Studies attempting to reverse brain damages induced by alcohol with the aim to find new strategies for treatment of patients with alcoholism

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ABSTRACT

Previous studies have shown that alcohol may induce apoptosis in both skeletal muscles and in the brain and it is well known that alcohol may affect the hippocampus in rat. Hippocampus is a brain area involved in memory, learning, mood and cognitive functions and previous studies have shown that also other abusing drugs, like central stimulants and opiates can trigger apoptosis and inhibit neuroregeneration in this area (Nyberg 2009, Rossbach et al. 2010). It is therefore of great interest to investigate if alcohol may have an influence on learning and memory and if damaged cells and tissue in this area could be repaired by triggering neuroregeneration and/or inhibiting apoptosis in the hippocampus.

It is established that pituitary derived GH are a necessity during childhood for normal growth, regulation of metabolism as for maintenance and repair of tissue (Chia and Rotwein 2010, Savine and Sönksen 2000, Kopchick and Andry 2000). GH has often been associated with effects on peripheral tissues and organs (Nyberg 2007) but in this study we try to define if it could have profound effects in the CNS upon alcohol exposure.

In the first stage of this study male rats were randomly selected into four groups. The groups were treated for six days with alcohol (3,2 g/kg) and saline, GH (1 IU/kg) and water, alcohol (3,2 g/kg) and GH (1 IU/kg) and well as water and saline as a control. An assignment was made in a Barnes maze to measure their memory ability and cognitive functions to investigate whether GH could have beneficial effects on these rats or not. After these studies, using tissue from the hippocampus area RNA was extracted and through qPCR the mRNA level of prodynorfin could be measured.

In this study, different proteins from the pituitary, namely STAT5, STAT3 and the GH-receptor were going to be investigated by using western blot, to try to see if there was any indication on GH action in the pituitary and if that in turn could be an indication of possibilities to find neuroregeneration upon GH treatment.
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INTRODUCTION

Chronic alcohol abuse has several times been shown to cause neurobiological abnormalities. For example, an up regulation of the N-methyl-D-aspartate (NMDA) receptors has been found which can result in neuronal damage due to a repetitive signaling from the NMDA receptors. One study on alcohol abuse among adolescent demonstrated significant smaller volume on the hippocampus compared to healthy controls, thus, it should also be mentioned that a smaller hippocampus could be a risk factor itself for an adolescent onset alcohol use disorder and one cannot exclude the fact that adolescence is more vulnerable for hippocampal toxicity (De Bellis et al. 2000). These results are interesting in many aspects and since the hippocampus is a brain area involved in memory, learning and cognitive functions (Nyberg 2009, Tang et al. 2011) one cannot elude that it is of significant interest to be able to avoid and treat individuals that suffers from the consequences caused by long term alcohol abuse, like decrease in cognitive function, learning, working-memory.

As far as until the early 1990s it has been stated that neurogenesis in the adult mammalian brain cannot take place. However, later studies have been convincing enough to reject these statements and data that confirms this have also been presented. It is now known that neurogenesis takes place in an adult brain and neurons retain its capacity for cell renewal throughout life in mammalian brain, including rodents, primates and human brain (Aberg, Brywe and Isgaard 2006).

Already in 1968 the first indications about a tropic effect of growth hormone (GH) on neural growth appeared and theories about the somatotrophic hormones rose (Aberg et al. 2006) and in 1945, bovine growth hormone was for the first time isolated (Kopchick and Andry 2000). In the brain GH is released to the circulation from the anterior pituitary and is a protein well known to control and affect a number of tissues and cell types. As the knowledge about GH and its functions in the body has increased the influence of the hormone on the brain has been more and more interesting. For example, more studies on the hormones effect on appetite, energy, cognitive functions, sleep, mood, neuroprotection and memory are being made (Nyberg 2000) and interestingly, growing body of evidence suggests that GH and insulin-like growth factor 1 (IGF-1) plays an important role in growth and development of the normal CNS (Aberg et al. 2006). Behavioral experiments have shown an improvement in learning and memory in aged rats after long-term GH/IGF-1 replacement and evidence for a neuroprotective effect of GH and IGF-1 is documented. Further, discussions about a future treatment of brain and spinal cord trauma have therefore devoted more attention and the positive effects of GH replacement therapy seen in GH deficient (GHD) patients have raised the discussion whether GH could have a potential clinic effect as a treatment for anti-aging processes as well (Nyberg 2007).

