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DETECTION OF SURROGATE MARKERS AFTER GAMMA-HYDROXYBUTYRATE (GHB) EXPOSURE

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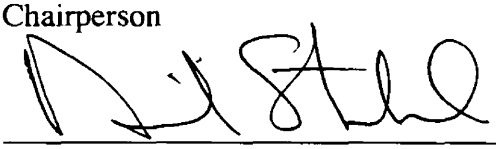

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Detection of Surrogate Markers after Gamma-hydroxybutyrate (GHB) Exposure

Chairperson: Mark Pershouse, Ph.D.

Gamma-hydroxybutyrate (GHB) is a substance naturally present in humans, as well as a drug of abuse. The use of GHB in date-rape sexual assaults has increased over the past few years. Currently different chromatography techniques are used to detect GHB in blood or urine, with a detection timeframe of around 12 hours. This limited window of detection causes many problems for law enforcement in preparing for these rape cases. In this study microarray technology is used in a mouse model to detect biomarkers in peripheral blood after GHB exposure. Epiregulin and Phosphoprotein enriched in astrocytes 15 (Pea-15) both had increased expression in GHB dosed mice (1g/kg) over control. The detection of these genes RNA was confirmed using a semi-quantitative RT-PCR assay. An intracellular flow cytometry assay was developed that could detect protein changes in peripheral blood in both of these potential biomarkers after GHB exposure. These results suggest that after further development, epiregulin and Pea-15 may prove to be significant surrogate markers in the indirect detection of GHB.

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Introduction

Gamma-hydroxybutyrate (GHB) is a powerful and rapidly active central nervous system depressant. It is a naturally occurring substance in the brain, first synthetically produced in 1960 as an anesthetic (Laborit, 1964). The majority of early research on GHB investigated its anesthetic properties.

GHB is both a metabolite of gamma-aminobutyric acid (GABA) and a precursor that can be degraded to produce a functional pool of GABA. Because of their chemical and metabolic relationship the two compounds have often been discussed and compared to each other. Exogenously administered GHB has many of the behavioral effects that are tied to GABA compounds, though many now consider GHB as a pharmacological entity that is unique and may act as a neurotransmitter (Bernasconi et al., 1999; Howard and Feigenbaum, 1997; Maitre, 1997; Tunnicliff, 1992; Vayer et al., 1987). Whether GHB acts as a neurotransmitter or neuromodulator is still the subject of much controversy in the literature.

GHB has recently been receiving more attention because of the potential therapeutic affects but also because the incidence of abuse has increased. The euphoric properties of GHB helped to foster the drug's emergence as a popular club drug in the late 1990's. In February of 2000, after it was termed a drug of abuse and a date rape drug, GHB was re-classified as a schedule I drug by the United States Drug Enforcement Administration (2000).

This introduction covers the chemical, pharmacological, behavioral, and toxicological background information on GHB. This introduction will also discuss

microarray technology and the use of surrogate markers. Additionally, the overall direction of this project will be presented.

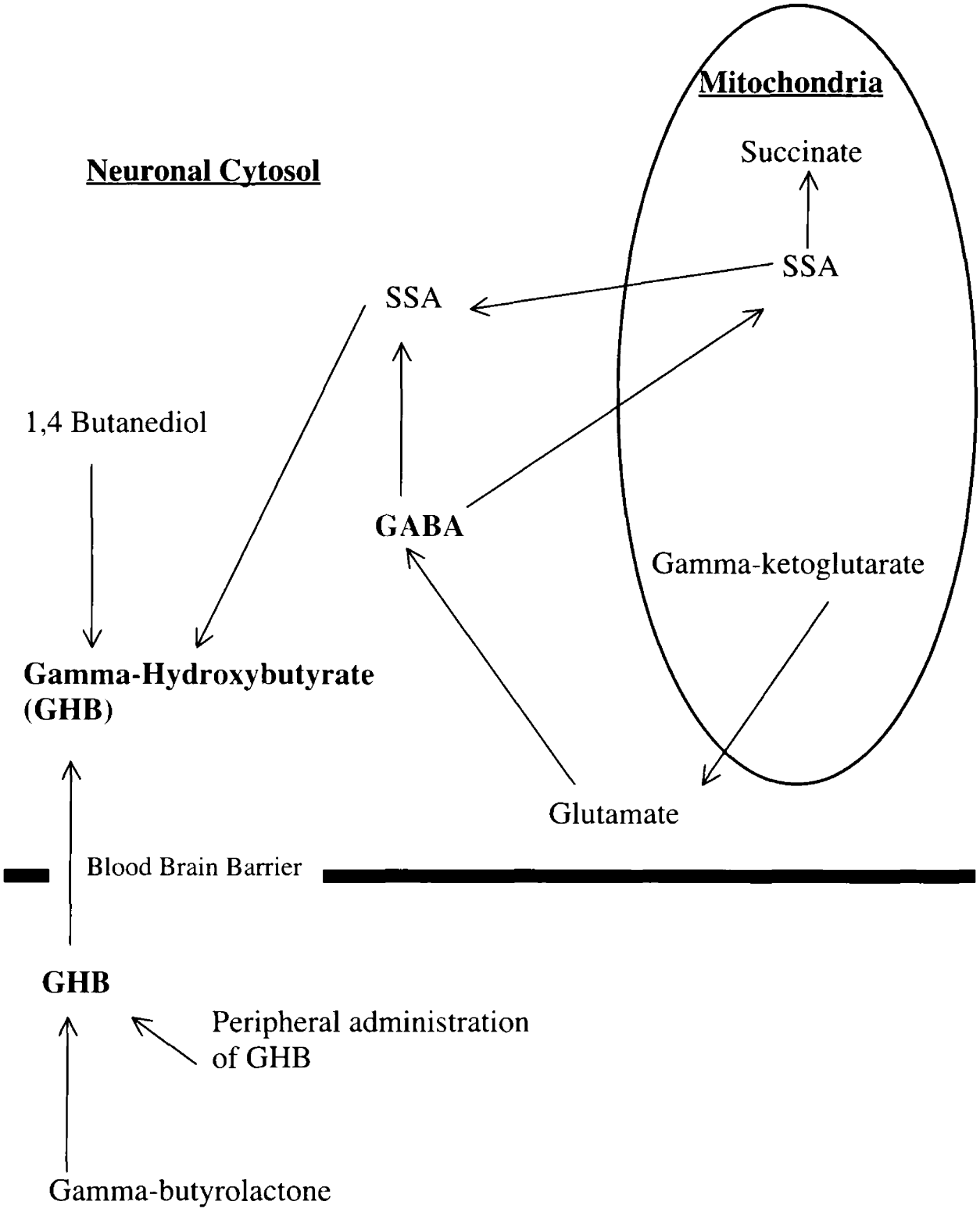
Metabolism and Distribution

GHB is distributed in distinct regions of the brain. It is found in the substantia nigra, striatum, hippocampus, and the frontal cortex. Higher concentrations of GHB have been found in peripheral organs such as the heart, kidney, liver, muscle, and fat though the biological significance of these concentrations is not known.

The metabolism of gamma-aminobutyrate (GABA) provides the primary source of GHB in the brain (Figure 1). This has been demonstrated by the administering of radiolabeled GABA into the rat brain and measuring its conversion to labeled GHB (Roth and Giarman, 1969). GABA is deaminated to succinic semialdehyde (SSA) by the enzyme GABA aminotransferase. The majority of SSA is converted to succinate and used in the Kreb's cycle, but a small portion is converted to GHB by SSA reductase (Cash et al., 1981; Gold and Roth, 1977). Experiments looking at the distribution of the SSA reductase enzyme have shown that it is present only in the cytoplasm of neurons. These neurons also contain the enzymes needed for the synthesis of GABA. It was concluded that GHB formation occurs in neurons that are also able to synthesize GABA.

Other precursors to GHB in the brain and in peripheral organs are 1,4-butanediol and gamma-butyrolacetone. Both of these are present in the rat brain at 10% the levels of GHB (Barker et al., 1985; Doherty et al., 1975). 1,4-butanediol is transformed rapidly into GHB in the brain (Snead and Liu, 1984; Snead et al., 1982) while gamma-butyrolacetone is probably metabolized before entry in the brain, through the blood-brain

FIGURE 1



(Adapted from Maitre, 1997)

Figure 1. Biosynthesis of GHB in brain

The main pathway for GHB synthesis is the metabolism of GABA. GABA is metabolized in the brain to succinic semialdehyde (SSA) and SSA eventually is broken down into GHB. Another route for GHB synthesis is through the reduction of 1,4 butanediol or gamma-butyrolactone. Both 1,4 butanediol and gamma-butyrolactone are present in low concentrations compared to GHB and GABA.

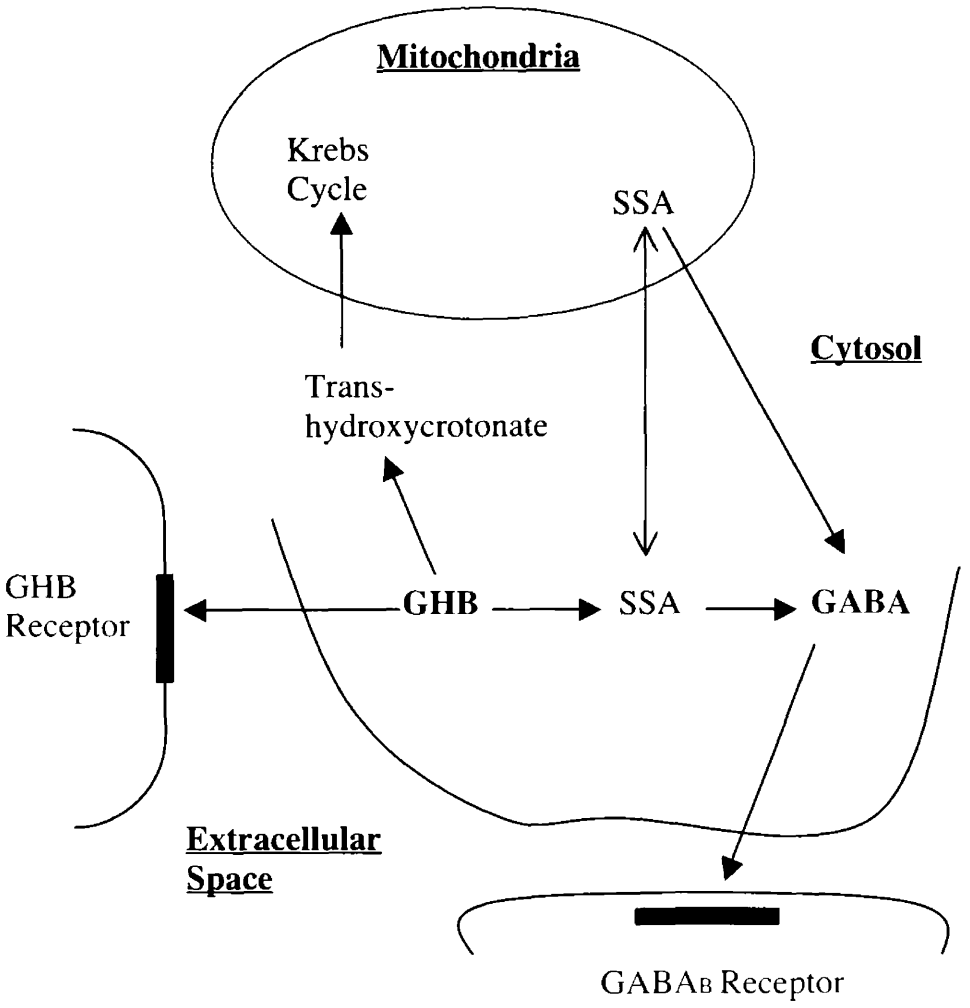
barrier by peripheral lactonases (Roth and Giarman, 1968). Gamma-butyrolactone has been used in many pharmacological studies because it is absorbed more easily in the body than GHB. It is also easier to obtain than GHB and can also be used as a drug of abuse.

Once GHB has bound to a receptor, released, and the activity terminated by uptake of the molecule from the synaptic cleft, it is then degraded. The degradative pathway of GHB starts with GHB dehydrogenase converting it to SSA. The SSA is then further broken down to succinate or GABA (Doherty et al., 1975; Kaufman and Nelson, 1991; Mohler et al., 1976). The succinate is further metabolized into carbon dioxide and water. The degradation of GHB into GABA can produce a functional pool of GABA (Figure 2).

GHB binding to GHB receptor and relationship with GABA$_B$ receptors

Many studies have been conducted examining the binding of GHB to a receptor so that pharmacological actions can take place. For years it was thought that GHB bound to the GABA$_B$ receptors exclusively. Eventually, research showed that GHB bound to a unique G-protein coupled receptor specific for the molecule (Maitre, 1997; Snead, 1994). Snead measured radiolabelled GHB binding patterns in a rat brain by autoradiography. He found that there was a different pattern of distribution of the labeled GHB from labeled GABA binding. It was also determined that there are both high and low affinity binding sites present in the brain. The GHB high affinity sites are absent from peripheral tissues like the liver, kidney, muscle and heart, even though there are significant amounts of endogenous levels of GHB in these organs (Snead and Liu, 1984). GHB-specific

FIGURE 2



(Adapted from Maitre, 1997)

Figure 2. Degradation of GHB in brain

The main route of GHB degradation is to succinic semialdehyde (SSA). SSA is then converted to GABA or oxidized through the Krebs cycle. GHB can also be converted to trans-hydroxycrotonate and further broken down through the Krebs cycle. The GABA pool formed from this pathway can be released and bind to $GABA_B$ receptors. Before degradation, the GHB can be released and bind to either the GHB receptor or $GABA_B$ receptors.

binding sites appear to exist only on neurons in different locations throughout the brain. Further evidence was gathered after the first antagonist for GHB binding (NCS-382) was discovered (Maitre et al., 1990). Maitre's research showed that this antagonist blocked GHB binding and many of the pharmacological and physiological effects of exogenous GHB administration.

