TENTATIVE LONG-TERM EFFECTS OF A NORADRENERGIC ANTIDEPRESSANT;

Affecting the number of glucose transporters.

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2 ABBREVIATIONS

CRF       Corticotrophin-releasing factor
ACTH      Adrenocorticotropic hormone
HPA       Hypothalamic-pituitary-adrenal
ECT       Electro convulsive therapy
CNS       Central nervous system
CSF       Cerebrospinal fluid
NE        Noradrenaline
5-HT      5-hydroxytryptamine, (Serotonin)
DA        Dopamine
MHPG      3-Methoxy-4-hydroxy-phenylglycol
5-HIAA    5-hydroxyindoleacetic acid
MAO I     Monoamine oxidase inhibitor
TCA       Tricyclic antidepressive
SSRI      Selective serotonin reuptake inhibitor
SNARI      Selective noradrenaline reuptake inhibitor
MR        Magnetic resonance
BDNF      Brain-derived neurotrophic factor
cAMP      cyclic adenosine monophosphate
BBB       Blood-brain barrier
TBV       Total blood volume
IDDM      Insulin-dependent diabetes mellitus
NIDDM     Non-insulin-dependent diabetes mellitus
MDD       Major depressive disorder
ACh       Acetylcholine
GFAP      Glial fibrillary acidic protein
GJ        Gap junction
GJC       Gap junctional communication
Cx        Connexin
NAT       Noradrenaline transporter
H&E staining   Hematoxylin and Eosin staining
¹⁸FDG   ¹⁸Fluorodeoxyglucose
3 SUMMARY

Major depressive disorder is an affective disorder affecting millions of people worldwide. Only in Europe at least 21 million are thought to be affected. Several theories have been developed during the years trying to explain the cause of depression.

This study is based on the theory where major depressive disorder is believed to be caused by impaired cerebral glucose metabolism, proposed by Hundal in 2006. The astroglia are thought to be the primary affected cells. The glucose transporters GLUT1 and GLUT3 are found to function in cerebral glucose metabolism. Noradrenergic influence is found to stimulate and increase the amount of several glucose transporters on the surface of different cell types.

In this study rats are given the antidepressives fluoxetine, which selectively inhibits serotonin reuptake, reboxetine, which selectively inhibits reuptake of noradrenaline and sibutramine, an anti-obesity drug, which inhibits both serotonin and noradrenaline reuptake. The aim of the study is to compare the different drugs to investigate if continuous noradrenergic stimulation is able to upregulate the amounts of GLUT1 and GLUT3 in rat brain.

The animals got the drugs dissolved in their drinking water for 18 weeks and their brain was analyzed for further investigation. Parts of the brain were cut into sections followed by immunohistochemical antibody staining.

However, blood samples revealed great variations in concentration of drugs within the groups of rats receiving the same drug and it is unsure if the blood sampling procedure was performed satisfyingly. The staining results also turned out quite variable and it was difficult to make any conclusions regarding the possible long-term effects of any of the drugs.
4 INTRODUCTION

4.1 Depression

Affective disorders are divided into two principal types; major depression and bipolar disorder. Major depression is the field discussed in this study, when depression is written.

Depression is a disabling disease and it causes a major burden for those affected. The economic impact of depression in Europe was estimated to be €118 billion in 2004, corresponding to 1 % of the total economy of Europe (GDP). Clearly, the disease poses a huge economic burden to the society in Europe and also in other parts of the world [1].

Familiar symptoms of depression are dysphoric mood, insomnia, loss of appetite, diminished sexual desire, fatigue, feelings of hopelessness, sadness, retardation of thought and action, withdrawal from life and social interactions, anhedonia, meaning that the depressed does not feel any pleasure and has lost interest of mostly everything, loss of self-esteem, guilt and loneliness. In the end suicide may seem like the best option [2, 3].

To be classified as a depression the symptoms have to persist during most of the day for 14 days. The symptoms are described as principal symptoms including a depressive mood, lack of interest or feeling of joy in activities and reduced energy. At least 2 of these symptoms are needed to qualify as a depressive episode according to ICD-10. The more additional symptoms that follow, the more severe is the depression. Additional symptoms are, as described above, lack of self esteem, feeling of guilt, suicidal thoughts to mention some. Psychotic symptoms also need to be considered [4].

There is a gender difference in the risk of developing depression, where men have a risk between 3 and 4 %, while women have a risk between 5-9 % [2]. It has also been found that men have a higher suicide rate. They often have atypical symptoms of depression, like aggressiveness, irritability and antisocial behaviour, which might not
be recognized as symptoms of depression, leaving the depression untreated [153, 154].

If depression is left untreated, in most cases the depression will improve in about 6 to 9 months. Episodes usually reoccur and could also increase in frequency. Studies show that there are several risk factors for developing affective disorders, like environmental stress, heredity and altered biological rhythms [2].

Several neurotransmitters will, in response to stress, regulate the release of corticotrophin-releasing factor (CRF) from hypothalamus. Adrenocorticotropic hormone (ACTH) release from the anterior pituitary into the blood is controlled by CRF. ACTH will in turn affect the adrenal cortex and increase the secretion of glucocorticoids, including cortisol. In healthy persons there is a negative feedback system where cortisol will reduce hypothalamic-pituitary-adrenal (HPA) activation [2].

![Figure 1 The HPA axis](image)

[5]
Some depressed patients have abnormal secretion of cortisol due to an increased release of ACTH. Both the pituitary and adrenal glands are found to be enlarged due to the hypersecretion, which again are thought to be caused by abnormal regulation of CRF by the hypothalamus. Depressed patients get reduced levels of CRF by both antidepressive medicines and electroconvulsive therapy (ECT) [2].

The circadian changes in blood cortisol levels are also found to be increased in depressed patients [2].

The dexamethasone suppression test can be used as a measure of the hypothalamic-pituitary-adrenocortical function. Dexamethasone is a synthetic glucocorticoid that normally suppresses the hypothalamus and pituitary. Cortisol levels will decrease due to reduced CRF and ACTH secretion. As mentioned earlier, depressed patients may have increased cortisol levels. If cortisol levels in the depressed are still increased after about 9 hours after an injection of dexamethasone, this implies a failure of suppression and hypersecretion of CRF and ACTH [2, 3, 6].

When it comes to heredity, studies imply that the more severe mood disorder, the stronger is the genetic contribution [2].

Altered sleep rhythms are very common symptoms of depression. The onset of sleep will be delayed and the REM periods will get shorter. The lack of deep sleep will also cause several awakenings during the night [2].

Several theories and hypotheses have been developed over the years trying to explain how depression develops and what causes it. One theory is where depression is believed to be caused by inflammation in the brain [7-12] and there is also one hypothesis where depression is believed to be caused by a failure in the purinergic signal system [13]. These theories will not be mentioned any further as the other theories are considered as more important to mention.

### 4.1.1 The monoamine hypothesis

In 1965 Schildkraut proposed the monoamine hypothesis, based on the assumption that reduced levels of monoamines in the central nervous system (CNS) could be
responsible for depressed mood whereas excess of monoamine transmitters would cause mania. Several pieces of evidence was put together to form the monoamine hypothesis. One observation was made with the blood pressure reducing drug reserpine, which would give depression as a side effect in a considerable amount of patients by preventing the packaging of the neurotransmitters dopamine (DA), noradrenaline (NA) and serotonin (5-HT) into vesicles. The monoamines would consequently be degraded by monoamine oxidase, MAO, leading to reduced levels of monoamines [2, 3].

Further, the mechanism of action of two types of antidepressives was tested, one monoamine oxidase inhibitor (MAOI) and one tricyclic antidepressive (TCA) respectively. These drugs had varied synaptic action, tricyclic antidepressants would block NA and 5-HT reuptake and MAOIs would increase the stores of NA and 5-HT. Both of these groups of drugs would acutely increase the function of NA or 5-HT or both and give an increased mood effect in depressed patients. Early data also found that depressed patients would have lowered levels of both the NA metabolite, 3-methoxy-4-hydroxy-phenylglycol (MHPG), and the 5-HT metabolite, 5-hydroxyindoleacetic acid (5-HIAA), in the plasma, cerebrospinal fluid (CSF) or urine, suggesting low utilization of the monoamines. These were some of the factors that formed the basis of the monoamine hypothesis [2, 3].

Several inconsistencies are found in this hypothesis; for example both amphetamine and cocaine enhance monoamine transmission, but they have no antidepressant effects. Also antidepressant drugs have a rapid biochemical effect in the brain, but the clinical antidepressant effect takes weeks to develop, suggesting that there are secondary adaptive changes in the brain, which takes time to develop, that results in the clinical improvement [3].

Over the years new research has lead to new hypotheses and some of these will now be considered.

4.1.2 The serotonin-noradrenaline hypothesis
In 1989 Sulser proposed a serotonin-noradrenaline hypothesis of depression based on chronic effects of antidepressants leading to down-regulation of β-receptors as well as
5-HT\textsubscript{2} receptors and further an enhanced physiological response to 5-HT. Noradrenaline and serotonin was found to modulate one another and Sulser suggested that noradrenaline function would involve several feedback loops that would use several different neurotransmitters, as for example 5-HT \cite{2, 14-17}.

Serotonin has influence on sensitivity to pain and emotionality and it also has effects on thermoregulation, eating and sleep. Serotonergic pathways are originating in the raphe nuclei of the brain. 5-HT turnover and function is usually determined by measuring its main metabolite 5-HIAA, usually in the CSF as this provides the best picture of CNS function. In general high levels of 5-HIAA reflects increased function of serotonergic neurons whereas low levels reflects the opposite. Reduced levels of 5-HIAA in the CSF of depressed patients and also lower levels of the metabolite have been found on post-mortem brains of depressed patients, mainly in suicide victims \cite{2}.

Low serotonergic activity is thought to give increased density of postsynaptic 5-HT\textsubscript{2} receptors most likely as a compensatory mechanism, in unmedicated depressed individuals. This coincides with studies which show that 5-HT\textsubscript{2} receptors are down-regulated by chronic antidepressant treatment \cite{2}.

Antidepressants can, acutely, increase 5-HT by inhibiting MAO or by blocking reuptake, which in turn will give increased 5-HT in the synaptic cleft followed by the 5-HT autoreceptors slowing the firing rate and thereby 5-HT synthesis and release is reduced. Chronic antidepressant treatment however, is shown to down-regulate the autoreceptors and reversed effects results and 5-HT in the synapse is increased \cite{2}.

