliant blue G250, TEMED, Tween 20, H_2O_2 (30 %), coumaric acid, hydrazine sulphate, methanol and ethanol were obtained from Sigma-Aldrich (UK). Fetal Bovine Serum (FBS), 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were obtained from Sigma- Aldrich (Germany). Tris base [Tris (hydroxymethyl) aminomethane], sodium hydroxide (NaOH), ammonium sulphates and glycerol were obtained from Fisher Scientific (UK). Ethylenediaminetetraacetic acid disodium salt (EDTA), isoamyl alcohol, phenol, phenylmethylsulfonyl fluoride (PMSF), phosphoric acid (85 % v/v) and ammonium persulfate were obtained from BDH Chemicals limited (Poole, England). Hydrochloric acid (HCl) and glacial acetic acid were obtained from Riedel - de Haeu (Germany). 30 % acrylamide/bisacrylamide solution was obtained from Severn Biotech. Ltd. 180mm long Immobiline ™ IPG gel strips (pH 3 - 10), Immobiline DryStrip Reswelling Tray with twelve reservoirs or channels and DryStrip cover fluid were obtained from Amersham Pharmacia Biotech (Sweden) and Pharmalyte 3 - 10 from Amersham Biosciences, UK. Glycine was obtained from Melford, isobutanol from Merck and agarose from Flowgen (UK). Trypsin Gold vial was obtained from Promega, USA. Bradford reagent was obtained from Bio-Rad, UK. Luminol and D-Fructose 1, 6-bisphosphate trisodium salt hydrate were obtained from Fluka.

2.1.4 PCR (Polymerase Chain Reaction) chemicals

Random Primers, PCR Nucleotide Mix (dNTP mix) (10 mM), M-MLV Reverse Transcriptase (200 units/µl), M-MLV Reverse Transcriptase Buffer (5 x), Flexi buffer (10 x), $MgCl_2$ (stock solution 25 mM), Taq. DNA polymerase (5 units /ul) and Nuclease free water were obtained from Promega, USA.

2.1.5 Oligonucleotide primers

Oligonucleotide primers for the amplification of PEA-15 (NM_003768.3), AKR1C3 (NM_003739), AKR1C4 (NM_001818) and GAPDH (NC_000012.11) were synthesised by Eurofins MWG operon (UK) while the oligonucleotides of epiregulin (NM_001432.2), HSD11B1 (NM_181755), DHRS9 (NM_005771) and AKR1C1 (NM_001353) primers are guaranteed primers called QuantiTect primer Assay obtained from Qiagen, Japan. The primers used were listed in table 2.1.

Table 2.1.: Oligonucleotide primers for real-time PCR Reaction

Oligonucleotide primer	Sequence	Annealing temperature (°C)	Amplicon size (bp)
PEA-15 (forward) PEA-15 (reverse)	5′-GCTCCAGAGGCGTCATGGCTG-3′ 5′-TGAGTAGGTCAGGACGGCGGG-3′	60 60	243
Epiregulin	Cat.No.QT00019194	60	122
HSD11B1	Cat.No.QT00030296	60	115
DHRS9	Cat.No.QT00027846	60	112
AKR1C1	Cat.No. QT01679804	60	133
AKR1C3 (forward) AKR1C3 (reverse)	5′-GTAAAGCTTTGG-AGGTCAC-3′ 5′-CACCCATCGTTTGTCTCGT-3′	60	590
AKR1C4(forward) AKR1C4 (reverse)	5′-CGAGGAACAGAGCTGTAGAGGTCAC-3′ 5′-GAGAGCCATTGGGAAATGAAGA-3′	60	278
GAPDH(forward) GAPDH(reverse)	5′-GGAGTCAACGGATTTGGT-3′ 5′-GTGATGGGATTTCCATTG-3′	46.4	206

PEA-15, AKR1C3, AKR1C4 and GAPDH oligonucleotides were synthesised by Eurofins MWG operon (UK). Epiregulin, HSD11B1, DHRS9 and AKR1C1 primers are obtained from Qiagen, Japan.

2.1.6 Cloning vector

The PCR 4-TOPO vector (Invitrogen) with a size of 3957 bp was used for general cloning (Figure 2.1.).

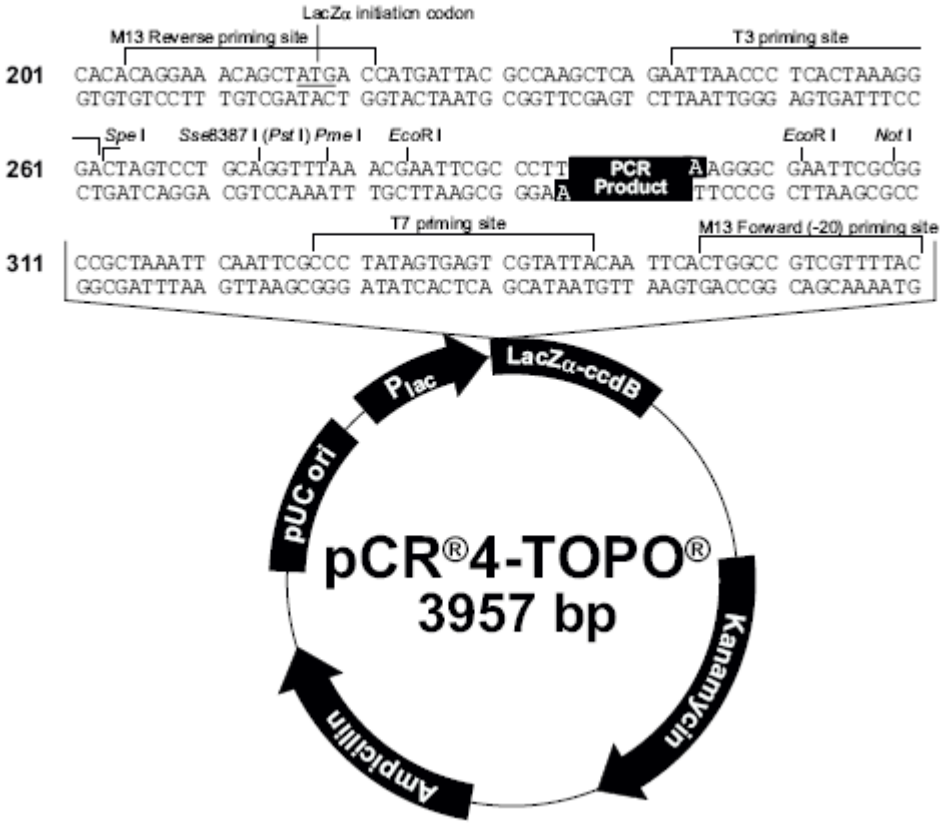


Figure 2.1.: Plasmid map of the TOPO vector including the sequence surrounding the cloning site.

2.1.7 Antibodies

Antibodies used for proteins detection are listed in table 2.2.

Table 2.2. Antibodies used for western blots.

Antibodies	**Catalogue number**	**Source/ Company**	**Predicted band size (KDa)**	**Dilution (µl)**
Rabbit polyclonal to GAPDH	LF-PA0018	Young in frontier	37	1:10000
mouse monoclonal to PEA-15 (H-3)	sc-166678	Santa Cruz Biotech.Inc	15	1:500
Rabbit polyclonal to Aldolase - N-terminal	ab71433	Abcam	36	1:100
Rabbit polyclonal to Hsp70	ab31010	Abcam	70	1:100
Goat anti-rabbit IgG secondary Ab Conjugated to horseradish peroxidase [HRP]).	166- 2408EDU	Bio-Rad Laboratories (Hertfordshire, UK)		1:3000
Anti-mouse IgG Biotin secondary Ab	ab6788	Abcam		1:3000

2.2 Methods

2.2.1 Cell culture

1321N1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Cat: D5796) supplemented with 10 % FBS and 1% penicillin-streptomycin (10,000 units penicillin; 10 mg streptomycin/ml 0.9 % NaCl) solution.

SH-SY5Y cells were grown in 1:1 mixture consisting of Ham's F-12 and Eagle's minimal essential medium (EMEM) (Cat: M2279), supplemented with 1% nonessential amino acids, 1% sodium pyruvate, 1 % L-glutamine, 100 units/ml each of penicillin and streptomycin, and 10 % fetal bovine serum.

THP-1 cells were grown in RPMI 1640 (Cat: R8758) media supplemented with 10 % FBS, 1 % L-glutamine and 1 % penicillin-streptomycin (10,000 units penicillin; 10 mg streptomycin/ml 0.9 % NaCl) solution. The cell culture hood was obtained from ICN Gelaine in England and the cell culture incubator was supplied by Heraeus in Germany.

The carbon dioxide (CO2) cylinder was supplied by BOC gases (UK) and micropipettes were obtained from Fisher Scientific (UK). All cells were grown in the incubator maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95 % air. Ethanol 70 % was used for sterilization.

2.2.1.1 Subculturing of cells

Cells were subcultured once they had reached 90% confluence, generally every 4-6 days. For the adherent cells, the old medium was aspirated and the SH-SY5Y cells were washed twice in phosphate-buffered saline (PBS: 137

mM NaCl, 10 mM sodium phosphate, 2.7 mM KCl, pH 7.4) while the 1321N1 cells were washed once with HBSS (Hank's balanced salt solution). Then the SH-SY5Y cells were detached using 1 ml of 1 X SSC (150 mM NaCl, 15 mM sodium citrate) and the 1321N1 cells were detached by 1 ml of 1 x trypsin-EDTA solution. Both cells were diluted in their media and collected by centrifugation at 1,000 x g for 5 min. The cell pellet was resuspended in an appropriate volume of media and the cells were seeded and placed into fresh sterile flasks (for a 75 cm2 flask, between 10 and 15 ml of medium). The flasks were placed in the incubator and the medium was changed every 3 days.

For the THP-1 cells, these were grown as a suspension of 50 ml in 75 cm^2 sterile flasks. The cells were split every 3-4 days by dividing 50 ml suspension into three flasks, each one were diluted into 50 ml by complete media.

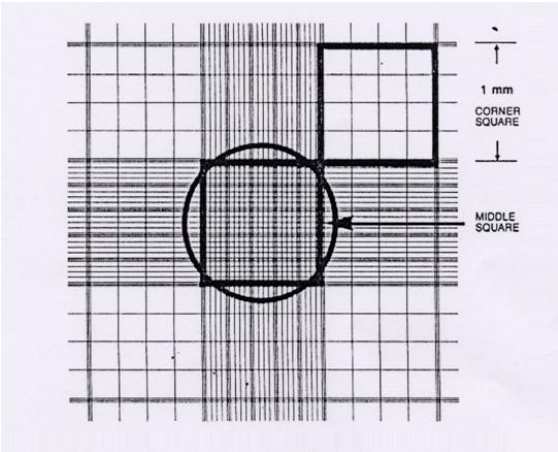
2.2.1.2 Cryopreservation of cells

1321N1 cells and SH-SY5Y cells were frozen in a mixture of their media and DMSO (dimethyl sulfoxide), 8 % DMSO for 1321N1 cells and 5 % DMSO for SH-SY5Y cells. THP-1 cells were frozen in a mixture of RPMI 1640 media supplemented with 20% FBS and 10 % DMSO. Cells were frozen in in liquid nitrogen for long time and in – 80 °C for short time.

2.2.2 Cell counting by Haemocytometer

Cells were detached and resuspended as outlined in the method above for splitting. 10 µl of the resuspended cells were diluted with an equal volume of medium. 10 µl of this diluted cell suspension were placed into a Bright-line

hemocytometer (Sigma-Aldrich-Germany) and cells counted using a microscope (Olympus CK40 from Olympus (Japan). Cells were counted in 4 of the corner quadrants and the mean number of cells per quadrant was taken.



The cell number per ml was calculated as below:

$C = n \times 10^4$

Where C = concentration of cells in the 1:1 dilution

n = Average cell count in 4 quadrants (each quadrant has a volume of 10^{-4} ml)

To determine how much medium was to be used in order to make a specific concentration of cells, the equation $C_1V_1=C_2V_2$ was used.

2.2.3 Cell viability study (MTT assay)

Principle

The MTT assay method was used for cell viability assessment (Mossman, 1983). The principle of this method based on the conversion of soluble yellow coloured tetrazolium salt, [3 (4, 5-dimethyldiazol (-2-yl) - 2, 5 diphenyltetrazolium bromide] (MTT) into an insoluble purple coloured

formazan by succinate dehydrogenase in the mitochondrion of bioenergetically active viable cells (figure 2.2.). The coloured compound formed has a characteristic absorption at 560 nm wavelength and absorbance at this wavelength was used to monitor the viability of the cells.

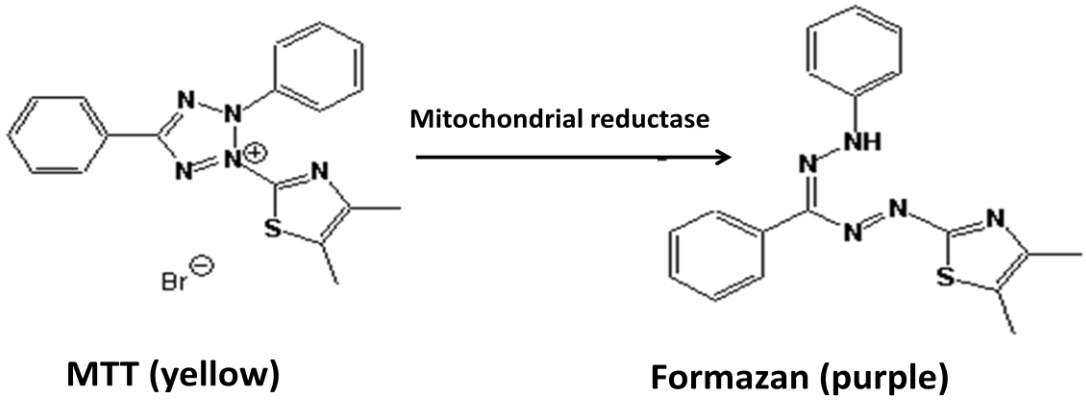


Figure 2.2. MTT reaction in cell viability study. The biochemistry of the reaction involves the reduction of a tetrazolium salt (MTT) by the action of intacellular reductases to form a purple coloured formazan.

Procedure

Cells were cultured in a 96 well plate at a concentration of 3 X 10^5 cells/ ml and allowed to attach for 24 hr. The cells were washed with phosphate buffered saline (PBS) and treated by the addition of 100 μl of media containing different concentrations of test compound (in this case GHB) dissolved in dimethylsulfoxide (DMSO). No more than 0.1% DMSO was added, and a negative control (DMSO and cells only) and positive control (Triton x100) were used as well as media-only control (no cells). Cells were incubated for 24 hr. At the end of the incubation period, 20 μl of MTT 1.2 mg/ml (1.2 mg of MTT dissolved in 1 ml of media freshly before use) were added to the media in each well, and incubated for a further 2 hr. Then the media were aspirated and 100 μl of DMSO was added to each well followed

with gentle shaking for 10 min in order to obtain complete dissolution. Absorbance was read at 560 nm using the Labsystems iEMS microplate spectrophotometer which was supplied by Labsystems and Life Sciences International Limited (UK). The background values (no cells control) were subtracted and the results were expressed as the mean value as a percentage of the DMSO and cells only control.

2.2.4 PCR (polymerase Chain Reaction)

2.2.4.1 RNA isolation

Total RNA was isolated with the SV Total RNA Isolation system (Cat.No Z3100) from Promega, USA, the components of which are listed in Table 2.3. below.

Preparation of solutions:

1- RNA Lysis Buffer

1 ml of β-mercaptoethanol was added to 50 ml of RNA Lysis Buffer.

2- RNA Wash Solution

100 ml of 95 % ethanol was added to 58.8 ml concentrated RNA Wash Solution.

3- DNase Stop Solution

8 ml of 95 % ethanol was added to the 5.3 ml DNase Stop Solution.

4- 95% ethanol: 95 ml ethanol was made up to 100 ml with sterile water.

Spin Column Assemblies

This consists of the Spin Basket and 2 ml Collection Tube. The Spin Column Assemblies were made by putting the Spin Basket in the Collection Tube.

Table 2.3. SV Total RNA Isolation System Components

Component	Volume/Pack
RNA Lysis Buffer (4 M guanidine isothiocyanate, GTC; 0.01 M Tris, pH 7.5)	50 ml
RNA Dilution Buffer (blue buffer)	20 ml
B- mercaptoethanol (48.7 %)	2 ml
DNase I (lyophilized)	1 vial
MnCl2 (0.09 M)	250 µl
Yellow Core Buffer	2.5 ml
DNase Stop Solution (concentrated) (5 M GTC; 10 Mm Tris-HCl, pH 7.5)	5.3 ml
RNA Wash Solution (concentrated) (162.8 mM potassium acetate; 27.1 mM Tris- HCl, pH7.5 at 25 °C)	58.8 ml
Nuclease-Free Water	13 ml
Spin Column Assemblies and Elution Tubes (5 each/pack)	50 packs

Procedure

Astrocytoma 1321N1 and neuroblastoma SH-SY5Y cells were cultured in 6 well plates at a concentration of 10^6 cells in each well and allowed to attach then treated with 1 µM and 100 µM GHB drug for 24 hr. The cells were washed with ice - cold, sterile 1xPBS and 175 µl of RNA Lysis Buffer was added to each well and the cells were scraped into sterile micro tubes. Monocytic leukaemia THP-1 cells were cultured and treated with 10 µM and 900 µM concentrations of GHB, NCS-382 or both for 24 hr, then the cells were centrifuged, washed with ice - cold, sterile 1x PBS and placed in sterile micro tubes, 175 µl of RNA Lysis Buffer was added to each tube. The cell lysates from all cells were passed through 20-gauge needles to shear the genomic DNA and 350 µl of RNA Dilution Buffer (blue) was added to the lysates and

mixed by inverting the tubes 3- 4 times. The tubes were incubated in a water bath at 70 °C for 3 min (longer than 3 min incubation may compromise the integrity of RNA) and then centrifuged at 12,000- 14,000 x *g* for 10 min at 20 - 25 °C. The clear supernatants were mixed (by pipetting 3- 4 times) with 200 µl of 95 % ethanol prepared in sterile water. The mixture was then transferred into the spin column assembly and centrifuged at 12,000- 14,000 x *g* for one minute. The filtrate was discarded and 600 µl of RNA Wash Solution was added and centrifuged at 12,000-14,000 x *g* for 1min. 50 µl freshly prepared DNA incubation mix (40 µl Yellow Core Buffer, 5 µl 0.09 M $MnCl_2$ and 5 µl DNase enzyme) per sample was added to the membrane inside the spin basket of the spin column assembly and incubated for 15 min at 20- 25°C. After the incubation, 200 µl of DNase Stop Solution was added to the spin basket and centrifuged at 12,000- 14,000 x *g* for 1minute. RNA in the spin basket was washed with 600 µl and then 250 µl of RNA wash solution. The spin basket was then placed on 1.5 ml Elution tube and 100 µl Nuclease-Free Water was added to the membrane of the spin basket and centrifuged at 12,000-14,000 x *g* for 1 min. The filtrate was retained as purified RNA and stored at - 80 °C.

2.2.4.2 Determination of RNA Yield and Quality for PCR

A Nanodrop 2000 UV-Vis Spectrophotometer (Thermo scientific (Leicestershire, UK)) was used for the determination of RNA yield at 260 nm. Each 1 absorbance unit (A_{260}) equals 40 ug of single - stranded RNA/ml. RNA purity was also estimated spectrophotometerically from the relative absorbance at 230, 260 and 280 nm (i.e., A_{260}/A_{280} and A_{260}/A_{230}). A_{280} is the absorbance due to protein contamination and A_{230} is the absorbance due to DNA contamination. Pure RNA has an A_{260}/A_{280} ratio between 1.7- 2.1 and an A_{260}/A_{230} ratio of 1.8- 2.2.

Isolated RNA was separated using TAE agarose gel electrophoresis to determine the integrity of the purified RNA (section 2.2.4.6).

2.2.4.3 First strand cDNA synthesis (Reverse transcriptase reaction)

The following PCR mixture was used:

RNA (isolated previously)……10 ul

Random Primers (500 μg/ml)……..1 ul

M-MLV Reverse Transcriptase Buffer (5x)……...5 ul

PCR Nucleotide Mix (dNTP mix) (10 mM)…….....1.25 ul

Neuclease free water……..….6.75 ul

M-MLV Reverse Transcriptase (200 u/μl)…….1 ul

Procedure

The PCR Sprint Thermal Cycler was obtained from Thermo Life sciences. 10 μl RNA (about 0.5 μg) and 1μl Random primer (46 ng final concentrations) was added together in a sterile eppendorf tube and incubated for 5 min at 70 °C. The mixture was quickly cooled on ice for 5 min after the incubation and 5 μl M-MLV reverse transcriptase buffer (5x); 1.25 μl PCR nucleotide mix (0.5 mM final concentration); 6.75 μl Nuclease free water and 1 μl M-MLV reverse transcriptase (at last) were added. The mixture was incubated for 10 min at 25 °C and the PCR reaction was performed in two steps: incubation at 42 °C for 50 min (step 1) and inactivation at 70 °C for 15 min (step 2). The cDNA produced was stored at -20 °C.

2.2.4.4 Polymerase Chain Reaction Amplification

The following PCR mixture was used:

cDNA from first strand reaction…… (1-10 ul)

Flexi buffer (10x)……10 ul

$MgCl_2$ (stock solution 25 mM)…….....3 ul

PCR Nucleotide Mix (dNTP mix) (10 mM)…….…1 ul

Forward primer (50 pM/ul)……....1 ul

Reverse primer (50 pM/ul)…….1 ul

Taq. DNA polymerase (5 units/ul)…….…0.5 ul

Neuclease free water to final volume of ……...……50 µl

Procedure

Amplification of cDNA was performed with the PCR Sprint Thermal Cycler from Thermo Life sciences. The above PCR components are mixed together (Taq. DNA polymerase added last) and the cycle conditions are the following:

Stage 1 step 1 Temp: 95 °C Time: 2.00 mins Initial denaturation

Stage 2 step 1 Temp: 95 °C Time: 0.30 seconds Denaturation

Step 2 Annealing temperature Time: 0.30 seconds Annealing

Step 3 Temp: 72 °C Time: 1.30 mins Extension

Stage 3 step 1 Temp: 72 °C Time: 10.00 mins Final Extension

The amplification was performed with 30 cycles.

2.2.4.5 Agarose Gel electrophoresis

Agarose gel electrophoresis tanks were supplied by Bethesda Research Laboratories (BRL). The regulated DC Power Source (Model PAB) was supplied by Telonic Instrument Limited (UK). The UV radiation source machine was supplied by Vilber Lourmat (France). The microwave oven was obtained from Electrolux (UK).

Preparation of 50x and 1x TAE buffer

0.5 M EDTA (pH 8.0) was prepared by dissolving 93.05 g EDTA disodium salt in 400ml deionised water and the pH was adjusted to 8.0 with NaOH. The solution was made up to 500 ml with water. 242 g Tris base (MW=121.14) was dissolved in 750 ml deionised water, 57.1 ml glacial acetic acid and 100 ml of the 0.5 M EDTA (pH 8.0). Then the solution was made up to 1L with deionised water to give 50 x TAE buffer. I ml of 50x TAE was made up to 50 ml with deionised water to prepare 1x TAE buffer.

Agarose (0.8 %) in 1x TAE buffer heated in a microwave oven until the agarose dissolved and then left to cool to about 50 °C and GelRed was added to make a final concentration of 0.5 μg /ml. The warm agarose was poured into the gel caster and a comb was inserted, after that the gel was left to set for about 30 min. The tank was filled with 1x TAE buffer and the comb was removed, then the RNA Samples were mixed with loading buffer (10x stock solution: 25% Ficoll, 0.25% Bromophenol blue) and the samples were loaded into the wells. The gel was run at 100 V and visualized under ultraviolet light.

2.2.4.6 DNA fragments purification from agarose gel

PCR products were run on agarose gel and the DNA bands were excised from the agarose gels with a clean, sharp scalpel. DNA was extracted using QIAEX II Gel Extraction Kit (cat. no. 20021). The gel slices were weighed in a colourless tube and 3 volumes of Buffer QX1 were added to 1 volume of gel for DNA fragments 100 bp - 4 kb. QIAEX II tube was resuspended by vortexing for 30 sec and 10 µl from it was added to each band and mixed. The mixtures were incubated at 50°C for 10 min (to solubilize the agarose and bind the DNA) with mixing by vortexing every 2 min (to keep QIAEX II in suspension). The colour of the mixture should be yellow; if the colour of the mixture was orange or purple, 10 µl of 3M sodium acetate, pH 5.0, was added, mixed and incubated for 5 min longer. Then the samples were centrifuged for 30 sec and the supernatants were removed carefully with a pipette. Then the pellets were washed once with 500 µl of Buffer QX1, twice with 500 µl of Buffer PE and air – dried for 10–15 min or until the pellet becomes white. The DNA was eluted by 20 µl of 10 mM Tris·Cl, pH 8.5 and incubated at room temp for 5 min, centrifuged for 30 sec and the supernatants were removed carefully with a pipette into a clean tube to be kept in -20°C freeze.

2.2.5 Preparation of competent cells

For the growth of the bacterium *Escherichia coli* strain NM522, the following media were prepared:

Luria-Bertani (LB) broth:

1.0% Tryptone, 0.5% Yeast extract, 0.5% NaCl made up in H2O (sterilised by autoclaving before use).

LB Agar: 1.0% Tryptone, 0.5% Yeast extract, 0.5% NaCl, and 2.0% Bacteriological Agar made up in H_2O (sterilised by autoclaving before use).

Cells were cultured on LB agar overnight. After that, a single colony was cultured in LB broth overnight and 1ml of this overnight culture was added into 100 ml of pre warmed LB broth and incubated at 37 ^oC on an orbital shaker (180 rpm) for approximately 1.5 - 2 hours (the OD_{600} of the culture should be between 0.2 and 0.4). This culture was then placed in cold room or on ice with shaking for 15 minutes.

After that, the culture was placed in sterile centrifuge tubes and centrifuged in a refrigerated centrifuge (4 ^oC) for 10 minutes at 3,500 rpm. Then the cells were re suspended in 10 ml of 75 mM calcium chloride and incubated on ice for 20 minutes. After that, the cells were centrifuged at 4^oC for 10 minutes at 3,500 rpm and the pellets were resuspended in 4 ml of TFB2 (10 mM MOPS pH = 7.0, 75 mM calcium chloride, 10 mM rubidium chloride and 15 % glycerol. All components of TFB2 were autoclaved except MOPS which is sterilised by filtration, then the resuspended cells kept on ice for about 20 minutes prior to use. The cells were stored in small aliquots (200 µl each) at – 80 ^oC.

2.2.6 Transformation of *Escherichia coli* cells

For the transformation of *E. coli* cells, the following solutions were prepared:

TMC: 10 mM TrisCl at a pH of 7.5, 10 mM MgCl, 10 mM CaCl (sterilised by filtration).

IPTG solution: 100 mM IPTG in water.

X-gal solution: 40 mg of x-gal were dissolved in 1 ml of N, N dimethyl formamide (DMF).

A master mix was prepared by taking 4 µl of PCR product, 1 µl of TOPO vector and 1 µl of salt solution. This mixture was incubated for 10 minutes at 22°C. An LB agar plate containing 50 ug/ml of ampicillin was spread with 40 µl IPTG and 40 µl x-gal solutions and set aside. Transformation of *E. coli* cells was carried out in sterile 1.5 ml micro centrifuge tubes. 6 µl of the above cloning mixture were mixed with 100 µl of TMC and 200 µl aliquots of *E. coli* cells, and the mixture was incubated on ice for 30 minutes. It was subsequently heated at 42 °C for 2 minutes and 1 ml of LB was added and incubated at 37 °C for 90 minutes in orbital shaker. Then the cells were centrifuged for 3 minutes, resuspended in the residual LB, plated out on an LB agar plate (with appropriate antibiotics) and the plate then incubated for 14 hours at 37°C. A single white colony was carefully picked in order to grow on LB broth for 14 hours at 37°C on an orbital shaker.

2.2.7 Plasmid Purification

Plasmid isolation was performed using a phenol chloroform extraction method based on Birnboim and Doly (1979). 1.5 ml of inoculant *E. coli* cells were transferred to a microcentrifuge tube and were centrifuged at 14.000 rpm for 3 minutes. The pellet was resuspended in 100 µl of TEG (Tris base pH 8, 10 mM EDTA and 50 mM glucose) by vortexing. The cells were subsequently lysed with the addition of 200 µl of lysis buffer (0.2 M NaOH with 1% SDS) mixed by inversion and incubated at 22°C for 5 minutes. 150 µl of sodium acetate (pH 5.2) was added, mixed by inversion and incubated on ice for 5 minutes and centrifuged for 10 minutes at 14.000 rpm. The supernatant was transferred to a clean tube and 500 µl of phenol, chloroform and isoamyl alcohol (a ratio of 50:50:1) was added. Following centrifugation for 3 minutes at 14.000 rpm, the aqueous layer was removed, transferred to a

clean tube and 500 µl of chloroform mixture with isoamyl alcohol (a ratio of 50:1) was added. Following another centrifugation for 3 minutes at 14,000 rpm, the aqueous layer was extracted and transferred to a clean tube, with the addition of 900 µl of 99% ethanol. The mixture was incubated for 5 minutes on ice and centrifuged for 10 minutes at 14,000 rpm. Any remaining ethanol was removed and after 10 minutes of drying, the pellets were resuspended in 50 µl sterile H2O and the plasmid stored at -20^{o}C.

The plasmid was sent for sequencing to ensure that the plasmid carries the gene of interest.

For high purity preparations, the plasmid was isolated using the Qiagen plasmid purification midi kit (Cat. No.12143).

2.2.8 Quantitative Real-Time PCR

2.2.8.1 Construction of standard curve

The Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Scientific (Leicestershire, UK)) was used for the determination of the concentration and purity of the plasmid and the corresponding copy number was calculated using the following equation (Whelan *et al.*, 2003)

$$\text{DNA (copy)} = \frac{6.02 \times 1023 \text{ (copy/mol)} \times \text{DNA (g)}}{\text{DNA length (dp)} \times 660 \text{ (g/mol/dp)}} = \text{DNA molecules in 1 ul}$$

DNA length (dp) = Amplicon size (bp) + TOPO vector size (3957bp)

A 10- fold serial dilution series of the plasmids or a DNA extracted from agarose gel ranging from 1×10^{5} to 1×10^{9} Copies/µl were used to construct the standard curves.

2.2.8.2 Quantitative Real- time PCR Amplification

Amplification of cDNA was performed with the PerfeCTa® SYBR Green FastMix (Quanta BioSciences, UK) using the LightCycler®2.0 System (Roche Diagnostics). PerfeCTa SYBR green FastMix (2X) contains 2X reaction buffer containing optimized concentrations of MgCl2, dNTPs (dATP, dCTP, dGTP, dTTP), AccuFast Taq DNA polymerase, SYBR Green I dye and stabilizers. The PCR mix contains: 10.0 µl PerfeCTa® SYBR Green FastMix (2 x) in a total volume of 20 ul, 2 µl primer mix (1 µl forward primer and 1 µl reverse primer, final concentration 0.5 µM) and 3 µl of nuclease free water. The components were mixed by gentle pipetting up and down and then transferred into LightCycler ®Capillary. 5 µl of cDNA or plasmid DNA was then added and the capillary sealed with a stopper (table 2.4). The capillary was spun with LC Carousel Centrifuge and then transferred into the LightCycler® Sample Carousel for PCR run.

Table 2.4. Quantitative PCR réaction mixture.

Component	Volume for 20µlmix	Final concentration
PerfeCTa® SYBR Green FastMix (2x)	10.0 ul	(1x)
Forward primer	1 ul	0.5 µM
Reverse primer	1 ul	0.5 µM
Nuclease free water	3 ul	
cDNA or plasmid DNA template	5 ul	variable
Final volume (ul)	20 ul	

LightCyler®2.0 System Protocol

The PCR parameters for a LightCycler®2.0 System PCR run with the PerfeCTa® SYBR Green FastMix (2x) are shown below in table 2.5.

Table 2.5. PCR programme for LightCycler®2.0 System

Analysis Mode	Cycles	Segment	Target Temperature	Hold Time	Acquisition mode
Denaturation					
None	1	1	95°C	30s	None
Amplification					
Quantification	45	Denaturation	95°C	0s	None
		Annealing	Primer dependent	5s	None
		Extension	72°C	Amplicon [bp]/25s	Single
Melting Curve					
Melting Curves	1	Denaturation	95°C	0s	None
		Annealing	65°C	15s	None
		Extension	95°C	0s	continuous
Cooling					
None	1		40°C	30s	None

The levels of the targeted mRNA were quantified using the standard curve method as described by Applied Biosystems (UK) and the amplification specificity was checked by melting curve.

2.2.9 Microarray Methods

2.2.9.1 RNA isolation

RNA was isolated from control untreated and treated (exposed to GHB for 24 hours) THP-1 cells. RNA isolation was performed using the SV Total RNA Isolation System (Cat. No Z3100) from Promega (USA), the details of method in section 2.2.4.1.

2.2.9.2 RNA quantity and quality

The quantity of RNA was measured using Nanodrop 2000 UV-Vis Spectrophotometer from Thermo scientific (Leicestershire, UK) and the quality of RNA for microarray analysis was measured by Experion Automated electrophoresis station (Bio-Rad) using Experion analysis kits containing the chips and reagents required for RNA separation and analysis. The chips consist of a plastic caddy with a small glass plate affixed to it. The glass plate is etched with numerous microchannels in an optimized network pattern. The electrophoresis station directs the samples through microchannels by controlling the applied voltage and current.

2.2.9.3 Preparation of One-Color Spike-Mix

The One-Color Spike-Mix stock solution was mixed vigorously on a vortex mixer and then heated at 37°C for 5 minutes, mixed on a vortex mixer once more and briefly centrifuged to drive contents to the bottom of the tube prior to opening and 2 μL of this stock were added to 38 μL of dilution buffer provided in the kit (1:20 dilution), then 2 μL of this diluted solution were added to 48 μL of dilution buffer to get (1:25 dilution) and 4 μL of the second dilution to 36 μL of dilution buffer to get (1:10 dilution). The last dilution is a 5000-fold final dilution, 2 μL of this last dilution mixture were added to 1.5

μL (200 ng) of total RNA of each sample, each tube now contains a total volume of 3.5 μL.

Briefly, 200 ng of total RNA and 2 μl (1:5000 dilution) of Agilent One-Colour RNA Spike-In RNA were labelled with the Agilent Low RNA Input Linear Amplification Kit PLUS, One-Colour (Agilent Technologies UK Ltd, Wokingham, UK) according to the manufacturer's instructions.

2.2.9.4 Preparation of labelling reaction

T7 Promoter Primer was mixed with nuclease- free water and 1.8 μL of this mixture was added to the 3.5 μL RNA mixture, each tube now contains a total of 5.3 μL. These primer and template mixtures were denatured by incubating them in 65°C for 10 min and then placed on ice for 5 min. The fluorescent Cy3-labeled cDNA was synthesised for 2 h at 40°C and denatured for 15 min at 70°C. The labelled cRNA product was purified with an RNeasy Mini Kit (Qiagen).

2.2.9.5 Hybridisation and scanning

Agilent Hybridisation Kit (catalogue number 5188–5242) was used in conjunction with Agilent SurePrint G3 Human gene expression 8x60K arrays (catalogue number G4851A, Agilent Technologies, Wokingham, UK), 600 ng of labelled cRNA was used in the hybridisation according to the Agilent One-Color Microarray-Based Gene Expression Analysis Protocol. The hybridization was performed for 17 h at 65°C and 10 rpm. After washing, microarrays were scanned using an Agilent DNA microarray scanner (Agilent Technologies). All procedures from labelling to scanning were performed according to the manufacturer's instructions. Intensity values of each scanned feature were quantified using Agilent Feature Extraction

Software, which performs background subtractions. Normalization was performed using Agilent GeneSpring GX software.

2.2.9.6 Bioinformatic analysis

Microarray data were analyzed by GeneSpring GX software and differentially expressed genes were identified. Agilent standard normalisations for FE1-colour arrays were applied to all data sets. A subset of genes for data interrogation was generated of probes that were present in at least 50% of the samples in each group, but which excluded spots of poor quality.

The relative expression of each probe in control and treated cells (exposed to 10 μM or 900 μM GHB drug for 24 h) was determined and the probes that were differentially expressed by greater than 2.0 fold were selected. T-Test (unpaired) was performed with a false discovery rate of 0.05. Microsoft Excel templates were prepared with the list of genes that were over - and under-expressed following treatment with 10 μM and 900 μM GHB drug.

2.2.9.7 Gene Ontology analysis

Gene Ontology analysis was performed using GOEAST -- Gene Ontology Enrichment Analysis Software Toolkit.

2.2.9.8 Bradford method for Determination of Protein contents

Principle

Protein determination was performed according to the Bradford method (1976). The method was based on the formation of dye- protein complex solution between Bradford reagent and protein with a characteristic absorbance at 595nm.

Bovine Serum Albumin (BSA) Solutions

One mg of BSA was dissolved in 1ml distilled water to get 1 mg/ml BSA stock solution and stored at - 20 °C. This stock solution was diluted 10-fold to 0.1 mg/ml. Serial dilution of the stock solution was prepared in distilled water as shown in table 2.6.below.

Table 2.6. BSA standard dilution for protein determination.

Volume (µl) of BSA 0.1 mg/ml solution	Amount (µg)	Distilled Water (µl)	Bradford Reagent (µl)
0	0	800	200
2	0.2	798	200
5	0.5	795	200
10	1.0	790	200
20	2.0	780	200
50	5.0	750	200
100	10.0	700	200
200	20.0	600	200
Sample (10ul)		790	200

Procedure

Samples of cell protein extracts or standards were diluted appropriately to allow the absorbance to fall within the standard range and 10µl were made up to 800µl with distilled water in an Eppendorf tube. 200µl of Bradford reagent (Bio - Rad, UK) was added to each tube and the mixtures were vortexed or inversed several times to allow for uniform solution. The mixtures were allowed to standard for at least 5 min and absorbance was taken at 595nm in a spectrophotometer. The amount of protein in the sample was extrapolated from the standard curve of absorbance versus microgram protein.

2.2.10 Protein gels and Western Blots

2.2.10.1 SDS - Polyacrylamide Gel Electrophoresis

Principle

In SDS-PAGE, proteins are separated based on their molecular weight in an applied electric field (Laemmli, 1970). SDS is an anionic detergent that denatures protein by disrupting their secondary, tertiary and quaternary structures thereby producing a linear polypeptide chain surrounded by negatively charged SDS molecules. Mercaptoethanol further helps in the denaturation process by reducing the disulfide bonds. The denatured proteins travel along the stationary phase (polyacrylamide gel) according to their molecular weight. The pore sizes retard the movement of the large protein while the smaller proteins travel further along the gel.

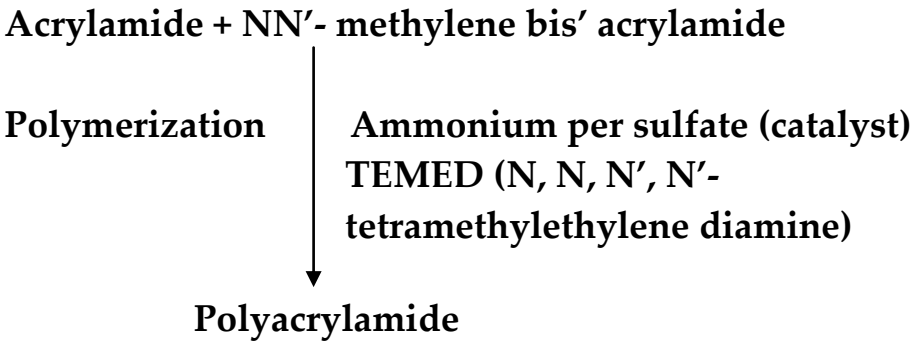


Figure 2.3: Biochemistry of acrylamide polymerisation. Acrylamide in the presence of ammonium persulfate catalyst and TEMED polymerised with bis - acrylamide to form polyacrylamide.

Protein Sample preparation

Astrocytoma 1321N1 and neuroblastoma SH-SY5Y cells were cultured in 6 well plates as described in section 2.2.1., at a concentration of 10^6 cells in each well and allowed to attach then treated with 1μM and 100μM GHB drug for 24 hr. Then the cells were washed with ice - cold, sterile 1x PBS and scraped using a rubber policeman into a microcentrifuge tube. Monocytic leukaemia THP-1 cells were cultured and treated with 10μM and 900μM GHB drug for 24 hr and then centrifuged, washed with ice - cold, sterile 1xPBS and placed in sterile micro centrifuge tube. The resultant pellets were then stored at -80°C before use. The pellets were resuspended in 100μl of 2x lysis buffer and pipette up and down by a syringe until foam formation, then boiled in a boiling water bath for 5min and centrifuged briefly for 1min at 1,000xg. Protein levels in the cell lysate were determined according to the protocol of Bradford (1976) using the bovine serum albumin (BSA) as standard (section 2.2.10).

For running SDS-PAGE, the following solutions were prepared:

Resolving buffer (4 x)

90.8g of tris base; 2g of SDS; made up to 450ml with distilled water and the pH was adjusted to 8.8 with concentrated HCl. The solution was then made up to 500ml with distilled water and filtered through the Whatman # 1 filter paper.

Stacking buffer (4 x)

15.14g Tris base; 1g SDS; made up to 200ml with distilled water and the pH was adjusted to 6.8 with concentrated HCl. The solution was made up to 250ml with distilled water and filtered through the Whatman # 1 filter paper.

Running buffer (10x) and (1x)

To get 10x buffer: 30.28 g Tris base; 144 g Glycine; 10 g SDS; made up to 1litre withdistilled water. 1x buffer was prepared by 1:10 dilution of 10x running buffer.

Lysis buffer (2x)

3.13ml Tris - Cl (pH 6.8); 2g SDS; 9ml Glycerol (heated to make it less viscous); 5ml Mercaptoethanol (added freshly on the day of use); 1ml Bromophenol (0.1%,v/v) (added freshly on the day of use); made up to 50ml with distilled water.

Procedures

Resolving Gel in the bottom (10% Gel)

30% acrylamide/bisacrylamide solution……..6.6ml
4 x resolving buffer…….5ml
Distilled water……..8.2ml
Ammonium persulfate (100mg/ml)……..100µl
TEMED……10µl

Stacking Gel in the top (5% Gel)

30% acrylamide/bisacrylamide solution……1.64ml
4 x stacking buffer ……. 2.5ml
Distilled water ……..5.86ml
Ammonium persulfate (100mg/ml)……...60µl
TEMED……...10µl

Gel plates were washed and cleaned with ethanol. The plates were then assembled using the rubber spacers in between plates. 10% resolving Gel was then poured into the assembled plates leaving about 1cm space at the top. The gel was then overlaid with water saturated isobutanol and left to polymerise. After polymerization, the isobutanol was poured out and the

space between the plates was dried up with paper towel. 5% Stacking Gel was then poured on top of the resolving gel and combs were inserted. The gel was then left to polymerise. After polymerization, the combs were removed and the plates were placed in ATTO gel apparatus (model - AE6450) from ATTO Corporation, Japan and the tank was filled with 1x running buffer. Each gel well was then loaded with approximately 30µg sample and the gel was run at 125V and 200mA.

2.2.10.2 Transfer and detection of proteins

The following solutions were prepared:

Transfer Buffer (1x)

14.4g glycine; 3.0g Tris base; 200ml Methanol; made up to 1litre with distilled water.

TBS (10x)

100ml of 1M Tris.Cl (pH 7.5); 375ml of 4M NaCl; made up to 1litre with distilled water.

TBStween (1x)

100ml of 10x TBS; 2ml of Tween 20 (0.2% v/v); made up to 1litre with distilled water.

Blocking Solution

5g dried skimmed milk powder was dissolved in 100ml 1x TBSTween.

Procedure

The gel containing proteins was transferred to a nitrocellulose membrane. For each gel to be transferred, 4pieces of What man 3MM paper and 1 piece of nitrocellulose membrane were cut to the size of the gel and together with 2

sponges were all soaked in 1x transfer buffer and then assembled in form of a sandwich (Fig. 2.4.). The sandwich was set up in such a way that the sponge was on the black (-ve) side of the plastic holder followed by 2 layers of Whatman paper, the gel containing the proteins, nitrocellulose membrane, 2 layers of Whatman paper and sponge in that order. The assembled sandwich was then placed in transfer tank filled with 1x Transfer Buffer and transfer was carried out for 2hr at 300mA. After the transfer, the nitrocellulose membrane was removed into a clean plastic container containing the blocking solution (5% dried schemed milk) and left for 1 hour or overnight in order to block the protein - binding sites present on the membranes. Then nitrocellulose membranes were incubated with primary antibodies (at dilutions as in table 2.2. in blocking solution) overnight at 4^{o}C. After that, the nitrocellulose membrane was washed with 1 x TBS tween for 4times (10min each). The secondary antibody diluted in blocking solution was added to the washed membrane and then incubated for at least 2 hours with gentle shaking. After the incubation, the membrane was washed 6 times (10 min each) in 1xTBSTween and once in TBS. The blot was then developed using ECL.

¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬ Plastic holder red top +ve
>>>>>>>>>>>>>>>>>>>>>>>> Sponge
= = = = = = = = = = = = = = = = 2layers of what man paper
---------- Nitrocellulose
~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ gel
= = = = = = = = = = = = = = = = 2layers of what man paper
>>>>>>>>>>>>>>>>>>>>>>>> Sponge
¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬ Plastic holder black bottom -ve

Figure 2.4: Western Blot transfer sandwich

2.2.10.3 Antibody Detection using Enhanced Chemiluminescence (ECL)

250mM Luminol

0.22g Luminol (Fluka, cat no 09253) was dissolved in 5ml DMSO. The solution was kept in the dark at -20 °C in 500µl aliquots.

90mM Coumaric acid

0.07g Coumaric acid (Sigma, cat no C- 9008) was dissolved in 5ml DMSO and the solution was kept in the dark at -20 °C in 500µl aliquots.

ECL 1 Solution

400ml of 250mM Luminol solution; 0.110ml of 90mM Coumaric acid solution; 4ml of 1M Tris - Cl (pH 8.5); was made up to 40ml with distilled water.

ECL 2 Solution

25.6µl of H2O2 (30%); 4ml of 1M Tris - Cl (pH 8.5); was made up to 40ml with distilled water.

Both solutions were kept in the dark at 4°C. Equal volumes of both solutions were mixed together just before use and poured over the membrane. Bands were detected using the computer assisted intelligent dark box image reader (LAS3000) from Fujifilm Company and images were stored as 8bits image format. The intensities of the bands were quantified with Image J analysis software (www.imagejdev.org) and compared relative to internal control.

2.2.11 Two- Dimentional gel electrophoresis (2-D gel)

2.2.11.1 Sample Preparation for 2- D Gel

Lysis Solution (L)

Lysis Solution contained 13.5g Urea; 0.5 ml Triton X-100; 0.5ml 2-Mercaptoethanol; 0.5ml Pharmalyte 3-10; 75mg Dithiothreitol (DTT) (added just before use); 35mg Phenylmethylsulfonyl fluoride (PMSF) (added freshly on the day of the experiment) and made up to 25 ml with ultra high quality water. The solution was stored as 1 ml aliquots in micro tubes at -20 °C.

Sample Solution (S)

Sample Solution contained 13.5g Urea; 0.13ml Triton X-00; 0.5ml 2-Mercaptoethanol; 0.5ml Pharmalyte 3-10; 75mg Dithiothreitol (DTT) (added freshly just before use); 35mg Phenylmethylsulfonyl fluoride (PMSF) (added freshly before use) and made up to 25ml with ultra high quality water. A few grains of bromophenol blue were added to the solution and the solution was stored as 1 ml aliquots in micro tubes at -20 °C.

Sample Preparation Procedure

Astrocytoma 1321N1 and neuroblastoma SH-SY5Y cells were cultured in 6 well plates as described in section 2.2.1., at a concentration of 10^6 cells in each well and allowed to attach then treated with 1μM and 100μM GHB drug for 24 hr. Then the cells were washed with ice - cold, sterile 1x PBS and scraped by a rubber policeman into a micro centrifuge tube. Monocytic leukaemia THP-1 cells were cultured and treated with 10μM and 900μM GHB drug for 24 hr and then centrifuged, washed with ice - cold, sterile 1xPBS and placed in sterile micro centrifuge tube. The resultant pellets were then stored at -

80°C before use. The pellets were resuspended in 200µl Lysis Solution (L) by vortexing and then centrifuged at 10,000 x g for 5 min.

The clear supernatant containing the cell extract was then transferred into a fresh micro centrrifuge tube and stored at - 80°C. Protein contents of the extracts were determined by Bradford method (as described in 2.2.10) and the cell extract was diluted in Sample Solution (S) to give a loading concentration of about 240 µg in a loading volume of 100 µl.

2.2.11.2 Rehydration of Immobiline DryStrips (pH 3- 10)

Rehydrating Solution

This solution contains 12.0 g Urea; 0.13 ml Triton X-100; 0.13 ml Pharmalyte 3-10; 50 mg DTT and made up to 25 ml with distilled water.

Procedure

The plastic cover of the immobiline ™ IPG DryStrips was peeled off from the acidic (+) end and then placed in such a way that the surface of the gel is downward into the channel of the reswelling tray which already contains 340µl rehydrating solution. The strip was placed in such way that the acidic end was placed against the slope end of the channel. Care was taken to avoid bubble formation under the strip. The channel was then overlaid with 2 ml of Dry Strip cover fluid to prevent evaporation of the rehydrating solution and urea crystallization and the lid of the reswelling tray was replaced. The Strip was allowed to rehydrate overnight or at least 10hours at room temperature.

2.2.11.3 Running First Dimension: Isoelectric Focussing (IEF)

Ettan IPGphor IEF system from Amersham Biosciences, UK was used in the first dimension isoelectric focusing for the separation of protein according to

their isoelectric point (IEP) on the rehydrated IPG strips. The IEF electrophoresis unit was connected to the Multi Temp TM III Thermostatic circulator set at 20 °C and 3 ml of DryStrip Cover Fluid was pipetted into the strip channels of the electrode plate. The rehydrated ImmobilineTM IPG DryStrips were removed from the reswelling tray and placed gel side up into the strip channel of the electrode plate with the acidic (+) ends toward the anode avoiding bubble formation. Care was taken to ensure that the strips were aligned in the electrode plate. IEF Electrode wicks were soaked in distilled water, blotted dry briefly and placed over both ends of the IPG strips. The electrodes were carefully lowered on top of the moist paper wicks at both ends of the strip to provide electrical contact. The sample cups were then placed on top of the IPG strips near the anodic end of the electrode with the cup touching the gel surface ensuring good contact to prevent leakage from the cups. 110 ml of DryStrip Cover Fluid was poured into the electrode plate to completely cover the cups. 100µl (240µg protein) of sample mixture was loaded into the sample cup and the electrophoresis unit was covered with the lid. The electrodes were then connected to the source of power and the first dimension separation was carried out according to the programme in table 2.7. below.

Table 2.7. Programming Ettan IPGphor IEF system for the first dimension separation.

Phase	Voltage mode	Voltage (V)	Time (hr:min)	Volthours(KVh)
1	Step and Hold	300	3:00	0.9
2	Gradient	1000	6:00	3.9
3	Gradient	8000	3:00	13.5
4	Step and Hold	8000	9:30	9.7
	Total		21.30	28.0

2.2.11.4 IPG Strip Equilibration

SDS Equilibration Buffer

This buffer can be prepared by mixing 6.7ml Tris (1.5M, pH8.8), 72.07g Urea, 69ml Glycerol (87%v/v), 4.0g SDS, a trace amount of Bromophenol blue and made up to 200ml with distilled water. This solution was stored at -20°C.

Equilibration solution I

This solution contains 1 %(w/v) DTT prepared freshly in SDS equilibration buffer.

Equilibration solution II

Contains 2.5% (w/v) iodoacetamide prepared freshly in SDS equilibration buffer.

Procedures

Each of the IPG strips from the first dimension process were soaked in approximately 10 ml equilibration solution I for about 15min and after that soaked in 10 ml of equilibration solution II.

2.2.11.5 Running Second Dimension: SDS- PAGE

Gel caster assembly

Gel plates were cleaned thoroughly with ethanol and then inserted into the gel caster in such a way that a thin space was left between the gel plate sets. The gel caster was laid lying flat to ease assembly. After the assembly of the plates, the other side of the gel caster was placed on top and the sides of the caster clamped together and the screw at the bottom was tightened to prevent leakage.

Electrophoresis buffer for second dimension

10 x SDS electrophoresis buffer (30.28 g Tris base; 144 g Glycine; 10 g SDS; made up to 1 litre with distilled water).

10% Acrylamide Gel Solution (450 ml)

The 10% Acrylamide Gel Solution were prepared using the following mixture:

30% Acrylamide/bisacrylamide solution 150ml

Tris (1.5M; pH 8.8)……. 113ml

Distilled water …….. 178ml

10% (w/v) SDS…… 4.5ml

10% (w/v) Ammonium persulfate……... 4.5ml

TEMED…… 77ul

450 ml of the 10% acrylamide solution were poured into the gel caster almost to the top leaving a 5mm gap at the top 1 ml of saturated isobutanol was poured on top of each gel and then left to set. After setting, the gel caster assembly was dismantled, and the top of each gel (25.5 x 20.5 cm, 1mm thick) was rinsed with distilled water. This was then allowed to drain briefly and the gel plates were laid flat with the shorter glass plate uppermost. The equilibrated IPG Strip was then laid on top of each gel in such a way that it just touched the gel with the IPG Strip gel side up and avoiding air bubble formation between the gel and the IPG Strip. The gel plates were then stood in an upright position and the IPG Strips was sealed with about 500 μl low melting agarose solution (0.5%).

Procedure

The electrophoresis system (EttanDaltSix; Amersham Biosciences, UK) was used for the second dimension separation of the proteins on the equilibrated

IPG strip from the first dimension separation. The gel plate containing the IPG strip was inserted into the EttanDaltSix electrophoresis unit and the unit was filled with SDS electrophoresis buffer (as shown below) and connected to the Multitemp cooler system at 20 °C. Separation of the protein was then carried out in two steps of 5W per gel for 30 min followed by 17W per gel for 4h.

Anode (lower chamber)	**Volume (L)**
1X SDS electrophoresis buffer	4.3

Cathode (upper chamber)	**Volume (L)**
2X SDS electrophoresis buffer	0.8

2.2.11.6 Staining the gel

The gel was removed from the plate after electrophoresis and placed gently into a plastic tray. Then fixing solution were added to the gel and kept for 60 min. The gel was then stained overnight with the staining solution and then transferred into the neutralisation solution for 1-3 min and washed with 25% (v/v) methanol for 2 h. After that the gels were soaked in stabilising solution for at least one day and then the staining steps can be repeated 3-5 times to get more clarity and sensitivity of the spot. The gel can remain in the staining or stabilising solution over the weekend without affecting the results.

Solutions

Fixing solution

The fixing solution contains 5 ml of o-Phosphoric acid (85%, v/v), 100 ml of methanol, made up to 500 ml with distilled water (DW).