GHB's mode of action in the brain appears through dual mechanisms (Wong et al., 2004). The neurobiological activity of GHB seems to be mediated through the GHB receptor, while the pharmacological effects of exogenously administered GHB seems to be partially mediated through the $GABA_B$ receptor. GHB may act directly on $GABA_B$ as a partial agonist or indirectly on $GABA_B$ receptor via GHB-derived GABA (Hechler et al., 1997). Evidence supporting this hypothesis comes from studies showing minimal binding of physiological levels of radiolabeled GHB to different $GABA_B$ receptor isoforms that were overexpressed in cells. When higher concentrations of the labeled GHB were used, there were low levels of binding to the $GABA_B$ receptors (Wu et al., 2004). This premise was strengthened when GHB-sensitive and GHB-resistant rats were dosed with GHB and baclofen (GABA agonist). Depending on dose, differences in motor coordination between the two groups of rats led the authors to conclude that high concentrations of GHB can lead to the activation of the GABAergic system (Lobina et al., 2005).

It seems that endogenous levels of GHB do not bind to, or activate, the $GABA_B$ receptors. The concentrations of GHB needed to stimulate these receptors are much higher than are found in the brain.

Release and membrane transport of GHB

It was determined that GHB plays a role in interneuronal signaling when it was discovered that there was localized release after cellular depolarization. Brain slices containing radiolabeled GHB gave results that were consistent with a depolarization-evoked and calcium-dependent release (Maitre et al., 1983; Maitre and Mandel, 1982).

Since exogenous GHB can pass through the blood-brain barrier and also be synthesized in the brain, an active transport system was postulated. It was discovered that there was a high-affinity uptake system that was dependent on both sodium and chloride ions (Benavides et al., 1982). Radiolabeled GHB accumulated in membrane vesicles in the brain with a linear dependence on the sodium ion concentration. Though there is still a small amount of uptake in vesicles that do not contain either the sodium or chloride ions, the ion's presence marks a large rise in uptake.

Role of GHB in brain function

Since GHB was synthesized as a substance to try and mimic GABA-like effects, the majority of early studies tried to understand the role of exogenous GHB activity. Humans need large doses of GHB (20-40 grams) to receive the sedative or anesthetic results, while endogenous levels of GHB in the brain are only in the 1-10 micromolar range (Vayer and Maitre, 1988). The role of GHB in daily brain function has yet to be determined.

The study of specific neuronal responses to GHB is hindered because it is metabolized to form GABA so rapidly. GHB administered to neuronal tissue (*in vivo*), brain slices, or neural cells can lead to a GHB-specific response mediated through either

the GHB receptor or the stimulation of the GABAergic system depending on dose. Past research has shown that low doses of GHB (micromolar range) can be blocked or reduced by specific GHB receptor antagonists. There is no change in signal with GABA receptor antagonists (Harris et al., 1989; Maitre et al., 1990; Osorio and Davidoff, 1979). Higher doses of GHB (millimolar) result in a blocked signal when using $GABA_B$ antagonists (Waldmeier, 1991; Xie and Smart, 1992). Other groups looked further into the GHB effect on the GABA system by inhibiting the ability of GHB to be metabolized (Hechler et al., 1997). When they inhibited the enzymes responsible for that action the formation of GABA from GHB was reduced. GHB was inhibited in displacing labeled GABA from its binding sites on $GABA_B$ receptors, further indicating that GHB was not a specific ligand for those receptors. These results have led many researchers to state that exogenous GHB metabolism is able to produce enough GABA to effect GABAergic signaling, but physiological levels of GHB have little affinity for the $GABA_B$ receptors.

Besides affecting the GABAergic system, exogenous GHB is known to affect the dopaminergic system. GHB initially inhibits the release of dopamine. This results in an accumulation of dopamine in presynaptic neurons (Hechler et al., 1991; Nissbrandt et al., 1994; Roth et al., 1980). After high doses of GHB there is a rapid increase in dopamine levels, followed by the release of this dopamine in certain regions of the brain (Hechler et al., 1993). This increase in dopamine levels is very high, reaching between 6-10 times baseline levels. Research has shown that changes in the dopamine system are regulated through the GHB receptors but that the GABAergic system is indirectly involved (Nissbrandt et al., 1994). These increases of dopaminergic activity may play a role in the euphoric effects and abuse potential of GHB.

It is also known that GHB affects the serotonin system, but whether this is through direct means or via another system (dopamine or GABA) is not known. GHB has been shown to increase serotonin turnover rates without altering absolute levels (Hedner and Lundborg, 1983; Waldmeier and Fehr, 1978). The mechanism for this is not known but it is thought that it may have to do with the elevated tryptophan (precursor to serotonin) levels associated with an increase in GHB levels (Maitre, 1997). Some researchers believe that this control on serotonin turnover is the result of the affects of GHB on the GABA system, since baclofen ($GABA_B$ agonist) has also been shown to alter serotonin turnover (Waldmeier and Fehr, 1978). However, the distributions of serotonin modulation are completely different between GHB and baclofen. This suggests a unique mechanism may exist for the affect on serotonin from GHB (Maitre, 1997).

Behavioral pharmacology of GHB

There have been many animal studies on the effects of GHB at the cellular level, but relatively few concerning the behavioral effects of the drug based on the therapeutic potential of GHB. The first behavioral effect found was anxiolytic effects. Given a non-sedative dose of GHB, mice showed a reduction in passive behaviors and increased interaction with littermates (Krsiak et al., 1974). This is similar to the loss of inhibitions humans show upon the ingestion of this drug. Further studies show that it is likely these effects are mediated through either the conversion of GHB to GABA and the subsequent binding to $GABA_A$ receptors (Hechler et al., 1997) or actual GHB stimulation of GABA release in certain sections of the brain (Gobaille et al., 1999; Goodwin et al., 2005).

There have also been studies looking at the reinforcing effects of self-administrated GHB preceding drug abuse. Colombo's group gave Wistar rats the option of water and a GHB solution. The rats always voluntarily consumed the GHB (Colombo et al., 1995a). Later, Colombo's group conducted experiments with alcohol-preferring rats (sP) and non-perferring (sNP) rats (Colombo et al., 1998). Presenting the GHB solution for fourteen days as the only water source and later giving the choice between the GHB solution and water both groups of rats showed an overwhelming preference for the GHB solution after the two-week time frame. The research suggested that the reinforcing properties of GHB were unmasked by the long time frame of GHB ingestion. When they repeated this experiment they only presented the GHB solution for three days instead of fourteen before giving the rats a choice. This time the sNP rats consumed very low amounts of the GHB solution when given the choice, while the sP rats continued to use large amounts of the GHB solution. The researchers theorized that the selectively bred high alcohol preference rats (sP) possessed a genetically higher sensitivity to the effects of alcohol, as well as GHB. They also concluded that GHB yields reinforcing properties similar to those of alcohol and that the two drugs share similar pathways at the cellular level.

Therapeutic uses of GHB

There are many potential therapeutic uses for GHB. It is currently being used for the treatment of narcolepsy and alcohol withdrawal. Studies have also considered GHB for the treatment of heroin dependence, anesthesia, anxiety, fibromyalgia, and other medical problems.

GHB's most important therapeutic use is to treat alcohol abuse. It has been used successfully in Russia and Western Europe for years to combat the effects of alcoholism and withdrawal symptoms, though this therapy has not been adopted in the United States. GHB is effective in treating alcoholism because it reduces alcohol cravings and alleviates the symptoms of alcohol withdrawal (Addolorato et al., 2000; Gallimberti et al., 2000). There are many pharmacological similarities between the two drugs and it has been proposed that GHB exerts its effects on alcohol dependence by duplicating the action of alcohol in the central nervous system (Gessa et al., 2000).

Alcohol withdrawal can cause an increase in heart and respiratory rates, nausea, depression, tremors, and seizures. Early studies looked at the ability of GHB to diminish these symptoms in a dose dependent manner. When high doses of GHB (1 g/kg) are administered, ethanol-dependent Sprague-Dawley rats were completely protected from tremors and seizures (Fadda et al., 1989). Many other similar studies have looked at these positive effects and all of them concluded that acute administration of GHB reduced the affects of ethanol withdrawal.

The currently accepted hypothesis is that GHB exerts effects on ethanol dependence through a substitution mechanism (Colombo et al., 1995b). There have been many observed pharmacological similarities on which this argument is based. Both low doses of GHB and ethanol have been reported to increase the release of dopamine in certain regions of the brain (Cheramy et al., 1977; Maitre et al., 1990) as well as to stimulate locomotor activity (Maitre, 1997). These findings are relevant because the activation of the dopamine system and increased locomotor activity are common in the reinforcing properties of drugs like alcohol (Bozarth, 1986; Wise and Bozarth, 1985). It

was also noted that doses of GHB producing anesthesia in naïve rats would only impose low levels of sedation in ethanol-dependant rats (Colombo et al., 1995d). These reports of cross-tolerance between GHB and ethanol suggest common neuronal changes by both drugs and could impose a problem in the use of GHB as a therapeutic drug.

The potential for abusing GHB is also the subject of much interest in the literature. Many studies have shown both a preference towards self-administration in rats (Colombo et al., 1995a; Colombo et al., 1998; Colombo et al., 1995c) and abuse by human beings (Addolorato et al., 2000); Centers for Disease Control, 1991; US Food and Drug Administration, 1991). These reports strengthen the idea that GHB possesses reinforcing properties similar to ethanol. Another similarity of GHB to ethanol is that it reduces anxiety. Many studies have looked at this property of alcohol (Becker and Flaherty, 1982; Blanchard et al., 1993; Colombo et al., 1995d) and only a few with GHB (Colombo et al., 1998; Krsiak et al., 1974). Measuring different levels of anti-anxiety behavior, both groups concluded that consumption of GHB produces effects similar to alcohol.

Because of the research conducted on alcohol withdrawal, the effects of GHB on the treatment of heroin dependence became a focus of several studies (Gallimberti et al., 1993; Gallimberti et al., 1994). Gallimberti's group performed a study to see if GHB was effective in suppressing withdrawal symptoms in heroin and methadone-dependent patients. They found that after treatment with GHB for over one week, the addicts showed fewer withdrawal symptoms than the placebo group. Their studies suggested the efficiency of GHB in suppressing opiate withdrawal in humans. Another study failed to demonstrate protection from opiate withdrawal symptoms by GHB (Rosen et al., 1996).

There were differences in the schedule and dosage of heroin; and this may explain the differential response. Even though there are discrepancies in the studies there is enough promising evidence to prompt continued study into GHB's efficiency in treating the withdrawal symptoms of heroin.

To legally purchase GHB, one must obtain a prescription (Xyrem) for the treatment of narcolepsy. Narcolepsy is a rare sleep disorder whose symptoms include excessive daytime sleepiness, sudden sleep attacks, sleep paralysis, hallucinations upon falling asleep, and the temporary loss of muscle tone. All these symptoms are created because of problems with REM sleep. The mechanism of narcolepsy is unknown, but there has been a substantial amount of work done categorizing symptoms patients' experience. Researchers believe these symptoms stem from the instability caused by both REM and non-REM sleep, an increased number of awakenings and long periods of time between sleep onset (Montplaisir et al., 1978; Zarcone, 1973; Zorick et al., 1986). The most common treatment for narcolepsy includes the use of a stimulant during the day to hold off the sudden sleep attacks, and later, an anti-REM sleep agent to ward off the more serious symptoms. The problem with this approach to treatment is the individual's drug tolerance, low compliance, and a low level of effectiveness. GHB was initially considered as a treatment for narcolepsy in the late 1970's (Broughton and Mamelak, 1979). After treating sixteen narcoleptic patients, researchers found that GHB produced a reduction in the severity of the symptoms including limiting hallucinations and sleep paralysis. They suggested that, GHB administered at night and certain amphetamine analogs given during the day, reduced the major symptoms of narcolepsy. The largest disadvantage of this treatment is the short duration of action requiring multiple

administrations. There have been many follow-up studies that have come to the same conclusion; GHB is an effective agent for the treatment of narcolepsy (Lammers et al., 1993; Mamelak et al., 1979; Scharf et al., 1985; Scrima et al., 1989; Scrima et al., 1990).

The mechanism by which GHB treats narcolepsy is not understood, but it is most likely a cumulative effect of many processes. First, the activation of GHB receptors is known to produce the sedative or sleep-inducing effects. This is known because the specific GHB antagonist NCS-382 can block this effect (Schmidt et al., 1991). GHB is also known to improve REM sleep efficiency and decrease latency (Entholzner et al., 1995; Lapierre et al., 1990). The GHB induced increase in opioid and dopamine response could be a factor in the anti-depressant effects found in GHB-treated patients. GHB also affects the release of acetylcholine, which has been implicated in animal models of narcolepsy (Nishino et al., 1995; Nitz et al., 1995).