Noradrenaline plays a role in attention and arousal and response to stress to mention some. Noradrenergic pathways are originating in the locus coeruleus of the brain. Threatening situations increase firing in this area whereas normal daily functions decrease firing. Levels of the main principal noradrenergic metabolite MHPG are in general found to be increased in patients using antidepressives indicating an improved noradrenaline turnover. By down-regulating both \(\beta\)-receptors and \(\alpha_2\)-autoreceptors, which chronic treatment with antidepressives has been found to do, these receptors will have opposite effect on adrenergic synapses. The \(\alpha_2\)-autoreceptors normally
decrease firing and reduce NA release, which in turn will lead to acutely reduced noradrenergic cell function. A down-regulation will have an opposite effect of both these cell functions. 1-3 weeks of antidepressive treatment leads to down-regulation of \( \beta \)-receptors, which is consistent with the onset of therapeutic response. MAOIs, TCAs, SSRIs and second generation antidepressants have been found to give these results as well. Electroconvulsive therapy is also believed to reduce \( \beta \)-receptors [2].

### 4.1.3 The glucocorticoid hypothesis

As new and alternative neurobiological models were tested, the glucocorticoid hypothesis developed. Stress-related neuroendocrine abnormalities linked to depression were at the focus of interest [18]. As described above, the increased secretion of glucocorticoids, including cortisol from the adrenal cortex is thought to be due to hypersecretion of ACTH from the pituitary, which again is a result of increased secretion of CRF from the hypothalamus. Normally the increased secretion of glucocorticoids in the blood has a negative feedback on both hippocampus and hypothalamus, which will lead to inhibition and reduced release of CRF. The CRF circuit is normally stimulated by the amygdala, the centre of emotional responses [2].

But however, when an individual is exposed to stress for prolonged periods of time, animal studies show that the hippocampal neurons are damaged as a result of the sustained increased levels of glucocorticoids. The damage includes reduced neurogenesis and decrease in dendritic branches and loss of dendritic spines on neurons. The hippocampal damage leads to decreased neuron sensitivity to the cortisol and the negative feedback loop leading to more cortisol in the blood and even more damage to the hippocampal cells. Some depressed patients have magnetic resonance (MR) imaging which show small reductions in hippocampal volume. It is also shown that CRF levels are reduced by ECT and antidepressant drugs. Some of the drugs are found to reverse the loss of dendrites as well as to increase neurogenesis in animals. Due to this hypothesis, research has been made to develop new antidepressants directed against CRF receptor antagonism [2, 18].

### 4.1.4 The BDNF hypothesis

Neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), are regulators needed during brain development. They also play an important role in
regulating changes in cells and survival in adult brains. Deficit in neurotrophic factors may be a reason for the hippocampal cell loss following stress [2, 19].

It is found that BDNF in the hippocampus is reduced in rats during chronic stress. Also, BDNF as well as monoamine transmission is increased both in humans and in animals in response to chronic antidepressant treatment, acute treatment does not have this effect. Antidepressants are also showed to prevent stress-induced reductions in BDNF and lead to increased survival and growth of the hippocampal cells as BDNF is thought to repair damaged cells and protect the cells from further damage [2].

The production of BDNF is dependent on the cAMP, second-messenger system. The monoamines NA and 5-HT is increased by acute antidepressant treatment and chronic antidepressant drug treatment will cause a down-regulation of their receptors, β-receptors and 5-HT receptors respectively. In response to the down-regulation the cAMP pathway is up-regulated, which in the end will give an increase in the protein synthesis of BDNF as well as other proteins [2, 19].

Depressed patients are found to have reduced levels of serum BDNF when depressed, which, at least partly, normalise during remission [155-158].

Based on this evidence the neurotrophic hypothesis proposes the theory that loss of dendritic branches and spines is caused by low BDNF and that antidepressant treatment may prevent the reduction in BDNF by protecting these cells [2, 19].

4.1.5 Major depressive disorder viewed as a dysfunction in astroglial bioenergetics

In 2007 Hundal proposed a hypothesis where depression is viewed as a dysfunction in astroglial bioenergetics and a cerebral hypoglycometabolic syndrome. Depressed patients are clinically diverse and respond differently to different drugs, which makes it likely to believe that the broad type of pathology leading to depression has a common denominator, which is believed to be impaired cerebral glucose metabolism [20].
Astroglia are a group of CNS cells, which has gained increasing interest over the last 10-15 years. These cells have an exceptional chance to modify neuron function considering their close interface with both brain capillaries and nerve cells. In the cycle of glutamate and glutamine, there is interplay between neurons and neighbouring astrocytes. Most glutamate released from neurons is taken up by neighbouring astrocytes, which will convert the glutamate to glutamine. Glutamine is then transported out of the astrocytes available for neurons, which will, after taken up, convert the glutamine back to glutamate [20]. Glutamate is also derived from the normal metabolic breakdown of glucose [21].

The term hypofrontality was first introduced in 1984, referring to local reduced glucose turnover in the frontal parts of the brain, as PET-scanning was available as a new imaging technique. $^{18}$Fluorodeoxyglucose ($^{18}$FDG) will be absorbed in the same way as glucose normally is absorbed in the brain, followed by phosphorylation. The resulting compound is not further metabolised and is trapped inside the cell. The $^{18}$FDG can be measured by PET-scanning. Several studies have found hypofrontality in depressed patients [22-24]. Depressed patients are found to have reduced cerebral blood flow, which can influence glucose metabolism, in the prefrontal cortex, anterior cingulate cortex and caudate nucleus [25]. Further, studies have found an epidemiologic correlation between depression and diabetes, where increased prevalence of depression amongst diabetics is found or opposite; there is an increased risk of developing diabetes after a depressed period [26-30].

The glucose metabolism in depressed, non-diabetic patients is found to be unbalanced. In one study the non-diabetics were found to have an insulin sensitivity of 35-56%, in the depressed period, of the levels that were found in remission. Also the elimination rate constant of glucose was increased 50% during remission [31]. Accordingly the glucose- and insulin turnover seems to be altered during depression and further, at least partly, normalised during remission.

CSF levels of insulin are in general constant and lower compared to serum levels, which are higher and more fluctuating depending on carbohydrate intake [32]. While astroglia are sensitive to insulin and thereby increase glucose uptake when insulin increases, neurons’ glucose uptake on the other hand is insensitive to insulin [33, 34].
This is seen in context with altered glucose- and insulin metabolism during depression.

Findings indicating increased insulin levels in serum when depressed, might contribute to assume that astroglia are the primary affected cells. This is based on the hypothesis that reduced brain glucose metabolism is counteracted by the enhanced insulin levels in serum as an attempt to increase CSF levels of insulin. However, increased insulin levels in serum may cause tissue damage and the peripheral insulin sensitivity is lowered to counteract the increased insulin levels in serum [20].

GLUT1 and GLUT3 are the main glucose transporters found in the brain. GLUT1 has two isoforms, one which is mainly found in the endothelial cells, i.e. by the blood-brain-barrier (bbb) (55 kDa) and one in the astroglia (45 kDa), whereas GLUT3 is a neuronal glucose transporter [35].

There are some data on the link between astrocytes and depression. One study found increased CSF levels of glutamine in depressed patients compared to the controls, suggesting a malfunction of the brain glial-neuronal glutamine-glutamate cycle [36]. Hasler et al found depressed patients to have reduced numbers of glial cells in the prefrontal cortex by the use of magnetic resonance spectroscopy [37] and also post mortem brains of depressed patients are found to have reduced density of glial cells [38, 39]. All in all this indicates that astroglia are the primary affected cells.

4.1.6 Current treatment of depression
Antidepressants can be divided into three major classes.

The first group is the monoamine oxidase inhibitors, MAOIs. These drugs can be a good alternative, with appropriate dietary restrictions, especially to patients who do not respond well to other drugs. Within the presynaptic terminal the enzyme MAO metabolizes the monoamine neurotransmitters that are not stored in vesicles. By inhibiting this enzyme, more of the transmitters is available for release into the synaptic cleft and NA, 5-HT as well as DA will get increased action at their receptors. The long-term effect will be a down-regulation of amine receptors and an up-regulation of second messengers, including the cAMP second-messenger system [2].
MAOIs are not the drug of choice in general as it produces many potentially dangerous side effects, so they were early superseded by the TCAs which are the second main group of antidepressants. These drugs inhibit the reuptake of NA and 5-HT into the presynaptic terminal and thereby prolonging the effect in the synaptic cleft. In the long run changes in both pre-and postsynaptic receptors are seen. Some TCAs are more effective in inhibiting reuptake of NA than 5-HT and vice versa, while some drugs are inhibiting reuptake of both neurotransmitters equally [2]. Anticholinergic side effects are common and TCAs may cause ventricular dysrhythmias, which may increase the risk of sudden cardiac death, especially when an overdose is taken. Alcohol is strongly potentiated by TCAs, increasing the risk of respiratory depression [2, 3].

As less toxic and more reliable antidepressants were needed, the third main group of drugs used in depression, the second-generation antidepressants, was introduced. They were made to give fewer side effects by being more selective in their action of increasing NA and/or 5-HT levels. Other than this, there are not too many differences from the older antidepressants but they are regarded as safer when it comes to taking an overdose [2].

The selective serotonin reuptake inhibitors (SSRIs) belong in this group. Their mechanism of action is to inhibit the presynaptic reuptake of 5-HT. TCA-induced side effects are absent as the SSRIs do not alter acetylcholine, histamine or NA levels. There are however other side effects related to the SSRIs, like nausea, insomnia, anorexia and sexual dysfunction [2, 3]. SSRIs should not be combined with other serotonergic agonists, like the MAOIs, as this may result in the “serotonin syndrome” characterized by hyperthermia, disorientation and cardiovascular collapse, which can be potentially life-threatening [2, 3].

ECT was introduced in 1938 and is still in use today in the treatment of severely depressed patients. The method involves stimulation through electrodes on the head to produce seizures while the patient is anesthetised. ECT is considered safe and effective in addition to giving few side effects, but confusion and memory loss lasting for weeks are reported. It is also a rather expensive method and normally therapy would last for four weeks with ECT three times a week to be fully effective [2, 3].
4.2 Drugs used in the study

4.2.1 Fluoxetine

Fluoxetine belongs in the group of SSRIs. In major depressive disorders the recommended dose for humans is 20 mg daily, but can be adjusted after 3-4 weeks if necessary. Active drug substances will persist in the body for weeks after ceasing the treatment. Weight loss is seen in some patients taking fluoxetine [40].