Stock staining solution A

The solution contains 9.5 ml of o-Phosphoric acid (85%, v/v), 40g ammonium sulphates (10%, w/v), made up to 400 ml with DW.

Stock staining solution B

2.5g of coomassie brilliant blue G250 (5% w/v), made up to 50 ml with DW.

Staining solution

10 ml of stock staining solution B was mixed with 400 ml of stock staining solution A and 100 ml of methanol added. This solution was prepared freshly each time.

Neutralisation solution

This solution contains 6 g of Tris- base (0.1M) dissolved in 500 ml DW and the pH was adjusted to 6.5 with o- Phosphoric acid.

Washing solution

This solution contains 125 ml methanol made up to 500 ml with DW.

Stabilising solution

This solution contains 100 g of ammonium sulphate made up to 500 ml by DW.

2.2.11.7 Imaging the gel

After repeated staining, the gel was photographed using the Fujifilm LAS-3000 Imaging system and the image was stored as 16-bit greyscale TIFF images.

2.2.11.8 Image analysis and spot-picking

For analysing the protein spots on the 2D-gel, the software called Progenesis SameSpots software (Nonlinear dynamics ltd, UK) was used. Then data from the Progenesis SameSpots software used as guide for picking spots of interest using a manual one touch spot picker (Web Scientific, UK). The picked spots kept in Eppendorf tubes at - 80°C for further analysis for protein identification.

2.2.11.9 Trypsin digestion of the picked spots

Gel slices were destained twice with 0.2 ml of 100 mM NH_4HCO_3/ 50% ACN for 45 min each treatment at 37 °C to remove the stain and then dehydrated for 5 minutes at room temperature in 100 µl 100% ACN. At this point, the gel slices are much smaller than their original size and are whitish or opaque in appearance. After that the gel slices were dried in a speed vacuum drier about 10–15 min at room temperature to remove the ACN.

Trypsin Gold vial was resuspended at 1µg/µl in 50 mM acetic acid and divided into aliquots of 10 ug each (to avoid freeze/ thaw times) and kept in -80 °C. Each aliquot of trypsin was diluted in 40 mM NH4HCO3 / 10% ACN to 20 µg / ml or 10 ug/0.5 ml and the gel slices were preincubated in about 10-20 µl of the trypsin solution at room temperature and should not exceed 30 °C for 1h, the slices will rehydrate during this time, if the gel slices appear white or opaque after 1h, an additional 10-20 µl of trypsin were added and incubated for another 1 h at room temperature. After that enough digestion buffer (40 mM NH_4HCO_3/10% ACN) about 50 µl were added to completely cover the gel slices. The tubes were capped tightly to avoid evaporation and incubated overnight at 37 °C.

At the next day, the gel slices were incubated in 150 µl of high purity grade water for 10 min with frequent vortex mixing and the liquid were removed in a new microcentrifuge tube and the gel slices were extracted twice, with 50 µl of 50% ACN/ 5% TFA with vortex mixing for 1h each time at room temperature and the extracts were pooled together with previous water extract and dried by speed vacuum drier at room temperature for 2– 4 h (do not exceed 30 °C). The dried extracted protein was kept in -80 °C to be analysed by electro spray ionization (ESI-TRAP) after purification by zip tips.

2.2.11.10 Protein identification by MASCOT

The results from electro spray ionization (ESI-TRAP) were analysed by MASCOT which is a powerful search engine uses mass spectrometry data to identify proteins from primary sequence databases.

2.2.12 Aldolase Enzyme assay

Principle

Aldolase activity was measured using a colorimetric assay according to Boyer's modification of the hydrazine assay (Jagannathan *et al*. 1956); 3-phosphoglyceraldehyde reacts with hydrazine to form a hydrazone which absorbs at 240 nm. Fructose-1, 6-bisphosphate was used as a substrate and hydrazine sulphate used as detection reagent for the formed 3-phosphoglyceraldehyde. One unit is described as a change in absorbance of 1.00 per minute at 25 °C, pH 7.5, and 240 nm wavelengths.

Procedures

Sample preparation

Monocytic leukaemia THP-1 cells were cultured and treated with 10 µM and 900 µM GHB drug for 24 hr and then centrifuged, washed with ice - cold,

sterile 1xPBS and placed in sterile micro centrifuge tube. The cell pellet was resuspended in 150 µl of 250 mM Tris buffer, pH 7.5. The resuspended cells were frozen at - 80 °C for 5 minutes and then directly transferred to 37 °C for 5 minutes. The process was repeated 3 times. After that, the cells were centrifuged at 10000 rpm for 5 minutes and the supernatant retained and store at – 80 °C until use. Protein levels in the supernatant were determined according to the protocol of Bradford (1976) using the bovine serum albumin (BSA) as standard (section 2.2.10).

Enzyme assay

For running of enzyme assay, the following solutions were prepared:

Substrate: 0.012 M Fructose-1, 6-bisphosphate pH 7.5

Detection reagent: 0.0001 M EDTA (pH 7.5) plus 0.0035 M hydrazine sulphate.

The assay was carried out in a reaction volume of 1 ml at 25 ◦C in quartz glass cuvettes obtained from Hellma. The reaction mixtures are shown in this table:

Table 2.8. The reaction mixtures for aldolase enzyme assay.

	Blank	Test
D-Fructose-1,6-bisphosphate	313 µl	313 µl
Hydrazine sulfate	625 µl	625 µl
Distilled water	31 µl	----
Record ΔA240 for 10 minutes		
Add enzyme	----	31 µl
Record ΔA240 for 10 minutes		

Then the ΔA240/min of the blank was subtracted from ΔA240/min of the test.

2.2.13 Statistical Analysis

Statistical analysis of results was performed with one-way analysis of variance (ANOVA) unless otherwise stated. Comparison between groups was performed with Dunnett's post-test. Statistical analysis was carried out using Excel and Prism software.

CHAPTER 3

GHB dependent changes in PEA-15 and Epiregulin Gene Expression In cell lines

3 GHB dependent changes in Pea-15 and Epiregulin Gene expression in cell lines

3.1 Introduction

The effect of GHB on the expression of a range of genes in various cell types has been investigated previously, and is described in the introduction. (Wisor *et al*., 2006; Larson *et al*., 2007; Schnakenberg *et al*., 2010; Kemmel *et al*., 2010).

The Larson study revealed that nine mRNA transcripts were up-regulated in mouse blood samples following 24 hours GHB exposure. Leading them to conclude that GHB induced changes in gene expression, and these changes depended on the time interval post exposure (Larson *et al*., 2007). Two of these upregulated mRNAs were epiregulin and PEA-15.

3.1.1 PEA-15

PEA-15 encodes a death effector domain (DED) -containing protein that is 130 amino acids in length. The N-terminal region contains the canonical death effecter domain that constitutes amino acids 1–80 and a nuclear export signal, and the C-terminal region contains two serine phosphorylation sites (Figure 3.1). PEA-15 is a major phosphoprotein in astrocytes, and is an endogenous substrate for protein kinase C. Unphosphorylated PEA-15 binds extracellular-signal-regulated kinase (ERK) and prevents accumulation of active ERK in the nucleus, thus blocking transcription and slowing proliferation. Upon phosphorylation at Ser-104, PEA-15 cannot bind ERK. Upon phosphorylation at Ser-116, PEA-15 binds Fas-associated death domain protein (FADD) and is thus recruited to the DISC (death-initiation signalling complex) in response to death receptor ligation. In this way, PEA-

15 can integrate ERK- and FADD-dependent signalling pathways (Renganathan *et al*., 2005) (Figure 3.2). Kubes *et al*., 1998 showed that PEA-15 is important in protection against cytokine-induced apoptosis.

3.1.2 Epiregulin

Epiregulin (EPR) is a member of the epidermal growth factor (EGF) family and is similar in structure to EGF. It is encoded by the *EREG* gene in humans. The EPR protein is 19 kDa, and can be phosphorylated. It can bind to the EGF receptor as well as other members of the ErbB receptor tyrosine kinase family but with less affinity that EGF. It induces tyrosine phosphorylation of the receptors to which it binds, thereby initiating intracellular signalling. Its function is not completely clear but it is known to be involved in mediating cell proliferation and angiogenesis in processes such as wound healing and oocyte maturation but also inhibits growth of several tumour derived epithelial cells (Toyoda *et al*., 1995).

The reasons why these two particular mRNAs are regulated by GHB are not clear, and require further investigation. In addition, a change in expression of these two genes has not been reported in human cells.

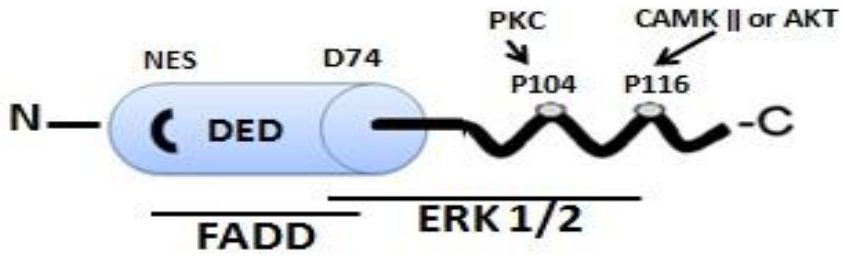


Figure 3.1: **A schematic illustration of PEA-15 protein**. It is 130 amino acids in length and comprised of an N terminal region containing a canonical death effecter domain (DED) that constitutes 1-80 amino acids and a C terminal region containing two serine phosphorylation sites, Ser-104 phosphorylated by protein kinase C (PKC) and Ser-116 phosphorylated by either CamKII or serine /threonine protein kinase (AKT). These phosphorylations are drawn by arrows. PEA-15 binding partners ERK andFADD are shown by lines, adapted from (Renganathan *et al*., 2005).

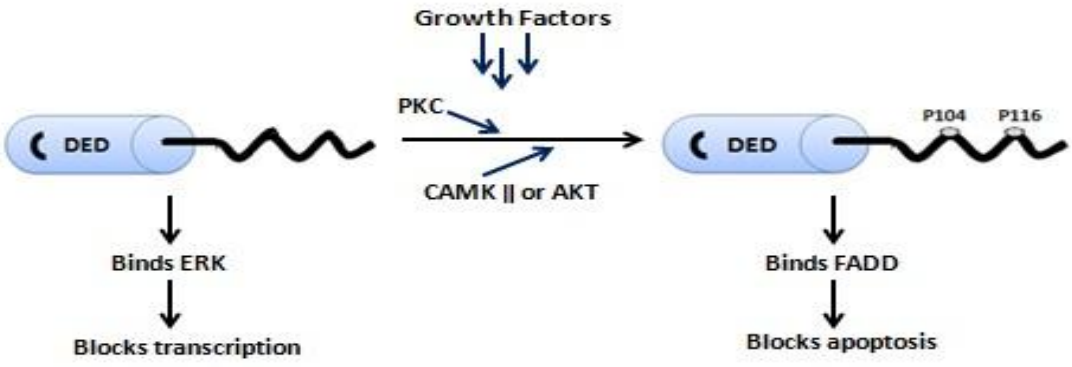


Figure 3.2: **A model for control of PEA-15 function by phosphorylation is depicted.** Phosphorylation status of PEA-15 alters its function in blocking transcription or blocking apoptosis. Unphosphorylated PEA-15 binds ERK and prevents accumulation of active ERK in the nucleus, blocking transcription and slowing proliferation. Upon phosphorylation at Ser-104, PEA-15 cannot bind ERK. Upon phosphorylation at Ser-116, PEA-15 binds FADD and is therefore recruited to the DISC in response to death receptor ligation. In this way, PEA-15 can integrate ERK- and FADD-dependent signalling pathways, adapted from (Renganathan *et al*., 2005).

3.2 Aim of this chapter

The overall aim of this chapter is to determine whether Epiregulin and PEA-15 expression can be used to extend the window of detection of GHB beyond 12 hours in human cells, and to understand the conditions under which these markers are expressed.

To achieve this aim, the effects of GHB on the levels of Epiregulin and PEA-15 mRNA and protein were studied using quantitative PCR and Western blots.

To investigate whether previous results from animal studies are true in human cells, three types of human cell lines were selected: SH-SY5Y cells and 1321N1 cells, which are neuronal and non neuronal cells respectively; and human monocytic leukaemia THP-1 cells.

3.3 Materials and Methods

Human astrocytoma 1321N1 cells (Clark *et al*., 1975), human neuroblastoma SH-SY5Y cells (Biedler *et al*., 1973; Biedler *et al*., 1978), and human monocytic leukemia THP-1 cell line (Tsuchiya *et al*., 1980) were used in this work.

Methods used in this chapter include MTT assay, RNA isolation, reverse transcriptase PCR, gene cloning, plasmid preparation, quantitative PCR and western blotting. Materials are methods are described in detail in chapter 2.

3.4 Results

Earlier studies had shown that two genes (PEA-15 and epiregulin) were upregulated in mouse blood cells after GHB administration (Larson *et al*.,

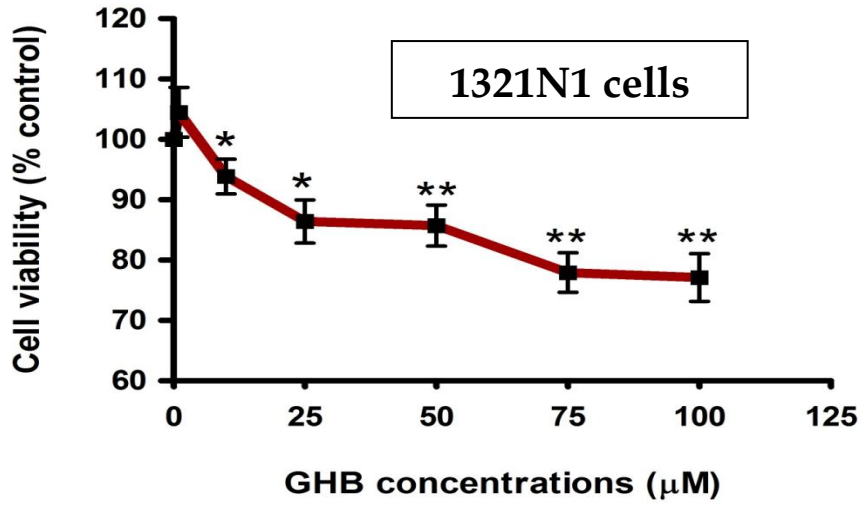
2007). In this Chapter, QPCR techniques were used to test whether GHB has an effect on PEA-15 and/or epiregulin gene expression in three human cell lines, human astrocytoma, human neuroblastoma and human monocytic leukaemia cell lines to see whether previous results are true in human cells.

Two types of human brain cells were selected: the non-neuronal astrocytoma 1321N1 cells and the neuronal-like neuroblastoma SH-SY5Y cells. In addition the human monocyte cell line THP-1 was also used as a model for blood cells (monocytes).

3.4.1 Determination of appropriate concentration of GHB

To determine an appropriate concentration of GHB to use, brain cells (1321N1 and SH-SY5Y cells) were treated with different concentrations of GHB for 24 hours and the effect on cell viability was measured with the MTT assay, which measures metabolic activity. The results show some decreased cell viability in the two cell lines at increasing concentrations of GHB (Fig 3.3). However the level of toxicity was not high and an IC_{50} could not be determined. Based on these results a safe non-toxic 1 μM dose and a slightly toxic dose 100 μM of GHB were selected for subsequent work. Only two doses were selected to find biomarkers for GHB exposure beyond 12 hours and then after confirmation of these markers further studies with many doses will be needed.

A)



B)

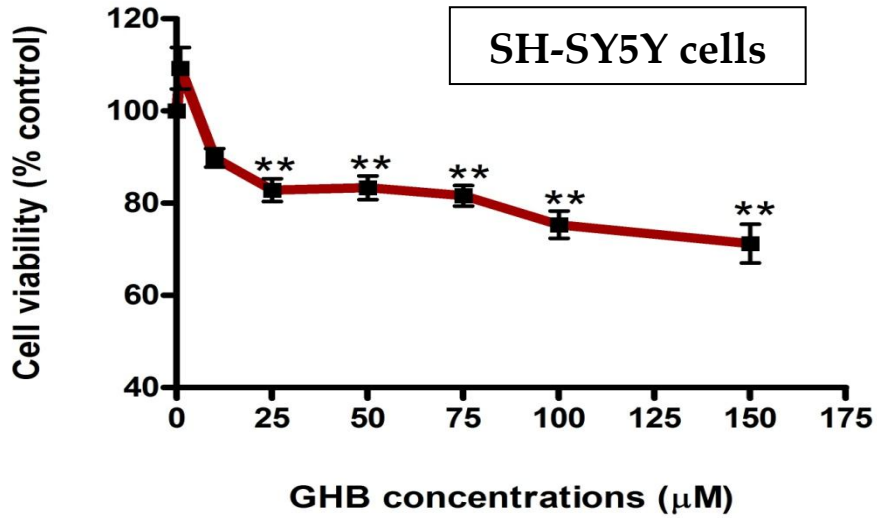


Figure 3.3: Effects of GHB on the viability of A) 1321N1 and B) SH-SY5Y cells. Cells at 60% confluence were exposed to 1, 10, 25, 50, 75, 100 and 150 μM GHB for 24hr and cell viability was assessed with the MTT assay as described in materials and methods. Values represent mean ± SEM (n=6) and are expressed as percentage of control (untreated) cells. Asterisks indicate significant compared with untreated control (**p<0.01 *p<0.05).

GHB concentrations for the blood cells (THP-1 cells) were selected based on a study carried out to compare the window of GHB detection in blood and saliva after a GHB dose of 60 mg/kg body weight (total of 4680 mg) administered orally on an empty stomach to a 54 years old male (78 kg). The results of that study indicated that the maximum GHB level in the blood was 991.3 μM reached after 20 min of GHB administration, and the level after about 5 hours was 79.3 μM (Kintz *et al*., 2001). Therefore in this study THP-1 cells were treated with a low concentration (10 μM) and high concentration (900 μM) of GHB to represent concentrations found following GHB exposure, to demonstrate the effect on PEA-15 mRNA and epiregulin mRNA levels.

3.4.2 Quality of RNA

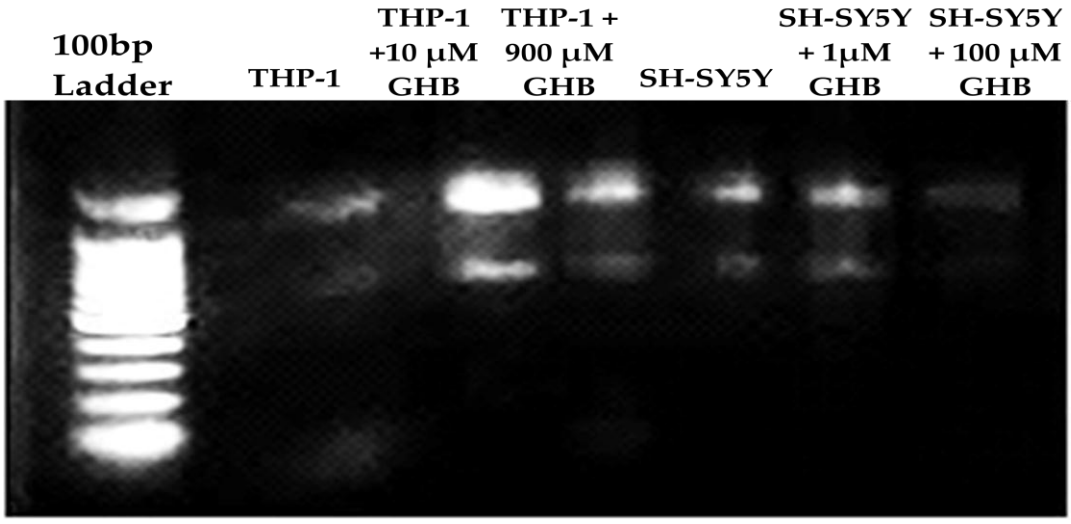
RNA was isolated for all of the three types of cells following treatment with specified doses of GHB using the SV Total RNA Isolation system. To determine the integrity of the purified RNA, the isolated RNA was run on 1% TAE agarose gel electrophoresis with DNA ladder (Figure 3.4). The results show that in all samples, two distinct RNA peaks are visible corresponding to 18S and 28S rRNA species found in eukaryotes.

3.4.3 Test amplification of PEA-15 and Epiregulin

To test whether the primers were able to amplify PEA-15 and epiregulin, cDNA was prepared from human astrocytoma 1321N1, human neuroblastoma SH-SY5Y and human monocytic leukaemia THP-1 cells as described in materials and methods, and a standard PCR reaction was carried out for control and treated cells. The PCR products were separated by electrophoresis along with 100kb DNA ladder for identifying the size of the

band (Fig 3.5). The results show that both cDNAs were present in the three cell types examined. The PEA-15 product size is about 243 bp and epiregulin product size about 122 bp.

A)



B)

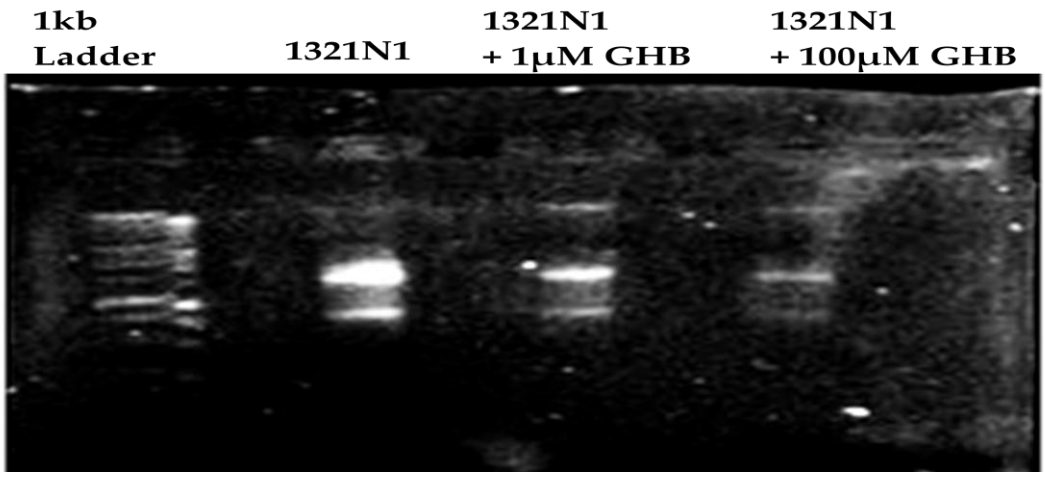
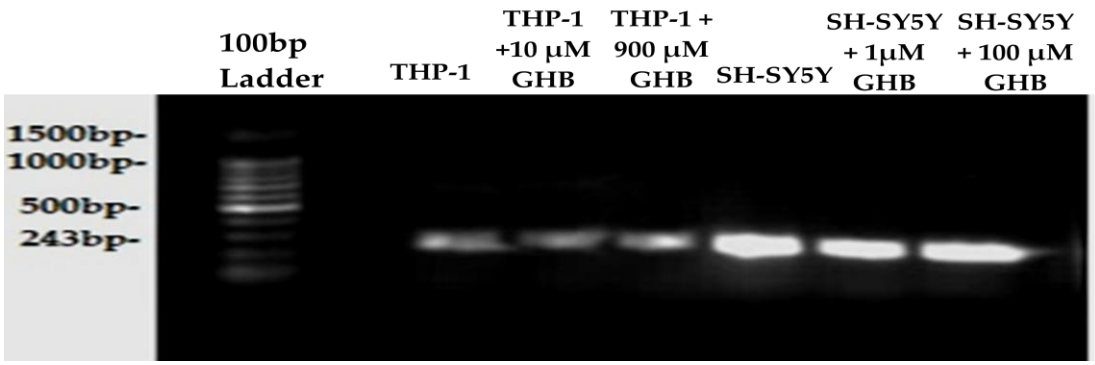
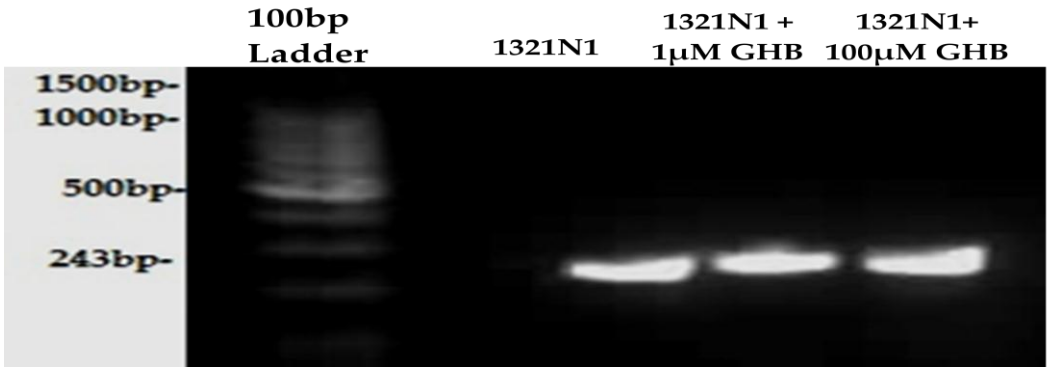


Figure 3.4: RNA (isolated by SV Total RNA Isolation system) was separated on 1% TAE agarose gel electrophoresis with A) gel red dye and B) Ethidium bromide dye using DNA ladder and the 18S and 28S rRNA bands are distinct.

A)



B)



C)

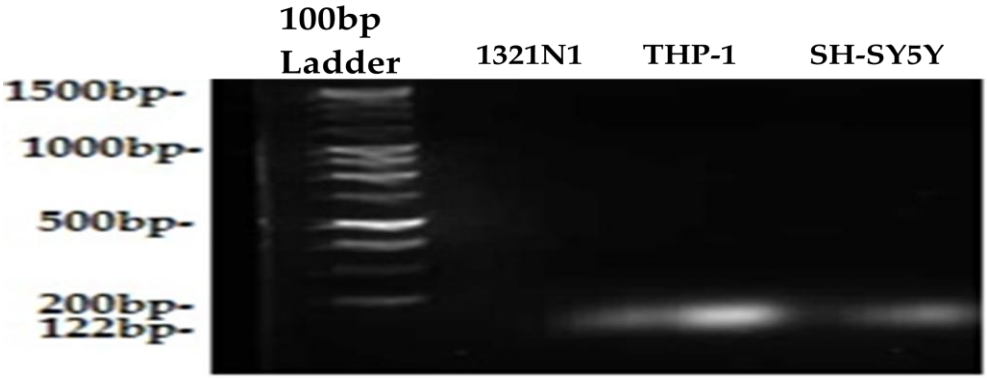


Figure 3.5: PCR amplification of PEA-15 gene (A and B), Epiregulin gene (C) from cDNA in 1321N1, SH-SY5Y and THP-1 cells. Total RNA was isolated from treated and untreated cells, and then PEA-15 and Epiregulin genes were amplified by RT-PCR using PEA-15 and Epiregulin specific primers. PCR products were run on 1% agarose gel electrophoresis along with DNA ladder. PEA-15 Product size is ~ 243 bp and Epiregulin Product size is ~ 122 bp.

3.4.4 Quantitation of PEA-15 and Epiregulin mRNA

To assess the effect of GHB exposure on epiregulin and PEA-15 mRNA levels in brain cells (1321N1 and SH-SY5Y) and blood cells (THP-1), the brain cells were exposed to 1 and 100 μM and the blood cells were exposed to 10 and 900 μM GHB for 24 hours and the levels of epiregulin and PEA-15 mRNA expression were measured by Quantitative RT-PCR using the specific oligonucleotide primers. The amplification specificity of each primer set was checked by melting and amplification curves analysis (Figures 3.6. and 3.7.).

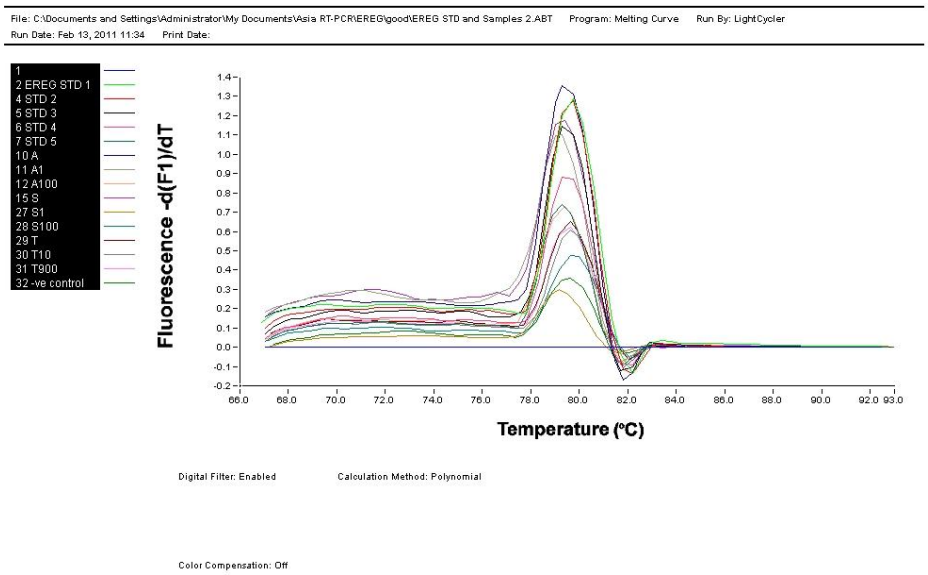
Standard curves were prepared using plasmid DNA containing either the cloned PEA-15 or epiregulin gene ranging from 10^5 to 10^9 copies/ μl and PCR reactions were performed simultaneously with the samples. The standard curves were linear in the range tested ($R^2 > 0.999$) by the triplicate reactions (Figure 3.8.).

Copy numbers were calculated by extrapolation with the standard samples from the standard curves and the copy numbers of the target genes (epiregulin and PEA-15 genes) were normalised with the reference gene (GAPDH gene) by using the formula.

$$\text{Normalised copy number} = \frac{\text{Copy number of target gene}}{\text{Copy number of reference gene}}$$

The normalised copy numbers of three independent readings were analysed using Excel and Prism software by the use of one-way analysis of variance (ANOVA) and the comparison between groups was performed with Dunnett's test.

A)



B)

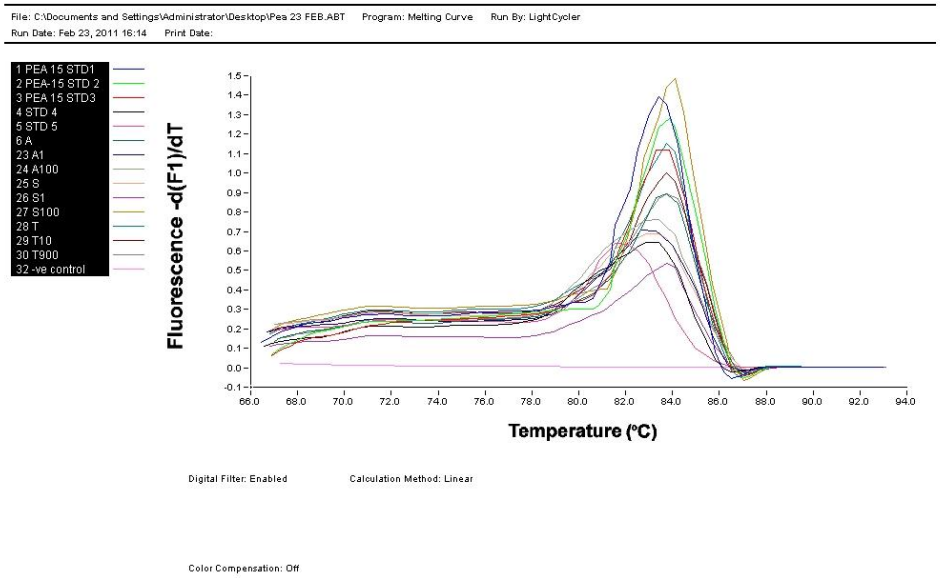
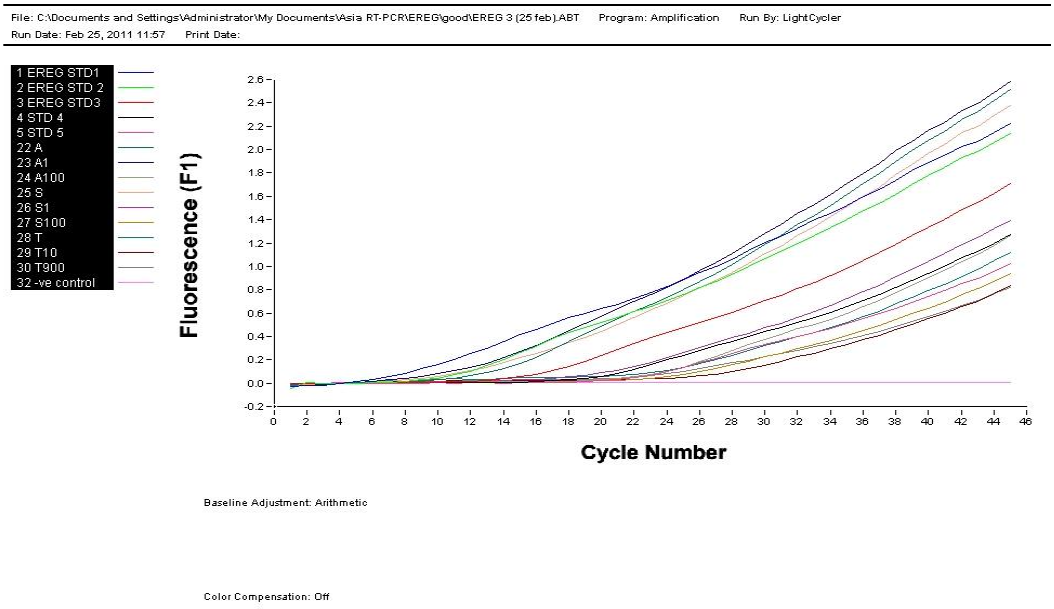


Figure 3.6: Melting curves of A) epiregulin and B) PEA-15. The melting temperature for both genes was 60°C and the amplicon sizes were 122bp and 243bp for epiregulin and PEA-15, respectively. 1321N1 cells (A), SH-SY5Y cells (S), THP-1 cells (T). (A1), (A100), (S1) and (S100) represents 1321N1 and SH-SY5Y cells treated with 1μM and 100 μM GHB respectively. (T10) and (T900) represent THP-1 cells treated with 10μM and 900 μM GHB respectively.

A)



B)

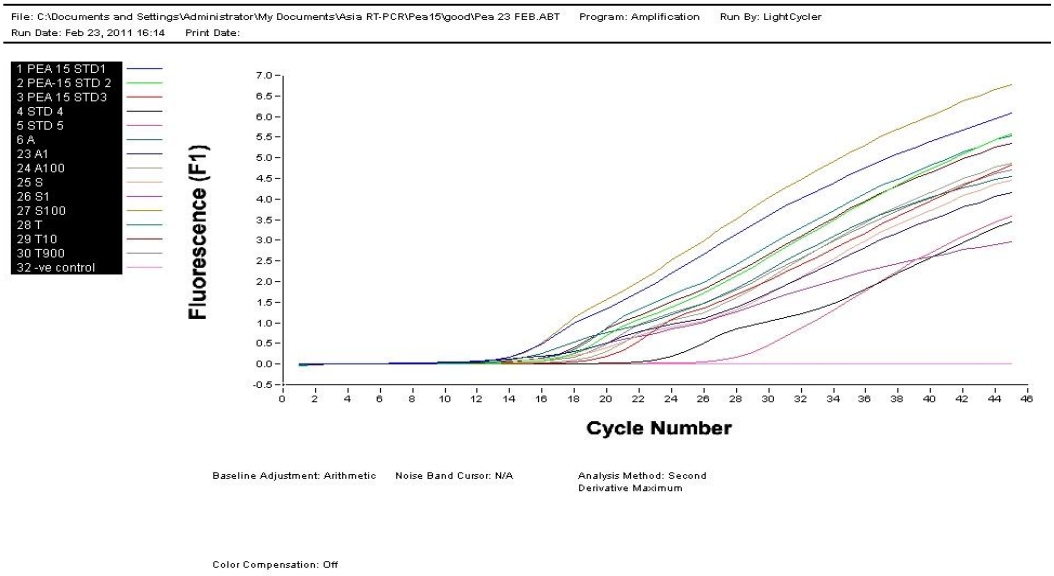
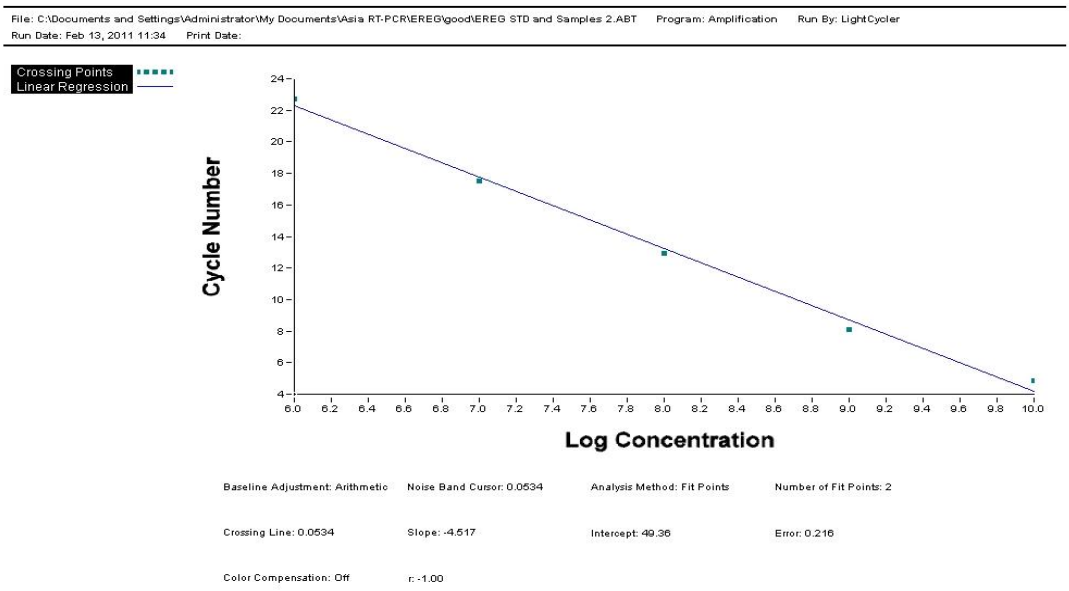


Figure 3.7: Amplification curves of A) epiregulin and B) PEA-15 genes. The melting temperature for both genes was 60°C and the amplicon sizes were 122bp and 243bp for epiregulin and PEA-15, respectively. 1321N1 cells (A), SH-SY5Y cells (S), THP-1 cells (T). (A1), (A100), (S1) and (S100) represents 1321N1 and SH-SY5Y cells treated with 1μM and 100 μM GHB respectively. (T10) and (T900) represent THP-1 cells treated with 10μM and 900 μM GHB respectively.

A)



B)

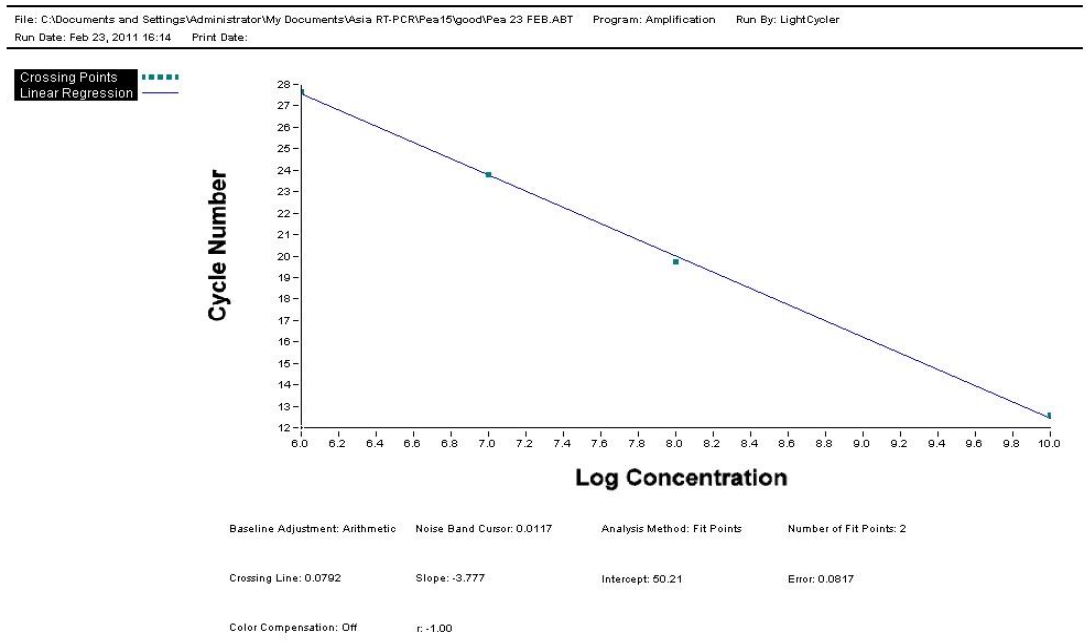
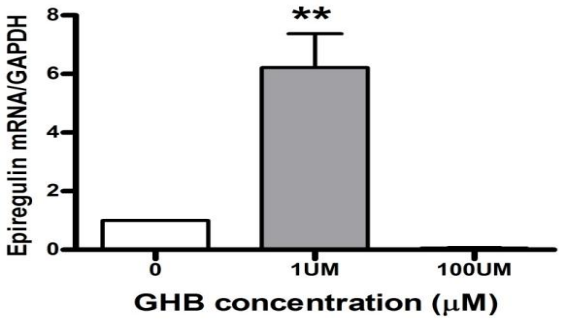


Figure 3.8: Standard curve for A) Epiregulin and B) PEA-15. The standard curves were constructed with serial 10-fold dilutions of the bacterial plasmid DNA ranging from 10^5 to 10^9copies/µl. Each standard dilution was amplified by real-time QPCR using specified primers.

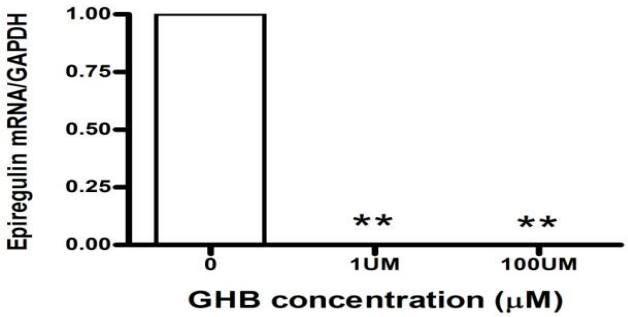
The results in Figure 3.9 showed that epiregulin mRNA levels were increased 6.22-fold in 1321N1 cells ($p<0.01$) and 5.18-fold in THP-1 cells ($p<0.005$) after treatment with low concentrations of GHB (1 μM for 1321N1 cells and 10 μM for THP-1 cells), with no significant changes following high concentration treatments with GHB. However epiregulin expression disappeared in SH-SY5Y after both treatments with GHB ($p<0.005$), as shown in Figure 3.9.

Regarding PEA-15 mRNA, the results showed that PEA-15 mRNA levels increased in 1321N1 and SH-SY5Y cells by 4.2 and 3.7-fold respectively after 100 μM GHB treatment ($p<0.01$ both), but with no significant change at low concentrations of GHB (1 μM). However, PEA-15 mRNA level was increased significantly in THP-1 cells by 40.8-fold after 10 μM GHB treatment ($p<0.01$) but with no significant change at high concentrations of GHB (900 μM), as shown in (Figure 3.10.).

A)



B)



C)

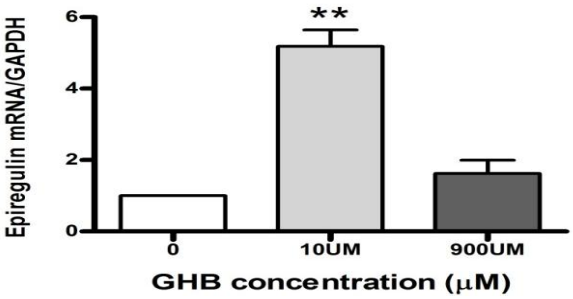
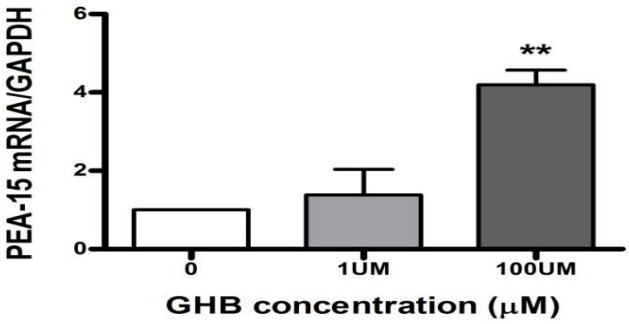


Figure 3.9: Epiregulin mRNA levels in control untreated and treated cells. Total RNA was isolated from A) 1321N1, B) SH-SY5Y and C) THP-1 cells (treated and untreated cells). Epiregulin mRNA level was quantified by QRT- PCR using the LightCyler SYBRgreen as described in Materials and Methods using and normalised to GAPDH mRNA, the fold change was calculated relative to the control. The levels of mRNA were calculated by the use of Standard curve method, standard curve were constructed with serial 10-fold dilutions of the plasmid DNA from E-coli N-M 522 after introduction of epiregulin gene. Data represent the mean value (n = 3 individual assays) relative to control ±SD. Asterisks indicate significant compared with untreated control (**$p<0.01$ and *$p<0.05$).

A)



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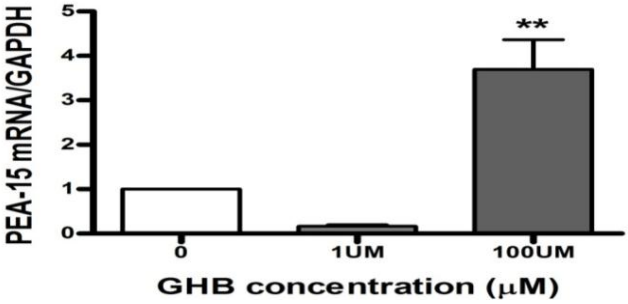


C)

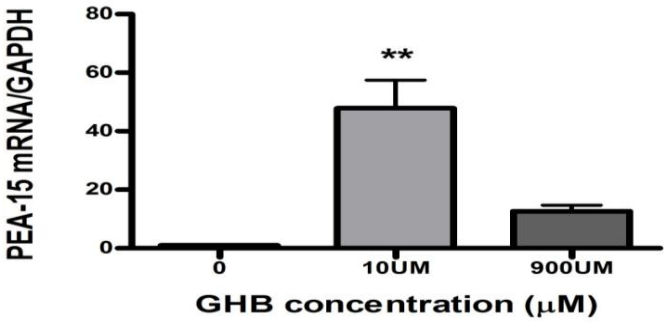


Figure 3.10: PEA-15 mRNA levels in control untreated and treated cells. Total RNA was isolated from A) 1321N1, B) SH-SY5Y and C) THP-1 cells (treated and untreated cells). PEA-15 mRNA level was quantified by QRT-PCR using the LightCyler SYBRgreen as described in Materials and Methods. Epiregulin mRNA was normalised to GAPDH mRNA and the fold change was calculated relative to the control. The levels of mRNA were calculated by the use of Standard curve method, standard curve were constructed with serial 10-fold dilutions of the plasmid DNA from E-coli N-M 522 after introduction of epiregulin gene. Data represent the mean value (n = 3 individual assays) relative to control ±SD. Asterisks indicate significant compared with untreated control (**$p<0.01$ and *$p<0.05$).

3.4.5 Secondary validation of PEA-15 protein expression after GHB exposure

Earlier reports had shown that the expression of two genes (PEA-15 and epiregulin) were changed in response to GHB and in this chapter QPCR technique has confirmed that the expression of these two genes at the mRNA level was changed after 24h exposure to GHB in human cells. In order to confirm whether GHB had an effect on PEA-15 protein expression in three cell lines, Western blotting analysis for protein was carried out on these cells.

Brain cells (1321N1 and SH-SY5Y cells) were treated with 1 μM and 100 μM concentrations of GHB and blood cells (THP-1 cells) were treated with 10 μM and 900 μM concentrations of GHB for 24 hours, extracts prepared and 40μg of protein were loaded onto SDS-PAGE. Western blots were carried out using PEA-15 (H-3) antibody (Santa Cruz Biotech.Inc sc-166678 catalogue number). GAPDH antibodies were used for normalisation. . Bands were quantified using ImageJ and expression levels were calculated relative to untreated control. The results showed that PEA-15 protein expression levels were increased significantly in blood cells (THP-1) by 1.9 and 1.6 fold ($p<0.001$) after 10μM and 900μM GHB treatment respectively, and were also increased significantly in neuronal cells (SH-SY5Y) by 1.6 fold ($p<0.05$) after 100 μM GHB treatment for 24h. However, there were no significant changes in PEA-15 protein expression level in 1321N1 cells after 24h of GHB exposure, as showed in (Figure 3.11.).

Regarding epiregulin expression, Western blots were carried out with two different epiregulin antibodies, but no bands were detectable.

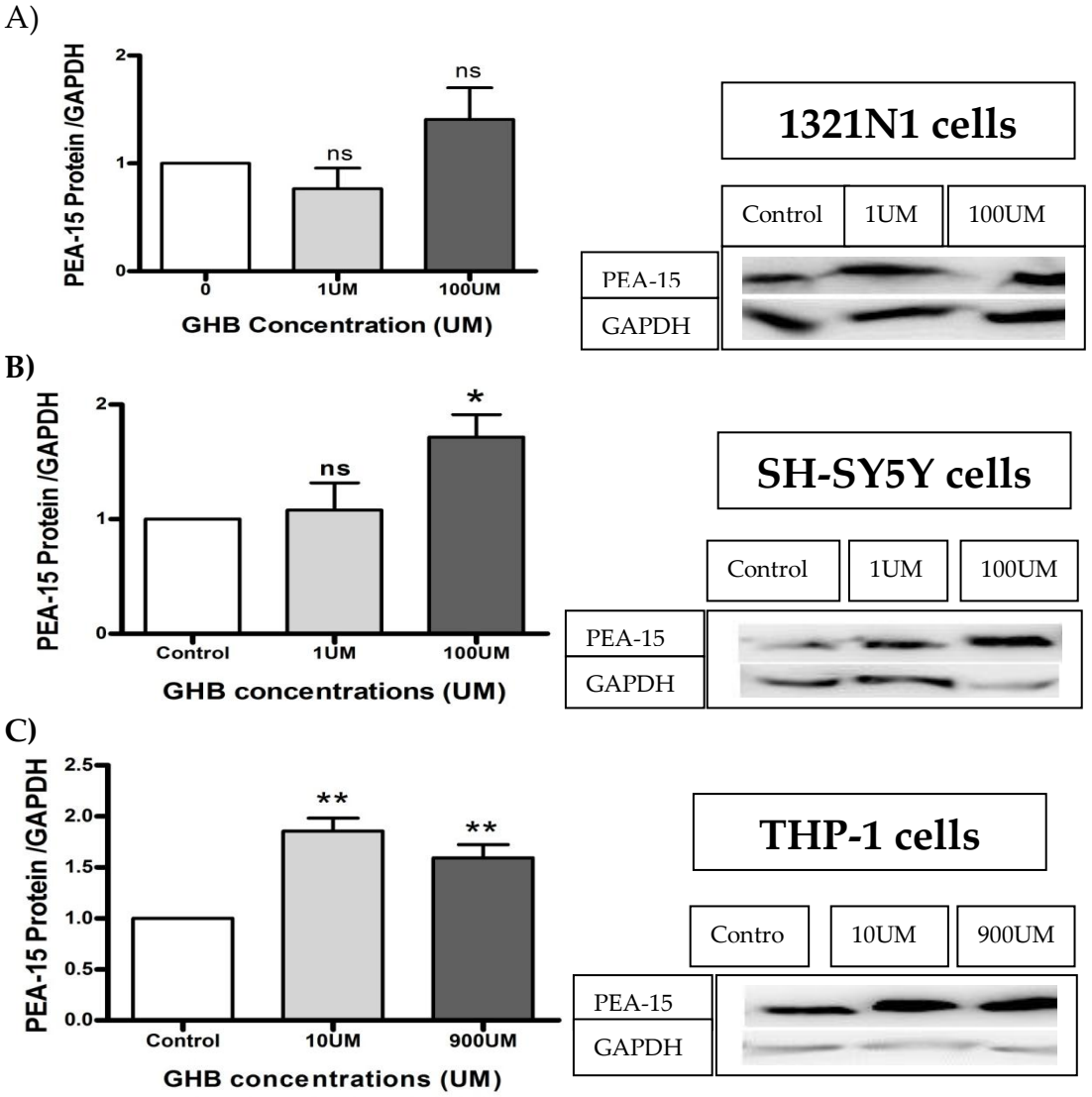


Figure 3.11: Change in PEA-15 protein expression in control untreated and treated cells after GHB exposure. A) 1321N and B) SH-SY5Y cells were treated with 1 or 100μM GHB and C) THP-1 cells were treated with 10 or 900μM GHB for 24 hours and western blots were carried out on whole cell extracts using PEA-15 antibody (Santa Cruz Biotech.Inc) with GAPDH used for the normalisation and loading on 10% SDS- PAGE gel. The protein concentrations of the loaded samples were determined by Bradford method and approximately 30μg of samples were loaded. Protein bands were quantified by Image J relative to untreated control (n = 3 individual assays). Relative PEA-15 expression was represented as histogram. Asterisks indicate significant compared with control (**$p<0.01$ and *$p<0.05$).

3.5 Discussion

Despite a great deal of discussion about GHB pharmacodynamics and the receptors involved in mediating GHB action, little is known about the changes in gene expression that may be involved.

3.5.1 GHB toxicity

In this chapter, the effect of different concentrations of GHB on the viability of two brain cell lines (SH-SY5Y and 1321N1 cells) was evaluated by the use of the MTT assay. The results presented revealed that toxicity of GHB was similar in both cell lines. GHB significantly decreases cell viability in a concentration dependent manner in both cell lines but is not significantly toxic at concentrations up to 150 μM. Our results are the first to show that GHB has the same effect on the viability of neuronal and non neuronal brain cells. This is interesting because in the brain astrocytes are known to provide metabolic and immune function support to neurones, and therefore might be considered to be more robust in terms of metabolic processes and ability to withstand toxic insult. However the results presented here indicate that both cell types are equally sensitive to GHB, which may indicate that metabolic processes are not involved in mediating any intrinsic protection.

The toxicity of GHB to THP-1 was not assessed, but previous results indicated that much higher concentrations of GHB could be tolerated by blood cells and the GHB concentrations for the blood cells (THP-1 cells) were selected based on a study carried out on 54 years old male (78 kg) administred 4.68 g GHB dose orally on an empty stomach and the results of that study indicated that the maximum GHB level in the blood was 991.3 μM

reached after 20 min of GHB administration, and the level after about 5 hours was 79.3 μM (Kintz *et al*., 2001).