GHB has been studied for its anesthesia properties as early as the 1960's and these studies continue today. Early research concluded that GHB treatment resulted in profound relaxation of jaw muscles, which helped facilitate upper airway surgery. There was no depression in the circulatory system, no need for other analgesic agents, and no major side effects (Solway and Sadove, 1965). Surgeries used in these studies have ranged from gastrectomy, pneumonectomy (Solway and Sadove, 1965), coronary artery bypass grafting (Kleinschmidt et al., 1997), and others. Despite a large amount of positive data, widespread acceptance has eluded GHB as an analgesic. This may be because of GHB's potentially addictive nature and its ability to produce seizures in animals that resemble petit mal epilepsy (Tunnicliff, 1992).

The current accepted hypothesis for the mechanism of schizophrenia is increased activity in the release of dopamine. While this theory has not been proven, there are several links to the idea. Drugs that treat schizophrenia block dopamine receptors, while drugs that increase dopaminergic activity usually aggravate symptoms. The interest in GHB comes from its ability to regulate dopaminergic activity. GHB is known to have conflicting effects on dopamine, causing both the stimulation and inhibition of the firing of dopamine. Some studies (Levy et al., 1983; Schulz et al., 1981) have looked into the efficacy of GHB with schizophrenia patients. The overall results of these studies showed that GHB had little to no antipsychotic effects and would not be helpful in the treatment of schizophrenia patients.

There have been many other studies conducted to further document the therapeutic effects of GHB, such as treatment for depression, anti-anxiety, and fibromyalgia. A study was performed with thirty females suffering depression who were injected with daily doses of GHB for 3-12 days. Depression severity was lowered in over 80% of patients (Rinaldi et al., 1967). As yet, there has been no follow up to this study and GHB is not used as an antidepressant today. GHB has also been tested as an anti-anxiety drug. Studies have shown that GHB can decrease anxiety and bring a level of calmness to subjects (Addolorato et al., 1999; Ferrara et al., 1999). The symptoms of fibromyalgia include chronic fatigue, muscular-skeleton pain, and non-restorative sleep. These symptoms are found in an estimated 2% of Americans (Wolfe et al., 1995). When GHB was administered to afflicted patients there was a significant decrease in the severity of the problem (Scharf et al., 1998). The mechanism involved is unknown, but it may be connected to the ability of GHB to increase slow-wave sleep.

Abuse Potential and Toxicology of GHB

GHB is absorbed rapidly and the onset of action is approximately fifteen minutes after ingestion (Vickers, 1969). Adverse effects associated with GHB are dose-dependent. Small doses (10 mg/kg) can cause euphoria, amnesia, nausea, vomiting, dizziness, confusion, drowsiness, and a lowered respiratory state. Large doses of the drug (50 mg/kg) can cause unconsciousness, coma, or death. These negative effects are produced from respiratory depression and can be difficult to treat. In fact, there is no real treatment for a GHB overdose. Supportive care is the usual action, since the effects of GHB toxicity usually wear off in a matter of hours. Physostigmine has been used in the past as a potential drug in treating GHB-related comas (Yates and Viera, 2000). The effectiveness of this treatment has recently been challenged in a new study that warned that physostigmine does not always arouse coma patients, but can cause physostigmine related toxicity (Bania and Chu, 2005). This drug acts as an acetlycholinesterase inhibitor and thus potentiates cholinergic transmission. These mechanisms are not clear, except that since GHB is a CNS depressant, the activation of the cholinergic system by physostigmine, should increase the excitatory pathways enough to overcome the inhibition from GHB.

GHB has been tested for genotoxicity in erythrocytes in the peripheral blood of mice (Dass and Ali, 2004). This study showed that GHB does not seem to be genotoxic, but future work must be done to assess any potential carcinogenicity. There is no evidence of tolerance, withdrawal, or physical dependence when GHB is used at or near therapeutic levels (3.5 g/day). Multiple studies of humans and rodents have supported these results (Addolorato et al., 2005; Addolorato et al., 1996; Maitre, 1997)). However,

there are reports of high volume, chronic users of the drug that have produced withdrawal symptoms similar to that of alcohol (Craig et al., 2000; Miotto et al., 2001). In 2000, the increased abuse of GHB led the DEA to re-classify the drug as a schedule 1 substance (2000).

Prevalence in Society Today

The prevalence of GHB in drug-facilitated rape is increasing and is now the most common substance used for this purpose (NDIC-2003 National Drug Threat Assessment). Drug facilitated rape or date rape occurs when the victim is physically unable to consent to a sexual activity. The ability of GHB to mentally and physically incapacitate a victim is further intensified when combined with alcohol. The combination of GHB and alcohol can cause an increased effect of symptoms during the time the alcohol is present in the body as compared to the effect of GHB alone (McCabe et al., 1971). GHB is quickly metabolized in the body by oxidative enzymes and the eventual byproducts are carbon dioxide and water. With rapid degradation, and no metabolite present, detecting this drug in victims is a serious problem. Many victims will not report an assault until days afterwards because of memory lapses or the physiological trauma associated with such an event. GHB is detectable in a person's system for only a limited amount of time; twelve hours in the urine (Kavanagh et al., 2001) and eight hours in blood (Ferrara et al., 1993) using currently available techniques. The most popular method so far is the use of gas chromatography-mass spectrometry (GC-MS) after the drug is extracted from the sample. New detection techniques are beginning to be developed that lower the sample preparation time and increase the sensitivity using both

GC-MS, liquid chromatography-tandem mass spectrometry, and capillary electrophoresis-electrospray ionization ion-trap mass spectrometry (Crookes et al., 2004; Gottardo et al., 2004; Wood et al., 2004)). With a window of less than 24 hours for detection of GHB, many perpetrators are acquitted for lack of evidence that the victim was drugged. This problem will be solved only by continued improvements in chromatography technology or new approaches to developing a test for the detection of GHB.

MicroArray Technology

Microarray analysis has become such a valuable technique because it generates useful data for the expression of thousands of well-annotated genes in a manner of days. Previously, this was either logistically impossible, or would have taken years of experiments. While microarray analysis was originally dismissed as unfocused, this bias is dissipating due to the realization that understanding a molecule, in this study GHB, in the context of its interactions with thousands of other genes, requires a high-throughput approach. The strength of a discovery-driven approach is that the results are not limited by the scope of an investigator's expectations. Results are often found that are unexpected and potentially more valuable than the expected results.

Modern microarrays contain 50-70 base pair long oligonucleotides designed to specifically detect thousands of RNA transcripts. Each spot on a chip correlates with a specific probe, and each probe corresponds to a specific gene transcript. These oligonucleotides are spotted multiple times on a chip to assess intra-array variability. The labeled pools of cDNA are then incubated with the slides and specifically bound to the

oligonucleotides. The fluorescent signal intensity is detected and this signal is indicative of the relative expression level of the gene versus control.

One concern with microarray experiments is the design of the experiments themselves. This procedure is often too expensive, time consuming, and requires more material than one tissue or animal yields. Pooling of samples from like animals can be done in some experimental designs. There are at least two types of pooling, complete and sub-pooling (Peng et al., 2003). Complete pooling occurs when all of the samples from one treatment group are pooled onto one array and the array is hybridized only once. This approach cannot be used for statistical analysis, but can be used to focus on potential targets or genes important to the treatment. Sub-pooling is similar to complete pooling except multiple arrays are used within each group. This addresses the variability found among array experiments. This approach can be used in statistical microarray experiments and is much more thorough, but it is also more expensive, time-consuming, and analysis is much more difficult. The experimental approach depends on the focus and aim of the study. This study used the complete pooling method. Some arrays were duplicated and the results from the genes of interest were consistent. Even after considering these potential concerns there is no other technology available that is as powerful and or rapid at uncovering novel biomarkers. This method provides a unique opportunity to identify thousands of genes expressed simultaneously in one set of experiments that can be directly compared.

The use of microarray technology to determine biomarkers is gaining acceptance in many different fields. Biomarkers are defined as a laboratory measurement that reflects the activity of a disease or drug process (Katz, 2004). Many studies have been conducted

to find markers for alcohol abuse (Chen et al., 2003; Helander, 2003; Montalto and Bean, 2003). These biomarkers have helped improve on knowledge of drinking patterns for treatment, monitored abstinence in outpatient treatment, and identified individuals at risk of alcohol abuse. Appropriate use of biochemical markers will facilitate early intervention and successful management of patients with alcohol use disorders. Biomarkers have also been developed to study the effects of heroin, cocaine, nicotine, and methamphetamines abuse (Al-Amri et al., 2004; Benowitz et al., 2003; Brenneisen et al., 2002; Elkashef and Vocci, 2003; Garcia-Fuster et al., 2003; Ishigami et al., 2003; Underner et al., 2004; Wiesner et al., 2004). By measuring apomorphine, researchers can assess central dopamine system alterations that are associated with chronic heroin consumption (Guardia et al., 2002). Other researchers have looked through neuroendocrine and neuroimaging studies to identify specific biological markers that could be used to characterize genes that are activated among chronic cocaine users (Elkashef and Vocci, 2003). In cancer diagnosis and treatment many surrogate markers have been discovered, and are currently being developed (Alvaro et al., 2005; Blay et al., 2005; Schmitt et al., 2004; Van den Eynden et al., 2004). Inflammatory breast cancer is an aggressive form of cancer with poor detection and prognosis. Surrogate markers such as the overexpression of E-Cadherin and RhoC GTPase proteins in breast tissue have been developed and are currently being used in laboratories (Van den Eynden et al., 2004). This is a relatively new field but one that is rapidly gaining acceptance.

Specific Aims

My hypothesis is that GHB will induce gene expression changes in a mouse model and these changes will be correlated with the time interval post exposure. In other words, GHB will induce acute gene expression changes that moderate over time. These gene expression changes may be of a longer duration than the time it takes to metabolize GHB, thus improving our window of detection.

Materials and Methods

Mice

Female DBA/2J mice (7-8 weeks old) were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed one week before GHB treatment, with a 12-hour light/dark cycle. The mice were maintained with 3 mice per cage and had access to mouse chow and water continuously. Animals used in this study were maintained in facilities consistent with the guidelines of American Association for the Accreditation of Laboratory Animal Care (AAA-LAC) and the Institutional Animal Care and Use Committee (IUCUC).

Administration of GHB and Blood Isolation

GHB (4-Hydroxybutyric acid sodium salt, Sigma-Aldrich, St Louis, MO) was kindly provided by the State of Montana Crime Lab. GHB was dissolved in distilled water (213 mg/ml) and injected intraperitoneally at a single dose of 1 g/kg. The injection size was 7.5 ml/kg per mouse. Animals were sacrificed on day 1,2, 4, or 7 post-injection by CO2 affixiation and exsanguinated by cardiac puncture. Blood used in microarrays was immediately frozen in liquid nitrogen, while blood used for RT-PCR or Flow Cytometer assays was placed in ice and immediately processed.

Microarray Experiments

RNA Isolation

Blood was pooled within the groups by days post-injection and total RNA was isolated using QIAamp RNA Blood Mini Kit (Qiagen, Valencia, CA). Brain tissue samples were

processed by Polytron homogenization in the presence of TRIzol reagent. RNA is then isolated following the manufacturer's protocol (GIBCO BRL, Grand Island, NY). Additional purification of the total RNA population was performed using the RNeasy kit (Qiagen, Valencia, CA). RNA quality is assessed by absorbance at 260/280 nm.

RNA Amplification

RiboAmp RNA Amplification Kit (Arcturus Biosciences, Mountain View, CA) was used to amplify the blood total RNA to high yields of aRNA. An average of 500-600 ng of total RNA was used perform a single round of amplification and the yield averaged 10 μg of mRNA.

Mouse 10K Microarrays

Microarray Array A oligonucleotides were purchased from MWG Biotech (High Point, NC) and consisted of 9853 mouse genes. The genes are represented by 50 bp oligos designed to specifically detect the gene of interest. The arrays also include 104 Arabidopsis controls. The represented genes were selected from various databases (e.g. EBI, NCBI, Ensembl, GoldenPath) and kept non-redundant in MWG's proprietary CodeSeq® database. Almost all genes on Array A have a known function or clearly defined protein domains.

Target Labeling and Hybridization

Labeled targets are made by incorporating aminoallyl-labeled dUTP (Ares Labeling Kit, Molecular Probes Inc., Eugene, OR) during first strand synthesis using 2 μg of amplified

RNA as template, reverse transcriptase (SuperScript II, GIBCO BRL, Grand Island, NY) and oligo dT (18) primers. Ares AlexaFluor 555 and AlexaFluor 647 dyes (Molecular Probes, Eugene, OR) are added by covalent attachment to the amino allyl group according to the manufacturer's protocol, and incubated for 60 minutes at room temperature. To insure sufficient labeling, the levels of the labeled cDNA were measured by spectrophotometer (Nanodrop,) before hybridization. The experimental or control target and the reference target were mixed and hybridized to the array at 46°C for 16 hours in a hybridization chamber. Arrays were washed once in 1 X SSC and 0.01% SDS, twice in 1X SSC, and twice in 10mM TE, then dried by centrifugation in a tabletop centrifuge fitted with a plate spinning rotor.