Since studies have shown a drastic decrease of GH and IGF-1 levels, as well as secretion, in spinal chord injury (Nyberg 2000) there could be reasons to believe that exogenous administrated GH and IGF-1 could work as a therapy in these injuries. The Signal
transducers and activators of transcription (STAT), in particular the STAT5B protein has been directly implicated in the GH-induced production of IGF-1 which is referred to as a mediator that give GH its anabolic effects (Nyberg 2007, Ajo et al. 2003).

**GH secretion and signaling mechanisms**

The human GH (hGH) gene has been found to be located on the long arm of chromosome 17 and is approximately 3000 nucleotides long. The hormone is synthesized as a precursor protein and its signal peptide is removed when secreted (Kopchick and Andry 2000). As mentioned earlier, GH is released in the brain from the anterior pituitary (Nyberg 2000) from acidophil GH secreting cells called somatotrophs. Its secretion and transcription is regulated by peptides deriving from hypothalamus, namely somatostatin that acts inhibitory and growth hormone-releasing-hormone (GHRH). GHRH acts stimulating on the somatotroph cell and when GHRH binds to its receptor on the somatotroph cell there is an increasing intracellular cAMP and/or Ca$^{2+}$ level which stimulates GH transcription (Kopchick and Andry 2000). The GH secretion is inhibited by somatostatin (Kopchick and Andry 2000, Nyberg 2000).

Recent studies have shown that the source of GH may be produced in various brain areas which may indicate that the hormone is not being restricted to the pituitary gland and there are reports saying GH gene expression occurs in many extrapituitary tissues like the hippocampal formation, an area referred to as the area of memory, learning and emotional experiences. However, in the literature evidence can be found that GH may reach the hippocampal formation trough the circulatory system (Nyberg 2007).

GH secretion is inhibited by somatostatin, interestingly, evidence show that in hypothalamus GH receptor are positioned together with somatostatin neurons which supports the theory that GH may be able to regulate is own secretion in this area (Nyberg 2000). It has also been suggested that rat GH (rGH) partly reduce dopamine synthesis and release in the median eminence in hypothalamus which leads to an increased release of the GH inhibiting somatostatin. Also, a reduced synthesis and turnover of noradrenalin has been seen which results in a reduced secretion of a GH-releasing factor.

The mechanism behind GH signaling in the brain is still not fully certain, however, it is anticipated that GH interacting with its receptor uses a similar mechanism to that one in peripheral cells (Nyberg 2009). In peripheral cells, GH signaling occurs in two steps; First part of the signaling consists of GH binding to the GH receptor (GHR) forming a hormone receptor complex. A second receptor binds to a different site on the hormone forming a receptor dimer bound to one GH molecule (Chia and Rotwein 2010, Nyberg 2000). A result of this event will lead to phosphorylation of Janus kinase 2 (JAK2) that in turn leads to phosphorylation of the intracellular part of the GHR that undergoes further autophosphorylation. This JAK2 kinase-GHR complex activates the earlier mentioned STAT proteins (Nyberg 2009). This protein dissociate from the receptor-docking site to dimerize by reciprocal interaction of a Src homology 2 domain (Sh2). It enters the nucleus where it binds as dimers to specific sites to the DNA (Chia and Rotwein 2010).

There is evidence that the STAT5 activation of GH requires phosphorylation of specific tyrosine residues within the GH receptor and studies on GH signaling in JAK2 deficient
cells have shown that STAT5 requires JAK2 activation (Herrington et al. 2000). Studies with green fluorescent protein (GFP)-STAT5 showed that a mutation which prevent binding of STAT5B to DNA abolish GH-stimulated nuclear localization (Herrington et al. 2000). This clearly emphasize that the STAT proteins plays an important role in the GH signaling pathway.