Following oral administration fluoxetine is absorbed from the gastro-intestinal tract and the bioavailability is not affected by food intake. In humans about 94.5 % of fluoxetine is bound to plasma proteins [41] and it has a volume of distribution of 20-40 l/kg. Maximum plasma concentration is normally achieved 6-8 hours after administration. After 4-5 weeks steady-state concentrations are achieved. A non-linear pharmacokinetic profile is seen with first pass liver effect, where fluoxetine is extensively metabolized by CYP2D6 to the active metabolite norfluoxetine. Fluoxetine has an elimination half-life of 4-6 days while norfluoxetine has an elimination half-life of 4-16 days and excretion mainly occurs via the kidneys [40].

![Fluoxetine structure](image)

**Figure 2a Fluoxetine**  
[42]

**Figure 2b Fluoxetine**  
IUPAC: N-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]-propan-1-amine  
Formula: C_{17}H_{18}F_{3}NO  
Mol. mass: 309.3 g/mol  
[41]

Studies have shown that fluoxetine is clinically effective in rats in doses of 10-20 mg/kg compared to humans in doses of 0.3-0.9 mg/kg [43-45]. But also lower doses, 0.67 mg/kg/day given orally, have shown to induce specific changes in rat forebrain excitatory synapses. After 15 days of treatment serum levels of drug were found to be 20.7±5.6 ng/mL. These levels were measured 6 hours after the last fluoxetine administration. An i.p. dose of 10 mg/kg for 15 days resulted in serum levels of
81±10.3 ng/mL [45]. In one study the pharmacokinetics of several drugs was compared using tail-vein, femoral-artery cannula- and retro-orbital sinus bleeding techniques in rats. An oral dose of 10 mg/kg of fluoxetine resulted in peak plasma concentrations (C\text{max}) of 110 ng/mL from the tail-vein bleeding technique, while 263 ng/mL and 291 ng/mL were measured from the cannula and retro-orbital sinus bleeding respectively [46]. A chronic dose of fluoxetine 10 mg/kg/day is shown to cause anorexia in rats [47].

4.2.2 Reboxetine

Reboxetine is an antidepressive which is considered to selectively inhibit reuptake of noradrenaline. It has a weak effect on 5-HT reuptake and no effect on dopamine reuptake. In humans reboxetine has a recommended therapeutic dose of 8 mg/day, which after 3-4 weeks can be increased to 10 mg/day. Maximum dose should not exceed 12 mg/day [40].

![Reboxetine Molecule](image)

**IUPAC:** 2-[(2-ethoxyphenoxy)-phenyl-methyl] morpholine

**Formula:** \( \text{C}_{19}\text{H}_{23}\text{N}_{0}\text{O}_{3} \)

**Mol. mass:** 313.391 g/mol

Figure 3 Reboxetine [42] [48]

In humans reboxetine is found to be 97% bound to plasma proteins and the drug seems to be distributed into total body water. Bioavailability is found to be at least 60%. An oral administration of 4 mg of reboxetine results in peak levels in the blood of approximately 130 ng/mL after 2 hours. Steady-state is seen within 5 days. Reboxetine plasma levels are found to decrease monoexponentially. In vitro studies indicate that reboxetine is metabolized by CYP3A4, has a half-life of about 13 hours and is excreted in urine [40].
In rats reboxetine is found to be 80% bound to plasma proteins [Personal communication Hundal]. Studies have shown that reboxetine is clinically effective in rats in doses of 20-40 mg/kg compared to humans in doses of 0.15 mg/kg [43, 49]. But also for reboxetine one study have shown that low doses, 0.128 mg/kg/day injected intramuscularly, induced specific changes in rat forebrain excitatory synapses [45].

4.2.3 Sibutramine

Reductil is an anti-obesity drug with no clinical effect on depression. One capsule of Reductil 15 mg contains 15 mg of sibutramine hydrochloride monohydrate which is equivalent to 12.55 mg of sibutramine. The recommended dose is one capsule administered daily [40].

![Figure 4a Sibutramine](image)

**Figure 4a Sibutramine**

**Formula**: C\(_{17}\)H\(_{26}\)ClN

**Mol. mass**: 279.85 g/mol

[42]

![Figure 4b Sibutramine](image)

**Figure 4b Sibutramine**

**IUPAC**: 1-(4-chlorophenyl)-N, N-dimethyl-a-(2-methylpropyl) - cyclobutanemethanamine

Therapeutic effect is mainly produced by its active secondary and primary amine metabolites, metabolite 1 and metabolite 2, which inhibit NA, 5-HT and DA reuptake. It has been found that the NA reuptake is inhibited by 73%, the serotonin reuptake is inhibited by 54% and the DA reuptake is inhibited by only 16% in humans. Administration of 20 mg of Reductil results in peak plasma levels after 1.2 hours and half-life is 1.1 hours. The active metabolites, metabolite 1 and metabolite 2, reach peak plasma levels after 3 hours and elimination half-lives are 14 and 16 hours.
respectively. Steady-state of the metabolites is seen within 4 days of drug administration. In the dose range 10-30 mg, linear kinetics has been demonstrated. Sibutramine and its active metabolites are eliminated by hepatic metabolism by CYP3A4, CYP2C9 and CYP1A2 [40].

Sibutramine administered to obese rats is found to reduce bodyweight gain. The weight loss is thought to be a result of enhanced satiety in addition to enhanced thermogenesis [51, 52]. This effect is thought to be mediated by inhibition of 5-HT and NA reuptake [40]. In one study sibutramine in doses of 0.9, 3 or 9 mg/kg i.p. were administered to rats to study cardiovascular effects [53]. Another study used doses of 10-30 mg/kg of sibutramine administered to rats to show that the animals had increased locomotor activity [54].

### 4.3 Diabetes

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycaemia and other metabolic disturbances due to insulin deficiency, often combined with insulin resistance [55]. The islets of Langerhans are the endocrine component of the pancreas and insulin is synthesized in the pancreatic β cells. If the production of insulin decreases or the insulin does not function properly, glucose levels in the blood will increase and eventually the renal threshold for glucose reabsorption will exceed, entailing glucose in the urine (glycosuria) and causing an osmotic diuresis (polyuria), which further will result in dehydration, thirst and increased drinking (polydipsia). Long-term effects of hyperglycaemia will lead to complications like neuropathy, cardiovascular disease, retinopathy, nephropathy as well as infections in general. Evidence is found that leucocyte function is impaired by blood glucose levels above 10-13 mmol/l [56]. Diabetes can be viewed as a disease where there is a failure in glucose turnover.

#### 4.3.1 Classification of diabetes

There are several types of diabetes but the two main types are type1 and type2 diabetes. Type1 diabetes is caused by an autoimmune reaction destroying the pancreatic islet beta-cells eventually leading to absolute insulin deficiency. It might affect people of all ages. The autoimmune process is a lingering process, which
untreated will lead to ketoacidosis, coma and death. Insulin is required for survival [55, 57].

Type2 diabetes is accompanied by both insulin resistance and by impaired insulin secretion. Also this type might affect people of all ages. Both heritance and life style might play a role in the development of this disease as obesity, hypertension and inactivity might be factors contributing to its development. Insulin resistance and compensatory hyperinsulinemia are often seen in non-diabetics with essential hypertension [55, 57].

4.3.2 Treatment of diabetes

Diabetes in general is treated with insulin injections and/or tablets to control glucose levels. Some people with diabetes type2 can manage to reduce glucose levels by weight reduction, exercise and a healthy diet but eventually most of them need tablets or even insulin injections. The dose has to be adjusted according to exercise and the amount and type of food. The aim is to stabilize blood glucose levels through the day and night between 4.5-10 mmol/l and the fasting glucose levels between 4.5-7 mmol/l [55, 57].

4.3.3 Depression and diabetes-is there a connection?

Increased prevalence of depression in persons with diabetes has been observed for a long time, but few studies were done to actually confirm the connection as depression was thought to simply be a secondary reaction followed by the difficulties associated with living with diabetes. In 1988 a study was done where an association between affective disorders and altered glucose utilization was recognized. An oral glucose test was performed on depressed, non-diabetic patients and healthy volunteers and the results indicated that insulin resistance appeared during major depressive illness [58]. In advance a study was done on depressed patients with painful diabetic neuropathy. Antidepressant treatment resulted in remission of both depression and pain in all the patients [59].

The first study to report that the probability of developing type2 diabetes was increased in depressed patients compared to non-depressed persons was published in
1996. This was a 13-year follow-up and population-based study which further confirmed that the earlier assumptions, where depression was seen as secondary to diabetes, were wrong [60]. Further studies have found an increased risk of developing type2 diabetes when depressed [61, 62] and progression of the disease and its complications are found to be increased both in type1 and type2 diabetes when depressed [63, 64]. Evidence strongly supports an association between depression and type2 diabetes, but the underlying mechanisms are still unclear.

It has been indicated that the prevalence of depression is increased in patients with diabetes [65]. One study found the odds of depression to be doubled in persons with diabetes compared to non-diabetics and diabetic women had a higher prevalence of depressive disorder (28%) compared to diabetic men (18%) [27].

Studies have been done to investigate possible negative effects of depression on glycemic control. A meta-analysis done in 2000, included 24 studies and found depression to be significantly associated with hyperglycemia in patients with both type1 and type2 diabetes [66]. A relationship between insulin resistance and depression has been observed, and depression treatment has been found to be associated with improvement in glycemic control. In one study treatment of depressive disorder with bupropion was tested on patients with type2 diabetes and major depressive disorder (MDD). The insulin sensitivity improved as the depression was treated and also remained improved during non-depressive periods [67]. Another study found impaired insulin sensitivity accompanied by hyperinsulinemia in patients with depression which improved after treatment of the depression [68]. Findings strongly suggest that treatment of depression improve insulin sensitivity, but also here further investigation is required [69].

It has been considered if depression increases the probability of developing diabetes complications like for example diabetic retinopathy, nephropathy, neuropathy and macrovascular complications. One meta-analysis included a total of 27 studies and found depression to be significantly associated with diabetes complications. Further studies are however needed to identify the pathways linking the association [70].
Research in the field of depression and diabetes suggest that there is a connection between these two diseases. This is an exciting area of research but as already mentioned further investigation is needed to find out if and how these conditions may be connected.