MicroArray Scanning and Analysis

The processed microarray is read with an Axon GenePix 4000B laser slide scanner. This scanner was fitted with two excitation lasers (532 nm and 635 nm) permitting simultaneous scanning of two color data without the need for multiple image registration. GenePix Pro 4.0 software was used to perform automatic delineation of spot boundaries, measurement of local background for each spot, and digitization of fluorescence intensity on the microarray. Tabular data was exported to GeneTraffic Duo Microarray Data Management and Analysis Software (Iobion Informatics, LLC, La Jolla, CA). GeneTraffic integrates the data and image files from GenePix into a browser-based interface that connects to an on-site Linux server. The software provides the user with an easy to use system for normalization, analysis, data backup and storage. The scanning parameters for successful array spots were set based on fluorescence intensity and detection levels. The brightest spots were adjusted to be at or just below saturation of the detector. Array spots with obvious defects were tagged and excluded from subsequent

analyses. Normalization was performed based on total red and green dye intensity levels throughout the spots on the array. This normalization compensates for differences in signal intensity based on extraction or labeling differences between samples. The fluorescence intensity values were log-transformed and replicates of the identical samples were checked for analogous readings. Ratios of gene expression for each feature (gene) were filtered to limit analysis to those outside of intra-array and experimentally induced variance. Genes that alter their expression between test populations and control by a factor of 2 (50% decrease or 100% increase) were then considered for confirmation by RT-PCR.

Semi-quantitative RT-PCR

The expression levels of epiregulin and Pea-15 mRNA were analyzed using reverse transcription-PCR method. Total RNA was isolated using QIAamp RNA Blood Mini Kit (Qiagen, Valencia, CA) from peripheral blood. The reverse transcription contained 50 ng RNA, oligo-dT primer and followed the protocol of Sensiscript RT (Qiagen, Valencia, CA). The cDNA was then subjected to gene-specific PCR.

Primer Name	Sequence	Size	Annealing Temperature	Cycles
Pea-15 (+)	5'-CCGTCCTGACCTCCTCACTAT-3'	233 bp.	61	29
Pea-15 (-)	5'-GGAAGGGAGTGGTCTGATGAA-3'	---	---	---
Epiregulin(+)	5'-ACACTGGTCTGCGATGTGAGC-3'	195 bp.	64	26
Epiregulin(-)	5'-TCCCCTGAGGTCACTCTCTCA-3'	---	---	---
β-actin (+)	5'-TTCTTTGCAGCTCCTTCGTTGCCG-3'	457 bp.	---	---
β-actin (-)	5'-TGGATGGCTACGTACATGGCTGGG-3'	---	---	---

Through electrophoresis, the amplified PCR product was resolved on a 10% polyacrylamide gel, stained with ethidium bromide, and the intensities were measured using the NIH Image Analysis program. The ratio of the gene of interest divided by the β-actin control was then determined. This ratio was log transformed and this data is presented.

Flow Cytometer

Peripheral blood was collected in a tube with EDTA (5mM final concentration). Whole blood (50μl) was added to 250 μl erythrocyte lysis Buffer EL (Qiagen, Valencia, CA). Cells were then washed two times with PAB (1X PBS, 1% BSA, 0.1% sodium azide). Cells were then fixed with a final concentration of 1.6% formaldehyde (Calbiochem, La Jolla, CA) for 10 minutes at room temperature and washed with PAB. The cells were then permeabilized with ice-cold methanol (J.T. Baker, Phillipsburg, NJ) for 15 minutes on ice and again washed with PAB. Cells were then incubated with 50 μl RatIgG (600 μg/ml) for 10 minutes on ice to block the Fc receptors and then washed with PAB. Goat anti-epiregulin antibody (1.5 μg) (R&D Systems, Minneapolis, MN) or goat anti-mouse Pea-15 (1.7 μg) (Santa Cruz Biotechnology, Santa Cruz, CA) was added and incubated on ice for 40 minutes. Cells were washed with PAB, resuspended, and then incubated with fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat Alexa flour 488 (Molecular Probes, Eugene, OR) for 20 minutes on ice. As a control, cells were incubated with anti-mouse CD45 (BD Biosciences Pharmingen, San Diego, CA), Epiregulin antibody pre-incubated with recombinant epiregulin (R&D Systems), or Pea-15 antibody

pre-incubated with a specific blocking peptide (Santa Cruz Technology). The cells were then washed two times with PAB. The labeled cells were analyzed on a Becton Dickenson FACSaria flow cytometer.

Statistical Methods

Statistical analysis was done using the software package PRISM, v. 3.03 (Graphpad, San Diego, CA) and Excel. Differences between control and GHB treated mouse groups in the flow cytometer experiments were assessed using a paired two-tailed t-test after the data was normalized to percent control. Differences between control and GHB treated mouse groups in the RT-PCR experiments were assessed using a paired two-tailed t-test after the log of the ratio between the gene specific band and the β-actin control band was determined. All values are reported as mean +/-SEM; $P \leq 0.01$ and $P \leq 0.05$ were considered significant.

Results

Microarray

Microarray technology was used to identify potential gene expression changes in peripheral blood from GHB injected DBA/2J mice. Total RNA was obtained from blood that was extracted from four GHB injected mice and pooled for each of the three time-points. There were also three saline injected control mice pooled for each of the time-points. Using the Mouse 10K Array set A from MWG we found nine genes that passed through the arbitrary cutoff 24 hours after a single acute GHB injection. Phosphoprotein enriched in Astrocytes 15 (Pea-15) showed a 20.9-fold increase in samples from day1 mice, an 8.5 fold increase in samples from day 4 mice, and a return to background levels by day 7 in those samples. Epiregulin increased 19.2-fold in day 1, 4.6-fold by day 4, and below control levels by day 7. Other genes that showed a large expression increase over control in day 1 mice were Dopamine receptor 2, Cholinergic receptor-muscarinic 3, Galanin, Nucleolar protein family A, Neurotensin receptor 2, Colony stimulating factor 2 receptor, and Histidine decarboxylase (Table 1). Based on the large increases of Pea-15 and epiregulin expression through 4 days post-GHB injection it was determined to look further at the affect of GHB on these genes.

TABLE 1

UNIQID	Name	Day1	Day4	Day7
Activated genes				
NM_008556	Phosphoprotein enriched astrocytes 15 (Pea-15)	20.9	8.5	.05
NM_007950	Epiregulin (Ereg)	19.2	4.6	-1.1
NM_033269	Cholinergic Receptor,Muscarinic 3 (Chrm3)	8.0	3.8	-.07
NM_010077	Dopamine Receptor 2 (Drd2)	7.8	.1	.02
NM_010253	Galanin (Gal)	6.5	N/A	.03
NM_026578	Nucleolar protein family A (Nola1)	5.5	3.2	1.1
NM_008747	Neurotensin receptor 2 (Ntsr2)	5.0	N/A	-.1
NM_007780	Colony Stimulating factor 2 receptor (Csf2rb1)	4.9	2.8	.03
NM_008230	Histidine Decarboxylase (Hdc)	4.5	3.1	-.24
Suppressed Genes				
NM_019578	Exostoses (multiple)-like 1 (Extl1)	-2.53	-1.58	-1.39
NM_025301	Mitochondrial Ribosomal protein L17 (Mrpl17)	-2.3	-1.37	-1.3
NM_011404	Solute carrier family 7 transporter (Slc7a5)	-1.8	-1.52	-1.23

Semi-quantitative RT-PCR

Semi-quantitative reverse transcription PCR (RT-PCR) was used to confirm the microarray data. Using gene specific primers, along with control primers (β-actin), differences in total mRNA levels can be distinguished. Total RNA was extracted from white blood cells and analyzed. Conditions were determined so that the gene specific RT-PCR product was still in the linear phase when the comparisons between that product and the control product were made. There were increased levels of Epiregulin expression in GHB injected mice (.4733 +/- .06) over the saline control group (.2857 +/- .05) after Day 1 (Figure 3). This increase lasted through the Day 2 experimental groups (GHB= .4324 +/- .067 and control= .2564 +/- .067) while by Day 4 there were small detectable changes between the groups (GHB= .2307 +/- .063 and control= .2784 +/- .05). While there were changes between the two groups they fall out of the statistical significant range.

Pea-15 expression was also increased through Day 1 (GHB= .1637 +/- .01 and control= .0339 +/- .009) and Day 2 (GHB= .2053 +/- .03 and control= .0614 +/- .003), while declining to control levels by Day 4 (GHB= .3035 +/- .06 and control= .2577 +/- .0024) (Figure 4).

The experimental design included 6 GHB-injected mice that were pooled into 3 groups during a timeframe of 24, 48, and 96 hours. Four saline-injected control mice were pooled into 2 groups during a timeframe of 24, 48, and 96 hours. All experiments were performed at least 2 times.

FIGURE 3

Fig. 3a

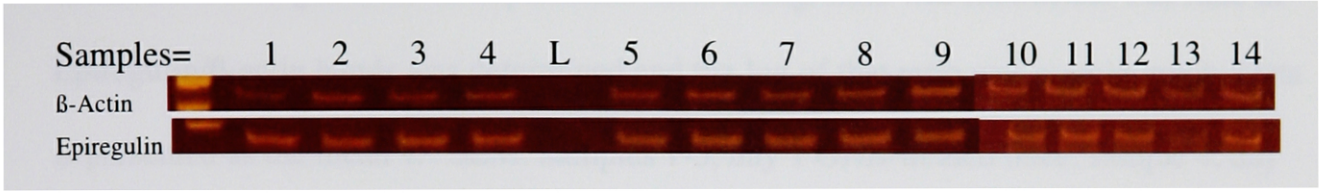


Fig. 3b

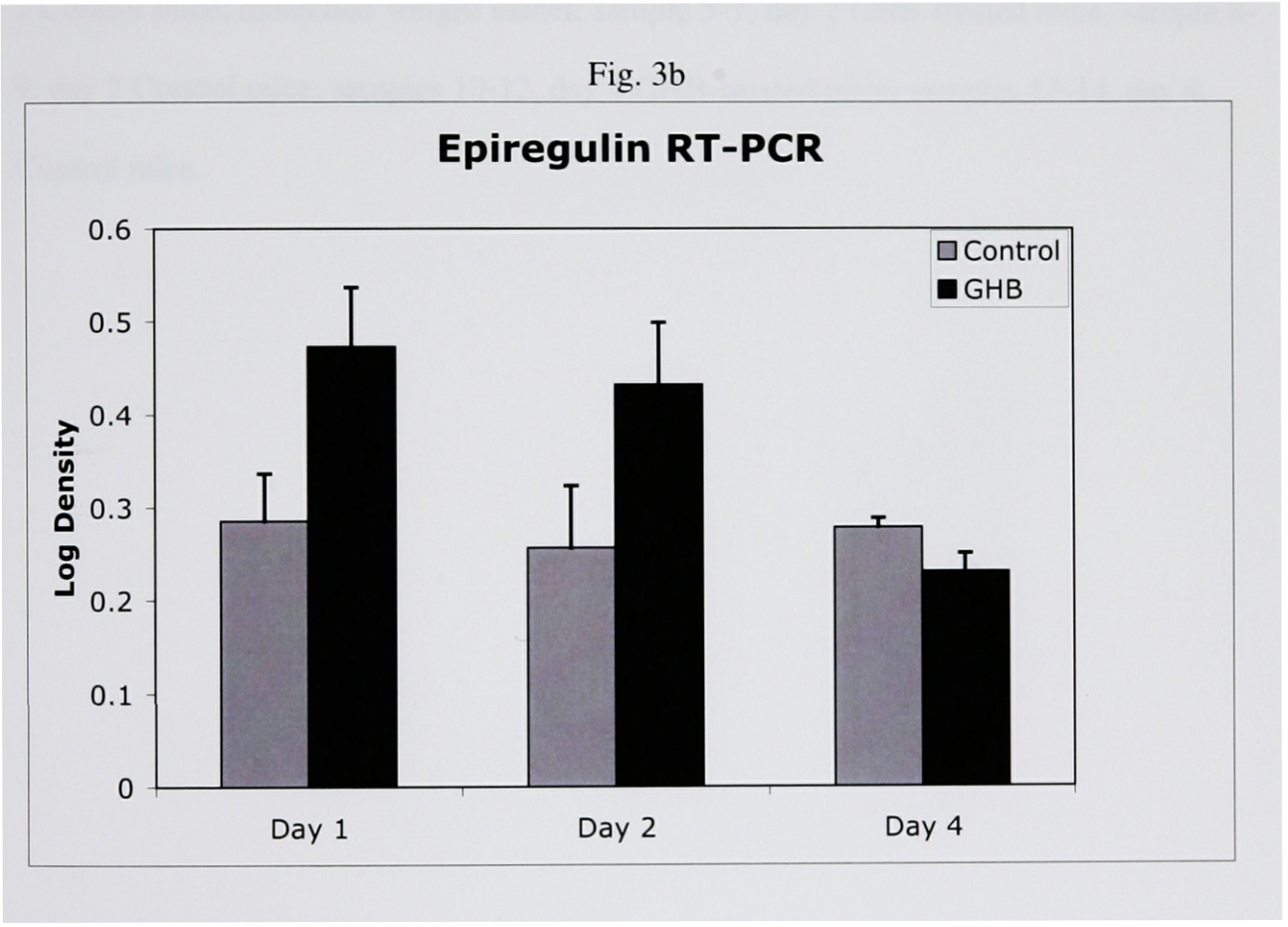


Figure 3. Epiregulin Semi-quantitative RT-PCR

(a) Representation of Epiregulin Semi-Quantitative RT-PCR experiment. (b) Densities of bands were averaged from all experiments after background was subtracted. The ratio of Epiregulin/β-actin bands was determined and the log of that ratio was used in graph. Data is presented as the mean +/- SEM. Samples 1-3, day 1 GHB-treated mice; sample 4, day 1 Control mice; molecular weight ladder; sample 5-7, day 2 GHB-treated mice; sample 8-9, day 2 Control mice; samples 10-12, day 4 GHB-treated mice; samples 13-14, day 4, Control mice.