**Signal transducers and activators of transcription (STAT)**

As just mentioned, one important transcription factor that mediates the action of GH is the STAT family (Chia and Rotwein 2010). The STAT proteins STAT1, STAT3 and STAT5 are cytoplasm proteins involved in the STAT-signaling pathway. The exact cellular distribution of STAT proteins is poorly understood but there is clear evidence that in response to GH, STAT proteins undergo a rapid accumulation in the nucleus and STAT binding to their gene targets is necessary for the actions of GH (Herrington et al. 2000, Nyberg 2007), although, they are said to be too big to diffuse through a nuclear pore and they lack a nuclear localization signal. Two different STAT5 genes have been found in mouse, human and rat, namely STAT5A and STAT5B. They are approximately 96% identical in their coding sequence (Strous et al. 1997) and it is primary in their COOH-terminal transcription activation domains they derive. The differences lies in their DNA binding specificities and they also seem to exhibit differences with respect to their tissue distribution. Thus, focusing at their role in GH signaling they are likely to have both distinct and overlapping functions (Herrington et al. 2000).

Independent studies have shown that STAT5B seem to be the responsible STAT protein for many GH linked physiological actions (Chia and Rotwein 2010) and the STAT5B protein has been seen to be directly implicated in the GH-induced production of IGF-1 which is thought to be a mediator that give GH its anabolic effects (Ajo et al. 2003). However, studies on gene expression in cultured cell lines, hepatocytes in primary culture and in vivo in liver have established that STAT5A is a critical mediator of GH-activated target gene expression. The genes that were found to be induced by GH was IGF-1, SOCS-2, CISH, IGFALS and SPI 2.1 while IGFBP-1 appeared to be acute inhibited by STAT5B (Chia and Rotwein 2010). Another indication that STAT proteins have a central role in GH signaling comes from a study where male dwarf rats were chronically infused with GH and where an increase in hypothalamic STAT5B could be seen. Further, with single infusion a phosphorylation of STAT5 proteins could be seen (Bennett et al. 2005) and in rats with congenital hypothyroidism (CHT) a decrease in GH and GHR expression in hippocampus accompanied of a corresponding decrease in phosphorylation of JAK2 and STAT5 which could indicate that Thyroid hormone (TH) could be an important regulator for GH synthesis and secretion in the periphery (Tang et al. 2011). It has also been suggested that the JAK/STAT pathway is involved in the mediation of events resulting in a calcium influx trough NMDA receptors in the brain (Nyberg 2009).

**GH and the Blood-Brain-Barrier**

The question whether GH is able to cross the blood-brain barrier (BBB) or not has been a topic for discussion for a long time. But, looking at a study on GH replacement therapy,
the beneficial effects that was found indicates that the hormone may find its way from the circulation into the CNS, further, during GH therapy of human adults an elevation of the hormone level in the cerebrospinal fluid (CSF) increased. Exact how peripheral GH is reaching CNS and broadcasting its effects are not fully understood but there are at least three pathways found to be discussed in the literature. One theory is based on an enzymatic degradation of GH in the circulation and trough mediators it reaches the CNS and interact with their receptor to exert effect. A second theory is that GH may bring out other mediators from the peripheral tissue that could cross the BBB and reach the CNS. Further, stimulation of the GHR in the brain may result in a release of brain-derived IGF-1 to exert some of its effects (Nyberg 2000). By many, the molecule size is considered too large for an easy passage trough the BBB, however, the GHR has been revealed to have a high density in the choroid plexus and together with that the present of GH in the CSF could speak for an ability to cross this barrier (Nyberg 2007, Nyberg 2000).