4.4 The astrocytes

Astrocytes are a type of glial cells existing in the glial tissue in the nervous system. They play a significant role in the nutrition of neurons [71].

Blood vessels deliver nutrients to cells and remove waste. Brain capillaries are different from the capillaries found throughout the body. Both types have endothelial cells forming the walls of the capillaries, but the walls of the brain capillaries are more closed as the adjoining edges of the endothelial cells are fused, forming tight junctions instead of fenestrations and intercellular clefts. Lipid-soluble materials can pass the capillaries but the water-soluble molecules and most materials are moved through the brain capillaries by special transporters, making the bbb selectively permeable. Further, the brain capillaries are surrounded by glial feet, which are extensions of the astrocytes. The position of the astrocytes makes a close interface with both brain capillaries and nerve cells providing them with a unique opportunity to adjust the function of the neurons [72].

Traditionally the presynaptic terminal and the postsynaptic neuron have been assumed to be the functionally important elements of the synapse, but the increasing interest and research on astrocytes has lead to the concept of the “tripartite synapse”, as the role of the astrocytes is added to the classical synapse function [73].

4.4.1 Functions of the astrocytes

There is a bidirectional communication system between neurons and astrocytes which affects neuronal excitability and synaptic transmission [74, 75]. Neurotransmitters released into the synaptic cleft activate nearby astrocytes by increasing cytosolic \([\text{Ca}^{2+}]\) (“calcium waves”). Neurone active compounds; transmitters, including glutamate and ATP, are then consequently released from the astrocytes as a signal back to the neurons [74]. This communication between neurons and astrocytes was
first demonstrated in vitro on cultured astrocytes and showed that many neurotransmitters, including glutamate, GABA, ATP, serotonin, adrenaline, ACh as well as a number of peptides, could induce increases in glial $[\text{Ca}^{2+}]_{\text{int}}$. Later studies on brain slices have confirmed glial responses also in vivo [76-78]. Transmitters released from glia will further be referred to as gliotransmitters. Gliotransmitter released from the astrocytes can decrease or increase further release of neurotransmitter presynaptically [78-81], and they are also able to stimulate postsynaptic neurons directly, and cause inhibitory or excitatory responses [78]. Gliotransmitters influencing neurons may also affect neighbouring astrocytes to be activated [82]. Peptides are thought to play a role in the regulation of different astrocytes functions like glucose uptake, glycogen metabolism, induction of calcium transients and calcium waves as well as control of internal pH [83].

In general astrocyte functions are diverse; sequestration and/or redistribution of $K^+$ during neural activity, synthesis of precursor for glutamate and GABA at synapses, removal of glutamate and GABA at synapses and ammonium detoxification. They are also thought to play a role in the providing of energy substrate, as lactate, to neurons, and brain water homeostasis as well as in the regulation of synaptogenesis and neurogenesis in adult brain [84].

The astrocytes can be seen as multifunctional housekeeping cells, which in addition to clearing neuronal waste, deliver glucose and provide metabolic substrates, and maintain control of local ion and pH homeostasis [85].

In addition to the influence on synaptic activity, recent studies have found that elevated $[\text{Ca}^{2+}]$ in astrocytes induced by neurons can affect perivascular astrocyte endfeet to secrete vasodilatory substances, indicating that cerebral blood flow regulation is astrocyte-mediated [86, 87].

At present synaptogenesis is believed to be a neuronal event. But studies have found that astrocytes might play a role in the regulation of CNS synaptogenesis and maintenance of the synapses. Different cell cultures, neuronal cell lines [88] and hippocampal neurons derived from stem cells of adult rats [89, 90] to mention some, have showed an increase in synaptogenesis induced by astrocytes. It could be that the
astrocytes were affecting the environment indirectly but glial support was required for optimal survival and maturation of the neurons [91].

All these findings strongly imply that astroglia play an even more important role than earlier assumed.

### 4.4.2 Four main types of human astrocytes

There are 1.4 astrocytes per neuron in the human cortex, compared to a typical ganglion in a leech which consists of 1 astrocyte per 25-30 neurons. The number of astrocytes per neuron is increasing with increased brain size and complexity [85, 92].

![Figure 5 Astrocytes and neurons in mouse cerebral cortex](image)

Figure 5 is illustrating the density of astrocytes and neurons in the cerebral cortex. The cell bodies of the neurons are coloured green and the astrocytes are coloured red/yellow. Throughout the image small red dots are reflecting processes of the astrocytes. Some of the astrocytic glial cells wrap themselves around blood vessels, which can be seen in black.

A typical astrocyte extends from five to eight major processes and each of these is further divided into finer branches. Golgi staining or immunolabelling make the astrocytes appear as star-like, which is how their name originated, as the glial fibrillary acidic protein (GFAP) is recognized. This can partly be illustrated in figure 5. GFAP is found in the cell body and the major processes of the astrocytes, but the expression of GFAP reflects only 15% of the total cell volume. The finer processes are however GFAP-negative and the morphology of the astrocytes are actually best defined as a polyhedron rather than a star [85, 94, 95].
The distribution of astrocytes in the adult CNS is highly organized and some are found to be separated in such a way that the cell bodies and the larger processes are not touching each other, thus they are making non-overlapping astrocytic domains and synapses located within a domain might be controlled by one single astrocyte [85, 96]. It is estimated that each astrocyte supports and modulates the function of about two million synapses [97].

Hitherto four main types of human astrocytes are described. Human protoplasmic astrocytes are the most abundant and reside in the deeper layers, layer 2-6, of the cortex. The GFAP-positive processes do not overlap. The astrocyte within one domain is able to cover up to five different blood vessels, eight neuronal cell bodies in addition to several synapses. The human protoplasmic astrocytes are symmetrical and larger, up to a 27-fold greater volume, than in rodents as well as being far more elaborated [97].

Human interlaminar astrocytes are found in layer 1 of the primate cortex along with numerous synapses. They send long, “cable-like” processes throughout the cortex and eventually terminate in layer 3 or 4 [97]. Some processes could sporadically extend to the vasculature [98]. These astrocytes are not organized in domains and they might be involved in long-distance signalling although their function is still unknown [97]. They are however found to be changed in pathologies like Alzheimer’s disease and Down’s syndrome [99, 100].

The third main type of human astrocytes is the polarized astrocytes which reside in layer 5-6 of the cortex near the white matter. Also these extend long processes, but still straighter and less branched than the protoplasmic astrocytes and they are GFAP-positive. Most of the processes extend to the neuropil while some extend to the vasculature. They are not organized in domains but rather the long processes pass through the domains of the protoplasmic astrocytes. Their function is unknown but it is speculated upon their role in long-distance communication [97].

The last type of human astrocytes found are the fibrous astrocytes located in the white matter. Compared to other glia the fibrous astrocytes are straighter and less branched and they have few primary GFAP-positive processes. In contrast to the protoplasmic
astrocytes, the fibrous astrocytes also tend to intermingle and overlap. Their functions are thought to be limited to metabolic support based on their relatively simple morphology [97].

4.4.3 Gap junctional communication in astrocytes

It is thought to be a connection between gap junctional communication (GJC) and the energetic metabolism in astrocytes [101], illustrated in figure 6 below. Gap junctions, (GJs) are manifold on the astrocytes and they make a direct pathway for intercellular communication available both between astrocytes, and between astrocytes and oligodendrocytes as well as neurons. [102]. Connexins (Cxs) are the molecular constituents forming the gap junctional channels and at least 11 different types have been identified [103, 104]. It has been found that the Cx channels in astrocytes can function as hemichannels where exchange of ions and small molecules between the extracellular space and the cytoplasm can take place [105]. In the supply of energy substrates to neurons the astrocytes can be seen as “cross-road” cells, as illustrated in figure 6 below, through which the energy substrates have to be taken up from the brain capillaries, travel further through astrocytic GJs and finally reach the synaptic cleft where they can be used by the neurons. The GJs are found to be permeable to several energy substrates like glucose, glucose-6-phosphate, and lactate [101]. The GJs have also been found to be permeable to glutamate and glutamine [106], inositol-trisphosphate [107] and ADP and ATP [108].
4.4.4 Glucose transporters, GLUT1 and GLUT3

Glucose is the main energy source for the mammalian brain and about 20% of the glucose in the blood is metabolized by the brain [122, 123]. Glucose is a polar substance which is unable to cross the bbb by itself.

The glucose transporter (GLUT) protein family mediates facilitative glucose transport, which is essential to supply the neurons and glia in the brain with glucose. So far thirteen members of the GLUT family have been described [124-133], where mainly two of them function in cerebral glucose metabolism, namely GLUT1 and GLUT3 [122].

GLUT1 is mainly found in vascular endothelial cells and is responsible for the transfer of glucose across the bbb. There are two isoforms of GLUT1, the 55-kDa form which is mainly found at endothelial cells and the 45-kDa form which is found in astrocytes [35, 123]. Expression of GLUT1 is regulated with metabolic demand and rate of cerebral glucose utilization [122].

Figure 6 The role of astrocytes in the supply of energy substrates to neurons
GLUT3 is the neuronal glucose transporter. The delivery of glucose to neurons is mediated by astrocytes and uptake of glucose to the neurons is mediated by GLUT3 [35, 122].

4.4.5 Glucose trafficking through astrocyte gap junctions

As mentioned above, the astrocyte GJ channels are permeable to glucose and also its derivatives like glucose-6-phosphate and lactate. It is also believed to be a link between the rate of uptake and the level of intercellular communication. This is based on the findings that inhibition of GJC will increase the uptake of glucose by the astrocytes [106].

When GJC is inhibited GLUT1 is translocated from the intracellular space to the plasma membrane and the $V_{max}$ of glucose uptake is increased [134]. Other mechanisms to increase the rate of glucose uptake in astrocytes are the glutamate released by neurons, which is proposed to activate the transporters [135-138]. When the astrocytes take up synaptic glutamate, glycolysis is activated and energy and lactate is produced. Lactate is further released from the astrocytes to nourish neurons and sustain neuronal activity [137].

GLUT3 expression is also found to be induced after inhibition of GJC [134] and combined with the upregulation of GLUT1; this is thought to be a mechanism to maintain the increased rate of astrocytic glucose uptake after inhibition of GJC [101].