FIGURE 4

Fig. 4a

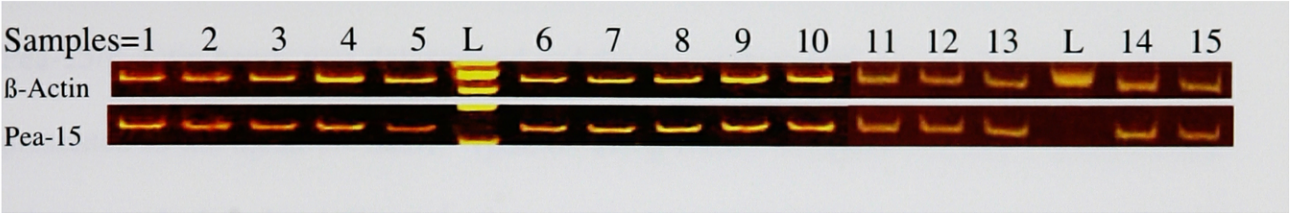


Fig. 4b

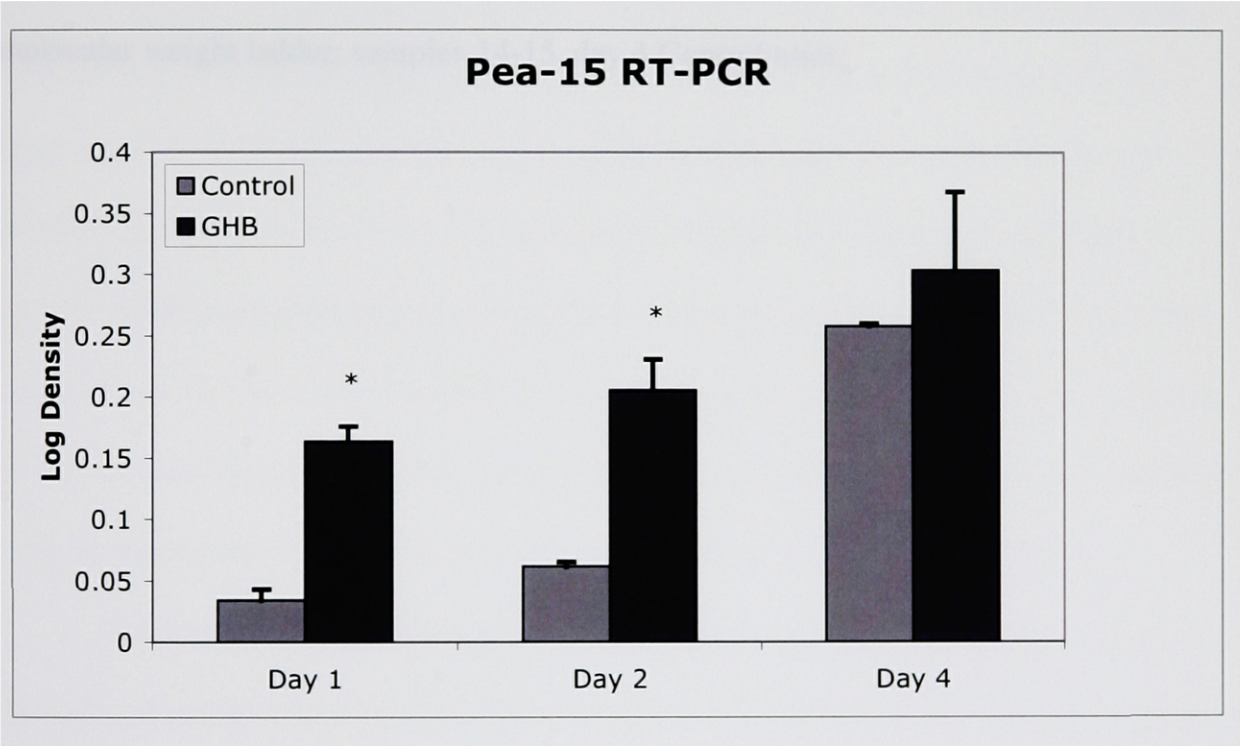


Figure 4. Pea-15 Semi-quantitative RT-PCR

(a) Representation of Pea-15 Semi-Quantitative RT-PCR experiment. (b) Densities of bands were averaged from all experiments and background was subtracted. The ratio of Pea-15/β-actin bands was determined and the log of that ratio was used in graph. Data is presented as the mean +/- SEM. *($p \leq 0.05$ using t-test). Samples 1-3, day 1 GHB-treated mice; sample 4-5, day 1 Control mice; molecular weight ladder; sample 6-8, day 2 GHB-treated mice; sample 9-10, day 2 Control mice; samples 11-13, day 4 GHB-treated mice; molecular weight ladder; samples 14-15, day 4 Control mice.

Intracellular Protein Staining

An intracellular flow cytometer assay was developed to look for protein differences between the GHB- treated and saline-treated control groups. Epiregulin was expressed both on the cell membrane and in the cytoplasm. Pea-15, however, is only found in the cytoplasm. This assay enabled us to use specific antibodies to detect the proteins in formaldehyde fixed white blood cells.

Epiregulin protein levels in GHB-treated mice were increased (256% +/- 39.6) over control 24 hours after injection (Figure 5). There was a smaller but statistically significant increase detected after day 2 (163% +/- 11), while levels returned to near baseline by day 4 (127% +/- 12). Co-incubation with a CD-45 specific lymphocyte-specific marker determined that the epiregulin antibody specifically stained white blood cells (Figure 6). Next, it was important to see if the antibodies were binding specifically to their respective proteins in the cell. Recombinant epiregulin protein was pre-incubated with the epiregulin antibody and blocked antibody binding by 67% (Figure 9a).

Pea-15 protein levels in GHB mice were increased (213% +/- 30.3) over control 24 hours after injection (Figure 7). Pea-15 levels were still increased after day 2 (208% +/- 32.2), while levels were near percent control by day 4 (112% +/- 6.6). Using a CD-45 lymphocyte specific marker it was determined that the Pea-15 antibody specifically stained white blood cells (Figure 8). A blocking peptide was used in correlation with the Pea-15 antibody to inhibit 54%-75% of antibody binding as assessed by median fluorescence (Figure 9b).

FIGURE 5

Fig. 5a

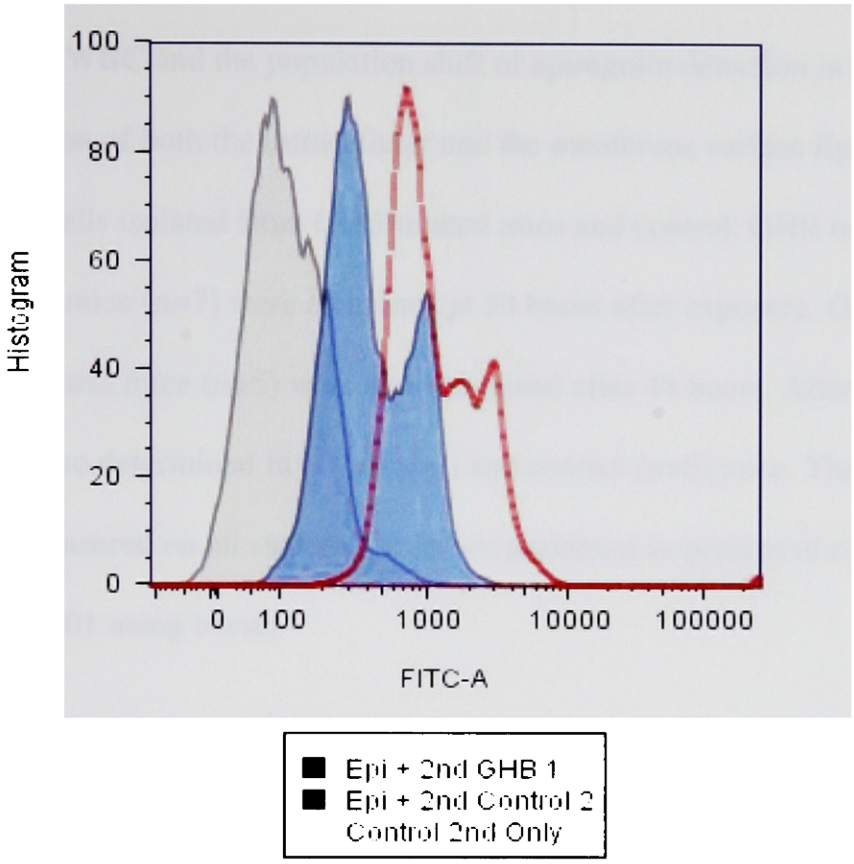


Fig. 5b

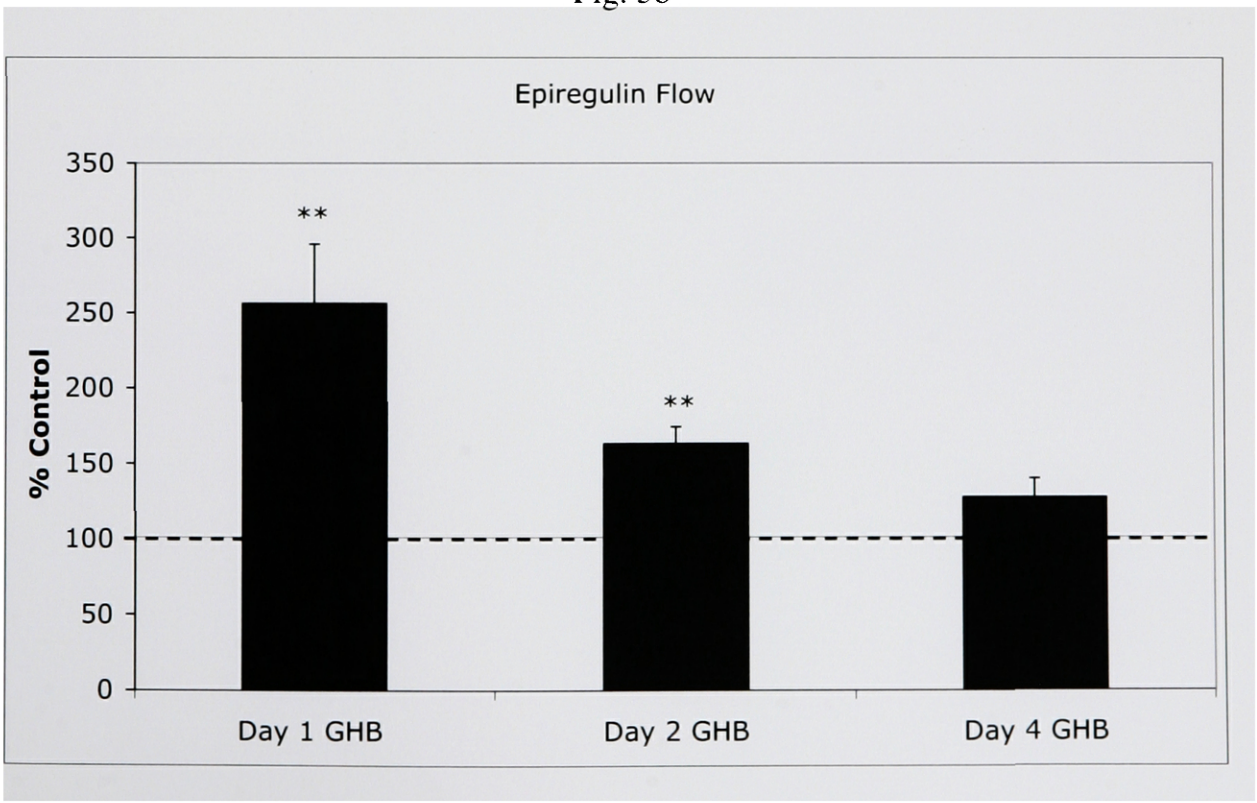
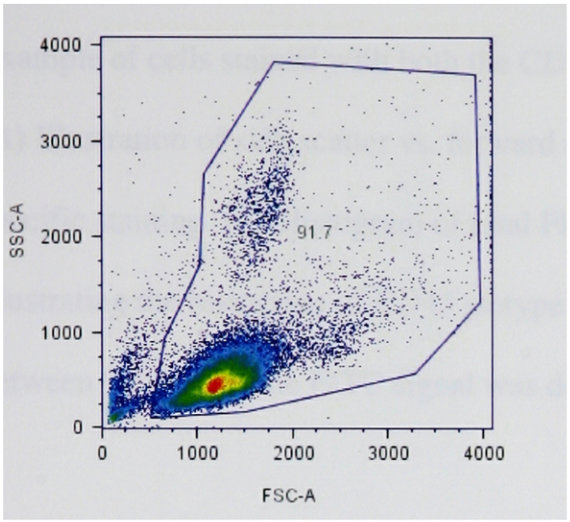