GH receptor
For more than 15 years ago the first evidences came for the existence of the GH-receptor in different brain regions. However, still very little is known about GHR regulation and transcript in the CNS. The GHR is a transmembrane protein from the cytokine family consisting of one polypeptide chain with 620 amino acid residues. The extracellular domain consists of two subunits, one interacting with the hormone and one associating with a second receptor forming a homodimer eventuating activation of the complex. GHR is distributed in several types of tissue besides the brain. In addition to liver it has been localized in kidney, lung, heart, lymphatic cells, pancreas, cartilage, skeletal muscle and testis (Nyberg 2000). The distribution of GHR in brain seem similar in rat as to that in human brain (Nyberg and Burman 1996) and the most dense area in rat brain has been found to be choroid plexus (Mustafa et al. 1994). Also, the receptor has been found in the hippocampus, thalamus, pituitary and hypothalamus. Related to this, a decrease in pituitary secretion of GH is parallel with an age-related decrease in the GHR density in the brain. Regulation of GHR levels can still not be fully explained, however, some inducers have been established, for example insulin and estrogen.

In addition to the transmembrane GH receptor a soluble form of the extracellular domain has been found, referred to as GH-binding protein (GHBP) (Nyberg 2000). This protein exists in several parts of the brain, including putamen, thalamus, pituitary, hippocampus and cortex (Tang et al. 2011). GHBP seem to have an important role in regulation of GH clearance since studies have shown a considerable faster clearance of GH in absence of GHBP and one theory is also that GHBP function as a GH reserve in the body in case of sudden reduced supply. The mechanism behind GH release from GHBP is still not fully understood (Nyberg 2000). Thus, the GHR gene from various parts of the rat brain (Nyberg 2007), human and mouse has recently been cloned (Talamantes 1994) and the gene transcript of the receptor in human choroid plexus has also been determined (Nyberg 2007) this could be essential for the future studies that has to be made about this receptor to get further knowledge about its mechanisms and functions.
Functions of GH

Pituitary derived GH plays a fundamental role in many physiological processes in human and other mammalian species. The presence of GH during childhood is a necessity for normal growth, for regulation of metabolism as well as maintenance and repair of tissue throughout life (Chia and Rotwein 2010, Savine and Sönksen 2000, Kopchick and Andry 2000). GH has often been associated with effects on peripheral tissues and organs (Nyberg 2007) and studies on transgenic rabbits that overexpress GH were found to develop enlarged bones, limbs, and organs, symptoms characteristic of conditions such as acromegaly (Costa et al. 1998). However, as mentioned earlier, already in 1968 the first indication appeared about a tropic effect of GH on neural growth (Aberg et al. 2006). Recent studies are suggesting a substantially effect on functions linked to the CNS and in the past decade it has become manifested that GH not only have profound effects in the CNS but that the GH-IGF-1 axis seem to play a role on cognition, mood and memory. There is now clear evidence that GH is present in the brain as well as in the CSF and its receptor has been detected in several areas in the CNS (Nyberg 2000). There are also indications that when administrated systemically it interacts with monoaminergic pathways in the brain (Andersson et al. 1983) and animal studies suggest that peripheral administrated GH may induce changes in glutamate transmission (Nyberg 2007). The knowledge about the direct effects and the functional aspects of GH on adult cell proliferation is confined but one study have shown GH seem to have protective effects on its own in the CNS administrated with in hours to a brain injury (Aberg et al. 2006).

Interesting data from clinical studies in patients with GHD show improvement in memory and cognitive functions (Nyberg 2007) as well as long-term and working memory after GH replacement therapy (Arwert et al. 2006). Other studies have shown improvements in mood, well-being, and alertness. In addition to this GH is known to affect several neuropeptides, monoamine metabolites and amino acids in the CSF. However, among the effects of GH in the brain there are also other effects caused by GH that acts through peripheral mediators (Nyberg 2000). The mechanisms underlying these effects are not fully known, thus, an increase in β-endorphin that have been detected after GH treatment can be one of the mechanisms behind the psychological well-being improvements. Further, the effect on memory and cognitive functions are likely to be mediated by the hormone (Nyberg 2007) and its receptor found in hippocampus (Nyberg 2000), a brain structure that plays an essential role in cognition, memory and mood as mentioned before (Rossbach et al. 2010). Besides this, data have shown increased anxiety, depression and attention disorders in children with GHD and in a study using Morris water maze to compare spatial learning and memory in male rats with multiple hormone deficiencies showed that GH treated rats had a significant better result than controls (Nyberg 2000). However, the mechanisms behind these variants of effects are not yet clearly understood. (Nyberg 2007)