3.5.2 GHB dependent changes in Epiregulin mRNA

The changes in epiregulin and PEA-15 mRNA levels after 24 hours exposure to GHB in two brain cell lines (neuronal and non-neuronal cells) and blood cell line were assessed using two different GHB concentrations. The results presented show that epiregulin mRNA levels do increase significantly in both 1321N1 cells and THP-1 cells but not in SH-SY5Y cells. The GHB dependent increase in epiregulin mRNA levels observed in monocytes supports the previous study which indicated an increase in epiregulin mRNA in mouse blood cells after GHB exposure (Larson *et al*., 2007).

Furthermore, epiregulin mRNA levels were increased significantly after exposure to 10μM GHB but not after 900 μM in THP-1 cells and also, after exposure to 1μM GHB but not after 100 μM in SH-SY5Y cells. The results of viability showed that GHB partially toxic at 100 μM to brain cells, this may explain the lower level of epiregulin mRNA. Furthermore, from the viability studies, 1μM GHB shows higher viability than the significantly lower viability value with 100μM GHB.

Epiregulin is a member of the epidermal growth factor family whose expression is known to be regulated by a variety of molecules, including hormones and peptide hormones. For example, previously epiregulin expression has been shown to be induced by follicle-stimulating hormone (FSH), which causes proliferation in ovarian cells (Sekiguchi *et al*., 2002). Luteinizing hormone (LH) also induces epiregulin expression in mouse ovaries (Sekiguchi *et al*., 2002), and epiregulin acts as a mediator of LH action

in ovarian follicular cells (Freimann *et al*., 2004). Human chorionic gonadotrophin (HCG) has also been shown to up-regulate epiregulin in ovrian follicles (Sayasith *et al*., 2013). This suggests a function for epiregulin in mediating hormonal changes. Epiregulin has also been shown to be induced by forskolin in granulosa cells, indicating the involvement of protein kinase A. In vascular smooth muscle cells, epiregulin mRNA was induced by angiotensin II, endothelin-1, and thrombin, molecules that bind the vasoactive GTP-binding protein-coupled receptor (Taylor *et al*., 1999; Deacon and Knox, 2010). Insulin has also been shown to upregulate epiregulin (Ornskov *et al*., 2006).

Regulation of epiregulin by molecules other than hormones has not been studied as extensively. Apart from the previous study that looked at regulation by GHB, epiregulin has been shown to be induced by 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) in mouse foetus (Choi *et al*., 2006), and this is thought to be mediated via the Aryl hydrocarbon receptor (AHR) and AHR nuclear translocator.

Epiregulin is known to be upregulated in physiologically-relevant conditions. For example, it was found to be upregulated in peripheral blood mononuclear cells in response to exercise (3.50-fold) along with other genes involved in stress, inflammation, and tissue repair (Connolly *et al*., 2004).

Epiregulin has also been found to be upregulated in a variety of inflammatory conditions, for example, in the epidermis in psoriasis (Shirakata *et al*., 2007). Also, epiregulin were found to be induced by Rhinovirus Infection (RV16) infection (Liu *et al*., 2008). However, the reasons for the observed changes in epiregulin expression in inflammatory conditions are not known.

The results showed a significant difference in the response between the astrocytoma 1321N1 cells and the neuroblastoma SH-SY5Y cells, with the neuronal-like cells being unresponsive. 1321N1 represent astroglial cells found in the central nervous system (CNS). Many functions including CNS immune system contribution are attributed to astroglial cells (Ridet *et al*., 1997). Hence, a difference in epiregulin expression between these two cell lines could represent the functional contribution of 1321N1 to immune responses.

Induction of expression of epiregulin in the human leukemic cell line (THP-1) was also noted. THP-1 cells have receptors for Fc portion (FcR) of immunoglobulin G (IgG) and for a C3b component of complement (C3bR) which have been known to play important roles in the immunologic mechanisms (Tsuchiya *et al*., 1982).

Overall, the results from QPCR suggested that the epiregulin gene was up-regulated by GHB in 1321N1 and THP-1 cells that both play a role in immune function. However, the large number of other factors that can also upregulate epiregulin may preclude its use as a useful blood-based biomarker for GHB exposure.

3.5.3 GHB dependent changes in PEA-15 mRNA

The results in this chapter also revealed that human PEA-15 mRNA levels were increased in 1321N1 and SH-SY5Y cells, and were also increased significantly in THP-1 cells after GHB treatment. Also, PEA-15 mRNA levels were increased significantly after exposure to 10μM GHB but not after 900μM in THP-1 cells. At the protein level, Western blotting showed that

PEA-15 protein expression was increased significantly in blood cells (THP-1) by GHB treatments and also increased significantly in neuronal cells (SH-SY5Y). However, there were no significant changes in PEA-15 protein expression level in 1321N1 cells.

PEA-15 is a major phosphoprotein in astrocytes that is known to block the inhibition of integrin activation mediated by Ras, and also modulates the ERK MAP kinase cascade. PEA-15 is known to be regulated post-translationally by phosphorylation in astrocytes by CaMKII (or a related kinase) and by protein kinase C in response to endothelin (Kubes *et al*., 1998).

Despite its name (phosphoprotein enriched in astrocytes) PEA-15 is ubiquitously expressed in tissues. In terms of function, studies in knockout mice indicate that PEA-15 protects astrocytes from TNF-induced apoptosis. It is known to be broadly antiapoptotic and controls caspase-3 function, thereby controlling cell survival. It is also overexpressed in type 2 diabetes mellitus, where it appears to influence glucose uptake and thereby contributes to insulin resistance. Another function appears to be in autophagy, as it can induce autophagy in myoblasts, and also differentiation (Iovino *et al*., 2012).

At the transcriptional and translational level, PEA-15 has been observed to be regulated by a range of conditions and factors. It is overexpressed in some cancer cell lines, and can be induced by exposure of cells to TGF-beta1 (Iovino *et al*., 2012). Morphine sensitization is known to lead to upregulation of PEA-15 (Ramos-Miguel *et al*., 2010). PEA-15 is also upregulated by 12-O-tetradecanoyl phorbol-13-acetate (TPA) in skin and keratinocytes, and this may explain its role in mediating TPA-induced effects on caspase 3 functions

and apoptosis sensitivity (Formisano *et al*., 2005). In contrast, leuprolide acetate, which is a gonadotropin-releasing hormone (GnRH) agonist, decreases Pea-15 expression level by 50% (Bifulco *et al*., 2004). Leuprolide acetate has an antiproliferative effects in uterine leiomyomas due to the suppression of the anti-apoptotic effects of Pea-15.

There is no apparent reason why PEA-15 would be regulated by GHB directly. However, it is known that GHB increases Growth Hormone secretion during sleep (Van Cauter *et al*., 1997), and it also known that Growth Hormone leads to a multitude of changes within the cell, so it is likely that the effect observed could be indirect.

3.5.4 Epiregulin and PEA-15: Evaluation as markers for GHB exposure

The results of present study show that GHB induces changes in PEA-15 and epiregulin mRNA levels and in PEA-15 protein expression levels in the human cell lines used and seems to support the previous study in mouse which suggests PEA-15 and epiregulin levels as possible surrogate markers for GHB administration beyond 12 hours (Larson *et al*., 2007). Our results are the first to show that GHB induces changes in PEA-15 and epiregulin gene levels in human cell lines.

Further experiments are needed to confirm that these results are a specific response to GHB exposure, by comparing epiregulin and Pea- 15 levels induced in cell lines against levels induced by combinations of GHB, GABA, and the specific antagonists NCS-382 (GHB receptor antagonist) and CGP-35348 ($GABA_B$ receptor antagonist).

CHAPTER 4

Transcriptome- based Gene Expression Signature of Gamma hydroxybutyric acid (GHB) exposure in Human monocytic leukaemia THP-1 cells

4 Transcriptome- based Gene Expression Signature of Gamma hydroxybutyric acid (GHB) exposure in Human monocytic leukaemia THP-1 cells

4.1 Introduction

In the previous chapter, the effect of GHB exposure on the expression of two specific genes, epiregulin and PEA-15, was investigated. There have been various studies on specific genes that are expressed in response to GHB in rat brain (Schmidt-Mutter *et al*., 1999; Wisor *et al*., 2006)., and in mouse brain (Schnackenberg *et al*., 2010).

Another approach has been to use global genome wide studies to investigate the effect of GHB exposure on gene expression. Microarray-based analysis has become a valuable tool because it produces reliable data on the expression of thousands of well-annotated genes within a few days. The use of microarray technology to find biomarkers is gaining acceptance in many different fields.

Two microarrays studies have been carried out previously to investigate the effect of GHB on gene expression in rat and mouse brain (Kemmel *et al*., 2010; Schnackenberg *et al*., 2010) as well as the original study on mouse blood (Larson *et al*., 2007). However there have been no previous microarray studies that have looked at global changes in human cells, and in particular there is no information on the global changes in human blood cells that can be readily monitored in forensic cases. A deeper knowledge of these changes will also help in the understanding of the mechanism by which GHB has its effects.

4.2 Aim of this chapter

The aim of this chapter is to use microarray technologies to identify novel and robust surrogate markers for GHB exposure beyond 12 hours. This is achieved through measuring GHB-dependent changes in gene expression in human monocytic leukaemia (THP-1) cell lines using THP-1 cells (Tsuchiya *et al.*, 1980). These were chosen as a model to study the effects of GHB on gene expression in blood cells.

4.3 Materials and methods

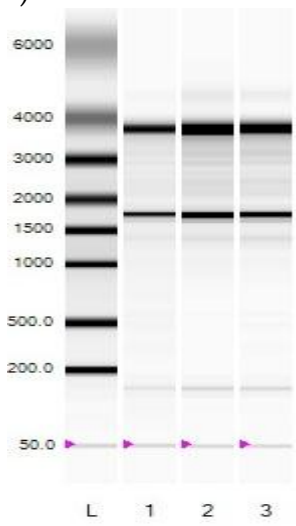
Human monocytic leukaemia (THP-1) cell line (Tsuchiya *et al.*, 1980) was used in this study. THP-1 cells were treated with two GHB concentrations 10μM and 900μM for 24 hours as described previously. Methods used in this chapter include Microarray methods (2.2.9.). Materials and methods are all described in chapter 2.

4.4 Results

4.4.1 RNA quality

RNA was isolated from THP-1 cells using SV Total RNA Isolation system following treatment with GHB. The RNA yield at 260 nm was determined by Nanodrop 2000 UV-Vis Spectrophotometer, and the quality of RNA was measured by Experion Automated electrophoresis station (Bio-Rad) using an Experion analysis kits containing the chips and reagents required to RNA separation and analysis. The results of RNA quality showed two distinct eukaryotic ribosomal RNA peaks corresponding to 18S and 28S rRNA species in all samples examined (Figure 4.1). This indicated good quality RNA had been isolated.

a)


6000
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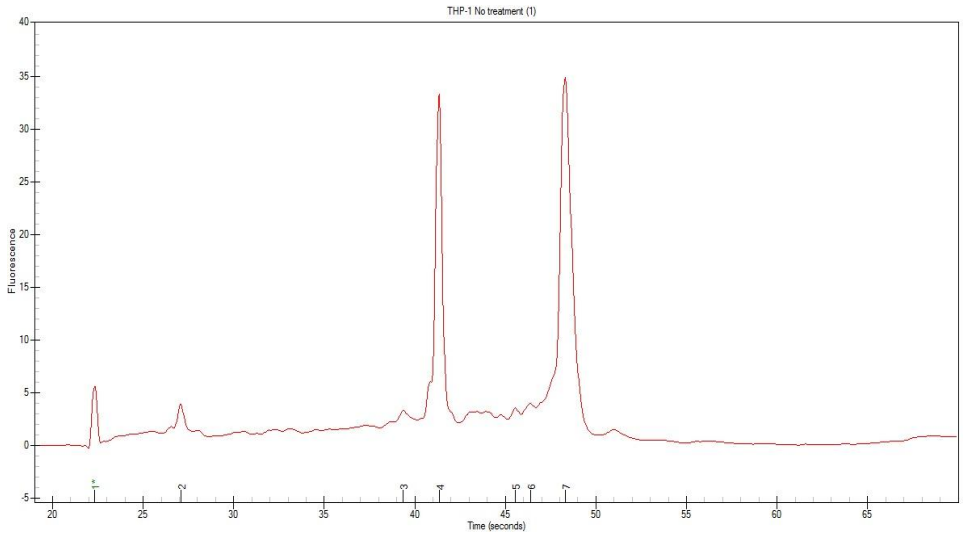
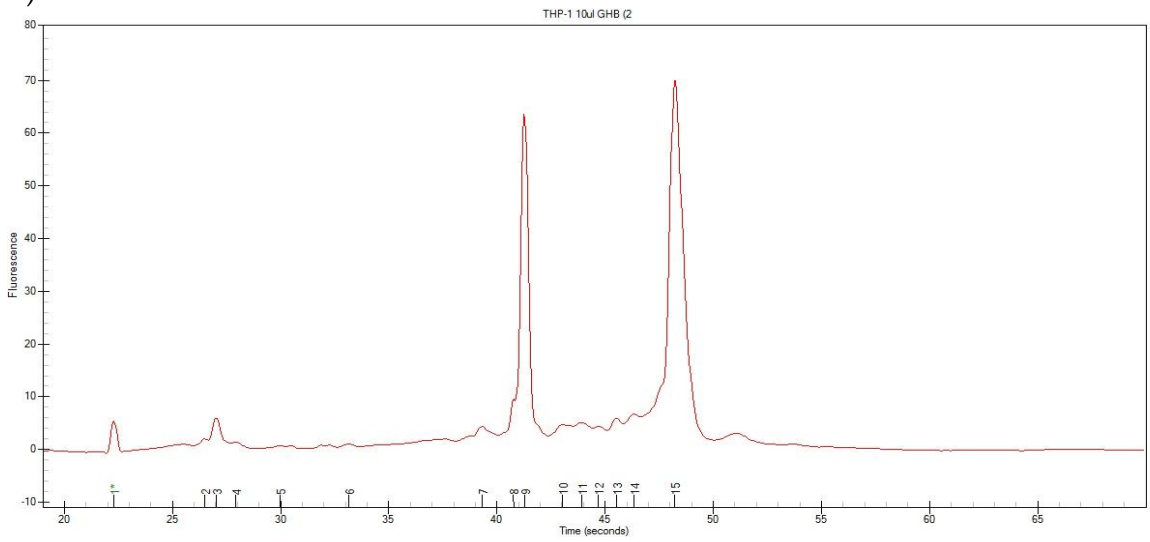
B)


THP-1 No treatment (1)
Fluorescence
Time (seconds)

C)



d)

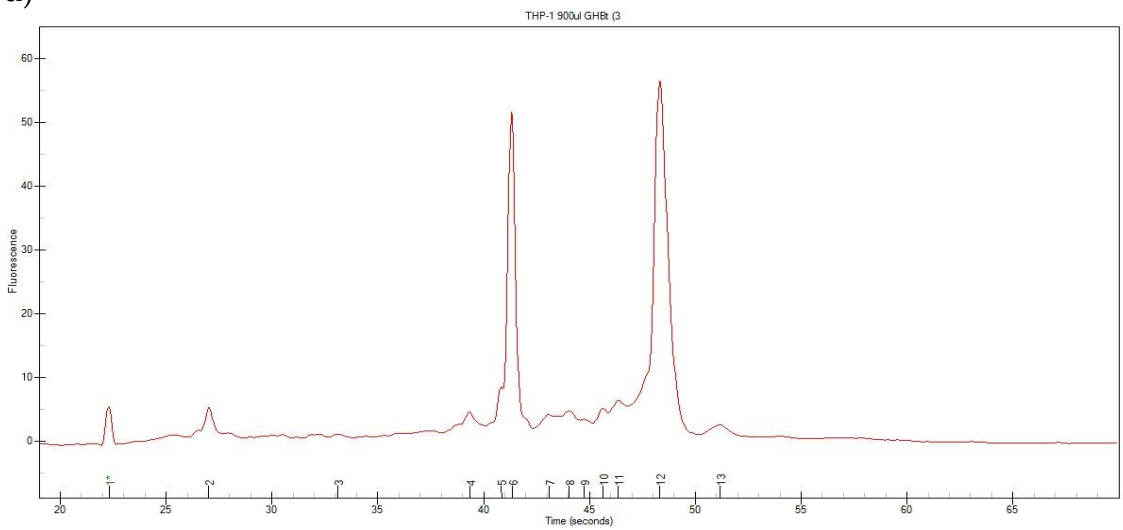


Figure 4.1: The quality of RNA (isolated by SV Total RNA Isolation System) measured using Exprion Automated electrophoresis station (biorad), a) represents the bands of 1(control THP-1 cells), 2(cells treated with 10 μM GHB) and 3(cells treated with 900 μM GHB), b), c) and d) represents the peaks of RNA of 1,2 and 3 samples, respectively.

4.4.2 Microarray Analysis of Gene expression after 24 h GHB exposure

In an attempt to identify all the genes whose expression changes after 24 hours exposure of THP-1 cells to 10μM or 900 μM GHB, the RNA prepared from the treated and control cells was reverse transcribed and labelled with Cy3 using the kits recommended by Agilent technologies (see Materials and Methods). The prepared labelled transcripts were then used to hybridize to Agilent SurePrint G3 Human gene expression 8x60K arrays.

Microarray transcription profiles were obtained and the results shown in the Appendix that 900 μM GHB significantly induces an alteration in 6676 genes, using $P<0.01$ as criteria of significance. This number of genes is reduced to 3197 using $P<0.01$ and a fold change of >2, and further reduced to 203 genes using $P<0.001$ and ≥3 fold change. Of these, 87 genes were down regulated and 116 were up regulated (Tables 4.3 and 4.4). Furthermore, 10 μM GHB significantly induces an alteration in 3288 genes using $P<0.05$ as criteria of significance, and this number is reduced to 11 using $P<0.001$ and ≥3 fold change. Of these, 8 genes were down regulated and 3 were up regulated (Tables 4.1 and 4.2). 20 genes are altered using $P<0.01$ and >2 fold change. These results confirmed that multiple genes were regulated by GHB treatment.

The highest absolute fold change after 24 h exposure to 10 μM GHB was for interleukin 7 (IL7) which was 5.37-fold, IL7 has an anti-apoptotic function. In addition, the highest upregulated gene was FAM19A4, which was upregulated 3.52 fold. FAM19A4 is a member of the TAFA family that act as regulators of immune and nervous cells (Tables 4.1 and 4.2).

Furthermore, the highest absolute fold change after 24 h exposure to 900 μM GHB were 11.5-fold in the down regulated genes for serpin peptidase inhibitor, clade B (ovalbumin), member 2 (SERPINB2) gene and 25.88-fold in the up regulated genes for SCML4 (sex comb on midleg-like 4 (Drosophila)) which encodes a member of the Polycomb group proteins. These proteins form the Polycomb repressive complexes which are involved in transcriptional repression (Tables 9.3 and 4.4).

Table 4.1: Significantly down regulated genes after 24 h exposure to 10 μM GHB (P<0.001 and ≥3 fold change).

	ProbeName	p-value	Fold change	Gene Symbol	Gene name
1	A_33_P3295917	4.53E-02	-5.37	IL7	interleukin 7
2	A_33_P3233749	9.88E-03	-3.97	LOC100128751	INM04
3	A_33_P3297415	1.24E-02	-3.65	NRP2	neuropilin 2
4	A_33_P3620832	4.36E-02	-3.63	ZNF483	zinc finger protein 483
5	A_23_P66739	1.76E-03	-3.33	SLC13A5	solute carrier family 13 (sodium-dependent citrate transporter), member 5
6	A_24_P921610	8.88E-03	-3.16	PAR5	Prader-Willi/Angelman syndrome-5
7	A_32_P204980	1.36E-02	-3.14	FBXO39	F-box protein 39
8	A_33_P3323904	4.41E-02	-3.04	WTH3DI	RAB6C-like

Table 4.2: Significantly up regulated genes after 24 h exposure to 10 μM GHB (P<0.001 and ≥3 fold change).

	ProbeName	p-value	Fold change	Gene Symbol	Gene name
1	A_32_P71571	4.86E-02	3.52	FAM19A4	family with sequence similarity 19 (chemokine (C-C motif)-like), member A4
2	A_24_P75963	4.63E-02	3.03	DSCR4	Down syndrome critical region gene 4
3	A_23_P88678	1.56E-02	3.00	C15orf27	chromosome 15 open reading frame 27

Table 4.3: Significantly down regulated genes after 24 h exposure to 900 μM GHB (P<0.001 and ≥3 fold change).

	ProbeName	p-value	FoldChange	Gene Symbol	Gene name
1	A_24_P245379	7.50E-06	-11.50	SERPINB2	Placental plasminogen activator inhibitor
2	A_32_P200308	7.89E-05	-10.06	LOC728724	Subcategory (RNA class): lncRNA
3	A_23_P21976	5.79E-05	-8.12	CSPG4	chondroitin sulfate proteoglycan 4
4	A_24_P356916	2.64E-04	-7.58	SLC13A3	solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3
5	A_33_P3286422	6.77E-06	-7.42	FANCA	Fanconi anemia, complementation group A
6	A_23_P2543	1.59E-04	-7.29	CUX2	cut-like homeobox 2
7	A_33_P3252048	4.80E-05	-6.90	DYSFIP1	protein phosphatase 1, regulatory subunit 27
8	A_33_P3287472	8.84E-06	-6.75	DYSFIP1	protein phosphatase 1, regulatory subunit 27
9	A_23_P36120	2.95E-05	-6.75	MS4A6A	membrane-spanning 4-domains, subfamily A, member 6A
10	A_33_P3272291	1.05E-05	-6.70	AKR1C4	aldo-keto reductase family 1, member C4
11	A_32_P351037	5.80E-05	-6.23	DNAH7	Dynein heavy chain-like protein
12	A_23_P203376	2.76E-04	-6.13	MS4A6A	membrane-spanning 4-domains, subfamily A, member 6A
13	A_23_P80295	2.03E-05	-5.98	SYN3	synapsin III
14	A_33_P3380383	3.12E-05	-5.98	TIFAB	TRAF-interacting protein with forkhead-associated domain, family member B
15	A_23_P209978	9.29E-04	-5.93	VSNL1	visinin-like 1
16	A_24_P152968	3.65E-05	-5.90	AKR1C1	aldo-keto reductase family 1, member C1
17	A_23_P340263	1.70E-05	-5.86	RNF175	ring finger protein 175
18	A_23_P126212	7.31E-04	-5.38	CLSPN	claspin
19	A_23_P56578	9.25E-07	-5.36	VIT	vitrin
20	A_23_P58266	9.82E-05	-5.31	S100P	S100 calcium binding protein P
21	A_23_P2492	5.94E-05	-5.17	C1S	complement component 1, s subcomponent
22	A_33_P3294372	2.63E-04	-5.05	LOC100128	uncharacterized LOC100128881 (RNA gene)
23	A_23_P257971	2.87E-04	-4.94	AKR1C1	aldo-keto reductase family 1, member C1
24	A_24_P190873	3.06E-04	-4.93	FAM163A	family with sequence similarity 163, member A
25	A_32_P105549	2.98E-05	-4.67	ANXA8L2	annexin A8-like 2
26	A_33_P3867534	9.66E-06	-4.64	MCM10	minichromosome maintenance complex 10
27	A_23_P360754	3.35E-05	-4.64	ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif, 4
28	A_23_P408955	8.05E-05	-4.62	E2F2	E2F transcription factor 2
29	A_23_P80032	2.10E-05	-4.59	E2F1	E2F transcription factor 1
30	A_33_P3289005	1.49E-04	-4.49	AP1S3	adaptor-related protein complex 1, sigma 3

					subunit
31	A_23_P370989	1.74E-04	-4.45	MCM4	minichromosome maintenance complex component 4
32	A_32_P183218	3.07E-04	-4.40	ZNF367	zinc finger protein 367
33	A_33_P3355185	1.67E-04	-4.37	BCL2	B-cell CLL/lymphoma 2
34	A_23_P251421	8.15E-05	-4.25	CDCA7	cell division cycle associated 7
35	A_23_P313981	4.17E-04	-4.19	SERPINB10	serpin peptidase inhibitor, clade B (ovalbumin), member 10
36	A_23_P208880	1.25E-04	-4.18	UHRF1	ubiquitin-like with PHD and ring finger domains 1
37	A_23_P37410	2.89E-05	-4.12	CYP19A1	cytochrome P450, family 19, subfamily A, polypeptide 1
38	A_33_P3399064	8.57E-04	-4.03	RN5-8S1	RNA5-8S5 RNA, 5.8S ribosomal 5
39	A_24_P105747	2.24E-04	-4.02	AP1S3	adaptor-related protein complex 1, sigma 3 subunit
40	A_23_P74449	1.22E-04	-4.01	HPDL	4-hydroxyphenylpyruvate dioxygenase-like
41	A_24_P299685	2.02E-04	-3.97	PDPN	podoplanin
42	A_33_P3303245	1.32E-04	-3.93	KIT	v-**kit** Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog[1] [2]
43	A_33_P3396214	4.33E-05	-3.84	KREMEN2	kringle containing transmembrane protein 2
44	A_23_P30976	2.12E-04	-3.84	GRM1	glutamate receptor, metabotropic 1
45	A_33_P3368830	3.76E-04	-3.73	LY9	lymphocyte antigen 9
46	A_23_P24903	2.37E-05	-3.71	P2RY2	purinergic receptor P2Y, G-protein coupled, 2
47	A_23_P101992	2.22E-04	-3.69	MARCO	macrophage receptor with collagenous structure
48	A_23_P324885	4.48E-04	-3.65	CCR2	chemokine (C-C motif) receptor 2
49	A_33_P3367301	1.12E-04	-3.63	GJD3	gap junction protein, delta 3, 31.9kDa
50	A_33_P3247022	2.98E-05	-3.61	CCNE2	cyclin E2
51	A_32_P780862	1.93E-04	-3.61	BMP8B	bone morphogenetic protein 8b
52	A_23_P209954	1.96E-04	-3.59	GNLY	Granulysin
53	A_23_P39955	2.76E-05	-3.57	ACTG2	actin, gamma 2, smooth muscle, enteric
54	A_33_P3232173	1.66E-04	-3.55	PSPC1	paraspeckle component 1
55	A_23_P57379	4.03E-05	-3.55	CDC45	cell division cycle 45 homolog (S. cerevisiae)
56	A_23_P352266	3.09E-04	-3.54	BCL2	B-cell CLL/lymphoma 2
57	A_33_P3378630	4.17E-04	-3.53	LOC1001-	uncharacterized LOC100131195
58	A_23_P129075	7.02E-04	-3.53	WDR76	WD repeat domain 76
59	A_33_P3209346	5.13E-04	-3.53	IARS	isoleucyl-tRNA synthetase
60	A_23_P33868	5.82E-04	-3.45	LPAR4	lysophosphatidic acid receptor 4
61	A_23_P148737	1.99E-04	-3.42	MYBPH	myosin binding protein H
62	A_23_P138541	1.18E-03	-3.39	AKR1C3	aldo-keto reductase family 1, member C3
63	A_23_P118246	5.56E-05	-3.36	GINS2	GINS complex subunit 2 (Psf2 homolog)

64	A_23_P81441	7.53E-04	-3.34	C5orf20	chromosome 5 open reading frame 20
65	A_33_P3275707	6.52E-05	-3.33	AP1S3	adaptor-related protein complex 1, sigma 3 subunit
66	A_23_P112554	1.74E-04	-3.32	COL15A1	collagen, type XV, alpha 1
67	A_23_P20443	4.39E-04	-3.31	LZTS1	leucine zipper, putative tumor suppressor 1
68	A_33_P3407424	2.47E-05	-3.28	CDC42EP1	CDC42 effector protein (Rho GTPase binding) 1
69	A_23_P56559	1.16E-04	-3.25	DHRS9	dehydrogenase/reductase(SDRfamily)member 9
70	A_24_P225616	9.85E-04	-3.20	RRM2	ribonucleotide reductase M2
71	A_23_P7873	3.35E-05	-3.15	MCM3	minichromosome maintenance complex component 3
72	A_24_P46130	4.41E-05	-3.15	ACPP	acid phosphatase, prostate
73	A_33_P3300267	3.49E-04	-3.14	VIT	vitrin
74	A_23_P250385	2.45E-04	-3.14	HIST1H1B	histone cluster 1, H1b
75	A_23_P215956	3.26E-04	-3.13	MYC	v-**myc** myelocytomatosis viral oncogene homolog (avian)
76	A_33_P3301524	2.49E-04	-3.12	XRCC3	X-ray repair complementing defective repair in Chinese hamster cells 3
77	A_23_P40956	3.74E-04	-3.11	GHRL	ghrelin/obestatin prepropeptide
78	A_23_P35871	8.69E-05	-3.08	E2F8	E2F transcription factor 8
79	A_23_P210425	4.51E-04	-3.07	MYL9	myosin, light chain 9, regulatory
80	A_23_P95640	5.59E-05	-3.05	C1orf186	chromosome 1 open reading frame 186
81	A_23_P130194	2.00E-04	-3.03	PYCR1	pyrroline-5-carboxylate reductase 1
82	A_33_P3267577	2.07E-04	-3.03	SEMG1	semenogelin I
83	A_33_P3351371	2.39E-04	-3.01	CYP19A1	cytochrome P450, family 19, subfamily A, polypeptide 1
84	A_33_P3235217	8.29E-05	-3.01	CTPS	CTP synthase 1
85	A_32_P103633	1.63E-05	-3.00	MCM2	minichromosome maintenance complex component 2
86	A_24_P186379	5.06E-05	-3.00	C10orf125	fucose mutarotase
87	A_23_P108751	3.14E-04	-3.00	FHL2	four and a half LIM domains 2

Table 4.4: Significantly up regulated genes after 24 h exposure to 900 μM GHB (P<0.001 and ≥3 fold change).

	Probe Name	p-value	FoldChange	Gene Symbol	Gene name
1	A_33_P3387691	1.06E-04	25.88	SCML4	sex comb on midleg-like 4 (Drosophila)
2	A_23_P146274	2.02E-04	20.85	STMN2	stathmin-like 2
3	A_33_P3535523	4.52E-04	19.38	LOC28606	uncharacterized LOC286068
4	A_33_P3416966	7.75E-05	17.23	C6orf168	failed axon connections homolog (Drosophila)

5	A_23_P360542	7.48E-04	12.87	C18orf2	charged multivesicular body protein 1B
6	A_23_P136671	1.38E-05	12.65	UGT2B7	UDP glucuronosyltransferase 2 family, polypeptide B7
7	A_33_P3789693	5.39E-05	12.45	MGC24103	uncharacterized MGC24103
8	A_33_P3441021	2.38E-04	12.30	TMEM233	transmembrane protein 233
9	A_24_P706953	1.97E-04	10.49	TMEM213	transmembrane protein 213
10	A_23_P125435	1.69E-04	9.55	GABRB1	gamma-aminobutyric acid (GABA) A receptor, beta 1
11	A_33_P3241071	1.95E-04	9.35	MANSC4	MANSC domain containing 4
12	A_24_P196528	2.25E-04	8.90	CRB1	crumbs homolog 1 (Drosophila)
13	A_33_P3330149	4.15E-04	8.72	PAX6	paired box 6
14	A_24_P234768	9.73E-04	8.08	HTR4	5-hydroxytryptamine (serotonin) receptor 4, G protein-coupled
15	A_24_P397817	1.50E-04	8.02	LEP	leptin
16	A_23_P29163	2.86E-04	7.90	GSTTP1	glutathione S-transferase theta pseudogene 1
17	A_24_P332081	4.51E-05	7.63	JAKMIP3	Janus kinase and microtubule interacting protein 3
18	A_33_P3245517	3.51E-04	7.57	LOC441666	zinc finger protein 91 pseudogene
19	A_24_P107859	1.19E-05	7.23	SPRED1	sprouty-related, EVH1 domain containing 1
20	A_33_P3312529	2.74E-04	7.01	OR4N3P	olfactory receptor, family 4, subfamily N, member 3 pseudogene
21	A_23_P84219	7.31E-04	6.72	LIPH	lipase, member H
22	A_23_P128281	3.23E-04	6.35	KLRC3	killer cell lectin-like receptor subfamily C, member 3
23	A_23_P435636	1.75E-04	6.34	DAND5	DAN domain family, member 5
24	A_23_P12363	5.85E-04	5.78	ROR1	receptor tyrosine kinase-like orphan receptor 1
25	A_33_P3290343	9.81E-05	5.72	CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1
26	A_24_P350546	9.87E-04	5.62	LOC646976	uncharacterized LOC646976
27	A_23_P30315	1.80E-04	5.55	TRIM7	tripartite motif containing 7
28	A_24_P113131	1.97E-05	5.53	BZRAP1	benzodiazapine receptor (peripheral) associated protein 1
29	A_23_P257003	1.31E-04	5.10	PCSK5	proprotein convertase subtilisin/kexin type 5
30	A_23_P121657	4.18E-04	4.70	HS3ST1	heparan sulfate (glucosamine) 3-O-sulfotransferase 1
31	A_23_P4254	6.19E-05	4.66	CRYBA1	crystallin, beta A1
32	A_23_P405885	8.91E-04	4.55	DPPA2	developmental pluripotency associated 2
33	A_33_P3331491	8.41E-05	4.52	LOC728392	uncharacterized LOC728392
34	A_24_P70906	9.09E-04	4.52	PDILT	protein disulfide isomerase-like, testis expressed
35	A_33_P3273777	6.53E-04	4.51	GJB7	gap junction protein, beta 7, 25kDa
36	A_33_P3351879	3.98E-05	4.37	LOC100128	uncharacterized LOC100128905

37	A_33_P3265679	8.01E-04	4.34	LOC389199	uncharacterized LOC389199
38	A_23_P89431	4.47E-05	4.33	CCL2	chemokine (C-C motif) ligand 2
39	A_23_P204640	7.41E-04	4.31	NANOG	Nanog homeobox
40	A_23_P216307	6.30E-04	4.30	RUNX1T1	runt-related transcription factor 1; translocated to, 1 (cyclin D-related)
41	A_33_P3370890	4.06E-05	4.27	C6orf225	chromosome 6 open reading frame 225
42	A_33_P3353027	5.01E-04	4.23	CHN2	chimerin (chimaerin) 2
43	A_23_P20363	4.93E-04	4.16	FAM164A	zinc finger, C2HC-type containing 1A
44	A_33_P3349395	6.55E-04	4.11	CECR2	cat eye syndrome chromosome region, candidate 2
45	A_24_P325520	4.05E-04	4.10	SORT1	sortilin 1
46	A_23_P209625	9.02E-06	4.08	CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1
47	A_33_P3379811	6.21E-04	4.04	C12orf77	chromosome 12 open reading frame 77
48	A_32_P703	1.86E-04	4.03	LOC646626	uncharacterized LOC646626
49	A_33_P3335624	3.29E-06	3.78	PSMD10	proteasome (prosome, macropain) 26S subunit, non-ATPase, 10
50	A_23_P100711	3.36E-04	3.78	PMP22	peripheral myelin protein 22
51	A_23_P82523	1.87E-04	3.74	ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1
52	A_33_P3337719	3.47E-05	3.74	LOC100134	uncharacterized LOC100134868
53	A_33_P3310232	4.12E-04	3.73	L2HGDH	L-2-hydroxyglutarate dehydrogenase
54	A_23_P171143	7.77E-04	3.71	TSPAN6	tetraspanin 6
55	A_33_P3522511	4.93E-04	3.69	KIAA0485	uncharacterized LOC57235
56	A_33_P3294961	2.55E-04	3.68	LAT2	linker for activation of T cells family, member 2
57	A_33_P3248863	2.52E-05	3.65	KRBA2	KRAB-A domain containing 2
58	A_33_P3269650	4.51E-04	3.65	LOC100128	uncharacterized LOC100128402
59	A_23_P124108	1.38E-04	3.60	ITGAM	integrin, alpha M (complement component 3 receptor 3 subunit)
60	A_23_P105963	8.59E-04	3.59	AK7	Adenylate kinase 7
61	A_33_P3274560	8.60E-04	3.58	SLC19A3	solute carrier family 19, member 3
62	A_23_P82651	5.81E-04	3.56	NPTX2	neuronal pentraxin II
63	A_23_P136978	1.74E-04	3.56	SRPX2	sushi-repeat containing protein, X-linked 2
64	A_33_P3380405	2.42E-04	3.53	CYTH1	cytohesin 1
65	A_23_P339818	1.04E-04	3.52	ARRDC4	arrestin domain containing 4
66	A_23_P428248	8.21E-04	3.52	TTC21A	tetratricopeptide repeat domain 21A
67	A_33_P3337415	1.58E-04	3.50	GNAL	guanine nucleotide binding protein (G protein), alpha activating activity polypeptide, olfactory type
68	A_33_P3276703	9.60E-06	3.49	VGF	VGF nerve growth factor inducible

69	A_23_P366453	9.11E-05	3.46	KHDRBS2	KH domain containing, RNA binding, signal transduction associated 2
70	A_33_P3365646	7.58E-04	3.44	LOC100128	uncharacterized LOC100128437
71	A_23_P111995	1.98E-05	3.40	LOXL2	lysyl oxidase-like 2
72	A_33_P3392123	3.18E-05	3.39	LOC100233	uncharacterized LOC100233209
73	A_33_P3354499	3.29E-04	3.39	LOC40148	uncharacterized LOC401480
74	A_33_P3380652	9.12E-04	3.37	ADAM28	ADAM metallopeptidase domain 28
75	A_33_P3400248	3.43E-04	3.35	FGF20	fibroblast growth factor 20
76	A_33_P3237135	6.39E-04	3.33	MMP2	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)
77	A_33_P3281333	5.04E-04	3.31	SNTB2	syntrophin, beta 2 (dystrophin-associated protein A1, 59kDa, basic component 2)
78	A_33_P3276068	1.35E-06	3.31	BET3L	BET3 like (S. cerevisiae)
79	A_32_P133840	7.60E-05	3.28	TMCC2	transmembrane and coiled-coil domain family 2
80	A_23_P7827	9.91E-05	3.28	FAM26F	family with sequence similarity 26, member F
81	A_23_P18055	3.30E-05	3.28	C3orf51	ERC2 intronic transcript 1 (non-protein coding)
82	A_24_P129588	6.59E-05	3.27	O3FAR1	omega-3 fatty acid receptor 1
83	A_23_P121926	6.73E-04	3.26	SEPP1	selenoprotein P, plasma, 1
84	A_23_P217228	1.31E-04	3.26	TRO	trophinin
85	A_33_P3465247	3.04E-04	3.24	KIF3A	kinesin family member 3A
86	A_33_P3333033	8.67E-04	3.23	SGSM2	small G protein signaling modulator 2
87	A_33_P3700860	8.12E-04	3.23	KIAA1661	KIAA1661 protein
88	A_23_P371824	5.97E-05	3.22	TUFT1	tuftelin 1
89	A_33_P3397795	9.65E-05	3.22	C14orf135	pecanex-like 4 (Drosophila)
90	A_33_P3893191	4.48E-04	3.22	DBIL5P2	diazepam binding inhibitor-like 5 pseudogene 2
91	A_33_P3385765	5.30E-04	3.21	ZNF763	zinc finger protein 763
92	A_24_P137522	8.77E-04	3.20	USP53	ubiquitin specific peptidase 53
93	A_33_P3252381	7.94E-04	3.19	PCA3	prostate cancer antigen 3 (non-protein coding)
94	A_33_P3235420	8.27E-05	3.18	RASSF6	Ras association (RalGDS/AF-6) domain family memb. 6
95	A_33_P3330991	5.49E-04	3.18	LOC100134	uncharacterized LOC100134237
96	A_33_P3321678	6.19E-05	3.17	RNF180	ring finger protein 180
97	A_23_P364625	6.02E-04	3.15	LRRC19	leucine rich repeat containing 19
98	A_33_P3219939	8.53E-04	3.15	CUBN	cubilin (intrinsic factor-cobalamin receptor)
99	A_23_P130764	3.86E-04	3.14	KCNJ14	potassium inwardly-rectifying channel, subfamily J, member 14
100	A_33_P3419938	4.69E-05	3.11	VHL	von Hippel-Lindau tumor suppressor, E3 ubiquitin protein ligase
101	A_32_P46214	9.61E-05	3.09	SLC9A9	solute carrier family 9, subfamily A (NHE9, cation proton antiporter 9), member 9

102	A_32_P54274	2.07E-04	3.09	DRD5	dopamine receptor D5
103	A_24_P41801	4.19E-05	3.08	LPA	lipoprotein, Lp(a)
104	A_33_P3322804	1.89E-04	3.07	NTRK2	neurotrophic tyrosine kinase, receptor, type 2
105	A_33_P3609431	2.28E-04	3.06	ERVFRD-2	endogenous retrovirus group FRD, member 2
106	A_33_P3305173	3.18E-04	3.06	RGAG4	retrotransposon gag domain containing 4
107	A_32_P83811	8.70E-04	3.05	FAM47E	family with sequence similarity 47, member E
108	A_33_P3418125	2.22E-04	3.05	GLIPR1	GLI pathogenesis-related 1
109	A_23_P13929	1.06E-04	3.03	NRIP2	nuclear receptor interacting protein 2
110	A_33_P3410831	8.27E-05	3.03	LOC729609	uncharacterized **LOC729609**
111	A_23_P45365	1.72E-04	3.02	COL4A5	collagen, type IV, alpha 5
112	A_23_P200260	3.35E-04	3.02	PCNXL2	pecanex-like 2 (Drosophila)
113	A_33_P3421219	3.42E-04	3.02	LOC647070	uncharacterized **LOC647070**
114	A_23_P40880	2.51E-05	3.01	CMTM8	CKLF-like MARVEL transmembrane domain containing 8
115	A_23_P168882	2.23E-04	3.00	TP53INP1	tumor protein p53 inducible nuclear protein 1
116	A_23_P333029	2.11E-04	3.00	C8orf47	chromosome 8 open reading frame 47

4.4.3 Altered Gene Families

The microarray results revealed that some members of well-known gene families were altered after 24 h of GHB exposure. These are:

1. Genes that encode steroid dehydrogenases. These genes were down regulated after 24 h of GHB exposure (Table 4.5).

2. Genes of the aldo-keto reductase family members were also significantly down regulated after 24 hours exposure to GHB (Table 4.6).

3. Genes of the hydroxysteroid dehydrogenases family members were significantly down regulated after 24 hours exposure to GHB (Table 4.7).

4. Genes that encode Heat Shock proteins were up-regulated after 24 h of GHB exposure (table 4.8).

5. Genes that encode Zinc Finger Proteins. These genes were up regulated after 24 h of GHB exposure (table 4.9).

Table 4.5: Genes that affect the expression of steroid dehydrogenases were significantly down regulated after 24 hours exposure to GHB.

Probe Name	Fold change		Gene Symbol	Gene Name
	10μM	900μM		
A_24_P152968	-1.12*	-5.9***	AKR1C1	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)
A_33_P3365117	0	-4.3***	AKR1C1	aldo-keto reductase family 1, member C1
A_23_P257971	-1.14*	-4.9***	AKR1C1	aldo-keto reductase family 1, member C1
A_23_P138541	0	-3.39***	AKR1C3	aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II)
A_33_P3272291	-1.12*	-6.7***	AKR1C4	aldo-keto reductase family 1, member C4 (chlordecone reductase; 3-alpha hydroxyl steroid dehydrogenase, type I; dihydrodiol dehydrogenase 4)
A_23_P63209	0	-2.96***	HSD11B1	hydroxysteroid (11-beta) dehydrogenase 1
A_23_P56559	0	-3.25***	DHRS9	dehydrogenase/reductase (SDR family) member 9

The results are mean values of n= 3 (** P < 0.01, *** P < 0.001 compared to control).

Table 4.6: Aldo-keto reductase family members that are significantly down regulated after 24 hours exposure to GHB

Probe Name	Fold change		Gene Symbol	Gene Name
	10μM	900μM		
A_24_P152968	-1.12*	-5.9***	AKR1C1	aldo-keto reductase family 1, member C1 (dihydrodiol dehydro genase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)
A_33_P3365117	0	-4.3***	AKR1C1	aldo-keto reductase family 1, member C1
A_23_P257971	-1.14*	-4.9***	AKR1C1	aldo-keto reductase family 1, member C1
A_23_P138541	0	-3.39***	AKR1C3	aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II)
A_33_P3272291	-1.12*	-6.7***	AKR1C4	aldo-keto reductase family 1, member C4 (chlordecone reductase ; 3-alpha hydroxyl steroid dehydrogenase, type I; dihydrodiol dehydrogenase 4)
A_24_P79755	0	-1.7*	AKR1A1	aldo-keto reductase family 1, member A1 (aldehyde reductase), dihydrodiol dehydrogenase 3, alcohol dehydrogenase [NADP(+)]
A_23_P316812	0	-1.5**	AKR7A2P1	aldo-keto reductase family 7, member A2 pseudogene
A_23_P115356	0	-1.4**	AKR7A2	aldo-keto reductase family 7, member A2(aflatoxin aldeh yde reductase) aflatoxin B1 aldehyde reductase memb. 2

The results are mean values of n= 3 (** P < 0.01, *** P < 0.001 compared to control).

Table 4.7: Hydroxy steroid dehydrogenases family members that are significantly down regulated after 24 hours exposure to GHB

Probe Name	Fold change		Gene Symbol	Gene Name
	10μM	900μM		
A_23_P63209	0	-2.95***	HSD11B1	hydroxysteroid (11-beta) dehydrogenase 1
A_23_P141992	0	-1.49**	HSD11B1L	hydroxysteroid (11-beta) dehydrogenase 1-like
A_33_P3335042	0	-1.62**	HSD17B12	hydroxysteroid (17-beta) dehydrogenase 12
A_23_P47377	0	-1.30***	HSD17B12	hydroxysteroid (17-beta) dehydrogenase 12
A_23_P11859	0	-1.66***	HSD17B7	hydroxysteroid (17-beta) dehydrogenase 7
A_33_P3276369	0	-1.80***	HSD17B7	hydroxysteroid (17-beta) dehydrogenase 7
A_33_P3225487	0	-1.62***	HSD17B7	hydroxysteroid (17-beta) dehydrogenase 7
A_23_P81973	0	-1.56**	HSD17B8	hydroxysteroid (17-beta) dehydrogenase 8
A_33_P3251776	0	-1.66**	HSD3B7	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7

The results are mean values of n= 3 (** P < 0.01, *** P < 0.001 compared to control).

Table 4.8: Genes encoding Heat Shock proteins that were up regulated after 24 h of GHB exposure

Probe Name	Fold change		Gene Symbol	Gene Name
	10μM	900μM		
A_23_P96497	0	1.79*	HSFX1	heat shock transcription factor family, X linked1
A_23_P62446	0	2.58***	HSFY2	heat shock transcription factor, Y linked 2
A_33_P3415430	0	1.27*	HSPA1B	heat shock 70kDa protein 1B
A_23_P363936	0	1.44***	HSPA4L	heat shock 70kDa protein 4-like
A_33_P3382331	0	1.85*	HSPA6	heat shock 70kDa protein 6 (HSP70B')
A_23_P114903	0	2.67***	HSPA6	heat shock 70kDa protein 6 (HSP70B')

The results are mean values, n = 3 and *P< 0.05, ** P < 0.01, *** P < 0.001 compared with the control cells.

Table 4.9: Genes encoding Zinc Finger Proteins up regulated after 24 h of GHB exposure

Probe Name	Fold change		p-value	Gene Symbol
	10μM	900μM		
A_24_P201125	0	5.33	5.53E-03	ZNF510
A_33_P3385765	0	3.21	5.30E-04	ZNF763

A_33_P3285122	0	3.07	3.04E-03	ZNF599
A_33_P3225696	0	2.35	1.06E-04	ZNF526
A_33_P3219720	0	2.34	4.11E-04	ZNF248
A_33_P3326927	0	2.30	8.97E-03	ZNF19
A_23_P82762	0	2.20	2.06E-03	ZNF596
A_33_P3371154	1.5	2.08	4.44E-03	ZNF365
A_33_P3228988	0	2.05	5.82E-04	ZNF799
A_23_P157022	0	2.00	1.65E-03	ZNF786
A_33_P3326672	0	1.98	2.64E-03	ZNF782
A_23_P93269	0	1.95	2.81E-03	ZNF165
A_33_P3238177	0	1.95	1.65E-04	ZNF37A
A_24_P941166	0	1.83	3.58E-03	ZNF425
A_23_P371011	0	1.78	7.45E-04	ZNF227
A_33_P3273324	0	1.77	7.57E-04	ZNF880
A_23_P345081	0	1.76	1.83E-03	ZNF655
A_23_P208325	0	1.74	3.02E-03	ZNF235
A_23_P321160	0	1.69	5.29E-04	ZNF594
A_23_P108342	0	1.68	4.77E-03	ZNF571
A_32_P206293	0	1.65	4.62E-03	ZNF322A
A_24_P333421	0	1.64	4.67E-04	ZNF862
A_33_P3296858	0	1.62	6.40E-04	ZNF37BP
A_23_P309865	0	1.61	5.18E-03	ZNF449
A_33_P3222744	0	1.60	4.84E-03	ZNF117
A_33_P3334108	0	1.60	2.33E-04	ZNF740
A_23_P208198	0	1.60	3.84E-04	ZNF577
A_33_P3296871	0	1.57	2.12E-04	ZNF33B
A_33_P3318596	0	1.57	8.63E-04	ZNF345
A_33_P3231695	0	1.57	6.38E-03	ZNF667
A_33_P3385750	0	1.56	7.14E-03	ZNF793
A_23_P209032	0	1.56	8.35E-03	ZNF302
A_33_P3259148	0	1.54	7.03E-04	ZNF286B
A_23_P311087	0	1.53	3.52E-03	ZNF281
A_23_P94911	0	1.50	1.42E-03	ZNF283
A_23_P304511	0	1.49	2.86E-03	ZNF397
A_33_P3414122	0	1.49	9.89E-03	ZNF260
A_33_P3222753	0	1.48	1.15E-03	ZNF137P
A_24_P247978	0	1.47	8.36E-03	ZNF589
A_23_P52176	0	1.46	8.57E-04	ZNF445
A_33_P3305158	0	1.46	4.82E-03	ZNF124
A_33_P3233774	0	1.46	9.81E-03	ZNF621

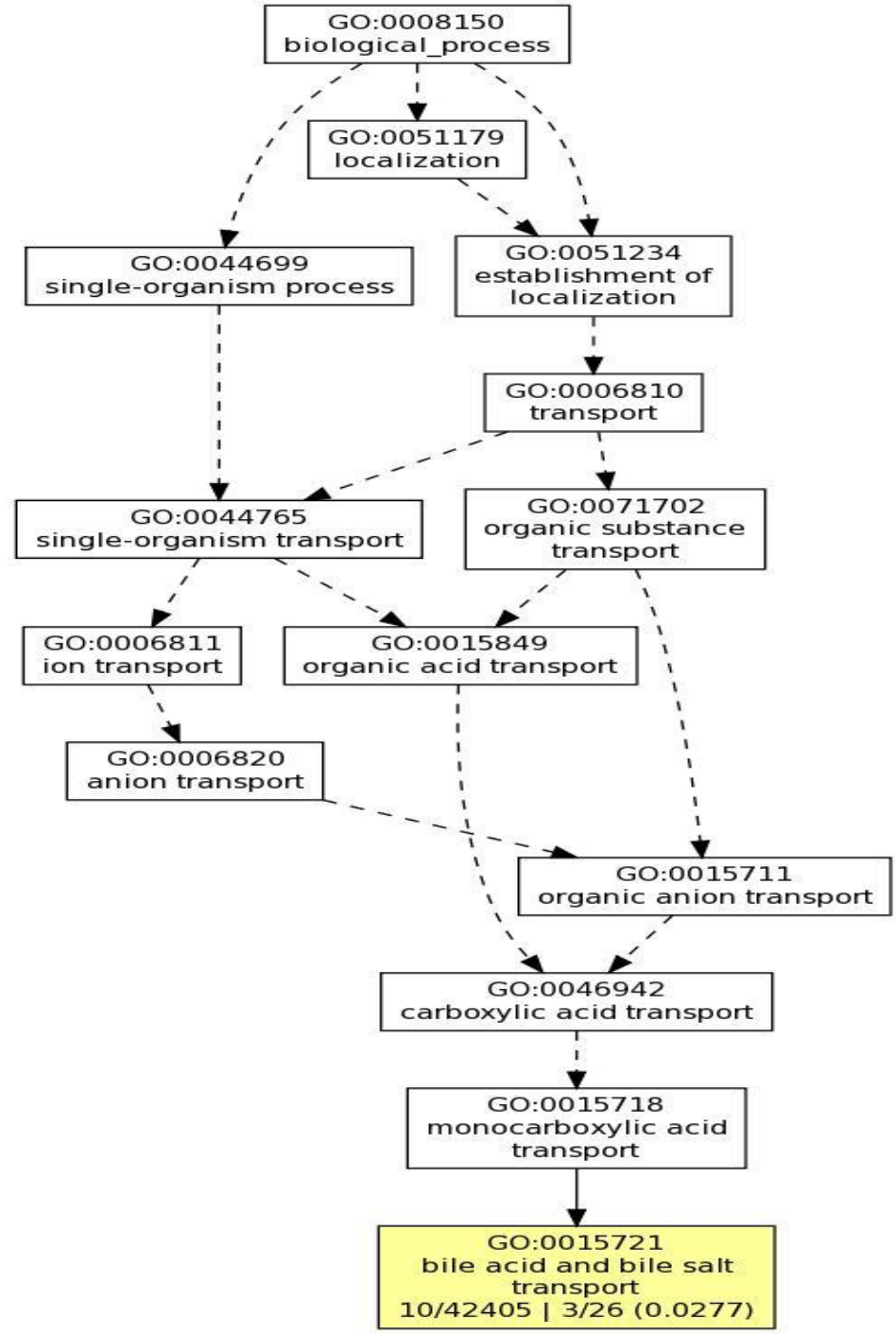
4.4.4 Gene ontology categorization

Gene Ontology (GO) enrichment analysis of the gene lists found that 59% and 82.5% of altered genes after exposure to 900 μM and 10μM GHB respectively were categorized as being involved in GO Biological Processes. Some of Biological Processes genes include those that were related to cell apoptosis, glucose transport and carbonic acid transport (Table 4.10 and Figure 4.2). In addition, 26% and 7% of altered genes after exposure to 900μM and 10μM GHB respectively were in the GO Cellular Component category. The largest numbers of these altered genes were in the Intracellular and Membrane components of the cell (Table 4.10 and Figure 4.3). Also 15% and 10.5% of altered genes after exposure to 900 μM and 10μM GHB respectively were in the GO Molecular Functions category (Table 4.10 and figure 4.4). The majority of these altered genes coded for proteins and nucleotide binding proteins and some of the altered genes encoded steroid dehydrogenase enzymes (Table 4.5). Gene ontology terms are listed in Tables 9.5 – 9.8 in the Appendix.

Table 4.10: Percentages of significantly enriched GO terms.