Figure 5. Epiregulin Specific Staining

(A) Overlay of background fluorescence from secondary antibody, epiregulin binding on control WBC and the population shift of epiregulin detection in GHB treated WBC. (B) Detection of both the intracellular and the membrane surface Epiregulin protein on white blood cells isolated from GHB treated mice and control. GHB treated mice (n=10) and control mice (n=7) were examined at 24 hours after exposure. GHB treated mice (n=7) and control mice (n=5) were also examined after 48 hours. After 96 hours protein levels were also determined in GHB (n=7) and control (n=5) mice. The FITC median intensity was measured on all samples. Data are presented as percent of control mean +/- SEM. **($p \leq 0.01$ using t-test)

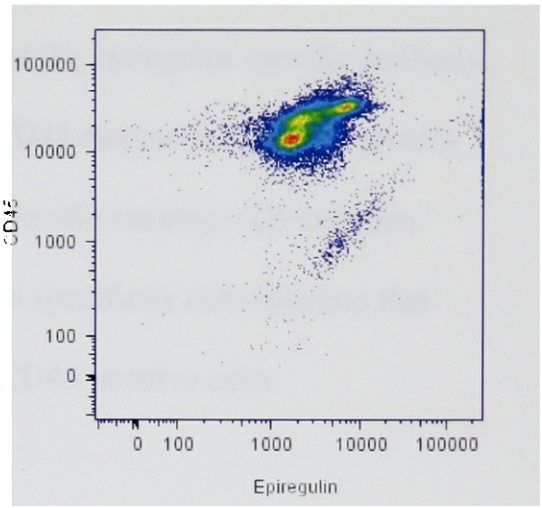
FIGURE 6

Fig. 6a

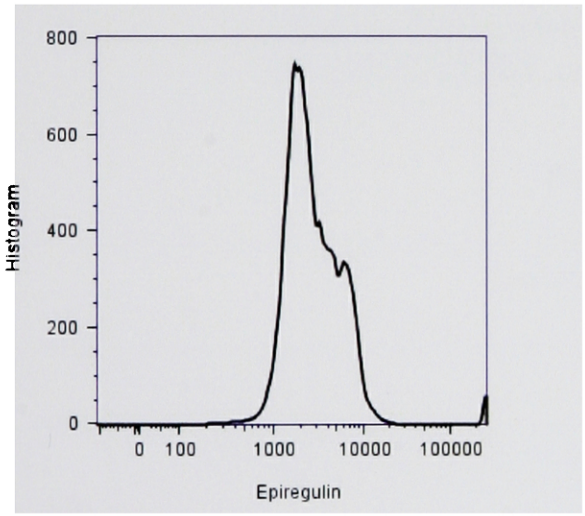


Epi + 2nd + GHB + CD45
Count 30000
Ungated

Fig. 6b

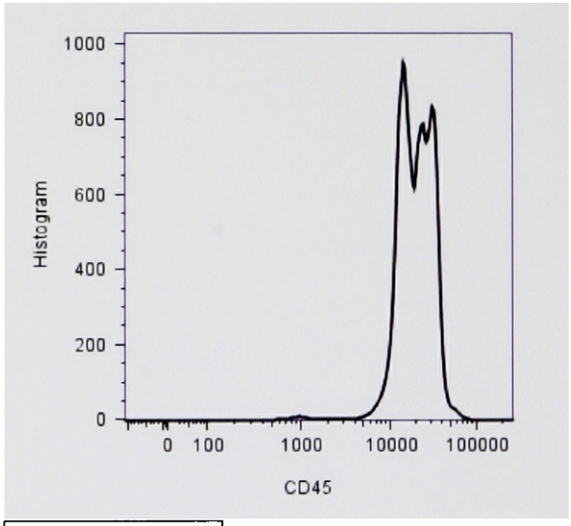


Epi + 2nd + GHB + CD45
Count 27518
Viable Cells



Epi + 2nd + GHB + CD45
Count 27518
Viable Cells

Fig. 6c



Epi + 2nd + GHB + CD45
Count 27518
Viable Cells

Fig. 6d

Figure 6. Epiregulin and CD45 Isotype Staining

Example of cells stained with both the CD45 isotype and the epiregulin specific antibody. (A) Illustration of side scatter vs. forward scatter. (B) CD45 isotype staining vs. protein specific staining. (C) Histogram of total FITC protein specific staining. (D) Diagram illustrating the histogram of CD45 isotype staining. This specificity demonstrates that between 90-98% of the FITC signal was derived from CD45 positive cells.

FIGURE 7

Fig. 7a

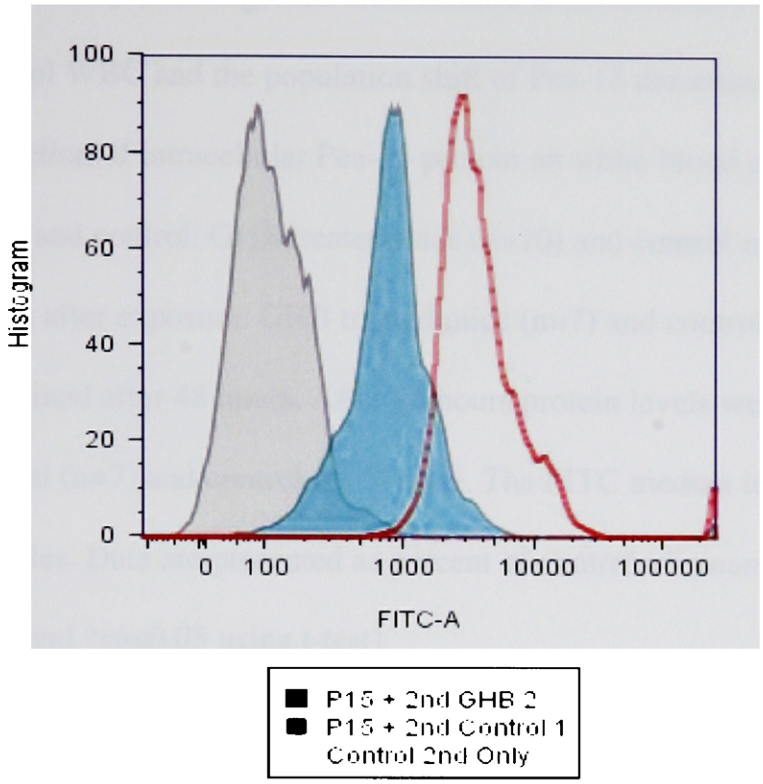


Fig. 7b

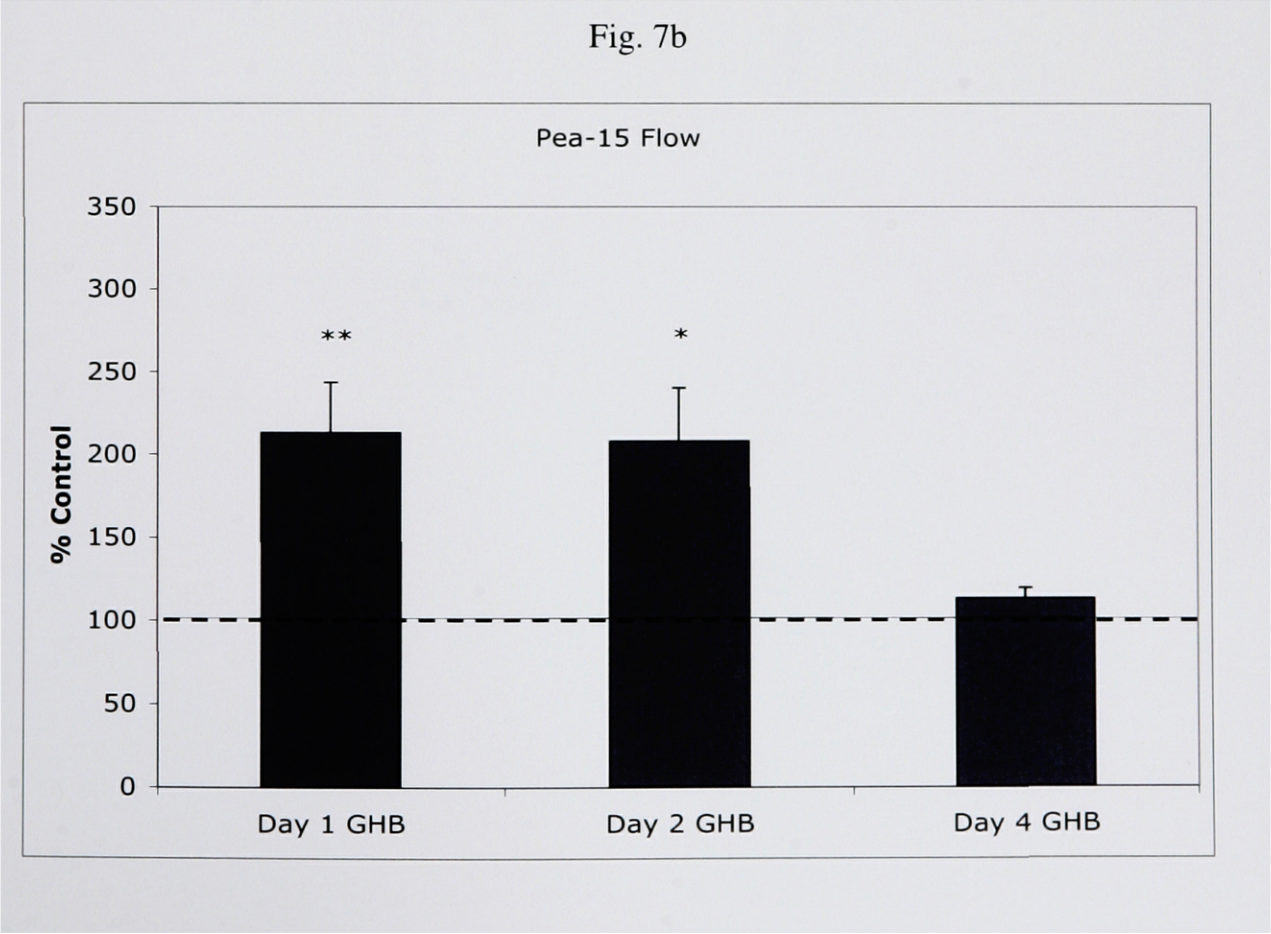
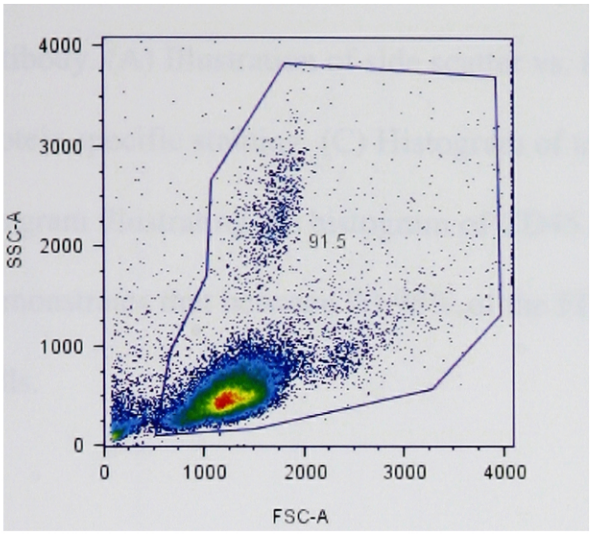


Figure 7. Pea-15 Specific Staining

(A) Overlay of background fluorescence from secondary antibody, Pea-15 binding on control WBC and the population shift of Pea-15 detection in GHB treated WBC. (B) Detection of intracellular Pea-15 protein on white blood cells isolated from GHB treated mice and control. GHB treated mice (n=10) and control mice (n=7) were examined at 24 hours after exposure. GHB treated mice (n=7) and control mice (n=5) were also examined after 48 hours. After 96 hours protein levels were also determined in GHB-treated (n=7) and control (n=5) mice. The FITC median intensity was measured on all samples. Data are presented as percent of control of mean +/- SEM. **($p \leq 0.01$ using t-test) and *($p \leq 0.05$ using t-test)