Contrary to the common opinion, lactate, not glucose, seems to be the preferred energy source for neurons [109] when excited. Astroglia are found, both in vitro [110] and in vivo [111] to have a lactate pool as part of their supportive role towards neurons [112-114]. Waagepetersen et al found in vitro that the cortical neurons absorb more lactate than the astrocytes [115, 116]. Findings of Ogawa et al [117] suggested that neurons in the resting state do not primarily rely upon lactate as the main energy source. Nehlig et al [118] used autoradiography combined with immunohistochemistry to investigate if astroglia or neurons would absorb [$^{16}$C] 2-deoxyglucose in resting state to the greatest extent. In 1998 Pellerin et al proposed the existence of an activity-dependent astrocyte-neuron lactate shuttle (the ANLS) [119].
Nehlig et al [118] investigated this question in vitro using a combination of high-resolution microautoradiographic imaging with $^{14}$C-trajectory ($^{14}$C-2-deoxyglucose) combined with immunohistochemistry. Of the recovered radioactivity, about 50% was found in astroglia while approximately 60% was found in neurons. But there were limitations to the method and the technique used and it was not possible to make any conclusions. There has been a scientific debate regarding the ANLS hypothesis [113]. Recently a compartmental mathematical model of the glucose and lactate metabolism in neurons and astroglia [120] was published, which strongly supports this hypothesis. A review on monocarboxylate transporters in astroglia and neurons also supports the hypothesis [121]

### 4.4.6 Glucose transporters and noradrenergic stimulation

In 1997 Kaloyianni et al published a study where metabolic effects and cellular volume responses induced by noradrenaline in nucleated erythrocytes was tested. Noradrenaline added to erythrocytes from the frog species *Rana ridibunda* led to a significant increase of cAMP and the cell volume was also significantly increased due to Na$^+$ influx, and thereby increased intracellular water content, and loss of K$^+$. The glucose concentration was significantly decreased in the presence of noradrenaline and lactate accumulation was seen. Noradrenaline was reported to induce an increase of up to 260% glucose consumption and up to 278% lactate formation. The stimulation of glycolysis was thought to be due to the increase of cAMP [139].

Another study was done to investigate the effects of noradrenaline in the cell-surface glucose transporters in cultured brown adipocytes. It was known that noradrenaline directly enhanced glucose uptake into brown adipocytes both in vivo and in vitro, independent of the action of insulin [140-143]. The study found that insulin was able to enhance the cell-surface GLUT4 and noradrenaline was found to increase the glucose transport by an increase in the cell-surface GLUT1 [144].

Noradrenaline transporter function and autonomic control of metabolism was investigated in one study. Postganglionic adrenergic neurons release noradrenaline which is involved in the regulation of blood pressure and energy metabolism. Noradrenaline transporters, NATs, on the neurons take up again most of the released noradrenaline, where it is repackaged for further release or it gets metabolized.
Clearly, if NAT function is changed this may have important effects on blood pressure and metabolism [145]. Healthy individuals were given 8 mg of reboxetine, the selective NAT inhibitor, or placebo. Systemic infusion of isoproterenol, a non-selective β-adrenoceptor agonist, was given in the presence of reboxetine or placebo and found glucose supply and metabolism to be significantly increased with reboxetine compared to the placebo group [145].

All of these articles indicate that noradrenergic influence may stimulate and increase the amount of glucose transporters on the surface of different cell types.

4.5 Immunohistochemistry

4.5.1 H&E Staining

H&E stain stands for hematoxylin and eosin stain and is a widely used staining method in histology. Application of hematoxylin, a basic dye, will color basophilic structures blue/purple while eosin Y, an alcohol based acid, will color eosinophilic structures bright pink. The terms basophilic and eosinophilic are based on affinity to the dyes and the colored structures do not necessarily have to be basic or acidic. Basophilic structures include nucleic acids in ribosomes, chromatin in the cell nucleus and RNA in regions of the cytoplasm. Eosinophilic structures are commonly made of intra- or extracellular proteins. Most of the cytoplasm is eosinophilic. Not all structures are stained well, hydrophobic structures, rich in fat, usually remain clear, like for example neuron axons, adipocytes and myelin [146].

H&E staining provides a good overview of the condition of the tissue. If cryostat sections are not well cut, this can be detected and new sections has to be cut before the next step of immunofluorescence staining can be performed.

4.5.2 Antibodies

Antibodies (immunoglobulins, Ig) are a group of proteins, consisting of two identical heavy (H) chains and two identical light (L) chains, linked together by disulfide bridges. There are 5 major classes of immunoglobulins; IgE, IgD, IgM, IgA and IgG [147].
The H chains (the long chains) determine the class and subclass of the molecule and their structural as well as their antigenic properties will differ. The L chains (the short chains) consist either of type lambda or of type kappa and the distribution of these chains will differ both in different species as well as in different immunoglobulin classes. IgM and IgG are the most frequently used antibodies in immunohistochemistry. In a newly immunized host, IgM will be the first humoral antibody to be detected. The period from the antigen introduction until the IgM antibodies appears is called the latent period lasting for approximately one week. IgG will be the most abundant antibody in the hyper immunized host. Within two weeks, after the introduction of the immunogen, IgG antibodies dominate [147].

In this study IgG antibodies are used and will here be considered in more detail. The IgG molecule consists of two gamma H chains, coloured blue in figure 8 below, and two L chains, coloured green, of type lambda or type kappa, forming a Y-structure. The molecule can be divided into domains, variable, coloured light blue and light green, and constant, coloured darker blue (marked as number 3) and dark green, containing 110 to 120 amino acids and one intrachain disulfide bond each. Variable domains of both the light (VL) and the heavy chain (VH) contain amino terminals of
the immunoglobulin molecule. One constant and one variable domain from each heavy and light chain form the antigen binding site, illustrated on figure 7 above as the antigen-binding fragment at the tip of the Y and also illustrated in figure 8 below marked as number 5. This region, where the antigen binds, is located on the Fab (fragment antigen binding) region of the molecule, marked as number 1 in figure 8. The rest of the two heavy chains is called the Fc (crystalline fragment)-region, marked as number 2 [147, 149].

Monoclonal antibodies are produced by one clone of plasma cells. Antibodies from one clone will be immunochemically identical. This means that they will react with a specific epitope on the antigen against which they are raised. There are several advantages when using monoclonal antibodies compared to polyclonal antibodies; absence of nonspecific antibodies, high homogeneity and no batch-to-batch variability. But there are many disadvantages as well, for example the targeted epitope must survive fixation. It has been shown that an antigen may survive formalin fixation, by using polyclonal antibodies, but the epitope interacting with the monoclonal antibody does not. The targeted epitope must also be unique to a given antigen. If the antibody is directed against an epitope shared by two or several dissimilar antigens, the specificity will be lost [147].
Polyclonal antibodies are produced by different cells and react with various epitopes on the antigen against which they are raised. Rabbit, goat, pig, sheep, horse and guinea pig are the most frequently used animals for production of polyclonal antibodies [147]. In this study polyclonal antibodies will be used.

To generate an immune response in animals the doses administered normally varies from 10 µg to 200 µg, depending on the immunogenicity of the antigen. To increase or maintain the antibody levels, booster shots are given normally once a month or whenever necessary when the titers are decreasing. Blood is harvested from the animal and polyclonal antibodies can be obtained after removal of cells. Different forms of polyclonal antibodies can be obtained [147].

The affinity of antibodies for the determinants they recognize on antigens will differ. The intrinsic affinity between an antibody and the epitope of the antigen is mainly determined by van der Waals forces, hydrogen bonding and ionic interactions. Hydrophobicity also seems to have a stabilizing effect. The antibody’s affinity can be measured by the association constant, $K_a$, of the binding between the antibody and its antigen. An antibody with high affinity requires lower concentration of antigen to saturate the binding sites of the antibody than antibodies with lower affinity [147].

Possible antibody cross-reactivity must be considered. Cross-reactivity is binding of an antibody to structurally related antigens [147].

How fast an antibody will react with its antigen is depending on many factors, for example tissue fixation. Overfixation will make it more difficult for antibodies and their complexes to penetrate the tissue. Antibody concentration, temperature and incubation time are other important factors to consider achieving desirable immunohistochemical staining. Elevated concentrations of high affinity primary and link antibodies may reduce the incubation time [147].

Antibody titers usually vary from 1:100 to 1:2000 for polyclonal antisera and are generally expressed as micrograms of antigen precipitated per milliliter of antiserum. Correct dilutions are important for the quality of staining. The best way to determine the correct dilution is by making a series of small, experimental dilutions until the
optimal dilution is found, using a fixed incubation time. The optimal antibody titer will result in the best specific staining with the least amount of background [147].

Incubation time may vary from 10-30 minutes and up to 48 hours depending on affinity and concentration and it is necessary for the antibody to react adequately with the bound antigen to gain a good quality and intensity of the staining [147].

4.5.3 Fixation
Fixation has a protective role and helps preserve and stabilize the cells and tissue, prevent autolysis and inhibit the growth of bacteria and molds. It may also cause artefacts like conformational changes and alteration of the chemical composition of the tissue, but it is however necessary to protect the tissue from the further handling of it. Concerning immunocytochemistry, cryostat sections will provide good antigen preservation. Acetone is a fine preservative but a poor penetrator and should only be used for smears and cryostat sections [147].

4.5.4 Staining method
There are several different staining methods to choose from. The direct method is the oldest; the enzyme-labelled primary antibody reacts with tissue antigen. Today the method is not good enough. The two-step indirect method is a more flexible and sensitive technique. A primary antibody is applied and binds to the tissue antigen as illustrated in figure 9 below. In this study a fluorescent-secondary antibody is added, which is directed against the primary antibody [147].

Photobleaching is a common problem when it comes to immunofluorescence staining techniques, which can cause loss of activity and colour. The problem can be solved by reducing light exposure and the time spent exposed to light while performing the procedure. The concentration of fluorescence could also be increased [151].
4.5.5 Controls

To validate the immunohistochemical staining results both reagent and tissue controls should be performed.

Reagent controls are done to determine that the primary and secondary antibodies bind specifically to their target antigens. To test the specificity of the primary antibody non-immune serum could be used as a control [147].