	900μM GHB			**10μM GHB**		
	Total	**Down regulated**	**Up regulated**	**Total**	**Down regulated**	**Up regulated**
Significantly enriched GO terms	100%	75%	25%	100%	3%	97%
Cellular Compartment	26%	17.5%	8.5%	7%	0%	7%
Molecular Function	15%	12%	3%	10.5%	1%	9.5%
Biological Processes	59%	46%	13%	82.5%	2%	80.5%

A)


GO:0008150
biological_process
GO:0051179
localization
GO:0044699
single-organism process
GO:0051234
establishment of
localization
GO:0006810
transport
GO:0044765
single-organism transport
GO:0071702
organic substance
transport
GO:0006811
ion transport
GO:0015849
organic acid transport
GO:0006820
anion transport
GO:0015711
organic anion transport
GO:0046942
carboxylic acid transport
GO:0015718
monocarboxylic acid
transport
GO:0015721
bile acid and bile salt
transport
10/42405 | 3/26 (0.0277)

B)

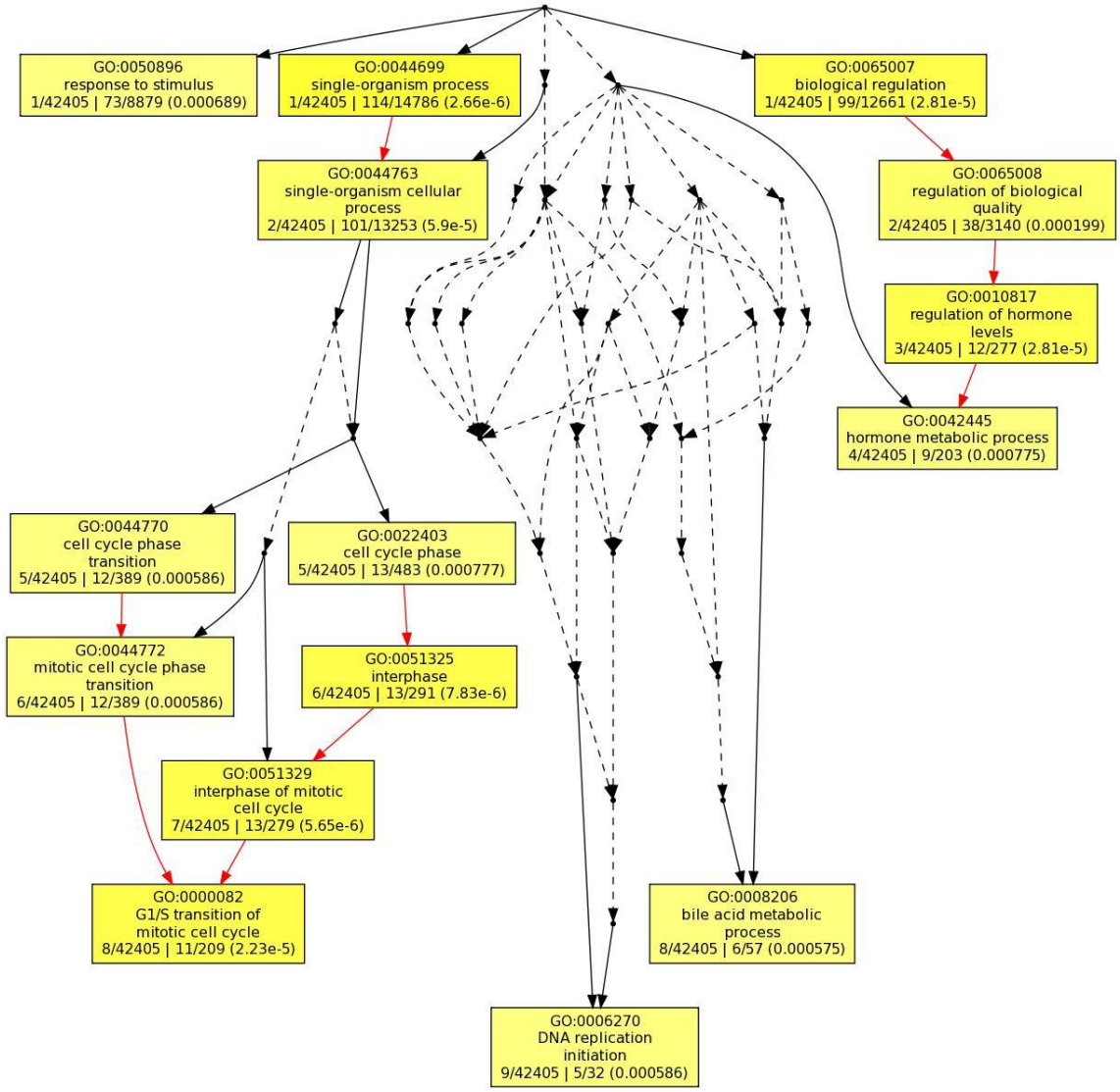
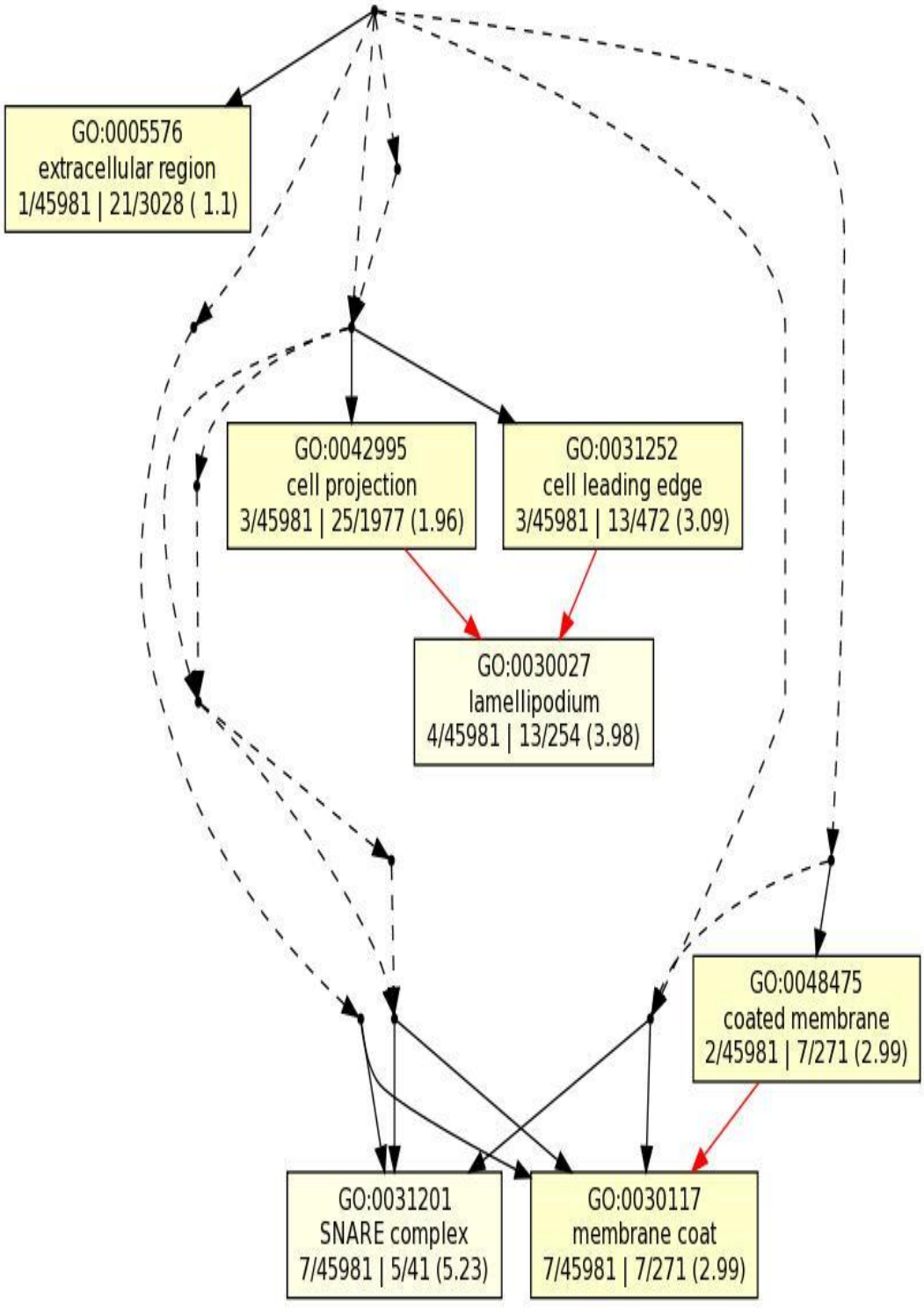


Figure 4.2: Gene Ontology Biological Processes of significantly altered genes after 24 hours exposure to A) 10μM and B) 900μM of GHB in THP-1 cells. Significantly enriched GO terms are marked yellow. The degree of colour saturation of each node positively correlates with the significance of GO term enrichment. Red edges stand between two enriched, black solid edges between enriched and unenriched, black dashed edges between two unenriched GO terms. Some of Biological Processes genes include those that were related to cell apoptosis, glucose transport and carbonic acid transport.

A)


GO:0005576
extracellular region
1/45981 | 21/3028 (1.1)
GO:0042995
cell projection
3/45981 | 25/1977 (1.96)
GO:0031252
cell leading edge
3/45981 | 13/472 (3.09)
GO:0030027
lamellipodium
4/45981 | 13/254 (3.98)
GO:0048475
coated membrane
2/45981 | 7/271 (2.99)
GO:0031201
SNARE complex
7/45981 | 5/41 (5.23)
GO:0030117
membrane coat
7/45981 | 7/271 (2.99)

B)

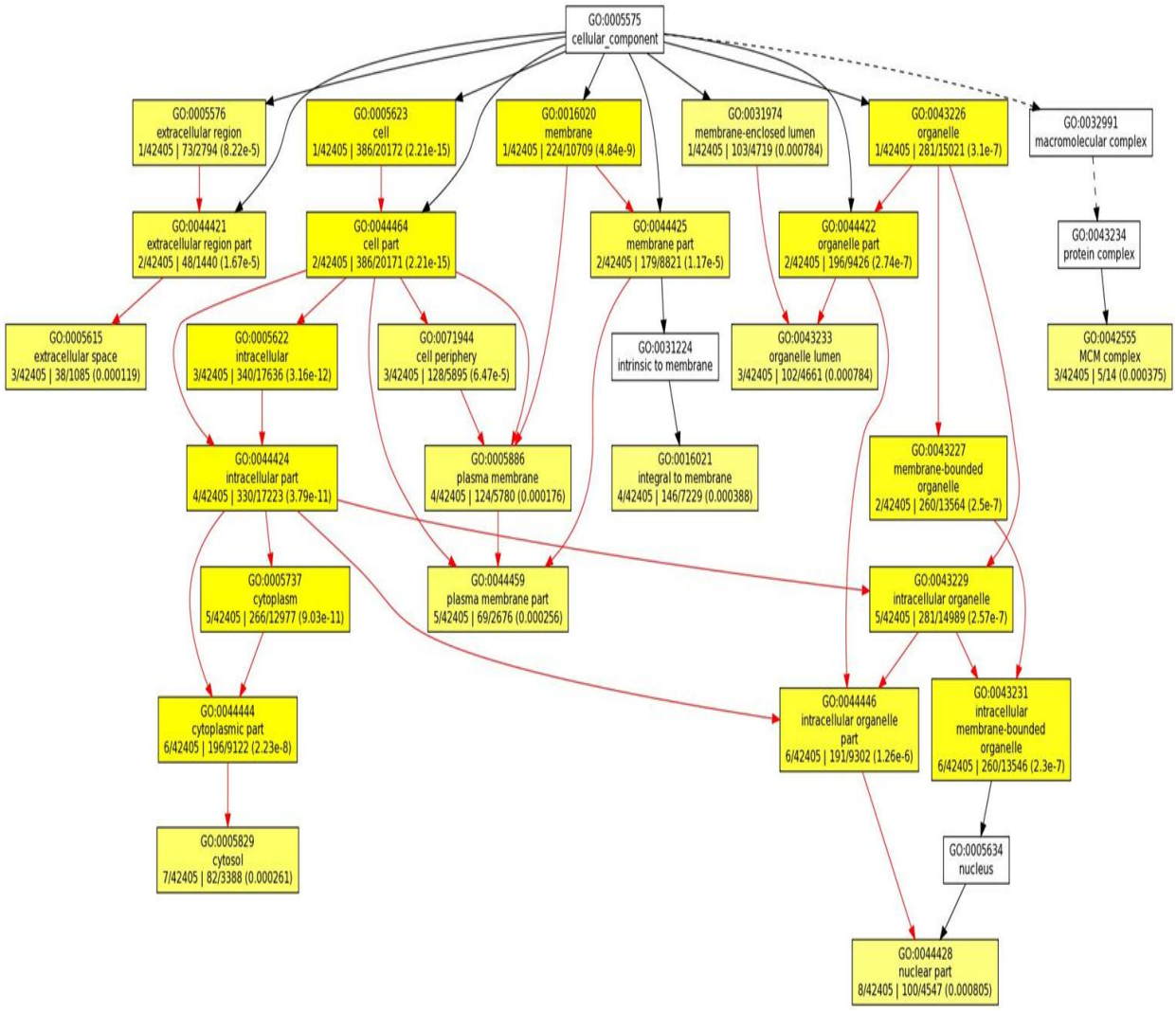
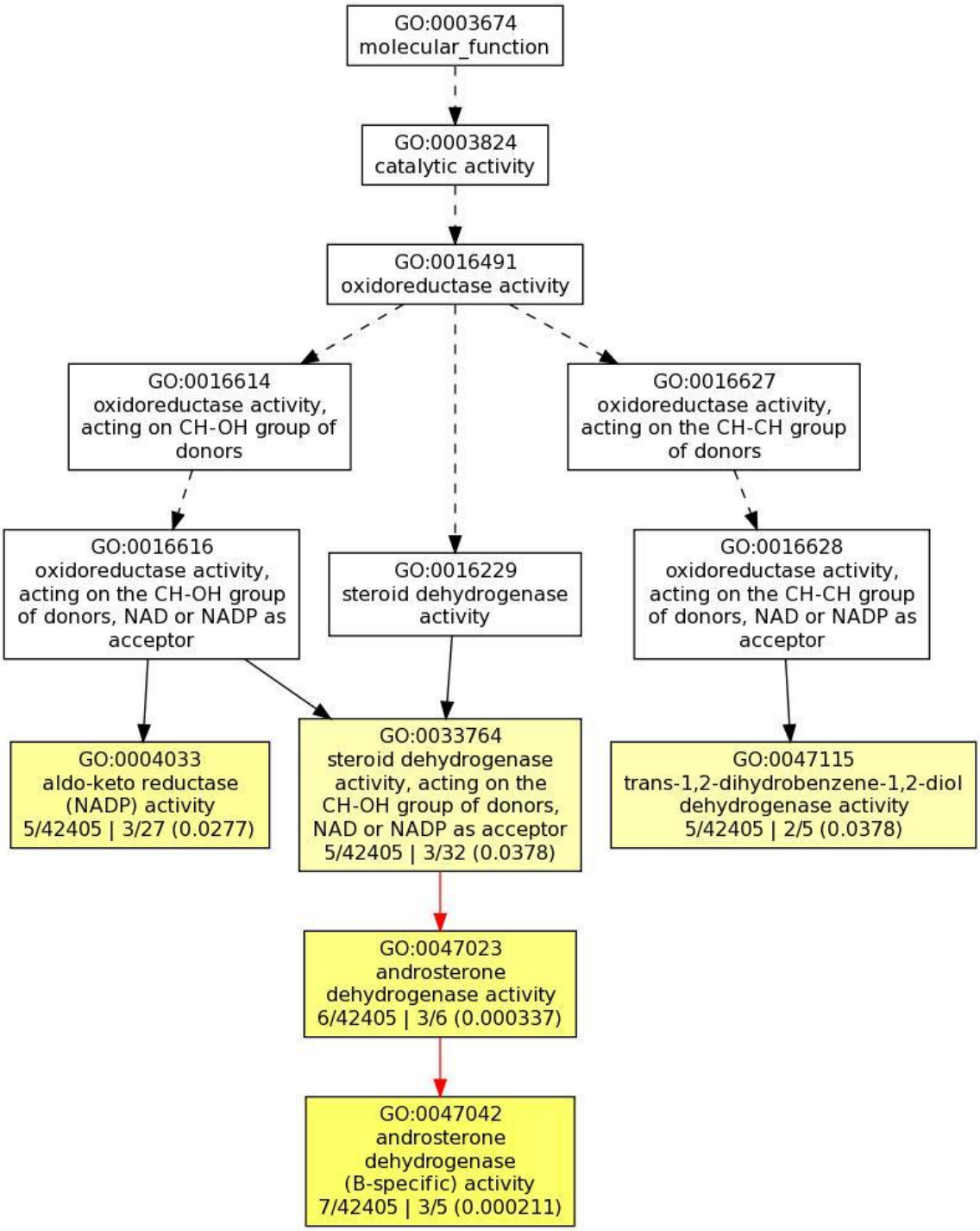


Figure 4.3: Gene Ontology Cellular Component of significantly altered genes after 24 hours exposure to A) 10μM and B) 900μM of GHB in THP-1 cells. Significantly enriched GO terms are marked yellow. The degree of colour saturation of each node positively correlates with the significance of GO term enrichment. Red edges stand between two enriched, black solid edges between enriched and unenriched, black dashed edges between two unenriched GO terms. The largest numbers of these altered genes were in the Intracellular and Membrane components of the cell.

A)


GO:0003674
molecular_function
GO:0003824
catalytic activity
GO:0016491
oxidoreductase activity
GO:0016614
oxidoreductase activity, acting on CH-OH group of donors
GO:0016627
oxidoreductase activity, acting on the CH-CH group of donors
GO:0016616
oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor
GO:0016229
steroid dehydrogenase activity
GO:0016628
oxidoreductase activity, acting on the CH-CH group of donors, NAD or NADP as acceptor
GO:0004033
aldo-keto reductase (NADP) activity
5/42405 | 3/27 (0.0277)
GO:0033764
steroid dehydrogenase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor
5/42405 | 3/32 (0.0378)
GO:0047115
trans-1,2-dihydrobenzene-1,2-diol dehydrogenase activity
5/42405 | 2/5 (0.0378)
GO:0047023
androsterone dehydrogenase activity
6/42405 | 3/6 (0.000337)
GO:0047042
androsterone dehydrogenase (B-specific) activity
7/42405 | 3/5 (0.000211)

B)

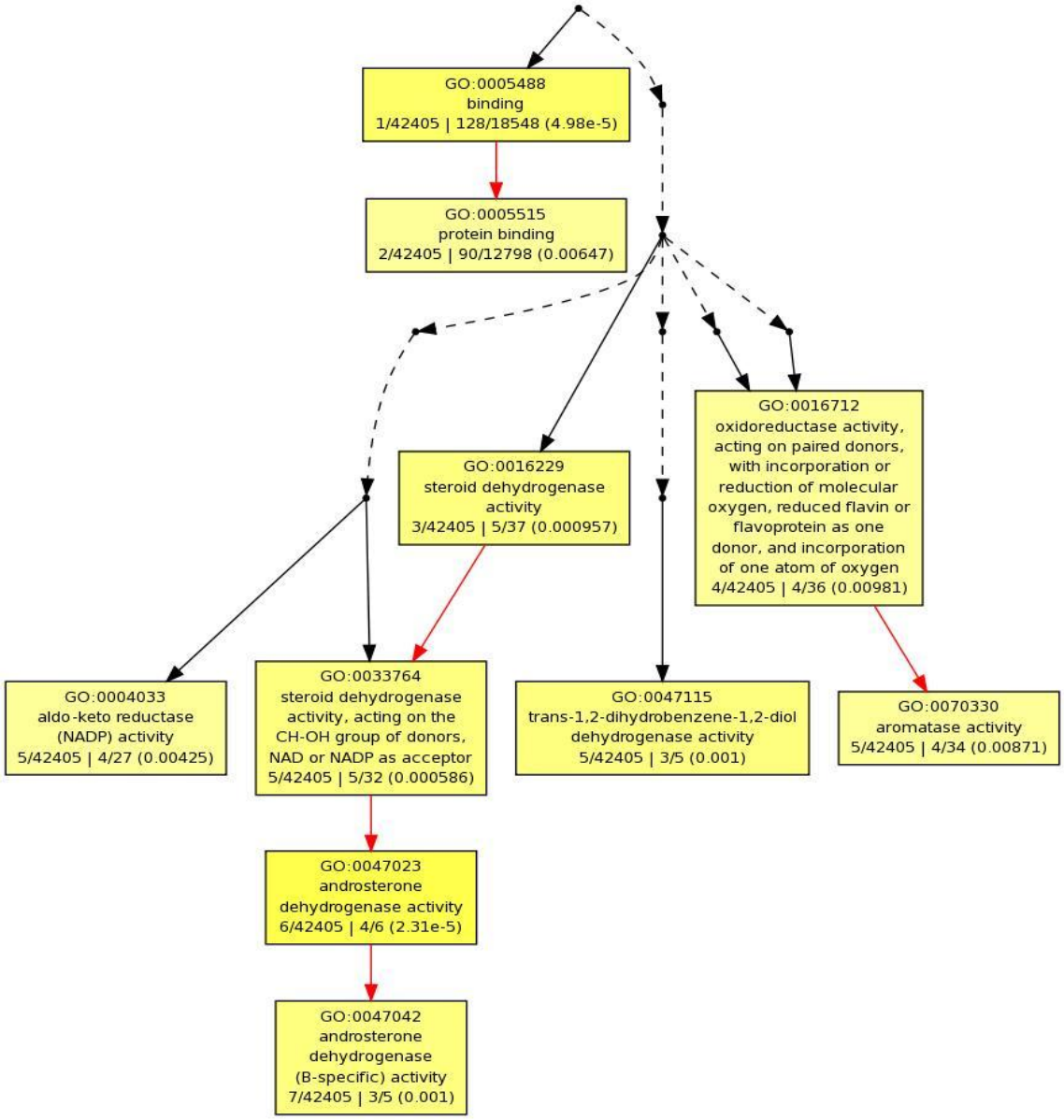


Figure 4.4: Gene Ontology Molecular Function of significantly altered genes after 24 hours exposure to A) 10μM and B) 900μM of GHB in THP-1 cells. Significantly enriched GO terms are marked yellow. The degree of colour saturation of each node positively correlates with the significance of GO term enrichment. Red edges stand between two enriched, black solid edges between enriched and unenriched, black dashed edges between two unenriched GO terms. The majority of these altered genes coded for proteins and nucleotide binding proteins and some of the altered genes encoded steroid dehydrogenase enzymes.

4.5 Discussion

In this study, microarray technology was used to identify biomarkers for GHB exposure. The human monocytic leukaemia (THP-1) cell line was used as blood cell model to study GHB dependent changes in gene expression after 24h exposure. It was found that GHB significantly induces an alteration in thousands of genes. These results seem to support the previous studies indicating that exogenously administered GHB can leave a gene expression signature after exposure (Larson *et al*., 2007; Schnakenberg *et al*., 2010; Kemmel *et al*., 2010; Wisor *et al*., 2006).

Gene ontology (GO) enrichment analysis revealed that the majority of altered genes were found in the GO Biological Processes category and the percentages of these genes were higher using 10μM than 900 μM concentration of GHB. These results suggest that the gene lists found in the GO Biological Processes category were more affected by lower concentrations of GHB and the major percentage of the altered genes were up regulated. Some of these up regulated genes were related to cells apoptosis, glucose transport and carbonic acid transport, similar to the function of PEA-15 which is a small anti-apoptotic protein, and indicates that GHB has profound effects on cell growth and metabolism.

Regarding the gene lists found in the GO Cellular Component, the results revealed that the percentage of the altered genes was higher using 900 μM than 10 μM concentration of GHB suggesting that the gene lists found in the GO Cellular Component were more affected by higher concentrations of GHB. The major percentage of the altered genes was down regulated and the largest numbers of the altered genes were in the intracellular and cell membrane parts.

In terms of GO Molecular Functions, the percentage of the altered genes was higher using 900 μM than 10 μM concentration of GHB suggesting that the gene lists found in the GO Molecular Functions were more affected by higher concentrations of GHB. The major percentage of the altered genes was down regulated and the majority of altered genes coded for proteins and nucleotide binding sites.

The microarray results revealed that members of well-known gene families were altered after 24 hour exposure to GHB, including aldo-ketoreductases, hydroxyl steroid dehydrogenases, heat-shock proteins and zinc finger proteins.

Of note, some of the altered genes encode steroid dehydrogenases. There is no obvious connection between steroid dehydrogenases and the known functions or metabolism of GHB, but there might be possible indirect connection. This aspect is investigated further in Chapter 5.

This preliminary research is the first step in developing clinically useful biomarkers for GHB exposure and further experiments are needed to confirm these microarray results, using quantitative PCR and Western blotting. Additional experiments are needed to confirm that results are a specific response to GHB exposure, by comparing levels of the altered genes in cell lines against levels induced by combinations of GHB, GABA, and the particular antagonists NCS-382 (GHB receptor antagonist) and CGP-35348 ($GABA_B$ receptor antagonist), as well as changes brought about by other drugs and/or hormones.

Chapter 5

Effects of GHB on

the expression of genes involved in

steroid hormone metabolism

5 Effects of GHB on the expression of genes involved in steroid hormone metabolism

5.1 Introduction

The microarray study (Chapter 4) aimed to extend the window of detection of GHB beyond 12 hours by measuring the GHB-dependent changes in gene expression using microarray technology on blood THP-1 cells.

One of the findings in Chapter 4 was that that GHB induces changes in expression of specific genes that encode steroid dehydrogenases from several different families. Those genes were AKR1C1, AKR1C3, AKR1C4, HSD11B1 and DHRS9 (Table 4.5). This Chapter will focus on the regulation of these genes by GHB in detail, and will also look at the effects of NCS-382, a structural analogue of GHB, on their expression.

5.1.1 Steroid hormones and regulation by hydroxysteroid dehydrogenases

Steroid hormones, whether they are natural or synthetic, exert their effects through binding to ligand-activated transcription factors that bind to the promoter regions of responsive genes to initiate transcription. At the pre-binding stage, steroid hormone action can be regulated in a process known as "intracrine modulation'' in which the concentration of the steroid hormone ligand is controlled by enzymes in the target tissue (Labrie *et al.*, 2001). Hydroxysteroid dehydrogenases (HSD) catalyze bidirectional reactions, and are involved in both the synthesis and inactivation of steroid hormones (Penning, 1997). Several HSD families exist, and include the aldo-keto reductases (AKR) family as well as the short chain dehydrogenase/reductase (SDR) family.

5.1.2 Aldo-Keto reductase (AKR) superfamily 1C gene

The hydroxysteroid dehydrogenases (HSDs) of the aldo-keto reductase (AKR) superfamily 1C group of enzymes are involved in the intracrine modulation of steroid hormones. The AKR1C group of enzymes are cytosolic HSD that catalyze the NADPH-dependent reduction of ketosteroids to hydroxysteroids. AKR1C isozymes catalyze the reduction of ketosteroids at the C3, C17, or C20 positions (Figure 5.1) and this effect causes these enzymes to regulate the concentration of active and inactive androgens, estrogens, and progestins in their target tissues (Penning, 1997; Dufort *et al*., 1999 and Dufort *et al*., 2001).

As well as regulating steroid hormone levels, human aldo-ketoreductases (AKRs), are also considered as Phase I drug-metabolizing enzymes and can also detoxify or activate xenobiotic compounds involved in the detoxification of electrophilic compounds, such as 4- hydroxy-trans-2-nonenal produced under oxidative stress conditions. Also, they are able to reduce a variety of lipophilic substrates, such as ketosteroids, ketoprostaglandins, and retinoids, which are hormone precursors (Penning and Drury, 2007).

There are four AKR1C isoforms present in human body: AKR1C1 (known as the human 20α-HSD) and AKR1C2 (known as the human 3 α-HSD type 3 and also known as bile acid binding protein), AKR1C3 (known as human 3 α-HSD type 2 or as human 17β- HSD type 5) and AKR1C4 (known as human 3 α-HSD type 1).

In spite of the high similarity in their sequence (>86%), these enzymes are involved in different physiological functions, with each human AKR1C enzyme preferring special positional and stereochemical substrates and also

having a distinctive tissue distribution pattern (Penning *et al*., 2000; Penning, 2003; Steckelbroeck *et al*., 2004).

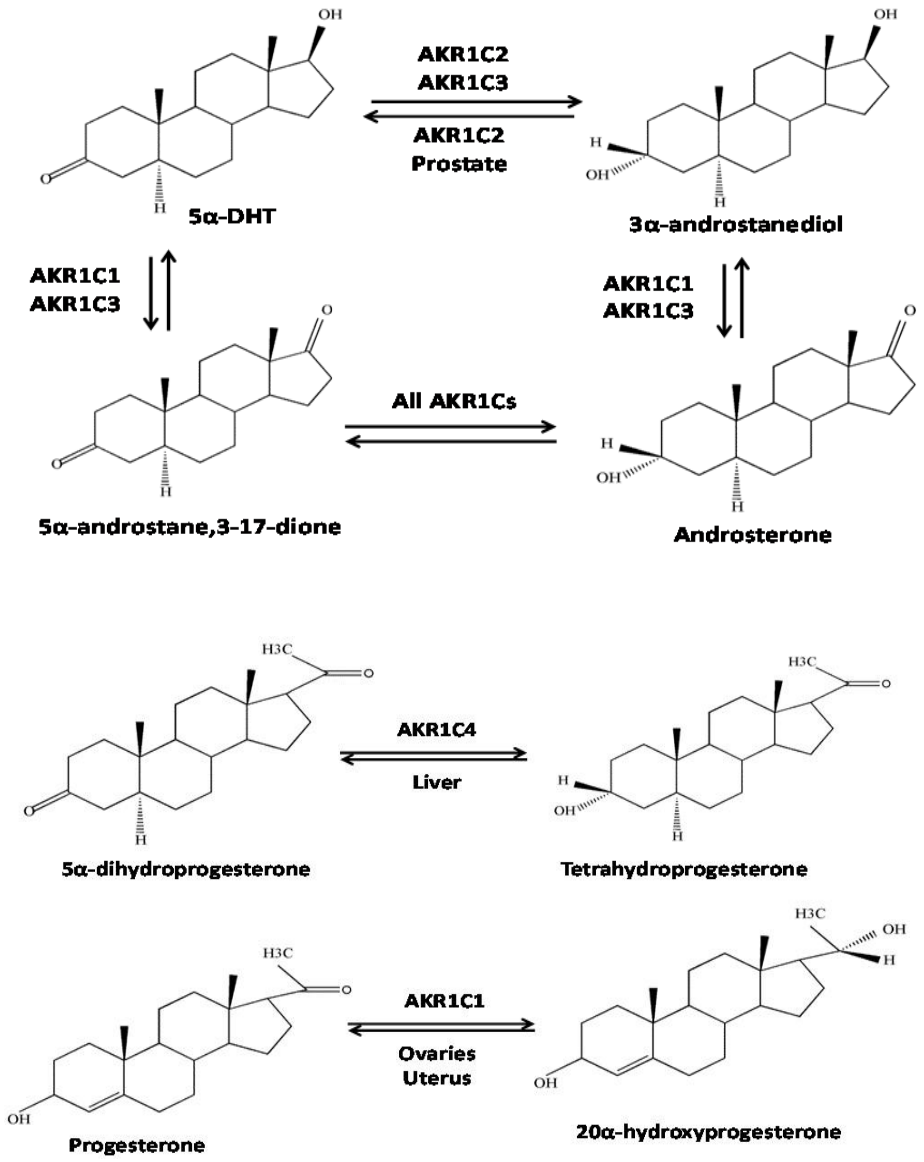
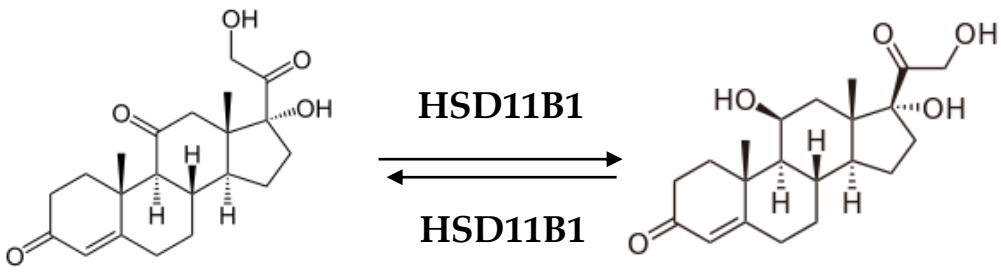


Figure 5.1.: **Illustration of steroid conversions which are catalyzed by the AKR1C family of enzymes**, some of AKR1C enzymes catalyzes oxidation-reduction reactions at the 3, 17, and 20 positions of the steroid molecule (Drasbek *et al*., 2006).

5.1.3 11β-Hydroxysteroid dehydrogenase type 1 (HSD11B1) gene

HSD11B1 is a hydroxysteroid dehydrogenase that belongs to the short chain dehydrogenase/reductase (SDR) family and is classified as SDR 26C, member 1. It functions as an NADPH-dependent reductase converting cortisone in humans to the active glucocorticoid cortisol (Figure 5.2.).

11β-Hydroxysteroid dehydrogenase isozymes catalyze the intracellular conversion of inert 11-keto glucocorticoids to physiologically active glucocorticoids in specific tissues, and vice versa. HSD11B1 mediates the pre-receptor activation of cortisone, which gives a mechanism for specific tissues to produce intracellular, non-adrenal cortisol, thereby locally amplifying glucocorticoid action (Seckl and Walker, 2001; Morton *et al*., 2001; Tomlinson *et al*., 2004).



Cortisone **Cortisol**

Figure 5.2: Interconversion of glucocorticoid catalyzed by 11β-HSDs

Human DHRS9 gene which is also called retinol dehydrogenase is a member of the short chain dehydrogenase/reductase (SDR) family, SDR9C4, is expressed in a variety of ciliated epithelial cell types. The product of the

DHRS9 gene is a retinol-metabolizing enzyme. DHRS9 is involved in the synthesis of all-trans retinoic acid from vitamin A. It has formerly been shown that DHRS9 gene expression is down-regulated in certain colon carcinomas, thus reducing the production of retinoic acid and limiting the inhibitory effects of retinoic acid on tumour cell growth (Jette *et al*., 2004; Duester, 2000).

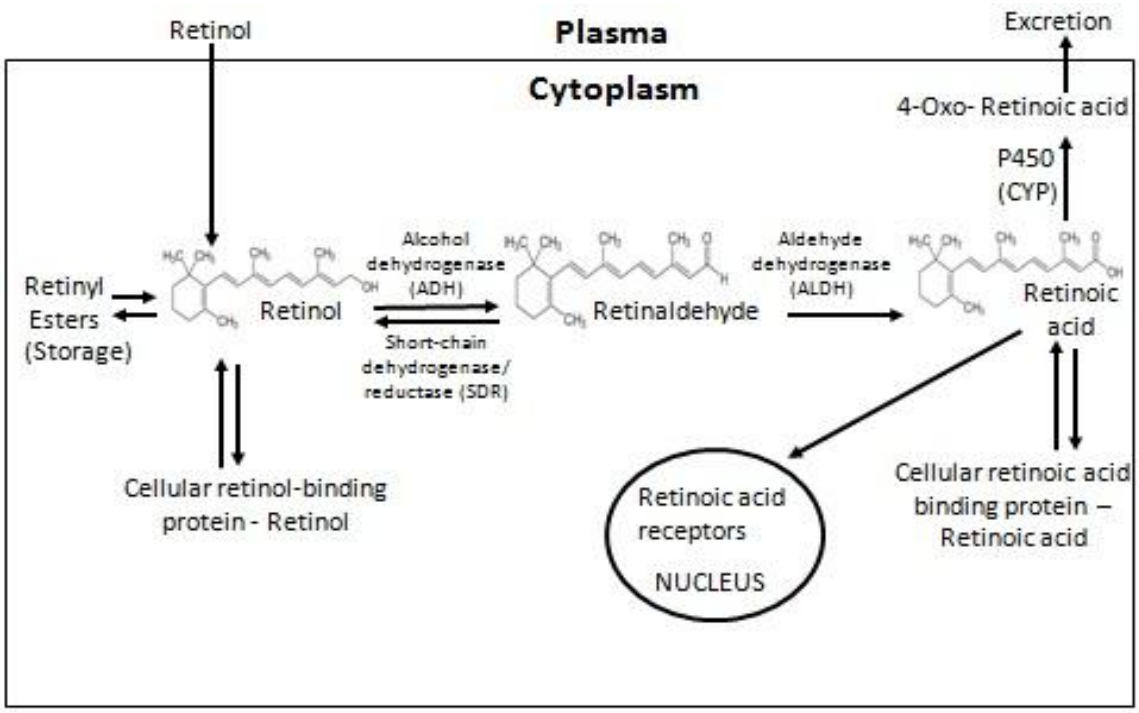
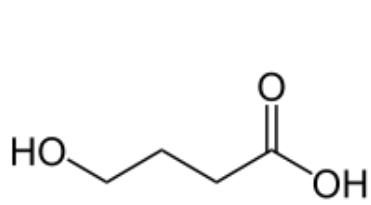


Figure 5.3: Retinol metabolism catalyzed by SDR enzymes. Adapted from (Molotkov *et al.*, 2004)

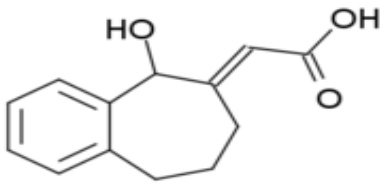
5.1.4 The GHB Structural Analogue NCS-382

GHB has been postulated to bind to specific GHB receptors as well as binding weakly to the $GABA_B$ receptor. So far, 6, 7, 8, 9-tetrahydro-5-hydroxy-5H-benzocyclohept-6-ylideneacetic acid (NCS-382), a compound structurally related to GHB, is the only compound reported to inhibit the

binding of GHB to the GHB receptor (Castelli *et al*., 2004). It does this by competitively binding to the GHB receptor (Maitre *et al*., 1990), and thereby antagonizes some of neuropharmacological effects of GHB (Maitre *et al*., 1990). NCS-382 is unlikely to be metabolized, but as a structural analogue of GHB it is likely to interact with cellular components and enzymes in a similar way to GHB.



GHB



NCS-382

Figure 5.4: Chemical structures of Gamma hydroxybutyric acid (GHB) and its analogue 6,7, 8, 9-tetrahydro-5-hydroxy-5H-benzocyclohept-6-ylideneacetic acid (NCS-382).

5.2 Aim of this chapter

The aim of this study is to validate the microarray results on the expression of specific genes encoding steroid dehydrogenases and aldo-keto reductases, and to investigate the mechanism by which GHB mediates changes in gene expression using the GHB structural analogue NCS-382.

5.3 Materials and methods

Human monocytic leukaemia (THP-1) cell line (Tsuchiya *et al*., 1980) was used in this study. THP-1 cells were treated with two concentrations (10μM and 900μM) of GHB, NCS- 382 or both for 24 hours. Methods used in this chapter include RNA isolation, reverse transcriptase PCR, DNA fragments purification from agarose gel and quantitative PCR. Materials and methods are described in chapter 2.

5.4 Results

5.4.1 RNA quality

RNA was isolated using SV Total RNA Isolation system from two cell lines before and after treatment with GHB, NCS - 382 or both for 24 hours. RNA yield at 260 nm was determined by Nanodrop 2000 UV-Vis Spectrophotometer.

5.4.2 PCR Amplification of cDNA and specific primers

A total of 5 genes encoding steroid dehydrogenases were investigated to validate the microarray results reported in Chapter 4. To test whether the primers were able to amplify AKR1C1, AKR1C3, AKR1C4, HSD11B1 and DHRS9, and the control GAPDH, cDNA was prepared from human monocytic leukaemia THP-1 mRNA and PCR reactions were carried out for control and GHB-treated cells. The PCR products were separated by electrophoresis along with 100kb DNA ladder to identify the size of the band (Fig 5.5). The results show that all five cDNA's were present in THP-1 cells and the predicted sizes of the products were 133, 590, 278, 115, 112 and 206bp for AKR1C1, AKR1C3, AKR1C4, HSD11B1, DHRS9 and GAPDH respectively.

5.4.3 Quantitative RT – PCR results

Once the primers of the five genes had been validated for use with these cells, quantitative RT-PCR was carried out to determine the relative levels of each mRNA following GHB treatment. In addition, the effect of the GHB structural analogue NCS-382 was also tested to show whether these effects were specific.

5.4.3.1 Effect of GHB treatment

The results show that treatment of THP-1 cells with GHB cause a significant reduction in the expression of AKR1C1, AKR1C3, AKR1C4, HSD11B1 and DHRS9 genes in blood THP-1 cells (Figures 5.6, 5.7 and 5.8 with Tables 5.1 and 5.2). The expression of these genes was found to be reduced significantly ($p<0.01$ and $p<0.05$) by between 2- and 9-fold and between 1.5- and 6-fold in blood THP-1 cells after the treatment with 900 and 10 μM of GHB respectively.

5.4.3.2 Effect of NCS-382 treatment

The results show that treatment of cells with NCS-382 also caused a significant reduction in the expression of AKR1C1, AKR1C3, AKR1C4 and DHRS9 genes in blood THP-1 cells (Figures 5.6, 5.7 and 5.8 with Table 5.2). The expression of these genes was found to be reduced significantly ($p<0.01$ and $p<0.05$) by between 3 and 42-fold and between 1.8- and 3.9-fold in blood THP-1 cells after treatment with 900 and 10 μM respectively of NCS-382 either alone or in combination with GHB at the same concentration.

The levels of HSD11B1 mRNA were found to be unchanged after treatment with NCS-382 alone or in combination with GHB.

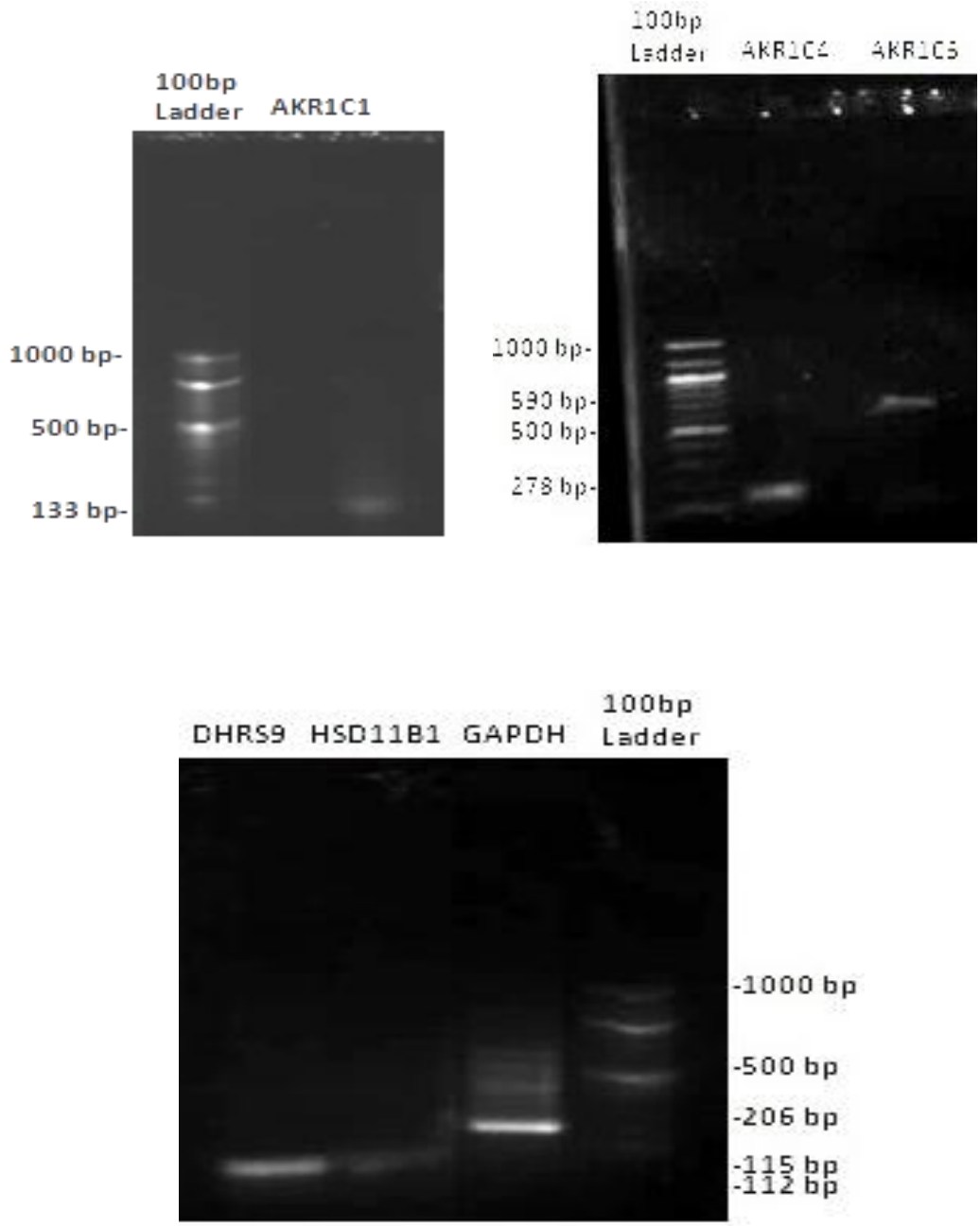
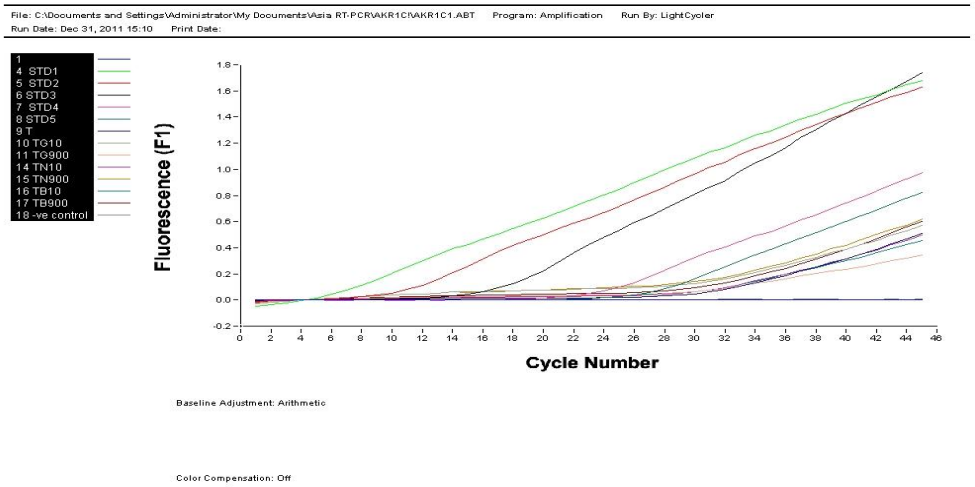


Figure 5.5: PCR amplification of AKR1C1, AKR1C3, AKR1C4, HSD11B1, DHRS9 and GAPDH from cDNA in THP-1 cells. Total RNA was isolated from treated and untreated cells, and then PEA-15 and Epiregulin genes were amplified by RT-PCR using specific primers. PCR products were run on 1% agarose gel electrophoresis along with DNA ladder. Products sizes were 133, 590, 278, 115, 112 and 206bp for AKR1C1, AKR1C3, AKR1C4, HSD11B1, DHRS9 and GAPDH respectively.

A)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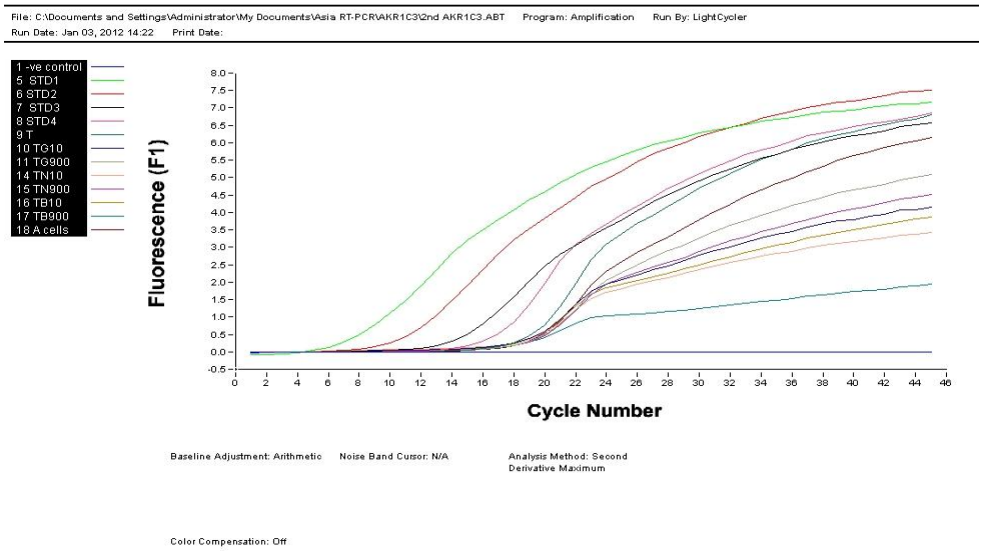
Run Date: Dec 31, 2011 15:10 Print Date:
1
4 STD1
5 STD2
6 STD3
7 STD4
8 STD5
9 T
10 TG10
11 TG900
14 TN10
15 TN900
16 TB10
17 TB900
18 -ve control
Fluorescence (F1)
Cycle Number
Baseline Adjustment: Arithmetic
Color Compensation: Off

B)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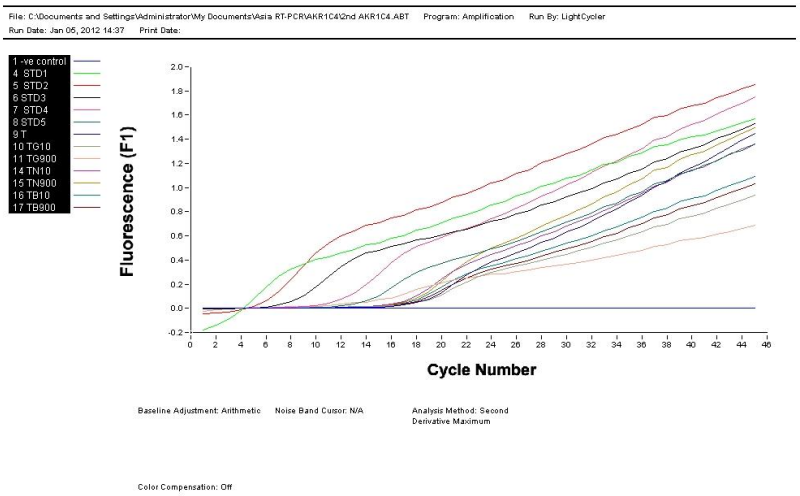
Run Date: Jan 03, 2012 14:22 Print Date:
1 -ve control
5 STD1
6 STD2
7 STD3
8 STD4
9 T
10 TG10
11 TG900
14 TN10
15 TN900
16 TB10
17 TB900
18 A cells
Fluorescence (F1)
Cycle Number
Baseline Adjustment: Arithmetic Noise Band Cursor: N/A Analysis Method: Second Derivative Maximum
Color Compensation: Off

C)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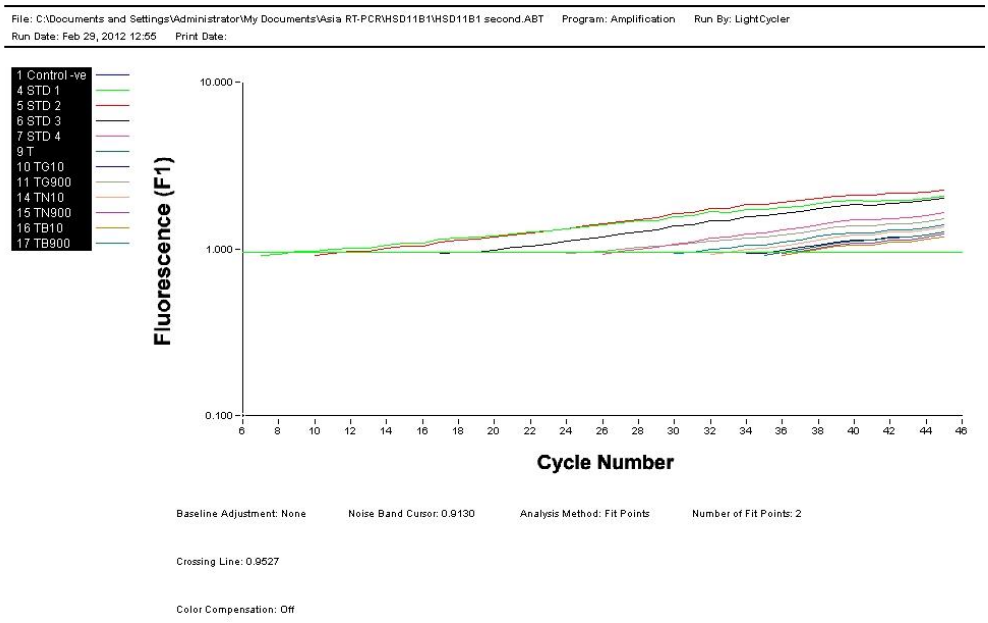
1 -ve control
4 STD1
5 STD2
6 STD3
7 STD4
8 STD5
9 T
10 TG10
11 TG900
14 TN10
15 TN900
16 TB10
17 TB900
Fluorescence (F1)
Cycle Number
Baseline Adjustment: Arithmetic Noise Band Cursor: N/A Analysis Method: Second Derivative Maximum
Color Compensation: Off

D)



E)

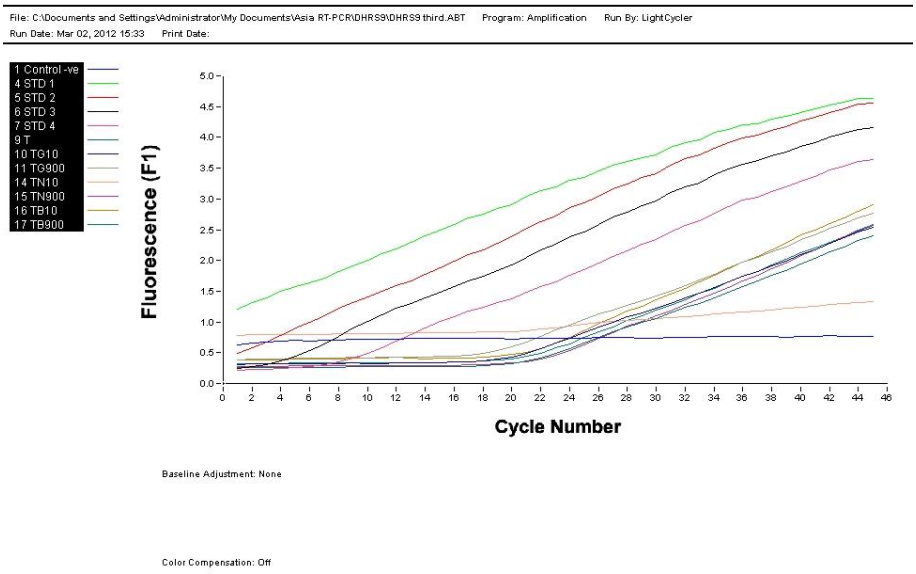
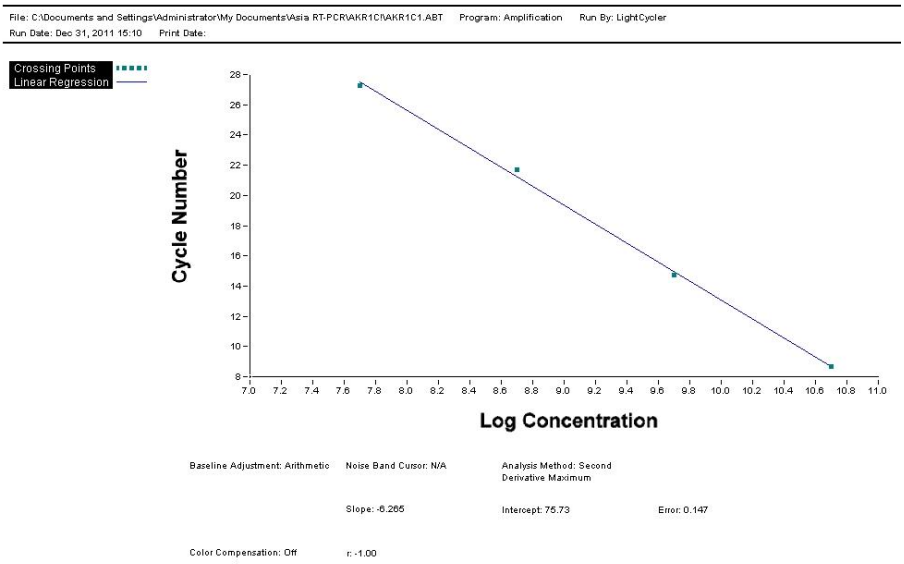


Figure 5.6: Amplification curves of A) AKR1C1, B) AKR1C3, C) AKR1C4, D) HSD11B1 and E) DHRS9 genes. The melting temperature for all genes was 60^{o}C and the amplicon sizes were 133, 590, 278, 115 and 112 bp for AKR1C1, AKR1C3, AKR1C4, HSD11B1 and DHRS9 respectively. (T) Represent untreated THP-1 cells, (TG10) and (TG900) represent THP-1 cells treated with 10μM and 900 μM GHB respectively, (TN10) and (TN900) represent THP-1 cells treated with 10μM and 900 μM NCS-382 respectively and finally, (TB10) and (TB900) represent THP-1 cells treated with either 10μM or 900 μM of both GHB and NCS-382.