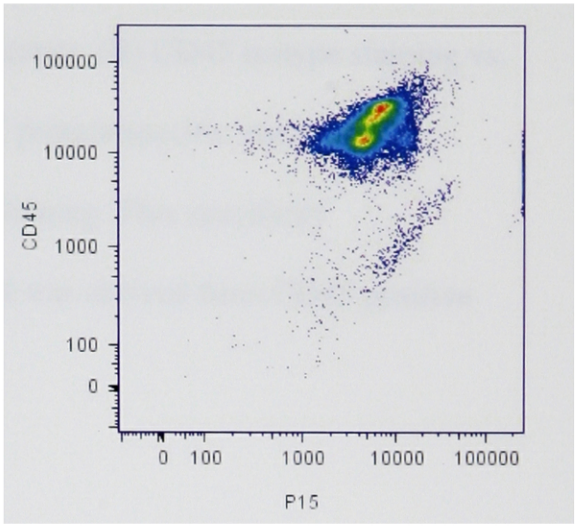
FIGURE 8

Fig. 8a

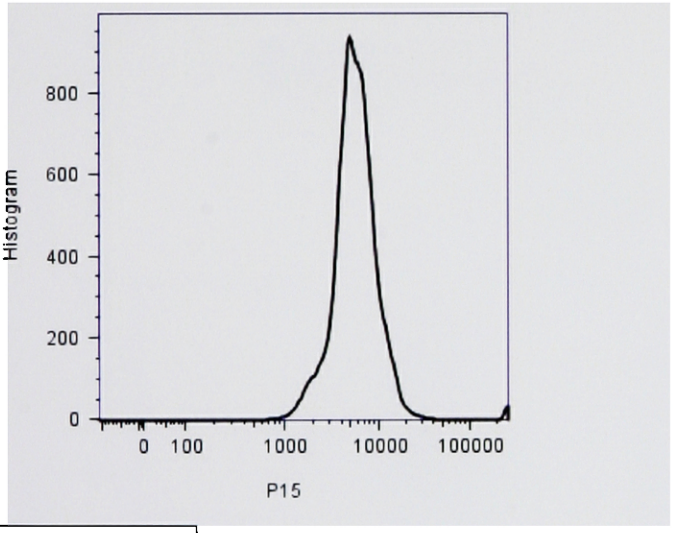


P15 + 2nd GHB + CD45
Count: 30000
Ungated

Fig. 8b

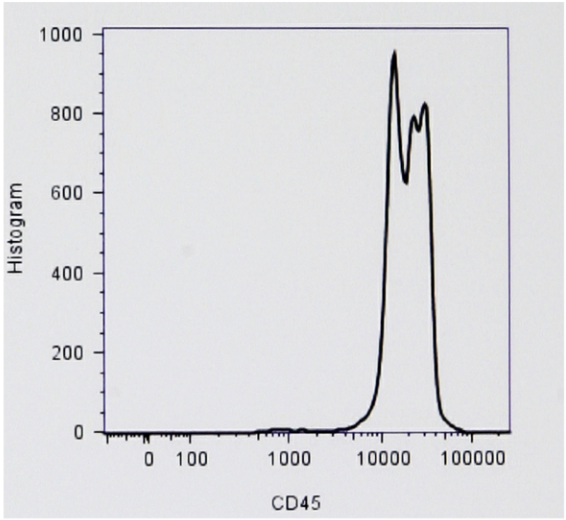


P15 + 2nd GHB + CD45
Count: 27466
Viable Cells



P15 + 2nd GHB + CD45
Count: 27466
Viable Cells

Fig. 8c



P15 + 2nd GHB + CD45
Count: 27466
Viable Cells

Fig. 8d

Figure 8. Pea-15 and CD45 Isotype Staining

Example of cells stained with both the CD45 isotype antibody and the Pea-15 specific antibody. (A) Illustration of side scatter vs. forward scatter. (B) CD45 isotype staining vs. protein specific staining. (C) Histogram of total FITC protein specific staining. (D) Diagram illustrating the histogram of CD45 isotype staining. This specificity demonstrates that between 90-98% of the FITC signal was derived from CD45 positive cells.

FIGURE 9

Fig. 9a

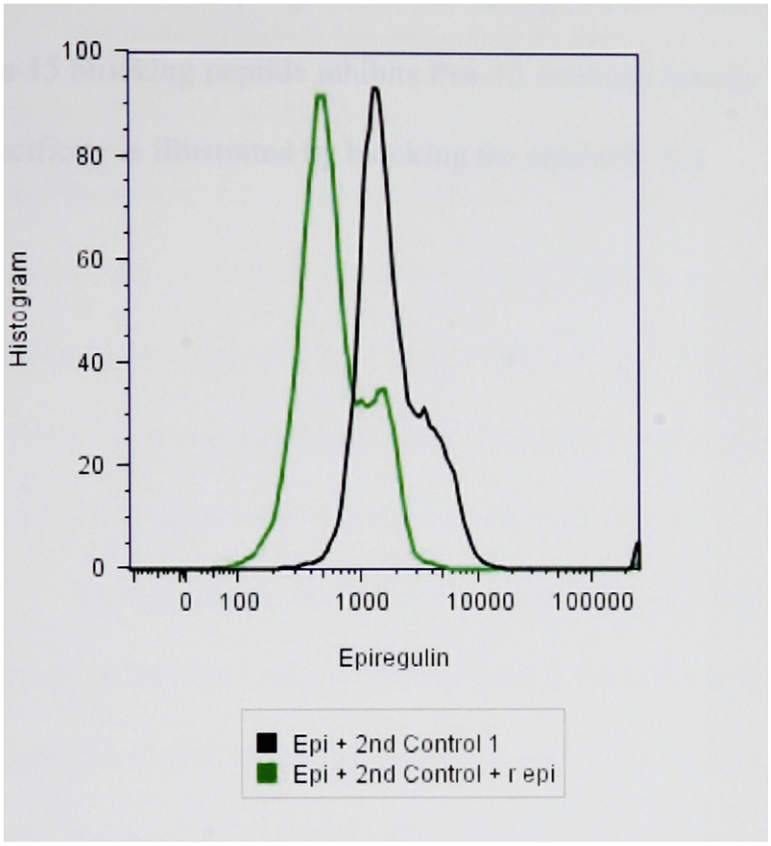


Fig. 9b

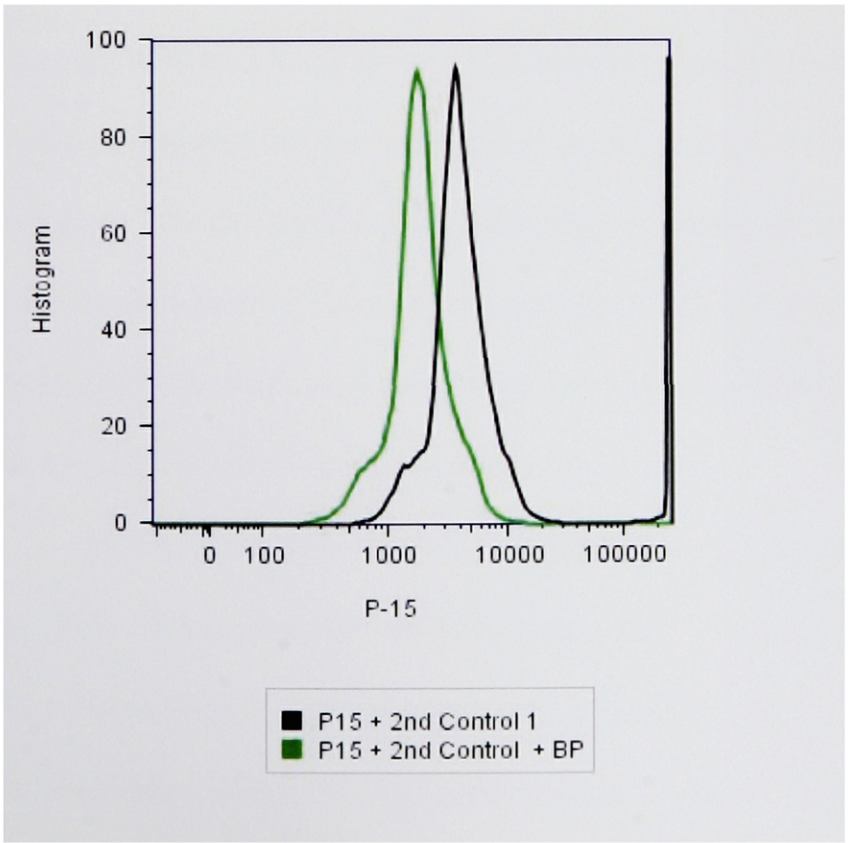


Figure 9. Antibody Specific Staining for Epiregulin and Pea-15

(A) Recombinant epiregulin blocks epiregulin antibody binding on white blood cells. (B) Pea-15 blocking peptide inhibits Pea-15 antibody binding on white blood cells. Antibody specificity is illustrated by blocking the antibody binding.

Discussion

General GHB use has risen dramatically over the last ten years. The use of GHB in drug-facilitated sexual assaults has increased the public awareness of the abuses of this drug. Because of the rapid metabolism of GHB and the short time frame it is present in the body, detection of the drug is very difficult. This has caused difficulties for clinics, forensic crime-labs, and general law enforcement in determining the overall prevalence of GHB use in sexual assault cases. This study examines potential surrogate markers that may be developed to improve the window of detection for GHB.

Epiregulin and Pea-15 were worthy of note because of their high levels of expression 24 hours after GHB exposure in the microarrays. The fact that 48 hours after injection both still had large increases over control made them candidates for further study. Neither Epiregulin nor Pea-15 was up regulated in microarrays performed with RNA from the brain on GHB dosed mice (data not shown). The idea that these genes were only activated in the blood is an important aspect of the development of a surrogate marker. It suggests that the event being measured in blood is indirectly related to the pathology. The fact that this effect takes time to develop and lasts for at least 48 hours works to our benefit. It rules out that the increased Pea-15 or Epiregulin production originated in the brain and escaped into systemic circulation. This is highly unlikely but the mechanism behind this increase in protein levels is not understood.

Epiregulin is a member of the epidermal growth factor (EGF) family (Toyoda et al., 1995). This protein was initially purified from media excreted from NIH3T3/clone T7, a mouse fibroblast-derived tumor cell line. The gene is mainly expressed in the placenta and on peripheral blood leukocytes in normal human tissues (Toyoda et al.,

1997). Overall, the expression level of epiregulin is low in normal adult tissues. It is also expressed in many different tumor cell lines. The highest levels of expression were in the bladder (T-24), lung (A-549), kidney (ACHN and TR-24), colon (Colo201 and HCT-15), and epidermal (HeLa and KB) carcinoma lines (Toyoda et al., 1997).

Various functional assays have suggested that epiregulin is a cell-signaling mediator that is involved in many biological systems. Originally thought to only act as a growth-inhibitor factor, epiregulin was later proven to act as a mitogen in some cell types. Its functions include a role in reproduction, liver regeneration, and as a vascular smooth muscle cell mitogen (Das et al., 1997; Park et al., 2004; Sekiguchi et al., 2002; Toyoda et al., 1997; Toyoda et al., 1995). Epiregulin expression is closely tied to bladder cancer survivors, but expression is also upregulated and active in the development of pancreatic and prostate cancer (Thogersen et al., 2001; Torring et al., 2000; Zhu et al., 2000). The expression of epiregulin is not needed in the development of intestinal tumors, but it is required for protection from intestinal damage (Lee et al., 2004). Epiregulin is a target molecule involved in tumorgenesis in Ki-Ras-mediated signaling in colon cancer cells (Baba et al., 2000). It has also been shown to be important in the immortalization of human fibroblasts by telomerase (Lindvall et al., 2003). Recently, epiregulin was found to play a role in the inflammatory response of keratinocytes and macrophages (Shirasawa et al., 2004).

The epiregulin protein is found in a membrane-bound form and also as a mature secreted form (Baba et al., 2000). The majority of the research conducted to date has focused on the mature form of the protein in the cytoplasm and later, as a type of cytokine through secretion. The membrane-bound epiregulin seems to act as a

proinflammatory cytokine that is produced in macrophages (Shirasawa et al., 2004). This shows that the soluble form and the membrane-bound form may have distinct functions.

Phosphoprotein enriched in astrocytes 15 (Pea-15) is a 15 kDa protein that was originally identified as an abundant protein located in the cytoplasm of brain astrocytes (Araujo et al., 1993). Pea-15 was later shown to be expressed in a wide range of tissues and to be conserved in various mammals (Danziger et al., 1995; Estelles et al., 1996; Ramos et al., 2000). Pea-15 structurally contains a N-terminal death effector domain (DED) and a C-terminal tail. It exists in three isoforms based on phosphorylation state. It can be present containing zero (N), one (Pa), or two (Pb) phosphorylated serine sites. The Pa isoform is phosphorylated (Serine-116) via calcium-calmodulin Kinase II, while protein kinase C controls the phosphorylation of the Pb (Serine 104) (Kubes et al., 1998).

Many studies have shown that Pea-15 regulates multiple cellular functions through various interactions. It has been shown that Pea-15 can bind to Fas associated death domain (FADD) and caspase-8. This results in the decrease of tumor necrosis factor- alpha (TNF) triggered apoptosis in astrocytes (Estelles et al., 1999; Kitsberg et al., 1999). Pea-15 also decreases Fas-induced apoptosis in fibroblasts (Condorelli et al., 1999). Pea-15 activates the extracellular signal receptor-activated kinase (ERK) MAP kinase pathway through Ras as well (Ramos et al., 2000). This function may serve to link the apoptotic pathways and the ERK MAP kinase pathway. Pea-15 also modifies ERK signaling by binding it in the nucleus and transporting it back into the cytoplasm (Formstecher et al., 2001). This limits the cell's ability to enter into the cell cycle.

The level of gene expression changes between the microarray and the RT-PCR analyses are very different with both Epiregulin and Pea-15. GHB induced increases in

Epiregulin expression 19.2 fold in the microarray, while expression was increased only 1.7 fold with the RT-PCR assay. Pea-15 expression was increased 20.9 fold in the microarray, but just 4.8 fold in the RT-PCR assay. There are some potential explanations for this large difference. The assays that measure these expression changes are very different. Microarrays involve hybridization of labeled cDNA to 50 base pair oligonucleotides probes. The microarray is based on the hybridization of the oligonucleotides to some internal region of the gene, while the RT-PCR assay is dependent on two independent oligonucleotides binding to the ends of the cDNA. The regions of the genes detected by the microarray oligonucleotides may have been better represented and more accessible for binding than those of the RT-PCR primers.

There was no statistically significant increase in Epiregulin RT-PCR product from GHB to control. There was a 1.6-fold increase on day 1 but because of a high standard error of the mean this increase fell below a significant change ($p \leq 0.08$). Pea-15 RT-PCR levels were increased 4.8 fold on day1 and 3.3-fold by day 2 in GHB-treated mice over control. While these increases are not as great as the increases by microarray, they do confirm that these genes are upregulated and increased transcription is detectable in peripheral blood.