4.5.6 Background staining

Background staining is a common problem in immunohistochemistry. The hydrophobicity of tissue proteins may result in cross-linking during fixation. Optimized fixation methods are important and also pre-treatment with blocking agents may contribute to less background staining. Antibodies, the immunoglobulins have hydrophobic properties, which may augment during storage. Addition of detergent to the diluents or addition of 1% bovine serum albumin (BSA) to the primary antibody is other efforts which may reduce non-specific binding [147].
5 AIM OF THE STUDY

This study is based upon the hypothesis that major depressive disorder is considered in coherence with a dysfunction in astroglial bioenergetics, proposed by Hundal in 2006 [20].

If depression is a result of failure in the glucose turnover in the astroglia of the brain, the right therapy is thought to be; improving the glucose turnover to counteract the failure.

Glucose transporters are found to be stimulated and up-regulated by noradrenergic influence [139, 144, 145] and thereby increasing the glucose uptake.

The main purpose of this study is to investigate if continuous noradrenergic stimulation of the brain will up-regulate the amount of glucose transporters GLUT1 and GLUT3 in the brain and thereby be able to counteract the failure in glucose metabolism caused by a dysfunction in astroglia. If the failure in glucose turnover is counteracted this is thought to improve the depression.

Findings may contribute to give new and important information about the function of the brain as depressive symptoms develop and it may contribute to developing new and improved antidepressant drugs.
6 MATERIALS AND METHODS

6.1 Materials

6.1.1 Animals
Forty *rattus norvegicus*, BD9, female rats, 2-3 months old, with body masses of 149-220 grams at the beginning of the experiment, were from Dyreavdelingen, Gades institutt, Haukeland Sykehus.

6.1.2 Medicines and placebo
Fluoxetine 20 mg tablets were from ratiopharm GmbH, Germany. Edronax (reboxetine) 4 mg tablets were from Pfizer AB, Sweden. Reductil (sibutramine) capsules 15 mg were from Abbot Scandinavia AB, Sweden.

Citric acid (*Acidum citricum monohydricum granulatum*) was from Apotekerproduksjon, Oslo, Norway.

6.1.3 Immunofluorescence staining products
Primary antibody Glut-1 (sc-1603) and Glut-3 (sc-31840), blocking peptides (sc-1603P and sc-31840P), PBS (sc-24946), normal blocking serum (sc-2044), secondary antibody (sc-2024), and mounting medium (sc-24941) were purchased from Santa Cruz Biotechnology, CA, USA.

6.2 Methods

6.2.1 Animal experiment
The 40 rats included in the study were divided into 4 main groups containing 10 animals each. There were two animals in each cage and one of them was marked with red colour on its tail to distinguish them. The rats were kept under 12/12 light/dark cycle in temperature controlled rooms (21-23°C). Food and drinking water were given *ad libitum* to all groups. The experimental protocol was approved by Aurora Brønstad, Doctor of Veterinary Medicine (DVM), Dyreavdelingene, Vivarium, Universitetet i Bergen, Haukeland Sykehus, protocol number 2007170.

Citric acid powder was dissolved in water to make concentrated citric acid water, 20 mg/mL. Fluoxetine tablets were dissolved in water, making a 400 mg/L solution.
Reboxetine (Edronax) tablets were dissolved in water, making a 40 mg/L solution, which after 12 weeks were upregulated to 100 mg/L. Sibutramine (Reductil) capsules were dissolved in water, making a 225 mg/L solution. 15 mL of concentrated citric acid water was added to all the solutions including the placebo solution consisting of pure water. After 2-3 days the solutions containing drug were filtrated and ready for use.

Samples were taken from the different solutions to measure the concentration of the drugs mixed in the drinking water. They were analyzed by Kjell Ove Fossan at Laboratorium for klinisk biokjemi, Haukeland Universitetssykehus. The concentration of drugs in the drinking water were based upon former pilot studies done by Hundal were the concentrations needed for use in rats for the different drugs were tested [personal communication Hundal].

The animals were acclimatized for 7 days before starting their treatment. During a period of 18 weeks the rats is group 1 received fluoxetine solution as their drinking water, group 2 received reboxetine solution and group 3 received sibutramine solution as their drinking water. Group 4, the placebo group, got diluted citric acid water 60mg/L.

The water intake, and thereby the drug intake, was measured by weighing the drinking bottles 5 days a week. The rats were weighed once a week to survey weight gain or weight loss.

6.2.2 Laboratory procedures

After 18 weeks the rats were put to death. They were fully anaesthetised using IsobaVet, isofluran. Movement reflexes were tested by making sound and pinching the rat between the toes. Cardiac puncture was performed using 10 ml syringes with needles size 0, 8 x 40 mm followed by breaking of the neck. Blood samples of 3-5 ml was collected from each rat and put into test tubes. The blood samples were centrifuged in a MegaFuge 1.0 R, Heraeus Sepatech with a speed of 3000 rpm, rotor time of 10 minutes and a temperature of 10°C. The top layer, the plasma, was
collected in labelled test tubes and stored frozen until further investigation at the laboratory; Laboratorium for klinisk biokjemi, Haukeland Universitetssykehus, by Kjell Ove Fossan. Blood concentrations of the drugs the different groups had been given was measured by HPLC; Agilent Technologies 1200 SL and MS; Agilent Technologies 6410 LC/MS/MS.

Following breaking of the neck, the fur covering the head was removed and the skull was opened and separated with a scissor. The brain was taken out and placed on a filter paper immersed in sodium chloride. 3 parts of the brain tissue were cut; 1 from the right ventricle frontal cortex, 1 from the hypothalamus area and 1 from the cerebellum. The cerebellum part was meant as a control, as abnormalities in this part of the brain never have been observed during depression. The 3 parts of the brain were immersed in isopentane cooled in liquid nitrogen for 30-45 seconds and stored at -80°C in a Sanyo Ultralow, MDF 592 freezer.

The tissue parts were cut into sections 5-8 microns thick by using a Kryostat 1720, Leitz, Mgw Landa. 6 slices from each of the 3 brain tissue parts were cut from each rat and put on Superfrost Plus microscope slides and stored in the -80°C freezer.

6.2.3 Staining methods

Hematoxylin and Eosin (H&E) staining was performed on 1 slide from each part of the brain from each of the 40 animals. The H&E staining was performed at Gades Institutt by the lab technicians Laila Vårdal and Edith Fick.

Immunofluorescence cell staining was performed on the tissue sections according to the procedure given from Santa Cruz Biotechnology.

Five slides from the right frontal cortex of each animal were used and five animals from each of the four main groups were tested.

The slides were fixed in cold acetone 50% and PBS 50% for 30 seconds followed by fixation in cold acetone 100% for 5 minutes. Then the slides were washed in 3 changes of PBS for 5 minutes each.
Incubation of the antibodies was performed at room temperature. Tissue sections were incubated with 10 % donkey serum, in PBS, for 20 minutes to suppress non-specific binding of IgG, and then washed with PBS.

From each animal 1 tissue section was incubated with no primary antibody (only 1.5% donkey serum), 2 tissue sections were incubated with primary goat polyclonal antibody GLUT1 and GLUT3 respectively and 2 tissue sections were incubated with blocking peptide mixed with GLUT1 and GLUT3 respectively, for 60 minutes.

The optimal primary antibody concentration was determined by titration, range 0, 5-20 µg/mL. 10 µg/mL of the primary antibody in PBS with 1.5 % donkey serum turned out to be the concentration giving the finest staining results, followed by washing with 3 changes of PBS.

By incubation with blocking peptides, 5 µl of GLUT1 or GLUT3 was mixed with 25 µl of blocking peptide GLUT1 or GLUT3 respectively and then mixed with 20 µl of PBS with 1.5 % donkey serum followed by an overnight incubation period at 4° C. The antibody/blocking peptide mixture was then diluted by adding 50 µl of PBS with 1.5 % donkey serum.

Sections were further incubated in a dark chamber for 45 minutes with the fluorochrome conjugated secondary antibody, donkey anti-goat IgG-FITC, 2.0 µg/mL, in PBS with 1.5 % donkey serum, followed by washing with 3 changes of PBS.

The sections were mounted with UltraCruz Mounting Medium and stored dark and refrigerated at 4°C. Finally the sections were examined by the use of a morfometry system including a fluorescence microscope, BX51 Olympus.

GLUT1 and GLUT3 staining results from the 4 main groups were blindly examined by Professor Roland Jonson, Brøegelmanns Laboratory, University of Bergen.
7 RESULTS

7.1 Drinking intake and weight gain

As shown in figure 10 below, the 4 main groups of rats had different drinking intake; the placebo group had the highest intake and the fluoxetine group had the lowest intake. All the groups increased their intake from week 1 to week 2 as there were less days measured in week 1. From the start the groups got pure water and then from week 2 and forward, with one week interval, the groups got medicines added in their drinking water and a fall in water intake can then be seen which further improves again. As the control group got no medicines, only citric acid, in their drinking water the curve does not seem to drop similarly. The citric acid does not seem to affect the water intake.

![Figure 10 Drinking intake](image-url)

<table>
<thead>
<tr>
<th>Group</th>
<th>Average weekly drinking intake per cage (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluoxetine</td>
<td>137</td>
</tr>
<tr>
<td>Reboxetine</td>
<td>266</td>
</tr>
<tr>
<td>Sibutramine</td>
<td>234</td>
</tr>
<tr>
<td>Control</td>
<td>292</td>
</tr>
</tbody>
</table>

Table 1 Average drinking intake
As figure 11 below shows, the different groups are gaining weight during the weeks registered, except the fluoxetine group which reduces weight after starting the medication, but then slowly gains some weight but still less than the other groups. The reboxetine group has the most even weight gain curve. Also the sibutramine group loses weight after 4 weeks and throughout the rest of the period of medication then slowly gains some weight. The control group has an even weight gain as well. Only 6 of the animals from this group started from week 1 and the remaining 4 animals were ready for the experiment from week 5, explaining the sudden drop in the curve during week 5.

![Figure 11 Weight gain](image)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Inhibitor of 5-HT reuptake</th>
<th>Inhibitor of NA reuptake</th>
<th>Clinical effect on depression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluoxetine</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Reboxetine</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sibutramine</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2 Reuptake inhibition and clinical effect on depression.
7.2 Laboratory results

Tests were run to measure concentration of drug mixed in the drinking water. As shown in table 3, the fluoxetine drinking water shows lower measured concentrations than the desired concentration of 400 mg/L. Similarly the measured and desired sibutramine concentrations deviate while the concentration of reboxetine measured in the drinking water is closest to the desired concentration.