A)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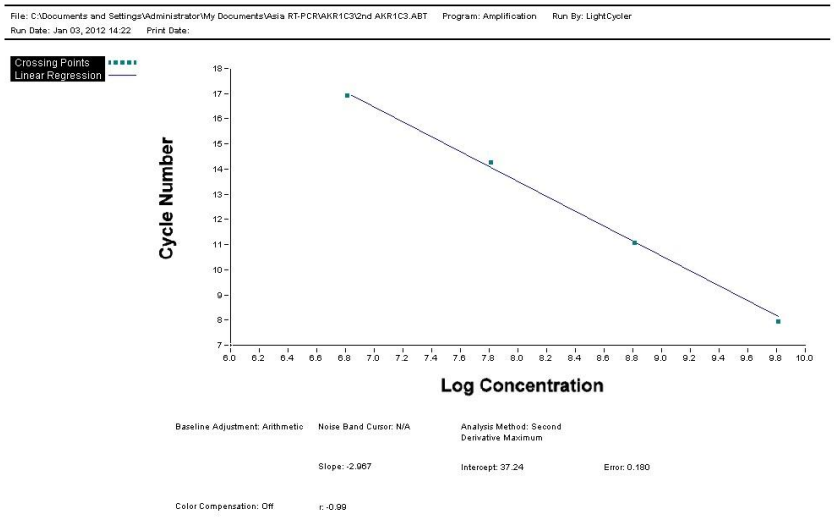
Crossing Points
Linear Regression
Cycle Number
Log Concentration
Baseline Adjustment: Arithmetic
Noise Band Cursor: N/A
Analysis Method: Second Derivative Maximum
Slope: -6.266
Intercept: 75.73
Error: 0.147
Color Compensation: Off
r: -1.00

B)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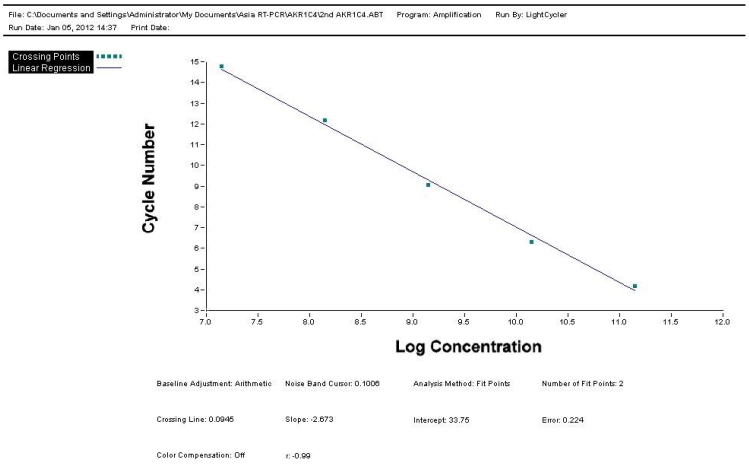
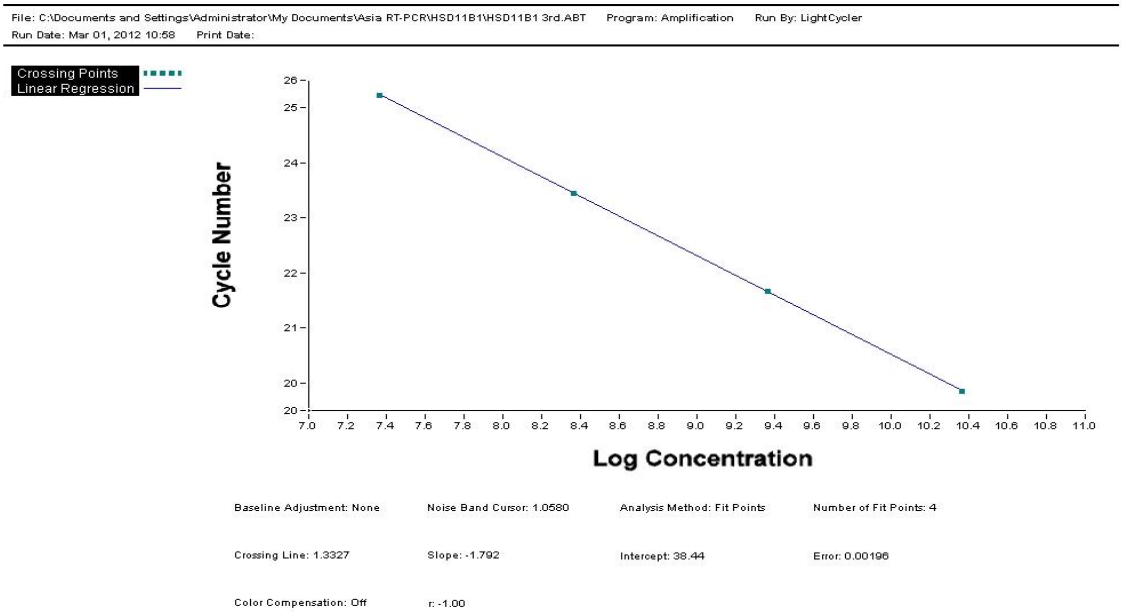
Run Date: Jan 03, 2012 14:22 Print Date:
Crossing Points
Linear Regression
Cycle Number
Log Concentration
Baseline Adjustment: Arithmetic
Noise Band Cursor: N/A
Analysis Method: Second Derivative Maximum
Slope: -2.967
Intercept: 37.24
Error: 0.180
Color Compensation: Off
r: -0.99

C)


File: C:\Documents and Settings\Administrator\My Documents\Asia RT-PCR\AKR1C4\2nd AKR1C4.ABT Program: Amplification Run By: LightCycler
Run Date: Jan 05, 2012 14:37 Print Date:
Crossing Points
Linear Regression
Cycle Number
Log Concentration
Baseline Adjustment: Arithmetic
Noise Band Cursor: 0.1005
Analysis Method: Fit Points
Number of Fit Points: 2
Crossing Line: 0.0945
Slope: -2.673
Intercept: 33.75
Error: 0.224
Color Compensation: Off
r: -0.99

D)



E)

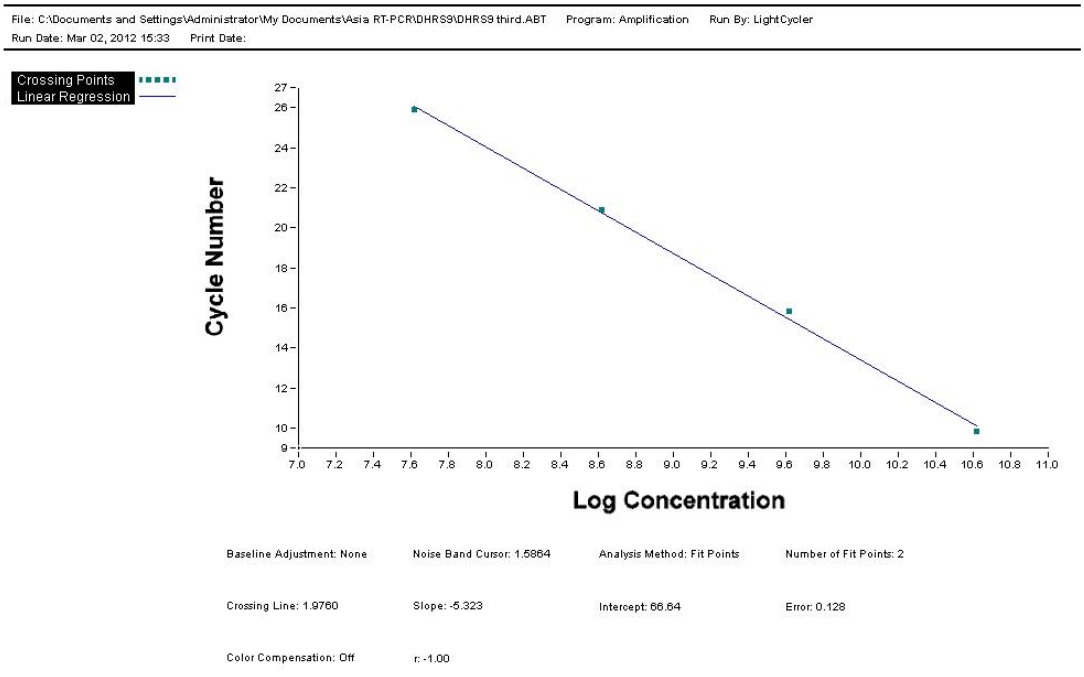


Figure 5.7: Standard curve for A) AKR1C1, B) AKR1C3, C) AKR1C4, D) HSD11B1 and E) DHRS9 genes. The melting temperature for all genes was 60^{o}C and the standard curves were constructed with serial 10-fold dilutions of the DNA (purified from agarose gel) ranging from 10^{5} to 10^{9}copies/µl. Each standard dilution was amplified by real-time QPCR using specified primers.

Table 5.1: Quantitative RT-PCR validation of microarray results for specific genes encoding for steroid dehydrogenases and aldo-keto reductases after 24 hour exposure to GHB

Gene Symbol	REFSEQ transcript	Fold-change: microarray		Fold-change: real-time PCR	
		10μM	900μM	10μM	900μM
AKR1C1	NM_001353	-1.14*	-5.9***	-3.28**	-5.98**
AKR1C3	NM_003739	0	-3.39***	-2.25**	-8.43**
AKR1C4	NM_001818	-1.12*	-6.7***	-5.88**	-9.18**
HSD11B1	NM_181755	0	-2.96***	-1.57**	-2.25**
DHRS9	NM_005771	0	-3.25***	-1.56	-2.07*

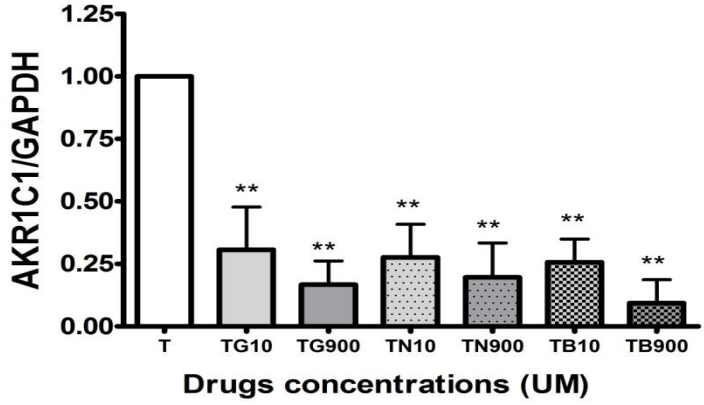
The results are mean values, n = 3 for microarrays and for real-time PCR.
*P< 0.05, ** P < 0.01, *** P < 0.001 compared with the control cells.

Table 5.2: Fold change in expression of specific genes encoding steroid dehydrogenases and aldo-keto reductases after 24 hour exposure to GHB, NCS-382 or both.

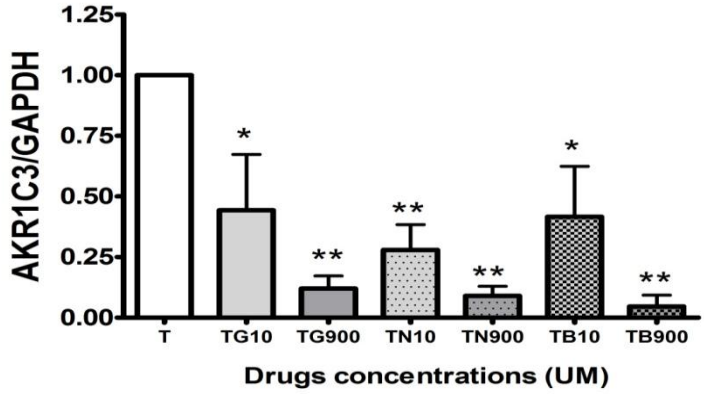
Gene Symbol	GHB		NCS-382		GHB+ NCS-382	
	10μM	900μM	10μM	900μM	10μM	900μM
AKR1C1	-3.28**	-5.98**	-3.63**	-5.15**	-3.89**	-10.79**
AKR1C3	-2.25*	-8.43**	-3.58**	-11.01**	-2.4*	-20.93**
AKR1C4	-5.88**	-9.18**	-1.76*	-2.98**	-2.57**	-42.71**
HSD11B1	-1.57	-2.25*	-1.38	-1.16	-1.30	0.94
DHRS9	-1.56	-2.07*	1.37	-6.44**	1.16	-5.19**

The results are mean values, n = 3 using real-time PCR.
*P< 0.05, ** P < 0.01, *** P < 0.001 compared with the control cells.

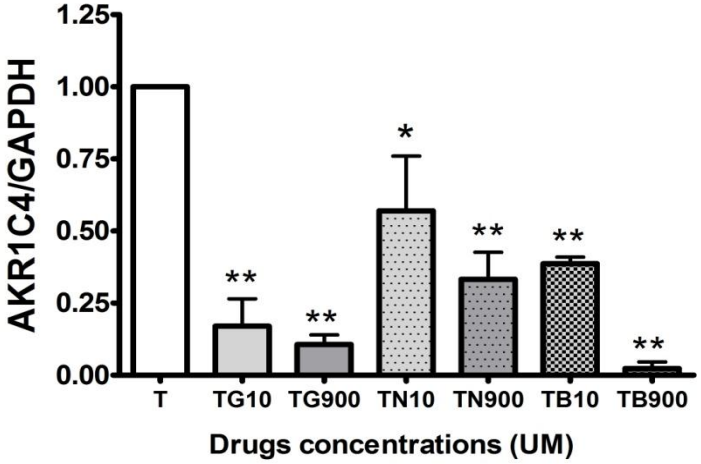
A)


AKR1C1/GAPDH
1.25
1.00
0.75
0.50
0.25
0.00
**
**
**
**
**
**
T
TG10
TG900
TN10
TN900
TB10
TB900
Drugs concentrations (UM)

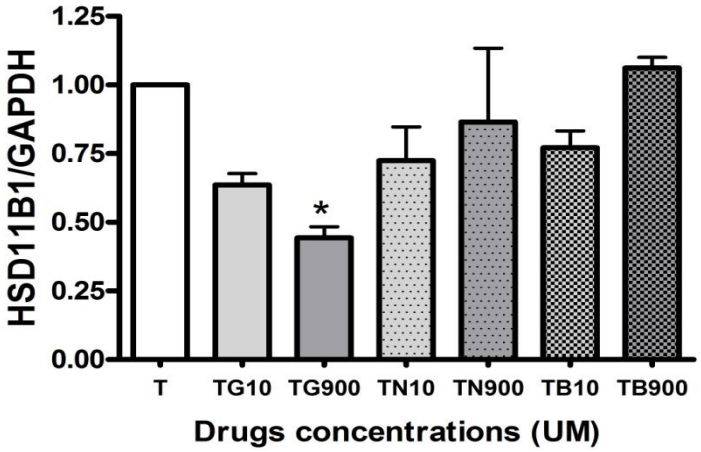
B)


AKR1C3/GAPDH
1.25
1.00
0.75
0.50
0.25
0.00
*
**
**
**
*
**
T
TG10
TG900
TN10
TN900
TB10
TB900
Drugs concentrations (UM)

C)


AKR1C4/GAPDH
1.25
1.00
0.75
0.50
0.25
0.00
**
**
*
**
**
**
T
TG10
TG900
TN10
TN900
TB10
TB900
Drugs concentrations (UM)

D)



E)

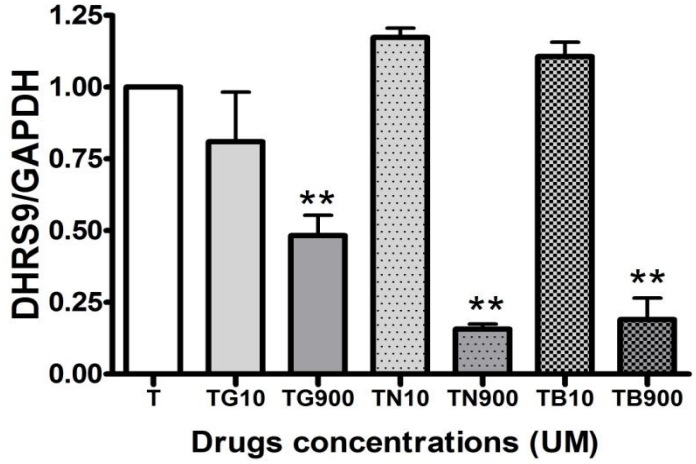


Figure 5.8: Fold change in mRNA levels of specific genes encoding for steroid dehydrogenases and aldo-keto reductases (a) AKR1C1 (b) AKR1C3 (c) AKR1C4 (d) HSD11B1 and (e) DHRS9 after 24 hour exposure to GHB, NCS - 382 or both. Gene expression was quantified by normalization to GAPDH as an internal control. (T) Represent untreated THP-1 cells, (TG10) and (TG900) represent THP-1 cells treated with 10μM and 900 μM GHB respectively, (TN10) and (TN900) represent THP-1 cells treated with 10μM and 900 μM NCS-382 respectively and finally, (TB10) and (TB900) represent THP-1 cells treated with either 10μM or 900 μM of both GHB and NCS-382. The untreated cells were used as control (**$p<0.01$, *$p<0.05$).

5.5 Discussion

In Chapter 4, the effect of two selected concentrations 10μM and 900μM of GHB on the gene expression profile of THP1 cells revealed that GHB induced changes in the expression of genes encoding steroid dehydrogenases in blood THP-1 cells.

This chapter aimed at validating the microarray results as well as investigating the mechanism by which GHB mediates changes in gene expression using the structural analogue NCS-382. The effect of GHB on the expression of these genes in blood THP-1 cells was studied using quantitative RT-PCR and the results show that treatment of cells with GHB lead to a significant reduction in the expression of AKR1C1, AKR1C3, AKR1C4 and DHRS9 gene expression in blood THP-1 cells, supporting the previous microarray data.

Our results are the first to show that GHB and NCS-382 induced changes in expression of genes encoding steroid dehydrogenases/aldo-keto reductases in blood THP-1 cells.

The effect of NCS-382 on expression of these genes was surprising. NCS-382 is known to bind to GHB receptor, but no previous study has examined its ability to cause changes within THP-1 cells. As THP-1 cells are not known to possess GHB receptors, and NCS-382 is known to not bind the GABA receptor this results indicates that the effect caused by NCS-382 is likely to be mediated intracellularly. This strongly suggests that either the GHB-induced changes are mediated via a GABA receptor, or are also likely to be mediated intracellularly in which case the role of an extracellular receptor may not be significant in THP-1 cells.

GHB is almost completely ionized at physiological pH, and does not easily cross cell membranes. However, it is possible that GHB could enter blood cells using transporter mechanisms such as the monocarboxylate transporters, which have been reported to transport GHB in rat kidney cortex membrane vesicles (Wang *et al*., 2006). This uptake is inhibited by moncarboxylates such as D-lactate. The uptake of MCT substrates in erythrocytes and cardiac myocytes can be inhibited by GHB, suggesting that it uses MCT transporters in these tissues as well (Poole and Halestrap, 1993). However, previous studies have not shown that THP-1 cells possess MCT transporters (Verhoeven and van Griensven, 2012).

The effects of GHB and NCS-382 appear to be additive for some genes and synergistic for others. For example, the effect of GHB on the expression of AKR1C1, AKR1C3, AKR1C4, and DHRS9 genes is not affected by NCS-382 treatment. The levels of HSD11B1 mRNA were found to be reduced significantly only after the treatment with 900μM GHB but neither after NCS-382 nor their combination.

No previous study has examined the effect of GHB on the level of steroid hormone dehydrogenases. Several other factors are known to induce AKR1C expression, including oxidative and chemical stress, and HSD11B1 is known to be induced by cortisone via a PPARγ–mediated mechanism and also by interleukin-1 (Yong *et al*., 2002). It is not known what compounds control expression of DHRS9. Dydrogesterone has been shown to significantly reduce SRD5A1, AKR1C2 and AKR1C3 expression level in Z-12 epithelial cells (Beranic and Rizner, 2012).

Further experiments are therefore needed to confirm that these results are a specific response to GHB exposure and to illustrate the mechanism by which GHB exerts these effects in more detail. Althought the results presented indicate that GHB may not be acting through a specific GHB receptor, its ability to enter THP-1 cells needs to be investigated thoroughly, through considering its use of the MCT transporters. This can be tested by cotreating with MCT transporter substrates such as D-lactate. In addition the role of the GABA receptors needs to be investigated, using specific molecules that bind the GABA receptor such as CGP-35348. These experiments would determine if the changes in gene expression levels are specific for GHB or give an indication that these changes are a consequence of a complex relationship between the GHB and GABAergic systems in THP-1 cells.

For consideration as specific biomarkers for GHB exposure, further studies are also needed to investigate genes expression after exposure to other abused drugs such as other CNS depressants, such as benzodiazepines, barbiturates and alcohol. No previous study has examined the effect of barbiturates on any of the genes encoding steroid dehydrogenases or aldo-keto reductases. However one study in rat revealed that 11β- HSD2 is induced in alcoholic liver disease (Ahmed *et al*., 2008), indicating that alcohol dependence may affect the usefulness of this gene as a biomarker.

CHAPTER 6

Proteomic Screen for GHB exposure In human Brain and Blood cells

6 Proteomic Screen for GHB exposure In human Brain and Blood cells

6.1 Introduction

In Chapter 3, the effect of GHB on cell viability and gene expression in SH-SY5Y human neuroblastoma cells and 1321N1 human astrocytoma cells was investigated and it was shown that GHB produces significant changes in specific investigated genes and on Pea-15 protein expression levels in brain cells and blood THP-1 cells. Furthermore, in Chapter 4 the effect of GHB on the gene expression profiles in blood THP-1 cells was investigated and this showed that GHB causes significant changes in the expression of various transcripts within the cell. These changes might have the potential of being used as markers of GHB exposure, if a clear and specific link can be demonstrated to GHB exposure.

In addition to investigating changes in mRNA levels, changes in protein levels have been adopted as a useful approach for examining drug-induced changes in protein expression to find novel biomarkers for drug exposure. In the middle of the 1970s several 2-dimentional gel electrophoresis (2-DE) techniques were developed for monitoring protein expression (MacGillivray and Rickwood, 1974; Klose, 1975; O'Farrell, 1975; Scheele, 1975; Iborra and Buhler, 1976). In the subsequent years, the 2-DE technique published by O'Farrell (1975) was the most commonly used for the comprehensive analysis of protein mixtures from different cells, tissues and biological samples in order to establish a protein profile. This technique separates proteins based on their isoelectric points (pI) in the first dimension step, followed by the second dimension step in which the proteins are separated on the basis of their molecular weight (MW) in an SDS-PAGE. Each spot of

the separated proteins corresponds to a single protein of different molecular mass (MW) and isoelectric point (pI) (Klose, 2009).

As detailed in the introduction, several previous studies have examined GHB-and GBL-dependent changes in protein expression in rat and mouse brain using a combination of proteomics, 2-D gels and Western blots (Van Nieuwenhuijzen *et al*., 2010; Zanata *et al*., 2002). This led to the identification of several potential markers of exposure including collapsin response mediator proteins and mouse stress induced phosphoprotein, as well as a glutathione S-transferases Yb4, neuronal pentraxin-1 and PEA-15 (Van Nieuwenhuijzen *et al*., 2010; Zanata *et al*., 2002).

However, these previous studies focused on changes occurring in the brain, which is not a suitable tissue for biomarkers use, and also no study has been carried out to look at the changes in human cells.

6.2 Aim of this chapter

The aim of this chapter is to use proteomics approaches to find novel and robust surrogate markers for detection of GHB beyond 12 hours. In order to gain further insight into the changes in the proteome induced by GHB, two-dimensional gel electrophoresis (2-D gel electrophoresis) was carried out on the whole cell extracts, and protein spots were picked and analysis by electro spray ionization (ESI-TRAP) and Protein identification by MASCOT software.

In this study, SH-SY5Y human neuroblastoma neuronal cells and 1321N1 human astrocytoma non neuronal cells were used as brain cells model and human monocytic leukaemia (THP-1) cell line was used as blood cell model

to study the effects of GHB on protein expression in blood cells because of their advantages mentioned in section 1.9.1.3.

6.3 Materials and methods

Human neuroblastoma SH-SY5Y cells (Biedler *et al*., 1973 and Biedler *et al*., 1978), human astrocytoma 1321N1 cells (Clark *et al*., 1975), and human monocytic leukemia THP-1 cell line (Tsuchiya *et al*., 1980) were used in this work.

Methods used in this chapter include cell culture, two dimensional gel electrophoresis (2-D gel), staining the gels, imaging the gels, analysis of gel images and spots picking, trypsin digestion of the picked spots, analysis by electro spray ionization (ESI-TRAP) and Protein identification by MASCOT software. Materials and methods are described in chapter 2.

6.4 Results

6.4.1 Protein separation by 2 – D electrophoresis from 1321N1, SH-SY5Y and THP-1 cell lines after 24 hour of GHB exposure

In order to determine the influence of 1μM and 100μM GHB concentrations on the proteomic profile of SH-SY5Y and 1321N1 cell lines and the influence of 10μM and 900μM GHB concentrations on the proteomic profile of THP-1 cell line after 24 hr exposure to GHB , 2-D gel electrophoresis was carried out on whole cell extracts using Immobiline TM IPG gel strips (based on isoelectric point, pI) for the first dimensional separation, and SDS-PAGE electrophoresis (based on molecular weight) for the second dimensional separation. As described in materials and methods the gels were stained with colloidal coomassie blue, photographed by Fujifilm LAS- 3000 Imaging

system and the pictures were analysed by Progenesis SameSpots software using a reference gel produced from control untreated cells.

More than 300 spots were detected and analysed in each gel by Progenesis SameSpots software comparing protein spots between gels produced from treated cells and control reference gel produced from untreated cells.

6.4.2 Changes in Protein expression in brain cells

The 2-D gel electrophoresis patterns of the proteomic profiles in brain cells SH-SY5Y and 1321N1 cells after 24hr exposure to 1μM and 100μM GHB concentrations are illustrated in Figures 6.1 and 6.2 respectively.

A total of 44 and 37 spots were altered in SH-SY5Y and 1321N1 cells respectively by at least 1.5 fold after 24h exposure to GHB. Some of these proteins were over-expressed and others were under-expressed after GHB exposure, as illustrated in table 6.1.

6.4.3 Changes in Protein expression in blood cells

The 2-D gel electrophoresis patterns of the proteomic profile for THP-1 cells after 24hr exposure to 10μM and 900μM GHB concentrations are shown in Figure 6.3.

A total of 27 spots were altered in THP-1 cells by at least 1.5 fold after 24h exposure to GHB. Some of these proteins were over-expressed and others were under-expressed after GHB exposure, as illustrated in table 6.1.

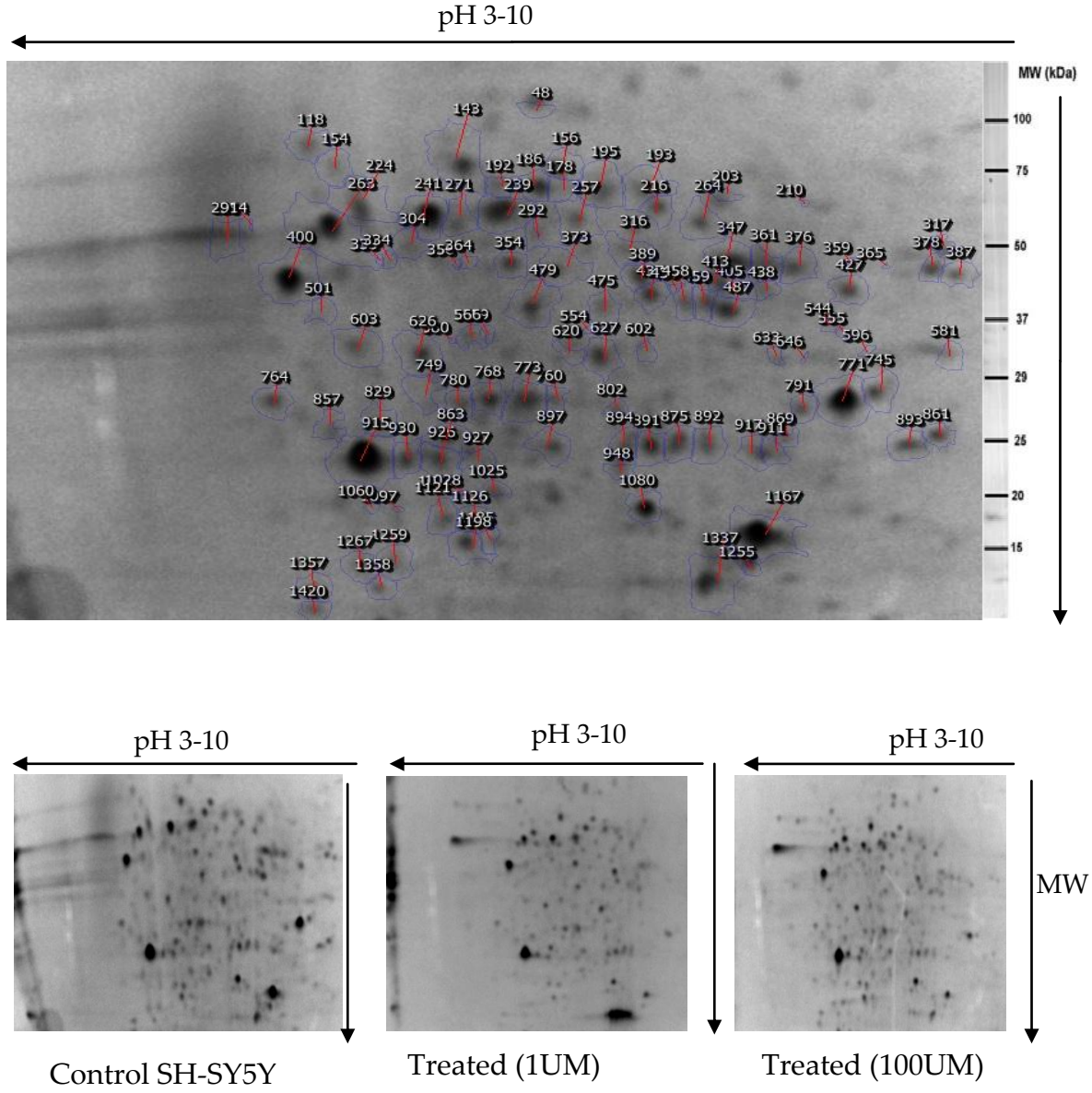


Figure 6.1: Two-dimensional gel electrophoresis of SH-SY5Y cells before and after 24 h treatment with GHB. SH-SY5Y cells were exposed to 1μM and 100μM GHB concentrations for 24hr and whole cell extracts were separated on a rehydrated immobilineTM IPG DryStrips (pH 6- 9) based on isoelectric point and then separated based on Molecular weight (MW) by SDS-PAGE electrophoresis, the gels were stained with colloidal coomassie blue, photographed by Fujifilm LAS- 3000 Imaging system and the pictures were analysed by progenesis samespote software using a reference gel produced from control untreated cells. The spots were numbered on control cells image by progenesis same spots software.

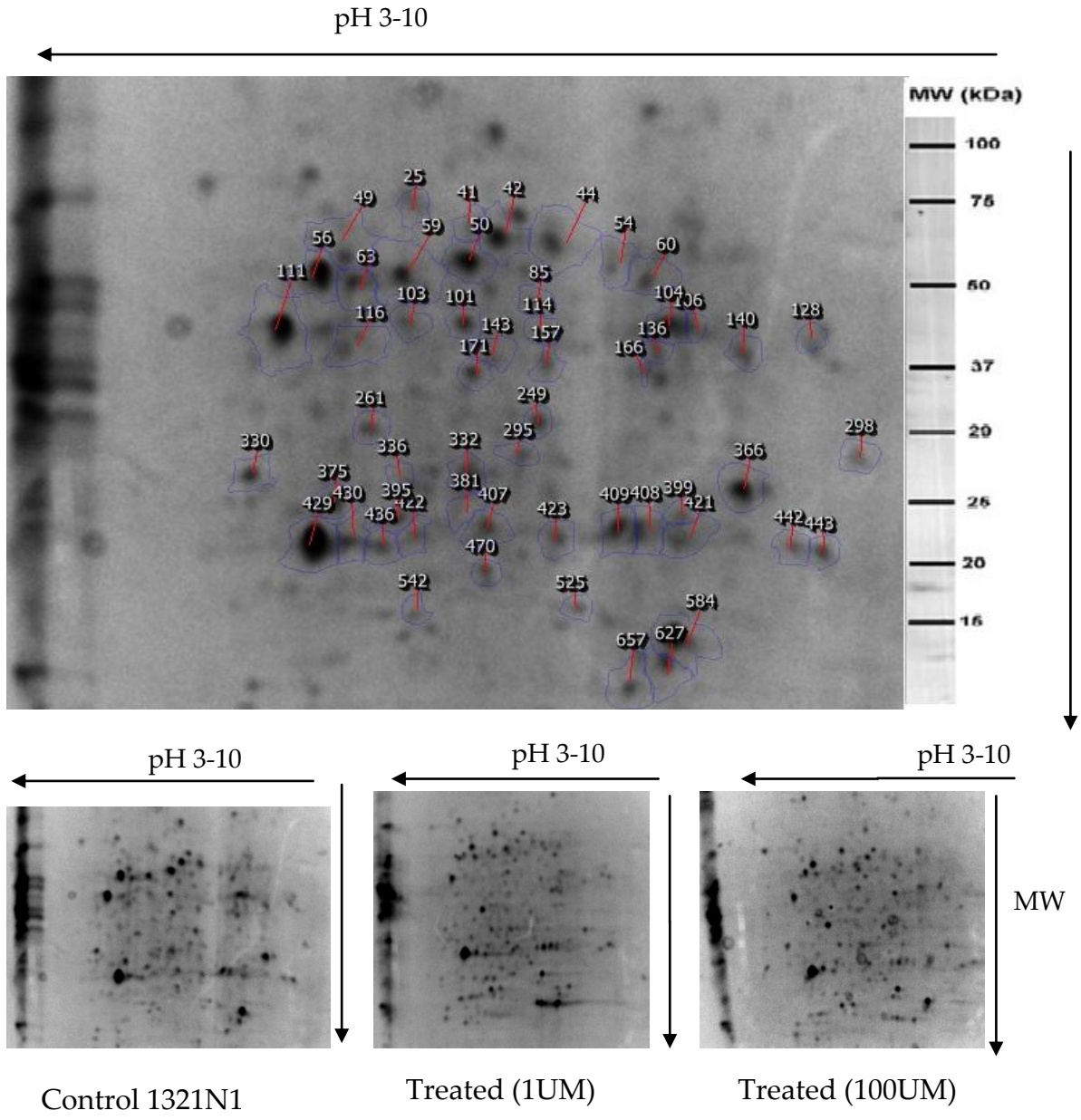


Figure 6.2: Two-dimensional gel electrophoresis of 1321N1 cells before and after 24 h treatment with GHB. 1321N1 cells were exposed to 1μM and 100μM GHB concentrations for 24hr and whole cell extracts were separated on a rehydrated immobilineTM IPG DryStrips (pH 6- 9) based on isoelectric point and then separated based on Molecular weight (MW) by SDS-PAGE electrophoresis, the gels were stained with colloidal coomassie blue, photographed by Fujifilm LAS- 3000 Imaging system and the pictures were analysed by progenesis samespote software using a reference gel produced from control untreated cells. The spots were numbered on control cells image by progenesis same spots software.

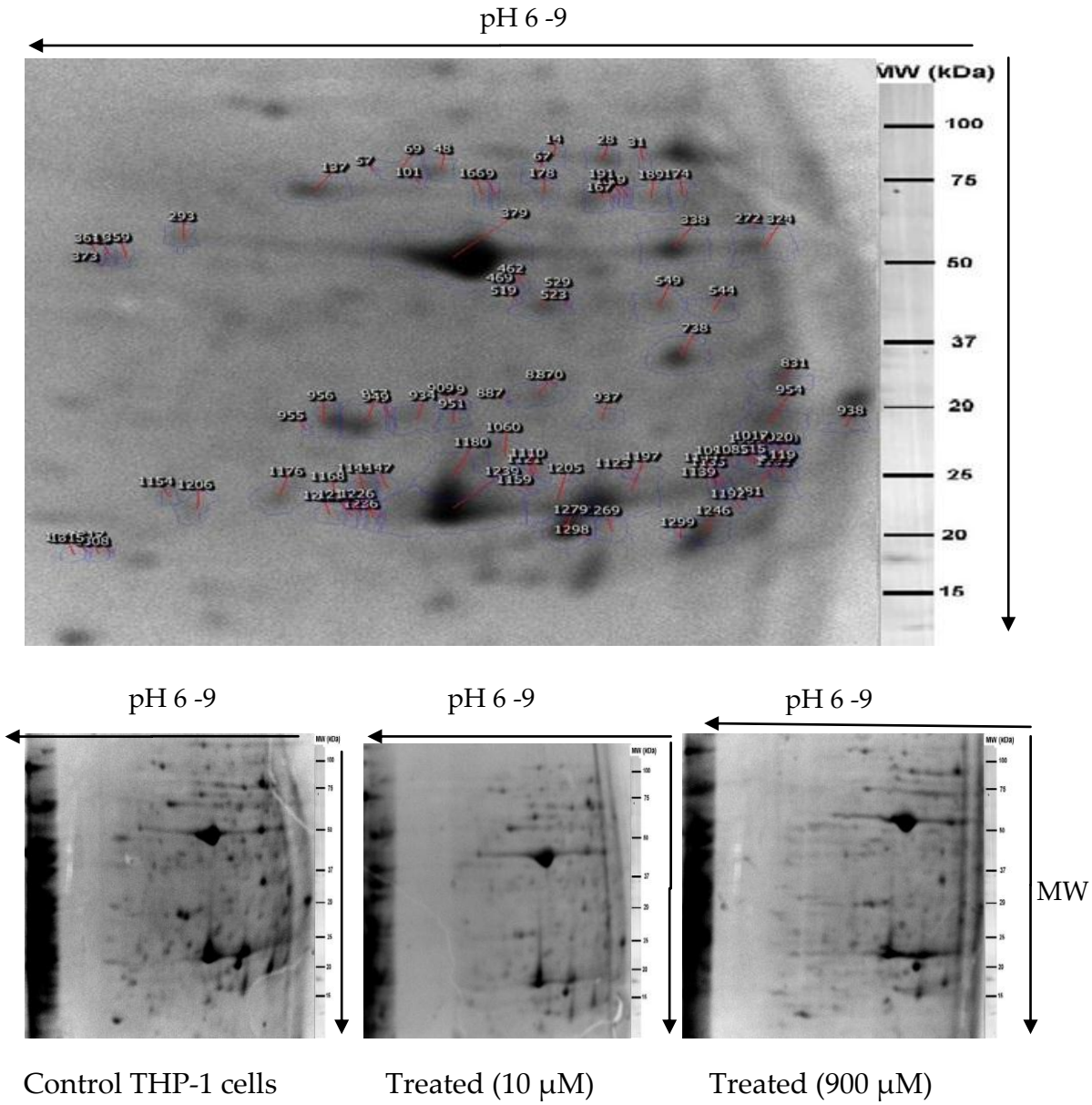


Figure 6.3: Two-dimensional gel electrophoresis of THP-1 cells before and after 24 h treatment with GHB. THP-1 cells were exposed to 10μM and 900μM GHB concentrations for 24hr and whole cell extracts were separated on a rehydrated immobilineTM IPG DryStrips (pH 6- 9) based on isoelectric point and then separated based on Molecular weight (MW) by SDS-PAGE electrophoresis, the gels were stained with colloidal coomassie blue, photographed by Fujifilm LAS- 3000 Imaging system and the pictures were analysed by progenesis samespote software using a reference gel produced from control untreated cells. The spots were numbered on control cells image by progenesis same spots software.

Table 6.1. The numbers of regulated proteins after 24h exposure to GHB.

Cell line	Low concentration *	High concentration **
SH-SY5Y cells	21 (I) 24 (R)	22 (I) 18 (R)
1321N1 cells	17 (I) 21 (R)	13 (I) 10 (R)
THP-1 cells	14 (I) 13 (R)	13 (I) 12 (R)

*Low concentration of GHB is 1μM for 1321N1 and SH-SY5Y cells and 10μM for THP-1 cells. ** High concentration of GHB is 100μM for 1321N1 and SH-SY5Y cells and 900μM for THP-1 cells. I= induced and R= repressed.

6.4.4 Changes in the identification of altered protein spots from GHB treated cells using ESI-TRAP and MASCOT

Significantly altered spots in the gels that produced from SH-SY5Y and 1321N1 brain cells and THP-1 blood cells were picked from the gels and the proteins were extracted from the spots by trypsin digestion and analysed using Electro spray ionization (ESI-TRAP) in Glasgow University. After that, MASCOT software was used to identify protein from primary sequence databases. The results of analysed spots from brain cells and blood cells were illustrated in Tables 6.2 and 6.3 respectively.

Table 6.2: The identification of altered protein spots from GHB treated brain cells SH-SY5Y and 1321N1 compared with control cells using ESI-TRAP and MASCOT.

Cell type	Spot no.	Score	Protein name	Molecular function	NCBI Reference	MW (Dalton)	pI	Fold change	
								10 uM	900 uM
1321N1	56	1498	ENO1 Isoform alpha-enolase	glycolysis	NP_001419.1	47481	7.01	1.5	1.2
1321N1	103	765	Moesin	leukocyte adhesion	NP_002435.1	66678	5.9	-1.8	-1.3
1321N1	366	584	Carbonic anhydrase 2	Reversible hydration of CO2	P00918.2	29285	6.87	-1.7	-1.3
1321N1	298	103	Heat shock protein-27 (HSPB1)	chaperone	NP_001531.1	22427	3.2	1.43	2.4
SHSY-5Y	1337	913	Chain A, Three Dimensional Structure Of Human Electron Transfer Flavop rotein To 2.1 A Resolution	electron carrier activity	GI:2781202	33418	6.95	2.08	2.13
SHSY-5Y	354	403	chaperonin (HSPD1 or HSP60)	chaperone	NP_001083970.1	61157	6.0	1.1	1.6
SHSY-5Y	257	844	HSP70-2	chaperone	GI:4529892	70267	5.48	1.2	1.9

Brain cells were exposed to 1μM and 100μM concentrations of GHB for 24hr and whole cell extracts were separated on a rehydrated immobilineTM IPG DryStrips (pH 3- 10) based on isoelectric point (pI) and then separated based on Molecular weight (MW) by SDS-PAGE electrophoresis, the gels were stained with colloidal coomassie blue, photographed by Fujifilm LAS- 3000 Imaging system and the pictures were analysed by progenesis samespote software using a reference gel produced from control gel. The altered spots were picked up from the gel, analysed by using Electro spray ionization (ESI-TRAP) and MASCOT software was used to identify protein from primary sequence databases.

Table 6.3: The identification of altered protein spots from GHB treated blood cells THP-1 compared with untreated cells using ESI-TRAP and MASCOT.

Spot no.	Score	Protein name	Molecular function	NCBI Reference	MW (Dalton)	pI	Fold change	
							10 uM	900 uM
137	670	Adenylyl cyclase-associated protein 1	regulates filament dynamics	NP_001099000.1	51752	8.27	1.5	2.0
272	205	ENO1 Isoform alpha-enolase	glycolysis	NP_001419.1	112357	7.58	1.6	1.8
293	761	ENO2 Isoform alpha-enolase	glycolysis	NP_001966.1	47467	7.01	1.5	1.7
544	91	HNRPDL heterogeneous nuclear ribonucleoprotein D-like	transcriptional regulator	NP_112740.1	57832	9.54	- 1.5	-1.9
956	86	ALDOA Fructose-bisphosphate aldolase A	glycolysis	GI:461187	96345	6.58	1.3	2.3
949	125	GAPDH 39 kDa protein	glycolysis	GI:378404906	38767	8.76	1.07	1.66
954	681	Heat shock protein-70 (HSP-70) protein 8	chaperone	NP_694881.1	53541	5.62	1.1	1.85
938	374	HSP-90 B1 Endoplasmin	chaperone	NP_003290.1	119548	5.68	1.06	1.53
1205	555	TPI-2 triosephosphate isomerase isoform 2	glycolysis	NP_001152759.1	31057	5.65	1.13	1.56
1298	1193	TPI-1 triosephosphate isomerase isoform 1	glycolysis	NP_000356.1	19035	5.25	1.4	2.4

THP-1cells were exposed to 10μM and 900μM concentrations of GHB for 24hr and whole cell extracts were separated on a rehydrated immobilineTM IPG DryStrips (pH 6- 9) based on isoelectric point and then separated based on Molecular weight (MW) by SDS-PAGE electrophoresis, the gels were stained with colloidal coomassie blue, photographed by Fujifilm LAS- 3000 Imaging system and the pictures were analysed by progenesis samespote software using a reference gel produced from control gel. The altered spots were picked up from the gel, analysed by using Electro spray ionization (ESI-TRAP) and MASCOT software was used to identify protein from primary sequence databases.

6.4.5 Validation of Protein changes by Western blotting

The results showed that the expression of several heat shock proteins such as HSP-27, HSP-60, HSP-70 and HSP-90 were changed after 24h exposure to GHB in cell lines. In order to validate the 2-D gel results about the effect of GHB on HSP-70 protein expression, Western blotting analysis for protein was carried out on blood and brain cell lines.

Brain cells (1321N1 and SH-SY5Y cells) were treated with 1 μM and 100 μM concentrations of GHB and blood cells (THP-1 cells) were treated with 10 μM and 900 μM concentrations of GHB for 24 hours. Western blots were carried out on whole cell extracts using HSP-70 antibody (Abcam) and GAPDH was used for the normalisation and loading on 10% SDS- PAGE gel. The protein concentrations of the loaded samples were determined by Bradford method and approximately 40μg of samples were loaded. Protein bands were quantified by image J relative to untreated control. The results of Western blots showed that HSP-70 protein expression levels were increased significantly by 2, 1.83 and 1.78 fold in THP-1, SH-SY5Y and 1321N1 cells respectively ($p<0.01$) after 24h exposure to 100 μM in brain cells and 900μM GHB in blood cells, as showed in (Figure 6.4.).

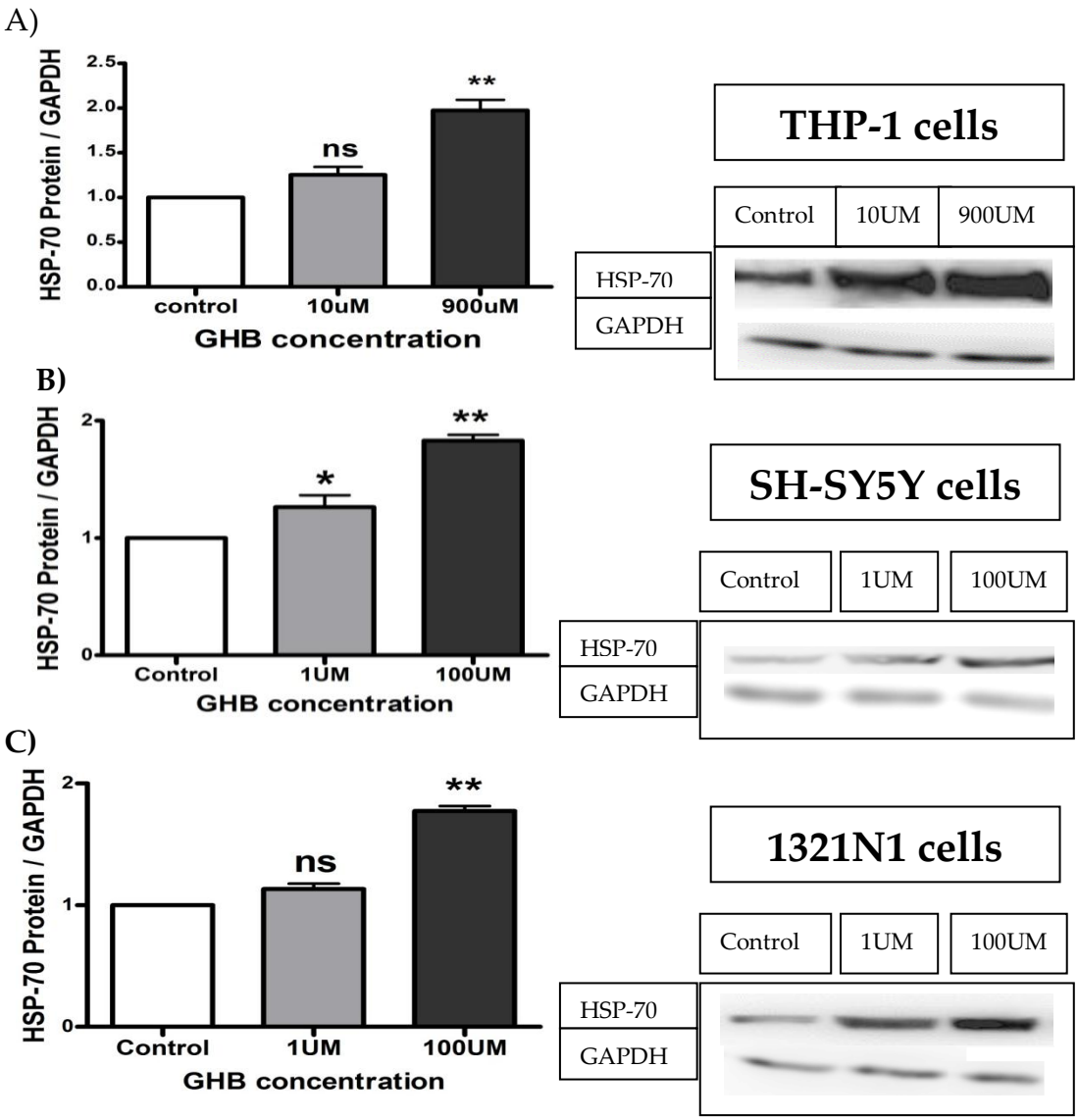


Figure 6.4: Change in HSP-70 protein expression in control untreated and treated cells after GHB exposure. A) THP-1, B) SH-SY5Y and C) 1321N1. Cells were treated for 24 hours with 10 or 900μM GHB for THP-1 cells and 1 or 100μM GHB for brain cells and then western blots were carried out on whole cell extracts using HSP-70 (70KDa) antibody (Abcam) with GAPDH (37KDa) used for the normalisation and loading on 10% SDS- PAGE gel. The protein concentrations of the loaded samples were determined by Bradford method and approximately 40μg of samples were loaded. Protein bands were quantified by Image J relative to untreated control (n = 3 individual assays). Relative HSP-70 expression was represented as histogram. Asterisks indicate significant compared with control (**p<0.01 and *p<0.05).

6.5 Discussion

The work carried out in this chapter aimed to examine the effect of GHB on the proteomic profile of human blood cell line and human brain cell lines (neuronal and non-neuronal cell lines) in order to identify robust surrogate biomarkers after 24hour of GHB exposure to improve the window of GHB detection.

Determining protein levels is an important aspect since the future research goals include developing a simplified assay for the detection of GHB exposure, and these are likely to be protein-based rather than mRNA-based.

The 2-D gel electrophoresis results revealed that a total of 27, 44 and 37 spots were altered by at least 1.5 fold in THP-1 cells, SH-SY5Y and 1321N1cells respectively after 24h exposure to two different concentrations of GHB. Among these altered proteins are the heat shock proteins (HSPs), HSP-27 was found to be up regulated in 1321N1 cells, HSP-60 and HSP-70 were found to be up regulated in SH-SY5Y cells and also, HSP-70 and HSP-90 were found to be up regulated in THP-1 cells.

There is several human heat shock proteins (HSPs) divided into groups according to their molecular weights. These comprise HSP-27, HSP-40, HSP-60, HSP-70, HSP-90 and HSP-110. Heat shock protein 70 is one of the major proteins induced by stress in the nervous system. It is important for cellular repair and is neuroprotective (Brown, 2007).

In this study, for the validation of some of the proteomic results, Western blotting analysis was performed using HSP-70 specific antibodies for the three cell lines. The results revealed that HSP-70 protein expression level was increased in all cell lines used in this study after 24h exposure to GHB. HSP-

70 is a molecular chaperone that has an antiapoptotic function and this antiapoptotic function of HSP-70 depends on its ability to interact with protein substrates that are not always associated with the chaperoning activity. Also it was revealed that HSP-70 is rapidly induced by physical and chemical stresses (Beere, 2004; Sabirzhanov *et al*., 2012).

The effect of acute administration of GHB on some parameters of oxidative stress was also investigated in a series of *in vitro* and *in vivo* studies in 15-day-old rats and the results showed that GHB and its precursor GBL induce oxidative stress by stimulating peroxidation and decreasing the non-enzymatic antioxidant defences in the cerebral cortex (Sgaravatti *et al*., 2007, 2009). Thus, it is possible that HSP-70 and other heat shock proteins were induced due to oxidative stress provoked by GHB.

In addition to that, the work in Chapter 3 showed that the antiapoptotic PEA-15 protein expression levels were increased significantly after 24 hours exposure to GHB in the same cells and at the same concentrations. Cell proliferation is a cellular response for abnormal conditions such as oxidative stress when low amount of reactive oxygen species (ROS) is produced (Martindale and Holbrook, 2002). As a result, it is possible that the cell proliferation response due to oxidative stress provoked by GHB needs an increase in the levels of an antiapoptotic proteins such as HSP-70 and PEA-15 proteins.