Since future research goals include developing an assay for the detection of GHB exposure, determining the protein levels are the most important aspect of these studies. The increases of the Epiregulin and Pea-15 protein in GHB-treated mice over control are imperative. These increases represent significant protein changes that are easily measured. It is not known whether these changes represent a "mechanism" biomarker of GHB exposure. It would be very interesting to determine if these alterations are the result

of random gene expression changes caused from GHB treatment or actual downstream effects induced from the drug.

There are no known data or mechanisms that connects epiregulin and GHB. Studies have been done that make a case for a possible connection. Epiregulin expression is induced by follicle-stimulating hormone (FSH), which causes ovarian cell proliferation (Sekiguchi et al., 2002). Epiregulin also acts as a mediator of luteinizing hormone (LH) action in mammalian ovulatory follicles (Freimann et al., 2004). It has also been found that LH stimulation induces the expression of epiregulin in mouse ovaries (Park et al., 2004). It is widely accepted that both FSH and LH are regulated by gonadotropin-releasing hormone (GnRH). Since there is a direct link of epiregulin involvement with these gonadotropin hormones in ovarian cells there may be a relationship between epiregulin and hormonal changes in other biological systems. It is accepted that GHB can directly increase human growth hormone in both humans and mice (Van Cauter et al., 1997; Volpi et al., 2000; Volpi et al., 1997). It may be that the endocrine system, via the release of hormones in the pituitary gland is the mechanism that explains the increase of epiregulin in peripheral blood after GHB exposure (Figure 10).

There is also very little data that elucidates a relationship between Pea-15 and GHB. A gonadotropin-releasing hormone (GnRH) agonist, leuprolide acetate, reduces the expression of Pea-15 by 50% in uterine leiomyomas (Bifulco et al., 2004). Leuprolide acetate contains anti-proliferative properties that reduce the leiomyoma volume. This is, in part, by suppressing the anti-apoptotic effects of Pea-15. GHB is known to increase the concentration of growth hormone, which can also decrease the rate of apoptosis (Baixeras

et al., 2001; Jeay et al., 2000). It is not known whether these mechanisms are connected (Figure 11).

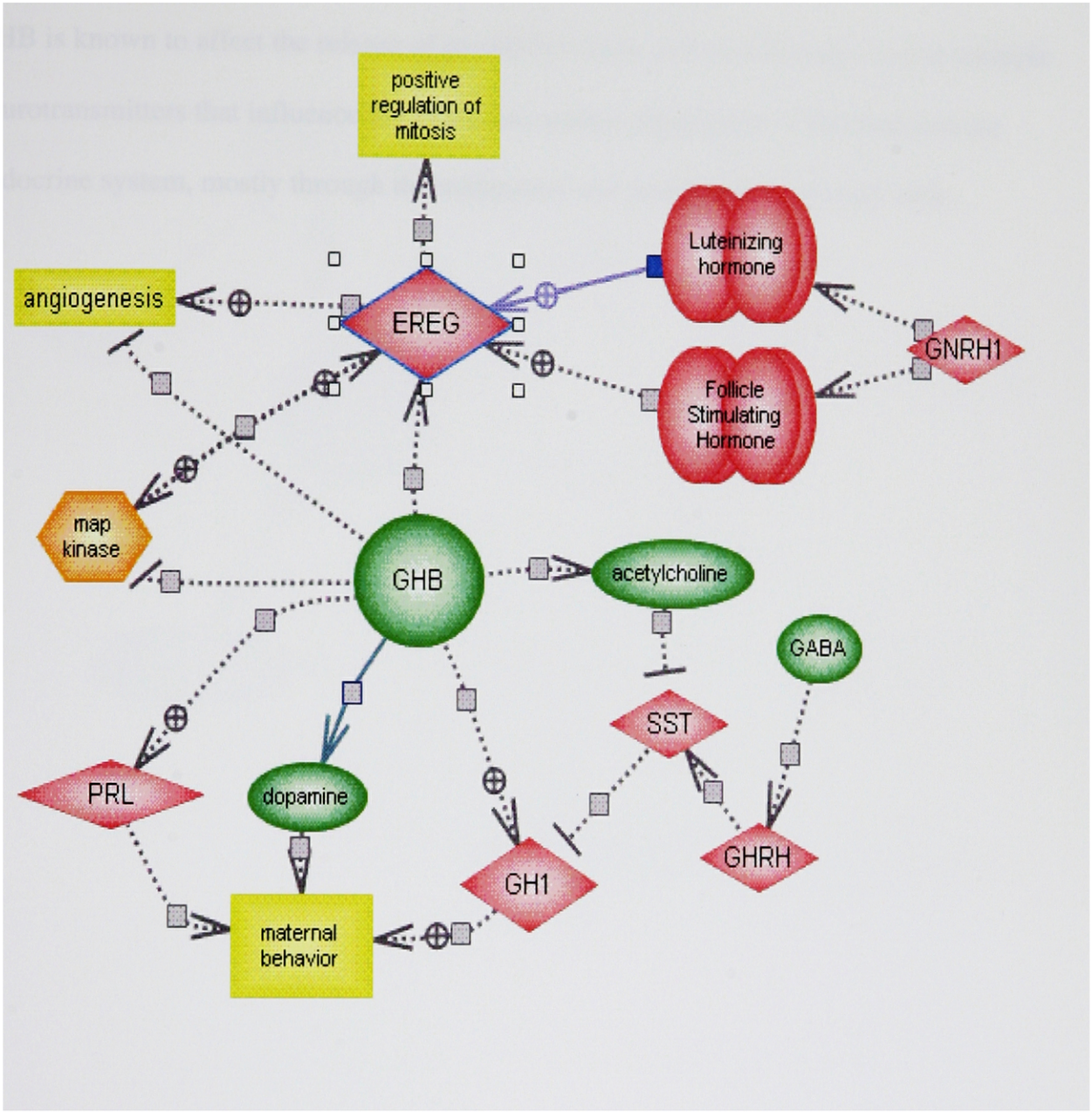
There is the potential that the information found in this study could be developed as a protein-based detection system for GHB use. Biomarkers are becoming increasingly important in the field of drug detection when there are issues involving their direct measurement. Instead of detecting the drug itself, surrogate markers, resulting in directly from the GHB exposure, would be detected. Biomarkers should accurately reflect the amount of GHB exposure over time. Both Epiregulin and Pea-15 protein levels are increased in this model. An important aspect of this study is that these increases are longer than the current detection limit of GHB using available techniques, which is around 12 hours. The development of an assay that can measure these proteins easily in a flow cytometer instrument is very advantageous based on the availability of these instruments in hospitals and clinics. It is also important that we have two independent markers. We have yet to validate the additional genes that were also up or down regulated by GHB. A successful assay may need to possess an optimal combination of multiple biomarkers as this could increase sensitivity and decrease false-positives.

Many future experiments would be needed to make sure these results are a specific response to GHB exposure. One of these experiments would include comparing Epiregulin and Pea-15 levels in DBA/2J mice against combinations of GHB, GABA, and the respective antagonists (NCS-382 and CGP-35348). This information would determine if the changes in protein levels are specific for GHB or give an indication that these changes are a by-product of complex relationship between the GABAergic and GHB systems. After determining which system these changes may be directed through, the

next step would be to determine if they are related to the dopaminergic or cholinergic systems. Both GHB and GABA affect these neurotransmitter levels and involvement of these molecules would be the most logical mechanism. Another study would need to compare Epiregulin and Pea-15 protein levels after exposure to other drugs of abuse. A comparison among other CNS depressant drugs such as benzodiazepines, barbiturates and alcohol would be useful. It is known that neither Epiregulin nor Pea-15 RNA expression levels are increased in microarray assays after a 1g/kg dose of ethanol (data not shown), but no protein information is available. Further development of this type of detection system would be to move from the current mouse model to a human model. Human baseline levels of Epiregulin and Pea-15 in peripheral blood would need to be determined. There are also no data on how tightly these genes are regulated in human white blood cells. There are many difficulties with this type of study that include getting approval for administering a schedule 1 drug to humans. Also vital would be to determine the influence of different variables such as age, sex, and ethnicity on the validity of the biomarkers.

In conclusion, this study begins the process needed to uncover and validate potential biomarkers that are specific to GHB exposure. Future work may provide an alternative testing system that would provide investigators with a longer timeframe for GHB detection.

FIGURE 10

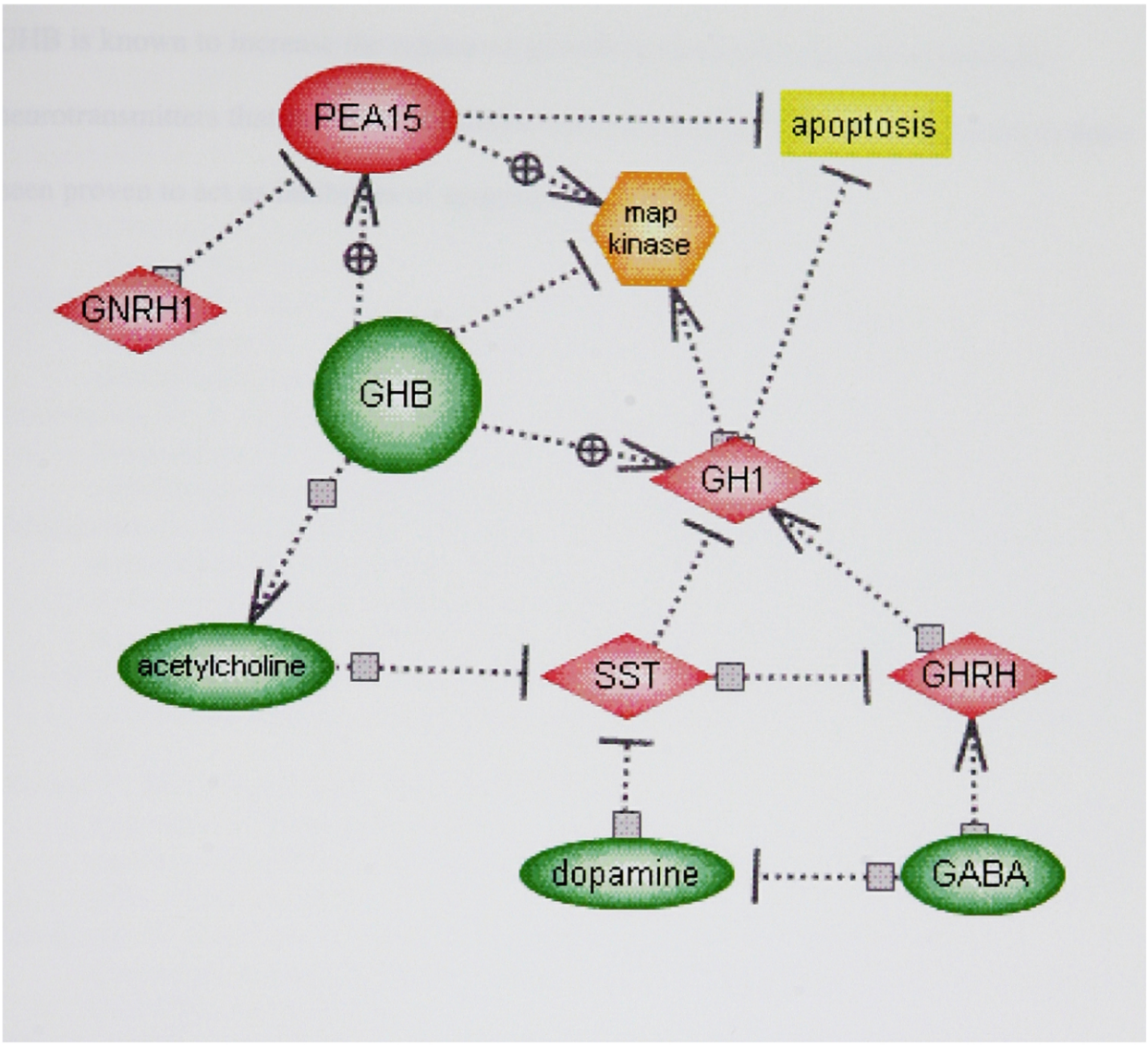


Abbreviations: Gonadotropin releasing hormone (GNRH1), Somatostatin (SST), Growth hormone (GH1), Growth hormone releasing hormone (GHRH), Prolactin (PRL), Gamma-hydroxybutyrate (GHB), Gamma-aminobutyric acid (GABA), Epiregulin (EREG).

Figure 10. Potential mechanism linking Epiregulin and GHB.

GHB is known to affect the release of growth hormone and also directly control multiple neurotransmitters that influence the endocrine system. Epiregulin is also tied into the endocrine system, mostly through the maturation and proliferation of ovary cells.

FIGURE 11



Abbreviations: Gonadotropin releasing hormone (GNRH1), Somatostatin (SST), Growth hormone (GH1), Growth hormone releasing hormone (GHRH), Gamma-hydroxybutyrate (GHB), Gamma-aminobutyric acid (GABA), Phosphoprotein enriched in Astrocytes 15 (Pea-15).

Figure 11. Potential mechanism linking Pea-15 and GHB

GHB is known to increase the release of growth hormone and also affect multiple neurotransmitters that influence the endocrine system. Pea-15 and growth hormone have been proven to act as inhibitors of apoptosis.

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