<table>
<thead>
<tr>
<th>Name drinking water</th>
<th>Fluoxetine (mg/L)</th>
<th>Reboxetine (mg/L)</th>
<th>Sibutramine (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluoxetine, 400 mg/L 13.09.07</td>
<td>237</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluoxetine, 400 mg/L 14.11.07</td>
<td>197</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluoxetine, 400 mg/L 13.12.07</td>
<td>186</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reboxetine, 40 mg/L 07.09.07</td>
<td></td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Reboxetine, 100 mg/L 23.11.07</td>
<td></td>
<td>119</td>
<td></td>
</tr>
<tr>
<td>Reboxetine, 100 mg/L 06.12.07</td>
<td></td>
<td>117</td>
<td></td>
</tr>
<tr>
<td>Reboxetine, 100 mg/L 27.12.07</td>
<td></td>
<td>119</td>
<td></td>
</tr>
<tr>
<td>Sibutramine, 225 mg/L 19.09.07</td>
<td></td>
<td></td>
<td>91</td>
</tr>
<tr>
<td>Sibutramine, 225 mg/L 09.11.07</td>
<td></td>
<td></td>
<td>93</td>
</tr>
<tr>
<td>Sibutramine, 225 mg/L 29.11.07</td>
<td></td>
<td></td>
<td>91</td>
</tr>
<tr>
<td>Sibutramine, 225 mg/L 19.12.07</td>
<td></td>
<td></td>
<td>86</td>
</tr>
</tbody>
</table>

Table 3 Concentration of drug in drinking water
Table 4 shows the results of the blood samples. F is short for fluoxetine, R is short for reboxetine and S is short for sibutramine, followed by the date the blood sample is taken. B is short for cage followed by cage number and M is short for marked (tail marked red) and U for unmarked rat. The blood samples show great variations in concentration of drug in the blood among rats within the same group.

<table>
<thead>
<tr>
<th>Rat name</th>
<th>Reboxetine (ng/mL)</th>
<th>Sibutramine (ng/mL)</th>
<th>Fluoxetine (ng/mL)</th>
<th>Norfluoxetine (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F 07.01.08., B1, M</td>
<td>214</td>
<td></td>
<td>4610</td>
<td></td>
</tr>
<tr>
<td>F 07.01.08., B1, U</td>
<td>347</td>
<td></td>
<td>9249</td>
<td></td>
</tr>
<tr>
<td>F 08.01.08., B2, M</td>
<td>152</td>
<td></td>
<td>4034</td>
<td></td>
</tr>
<tr>
<td>F 08.01.08., B2, U</td>
<td>288</td>
<td></td>
<td>6808</td>
<td></td>
</tr>
<tr>
<td>F 08.01.08., B3, M</td>
<td>309</td>
<td></td>
<td>8414</td>
<td></td>
</tr>
<tr>
<td>F 08.01.08., B3, U</td>
<td>497</td>
<td></td>
<td>12711</td>
<td></td>
</tr>
<tr>
<td>F 09.01.08., B4, M</td>
<td>212</td>
<td></td>
<td>4782</td>
<td></td>
</tr>
<tr>
<td>F 09.01.08., B4, U</td>
<td>184</td>
<td></td>
<td>4762</td>
<td></td>
</tr>
<tr>
<td>F 09.01.08., B5, M</td>
<td>453</td>
<td></td>
<td>11342</td>
<td></td>
</tr>
<tr>
<td>F 09.01.08., B5, U</td>
<td>357</td>
<td></td>
<td>8601</td>
<td></td>
</tr>
<tr>
<td>R 14.01.08., B10, M</td>
<td>0,55</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R 14.01.08., B10, U</td>
<td>3,89</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R 14.01.08., B6, M</td>
<td>0,43</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R 14.01.08., B6, U</td>
<td>12,52</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R 14.01.08., B7, M</td>
<td>0,10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R 14.01.08., B8, M</td>
<td>0,29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R 14.01.08., B8, U</td>
<td>16,03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R 14.01.08., B9, M</td>
<td>0,50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R 14.01.08., B9, U</td>
<td>0,08</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S 21.01.08., B11, U</td>
<td>0,93</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S 21.01.08., B11, M</td>
<td>2,06</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S 21.01.08., B12, M</td>
<td>10,39</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S 21.01.08., B12, U</td>
<td>1,27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S 21.01.08., B13, M</td>
<td>0,91</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S 21.01.08., B13, U</td>
<td>0,72</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>S 21.01.08., B14, M</td>
<td>2,56</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S 21.01.08., B14, U</td>
<td>14,45</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S 21.01.08., B15, M</td>
<td>1,09</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S 21.01.08., B15, U</td>
<td>1,61</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4 Blood sample results
7.3 Staining results

Samples of H&E staining showed that the brain tissue was intact and approved for further investigation. One sample of the cryostat sections is shown below in figure 12.

Figure 12  H&E staining result
The main results from the immunofluorescence staining procedure are seen in figure 13 below. 5 series was made in each of the 4 main groups of rats. The first slide in each of the series, slide 1, 6, 11, 14 and 17, contained no primary antibody (-Pr), followed by the second slide in each series, slide 2, 7, 12, 15 and 18 containing GLUT1 primary antibody (G1). The third slide in each series, slide 3, 8, 13, 16 and 19 contained GLUT3 primary antibody (G3). In addition, the two first series in each main group included two slides with antibody/blocking peptide mixture GLUT1 and GLUT3 each. Slide 4 and 9 contained GLUT1 antibody/blocking peptide mixture, (G1+bl), and slid 5 and 10 contained GLUT3 antibody/blocking peptide mixture, (G3+bl).

Slides colored blue refers to a negative result with no staining, slides colored yellow were weakly positive and the slides colored red were positive.
<table>
<thead>
<tr>
<th>Group 1 Fluoextine</th>
<th>Group 2 Reboextine</th>
</tr>
</thead>
<tbody>
<tr>
<td>-PR 1 G1 2 G3 3 G1 +bl 4 G3 +bl 5</td>
<td></td>
</tr>
<tr>
<td>-PR 6 G1 7 G3 8 G1 +bl 9 G3 +bl 10</td>
<td></td>
</tr>
<tr>
<td>-PR 11 G1 12 G3 13</td>
<td></td>
</tr>
<tr>
<td>-PR 14 G1 15 G3 16</td>
<td></td>
</tr>
<tr>
<td>-PR 17 G1 18 G3 19</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group 3 Sibutramine</th>
<th>Group 4 Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>-PR 1 G1 2 G3 3 G1 +bl 4 G3 +bl 5</td>
<td></td>
</tr>
<tr>
<td>-PR 6 G1 7 G3 8 G1 +bl 9 G3 +bl 10</td>
<td></td>
</tr>
<tr>
<td>-PR 11 G1 12 G3 13</td>
<td></td>
</tr>
<tr>
<td>-PR 14 G1 15 G3 16</td>
<td></td>
</tr>
<tr>
<td>-PR 17 G1 18 G3 19</td>
<td></td>
</tr>
</tbody>
</table>

Figure 13 Immunofluorescence staining results
Table 5 shows the connection between concentration of drug in the blood and the staining results. Bl.conc. is short for blood concentration.

<table>
<thead>
<tr>
<th>Rat name</th>
<th>Fluoxetine Bl.conc (ng/mL)</th>
<th>Reboxetine Bl.conc (ng/mL)</th>
<th>Sibutramine Bl.conc (ng/mL)</th>
<th>GLUT1</th>
<th>GLUT3</th>
</tr>
</thead>
<tbody>
<tr>
<td>B3U</td>
<td>497</td>
<td></td>
<td></td>
<td>Weak</td>
<td>Positive</td>
</tr>
<tr>
<td>B4M</td>
<td>212</td>
<td></td>
<td></td>
<td>Weak</td>
<td>Positive</td>
</tr>
<tr>
<td>B5U</td>
<td>357</td>
<td></td>
<td></td>
<td>Weak</td>
<td>Weak</td>
</tr>
<tr>
<td>B2M</td>
<td>152</td>
<td></td>
<td></td>
<td>Weak</td>
<td>Weak</td>
</tr>
<tr>
<td>B2U</td>
<td>288</td>
<td></td>
<td></td>
<td>Weak</td>
<td>Negative</td>
</tr>
<tr>
<td>B6M</td>
<td>0.43</td>
<td></td>
<td></td>
<td>Positive</td>
<td>Weak</td>
</tr>
<tr>
<td>B7U</td>
<td>-</td>
<td></td>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>B8M</td>
<td>0.29</td>
<td></td>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>B6U</td>
<td>12.52</td>
<td></td>
<td></td>
<td>Weak</td>
<td>Weak</td>
</tr>
<tr>
<td>B10M</td>
<td>0.55</td>
<td></td>
<td></td>
<td>Negative</td>
<td>Weak</td>
</tr>
<tr>
<td>B14M</td>
<td>2.56</td>
<td></td>
<td></td>
<td>Weak</td>
<td>Positive</td>
</tr>
<tr>
<td>B14U</td>
<td>14.45</td>
<td></td>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>B12U</td>
<td>1.27</td>
<td></td>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>B11U</td>
<td>0.93</td>
<td></td>
<td></td>
<td>Negative</td>
<td>Weak</td>
</tr>
<tr>
<td>B13U</td>
<td>0.72</td>
<td></td>
<td></td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Table 5 Blood concentrations and staining results
Figure 14 shows the best picture of the immunofluorescence staining of GLUT1. Compared to GLUT3, GLUT1 appeared as small clusters.

Figure 14 GLUT1, control

Figure 15 shows the best picture of GLUT3. The appearance was quite distinctive for this glucose transporter.

Figure 15 GLUT3, control
Figure 16 No primary antibody, reboxetine

Figure 17 GLUT1, fluoxetine

Figure 18 GLUT3, reboxetine
Figure 16-20 illustrate the typical images seen in the microscope. Slides with no primary antibody added, as illustrated in figure 16 from the reboxetine group, appeared rather dark and no staining was seen. Figure 17 shows a weak staining of GLUT1 from the fluoxetine group while figure 18 shows GLUT3 staining from the reboxetine group. Figure 19 and 20, both from the sibutramine group; illustrate images where antibody/blocking peptide mixture is added, GLUT1 and GLUT3 respectively. These slides typically showed suppressed or no staining at all.
8 DISCUSSION

8.1 Discussion of rat model and materials

In this study rats were considered the best option to use, as most of the research literature on astroglia reports on the use of rats. Thus the study can be compared to existing literature. Rats are preferred to mice as the brain is bigger and easier to handle.