The results of this study also revealed that GHB cause an increase in the level of some glycolytic enzymes. ENO1, Isoform alpha-enolase was found to be up-regulated in THP-1 and 1321N1 cells; ENO2, Isoform alpha-enolase, ALDOA, Fructose-bisphosphate aldolase A, TPI-1 triosephosphate isomerase

isoform 1, TPI-2 triosephosphate isomerase isoform 2 and GAPDH 39 kDa protein were also found to be up regulated in THP-1 cells.

The validation of the GHB effect on glycolytic enzymes in particular ALDOA enzyme will be examined in detail in Chapter 7.

CHAPTER 7

Effects of GHB on the expression and Specific activity of Aldolase enzyme in THP-1 cells

7 Effects of GHB on the expression and Specific activity of Aldolase enzyme in THP-1 cells

7.1 Introduction

In chapter 6, the proteomic screen for GHB exposure in SH-SY5Y, 1321N1 and THP-1 cells, and the analysis of picked spots by electro spray ionization (ESI-TRAP) and then protein identification by MASCOT software was carried out and revealed that GHB induces changes in the expression of some glycolytic enzymes. These include Isoform alpha-enolase (ENO1) which was found to be up regulated by GHB in THP-1 and 1321N1 cells; Isoform alpha-enolase (ENO2); Fructose-bisphosphate aldolase A (ALDOA); triosephosphate isomerase isoform 1(TPI-1), triosephosphate isomerase isoform 2 (TPI-2), and glyceraldehydes-3-phosphate dehydrogenase (GAPDH 39 KDa); were found to be up regulated in THP-1 cells.

7.1.1 Glycolysis

Glycolysis is one of the most important metabolic pathways in the body, mainly concerned with the process of glucose degradation, which includes glycolysis, aerobic oxidation and pentose phosphate metabolism. Glucose metabolism supplies energy for physical activity and also mediates a variety of physiological processes throughout the formation of complex signalling networks with metabolic substrates. Glycolysis includes the conversion of one molecule of glucose into pyruvate to produce two adenosine triphosphate (ATP) molecules.

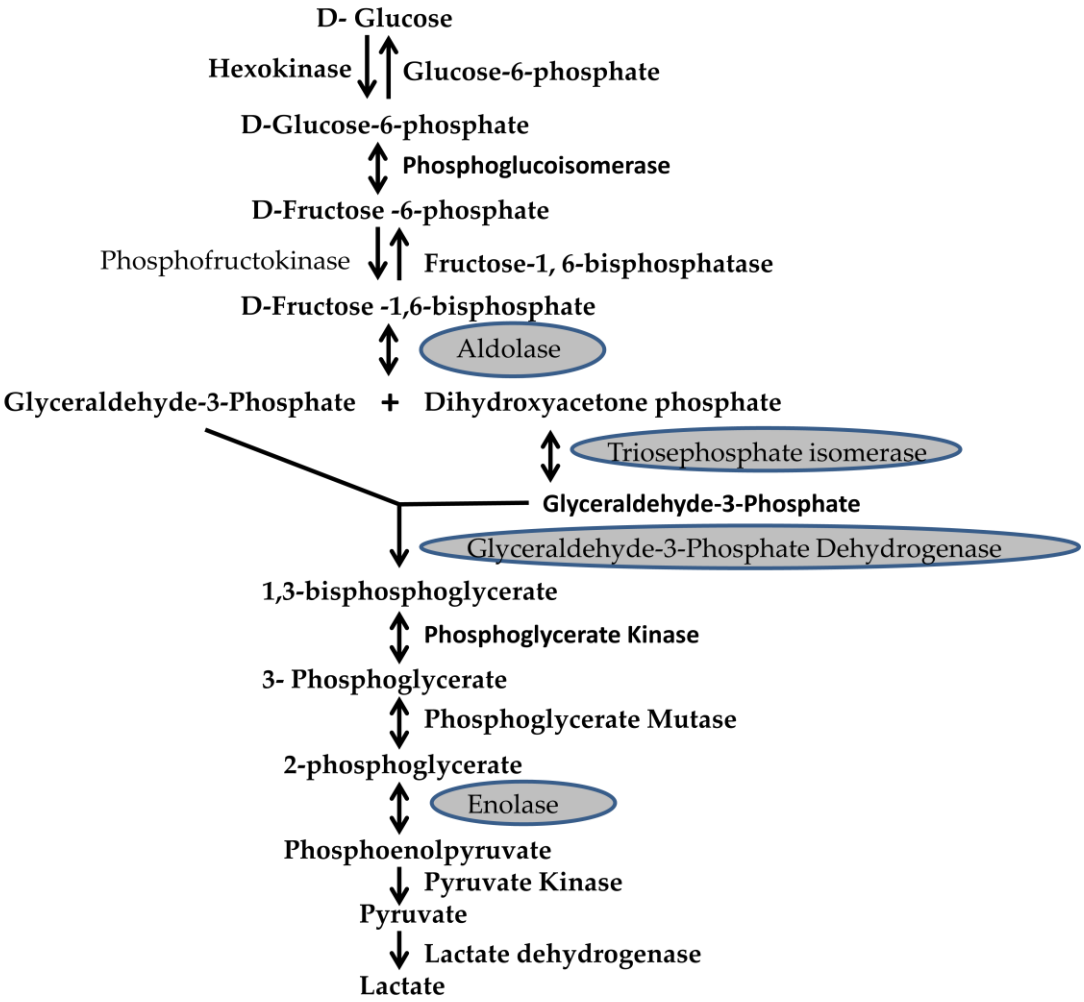


Figure 7.1: Scheme of glycolysis and gluconeogenesis. In the cytosol, glucose is phosphorylated and converted into pyruvate by glycolysis. Adapted from (Koeck *et al*., 2004).

The complete oxidation of glucose includes the conversion of one molecule of glucose into CO_2 and H_2O and generates 38 or 36 ATP molecules. Even though both glycolysis and the complete oxidation of glucose are important ways to generate ATP, the pathways that produce energy usually are connected to the physiological and pathological processes taking place in tissues (Rutz, 2004; Koeck *et al*., 2004). The role of aldolase, enolase, triose -

phosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase enzymes in glycolysis is illustrated in (Figure 7.1).

7.1.2 Glycolytic enzymes

Glycolytic enzymes are often thought of as 'dull' enzymes due to their conserved nature over millions of year. They have been considered as enzymes with no advanced controlling properties. In spite of this, these enzymes are well studied and details of their structures might give a robust basis to understanding some of the essential features in biochemistry (Pancholi, 2001). Some of these glycolytic enzymes are described below.

Enolase (ENO)

ENO was discovered in 1934 by Lohman and Mayerhof when they were studying the conversion of 3- phosphoglycerate to pyruvate in muscle extracts (Pancholi, 2001). It was initially characterized as glycolytic enzyme.

In vertebrates, three distinct genetic loci, α, β and γ, encode three isoforms of ENO enzyme and the expression of these isoforms is adjusted in a tissue-specific manner (Marangos *et al*., 1978). Alpha-ENO (ENO1) exists in a variety of tissues, virtually in all adult tissues, while β-ENO (ENO3) is mostly found in muscle tissues and γ -ENO (ENO2) exist in neurons and neuro endocrine tissues (Pancholi, 2001). Enolase converts 2-phosphoglycerate into phosphoenolpyruvate in glycolysis (Pancholi, 2001). ENO1 may act as a stress protein that promotes hypoxic tolerance in tumor cells by increasing anaerobic metabolism (Jiang *et al*., 1997). Furthermore, ENO1 may have a role as a plasminogen receptor on the surface of a variety of hematopoetic, epithelial and endothelial cells (Wygrecka, 2009; Dudani *et al*., 1993).

Many cancer cells exhibit increased glycolysis, with much of the pyruvate being converted to lactate, as opposed to entry into the mitochondria and conversion to acetyl CoA. This is known as the Warburg effect (Ramsay *et al*., 2011).

Recently, many indications have suggested that ENO1 might contribute to tumor malignancy (Dudani *et al*., 1993; Altenberg and Greulich, 2004; Chang *et al*., 2006; Hennipman *et al*., 1988; Tsai *et al*., 2010, Peebles *et al*., 2003; Wu *et al*., 2002; Zhang *et al*., 2000; Takashima *et al*., 2005; Takikita *et al*., 2009). Several highly tumorigenic or metastatic cell lines have displayed up regulation of the ENO1 gene (Peebles *et al*., 2003; Wu *et al*., 2002; Zhang *et al*., 2000) and enzymatic activities in breast cancer indicated a role for ENO1 in tumor progression (Hennipman *et al*., 1988). Many cancer cells exhibit increased glycolysis, with much of the pyruvate being converted to lactate, as opposed to entry into the mitochondria and conversion to acetyl CoA. This is known as the Warburg effect (Ramsay *et al*., 2011).

Aldolase (ALDO)

Fructose-1,6 -bisphosphate aldolase or aldolase is a glycolytic enzyme that catalyzes the reversible conversion of fructose 1,6- bisphosphate to dihydroxyacetone phosphate and glyceraldehyde 3-phosphate by an aldol cleavage reaction. In addition, fructose 1-phosphate can also be a substrate in the aldol cleavage reaction.

In vertebrates, there are three tissue-specific isozymic forms that can be distinguished by their electrophoretic and catalytic features: aldolases A, B, and C. Aldolase A dominates in skeletal muscle and red blood cells; aldolase B exists in liver, kidney, and small intestine; and aldolase C in neuronal

tissues and smooth muscle (Kishi *et al*., 1987; Rottmann *et al*., 1984; Kim *et al*., 1998; Koeck *et al*., 2004). All of the aldolase isozymes are identified as the nitrated proteins. Protein nitration occurs mostly at tyrosine residues in vivo as a result of oxidative stress. Changes in tissue distribution and/or reduction in catalytic activity of aldolases are involved in many diseases such as hereditary fructose intolerance; glycogenosis type XII and cancer (Koeck *et al*., 2004).

Human triosephosphate isomerise (TPI)

Triosephosphate isomerase is a glycolytic enzyme that catalyzes the interconversion of D-glyceraldehyde 3-phosphate to dihydroxyacetone phosphate (Mande *et al*., 1994). It is an important part of the glycolytic pathway. Deficiency of TPI has been shown to increase the levels of dihydroxyacetone phosphate in human results in chronic anemia and neuromuscular impairment (Daar *et al*., 1986; Eber *et al*., 1991).

TPI, because of its important role in glycolysis, it is also considered as a possible target for drug design against parasites that can survive in the mammalian bloodstream. These parasites can cause various diseases that can sweep the world, such as malaria and sleeping sickness (Mande *et al*., 1994; Ostoa-Saloma *et al*.,, 1997; Velanker *et al*., 1997; Rodriguez-Romero *et al*., 2002).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

GAPDH is a traditional protein with huge biochemical and biophysical interests due to its functional significance in glucose metabolism and energy production. Recent progress in studying the cancer related roles of GAPDH together with the sensitivity of tumor cells to its inhibition assigns GAPDH

as a possible molecular target in cancer treatment. A number of elegant reviews have outlined the functional diversity of GAPDH, especially its role in cell death and survival mechanisms (Sirover, 2005, 2011 and 2012; Colell, 2007 and 2009; Ganapathy-Kanniappan, 2012).

7.2 Aim of this chapter

The aim of this chapter is to validate the proteomic results and to gain further insight into the GHB dependent changes in glycolytic enzymes expression level and activity, Fructose-bisphosphate aldolase A (ALDOA) enzyme was selected s a glycolytic enzyme and the study of GHB dependent changes in the expression and activity of aldolase enzyme were performed. Furthermore, to investigate the mechanism by which GHB mediates changes in ALDOA expression level and activity, the GHB structural analogue was used in this study.

7.3 Materials and methods

Human monocytic leukaemia (THP-1) cell line (Tsuchiya *et al*., 1980) was used in this study. THP-1 cells were treated with two concentrations (10μM and 900μM) of GHB, NCS- 382 or both treatments for 24 hours. Methods used in this chapter include Western blotting and aldolase enzyme assay (chapter 2).

7.4 Results

7.4.1 Effect of GHB on aldolase protein expression level

In chapter 6, GHB was observed to cause a significant increase of 2.3 fold in the expression of ALDOA in blood THP-1 cells. In order to validate these results, Western blotting analysis for protein was carried out on THP-1 cell extracts. As previous, THP-1 cells were treated with 10 μM or 900 μM GHB for 24h. Cell extracts were prepared and separated on SDS-PAGE before blotting to nylon membrane and probing with specific aldolase antibodies.

The results of Western blotting analysis revealed that GHB cause a significant ($p<0.01$) increase of 2.1 fold in the expression level of aldolase protein after 24 hour exposure to 900 μM GHB (Figure 7.2).

7.4.2 Effect of NCS-382 on aldolase protein expression level

The levels of ALDOA protein expression were found to be unchanged after treatment with the GHB analogue NCS-382 alone. Importantly though NCS-382 was able to inhibit the GHB-dependent induction of aldolase. This suggests that NCS-382 is competing for binding with GHB to a component within the cells.

	T	TG10	TG900	TN10	TN900	TB10	TB900
GHB (uM)	0	10	900	0	0	10	900
NCS-382 (uM)	0	0	0	10	900	10	900
Aldolase							
GAPDH							

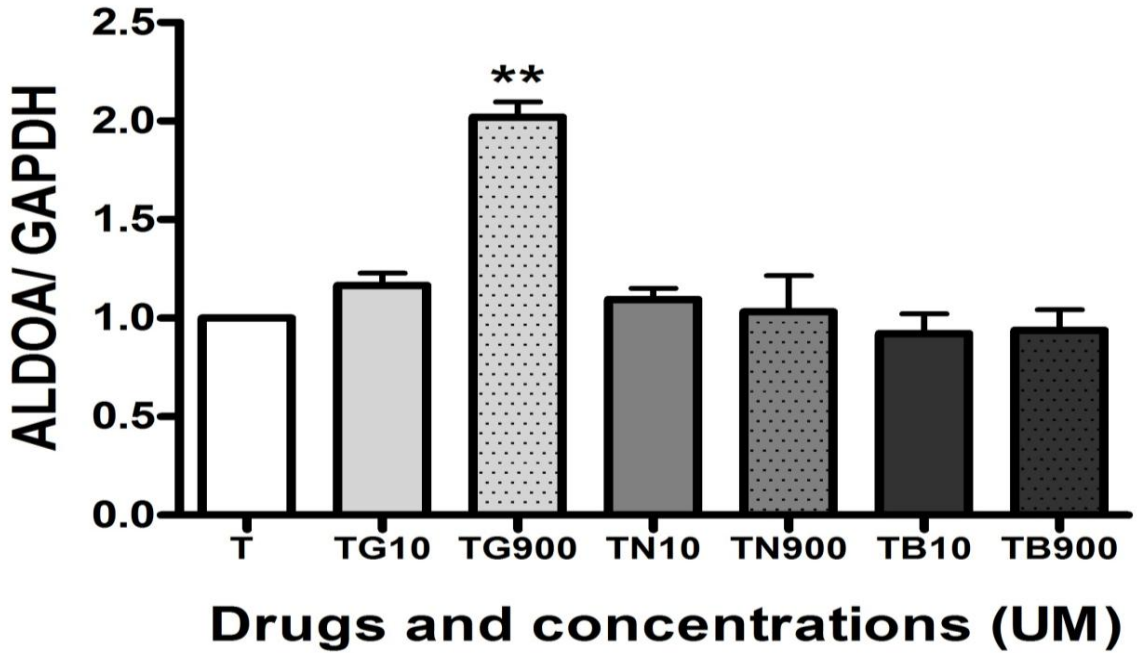


Figure 7.2: Change in ALDOA protein expression in THP-1 cells after GHB exposure. Cells were treated for 24 hours with 10 or 900μM of GHB, NCS-382 or both and then western blots were carried out on whole cell extracts using ALDOA (36KDa) antibody (Abcam) with GAPDH (37KDa) used for the normalisation. The protein concentrations were determined by Bradford method and approximately 40μg of samples were loaded on 10% SDS- PAGE gel. Protein bands were quantified by Image J relative to untreated control (n = 3 individual assays). (T) Represent untreated THP-1 cells, (TG10) and (TG900) represent THP-1 cells treated with 10μM and 900 μM GHB respectively, (TN10) and (TN900) represent THP-1 cells treated with 10μM and 900 μM NCS-382 respectively and finally, (TB10) and (TB900) represent THP-1 cells treated with either 10μM or 900 μM of both GHB and NCS-382. Relative ALDOA expression was represented as histogram. Asterisks indicate significant compared to control (**$p<0.01$).

7.4.3 Effect of GHB on aldolase enzyme activity

In order to further validate our 2 D gel results about the effect of GHB on ALDOA enzyme activity, an aldolase enzyme assay was carried out on THP-1 cells using a colorimetric assay according to Boyer's modification of the hydrazine assay (Jagannathan *et al*. 1956). Fructose-1, 6-bisphosphate was used as a substrate and hydrazine sulphate used as detection reagent for the formed 3-phosphoglyceraldehyde.

The results of the aldolase enzyme assay revealed that GHB cause a significant ($p<0.01$) increase of 1.55 fold in the aldolase enzyme specific activity after 24 hour exposure to 900 μM GHB (Figure 7.3). This correlates well with the increase in protein expression observed in Figure 7.2.

7.4.4 Effect of NCS-382 on aldolase enzyme activity

The results of aldolase enzyme assay revealed that the enzyme activity was unchanged after treatment with NCS-382 alone.

Similar to the Western blot result, NCS-382 was able to inhibit the GHB-dependent induction of aldolase activity, again indicating an interaction of NCS-382 with a component involved in mediating the GHB-dependent effects.

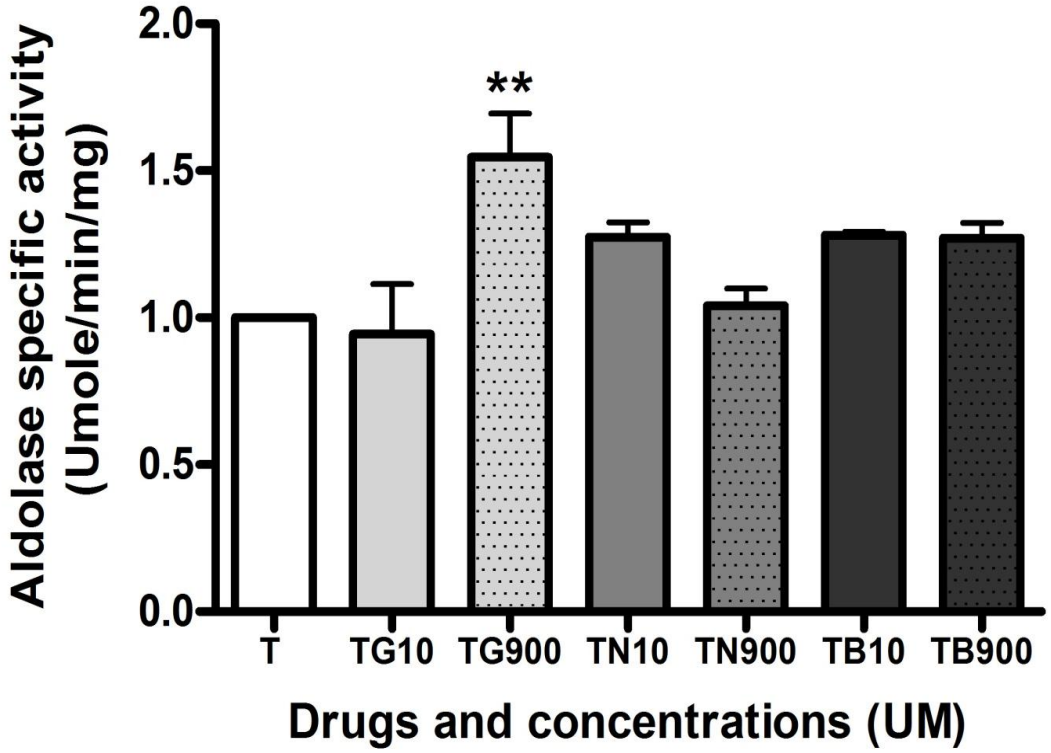


Figure 7.3: Change in Aldolase enzyme specific activity in THP-1 cells after GHB exposure. Cells were treated for 24 hours with 10 or 900μM of GHB, NCS-382 or both and then aldolase enzyme assay was performed by using colorimetric assay according to Boyer's modification of the hydrazine assay to measure the change in aldolase specific activity after GHB exposure. Hydrazine sulphate used as a detection reagent for the formed 3-phosphoglyceraldehyde to form a hydrazone which absorbs at 240 nm. (T) Represent untreated THP-1 cells, (TG10) and (TG900) represent THP-1 cells treated with 10μM and 900 μM GHB respectively, (TN10) and (TN900) represent THP-1 cells treated with 10μM and 900 μM NCS-382 respectively and finally, (TB10) and (TB900) represent THP-1 cells treated with either 10μM or 900 μM of both GHB and NCS-382. The untreated cells were used as control (**$p<0.01$).

7.5 Discussion

In chapter 6, the effect of two selected concentrations 10μM and 900μM of GHB on the protein expression profile of THP1 cells revealed that GHB induced changes in expression of a number of enzymes involved in glycolysis in blood THP-1 cells.

This study aimed at validation of some of the proteomic results on the expression of glycolytic enzymes in THP-1 cells by measuring the effect of GHB exposure on aldolase enzyme expression level and specific activity and also, to investigate the mechanism by which GHB mediates changes in aldolase protein expression level and specific activity using the GHB analogue NCS-382.

Aldolase enzyme expression levels and specific activity were found to be increased only after the treatment with 900μM GHB but not after NCS-382 or their combination in THP-1 cells. It is possible that the effect of GHB on the expression level and specific activity of aldolase enzyme may be mediated through a specific receptor or transporter to which both GHB and NCS-382 bind. Although it is not known that THP-1 cells pocesses a specific GHB receptor, results from the previous chapters indicate that GHB may enter cells, possibly via MCT transporters. It was revealed that MCT4 is mainly expressed in cells with a high glycolytic rate such as white blood cells, where it is involved in the elimination of lactic acid produced from glycolysis (Juel and Halestrap, 1999; Manning Fox *et al*., 2000).

Our results are the first to show the effect of GHB and NCS-382 on the expression level and specific activity of aldolase enzyme in THP-1 cells.

The effect of acute administration of GHB on some parameters of oxidative stress was investigated in a series of in vitro and in vivo studies in 15-day-old rats and the results showed that GHB and its precursor GBL induce oxidative stress by stimulating peroxidation and decreasing the non-enzymatic antioxidant defences in the cerebral cortex (Sgaravatti *et al*., 2007, 2009). Thus, it is possible that aldolase enzyme expression level and specific activity are increased due to the oxidative stress provoked by GHB.

In addition the work in previous chapters show that the antiapoptotic proteins such as PEA-15 and HSP70 expression levels were increased significantly in THP-1 cells after 24h exposure to GHB. Also, it is known that cell proliferation causes an increase in glycolysis, with much of the pyruvate being converted to lactate (Ramsay *et al*., 2011). So, the increase in aldolase enzyme expression level and specific activity might be due to increase in cell proliferation caused by GHB exposure.

Glucose metabolism plays a significant role in diseases such as diabetes, schizophrenia, stroke, and drug abuse (Rutz, 2004). Thus, it is possible that glycolytic enzymes including aldolase enzyme expression level will increase after exposure to GHB as an abused drug. Previous study revealed that treatment of rats with morphine (10 mg/kg) resulted in activation of glycolytic enzymes (Lelevich, 2011).

For consideration as biomarkers for GHB exposure, glycolytic enzymes are not particularly suitable for the detection of the presence of a drug, because of their sensitivity to many external and internal changes in the body. However, the clear induction of aldolase observed in this Chapter has not been reported for any other drug treatment, and may represent a useful marker.

CHAPTER 8

Summary and General Discussion

8 Summary and General discussion

GHB is an important compound in several respects: it is found to be present endogenously (Bessman and Fishbein, 1963; Nelson *et al.*, 1981); it is prescribed for therapeutic purposes (Addolorato *et al.*, 1999; Carter *et al.*, 2009; Fuller and Hornfeldt, 2003); it has been sold as an anabolic supplement over the counter drug (Van Cauter *et al.*, 1997; Miotto *et al.*, 2001); and unfortunately, GHB gained a reputation as a "date-rape drug" being involved in drug facilitated sexual assault (Stillwell, 2002; Elsohly and Salamone, 1999; Rodgers *et al.*, 2004). GHB has a rapid rate of metabolism; it can disappear from the body within 12 hours after exposure and the criminal date rape cases are often difficult to prosecute. This study aimed at extending the window of GHB detection beyond 12 hours.

8.1 Summary of results

The first part of this study (Chapter 3) focused on two candidate genes epiregulin and PEA-15, which represent possible surrogate markers for GHB exposure in human cell lines. Also, the effect of GHB on the viability of brain cells were measured and the results show that GHB significantly decreases cell viability in a concentration dependent manner in brain cells but it is not significantly toxic at concentrations up to 150 μM. Our results are the first to show that GHB has similar effects on the viability of neuronal and non neuronal brain cells.

Also, the results presented in Chapter 3 show that epiregulin mRNA levels were increased significantly in both 1321N1 astrocytoma cells and THP-1

blood cells but not in SH-SY5Y neuroblastoma cells, and PEA-15 mRNA levels were increased in all cells after 24h of GHB exposure. Furthermore, protein analysis revealed that PEA-15 protein expression was increased in THP-1 and SH-SY5Y cells but not changed in 1321N1 cells after 24h of GHB exposure. These results seem to support the previous study in mouse which suggests that PEA-15 and epiregulin levels are possible surrogate markers for GHB administration beyond 12 hours (Larson *et al*., 2007). Our results are the first to show that GHB induces changes in PEA-15 and epiregulin gene levels in human cell lines.

The second part (Chapters 4 and 5) of this study describes microarray-based analysis with quantitative PCR validation to identify novel surrogate markers for GHB exposure, as well as to investigate the mechanism by which GHB mediates changes in gene expression, using the structural analogue of GHB NCS-382. The results of this part revealed that the expression of members of well-known gene families were altered in blood THP-1 cells after 24 h exposure to GHB, including aldo-keto reductases, hydroxysteroid dehydrogenases, heat-shock proteins and zinc finger proteins. The most important finding was the effect of GHB and also NCS-382 on genes encoding steroid dehydrogenases. The effect appears to be additive for some genes and synergistic for others. For example, the effect of GHB on the levels of mRNA encoding AKR1C1, AKR1C3, AKR1C4, and DHRS9 is not affected by co-treatment with the analogue NCS-382. The levels of HSD11B1 mRNA were found to be reduced significantly after treatment with 900μM GHB, but not after NCS-382 or the combination of the two compounds.

This study is the first to measure the changes in mRNA levels after 24h exposure to GHB in human cell lines and also to specifically examine the

effect of GHB and NCS-382 on the level of steroid hormone dehydrogenase mRNA levels.

The third part of this study (Chapters 6 and 7) focused on proteomic analysis carried out on blood and brain cells to find novel surrogate protein markers of GHB exposure as well as investigating the mechanism by which GHB mediates changes in gene expression using the structural analogue NCS-382. The results of this part revealed that GHB induced alteration in protein expression in the three types of cells used in this study, and the most important results was the GHB-dependent increase of specific glycolytic enzymes and the heat-shock protein HSP-70 in blood and brain cells. Furthermore, aldolase enzyme expression levels and specific activity were found to be increased, but only after treatment with GHB and not after NCS-382 or the combination of GHB and NCS-382 in THP-1 cells.

These results are the first to show the effect of GHB on protein expression in blood and brain cells and in addition to the effect of GHB and NCS-382 on the expression level and specific activity of aldolase enzyme in THP-1 cells, and opens the potential for the use of these changes as biomarkers.

8.2 General discussion

Even though there has been a considerable discussion about GHB pharmacodynamics and the receptors involved in mediating GHB action, little is known about the changes in gene expression that may be involved after GHB exposure.

The microarray results identified members of the aldo-ketoreductase, hydroxyl steroid dehydrogenases, heat-shock proteins and zinc finger proteins as being altered following exposure to GHB.

8.2.1 Steroid Dehydrogenases

There is no obvious connection between steroid dehydrogenases and the known functions or metabolism of GHB and it is not known whether the effect observed is direct or indirect.

Although previous studies have not previously investigated the effect of GHB on steroid hormone dehydrogenases, several factors are known to induce the expression of certain steroid dehydrogenases. For example, members of the AKR1C (including AKR1C2) are known to be regulated by oxidative and chemical stress (Burczynski et al., 1999). One hypothesis to explain the effect of GHB on AKR1C expression is that GHB induces oxidative or metabolic stress. There is evidence to support this hypothesis. For example, the effect of acute administration of GHB on certain parameters of oxidative stress has been investigated in a series of in vitro and in vivo studies in 15-day-old rats. The results from this work showed that GHB and its precursor GBL induce oxidative stress by stimulating peroxidation and decreasing the non-enzymatic antioxidant defences in the cerebral cortex (Sgaravatti *et al*., 2007, 2009). It is therefore possible that AKR1C mRNAs are induced due to oxidative stress induced by GHB. To test this hypothesis, the effect of GHB on markers of oxidative and metabolic stress could be investigated in the cell lines under study.

Another HSD, HSD11B1 is known to be induced by cortisone via a PPARγ–mediated mechanism and also by interleukin-1 (Yong *et al*., 2002). It is not known what compounds regulate expression of DHRS9.

Dydrogesterone (a steroid progestin) has been shown to significantly lower the expression of SRD5A1, AKR1C2 and AKR1C3 expression level in Z-12 epithelial cells (Beranic and Rizner, 2012). It would be interesting to see whether these hormones give similar results to GHB in the three cell lines being investigated in this study.

In order to be considered as specific biomarkers for GHB exposure, further studies are needed to investigate the full range of factors that could potentially regulate the expression of these steroid hormone dehydrogenases. In particular, analysis of gene expression in cells after exposure to other abused drugs such as other CNS depressants, including benzodiazepines, barbiturates and alcohol, needs to be carried out. No previous study has examined the effect of barbiturates on any of the genes encoding steroid dehydrogenases or aldo-keto reductases. However one study in rat revealed that 11β- HSD2 is induced in alcoholic liver disease (Ahmed *et al*., 2008) indicating that alcohol dependence may affect the usefulness of this gene as a biomarker.

8.2.2 Heat-shock Proteins

Heat-shock proteins (including HSP70) were seen to be altered in both the microarray and proteomic studies and also when being altered in both brain and blood cells measured directly, HSP70 is one of the major proteins induced by stress in the nervous system. It is important for cellular repair and is known to be neuroprotective (Brown, 2007).

No previous study has examined the effect of GHB on the level of Heat-Shock Proteins. However HSP70 is known to be induced by oxidative and other stresses, including glucose-deprivation which can mediate metabolic

oxidative stress (Lee and Corry, 1998). This induction is thought to be mediated by stress-activated protein kinase (SAPK) pathway (Lee and Corry, 1998). Similar to the AKR1C mRNA expression, it is possible that HSP-70 and other heat shock proteins were induced due to oxidative stress provoked by GHB. Testing the effect of GHB on markers of oxidative stress would confirm this hypothesis.

In THP-1 cells, GHB induced an alteration in expression of PEA-15; both of PEA-15 and HSP70 are antiapoptotic proteins (Rerole et al., 2011). An alternative hypothesis for the regulation of HSP70 by GHB is that GHB causes changes in cell proliferation and apoptosis, through either an indirect or direct mechanism. It is known that GHB leads to an increase in Growth Hormone secretion during sleep (Van Cauter *et al*., 1997), and it also known that Growth Hormone leads to changes in cell proliferation, so it is possible that the effect of GHB in HSP70 expression is indirect. This could be tested by measuring cell proliferation rates and GH secretion from the cells lines being investigated.

Similarly, studies are also needed to investigate HSP70 gene expression after exposure to other abused drugs including benzodiazepines, barbiturates and alcohol, as there is limited information available. One previous study investigated the effect of benzodiazepines on leukaemia cells, and this revealed that clonazapine induces agranulocytosis by increasing the expression level of HSPA1A mRNA which encodes HSP70 (Yang *et al*., 2011). Another study revealed that the thiobarbiturate drug thiopental induces HSP70 expression in human T lymphocytes (Roesslein et al., 2008). Hence it is important to test whether these drugs influence HSP70 expression in the three cell lines used in this study.

8.2.3 Zinc Finger Proteins

No previous study has examined the effect of GHB on the level of zinc finger proteins but there are plenty of factors that influence expression of these important regulatory proteins. Some are known to be influence by oxidative stress, hormones, apoptosis, and so a careful analysis is needed to test extraneous influences on expression. Also, no previous study has examined the effect of benzodiazepines, barbiturates or alcohols on zinc finger proteins expression, so this requires to be investigated in detail in the cell lines chosen.

8.2.4 Metabolic Enzymes

Carbonic anhydrase 2 expression levels were found to be increased by GHB in both the microarray and proteomic studies. There is very little information on factors that influence the expression of carbonic anhydrase 2, but the gene is thought to be upregulated in some tumours through an unknown mechanism. No previous study has examined the effect of GHB on the level of this enzyme and also, no previous study has examined the effect of benzodiazepines, barbiturates or alcohols on carbonic anhydrase expression.

The proteomic results revealed that GHB caused an increase in the level of some glycolytic enzymes such as ENO1, ENO2, ALDOA, TPI-1 triosephosphate isomerase isoform 1, TPI-2 triosephosphate isomerase isoform 2 and GAPDH 39 kDa protein. This is an interesting result, and although GHB is thought to influence cellular metabolism, no previous study has examined the effect of GHB on the level of these enzymes. However previous studies have revealed that treatment of rats with morphine (10 mg/kg) also results in activation of glycolytic enzymes (Lelevich, 2011).

Many glycolytic enzymes including Enolase I, encoded by ENO1 are thought to be important in tumour malignancy. This is because many cancer cells exhibit increased glycolysis, with much of the pyruvate being converted to lactate, as opposed to entry into the mitochondria and conversion to acetyl CoA. This is known as the Warburg effect (Ramsay *et al*., 2011). Glucose metabolism also plays a significant role in diseases such as diabetes, schizophrenia, stroke, and drug abuse (Rutz, 2004). As GHB is known to affect the metabolic status of cells, it is possible that glycolytic enzymes including aldolase enzyme expression level will increase after exposure to GHB.

For consideration as biomarkers for GHB exposure, at first glance glycolytic enzymes do not appear particularly suitable for the detection of the presence of a drug, because of their sensitivity to many external and internal changes in the body. However, the clear induction of aldolase observed after 24h exposure to GHB has not been reported for any other drug treatment, and may represent a useful marker.

8.2.5 Drug Profiling

Previously, in vivo microarray studies have been carried out using certain other antipsychotic drugs, such as diazepam (30mg/kg dose given to mice). The microarray analysis was performed on mouse brain (Huopaniemi *et al*., 2004). Also, oxazepam (2500 p.p.m.) given to mice to induce liver cancer and the microarray analysis was performed on mice liver (Lida *et al*., 2003) and also haloperidol 21 mg/kg subcutaneously or olanzapine 240 mg/kg subcutaneously were given to rats once per week for four weeks and the microarray analysis was performed on multiple brain regions of rat (Girgenti

et al., 2010). None of these studies found similar profiles of microarray results that our study found for GHB. It is worth noting that these studies on mice or rat brains while our study on human blood cell line.

8.3 Future work

The preliminary research described in this thesis is the first step in developing clinically useful biomarkers for GHB exposure. As indicated, further studies are needed to investigate the expression of these genes and proteins after exposure to other abused drugs such as other CNS depressants, like benzodiazepines, barbiturates and alcohol in order to determine whether the effects observed are specific for GHB.

Additional experiments are also needed to confirm that results are a specific response to GHB exposure, by comparing levels of the altered genes in cell lines against levels induced by combinations of GHB, GABA, and CGP-35348 ($GABA_B$ receptor antagonist).

In addition, from the above presented useful candidates biomarkers have been identified and require further validation using human blood samples from people exposed to GHB to develop robust markers for use in forensic toxicology.

Appendix

9 Appendix

9.1 Published Research Outputs

I. **Abdullah AS**, Ellis EM (2011). Surrogate Markers of Gamma-hydroxybutyrate (GHB) exposure in cell lines.

- *In Proceedings: British Pharmacological Society: James Black Meeting - Biologics for the New Millennium,* 19-20 September 2011 in Cambridge.
  http://www.pa2online.org/abstracts/vol9issue2abst002p.pdf
- University of Strathclyde research day, June 2011.

II. **Abdullah AS**, Ellis EM (2012). Gene expression signature of Gamma hydroxybutyric acid (GHB) exposure in Human monocytic leukaemia THP-1 cells.

- *In Proceedings: The Physiological Society: The Biomedical Basis of Elite Performance Meeting,* 19-21 March 2012 at The Queen Elizabeth II Conference Centre, London, UK.
  http://www.physoc.org/proceedings/abstract/Proc%20Physiol%20Soc%2026PC80
- University of Strathclyde research day, June 2012.

III. **Abdullah AS**, Ellis EM (2012). Proteomic screen for Gamma hydroxybutyric acid (GHB) exposure in Human monocytic leukaemia THP-1 cells. *In Proceedings: EuPA/BSPR 2012 Scientific Congress, New Horizons and Applications for Proteomics,* 9 - 12 July 2012, The Glasgow Royal Concert Hall.

IV. **Abdullah AS**, Ellis EM (2012). Effects of GHB, NCS- 382 and their combination on the expression of specific genes that affect the catalytic activity of steroid dehydrogenases.

In Proceedings: British Pharmacological Society Winter Meeting, London 2012.

http://www.pa2online.org/abstract/abstract.jsp?abid=30969&kw=GHB&author=Abdullah&cat=-1&period=-1

9.2 Microarray Analysis of Gene expression after 24 h GHB exposure (with chapter 4).

Table 9.1: Significantly down regulated genes after 24 h exposure to 10 μM GHB

	ProbeName	p-value	Fold change	Gene Symbol	Gene name
1	A_33_P3295917	4.53E-02	-5.37	IL7	interleukin 7
2	A_33_P3233749	9.88E-03	-3.97	LOC100128751	INM04
3	A_33_P3297415	1.24E-02	-3.65	NRP2	neuropilin 2
4	A_33_P3620832	4.36E-02	-3.63	ZNF483	zinc finger protein 483
5	A_23_P66739	1.76E-03	-3.33	SLC13A5	solute carrier family 13 (sodium-dependent citrate transporter), member 5
6	A_24_P921610	8.88E-03	-3.16	PAR5	Prader-Willi/Angelman syndrome-5
7	A_32_P204980	1.36E-02	-3.14	FBXO39	F-box protein 39
8	A_33_P3323904	4.41E-02	-3.04	WTH3DI	RAB6C-like
9	A_33_P3320079	4.23E-02	-2.98	NFIB	nuclear factor I/B
10	A_24_P228796	2.44E-02	-2.75	GAGE7	G antigen 7
11	A_33_P3400482	2.00E-02	-2.75	LOC728158	hCG2044975
12	A_33_P3252661	2.03E-02	-2.62	POTEA	POTE ankyrin domain family, member A
13	A_33_P3407314	3.26E-02	-2.62	LOC100132707	uncharacterized **LOC100132707**
14	A_23_P94434	2.09E-02	-2.62	HRCT1	histidine rich carboxyl terminus 1

15	A_33_P3741835	2.52E-02	-2.61	ZCCHC23	zinc finger, CCHC domain containing 23
16	A_23_P135722	2.81E-02	-2.53	BTC	betacellulin
17	A_23_P415395	1.17E-03	-2.48	MBD3L1	methyl-CpG binding domain protein 3-like 1
18	A_33_P3243307	3.14E-02	-2.46	LOC100128281	uncharacterized **LOC100128281**
19	A_33_P3378630	1.24E-02	-2.46	LOC100131195	uncharacterized **LOC100131195**
20	A_33_P3293511	4.27E-02	-2.42	KIAA1984	KIAA1984
21	A_33_P3215676	3.89E-02	-2.40	MANSC4	MANSC domain containing 4
22	A_24_P7790	6.98E-03	-2.40	WFDC10B	WAP four-disulfide core domain 10B
23	A_33_P3795524	8.82E-03	-2.39	LOC100506485	uncharacterized LOC100506485
24	A_33_P3465440	1.94E-02	-2.39	PRO1596	uncharacterized LOC29013
25	A_33_P3294459	2.59E-02	-2.36	COPG2IT1	COPG2 imprinted transcript 1 (non-protein coding)
26	A_23_P111311	1.85E-02	-2.33	AKAP12	A kinase (PRKA) anchor protein 12
27	A_23_P415470	4.10E-02	-2.33	PCDHGB6	protocadherin gamma subfamily B, 6
28	A_23_P394972	1.08E-02	-2.31	TSPEAR	thrombospondin-type laminin G domain and EAR repeats
29	A_23_P52121	2.50E-02	-2.29	PDZK1	PDZ domain containing 1
30	A_33_P3283515	8.13E-03	-2.28	PDZD2	PDZ domain containing 1
31	A_33_P3405888	1.17E-02	-2.26	LOC100133130	PRO1102
32	A_33_P3349576	8.34E-03	-2.26	TSTD2	thiosulfate sulfurtransferase (rhodanese)-like domain containing 2
33	A_23_P92120	4.14E-02	-2.26	SNTN	sentan, cilia apical structure protein
34	A_33_P3281567	8.48E-03	-2.21	CMAHP	cytidine monophospho-N acetylneur - aminic acid hydroxylase, pseudogene
35	A_23_P360079	3.73E-02	-2.20	NCKAP5	NCK-associated protein 5
36	A_33_P3297020	4.32E-02	-2.20	PSORS1C3	psoriasis susceptibility 1 candidate 3 (non-protein coding)
37	A_24_P391868	3.53E-02	-2.17	CPLX2	complexin 2
38	A_33_P3277532	1.03E-02	-2.17	LAMC3	laminin, gamma 3
39	A_24_P109071	4.26E-02	-2.15	KIF27	kinesin family member 27
40	A_24_P280148	3.71E-02	-2.14	GPR82	G protein-coupled receptor 82
41	A_33_P3212102	2.59E-02	-2.14	SLC10A6	solute carrier family 10 (sodium/bile acid cotransporter family), member 6
42	A_23_P33664	2.87E-02	-2.14	ELSPBP1	epididymal sperm binding protein 1
43	A_32_P232192	3.27E-02	-2.12	DIS3L2	DIS3 mitotic control homolog (S.

					cerevisiae)-like 2
44	A_24_P52887	4.90E-02	-2.12	ENDOU	endonuclease, polyU-specific
45	A_33_P3345414	2.62E-02	-2.07	RPS4X	ribosomal protein S4, X-linked
46	A_33_P3315263	3.83E-02	-2.05	KRT79	keratin 79
47	A_23_P217498	2.75E-02	-2.04	GDPD2	glycerophosphodiester phosphodiesterase domain containing 2
48	A_32_P516818	4.64E-02	-2.03	C3orf55	chromosome 3 open reading frame 55
49	A_24_P943949	4.54E-02	-2.02	LRRC8B	chromosome 3 open reading frame 55
50	A_23_P133739	1.70E-02	-2.02	HUS1B	HUS1 checkpoint homolog b (S. pombe)
51	A_33_P3400334	3.62E-02	-2.01	LOC100130372	uncharacterized **LOC100130372**
52	A_23_P98070	2.46E-02	-2.01	PDE6C	phosphodiesterase 6C, cGMP-specific, cone, alpha prime
53	A_24_P152968	1.98E-02	-1.14	AKR1C1	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)
54	A_33_P3272291	1.75E-02	-1.12	AKR1C4	aldo-keto reductase family 1, member C4 (chlordecone reductase; 3-alpha hydroxysteroid dehydrogenase 4) dehydrogenase, type I; dihydrodiol dehydrogenase 4)
55	A_23_P257971	4.65E-02	-1.06	AKR1C1	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)

Table 9.2: Significantly up regulated genes after 24 h exposure to 10 μM GHB.

	ProbeName	**p-value**	**Fold change**	**Gene Symbol**	**Gene name**
1	A_32_P71571	4.86E-02	3.52	FAM19A4	family with sequence similarity 19 (chemokine (C-C motif)-like), member A4
2	A_24_P75963	4.63E-02	3.03	DSCR4	Down syndrome critical region gene 4
3	A_23_P88678	1.56E-02	3.00	C15orf27	chromosome 15 open reading frame 27
4	A_24_P332081	1.63E-02	2.94	JAKMIP3	Janus kinase and microtubule interacting protein 3
5	A_23_P41629	3.35E-02	2.90	ADAMTS16	ADAM metallopeptidase with thrombospondin type 1 motif, 16
6	A_23_P208870	7.03E-04	2.89	AKT2	v-akt murine thymoma viral oncogene homolog 2
7	A_24_P941167	4.12E-02	2.70	APOL6	apolipoprotein L, 6
8	A_33_P3304501	3.33E-02	2.67	CDX2	caudal type homeobox 2
9	A_23_P126363	3.19E-02	2.66	ADAM30	ADAM metallopeptidase domain 30
10	A_33_P3522511	2.02E-02	2.49	KIAA0485	uncharacterized LOC57235

11	A_33_P3270104	1.50E-02	2.45	FAM26D	family with sequence similarity 26, member D
12	A_33_P3378136	4.56E-02	2.40	OR5D14	olfactory receptor, family 5, subfamily D, member 14
13	A_23_P364625	2.36E-02	2.40	LRRC19	leucine rich repeat containing 19
14	A_24_P42446	4.36E-02	2.39	PURG	purine-rich element binding protein G
15	A_24_P353794	2.25E-03	2.38	GALNT2	UDP-N-acetyl-alpha-D-galactosamine: polypeptide N-acetylgalactosaminyl transferase 2 (GalNAc-T2)
16	A_32_P101031	1.09E-02	2.37	LYPD1	LY6/PLAUR domain containing 1
17	A_33_P3294153	1.64E-03	2.37	CALY	calcyon neuron-specific vesicular protein
18	A_24_P92451	8.67E-03	2.31	LOC729444	uncharacterized **LOC729444**
19	A_23_P50241	5.32E-03	2.31	CLPTM1	cleft lip and palate associated transmembrane protein 1
20	A_33_P3290709	9.20E-03	2.29	EGFL6	EGF-like-domain, multiple 6
21	A_23_P42931	3.12E-02	2.28	CLEC2L	C-type lectin domain family 2, member L
22	A_33_P3271284	2.22E-02	2.26	C20orf112	chromosome 20 open reading frame 112
23	A_23_P121976	1.59E-02	2.21	POU4F3	POU class 4 homeobox 3
24	A_23_P159305	8.38E-03	2.18	TAF15	**TAF15** RNA polymerase II, TATA box binding protein (TBP)-associated factor, 68kDa
25	A_33_P3364463	9.17E-03	2.14	NKAIN3	Na+/K+ transporting ATPase interacting 3
26	A_33_P3417582	4.77E-02	2.13	SNX24	sorting nexin 24
27	A_33_P3791118	1.59E-02	2.11	ATP5L2	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit G2
28	A_33_P3422124	1.30E-02	2.11	IL22RA2	interleukin 22 receptor, alpha 2
29	A_24_P253827	3.64E-03	2.09	AP2B1	adaptor-related protein complex 2, beta 1 subunit
30	A_32_P217140	3.00E-02	2.07	ISX	intestine-specific homeobox
31	A_33_P3235043	4.39E-03	2.07	TRIM67	tripartite motif containing 67
32	A_23_P86599	1.80E-02	2.07	DMBT1	deleted in malignant brain tumors 1
33	A_33_P3281985	5.89E-03	2.06	CR2	complement component (3d/Epstein Barr virus) receptor 2

Table 9.3: Significantly down regulated genes after 24 h exposure to 900 μM GHB.