Growth Hormone (Tang et al. 2011) together with IGF-1 has been found to be significant in the role of development in the brain, neural growth and differentiation. It has been suggested that GH may function through the release of IGF-1 (Kopchick and Andry 2000, Ajo et al. 2003) and a correlation between increasing age and decreased GH secretion has been found, further, simultaneously a decrease in IGF-1 levels in the plasma could be
seen (Nyberg 2000). As for GHR, the IGF-1 receptor (IGF-1R) has been identified in the mature brain and it has been shown to be highly expressed in the hippocampus where it seem to be involved in pathways essential for cognition (Tang et al. 2011, Nyberg 2000). Binding studies have also shown binding sites in several other brain structures in rat, like choroid plexus of the lateral and third ventricles and thalamus. Further, focus has been addressed to the age-related decrease in serum GH and IGF-1 as a potential mechanism that could influence cognitive functions in elderly, which is an another aspect in how these two hormones could work together, and therefore there could be reasons to believe that IGF-1 plays an important role in the endogenous neuroprotective system (Nyberg 2000). Thus, the IGF-1 gene lacks promoter-associated STAT5B response element which suggests that IGF-1 is dependent of GH to promote growth (Chia and Rotwein 2010). IGF-1 and GH have been successful in reducing damage after ischemic lesion in animal models. Treating normal animals with GH or IGF-1 for more than 24h poses problems due to the endogenous GH from the pituitary is decreased. So far, GH and IGF-1 seem to work together and influence many important mechanisms in the body including the brain. Both GH and IGF-1 activity are shown to decrease with age (Aberg et al. 2006) which is another, among many, evidence that these two hormones have significant effects throughout life but the knowledge is limited and manifests that further studies should be done.

**Neuroprotection**

One study in rat with a prolonged administration of synthetic GH showed a lowering in the neurological motor dysfunction three weeks after trauma (Nyberg 2000). GH and/or IGF-1 replacement increases neurogenesis and vascular density. Studies have shown effects from both locally produced and circulating IGF-1. For instance, IGF-1 is shown to recruit oligodendrocytes and newborn endothelial phenotype cells in the hippocampus and IGF-1 could be an explanation that upon GH treatment an increase in cerebral arteriole density was observed (Nyberg 2007). There are two areas responsible for the highest density of proliferation progenitor cells in adult brain, namely the subgranular zone (SGZ) of the dentate gyrus of the hippocampal formation and the subventricular zone. Demonstrations that adult neurogenesis could be up and down regulated by different stimuli raised the possibility that adult hippocampal neurogenesis could be important for cognition in higher animals (Aberg et al. 2006).
MATERIALS AND METHODS

Materials
Hippocampal and liver tissue from male Wistar rat.
Antibodies: STAT5a (1:1000, sc-1081, Santa Cruz), p-STAT5 (1:200, Tyr 697, sc-11761, Santa Cruz), STAT3 (1:200, sc-482, Santa Cruz) GHR (1:1000, sc-20747, Santa Cruz) and actin (1:1000, sc-1615, Santa Cruz). Secondary antibodies: Horseradish peroxidase (HRP) conjugated antibodies (Santa Cruz). ECL Detection Reagent (GE Healthcare), SeeBlue® Prestained (Invitrogen) and nitrocellulose membrane (Amersham Hybond-P ECL, GE Healthcare).