	ProbeName	p-value	Fold change	Gene Symbol	Gene name
1	A_24_P245379	7.50E-06	-11.50	SERPINB2	Placental plasminogen activator inhibitor
2	A_32_P200308	7.89E-05	-10.06	LOC728724	Subcategory (RNA class): lncRNA

3	A_23_P21976	5.79E-05	-8.12	CSPG4	chondroitin sulfate proteoglycan 4
4	A_24_P356916	2.64E-04	-7.58	SLC13A3	solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3
5	A_33_P3286422	6.77E-06	-7.42	FANCA	Fanconi anemia, complementation group A
6	A_23_P2543	1.59E-04	-7.29	CUX2	cut-like homeobox 2
7	A_33_P3252048	4.80E-05	-6.90	DYSFIP1	protein phosphatase 1, regulatory subunit 27
8	A_33_P3287472	8.84E-06	-6.75	DYSFIP1	protein phosphatase 1, regulatory subunit 27
9	A_23_P36120	2.95E-05	-6.75	MS4A6A	membrane-spanning 4-domains, subfamily A, member 6A
10	A_33_P3272291	1.05E-05	-6.70	AKR1C4	aldo-keto reductase family 1, member C4 (3-alpha hydroxysteroid dehydrogenase, type I
11	A_32_P351037	5.80E-05	-6.23	DNAH7	Dynein heavy chain-like protein
12	A_23_P203376	2.76E-04	-6.13	MS4A6A	membrane-spanning 4-domains, subfamily A, member 6A
13	A_23_P80295	2.03E-05	-5.98	SYN3	synapsin III
14	A_33_P3380383	3.12E-05	-5.98	TIFAB	TRAF-interacting protein with forkhead-associated domain, family member B
15	A_23_P209978	9.29E-04	-5.93	VSNL1	visinin-like 1
16	A_24_P152968	3.65E-05	-5.90	AKR1C1	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)
17	A_23_P340263	1.70E-05	-5.86	RNF175	ring finger protein 175
18	A_23_P126212	7.31E-04	-5.38	CLSPN	claspin
19	A_23_P56578	9.25E-07	-5.36	VIT	vitrin
20	A_23_P58266	9.82E-05	-5.31	S100P	S100 calcium binding protein P
21	A_23_P2492	5.94E-05	-5.17	C1S	complement component 1, s subcomponent
22	A_33_P3294372	2.63E-04	-5.05	LOC100128881	uncharacterized **LOC100128881 (RNA gene)**
23	A_23_P257971	2.87E-04	-4.94	AKR1C1	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)
24	A_24_P190873	3.06E-04	-4.93	FAM163A	family with sequence similarity 163, member A
25	A_32_P105549	2.98E-05	-4.67	ANXA8L2	annexin A8-like 2
26	A_33_P3867534	9.66E-	-4.64	MCM10	minichromosome maintenance complex

		06			component 10
27	A_23_P360754	3.35E-05	-4.64	ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif, 4
28	A_23_P408955	8.05E-05	-4.62	E2F2	E2F transcription factor 2
29	A_23_P80032	2.10E-05	-4.59	E2F1	E2F transcription factor 1
30	A_33_P3289005	1.49E-04	-4.49	AP1S3	adaptor-related protein complex 1, sigma 3 subunit
31	A_23_P370989	1.74E-04	-4.45	MCM4	minichromosome maintenance complex component 4
32	A_32_P183218	3.07E-04	-4.40	ZNF367	zinc finger protein 367
33	A_33_P3355185	1.67E-04	-4.37	BCL2	B-cell CLL/lymphoma 2
34	A_23_P251421	8.15E-05	-4.25	CDCA7	cell division cycle associated 7
35	A_23_P313981	4.17E-04	-4.19	SERPINB10	serpin peptidase inhibitor, clade B (ovalbumin), member 10
36	A_23_P208880	1.25E-04	-4.18	UHRF1	ubiquitin-like with PHD and ring finger domains 1
37	A_23_P37410	2.89E-05	-4.12	CYP19A1	cytochrome P450, family 19, subfamily A, polypeptide 1
38	A_33_P3399064	8.57E-04	-4.03	RN5-8S1	RNA5-8S5 RNA, 5.8S ribosomal 5
39	A_24_P105747	2.24E-04	-4.02	AP1S3	adaptor-related protein complex 1, sigma 3 subunit
40	A_23_P74449	1.22E-04	-4.01	HPDL	4-hydroxyphenylpyruvate dioxygenase-like
41	A_24_P299685	2.02E-04	-3.97	PDPN	podoplanin
42	A_33_P3303245	1.32E-04	-3.93	KIT	v-**kit** Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog[1] [2]
43	A_33_P3396214	4.33E-05	-3.84	KREMEN2	kringle containing transmembrane protein 2
44	A_23_P30976	2.12E-04	-3.84	GRM1	glutamate receptor, metabotropic 1
45	A_33_P3368830	3.76E-04	-3.73	LY9	lymphocyte antigen 9
46	A_23_P24903	2.37E-05	-3.71	P2RY2	purinergic receptor P2Y, G-protein coupled, 2
47	A_23_P101992	2.22E-04	-3.69	MARCO	macrophage receptor with collagenous structure
48	A_23_P324885	4.48E-04	-3.65	CCR2	chemokine (C-C motif) receptor 2
49	A_33_P3367301	1.12E-04	-3.63	GJD3	gap junction protein, delta 3, 31.9kDa
50	A_33_P3247022	2.98E-05	-3.61	CCNE2	cyclin E2

51	A_32_P780862	1.93E-04	-3.61	BMP8B	bone morphogenetic protein 8b
52	A_23_P209954	1.96E-04	-3.59	GNLY	Granulysin
53	A_23_P39955	2.76E-05	-3.57	ACTG2	actin, gamma 2, smooth muscle, enteric
54	A_33_P3232173	1.66E-04	-3.55	PSPC1	paraspeckle component 1
55	A_23_P57379	4.03E-05	-3.55	CDC45	cell division cycle 45 homolog (S. cerevisiae)
56	A_23_P352266	3.09E-04	-3.54	BCL2	B-cell CLL/lymphoma 2
57	A_33_P3378630	4.17E-04	-3.53	LOC100131195	uncharacterized **LOC100131195**
58	A_23_P129075	7.02E-04	-3.53	WDR76	WD repeat domain 76
59	A_33_P3209346	5.13E-04	-3.53	IARS	isoleucyl-tRNA synthetase
60	A_23_P33868	5.82E-04	-3.45	LPAR4	lysophosphatidic acid receptor 4
61	A_23_P148737	1.99E-04	-3.42	MYBPH	myosin binding protein H
62	A_23_P138541	1.18E-03	-3.39	AKR1C3	aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase type II
63	A_23_P118246	5.56E-05	-3.36	GINS2	GINS complex subunit 2 (Psf2 homolog)
64	A_23_P81441	7.53E-04	-3.34	C5orf20	chromosome 5 open reading frame 20
65	A_33_P3275707	6.52E-05	-3.33	AP1S3	adaptor-related protein complex 1, sigma 3 subunit
66	A_23_P112554	1.74E-04	-3.32	COL15A1	collagen, type XV, alpha 1
67	A_23_P20443	4.39E-04	-3.31	LZTS1	leucine zipper, putative tumor suppressor 1
68	A_33_P3407424	2.47E-05	-3.28	CDC42EP1	CDC42 effector protein (Rho GTPase binding) 1
69	A_23_P56559	1.16E-04	-3.25	DHRS9	dehydrogenase/reductase (SDR family) member 9
70	A_24_P225616	9.85E-04	-3.20	RRM2	ribonucleotide reductase M2
71	A_23_P7873	3.35E-05	-3.15	MCM3	minichromosome maintenance complex component 3
72	A_24_P46130	4.41E-05	-3.15	ACPP	acid phosphatase, prostate
73	A_33_P3300267	3.49E-04	-3.14	VIT	vitrin
74	A_23_P250385	2.45E-04	-3.14	HIST1H1B	histone cluster 1, H1b
75	A_23_P215956	3.26E-04	-3.13	MYC	v-**myc** myelocytomatosis viral oncogene homolog (avian)

76	A_33_P3301524	2.49E-04	-3.12	XRCC3	X-ray repair complementing defective repair in Chinese hamster cells 3
77	A_23_P40956	3.74E-04	-3.11	GHRL	ghrelin/obestatin prepropeptide
78	A_23_P35871	8.69E-05	-3.08	E2F8	E2F transcription factor 8
79	A_23_P210425	4.51E-04	-3.07	MYL9	myosin, light chain 9, regulatory
80	A_23_P95640	5.59E-05	-3.05	C1orf186	chromosome 1 open reading frame 186
81	A_23_P130194	2.00E-04	-3.03	PYCR1	pyrroline-5-carboxylate reductase 1
82	A_33_P3267577	2.07E-04	-3.03	SEMG1	semenogelin I
83	A_33_P3351371	2.39E-04	-3.01	CYP19A1	cytochrome P450, family 19, subfamily A, polypeptide 1
84	A_33_P3235217	8.29E-05	-3.01	CTPS	CTP synthase 1
85	A_32_P103633	1.63E-05	-3.00	MCM2	minichromosome maintenance complex component 2
86	A_24_P186379	5.06E-05	-3.00	C10orf125	fucose mutarotase
87	A_23_P108751	3.14E-04	-3.00	FHL2	four and a half LIM domains 2
88	A_33_P3402615	3.24E-04	-2.97	SLC6A9	solute carrier family 6 (neurotransmitter transporter, glycine), member 9
89	A_33_P3293913	6.10E-04	-2.97	BICC1	bicaudal C homolog 1 (Drosophila)
90	A_23_P63209	8.71E-05	-2.95	HSD11B1	hydroxysteroid (11-beta) dehydrogenase 1
91	A_23_P110253	2.47E-04	-2.94	KIT	v-**kit** Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
92	A_32_P180265	1.03E-05	-2.94	LOC100506845	LOC100506845 hypothetical LOC100506845
93	A_23_P212354	1.00E-04	-2.93	CCR2	chemokine (C-C motif) receptor 2
94	A_23_P23048	2.79E-05	-2.92	S100A9	S100 calcium binding protein A9
95	A_23_P62336	4.75E-04	-2.91	CPXCR1	CPX chromosome region, candidate 1
96	A_33_P3734378	3.83E-04	-2.89	LOC285954	INHBA-AS1 INHBA antisense RNA 1
97	A_23_P340848	2.61E-04	-2.89	PTGIR	prostaglandin I2 (prostacyclin) receptor (IP)
98	A_23_P119042	4.84E-04	-2.88	NKG7	natural killer cell group 7 sequence
99	A_24_P211151	8.25E-04	-2.85	EXOSC5	exosome component 5
100	A_33_P3293446	2.34E-04	-2.82	KIAA1462	junctional protein associated with coronary artery disease

101	A_23_P8640	5.84E-07	-2.79	GPER	G protein-coupled estrogen receptor 1
102	A_24_P402825	1.04E-04	-2.79	CACNA2D3	calcium channel, voltage-dependent, alpha 2/delta subunit 3
103	A_33_P3672756	5.38E-05	-2.79	LOC284561	LOC284561 uncharacterized LOC284561
104	A_33_P3531828	9.01E-04	-2.77	LARS	leucyl-tRNA synthetase
105	A_24_P181055	6.13E-04	-2.77	ST3GAL4	ST3 beta-galactoside alpha-2,3-sialyltransferase 4
106	A_23_P163099	9.01E-05	-2.76	POLE2	polymerase (DNA directed), epsilon 2, accessory subunit
107	A_23_P415411	1.79E-04	-2.74	HIST1H4E	histone cluster 1, H4e
108	A_23_P434809	5.64E-05	-2.74	S100A8	S100 calcium binding protein A8
109	A_23_P19333	4.60E-04	-2.71	TREM1	triggering receptor expressed on myeloid cells 1
110	A_24_P106542	4.60E-04	-2.69	RSPO3	R-spondin 3
111	A_33_P3408320	8.75E-05	-2.69	CERS1	ceramide synthase 1
112	A_23_P110851	1.34E-04	-2.68	TERT	telomerase reverse transcriptase
113	A_23_P202245	1.43E-04	-2.67	RET	**ret** proto-oncogene
114	A_24_P397107	2.13E-04	-2.67	CDC25A	cell division cycle 25 homolog A (S. pombe)
115	A_23_P5903	8.29E-04	-2.67	SLCO4A1	solute carrier organic anion transporter family, member 4A1
116	A_23_P391396	5.91E-05	-2.65	EBF3	early B-cell factor 3
117	A_23_P40315	5.84E-04	-2.63	C20orf12	double zinc ribbon and ankyrin repeat domains 1
118	A_24_P386622	9.28E-04	-2.63	ARRB1	arrestin, beta 1
119	A_23_P90612	3.94E-04	-2.62	MCM6	minichromosome maintenance complex component 6
120	A_23_P93690	3.56E-04	-2.62	MCM7	minichromosome maintenance complex component 7
121	A_23_P41267	9.13E-04	-2.60	LOC401127	WD repeat domain 5 pseudogene
122	A_23_P37892	1.15E-04	-2.59	GPT2	glutamic pyruvate transaminase (alanine aminotransferase) 2
123	A_33_P3407529	2.14E-04	-2.59	PRRT4	proline-rich transmembrane protein 4
124	A_24_P335620	1.24E-04	-2.59	SLC7A5	solute carrier family 7 (amino acid transporter light chain, L system), member 5

125	A_23_P92132	3.17E-04	-2.58	IFRD2	interferon-related developmental regulator 2
126	A_33_P3420816	9.32E-04	-2.58	GDF1	growth differentiation factor 1
127	A_33_P3301940	8.23E-04	-2.58	PDE7B	phosphodiesterase 7B
128	A_23_P161156	2.79E-04	-2.57	ZNF438	zinc finger protein 438
129	A_23_P156739	8.54E-06	-2.57	C6orf125	mitochondrial nucleoid factor 1
130	A_33_P3326210	1.04E-06	-2.56	ESCO2	establishment of cohesion 1 homolog 2 (S. cerevisiae)
131	A_23_P259692	2.72E-04	-2.55	PSAT1	phosphoserine aminotransferase 1
132	A_24_P331128	8.03E-04	-2.54	GNA15	guanine nucleotide binding protein (G protein), alpha 15 (Gq class)
133	A_23_P41804	5.76E-05	-2.54	NKD2	naked cuticle homolog 2 (Drosophila)
134	A_33_P3365810	1.34E-04	-2.54	MRPL12	mitochondrial ribosomal protein L12
135	A_23_P399078	8.44E-05	-2.54	TIMP3	TIMP metallopeptidase inhibitor 3
136	A_24_P183128	7.27E-05	-2.54	PLAC8	placenta-specific 8
137	A_23_P62227	7.41E-04	-2.53	CXorf21	chromosome X open reading frame 21
138	A_23_P200138	6.49E-04	-2.52	SLAMF8	SLAM family member 8
139	A_23_P57306	1.52E-04	-2.51	CHAF1B	chromatin assembly factor 1, subunit B (p60)
140	A_24_P280868	8.64E-04	-2.50	FAM86B2	family with sequence similarity 86, member B2
141	A_33_P3269740	8.70E-04	-2.50	FAM86B2	family with sequence similarity 86, member B2
142	A_24_P940434	4.12E-04	-2.49	LOC439914	uncharacterized LOC439914
143	A_23_P147025	1.98E-04	-2.49	RAB33A	RAB33A, member RAS oncogene family
144	A_23_P253321	2.27E-04	-2.49	PNOC	prepronociceptin
145	A_33_P3360216	4.61E-04	-2.48	HIST1H2AI	histone cluster 1, H2ai
146	A_24_P82880	2.53E-04	-2.47	TPM4	tropomyosin 4
147	A_23_P91390	6.58E-06	-2.47	THBD	thrombomodulin
148	A_24_P190168	4.26E-04	-2.47	TMEM97	transmembrane protein 97
149	A_33_P3578325	3.45E-05	-2.45	SNORD15A	small nucleolar RNA, C/D box 15A

150	A_33_P3245321	1.04E-04	-2.43	CENPP	centromere protein P
151	A_23_P39364	2.61E-04	-2.43	HOMER3	homer homolog 3 (Drosophila)
152	A_23_P302550	2.27E-04	-2.42	RGS18	regulator of G-protein signaling 18
153	A_24_P91991	5.41E-04	-2.42	NAT8L	N-acetyltransferase 8-like (GCN5-related, putative)
154	A_33_P3233645	5.00E-05	-2.41	MT1G	metallothionein 1G
155	A_24_P362193	9.25E-04	-2.41	CD84	CD84 molecule
156	A_32_P203528	1.28E-04	-2.40	SYCE2	synaptonemal complex central element protein 2
157	A_33_P3318946	9.20E-05	-2.40	HAPLN2	hyaluronan and proteoglycan link protein 2
158	A_33_P3376971	6.25E-04	-2.39	CHAC1	ChaC, cation transport regulator homolog 1 (E. coli)
159	A_23_P130585	7.52E-05	-2.39	CIB3	calcium and integrin binding family member 3
160	A_33_P3323847	1.50E-04	-2.39	RECQL4	RecQ protein-like 4
161	A_33_P3397443	2.55E-05	-2.38	PKMYT1	protein kinase, membrane associated tyrosine/threonine 1
162	A_33_P3357580	9.41E-06	-2.37	MRTO4	mRNA turnover 4 homolog (S. cerevisiae)
163	A_24_P64653	3.40E-04	-2.37	METTL7B	methyltransferase like 7B
164	A_23_P212400	2.60E-04	-2.36	NAT6	N-acetyltransferase 6 (GCN5-related)
165	A_23_P21706	2.27E-04	-2.36	CTPS	CTP synthase 1
166	A_23_P206612	3.62E-04	-2.36	USP31	ubiquitin specific peptidase 31
167	A_23_P421011	3.64E-04	-2.35	KAZALD1	Kazal-type serine peptidase inhibitor domain 1
168	A_33_P3344229	7.86E-05	-2.35	HIST1H4A	histone cluster 1, H4a
169	A_23_P4611	2.67E-04	-2.35	SLC27A5	solute carrier family 27 (fatty acid transporter), member 5
170	A_23_P31135	5.32E-05	-2.35	ACAT2	acetyl-CoA acetyltransferase 2
171	A_33_P3310430	1.67E-04	-2.35	FAM86B2	family with sequence similarity 86, member B2
172	A_33_P3340025	2.19E-05	-2.34	GINS1	GINS complex subunit 1 (Psf1 homolog)
173	A_24_P99795	2.44E-04	-2.34	ISOC2	isochorismatase domain containing 2
174	A_33_P3351175	8.55E-	-2.34	WNK2	WNK lysine deficient protein kinase 2

		05			
175	A_23_P93258	3.31E-04	-2.34	HIST1H3B	histone cluster 1, H3b
176	A_23_P10385	9.63E-04	-2.33	DTL	denticleless E3 ubiquitin protein ligase homolog (Drosophila)
177	A_23_P211572	5.22E-04	-2.33	Sep-03	SEPT3 septin 3
178	A_23_P251695	1.99E-04	-2.32	NXT1	NTF2-like export factor 1
179	A_24_P404245	8.21E-04	-2.32	PCYT2	phosphate cytidylyltransferase 2, ethanolamine
180	A_24_P229025	2.94E-04	-2.32	GRIA3	glutamate receptor, ionotropic, AMPA 3
181	A_33_P3229863	5.70E-04	-2.32	LOC100128714	uncharacterized LOC100128714
182	A_23_P160537	6.43E-04	-2.31	C1orf135	aurora kinase A and ninein interacting protein
183	A_33_P3265374	3.70E-04	-2.30	HOMER3	homer homolog 3 (Drosophila)
184	A_23_P88630	4.11E-04	-2.30	BLM	Bloom syndrome, RecQ helicase-like
185	A_23_P22761	2.97E-04	-2.30	SHOX	short stature homeobox
186	A_23_P28886	6.77E-04	-2.29	PCNA	proliferating cell nuclear antigen
187	A_32_P1701	3.79E-04	-2.28	POLA1	polymerase (DNA directed), alpha 1, catalytic subunit
188	A_23_P64372	2.35E-05	-2.28	TCN1	transcobalamin I (vitamin B12 binding protein, R binder family)
189	A_33_P3240702	1.59E-04	-2.27	RBBP8	retinoblastoma binding protein 8
190	A_23_P74115	4.93E-04	-2.27	RAD54L	RAD54-like (S. cerevisiae)
191	A_23_P416036	6.76E-04	-2.27	HAUS7	HAUS augmin-like complex, subunit 7
192	A_33_P3562537	1.57E-04	-2.27	RET	**ret** proto-oncogene
193	A_23_P9523	6.66E-05	-2.27	RBKS	ribokinase
194	A_24_P53519	5.65E-05	-2.26	CHAF1A	chromatin assembly factor 1, subunit A (p150)
195	A_23_P160934	9.82E-05	-2.26	ANP32E	acidic (leucine-rich) nuclear phosphoprotein 32 family, member E
196	A_23_P170352	2.54E-04	-2.25	MRPL12	mitochondrial ribosomal protein L12
197	A_23_P75978	4.31E-04	-2.25	CLPB	ClpB caseinolytic peptidase B homolog (E. coli)
198	A_33_P3417452	5.10E-04	-2.24	ZGLP1	zinc finger, GATA-like protein 1

199	A_23_P252740	9.99E-04	-2.24	DSCC1	defective in sister chromatid cohesion 1 homolog (S. cerevisiae)
200	A_24_P319374	9.92E-05	-2.23	GPA33	glycoprotein A33 (transmembrane)
201	A_33_P3217480	1.08E-04	-2.23	HIRIP3	HIRA interacting protein 3
202	A_33_P3368188	1.30E-04	-2.23	Sep-03	SEPT3 septin 3
203	A_23_P74269	7.88E-04	-2.23	SRM	spermidine synthase
204	A_32_P37867	4.16E-04	-2.22	KIAA1644	KIAA1644
205	A_24_P335358	6.77E-04	-2.22	PUS1	pseudouridylate synthase 1
206	A_33_P3632937	9.24E-04	-2.21	LOC100131262	uncharacterized LOC100131262
207	A_23_P122863	2.75E-05	-2.21	GRB10	growth factor receptor-bound protein 10
208	A_23_P60517	2.86E-04	-2.21	FXN	frataxin
209	A_24_P147461	9.64E-04	-2.20	SERPINB8	serpin peptidase inhibitor, clade B (ovalbumin), member 8
210	A_32_P473302	8.16E-04	-2.19	FLJ35024	uncharacterized LOC401491
211	A_23_P302672	1.19E-04	-2.19	DDIT4L	DNA-damage-inducible transcript 4-like
212	A_23_P166306	7.24E-05	-2.19	CBS	cystathionine-beta-synthase
213	A_33_P3372099	3.13E-04	-2.19	DDIT4L	DNA-damage-inducible transcript 4-like
214	A_23_P348121	6.79E-04	-2.18	FOSL2	FOS-like antigen 2
215	A_23_P86731	6.83E-04	-2.18	ZNF239	zinc finger protein 239
216	A_23_P22224	9.64E-04	-2.17	EIF4EBP1	eukaryotic translation initiation factor 4E binding protein 1
217	A_33_P3386262	4.85E-04	-2.16	CDT1	chromatin licensing and DNA replication factor 1
218	A_23_P24784	1.10E-04	-2.15	TNNI2	troponin I type 2 (skeletal, fast)
219	A_24_P264943	7.29E-04	-2.15	COMP	cartilage oligomeric matrix protein
220	A_33_P3387831	5.30E-06	-2.14	CENPM	centromere protein M
221	A_23_P107421	4.49E-04	-2.13	TK1	thymidine kinase 1, soluble
222	A_23_P386320	6.06E-04	-2.13	MFI2	antigen p97 (melanoma associated) identified by monoclonal antibodies 133.2 and 96.5
223	A_33_P3216694	3.09E-04	-2.13	HIVEP3	human immunodeficiency virus type I enhancer binding protein 3

224	A_33_P3404651	1.47E-04	-2.13	TTC7A	tetratricopeptide repeat domain 7A
225	A_23_P50455	7.07E-05	-2.13	POLD1	polymerase (DNA directed), delta 1, catalytic subunit
226	A_33_P3265301	1.98E-04	-2.12	GJD3	gap junction protein, delta 3, 31.9kDa
227	A_23_P162547	5.76E-04	-2.12	MYL2	myosin, light chain 2, regulatory, cardiac, slow
228	A_33_P3339212	4.06E-04	-2.12	TRIP13	thyroid hormone receptor interactor 13
229	A_32_P103291	2.72E-05	-2.12	SMYD3	SET and MYND domain containing 3
230	A_33_P3423570	1.03E-04	-2.11	METRN	meteorin, glial cell differentiation regulator
231	A_23_P39116	2.67E-05	-2.11	LIG1	ligase I, DNA, ATP-dependent
232	A_23_P7976	5.84E-05	-2.11	HIST1H1E	histone cluster 1, H1e
233	A_23_P116173	2.66E-04	-2.11	C11orf93	chromosome 11 open reading frame 93
234	A_23_P53276	5.20E-04	-2.11	TIMELESS	**timeless** homolog (Drosophila)
235	A_23_P43557	8.35E-04	-2.10	DENND1A	DENN/MADD domain containing 1A
236	A_23_P57709	7.58E-05	-2.10	PCOLCE2	procollagen C-endopeptidase enhancer 2
237	A_23_P16275	5.63E-04	-2.10	TSKS	testis-specific serine kinase substrate
238	A_33_P3285260	3.55E-04	-2.09	C21orf33	chromosome 21 open reading frame 33
239	A_23_P360302	8.81E-04	-2.09	GUCY2F	guanylate cyclase 2F, retinal
240	A_24_P399888	6.40E-04	-2.09	CENPM	centromere protein M
241	A_23_P161918	8.90E-04	-2.08	CCDC86	coiled-coil domain containing 86
242	A_23_P8981	2.46E-04	-2.08	STAR	steroidogenic acute regulatory protein
243	A_24_P390583	9.44E-04	-2.07	USP31	ubiquitin specific peptidase 31
244	A_23_P31073	2.69E-04	-2.06	MYB	v-**myb** myeloblastosis viral oncogene homolog (avian)
245	A_33_P3329187	5.84E-04	-2.06	DNMT1	DNA (cytosine-5-)-methyltransferase 1
246	A_33_P3220643	4.32E-04	-2.05	PTRH1	peptidyl-tRNA hydrolase 1 homolog (S. cerevisiae)
247	A_23_P152838	4.43E-04	-2.05	CCL5	chemokine (C-C motif) ligand 5
248	A_23_P88731	7.92E-04	-2.05	RAD51	RAD51 homolog (S. cerevisiae)
249	A_23_P360626	5.54E-	-2.05	PLD6	phospholipase D family, member 6

		04			
250	A_33_P3383955	8.34E-04	-2.04	DDB2	damage-specific DNA binding protein 2, 48kDa
251	A_23_P152984	8.11E-04	-2.04	THOC4	THOC3 THO complex 4
252	A_33_P3384932	4.98E-04	-2.03	NUDT8	nudix (nucleoside diphosphate linked moiety X)-type motif 8
253	A_23_P301521	7.10E-05	-2.03	KIAA1462	KIAA1462
254	A_23_P24997	2.46E-04	-2.02	CDK4	cyclin-dependent kinase 4
255	A_23_P97265	4.25E-04	-2.02	GPATCH4	G patch domain containing 4
256	A_23_P87257	2.01E-04	-2.02	MRPL17	mitochondrial ribosomal protein L17
257	A_24_P311771	5.53E-04	-2.02	ZFR	zinc finger RNA binding protein
258	A_24_P31235	3.81E-04	-2.02	EIF5A	eukaryotic translation initiation factor 5A
259	A_23_P209700	3.66E-04	-2.01	NMUR1	neuromedin U receptor 1
260	A_33_P3349536	3.27E-04	-2.01	CHEK1	checkpoint kinase 1
261	A_23_P145694	8.60E-04	-2.01	ASNS	asparagine synthetase (glutamine-hydrolyzing)
262	A_23_P213166	3.11E-05	-2.01	C4orf21	chromosome 4 open reading frame 21
263	A_33_P3390868	3.98E-04	-2.01	SYNPO2	synaptopodin 2
264	A_33_P3803667	7.03E-04	-2.01	FLJ38122	uncharacterized LOC401289
265	A_33_P3272493	7.21E-05	-2.01	CD209	CD209 molecule
266	A_23_P47790	1.57E-04	-2.00	METTL1	methyltransferase like 1
267	A_23_P141974	3.55E-04	-2.00	TPM4	tropomyosin 4
268	A_23_P86653	6.75E-04	-2.00	SRGN	serglycin
269	A_33_P3474319	5.27E-04	-2.00	SLC37A2	solute carrier family 37 (glycerol-3-phosphate transporter), member 2

Table 9.4: Significantly up regulated genes after 24 h exposure to 900 μM GHB.

	Probe Name	p-value	Fold change	Gene Symbol	Gene name
1	A_33_P3387691	1.06E-04	25.88	SCML4	sex comb on midleg-like 4 (Drosophila)
2	A_23_P146274	2.02E-04	20.85	STMN2	stathmin-like 2
3	A_33_P3535523	4.52E-04	19.38	LOC286068	uncharacterized LOC286068
4	A_33_P3416966	7.75E-05	17.23	C6orf168	failed axon connections homolog (Drosophila)
5	A_23_P360542	7.48E-04	12.87	C18orf2	charged multivesicular body protein 1B
6	A_23_P136671	1.38E-05	12.65	UGT2B7	UDP glucuronosyltransferase 2 family, polypeptide B7
7	A_33_P3789693	5.39E-05	12.45	MGC24103	uncharacterized MGC24103
8	A_33_P3441021	2.38E-04	12.30	TMEM233	transmembrane protein 233
9	A_24_P706953	1.97E-04	10.49	TMEM213	transmembrane protein 213
10	A_23_P125435	1.69E-04	9.55	GABRB1	gamma-aminobutyric acid (GABA) A receptor, beta 1
11	A_33_P3241071	1.95E-04	9.35	MANSC4	MANSC domain containing 4
12	A_24_P196528	2.25E-04	8.90	CRB1	crumbs homolog 1 (Drosophila)
13	A_33_P3330149	4.15E-04	8.72	PAX6	paired box 6
14	A_24_P234768	9.73E-04	8.08	HTR4	5-hydroxytryptamine (serotonin) receptor 4, G protein-coupled
15	A_24_P397817	1.50E-04	8.02	LEP	leptin
16	A_23_P29163	2.86E-04	7.90	GSTTP1	glutathione S-transferase theta pseudogene 1
17	A_24_P332081	4.51E-05	7.63	JAKMIP3	Janus kinase and microtubule interacting protein 3
18	A_33_P3245517	3.51E-04	7.57	LOC441666	zinc finger protein 91 pseudogene
19	A_24_P107859	1.19E-05	7.23	SPRED1	sprouty-related, EVH1 domain containing 1
20	A_33_P3312529	2.74E-04	7.01	OR4N3P	olfactory receptor, family 4, subfamily N, member 3 pseudogene
21	A_23_P84219	7.31E-04	6.72	LIPH	lipase, member H
22	A_23_P128281	3.23E-04	6.35	KLRC3	killer cell lectin-like receptor subfamily C, member 3

23	A_23_P435636	1.75E-04	6.34	DAND5	DAN domain family, member 5
24	A_23_P12363	5.85E-04	5.78	ROR1	receptor tyrosine kinase-like orphan receptor 1
25	A_33_P3290343	9.81E-05	5.72	CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1
26	A_24_P350546	9.87E-04	5.62	LOC646976	uncharacterized LOC646976
27	A_23_P30315	1.80E-04	5.55	TRIM7	tripartite motif containing 7
28	A_24_P113131	1.97E-05	5.53	BZRAP1	benzodiazapine receptor (peripheral) associated protein 1
29	A_23_P257003	1.31E-04	5.10	PCSK5	proprotein convertase subtilisin/kexin type 5
30	A_23_P121657	4.18E-04	4.70	HS3ST1	heparan sulfate (glucosamine) 3-O-sulfotransferase 1
31	A_23_P4254	6.19E-05	4.66	CRYBA1	crystallin, beta A1
32	A_23_P405885	8.91E-04	4.55	DPPA2	developmental pluripotency associated 2
33	A_33_P3331491	8.41E-05	4.52	LOC728392	uncharacterized LOC728392
34	A_24_P70906	9.09E-04	4.52	PDILT	protein disulfide isomerase-like, testis expressed
35	A_33_P3273777	6.53E-04	4.51	GJB7	gap junction protein, beta 7, 25kDa
36	A_33_P3351879	3.98E-05	4.37	LOC100128905	uncharacterized LOC100128905
37	A_33_P3265679	8.01E-04	4.34	LOC389199	uncharacterized LOC389199
38	A_23_P89431	4.47E-05	4.33	CCL2	chemokine (C-C motif) ligand 2
39	A_23_P204640	7.41E-04	4.31	NANOG	Nanog homeobox
40	A_23_P216307	6.30E-04	4.30	RUNX1T1	runt-related transcription factor 1; translocated to, 1 (cyclin D-related)
41	A_33_P3370890	4.06E-05	4.27	C6orf225	chromosome 6 open reading frame 225
42	A_33_P3353027	5.01E-04	4.23	CHN2	chimerin (chimaerin) 2
43	A_23_P20363	4.93E-04	4.16	FAM164A	zinc finger, C2HC-type containing 1A
44	A_33_P3349395	6.55E-04	4.11	CECR2	cat eye syndrome chromosome region, candidate 2
45	A_24_P325520	4.05E-04	4.10	SORT1	sortilin 1
46	A_23_P209625	9.02E-06	4.08	CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1

47	A_33_P3379811	6.21E-04	4.04	C12orf77	chromosome 12 open reading frame 77
48	A_32_P703	1.86E-04	4.03	LOC646626	uncharacterized LOC646626
49	A_33_P3335624	3.29E-06	3.78	PSMD10	proteasome (prosome, macropain) 26S subunit, non-ATPase, 10
50	A_23_P100711	3.36E-04	3.78	PMP22	peripheral myelin protein 22
51	A_23_P82523	1.87E-04	3.74	ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1
52	A_33_P3337719	3.47E-05	3.74	LOC100134868	uncharacterized LOC100134868
53	A_33_P3310232	4.12E-04	3.73	L2HGDH	L-2-hydroxyglutarate dehydrogenase
54	A_23_P171143	7.77E-04	3.71	TSPAN6	tetraspanin 6
55	A_33_P3522511	4.93E-04	3.69	KIAA0485	uncharacterized LOC57235
56	A_33_P3294961	2.55E-04	3.68	LAT2	linker for activation of T cells family, member 2
57	A_33_P3248863	2.52E-05	3.65	KRBA2	KRAB-A domain containing 2
58	A_33_P3269650	4.51E-04	3.65	LOC100128402	uncharacterized LOC100128402
59	A_23_P124108	1.38E-04	3.60	ITGAM	integrin, alpha M (complement component 3 receptor 3 subunit)
60	A_23_P105963	8.59E-04	3.59	AK7	Adenylate kinase 7
61	A_33_P3274560	8.60E-04	3.58	SLC19A3	solute carrier family 19, member 3
62	A_23_P82651	5.81E-04	3.56	NPTX2	neuronal pentraxin II
63	A_23_P136978	1.74E-04	3.56	SRPX2	sushi-repeat containing protein, X-linked 2
64	A_33_P3380405	2.42E-04	3.53	CYTH1	cytohesin 1
65	A_23_P339818	1.04E-04	3.52	ARRDC4	arrestin domain containing 4
66	A_23_P428248	8.21E-04	3.52	TTC21A	tetratricopeptide repeat domain 21A
67	A_33_P3337415	1.58E-04	3.50	GNAL	guanine nucleotide binding protein (G protein), alpha activating activity polypeptide, olfactory type
68	A_33_P3276703	9.60E-06	3.49	VGF	VGF nerve growth factor inducible
69	A_23_P366453	9.11E-05	3.46	KHDRBS2	KH domain containing, RNA binding, signal transduction associated 2
70	A_33_P3365646	7.58E-04	3.44	LOC100128437	uncharacterized LOC100128437

71	A_23_P111995	1.98E-05	3.40	LOXL2	lysyl oxidase-like 2
72	A_33_P3392123	3.18E-05	3.39	LOC100233209	uncharacterized LOC100233209
73	A_33_P3354499	3.29E-04	3.39	LOC401480	uncharacterized LOC401480
74	A_33_P3380652	9.12E-04	3.37	ADAM28	ADAM metallopeptidase domain 28
75	A_33_P3400248	3.43E-04	3.35	FGF20	fibroblast growth factor 20
76	A_33_P3237135	6.39E-04	3.33	MMP2	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)
77	A_33_P3281333	5.04E-04	3.31	SNTB2	syntrophin, beta 2 (dystrophin-associated protein A1, 59kDa, basic component 2)
78	A_33_P3276068	1.35E-06	3.31	BET3L	BET3 like (S. cerevisiae)
79	A_32_P133840	7.60E-05	3.28	TMCC2	transmembrane and coiled-coil domain family 2
80	A_23_P7827	9.91E-05	3.28	FAM26F	family with sequence similarity 26, member F
81	A_23_P18055	3.30E-05	3.28	C3orf51	ERC2 intronic transcript 1 (non-protein coding)
82	A_24_P129588	6.59E-05	3.27	O3FAR1	omega-3 fatty acid receptor 1
83	A_23_P121926	6.73E-04	3.26	SEPP1	selenoprotein P, plasma, 1
84	A_23_P217228	1.31E-04	3.26	TRO	trophinin
85	A_33_P3465247	3.04E-04	3.24	KIF3A	kinesin family member 3A
86	A_33_P3333033	8.67E-04	3.23	SGSM2	small G protein signaling modulator 2
87	A_33_P3700860	8.12E-04	3.23	KIAA1661	KIAA1661 protein
88	A_23_P371824	5.97E-05	3.22	TUFT1	tuftelin 1
89	A_33_P3397795	9.65E-05	3.22	C14orf135	pecanex-like 4 (Drosophila)
90	A_33_P3893191	4.48E-04	3.22	DBIL5P2	diazepam binding inhibitor-like 5 pseudogene 2
91	A_33_P3385765	5.30E-04	3.21	ZNF763	zinc finger protein 763
92	A_24_P137522	8.77E-04	3.20	USP53	ubiquitin specific peptidase 53
93	A_33_P3252381	7.94E-04	3.19	PCA3	prostate cancer antigen 3 (non-protein coding)
94	A_33_P3235420	8.27E-05	3.18	RASSF6	Ras association (RalGDS/AF-6) domain family member 6
95	A_33_P3330991	5.49E-04	3.18	LOC100134237	uncharacterized LOC100134237

96	A_33_P3321678	6.19E-05	3.17	RNF180	ring finger protein 180
97	A_23_P364625	6.02E-04	3.15	LRRC19	leucine rich repeat containing 19
98	A_33_P3219939	8.53E-04	3.15	CUBN	cubilin (intrinsic factor-cobalamin receptor)
99	A_23_P130764	3.86E-04	3.14	KCNJ14	potassium inwardly-rectifying channel, subfamily J, member 14
100	A_33_P3419938	4.69E-05	3.11	VHL	von Hippel-Lindau tumor suppressor, E3 ubiquitin protein ligase
101	A_32_P46214	9.61E-05	3.09	SLC9A9	solute carrier family 9, subfamily A (NHE9, cation proton antiporter 9), member 9
102	A_32_P54274	2.07E-04	3.09	DRD5	dopamine receptor D5
103	A_24_P41801	4.19E-05	3.08	LPA	lipoprotein, Lp(a)
104	A_33_P3322804	1.89E-04	3.07	NTRK2	neurotrophic tyrosine kinase, receptor, type 2
105	A_33_P3609431	2.28E-04	3.06	ERVFRD-2	endogenous retrovirus group FRD, member 2
106	A_33_P3305173	3.18E-04	3.06	RGAG4	retrotransposon gag domain containing 4
107	A_32_P83811	8.70E-04	3.05	FAM47E	family with sequence similarity 47, member E
108	A_33_P3418125	2.22E-04	3.05	GLIPR1	GLI pathogenesis-related 1
109	A_23_P13929	1.06E-04	3.03	NRIP2	nuclear receptor interacting protein 2
110	A_33_P3410831	8.27E-05	3.03	LOC729609	uncharacterized **LOC729609**
111	A_23_P45365	1.72E-04	3.02	COL4A5	collagen, type IV, alpha 5
112	A_23_P200260	3.35E-04	3.02	PCNXL2	pecanex-like 2 (Drosophila)
113	A_33_P3421219	3.42E-04	3.02	LOC647070	uncharacterized **LOC647070**
114	A_23_P40880	2.51E-05	3.01	CMTM8	CKLF-like MARVEL transmembrane domain containing 8
115	A_23_P168882	2.23E-04	3.00	TP53INP1	tumor protein p53 inducible nuclear protein 1
116	A_23_P333029	2.11E-04	3.00	C8orf47	chromosome 8 open reading frame 47
117	A_33_P3524912	3.41E-05	2.99	LOC283038	uncharacterized **LOC283038**
118	A_23_P415021	2.31E-04	2.99	METTL7A	methyltransferase like 7A
119	A_33_P3272508	9.44E-06	2.98	MRP63	mitochondrial ribosomal protein 63
120	A_33_P3702281	1.17E-06	2.97	LOC284014	uncharacterized **LOC284014**

121	A_33_P3401317	5.85E-04	2.97	POTEM	POTE ankyrin domain family, member M
122	A_33_P3351934	4.37E-05	2.97	MSTO2P	misato homolog 2 pseudogene
123	A_33_P3211569	4.03E-06	2.96	ERBB3	Receptor tyrosine-protein kinase erbB-3
124	A_23_P359897	5.84E-04	2.96	JHDM1D	jumonji C domain containing histone demethylase 1 homolog D (S. cerevisiae)
125	A_32_P104746	5.97E-04	2.95	ZFYVE28	zinc finger, FYVE domain containing 28
126	A_24_P476086	3.60E-04	2.95	KPNA5	karyopherin alpha 5 (importin alpha 6)
127	A_33_P3537875	6.70E-06	2.95	MGC10814	uncharacterized protein **MGC10814**
128	A_32_P711043	9.84E-05	2.93	LOC100507191	uncharacterized **LOC100507191**
129	A_23_P167983	1.82E-04	2.91	HIST1H2AC	histone cluster 1, H2ac
130	A_24_P303097	6.71E-04	2.90	SNX25	sorting nexin 25
131	A_23_P14649	5.78E-06	2.90	NCRNA00321	ANP32A intronic transcript 1 (non-protein coding)
132	A_33_P3273000	5.30E-04	2.90	PTK2B	PTK2B protein tyrosine kinase 2 beta
133	A_33_P3310047	2.29E-04	2.89	SHROOM4	shroom family member 4
134	A_33_P3292179	4.27E-04	2.88	ABCA9	ATP-binding cassette, sub-family A (ABC1), member 9
135	A_33_P3265606	2.64E-04	2.86	GCLM	glutamate-cysteine ligase, modifier subunit
136	A_23_P387184	7.49E-04	2.86	NHSL1	NHS-like 1
137	A_33_P3338121	8.51E-05	2.86	LAMB3	laminin, beta 3
138	A_23_P11543	3.28E-04	2.85	FUCA1	fucosidase, alpha-L- 1, tissue
139	A_33_P3400708	2.95E-04	2.85	BNIPL	BCL2/adenovirus E1B 19kD interacting protein like
140	A_23_P91697	3.02E-04	2.84	LARGE	like-glycosyltransferase
141	A_33_P3409675	9.46E-05	2.83	LOC442132	golgin A6 family-like 1 pseudogene
142	A_23_P157283	9.64E-04	2.82	C7orf23	chromosome 7 open reading frame 23
143	A_23_P387000	4.09E-04	2.82	XKR6	XK, Kell blood group complex subunit-related family, member 6
144	A_23_P255376	2.47E-04	2.81	CCDC109B	coiled-coil domain containing 109B

145	A_33_P3382769	6.70E-04	2.80	UBE2Q2P3	ubiquitin-conjugating enzyme E2Q family member 2 pseudogene 3
146	A_23_P123848	8.92E-04	2.79	DAB2IP	DAB2 interacting protein
147	A_23_P34644	6.50E-04	2.79	FCGR2B	Fc fragment of IgG, low affinity IIb, receptor (CD32)
148	A_33_P3219697	3.93E-04	2.79	ANKRD45	ankyrin repeat domain 45
149	A_33_P3313929	6.48E-06	2.78	CCR6	chemokine (C-C motif) receptor 6
150	A_23_P208493	4.89E-04	2.78	LILRB2	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 2
151	A_23_P168771	6.13E-05	2.76	CCDC146	coiled-coil domain containing 146
152	A_33_P3559138	9.31E-04	2.75	FLJ42200	FLJ42200 protein
153	A_23_P166929	9.30E-04	2.74	SERPINI1	serpin peptidase inhibitor, clade I (neuroserpin), member 1
154	A_33_P3365720	7.11E-04	2.73	FLJ35424	uncharacterized FLJ35424
155	A_23_P151565	2.10E-04	2.73	RALGAPA1	Ral GTPase activating protein, alpha subunit 1 (catalytic)
156	A_33_P3282688	6.89E-04	2.73	LOC100130027	uncharacterized LOC100130027
157	A_23_P109907	1.03E-04	2.73	ILDR1	immunoglobulin-like domain containing receptor 1
158	A_23_P142574	7.18E-04	2.72	MOGAT1	monoacylglycerol O-acyltransferase 1
159	A_33_P3420204	7.23E-04	2.72	CRTC1	CREB regulated transcription coactivator 1
160	A_33_P3337044	8.58E-07	2.71	SLC2A6	solute carrier family 2 (facilitated glucose transporter), member 6
161	A_23_P51397	1.37E-05	2.70	ENAH	enabled homolog (Drosophila)
162	A_33_P3734384	9.53E-05	2.69	LOC285957	uncharacterized LOC285957
163	A_23_P205867	1.34E-04	2.68	NR2E3	nuclear receptor subfamily 2, group E, member 3
164	A_23_P40096	6.01E-04	2.68	PROC	protein C (inactivator of coagulation factors Va and VIIIa)
165	A_23_P38795	1.72E-04	2.67	FPR1	formyl peptide receptor 1
166	A_23_P89812	1.80E-04	2.67	CNDP2	CNDP dipeptidase 2 (metallopeptidase M20 family)
167	A_33_P3383551	3.30E-04	2.67	SEC14L3	SEC14-like 3 (S. cerevisiae)
168	A_33_P3327697	5.53E-	2.66	LOC14547	uncharacterized LOC145474

		05		4	
169	A_33_P3220437	3.88E-05	2.66	TNFAIP8L1	tumor necrosis factor, alpha-induced protein 8-like 1
170	A_33_P3213317	1.96E-04	2.65	SPINLW1	EPPIN-WFDC6 readthrough
171	A_24_P267664	1.50E-04	2.65	C21orf88	chromosome 21 open reading frame 88
172	A_23_P5392	1.69E-04	2.64	TP53I3	tumor protein p53 inducible protein 3
173	A_33_P3375613	7.71E-05	2.62	C8orf44-SGK3	C8orf44-SGK3 readthrough
174	A_33_P3338166	1.60E-04	2.62	NUAK2	NUAK family, SNF1-like kinase, 2
175	A_23_P128174	3.71E-05	2.62	RAB3IP	RAB3A interacting protein (rabin3)
176	A_23_P203920	3.44E-04	2.60	SSPN	sarcospan
177	A_32_P151544	7.79E-04	2.60	KRT18	keratin 18
178	A_23_P144531	2.07E-04	2.59	ALPK1	alpha-kinase 1
179	A_33_P3298128	1.70E-04	2.57	ITPR2	inositol 1,4,5-trisphosphate receptor, type 2
180	A_23_P4536	3.06E-04	2.57	EPB41L3	erythrocyte membrane protein band 4.1-like 3
181	A_33_P3255499	3.22E-04	2.57	MCART1	solute carrier family 25, member 51
182	A_33_P3322430	1.40E-04	2.56	CTNS	cystinosin, lysosomal cystine transporter
183	A_23_P422831	4.38E-05	2.56	FAM189A2	family with sequence similarity 189, member A2
184	A_23_P59138	5.58E-04	2.55	POU5F1	POU class 5 homeobox 1
185	A_33_P3762918	6.87E-04	2.54	LOC100216546	uncharacterized **LOC100216546**
186	A_33_P3257903	3.99E-04	2.53	GSTA4	glutathione S-transferase alpha 4
187	A_33_P3365134	8.30E-04	2.53	PPP1R13B	protein phosphatase 1, regulatory subunit 13B
188	A_23_P167096	2.86E-04	2.52	VEGFC	vascular endothelial growth factor C
189	A_33_P3379916	3.19E-04	2.51	GLS	glutaminase
190	A_23_P379649	1.51E-04	2.50	BMF	Bcl2 modifying factor
191	A_33_P3392077	4.10E-04	2.50	TP53I3	tumor protein p53 inducible protein 3
192	A_33_P3311498	4.72E-04	2.49	LOC283392	TRHDE antisense RNA 1
193	A_23_P112874	2.75E-04	2.49	GPC5	glypican 5

194	A_33_P3358163	9.32E-04	2.49	C15orf2	nuclear pore associated protein 1
195	A_33_P3234267	6.51E-04	2.49	LOC100130442	uncharacterized **LOC100130442**
196	A_33_P3365760	2.28E-04	2.48	STAP1	signal transducing adaptor family member 1
197	A_24_P332218	6.14E-04	2.46	GPR149	G protein-coupled receptor 149
198	A_23_P145114	6.63E-04	2.46	GCLC	glutamate-cysteine ligase, catalytic subunit
199	A_33_P3326904	4.04E-04	2.46	LOC441268	uncharacterized **LOC441268**
200	A_23_P15108	7.39E-04	2.45	YPEL3	yippee-like 3 (Drosophila)
201	A_23_P48217	4.00E-04	2.45	APOLD1	apolipoprotein L domain containing 1
202	A_23_P110941	4.55E-04	2.45	GSTA4	glutathione S-transferase alpha 4
203	A_33_P3360887	3.13E-04	2.44	PRIC285	peroxisomal proliferator-activated receptor A interacting complex 285
204	A_33_P3567967	5.28E-04	2.44	FLJ11292	uncharacterized protein **FLJ11292**
205	A_33_P3397073	6.43E-04	2.44	LOC100507547	uncharacterized **LOC100507547**
206	A_33_P3381454	1.10E-04	2.44	C1orf56	chromosome 1 open reading frame 56
207	A_33_P3353496	5.15E-05	2.43	GJD4	gap junction protein, delta 4, 40.1kDa
208	A_33_P3281613	1.57E-04	2.43	KCNT1	potassium channel, subfamily T, member 1
209	A_33_P3399318	4.41E-05	2.43	GNG12	guanine nucleotide binding protein (G protein), gamma 12
210	A_23_P329261	9.51E-04	2.43	KCNJ2	potassium inwardly-rectifying channel, subfamily J, member 2
211	A_23_P150053	4.49E-04	2.43	ACTA2	actin, alpha 2, smooth muscle, aorta
212	A_33_P3338928	3.51E-04	2.43	DAB2	disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)
213	A_23_P112846	5.02E-04	2.42	MTHFD2L	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2-like
214	A_33_P3288074	3.45E-04	2.40	LOC730202	uncharacterized **LOC730202**
215	A_23_P133543	2.57E-04	2.39	KLHL3	kelch-like 3 (Drosophila)
216	A_23_P108823	6.88E-04	2.39	OSBPL6	oxysterol binding protein-like 6
217	A_33_P3301381	3.09E-04	2.39	RBM43	RNA binding motif protein 43
218	A_23_P112634	1.81E-04	2.38	C4orf34	chromosome 4 open reading frame 34

219	A_23_P94546	7.68E-04	2.38	GKAP1	G kinase anchoring protein 1
220	A_32_P30831	2.70E-04	2.37	LOC100505648	uncharacterized **LOC100505648**
221	A_33_P3232294	6.20E-04	2.36	ITPR2	inositol 1,4,5-trisphosphate receptor, type 2
222	A_23_P362191	1.79E-04	2.35	NCRNA00324	long intergenic non-protein coding RNA 324
223	A_33_P3225696	1.06E-04	2.35	ZNF526	zinc finger protein 526
224	A_33_P3219720	4.11E-04	2.34	ZNF248	zinc finger protein 248
225	A_23_P381714	8.36E-04	2.33	CA13	carbonic anhydrase XIII
226	A_33_P3230594	1.74E-04	2.33	LOC100507547	uncharacterized **LOC100507547**
227	A_23_P17103	7.43E-04	2.32	TSGA10	testis specific, 10
228	A_33_P3296205	2.66E-04	2.31	TSC22D1	TSC22 domain family, member 1
229	A_23_P82351	5.12E-04	2.31	BBS9	Bardet-Biedl syndrome 9
230	A_33_P3324333	3.89E-04	2.31	ANKRD20A5P	ankyrin repeat domain 20 family, member A5, pseudogene
231	A_23_P500501	2.48E-04	2.30	FGFR3	fibroblast growth factor receptor 3
232	A_23_P30294	4.87E-04	2.30	CDO1	cysteine dioxygenase, type I
233	A_33_P3330443	1.90E-04	2.29	FAM110B	family with sequence similarity 110, member B
234	A_24_P408047	4.05E-04	2.29	PLEKHA4	pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 4
235	A_23_P201319	1.94E-04	2.28	DISP1	dispatched homolog 1 (Drosophila)
236	A_23_P329353	8.00E-04	2.28	CNRIP1	cannabinoid receptor interacting protein 1
237	A_24_P110983	2.01E-04	2.27	AKT3	v-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma)
238	A_33_P3384442	3.34E-05	2.26	LAMA5	laminin, alpha 5
239	A_33_P3223056	3.65E-04	2.25	ADAMTS10	ADAM metallopeptidase with thrombospondin type 1 motif, 10
240	A_32_P205241	5.88E-05	2.25	GJA3	gap junction protein, alpha 3, 46kDa
241	A_33_P3267248	8.42E-04	2.25	TDRD10	tudor domain containing 10
242	A_33_P3252206	2.17E-04	2.25	LEPROTL1	leptin receptor overlapping transcript-like 1
243	A_23_P421054	1.02E-	2.25	C6orf165	chromosome 6 open reading frame 165

		04			
244	A_24_P250535	5.63E-05	2.25	TMX4	thioredoxin-related transmembrane protein 4
245	A_33_P3274528	8.27E-04	2.25	C9orf153	chromosome 9 open reading frame 153
246	A_23_P375147	3.73E-04	2.24	RC3H2	ring finger and CCCH-type domains 2
247	A_23_P217528	7.19E-04	2.24	KLF8	Kruppel-like factor 8
248	A_33_P3372526	4.04E-04	2.24	MDH1B	malate dehydrogenase 1B, NAD (soluble)
249	A_33_P3549091	3.72E-04	2.24	LOC100131347	RAD52 motif 1 pseudogene
250	A_23_P95130	3.80E-04	2.24	SLC37A3	solute carrier family 37 (glycerol-3-phosphate transporter), member 3
251	A_24_P131522	8.80E-04	2.23	ANTXR1	anthrax toxin receptor 1
252	A_24_P205130	1.14E-05	2.23	FNBP1	formin binding protein 1
253	A_33_P3283231	5.88E-05	2.23	YY2	**YY2** transcription factor
254	A_33_P3257993	1.64E-04	2.23	RNF125	ring finger protein 125, E3 ubiquitin protein ligase
255	A_33_P3380837	8.88E-04	2.21	AMZ1	archaelysin family metallopeptidase 1
256	A_23_P166297	7.46E-04	2.21	ABCG1	ATP-binding cassette, sub-family G (WHITE), member 1
257	A_23_P19226	6.66E-04	2.20	DSE	dermatan sulfate epimerase
258	A_23_P204885	7.34E-04	2.20	PCDH20	protocadherin 20
259	A_33_P3239267	1.36E-04	2.20	C3orf54	family with sequence similarity 212, member A
260	A_23_P501538	2.01E-04	2.19	HOXA3	homeobox A3
261	A_23_P259442	1.64E-04	2.19	CPE	carboxypeptidase E
262	A_23_P164022	2.34E-04	2.19	MYO1C	myosin IC
263	A_33_P3787904	2.07E-04	2.18	IKBKB	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
264	A_23_P126836	2.56E-04	2.17	TNFSF4	tumor necrosis factor (ligand) superfamily, member 4
265	A_33_P3711318	9.47E-04	2.17	LOC257152	uncharacterized **LOC257152**
266	A_23_P417282	9.25E-04	2.17	IGF1R	insulin-like growth factor 1 receptor
267	A_23_P39766	1.53E-04	2.15	GLS	glutaminase

268	A_23_P206501	3.58E-04	2.15	CLEC18B	C-type lectin domain family 18, member B
269	A_23_P32454	3.35E-04	2.14	TG	thyroglobulin
270	A_33_P3270192	1.95E-04	2.14	DIS3L2	DIS3 mitotic control homolog (S. cerevisiae)-like 2
271	A_33_P3311439	3.19E-06	2.13	GCH1	GTP cyclohydrolase 1
272	A_23_P6914	1.04E-04	2.13	OSBPL11	oxysterol binding protein-like 11
273	A_23_P59637	5.46E-05	2.12	DOCK4	dedicator of cytokinesis 4
274	A_23_P376188	4.49E-04	2.12	BEND7	BEN domain containing 7
275	A_23_P99044	9.84E-04	2.12	KRT71	keratin 71
276	A_23_P304171	5.82E-04	2.12	KIAA0226	KIAA0226
277	A_33_P3359084	1.76E-04	2.12	TAS1R3	taste receptor, type 1, member 3
278	A_24_P268993	5.18E-04	2.11	LEAP2	liver expressed antimicrobial peptide 2
279	A_33_P3247678	8.19E-04	2.11	LOC100130876	uncharacterized **LOC100130876**
280	A_33_P3539345	5.44E-04	2.10	MYO6	myosin VI
281	A_23_P71328	5.43E-04	2.10	MATN2	matrilin 2
282	A_24_P28977	3.33E-04	2.09	TRPC1	transient receptor potential cation channel, subfamily C, member 1
283	A_33_P3326588	3.41E-04	2.09	TNFRSF10D	tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain
284	A_23_P207564	5.40E-04	2.09	CCL4	chemokine (C-C motif) ligand 4
285	A_23_P200801	5.60E-04	2.09	PDE4DIP	phosphodiesterase 4D interacting protein
286	A_23_P423108	7.83E-05	2.09	TDRD10	tudor domain containing 10
287	A_33_P3212615	8.05E-04	2.09	TFPI	ue factor pathway inhibitor (lipoprotein-ɔciated coagulation inhibitor
288	A_23_P359647	1.02E-04	2.09	NFAT5	nuclear factor of activated T-cells 5, tonicity-responsive
289	A_23_P157926	5.57E-04	2.09	LINGO2	leucine rich repeat and Ig domain containing 2
290	A_33_P3307955	3.76E-04	2.09	ABCD3	ATP-binding cassette, sub-family D (ALD), member 3
291	A_33_P3223660	5.43E-05	2.08	C9orf130	long intergenic non-protein coding RNA 476

292	A_23_P210210	2.01E-04	2.08	EPAS1	endothelial PAS domain protein 1
293	A_23_P160546	3.87E-04	2.08	FAM63A	family with sequence similarity 63, member A
294	A_32_P358887	7.74E-04	2.07	SLC4A4	solute carrier family 4, sodium bicarbonate cotransporter, member 4
295	A_23_P46315	3.80E-04	2.07	DENND2C	DENN/MADD domain containing 2C
296	A_24_P89891	9.92E-05	2.07	TRAF1	TNF receptor-associated factor 1
297	A_24_P151834	4.94E-05	2.07	ANKFY1	ankyrin repeat and FYVE domain containing 1
298	A_23_P207319	3.89E-04	2.07	MAP3K14	mitogen-activated protein kinase kinase kinase 14
299	A_23_P206371	5.54E-05	2.07	NOL3	nucleolar protein 3 (apoptosis repressor with CARD domain)
300	A_23_P24004	1.77E-04	2.06	IFIT2	interferon-induced protein with tetratricopeptide repeats 2
301	A_23_P156049	6.50E-04	2.06	HEXB	hexosaminidase B (beta polypeptide)
302	A_23_P95060	7.03E-04	2.06	EPHB3	Tyrosine-protein kinase TYRO6
303	A_24_P398810	7.86E-04	2.06	EIF5	eukaryotic translation initiation factor 5
304	A_33_P3827416	5.99E-04	2.06	LOC399753	uncharacterized **LOC399753**
305	A_23_P117782	1.00E-04	2.05	LARP6	La ribonucleoprotein domain family, member 6
306	A_33_P3284508	4.72E-05	2.05	CD14	**CD14** molecule
307	A_33_P3494875	2.94E-04	2.05	LOC100506748	uncharacterized **LOC100506748**
308	A_23_P212696	2.10E-04	2.05	FSTL1	follistatin-like 1
309	A_33_P3228988	5.82E-04	2.05	ZNF799	zinc finger protein 799
310	A_33_P3373892	9.68E-04	2.04	NAA35	N(alpha)-acetyltransferase 35, NatC auxiliary subunit
311	A_23_P218463	1.64E-04	2.04	SERTAD1	SERTA domain containing 1
312	A_33_P3678883	2.84E-04	2.04	KIAA1654	**KIAA1654** protein
313	A_24_P943472	3.30E-04	2.02	NR1D2	nuclear receptor subfamily 1, group D, member 2
314	A_33_P3275959	6.35E-05	2.02	SLC25A16	solute carrier family 25 (mitochondrial carrier; Graves disease autoantigen), member 16
315	A_23_P328206	4.89E-04	2.01	DNMBP	dynamin binding protein

316	A_23_P143845	1.54E-04	2.01	TIPARP	TCDD-inducible poly(ADP-ribose) polymerase
317	A_23_P85716	9.06E-05	2.01	FCGR2A	Fc fragment of IgG, low affinity IIa, receptor (CD32)
318	A_33_P3287348	8.66E-04	2.01	CHN2	chimerin (chimaerin) 2
319	A_24_P941217	3.69E-04	2.00	SGPP2	sphingosine-1-phosphate phosphatase 2

9.3 Gene Ontology tables (with chapter 4)

Table 9.5: Information of enriched GO terms for significantly down regulated genes after 24 hours exposure to 10μM GHB.

Go ID	Ontology	Term	Number of altered genes
GO:0006820	biological process	anion transport	6
GO:0015711	biological process	organic anion transport	6
GO:0015718	biological process	monocarboxylic acid transport	4
GO:0015849	biological process	organic acid transport	5
GO:0046942	biological process	carboxylic acid transport	5
GO:0008206	biological process	bile acid metabolic process	3
GO:0015721	biological process	bile acid and bile salt transport	3
GO:0030299	biological process	intestinal cholesterol absorption	2
GO:0044241	biological process	lipid digestion	2
GO:0004033	molecular function	aldo-keto reductase (NADP) activity	3
GO:0032052	molecular function	bile acid binding	2
GO:0016229	molecular function	steroid dehydrogenase activity	3
GO:0033764	molecular function	steroid dehydrogenase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	3
GO:0047023	molecular function	androsterone dehydrogenase activity	3
GO:0047042	molecular function	androsterone dehydrogenase (B-specific) activity	3

Table 9.6: Information of enriched GO terms for significantly up regulated genes after 24 hours exposure to 10μM GHB.

GOID	Ontology	Term	Number of genes
GO:0016020	cellular_component	membrane	30
GO:0005654	cellular_component	nucleoplasm	9
GO:0016023	cellular_component	cytoplasmic membrane-bounded vesicle	12
GO:0031410	cellular_component	cytoplasmic vesicle	12
GO:0031982	cellular_component	vesicle	12
GO:0031988	cellular_component	membrane-bounded vesicle	12
GO:0030141	cellular_component	secretory granule	9
GO:0030118	cellular_component	clathrin coat	3
GO:0030119	cellular_component	AP-type membrane coat adaptor complex	3
GO:0030131	cellular_component	clathrin adaptor complex	3
GO:0030027	cellular_component	lamellipodium	8
GO:0031252	cellular_component	cell leading edge	8
GO:0032593	cellular_component	insulin-responsive compartment	8
GO:0048583	biological_process	regulation of response to stimulus	12
GO:0048584	biological_process	positive regulation of response to stimulus	10
GO:0006357	biological_process	regulation of transcription from RNA polymerase II promoter	10
GO:0009892	biological_process	negative regulation of metabolic process	9
GO:0010605	biological_process	negative regulation of macromolecule metabolic process	9
GO:0031324	biological_process	negative regulation of cellular metabolic process	9
GO:0045934	biological_process	negative regulation of nucleobase-containing compound metabolic process	9
GO:0051172	biological_process	negative regulation of nitrogen compound metabolic process	9
GO:0051253	biological_process	negative regulation of RNA metabolic process	9
GO:0030154	biological_process	cell differentiation	14
GO:0048869	biological_process	cellular developmental process	14
GO:0051179	biological_process	localization	17
GO:0051641	biological_process	cellular localization	12
GO:0048468	biological_process	cell development	11
GO:0009891	biological_process	positive regulation of biosynthetic process	9
GO:0010557	biological_process	positive regulation of macromolecule biosynthetic process	9
GO:0010628	biological_process	positive regulation of gene expression	9
GO:0031328	biological_process	positive regulation of cellular biosynthetic process	9
GO:0045893	biological_process	positive regulation of transcription, DNA-dependent	9
GO:0045935	biological_process	positive regulation of nucleobase-containing compound metabolic process	9

GO:0051173	biological_process	positive regulation of nitrogen compound metabolic process	9
GO:0051254	biological_process	positive regulation of RNA metabolic process	9
GO:0080134	biological_process	regulation of response to stress	10
GO:0008104	biological_process	protein localization	13
GO:0033036	biological_process	macromolecule localization	14
GO:0009719	biological_process	response to endogenous stimulus	9
GO:0009725	biological_process	response to hormone stimulus	9
GO:0034613	biological_process	cellular protein localization	11
GO:0070727	biological_process	cellular macromolecule localization	11
GO:0003008	biological_process	system process	11
GO:0019226	biological_process	transmission of nerve impulse	10
GO:0035637	biological_process	multicellular organismal signaling	10
GO:0050877	biological_process	neurological system process	11
GO:0006900	biological_process	membrane budding	3
GO:0006901	biological_process	vesicle coating	3
GO:0016044	biological_process	cellular membrane organization	11
GO:0016050	biological_process	vesicle organization	3
GO:0048268	biological_process	clathrin coat assembly	3
GO:0061024	biological_process	membrane organization	11
GO:0048488	biological_process	synaptic vesicle endocytosis	2
GO:0022008	biological_process	neurogenesis	10
GO:0009966	biological_process	regulation of signal transduction	10
GO:0016740	molecular_function	transferase activity	15
GO:0006915	biological_process	apoptotic process	9
GO:0008219	biological_process	cell death	9
GO:0012501	biological_process	programmed cell death	9
GO:0016265	biological_process	death	9
GO:0033081	biological_process	regulation of T cell differentiation in thymus	2
GO:0006970	biological_process	response to osmotic stress	8
GO:0009628	biological_process	response to abiotic stimulus	8
GO:0009967	biological_process	positive regulation of signal transduction	8
GO:0010647	biological_process	positive regulation of cell communication	8
GO:0023056	biological_process	positive regulation of signaling	8
GO:0002028	biological_process	regulation of sodium ion transport	8
GO:0010765	biological_process	positive regulation of sodium ion transport	8
GO:0010959	biological_process	regulation of metal ion transport	8
GO:0032879	biological_process	regulation of localization	9
GO:0043269	biological_process	regulation of ion transport	8
GO:0043270	biological_process	positive regulation of ion transport	8
GO:0051049	biological_process	regulation of transport	9
GO:0051050	biological_process	positive regulation of transport	9
GO:0014823	biological_process	response to activity	8
GO:0014850	biological_process	response to muscle activity	8
GO:0030334	biological_process	regulation of cell migration	8

GO:0030335	biological_process	positive regulation of cell migration	8
GO:0040012	biological_process	regulation of locomotion	8
GO:0040017	biological_process	positive regulation of locomotion	8
GO:0051270	biological_process	regulation of cellular component movement	8
GO:0051272	biological_process	positive regulation of cellular component movement	8
GO:2000145	biological_process	regulation of cell motility	8
GO:2000147	biological_process	positive regulation of cell motility	8
GO:0001508	biological_process	regulation of action potential	8
GO:0006873	biological_process	cellular ion homeostasis	8
GO:0007009	biological_process	plasma membrane organization	8
GO:0007272	biological_process	ensheathment of neurons	8
GO:0007422	biological_process	peripheral nervous system development	8
GO:0008366	biological_process	axon ensheathment	8
GO:0010001	biological_process	glial cell differentiation	8
GO:0014037	biological_process	Schwann cell differentiation	8
GO:0014044	biological_process	Schwann cell development	8
GO:0019228	biological_process	regulation of action potential in neuron	8
GO:0019725	biological_process	cellular homeostasis	8
GO:0021782	biological_process	glial cell development	8
GO:0022011	biological_process	myelination in peripheral nervous system	8
GO:0032287	biological_process	peripheral nervous system myelin maintenance	8
GO:0032292	biological_process	peripheral nervous system axon ensheathment	8
GO:0042063	biological_process	gliogenesis	8
GO:0042391	biological_process	regulation of membrane potential	8
GO:0042552	biological_process	myelination	8
GO:0042592	biological_process	homeostatic process	8
GO:0043217	biological_process	myelin maintenance	8
GO:0048878	biological_process	chemical homeostasis	8
GO:0050801	biological_process	ion homeostasis	8
GO:0055082	biological_process	cellular chemical homeostasis	8
GO:0010243	biological_process	response to organic nitrogen	8
GO:0032868	biological_process	response to insulin stimulus	8
GO:0032869	biological_process	cellular response to insulin stimulus	8
GO:0032870	biological_process	cellular response to hormone stimulus	8
GO:0043434	biological_process	response to peptide hormone stimulus	8
GO:0071310	biological_process	cellular response to organic substance	8
GO:0071375	biological_process	cellular response to peptide hormone stimulus	8
GO:0071417	biological_process	cellular response to organic nitrogen	8
GO:0071495	biological_process	cellular response to endogenous stimulus	8
GO:1901652	biological_process	response to peptide	8
GO:1901653	biological_process	cellular response to peptide	8
GO:0033119	biological_process	negative regulation of RNA splicing	8
GO:0043484	biological_process	regulation of RNA splicing	8

GO:0001932	biological_process	regulation of protein phosphorylation	9
GO:0001934	biological_process	positive regulation of protein phosphorylation	8
GO:0010562	biological_process	positive regulation of phosphorus metabolic process	8
GO:0019220	biological_process	regulation of phosphate metabolic process	9
GO:0031399	biological_process	regulation of protein modification process	9
GO:0031401	biological_process	positive regulation of protein modification process	8
GO:0032268	biological_process	regulation of cellular protein metabolic process	9
GO:0032270	biological_process	positive regulation of cellular protein metabolic process	8
GO:0033135	biological_process	regulation of peptidyl-serine phosphorylation	8
GO:0033138	biological_process	positive regulation of peptidyl-serine phosphorylation	8
GO:0042325	biological_process	regulation of phosphorylation	9
GO:0042327	biological_process	positive regulation of phosphorylation	8
GO:0045937	biological_process	positive regulation of phosphate metabolic process	8
GO:0051174	biological_process	regulation of phosphorus metabolic process	9
GO:0051246	biological_process	regulation of protein metabolic process	9
GO:0051247	biological_process	positive regulation of protein metabolic process	8
GO:0010941	biological_process	regulation of cell death	8
GO:0042981	biological_process	regulation of apoptotic process	8
GO:0043066	biological_process	negative regulation of apoptotic process	8
GO:0043067	biological_process	regulation of programmed cell death	8
GO:0043069	biological_process	negative regulation of programmed cell death	8
GO:0060548	biological_process	negative regulation of cell death	8
GO:0010466	biological_process	negative regulation of peptidase activity	8
GO:0010951	biological_process	negative regulation of endopeptidase activity	8
GO:0043086	biological_process	negative regulation of catalytic activity	8
GO:0043154	biological_process	negative regulation of cysteine-type endopeptidase activity involved in apoptotic process	8
GO:0043281	biological_process	regulation of cysteine-type endopeptidase activity involved in apoptotic process	8
GO:0044092	biological_process	negative regulation of molecular function	8
GO:0050790	biological_process	regulation of catalytic activity	8
GO:0051336	biological_process	regulation of hydrolase activity	8
GO:0051346	biological_process	negative regulation of hydrolase activity	8
GO:0052547	biological_process	regulation of peptidase activity	8
GO:0052548	biological_process	regulation of endopeptidase activity	8
GO:2000116	biological_process	regulation of cysteine-type endopeptidase activity	8
GO:2000117	biological_process	negative regulation of cysteine-type endopeptidase activity	8

GO:0007243	biological_process	intracellular protein kinase cascade	8
GO:0043491	biological_process	protein kinase B signaling cascade	8
GO:0045428	biological_process	regulation of nitric oxide biosynthetic process	8
GO:0045429	biological_process	positive regulation of nitric oxide biosynthetic process	8
GO:0045944	biological_process	positive regulation of transcription from RNA polymerase II promoter	8
GO:0010627	biological_process	regulation of intracellular protein kinase cascade	9
GO:0032872	biological_process	regulation of stress-activated MAPK cascade	8
GO:0043408	biological_process	regulation of MAPK cascade	8
GO:0046328	biological_process	regulation of JNK cascade	8
GO:0070302	biological_process	regulation of stress-activated protein kinase signaling cascade	8
GO:0080135	biological_process	regulation of cellular response to stress	8
GO:0032101	biological_process	regulation of response to external stimulus	8
GO:0032103	biological_process	positive regulation of response to external stimulus	8
GO:0048520	biological_process	positive regulation of behavior	8
GO:0050795	biological_process	regulation of behavior	8
GO:0050920	biological_process	regulation of chemotaxis	8
GO:0050921	biological_process	positive regulation of chemotaxis	8
GO:0050926	biological_process	regulation of positive chemotaxis	8
GO:0050927	biological_process	positive regulation of positive chemotaxis	8
GO:0072657	biological_process	protein localization to membrane	8
GO:0010827	biological_process	regulation of glucose transport	8
GO:0010828	biological_process	positive regulation of glucose transport	8
GO:0046324	biological_process	regulation of glucose import	8
GO:0046326	biological_process	positive regulation of glucose import	8
GO:1900076	biological_process	regulation of cellular response to insulin stimulus	8
GO:1900078	biological_process	positive regulation of cellular response to insulin stimulus	8
GO:2001273	biological_process	regulation of glucose import in response to insulin stimulus	8
GO:2001275	biological_process	positive regulation of glucose import in response to insulin stimulus	8
GO:0005488	molecular_function	binding	45
GO:0019899	molecular_function	enzyme binding	10
GO:0008565	molecular_function	protein transporter activity	3
GO:0030276	molecular_function	clathrin binding	3
GO:0005543	molecular_function	phospholipid binding	17
GO:0008289	molecular_function	lipid binding	18
GO:0019900	molecular_function	kinase binding	9
GO:0004672	molecular_function	protein kinase activity	12
GO:0004674	molecular_function	protein serine/threonine kinase activity	11
GO:0016301	molecular_function	kinase activity	12
GO:0016772	molecular_function	transferase activity, transferring	12

		phosphorus-containing groups	
GO:0016773	molecular_function	phosphotransferase activity, alcohol group as acceptor	12
GO:0005080	molecular_function	protein kinase C binding	8
GO:0019901	molecular_function	protein kinase binding	8

Table 9.7: Information of enriched GO terms for significantly down regulated genes after 24 hours exposure to 900μM GHB.

GOID	Ontology	Term	Number of genes
GO:0005576	cellular_component	extracellular region	33
GO:0031012	cellular_component	extracellular matrix	10
GO:0044421	cellular_component	extracellular region part	23
GO:0005615	cellular_component	extracellular space	17
GO:0016020	cellular_component	membrane	87
GO:0016021	cellular_component	integral to membrane	57
GO:0044425	cellular_component	membrane part	69
GO:0005622	cellular_component	intracellular	181
GO:0005623	cellular_component	cell	196
GO:0005737	cellular_component	cytoplasm	137
GO:0044424	cellular_component	intracellular part	176
GO:0044464	cellular_component	cell part	196
GO:0005829	cellular_component	cytosol	48
GO:0044444	cellular_component	cytoplasmic part	103
GO:0005634	cellular_component	nucleus	99
GO:0043226	cellular_component	Organelle	150
GO:0043227	cellular_component	membrane-bounded organelle	138
GO:0043229	cellular_component	intracellular organelle	150
GO:0043231	cellular_component	intracellular membrane-bounded organelle	138
GO:0005730	cellular_component	nucleolus	31
GO:0031974	cellular_component	membrane-enclosed lumen	65
GO:0031981	cellular_component	nuclear lumen	57
GO:0043228	cellular_component	non-membrane-bounded organelle	74
GO:0043232	cellular_component	intracellular non-membrane-bounded	74

		organelle	
GO:0043233	cellular_component	organelle lumen	64
GO:0044422	cellular_component	organelle part	114
GO:0044428	cellular_component	nuclear part	65
GO:0044446	cellular_component	intracellular organelle part	113
GO:0070013	cellular_component	intracellular organelle lumen	63
GO:0005654	cellular_component	Nucleoplasm	39
GO:0032991	cellular_component	macromolecular complex	50
GO:0043234	cellular_component	protein complex	44
GO:0005739	cellular_component	mitochondrion	25
GO:0005886	cellular_component	plasma membrane	50
GO:0071944	cellular_component	cell periphery	51
GO:0005887	cellular_component	integral to plasma membrane	21
GO:0031226	cellular_component	intrinsic to plasma membrane	21
GO:0044459	cellular_component	plasma membrane part	28
GO:0030425	cellular_component	dendrite	10
GO:0042555	cellular_component	MCM complex	5
GO:0012505	cellular_component	endomembrane system	29
GO:0005955	cellular_component	calcineurin complex	2
GO:0005635	cellular_component	nuclear envelope	10
GO:0031965	cellular_component	nuclear membrane	7
GO:0016023	cellular_component	cytoplasmic membrane-bounded vesicle	16
GO:0031410	cellular_component	cytoplasmic vesicle	16
GO:0031982	cellular_component	vesicle	16
GO:0031988	cellular_component	membrane-bounded vesicle	16
GO:0000793	cellular_component	condensed chromosome	6
GO:0005694	cellular_component	chromosome	26
GO:0044427	cellular_component	chromosomal part	24
GO:0000785	cellular_component	chromatin	14
GO:0000228	cellular_component	nuclear chromosome	11
GO:0005657	cellular_component	replication fork	7
GO:0005658	cellular_component	alpha DNA polymerase:primase complex	2
GO:0030894	cellular_component	replisome	3

GO:0032993	cellular_component	protein-DNA complex	6
GO:0043596	cellular_component	nuclear replication fork	4
GO:0043601	cellular_component	nuclear replisome	3
GO:0044454	cellular_component	nuclear chromosome part	10
GO:0034399	cellular_component	nuclear periphery	5
GO:0005856	cellular_component	cytoskeleton	29
GO:0044430	cellular_component	cytoskeletal part	23
GO:0030136	cellular_component	clathrin-coated vesicle	7
GO:0044456	cellular_component	synapse part	10
GO:0030665	cellular_component	clathrin-coated vesicle membrane	5
GO:0030016	cellular_component	myofibril	8
GO:0030017	cellular_component	Sarcomere	8
GO:0043292	cellular_component	contractile fiber	8
GO:0044449	cellular_component	contractile fiber part	8
GO:0005862	cellular_component	muscle thin filament tropomyosin	2
GO:0005865	cellular_component	striated muscle thin filament	3
GO:0001725	cellular_component	stress fiber	4
GO:0032432	cellular_component	actin filament bundle	4
GO:0042641	cellular_component	actomyosin	4
GO:0000794	cellular_component	condensed nuclear chromosome	4
GO:0000795	cellular_component	synaptonemal complex	3
GO:0000800	cellular_component	lateral element	2
GO:0000323	cellular_component	lytic vacuole	8
GO:0005764	cellular_component	lysosome	8
GO:0005905	cellular_component	coated pit	4
GO:0033186	cellular_component	CAF-1 complex	2
GO:0043197	cellular_component	dendritic spine	7
GO:0044309	cellular_component	neuron spine	7
GO:0097060	cellular_component	synaptic membrane	7
GO:0012510	cellular_component	trans-Golgi network transport vesicle membrane	3
GO:0030125	cellular_component	clathrin vesicle coat	3
GO:0030130	cellular_component	clathrin coat of trans-Golgi network vesicle	3

GO:0030140	cellular_component	trans-Golgi network transport vesicle	3
GO:0030660	cellular_component	Golgi-associated vesicle membrane	3
GO:0007275	biological_process	multicellular organismal development	55
GO:0032501	biological_process	multicellular organismal process	80
GO:0032502	biological_process	developmental process	60
GO:0044699	biological_process	single-organism process	111
GO:0044707	biological_process	single-multicellular organism process	80
GO:0048731	biological_process	system development	45
GO:0048856	biological_process	anatomical structure development	52
GO:0009987	biological_process	cellular process	182
GO:0030154	biological_process	cell differentiation	33
GO:0048869	biological_process	cellular developmental process	34
GO:0007154	biological_process	cell communication	55
GO:0007165	biological_process	signal transduction	48
GO:0023052	biological_process	signaling	52
GO:0044700	biological_process	single organism signaling	52
GO:0050789	biological_process	regulation of biological process	129
GO:0050794	biological_process	regulation of cellular process	116
GO:0050896	biological_process	response to stimulus	99
GO:0051716	biological_process	cellular response to stimulus	84
GO:0065007	biological_process	biological regulation	146
GO:0006082	biological_process	organic acid metabolic process	23
GO:0006139	biological_process	nucleobase-containing compound metabolic process	65
GO:0006520	biological_process	cellular amino acid metabolic process	14
GO:0006725	biological_process	cellular aromatic compound metabolic process	72
GO:0006807	biological_process	nitrogen compound metabolic process	83
GO:0008152	biological_process	metabolic process	121
GO:0009058	biological_process	biosynthetic process	70
GO:0009059	biological_process	macromolecule biosynthetic process	51
GO:0019752	biological_process	carboxylic acid metabolic process	23
GO:0034641	biological_process	cellular nitrogen compound metabolic process	75

GO:0034645	biological_process	cellular macromolecule biosynthetic process	51
GO:0043170	biological_process	macromolecule metabolic process	85
GO:0043436	biological_process	oxoacid metabolic process	23
GO:0044237	biological_process	cellular metabolic process	110
GO:0044238	biological_process	primary metabolic process	114
GO:0044249	biological_process	cellular biosynthetic process	69
GO:0044260	biological_process	cellular macromolecule metabolic process	82
GO:0044281	biological_process	small molecule metabolic process	31
GO:0044710	biological_process	single-organism metabolic process	116
GO:0046483	biological_process	heterocycle metabolic process	72
GO:0071704	biological_process	organic substance metabolic process	116
GO:0090304	biological_process	nucleic acid metabolic process	59
GO:1901360	biological_process	organic cyclic compound metabolic process	80
GO:1901564	biological_process	organonitrogen compound metabolic process	28
GO:1901576	biological_process	organic substance biosynthetic process	70
GO:0000278	biological_process	mitotic cell cycle	35
GO:0007049	biological_process	cell cycle	51
GO:0022402	biological_process	cell cycle process	41
GO:0022403	biological_process	cell cycle phase	40
GO:0051325	biological_process	interphase	28
GO:0051329	biological_process	interphase of mitotic cell cycle	28
GO:0006355	biological_process	regulation of transcription, DNA-dependent	45
GO:0006357	biological_process	regulation of transcription from RNA polymerase II promoter	18
GO:0009889	biological_process	regulation of biosynthetic process	59
GO:0010468	biological_process	regulation of gene expression	53
GO:0010556	biological_process	regulation of macromolecule biosynthetic process	58
GO:0019219	biological_process	regulation of nucleobase-containing compound metabolic process	52

GO:0019222	biological_process	regulation of metabolic process	83
GO:0031323	biological_process	regulation of cellular metabolic process	72
GO:0031326	biological_process	regulation of cellular biosynthetic process	57
GO:0048519	biological_process	negative regulation of biological process	43
GO:0048523	biological_process	negative regulation of cellular process	38
GO:0051171	biological_process	regulation of nitrogen compound metabolic process	52
GO:0051252	biological_process	regulation of RNA metabolic process	45
GO:0060255	biological_process	regulation of macromolecule metabolic process	74
GO:0080090	biological_process	regulation of primary metabolic process	72
GO:2000112	biological_process	regulation of cellular macromolecule biosynthetic process	54
GO:2001141	biological_process	regulation of RNA biosynthetic process	45
GO:0008283	biological_process	cell proliferation	16
GO:0009891	biological_process	positive regulation of biosynthetic process	20
GO:0009893	biological_process	positive regulation of metabolic process	31
GO:0010557	biological_process	positive regulation of macromolecule biosynthetic process	20
GO:0010604	biological_process	positive regulation of macromolecule metabolic process	30
GO:0010628	biological_process	positive regulation of gene expression	19
GO:0031325	biological_process	positive regulation of cellular metabolic process	31
GO:0031328	biological_process	positive regulation of cellular biosynthetic process	20
GO:0048518	biological_process	positive regulation of biological process	46
GO:0048522	biological_process	positive regulation of cellular process	41
GO:0051726	biological_process	regulation of cell cycle	27
GO:0000082	biological_process	G1/S transition of mitotic cell cycle	21
GO:0003677	molecular_function	DNA binding	46
GO:0018130	biological_process	heterocycle biosynthetic process	26
GO:0019438	biological_process	aromatic compound biosynthetic process	24

GO:0034654	biological_process	nucleobase-containing compound biosynthetic process	23
GO:0044271	biological_process	cellular nitrogen compound biosynthetic process	27
GO:1901362	biological_process	organic cyclic compound biosynthetic process	32
GO:0000003	biological_process	reproduction	23
GO:0022414	biological_process	reproductive process	23
GO:0044702	biological_process	single organism reproductive process	19
GO:0008284	biological_process	positive regulation of cell proliferation	14
GO:0042127	biological_process	regulation of cell proliferation	20
GO:0065008	biological_process	regulation of biological quality	49
GO:0010033	biological_process	response to organic substance	34
GO:0033993	biological_process	response to lipid	14
GO:0042221	biological_process	response to chemical stimulus	42
GO:0070887	biological_process	cellular response to chemical stimulus	25
GO:0071310	biological_process	cellular response to organic substance	18
GO:0006950	biological_process	response to stress	58
GO:0009628	biological_process	response to abiotic stimulus	24
GO:0033554	biological_process	cellular response to stress	33
GO:0000083	biological_process	regulation of transcription involved in G1/S phase of mitotic cell cycle	6
GO:0007346	biological_process	regulation of mitotic cell cycle	11
GO:0010564	biological_process	regulation of cell cycle process	19
GO:0000075	biological_process	cell cycle checkpoint	17
GO:0006974	biological_process	response to DNA damage stimulus	26
GO:0031570	biological_process	DNA integrity checkpoint	8
GO:0071156	biological_process	regulation of cell cycle arrest	17
GO:0009605	biological_process	response to external stimulus	18
GO:0009611	biological_process	response to wounding	20
GO:0002376	biological_process	immune system process	22
GO:0006968	biological_process	cellular defense response	4
GO:0007166	biological_process	cell surface receptor signaling pathway	30
GO:0019220	biological_process	regulation of phosphate metabolic process	25

GO:0050790	biological_process	regulation of catalytic activity	29
GO:0051174	biological_process	regulation of phosphorus metabolic process	25
GO:0065009	biological_process	regulation of molecular function	32
GO:0006873	biological_process	cellular ion homeostasis	12
GO:0006874	biological_process	cellular calcium ion homeostasis	8
GO:0006875	biological_process	cellular metal ion homeostasis	12
GO:0019725	biological_process	cellular homeostasis	14
GO:0030003	biological_process	cellular cation homeostasis	12
GO:0042592	biological_process	homeostatic process	24
GO:0048878	biological_process	chemical homeostasis	17
GO:0050801	biological_process	ion homeostasis	12
GO:0055065	biological_process	metal ion homeostasis	12
GO:0055074	biological_process	calcium ion homeostasis	8
GO:0055080	biological_process	cation homeostasis	12
GO:0055082	biological_process	cellular chemical homeostasis	14
GO:0072503	biological_process	cellular divalent inorganic cation homeostasis	8
GO:0072507	biological_process	divalent inorganic cation homeostasis	8
GO:0035556	biological_process	intracellular signal transduction	20
GO:0051704	biological_process	multi-organism process	68
GO:0001974	biological_process	blood vessel remodeling	3
GO:0002407	biological_process	dendritic cell chemotaxis	3
GO:0006928	biological_process	cellular component movement	18
GO:0016477	biological_process	cell migration	13
GO:0030595	biological_process	leukocyte chemotaxis	4
GO:0036336	biological_process	dendritic cell migration	3
GO:0048870	biological_process	cell motility	14
GO:0050900	biological_process	leukocyte migration	8
GO:0051179	biological_process	localization	50
GO:0051674	biological_process	localization of cell	14
GO:0002827	biological_process	positive regulation of T-helper 1 type immune response	2
GO:0002829	biological_process	negative regulation of type 2 immune response	2

GO:0051239	biological_process	regulation of multicellular organismal process	27
GO:0010819	biological_process	regulation of T cell chemotaxis	3
GO:0010820	biological_process	positive regulation of T cell chemotaxis	3
GO:0030334	biological_process	regulation of cell migration	9
GO:0030335	biological_process	positive regulation of cell migration	9
GO:0040012	biological_process	regulation of locomotion	10
GO:0040017	biological_process	positive regulation of locomotion	9
GO:0048520	biological_process	positive regulation of behavior	4
GO:0051270	biological_process	regulation of cellular component movement	9
GO:0051272	biological_process	positive regulation of cellular component movement	9
GO:1901623	biological_process	regulation of lymphocyte chemotaxis	3
GO:2000145	biological_process	regulation of cell motility	9
GO:2000147	biological_process	positive regulation of cell motility	9
GO:2000401	biological_process	regulation of lymphocyte migration	3
GO:2000403	biological_process	positive regulation of lymphocyte migration	3
GO:2000404	biological_process	regulation of T cell migration	3
GO:2000406	biological_process	positive regulation of T cell migration	3
GO:0050793	biological_process	regulation of developmental process	20
GO:2000026	biological_process	regulation of multicellular organismal development	18
GO:0032743	biological_process	positive regulation of interleukin-2 production	2
GO:0045123	biological_process	cellular extravasation	3
GO:0048247	biological_process	lymphocyte chemotaxis	3
GO:0072676	biological_process	lymphocyte migration	3
GO:0042534	biological_process	regulation of tumor necrosis factor biosynthetic process	3
GO:0051246	biological_process	regulation of protein metabolic process	29
GO:0051247	biological_process	positive regulation of protein metabolic process	17
GO:0043301	biological_process	negative regulation of leukocyte	2

		degranulation	
GO:0050865	biological_process	regulation of cell activation	9
GO:0051051	biological_process	negative regulation of transport	7
GO:0042063	biological_process	gliogenesis	6
GO:0043615	biological_process	astrocyte cell migration	2
GO:0002696	biological_process	positive regulation of leukocyte activation	7
GO:0032944	biological_process	regulation of mononuclear cell proliferation	6
GO:0032946	biological_process	positive regulation of mononuclear cell proliferation	6
GO:0042102	biological_process	positive regulation of T cell proliferation	4
GO:0046634	biological_process	regulation of alpha-beta T cell activation	4
GO:0046635	biological_process	positive regulation of alpha-beta T cell activation	4
GO:0046640	biological_process	regulation of alpha-beta T cell proliferation	3
GO:0046641	biological_process	positive regulation of alpha-beta T cell proliferation	3
GO:0050670	biological_process	regulation of lymphocyte proliferation	6
GO:0050671	biological_process	positive regulation of lymphocyte proliferation	6
GO:0050867	biological_process	positive regulation of cell activation	8
GO:0051251	biological_process	positive regulation of lymphocyte activation	7
GO:0070663	biological_process	regulation of leukocyte proliferation	6
GO:0070665	biological_process	positive regulation of leukocyte proliferation	6
GO:0031347	biological_process	regulation of defense response	10
GO:0080134	biological_process	regulation of response to stress	15
GO:0070098	biological_process	chemokine-mediated signaling pathway	2
GO:0090025	biological_process	regulation of monocyte chemotaxis	2
GO:0090026	biological_process	positive regulation of monocyte chemotaxis	2
GO:0002691	biological_process	regulation of cellular extravasation	2
GO:0006259	biological_process	DNA metabolic process	44

GO:0006260	biological_process	DNA replication	29
GO:0006261	biological_process	DNA-dependent DNA replication	16
GO:0006268	biological_process	DNA unwinding involved in replication	5
GO:0032392	biological_process	DNA geometric change	6
GO:0032508	biological_process	DNA duplex unwinding	6
GO:0071103	biological_process	DNA conformation change	11
GO:0006270	biological_process	DNA replication initiation	9
GO:0000084	biological_process	S phase of mitotic cell cycle	16
GO:0051320	biological_process	S phase	16
GO:0000216	biological_process	M/G1 transition of mitotic cell cycle	10
GO:0006271	biological_process	DNA strand elongation involved in DNA replication	12
GO:0022616	biological_process	DNA strand elongation	13
GO:0016043	biological_process	cellular component organization	57
GO:0071840	biological_process	cellular component organization or biogenesis	59
GO:0001101	biological_process	response to acid	6
GO:0003006	biological_process	developmental process involved in reproduction	11
GO:0007548	biological_process	sex differentiation	7
GO:0008406	biological_process	gonad development	7
GO:0045137	biological_process	development of primary sexual characteristics	7
GO:0048608	biological_process	reproductive structure development	7
GO:0061458	biological_process	reproductive system development	7
GO:0001655	biological_process	urogenital system development	7
GO:0001657	biological_process	ureteric bud development	5
GO:0001836	biological_process	release of cytochrome c from mitochondria	3
GO:0006996	biological_process	organelle organization	39
GO:0007005	biological_process	mitochondrion organization	7
GO:0002244	biological_process	hematopoietic progenitor cell differentiation	4
GO:0002320	biological_process	lymphoid progenitor cell differentiation	4
GO:0002520	biological_process	immune system development	10

GO:0030097	biological_process	hemopoiesis	9
GO:0048534	biological_process	hematopoietic or lymphoid organ development	9
GO:0002326	biological_process	B cell lineage commitment	2
GO:0002521	biological_process	leukocyte differentiation	8
GO:0002360	biological_process	T cell lineage commitment	2
GO:0003008	biological_process	system process	29
GO:0006796	biological_process	phosphate-containing compound metabolic process	26
GO:0043412	biological_process	macromolecule modification	31
GO:0006979	biological_process	response to oxidative stress	8
GO:0030029	biological_process	actin filament-based process	9
GO:0044706	biological_process	multi-multicellular organism process	6
GO:0007568	biological_process	aging	7
GO:0007569	biological_process	cell aging	4
GO:0007584	biological_process	response to nutrient	7
GO:0009991	biological_process	response to extracellular stimulus	10
GO:0031667	biological_process	response to nutrient levels	10
GO:0008584	biological_process	male gonad development	7
GO:0046546	biological_process	development of primary male sexual characteristics	7
GO:0046661	biological_process	male sex differentiation	7
GO:0009314	biological_process	response to radiation	17
GO:0009636	biological_process	response to toxin	10
GO:0010035	biological_process	response to inorganic substance	11
GO:0010038	biological_process	response to metal ion	11
GO:0010039	biological_process	response to iron ion	3
GO:0009411	biological_process	response to UV	7
GO:0009416	biological_process	response to light stimulus	11
GO:0010224	biological_process	response to UV-B	2
GO:0010212	biological_process	response to ionizing radiation	7
GO:0032268	biological_process	regulation of cellular protein metabolic process	24
GO:0014041	biological_process	regulation of neuron maturation	2
GO:0045664	biological_process	regulation of neuron differentiation	9

GO:0050767	biological_process	regulation of neurogenesis	12
GO:0051094	biological_process	positive regulation of developmental process	13
GO:0051960	biological_process	regulation of nervous system development	13
GO:0060284	biological_process	regulation of cell development	12
GO:0014910	biological_process	regulation of smooth muscle cell migration	4
GO:0014911	biological_process	positive regulation of smooth muscle cell migration	4
GO:0018107	biological_process	peptidyl-threonine phosphorylation	3
GO:0018210	biological_process	peptidyl-threonine modification	3
GO:0021548	biological_process	pons development	2
GO:0030279	biological_process	negative regulation of ossification	4
GO:0051241	biological_process	negative regulation of multicellular organismal process	9
GO:0043900	biological_process	regulation of multi-organism process	29
GO:0051128	biological_process	regulation of cellular component organization	25
GO:0009719	biological_process	response to endogenous stimulus	19
GO:0010243	biological_process	response to organic nitrogen	17
GO:0014070	biological_process	response to organic cyclic compound	15
GO:0014074	biological_process	response to purine-containing compound	6
GO:0031000	biological_process	response to caffeine	3
GO:0043279	biological_process	response to alkaloid	6
GO:0032847	biological_process	regulation of cellular pH reduction	2
GO:0033033	biological_process	negative regulation of myeloid cell apoptotic process	2
GO:0010044	biological_process	response to aluminum ion	2
GO:0010523	biological_process	negative regulation of calcium ion transport into cytosol	2
GO:0001932	biological_process	regulation of protein phosphorylation	15
GO:0031399	biological_process	regulation of protein modification process	18
GO:0032270	biological_process	positive regulation of cellular protein	15

		metabolic process	
GO:0042325	biological_process	regulation of phosphorylation	17
GO:0033273	biological_process	response to vitamin	6
GO:0033591	biological_process	response to L-ascorbic acid	2
GO:0070661	biological_process	leukocyte proliferation	4
GO:0042493	biological_process	response to drug	12
GO:0043497	biological_process	regulation of protein heterodimerization activity	2
GO:0043627	biological_process	response to estrogen stimulus	7
GO:0045471	biological_process	response to ethanol	5
GO:0097305	biological_process	response to alcohol	7
GO:0046688	biological_process	response to copper ion	3
GO:0006810	biological_process	transport	38
GO:0014075	biological_process	response to amine stimulus	6
GO:0043200	biological_process	response to amino acid stimulus	6
GO:0051593	biological_process	response to folic acid	4
GO:0051900	biological_process	regulation of mitochondrial depolarization	2
GO:0070059	biological_process	intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress	3
GO:0001656	biological_process	metanephros development	4
GO:0043549	biological_process	regulation of kinase activity	11
GO:0045859	biological_process	regulation of protein kinase activity	11
GO:0051338	biological_process	regulation of transferase activity	12
GO:0071900	biological_process	regulation of protein serine/threonine kinase activity	10
GO:0002318	biological_process	myeloid progenitor cell differentiation	2
GO:0002573	biological_process	myeloid leukocyte differentiation	5
GO:0006629	biological_process	lipid metabolic process	18
GO:0007286	biological_process	spermatid development	4
GO:0048610	biological_process	cellular process involved in reproduction	11
GO:0031272	biological_process	regulation of pseudopodium assembly	3
GO:0031274	biological_process	positive regulation of pseudopodium	3

		assembly	
GO:0031344	biological_process	regulation of cell projection organization	9
GO:0031346	biological_process	positive regulation of cell projection organization	8
GO:0043902	biological_process	positive regulation of multi-organism process	14
GO:0051130	biological_process	positive regulation of cellular component organization	13
GO:0035234	biological_process	germ cell programmed cell death	2
GO:0051301	biological_process	cell division	11
GO:0044057	biological_process	regulation of system process	11
GO:0048170	biological_process	positive regulation of long-term neuronal synaptic plasticity	2
GO:0050769	biological_process	positive regulation of neurogenesis	5
GO:0010517	biological_process	regulation of phospholipase activity	5
GO:0010518	biological_process	positive regulation of phospholipase activity	5
GO:0010863	biological_process	positive regulation of phospholipase C activity	4
GO:0051336	biological_process	regulation of hydrolase activity	18
GO:0051345	biological_process	positive regulation of hydrolase activity	11
GO:0060191	biological_process	regulation of lipase activity	5
GO:0060193	biological_process	positive regulation of lipase activity	5
GO:1900274	biological_process	regulation of phospholipase C activity	4
GO:0014068	biological_process	positive regulation of phosphatidylinositol 3-kinase cascade	3
GO:0050731	biological_process	positive regulation of peptidyl-tyrosine phosphorylation	5
GO:0000279	biological_process	M phase	16
GO:0006281	biological_process	DNA repair	21
GO:0071897	biological_process	DNA biosynthetic process	4
GO:0006273	biological_process	lagging strand elongation	2
GO:0006302	biological_process	double-strand break repair	7
GO:0000076	biological_process	DNA replication checkpoint	4
GO:0010389	biological_process	regulation of G2/M transition of mitotic	4

		cell cycle	
GO:0010948	biological_process	negative regulation of cell cycle process	5
GO:0010972	biological_process	negative regulation of G2/M transition of mitotic cell cycle	4
GO:0045930	biological_process	negative regulation of mitotic cell cycle	4
GO:0000722	biological_process	telomere maintenance via recombination	5
GO:0000723	biological_process	telomere maintenance	7
GO:0006310	biological_process	DNA recombination	12
GO:0006312	biological_process	mitotic recombination	6
GO:0032200	biological_process	telomere organization	7
GO:0051276	biological_process	chromosome organization	22
GO:0060249	biological_process	anatomical structure homeostasis	7
GO:0010833	biological_process	telomere maintenance via telomere lengthening	6
GO:0032201	biological_process	telomere maintenance via semi-conservative replication	5
GO:0000724	biological_process	double-strand break repair via homologous recombination	4
GO:0000725	biological_process	recombinational repair	4
GO:0007126	biological_process	meiosis	8
GO:0051321	biological_process	meiotic cell cycle	8
GO:0051327	biological_process	M phase of meiotic cell cycle	8
GO:0042060	biological_process	wound healing	13
GO:0001501	biological_process	skeletal system development	9
GO:0010817	biological_process	regulation of hormone levels	8
GO:0071702	biological_process	organic substance transport	26
GO:0071705	biological_process	nitrogen compound transport	11
GO:1901566	biological_process	organonitrogen compound biosynthetic process	13
GO:0006241	biological_process	CTP biosynthetic process	2
GO:0009208	biological_process	pyrimidine ribonucleoside triphosphate metabolic process	2
GO:0009209	biological_process	pyrimidine ribonucleoside triphosphate biosynthetic process	2
GO:0046036	biological_process	CTP metabolic process	2

GO:0046134	biological_process	pyrimidine nucleoside biosynthetic process	3
GO:0006541	biological_process	glutamine metabolic process	3
GO:0009064	biological_process	glutamine family amino acid metabolic process	4
GO:1901605	biological_process	alpha-amino acid metabolic process	9
GO:0015949	biological_process	nucleobase-containing small molecule interconversion	3
GO:0031572	biological_process	G2/M transition DNA damage checkpoint	4
GO:0031576	biological_process	G2/M transition checkpoint	4
GO:0022607	biological_process	cellular component assembly	24
GO:0044085	biological_process	cellular component biogenesis	26
GO:0033261	biological_process	regulation of S phase	3
GO:0006275	biological_process	regulation of DNA replication	5
GO:0051052	biological_process	regulation of DNA metabolic process	8
GO:0006325	biological_process	chromatin organization	12
GO:0031056	biological_process	regulation of histone modification	4
GO:0031058	biological_process	positive regulation of histone modification	3
GO:0031062	biological_process	positive regulation of histone methylation	2
GO:0033044	biological_process	regulation of chromosome organization	5
GO:0051571	biological_process	positive regulation of histone H3-K4 methylation	2
GO:2001252	biological_process	positive regulation of chromosome organization	3
GO:0051570	biological_process	regulation of histone H3-K9 methylation	2
GO:0008202	biological_process	steroid metabolic process	9
GO:0008206	biological_process	bile acid metabolic process	7
GO:0032787	biological_process	monocarboxylic acid metabolic process	9
GO:0006820	biological_process	anion transport	10
GO:0015711	biological_process	organic anion transport	9
GO:0015721	biological_process	bile acid and bile salt transport	4
GO:0015849	biological_process	organic acid transport	9

GO:0046942	biological_process	carboxylic acid transport	9
GO:0030299	biological_process	intestinal cholesterol absorption	2
GO:0051259	biological_process	protein oligomerization	8
GO:0065003	biological_process	macromolecular complex assembly	15
GO:0042445	biological_process	hormone metabolic process	7
GO:0006865	biological_process	amino acid transport	5
GO:0072215	biological_process	regulation of metanephros development	3
GO:0072216	biological_process	positive regulation of metanephros development	2
GO:0072298	biological_process	regulation of metanephric glomerulus development	2
GO:0072300	biological_process	positive regulation of metanephric glomerulus development	2
GO:0090183	biological_process	regulation of kidney development	3
GO:0090184	biological_process	positive regulation of kidney development	3
GO:0090193	biological_process	positive regulation of glomerulus development	2
GO:0019985	biological_process	translesion synthesis	2
GO:0003012	biological_process	muscle system process	7
GO:0006936	biological_process	muscle contraction	7
GO:0030048	biological_process	actin filament-based movement	4
GO:0030049	biological_process	muscle filament sliding	4
GO:0033275	biological_process	actin-myosin filament sliding	4
GO:0070252	biological_process	actin-mediated cell contraction	4
GO:0006287	biological_process	base-excision repair, gap-filling	2
GO:0006289	biological_process	nucleotide-excision repair	5
GO:0006297	biological_process	nucleotide-excision repair, DNA gap filling	4
GO:0006283	biological_process	transcription-coupled nucleotide-excision repair	4
GO:0006879	biological_process	cellular iron ion homeostasis	4
GO:0065004	biological_process	protein-DNA complex assembly	7
GO:0071824	biological_process	protein-DNA complex subunit organization	7

GO:0006323	biological_process	DNA packaging	6
GO:0006333	biological_process	chromatin assembly or disassembly	7
GO:0006334	biological_process	nucleosome assembly	6
GO:0031497	biological_process	chromatin assembly	6
GO:0034723	biological_process	DNA replication-dependent nucleosome organization	2
GO:0034728	biological_process	nucleosome organization	6
GO:0071214	biological_process	cellular response to abiotic stimulus	5
GO:0006563	biological_process	L-serine metabolic process	2
GO:0008652	biological_process	cellular amino acid biosynthetic process	6
GO:0016053	biological_process	organic acid biosynthetic process	10
GO:0044283	biological_process	small molecule biosynthetic process	10
GO:0044711	biological_process	single-organism biosynthetic process	10
GO:0046394	biological_process	carboxylic acid biosynthetic process	10
GO:1901607	biological_process	alpha-amino acid biosynthetic process	5
GO:0006694	biological_process	steroid biosynthetic process	6
GO:0006703	biological_process	estrogen biosynthetic process	2
GO:0034754	biological_process	cellular hormone metabolic process	5
GO:0042446	biological_process	hormone biosynthetic process	4
GO:0008209	biological_process	androgen metabolic process	4
GO:0010164	biological_process	response to cesium ion	2
GO:0018879	biological_process	biphenyl metabolic process	2
GO:0018894	biological_process	dibenzo-p-dioxin metabolic process	3
GO:0018963	biological_process	phthalate metabolic process	2
GO:0030186	biological_process	melatonin metabolic process	2
GO:0008207	biological_process	C21-steroid hormone metabolic process	4
GO:0042180	biological_process	cellular ketone metabolic process	3
GO:0042448	biological_process	progesterone metabolic process	3
GO:0032354	biological_process	response to follicle-stimulating hormone stimulus	3
GO:0034698	biological_process	response to gonadotropin stimulus	3
GO:0051599	biological_process	response to hydrostatic pressure	2
GO:0071462	biological_process	cellular response to water stimulus	2
GO:0071464	biological_process	cellular response to hydrostatic pressure	2

GO:0010923	biological_process	negative regulation of phosphatase activity	4
GO:0051346	biological_process	negative regulation of hydrolase activity	7
GO:0006699	biological_process	bile acid biosynthetic process	3
GO:0002544	biological_process	chronic inflammatory response	3
GO:0050690	biological_process	regulation of defense response to virus by virus	3
GO:0000079	biological_process	regulation of cyclin-dependent protein kinase activity	5
GO:0016264	biological_process	gap junction assembly	2
GO:0009994	biological_process	oocyte differentiation	3
GO:0048599	biological_process	oocyte development	3
GO:0050667	biological_process	homocysteine metabolic process	2
GO:0000733	biological_process	DNA strand renaturation	2
GO:0006390	biological_process	transcription from mitochondrial promoter	2
GO:0003676	molecular_function	nucleic acid binding	55
GO:0005488	molecular_function	binding	194
GO:0097159	molecular_function	organic cyclic compound binding	93
GO:1901363	molecular_function	heterocyclic compound binding	89
GO:0005515	molecular_function	protein binding	147
GO:0000166	molecular_function	nucleotide binding	46
GO:0036094	molecular_function	small molecule binding	48
GO:1901265	molecular_function	nucleoside phosphate binding	46
GO:0003824	molecular_function	catalytic activity	89
GO:0016874	molecular_function	ligase activity	11
GO:0001882	molecular_function	nucleoside binding	39
GO:0001883	molecular_function	purine nucleoside binding	38
GO:0005524	molecular_function	ATP binding	34
GO:0017076	molecular_function	purine nucleotide binding	39
GO:0030554	molecular_function	adenyl nucleotide binding	34
GO:0032549	molecular_function	ribonucleoside binding	38
GO:0032550	molecular_function	purine ribonucleoside binding	38
GO:0032553	molecular_function	ribonucleotide binding	38
GO:0032555	molecular_function	purine ribonucleotide binding	38

GO:0032559	molecular_function	adenyl ribonucleotide binding	34
GO:0035639	molecular_function	purine ribonucleoside triphosphate binding	38
GO:0043167	molecular_function	ion binding	95
GO:0043168	molecular_function	anion binding	47
GO:0043169	molecular_function	cation binding	62
GO:0046872	molecular_function	metal ion binding	62
GO:0016740	molecular_function	transferase activity	31
GO:0016772	molecular_function	transferase activity, transferring phosphorus-containing groups	18
GO:0004872	molecular_function	receptor activity	24
GO:0001664	molecular_function	G-protein coupled receptor binding	8
GO:0048020	molecular_function	CCR chemokine receptor binding	3
GO:0042802	molecular_function	identical protein binding	21
GO:0042803	molecular_function	protein homodimerization activity	15
GO:0046983	molecular_function	protein dimerization activity	17
GO:0003678	molecular_function	DNA helicase activity	5
GO:0004386	molecular_function	helicase activity	8
GO:0016462	molecular_function	pyrophosphatase activity	18
GO:0016787	molecular_function	hydrolase activity	33
GO:0016817	molecular_function	hydrolase activity, acting on acid anhydrides	18
GO:0016818	molecular_function	hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	18
GO:0017111	molecular_function	nucleoside-triphosphatase activity	18
GO:0003697	molecular_function	single-stranded DNA binding	5
GO:0043566	molecular_function	structure-specific DNA binding	9
GO:0004003	molecular_function	ATP-dependent DNA helicase activity	5
GO:0008026	molecular_function	ATP-dependent helicase activity	5
GO:0008094	molecular_function	DNA-dependent ATPase activity	7
GO:0070035	molecular_function	purine NTP-dependent helicase activity	5
GO:0031625	molecular_function	ubiquitin protein ligase binding	5
GO:0044389	molecular_function	small conjugating protein ligase binding	5
GO:0051400	molecular_function	BH domain binding	2

GO:0051434	molecular_function	BH3 domain binding	2
GO:0070513	molecular_function	death domain binding	2
GO:0003887	molecular_function	DNA-directed DNA polymerase activity	4
GO:0016779	molecular_function	nucleotidyltransferase activity	6
GO:0034061	molecular_function	DNA polymerase activity	5
GO:0005509	molecular_function	calcium ion binding	19
GO:0004033	molecular_function	aldo-keto reductase (NADP) activity	3
GO:0016614	molecular_function	oxidoreductase activity, acting on CH-OH group of donors	6
GO:0016616	molecular_function	oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	6
GO:0032052	molecular_function	bile acid binding	2
GO:0016229	molecular_function	steroid dehydrogenase activity	5
GO:0033764	molecular_function	steroid dehydrogenase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	5
GO:0047023	molecular_function	androsterone dehydrogenase activity	3
GO:0047042	molecular_function	androsterone dehydrogenase (B-specific) activity	3
GO:0047115	molecular_function	trans-1,2-dihydrobenzene-1,2-diol dehydrogenase activity	2
GO:0005342	molecular_function	organic acid transmembrane transporter activity	6
GO:0008514	molecular_function	organic anion transmembrane transporter activity	6
GO:0015171	molecular_function	amino acid transmembrane transporter activity	5
GO:0046943	molecular_function	carboxylic acid transmembrane transporter activity	6
GO:0003684	molecular_function	damaged DNA binding	4
GO:0008307	molecular_function	structural constituent of muscle	5
GO:0000405	molecular_function	bubble DNA binding	2
GO:0043138	molecular_function	3'-5' DNA helicase activity	2
GO:0043140	molecular_function	ATP-dependent 3'-5' DNA helicase activity	2

Table 9.8: Information of enriched GO terms for significantly up regulated genes after 24 hours exposure to 900μM GHB.

GOID	Ontology	Term	Number of genes
GO:0005623	cellular_component	cell	189
GO:0005886	cellular_component	plasma membrane	74
GO:0016020	cellular_component	membrane	137
GO:0044464	cellular_component	cell part	189
GO:0071944	cellular_component	cell periphery	77
GO:0016021	cellular_component	integral to membrane	89
GO:0031224	cellular_component	intrinsic to membrane	90
GO:0044425	cellular_component	membrane part	110
GO:0044459	cellular_component	plasma membrane part	41
GO:0005737	cellular_component	cytoplasm	129
GO:0044444	cellular_component	cytoplasmic part	98
GO:0005576	cellular_component	extracellular region	40
GO:0005615	cellular_component	extracellular space	21
GO:0044421	cellular_component	extracellular region part	25
GO:0003008	biological_process	system process	38
GO:0032501	biological_process	multicellular organismal process	87
GO:0044699	biological_process	single-organism process	123
GO:0044707	biological_process	single-multicellular organism process	87
GO:0009987	biological_process	cellular process	165
GO:0050789	biological_process	regulation of biological process	124
GO:0050794	biological_process	regulation of cellular process	119
GO:0065007	biological_process	biological regulation	132
GO:0042221	biological_process	response to chemical stimulus	48
GO:0050896	biological_process	response to stimulus	110
GO:0051179	biological_process	localization	70
GO:0051716	biological_process	cellular response to stimulus	76
GO:0065008	biological_process	regulation of biological quality	44
GO:0007154	biological_process	cell communication	81
GO:0007165	biological_process	signal transduction	66
GO:0007167	biological_process	enzyme linked receptor protein signaling pathway	19
GO:0023052	biological_process	signaling	76
GO:0044700	biological_process	single organism signaling	76
GO:0009653	biological_process	anatomical structure morphogenesis	39
GO:0032502	biological_process	developmental process	67
GO:0007169	biological_process	transmembrane receptor protein tyrosine kinase signaling pathway	17
GO:0001666	biological_process	response to hypoxia	10

GO:0036293	biological_process	response to decreased oxygen levels	10
GO:0070482	biological_process	response to oxygen levels	10
GO:0010646	biological_process	regulation of cell communication	37
GO:0023051	biological_process	regulation of signaling	37
GO:0001525	biological_process	angiogenesis	13
GO:0001568	biological_process	blood vessel development	16
GO:0001944	biological_process	vasculature development	16
GO:0048514	biological_process	blood vessel morphogenesis	15
GO:0048646	biological_process	anatomical structure formation involved in morphogenesis	33
GO:0072358	biological_process	cardiovascular system development	20
GO:0072359	biological_process	circulatory system development	20
GO:0006810	biological_process	transport	55
GO:0051234	biological_process	establishment of localization	56
GO:0007267	biological_process	cell-cell signaling	22
GO:0010817	biological_process	regulation of hormone levels	10
GO:0042445	biological_process	hormone metabolic process	8
GO:0006518	biological_process	peptide metabolic process	7
GO:0006520	biological_process	cellular amino acid metabolic process	13
GO:0006575	biological_process	cellular modified amino acid metabolic process	11
GO:0006749	biological_process	glutathione metabolic process	6
GO:0006790	biological_process	sulfur compound metabolic process	10
GO:0043603	biological_process	cellular amide metabolic process	8
GO:0014047	biological_process	glutamate secretion	4
GO:0008652	biological_process	cellular amino acid biosynthetic process	8
GO:0000096	biological_process	sulfur amino acid metabolic process	4
GO:0044272	biological_process	sulfur compound biosynthetic process	6
GO:0042398	biological_process	cellular modified amino acid biosynthetic process	5
GO:0043043	biological_process	peptide biosynthetic process	4
GO:0006534	biological_process	cysteine metabolic process	3
GO:0005488	molecular_function	binding	191
GO:0005515	molecular_function	protein binding	132
GO:0004871	molecular_function	signal transducer activity	33
GO:0060089	molecular_function	molecular transducer activity	33
GO:0043167	molecular_function	ion binding	93
GO:0008289	molecular_function	lipid binding	16
GO:0019898	cellular_component	extrinsic to membrane	16
GO:0004714	molecular_function	transmembrane receptor protein tyrosine kinase activity	6

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