Western Blot analysis
Aproxematley 85 mg of hippocampal tissue and 0.19 g liver tissue from rat was homogenized separately in 4 ml ice-cold lysis buffer per gram tissue in 1 min. The lysate was centrifuged at 14,000 rpm 4°C in 10 min. Protein concentration was determined with the Lowry procedure with bovine serum albumin as the standard. 30 µg protein was loaded and resolved on 7% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. Blots were incubated with primary antibodies against STAT5a, p-STAT5, STAT3, GHR and actin for 1 h in 3% BSA/PBS-T 0.1% in room temperature (RT). The membranes were subsequently incubated with the corresponding secondary horseradish peroxidase conjugated antibodies for 1 h RT. Immunoblots were visualized using chemiluminescent ECL and detected by size, with SeeBlue® Prestained as standard.
Membranes were stripped in stripping solution; 1.4 ml 2-mercaptoethanol [14.3 M], 25 ml 0.5 M Tris-HCL, pH 6.8, 40 ml 10% SDS, 133.6 ml ddH2O. The membranes were rebotted.

RESULTS

Western blot analysis
To determine the protein level of STAT3 and STAT5A in rat pituitary these proteines were analysed through Western blot. Both STAT3 and STAT5A could be detected. The supernatant were loaded in wells containing 30 ng and 10 ng protein which seemed to be good levels for detection. GHR and p-STAT could not be detected in this study.
In liver the STAT3, STAT5A, GHR and p-STAT proteins could not be detected but using Actin as a control a successful separation and transferring on to the membrane could be established.
Fig. 1. Protein levels of STAT3, STAT5a and GHR in rat pituitary were analyzed by Western blot. STAT3 and STAT5A could be detected. No protein level from GHR could be seen.

DISKUSSION

In this study it has been demonstrated that STAT5A and STAT3 is expressed in rat pituitary. This probate earlier studies showing that STAT5B is present in Hippocampus. Therefore there could be reason to believe that stimulation of the GH receptor in hippocampus could, with the same signal transduction, result in gene transcription and growth in the hippocampus area.

In this study GHR in rat pituitary or rat liver could not be detected. Since the GHR is a transmembrane protein consisting of one polypeptide chain containing approximately just over 620 amino acid residues there are reason to believe that the GHR did stay attached to the cell membrane and were to be found in the pellet after centrifugation instead of the supernatant that were loaded on to gels in these studies. The GHR may have to be separated from the cell membrane by detergents so the protein could be loaded and separated properly in gels. The primary antibody that were used was against amino acid residues 339-638, a part of the receptor that may be considered outside the cell membrane in an intracellular position, thus, one may not exclude that important binding sites for the antibody could be blocked by the cell membrane. One other explanation could simply be too low concentration of GHR in the samples so that the protein could not be detected.

The activated form of STAT5, namely p-STAT5, has not been detected in rat pituitary or rat liver in these trials. There are reasons to believe that our antibody did not work properly which can explain the absense of protein band on the exposed film since there are earlier evidence that p-STAT is found in these tissues.
Further conclusions are, it appeared in these trials that primary antibody could be diluted even more than the recommendations from the manufactures. The best results were given with a dilution of 1:3000 for STAT5A, STAT3 and 1:10,000 for Actin. Little can be established regarding primary antibody against p-STAT5 since nothing could be seen on these exposed films even when trials were repeated. Corresponding HRP labeled secondary antibody were also diluted to a considerable lower concentration than manufactures recommendation.

The exact mechanism for GH signaling and its specific effects in the CNS is still not fully understood and further studies has to be made on the hormone, its receptor and mediators to clarify the exact signaling pathway and affecting mediators. Although GH is strongly associated with cognitive function, memory and learning further studies can be made regarding this hormone, for example, GH may be an interesting hormone in the treatment of elderly suffering from e.g. dementia. Also, more studies must be focused on whether the outcome of GH secretion occur direct on targets or through other facilitators and which of these that could be central for growth and neuroregeneration if this was to be discovered. The importance of GH and IGF-1 in therapeutic aspects should be considered for further clinical research and in hope to reach more progress in the study on brain damage induced by alcohol the research in this area should continue.

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