When a patient is treated with an antidepressant for the first time it may take 3-8 weeks until clinical effect is gained. The time range of this experiment was based on this fact. To be quite sure that the rats were treated for a satisfyingly long enough period of time, the medication period was determined to last for at least 18 weeks. The use of cell culture would not be considered an option as it is difficult to keep the tissue in culture for such an extended period of time. It would also be uncertain if all the tentative links between pharmacological effect and influence of glucose transporters would be maintained sufficiently. In addition, tissue was needed where 3-dimensional integrity and all the necessary functions were intact.

Similar studies dealing with such a pharmacological experiment where noradrenergic medicines were used to study the noradrenergic effect on the regulation of glucose transporters was not found ahead of this study. Only studies where noradrenaline itself were used, was found. Thus the amount of animals needed for the study was arbitrary chosen. 10 rats in each group were considered sufficient for the analyzing of the results and at the same time ethics concerning animal research was considered, by using as small amounts of animals as possible.

As table 2 shows, fluoxetine inhibits reuptake of 5-HT and it has clinical effect on depression, while reboxetine inhibits reuptake of NA and it has clinical effect on depression. Sibutramine on the other hand, inhibits reuptake of both 5-HT and NA but has no effect on depression. Concerning the existing documentation indicating that noradrenergic influence can stimulate and increase the amount of glucose transporters on the surface of different cell types [139, 144, 145]; the aim is to compare reboxetine and sibutramine. If they both get an increase in the amount of glucose transporters, it could indicate that this increase is not the reason why only reboxetin has a clinical
effect on depression. But if only reboxetine results in an increase in glucosetransporters and sibutramine, or fluoxetine, do not, this could support the theory that the effect on depression is related to the increased amount of glucosetransporters as an attempt to counteract the dysfunction in glucose turnover in the brain.

Sibutramine turned out difficult to dissolve in pure water with dissolution of 18 mg/L by neutral pH [Personal communication Hundal, based on earlier pilot study]. The pH was lowered by adding concentrated citric acid solution into the solution to lower the pH and thereby increasing the dissolution of sibutramine. To make the conditions of the research as similar as possible, 15 ml of concentrated citric acid water was added to the drinking water of all the 4 groups. The adding of citric acid was discussed with Aurora Brønstad and was considered to do no harm to the rats. Also earlier experiments had found that the added amount of citric acid was sufficient to lower pH adequately and thus dissolving the sibutramine [Personal communication Hundal].

It was decided to place two rats in each cage based on animal ethics concerning the welfare of the rats.

### 8.2 Discussion of method and results

As shown in figure 10 and table 1, the drinking intake of the different groups varies, and the fluoxetine group had the lowest intake of all the groups, under half of the amount of the control group. It might be that the former group was dehydrated, which could affect the blood and their blood values. Tests were however not taken to measure signs of dehydration. The flavor of the drinking water could be affected by dissolving medicines in it and could be a reason why all the groups had lower drinking intake compared to the control group.

The fluoxetine group similarly had the lowest weight and weight gain during the testing period, which seemed to stabilize on about 210 grams compared to the sibutramine group which seemed to stabilize on about 240 grams. The reboxetine and control group both seemed to stabilize on quite similar weights, about 260 and 265 grams respectively, as shown in figure 11. In this study the fluoxetine group got an
average daily dose of 16 mg/kg of fluoxetine per cage containing two animals, based on their drinking intake. Former studies have found that rats receiving 10 mg/kg/day of fluoxetine developed anorexia [47]. This could also be the case in this study and could be a factor explaining the low weight in the fluoxetine group. The reboxetine group received an average daily dose of 4.52 mg/kg of reboxetine per cage, which were increased to 12.74 mg/kg after 12 weeks. Other studies have used this drug in concentrations from 0.128 mg/kg/day to 20-40 mg/kg/day [43, 45, 49], suggesting that the doses in this study are within the range of doses normally used in rat experiments. This is also the case regarding sibutramine, where studies have used concentrations ranging from 0.9-30 mg/kg/day [53, 54]. In this study the average daily dose of sibutramine was 12.41 mg/kg per cage.

Regarding therapeutic concentrations of fluoxetine, reboxetine or sibutramine in rats, there are no such concentrations existing [personal communication Professor Arne Reimers, seksjonsoverlege, spes.klin. farmakologi, St.Olavs Hospital]. The concentrations used in this study are therefore compared to doses of the drugs used in other studies. The average daily doses of drugs in each of the main groups are written as doses per cage, as it cannot be assumed that the two rats in each cage are drinking equally amounts of water. But it has to be kept in mind that the dose is shared by two rats. The average daily dose of drug in the three main groups is based on their measured average drinking intake found in table 1 and the actual measured concentration of drug found in their drinking water.

Tests were run to measure the concentration of drug in the drinking water, illustrated in table 3. Test results showed deviations from the desired concentrations for both fluoxetine and sibutramine, both having about half, or less, of the wanted concentration. This may have affected the drugs clinical effect on the rats. All the solutions containing drugs were filtrated to get a clear solution of medicine and water. The filtration was done 2-3 days after the addition of drug so it would be dissolved satisfyingly. However, it might be that some of the drug was removed by filtration.

The reboxetine group got increased concentration of drug in their drinking water during the medication period due to results from Hundal’s pilot study showing that these rats had too low concentrations of drugs in their blood [Personal communication
Hundal]. The dose was increased from 40 to 100 mg/L after 12 weeks, when only 6 weeks remained of the medication period. This may have affected the drugs clinical effect on the rats as well and the period may have been too short to potentially increase the amount of glucose transporters to a maximum extent. The concentration of reboxetine in the drinking water was doubled compared to former pilot studies performed by Hundal [Personal communication Hundal], but still the blood results show low concentrations of the drug in the blood.

Alternatively, the rats could be anaesthetized while they were given drugs administered into the blood instead of mixing the drugs in their drinking water. In this way it is possible that their water intake and possibly their weight gain would be more similar to each other and the blood test results might have been more even within the groups. However, there are more practical methods which could be considered. One study administered fluoxetine by mouth through a plastic tube and reboxetine was injected intramuscularly [45] while other studies have injected drugs intraperitoneally [53]. By using some of these techniques it would be made sure that each rat got the desired daily dose of drug administered into the body. In this study it was considered to be less stressful for the rats to receive the drugs in their drinking water.

Table 4 shows great variations in blood concentrations of drugs measured within the groups. This may further affect the staining results. The blood tests were taken by cardiac puncture, which is a rather difficult procedure and much practice is needed to accomplish the right technique. The tests need to be taken relatively quickly and carefully, ideally heart puncture should be avoided, to avoid hemolysis of the blood and redistribution. Blood tests from a living animal would give more reliable results as redistribution is a very important issue regarding antidepressants as well as antipsychotics [Personal communication Kjell Ove Fossan].

This study had to be done within a limited period of time and therefore time was limited to rehearse the cardiac puncture technique. Heart puncture may have happened and animals may have died during the procedure. These are factors which may have contributed to the varying blood test results.
Ideally the drinking intake to each single rat should be measured. In this study there were two animals in each cage drinking from the same bottle; consequently it is unknown how much each of the rats were drinking and it cannot be assumed that they were drinking the same amounts of water. The rats would then be administered uneven daily doses of drugs.

Blood samples could have been taken from other parts of the body, for example the tail vein or it could be interesting to measure the concentration of drugs in defined areas of the brain tissue, to get more reliable drug concentration results.

Immunofluorescence staining results presented in figure 13 were quite varying. All the slides with no primary antibody showed no staining, which indicated that there were little unspecific binding of the secondary antibodies. Also the mixture of GLUT1 and blocking peptide and the mixture of GLUT3 and blocking peptide showed suppressed or no staining, demonstrating specificity of the observed staining. In the fluoxetine group most GLUT1 slides were positive, while the reboxetine group had one slide stained weakly positive but in addition had one positive slide. The sibutramine group had only one slide that stained weakly positive of GLUT1 and the control group had one positive slide of GLUT1.

Overall, more staining was seen on the GLUT3 slides in all the groups. The fluoxetine group had 2 positive slides and 2 weakly positive slides, while the reboxetine group had 2 positive slides and 3 weakly positive slides. The sibutramine group had 3 positive slides and 1 weakly positive, whereas the control group had the best staining of 4 positive slides and 1 weakly positive slide.

In general the results are quite variable and it is difficult to make any conclusions regarding possible long-term effects of any of the drugs. Figure 13 actually shows that the control group had the best staining results of GLUT3. It appears like the staining method is working, based on the fact that staining is only seen on the GLUT1 and the GLUT3 slides. At the starting point of the study, the aim was to be able to count the amounts of glucose transporters through the microscope. This turned out to be difficult by using this method. The aim was changed into looking at the amount and strength of immunofluorescence staining instead.
Controls were used to validate the staining results. As mentioned above the first slide in each series contained no primary antibody as a specificity test. The entire immunohistochemical procedure was carried out, except the addition of primary antibody and no staining was seen in these slides, indicating that the secondary antibodies were specific in their binding.

Positive controls contain the target protein. In this study the controls were mixed GLUT1 or GLUT3 with their corresponding blocking peptides in such a concentration that they would block the binding of the primary antibody to the tissue antigen, which in this study resulted in no staining.

Western blotting could be an option to detect specific proteins after separation of the proteins by the use of gel electrophoresis. This was not an option due to the limited time range of this project.

Based on the varying staining results it was decided that five animals in each group was enough to do research on. It was not thought that further staining would give any better staining results. The staining was only done on one part of the brain, the right frontal cortex as the time range of the project was limited.

Also the possibility was examined that the rats with the highest concentration of drug in their blood would give the best staining results, but as showed in table 5 this was not the case. In the fluoxetine group the highest blood concentration measured was 497 ng/mL which resulted in weak staining of GLUT1 and positive staining of GLUT3. The same staining result was seen from a rat with a blood concentration of 212 ng/mL, which is more than two times lower than the concentration of the former. At the same time a concentration of 357 ng/mL only resulted in weak staining of both GLUT1 and GLUT3.

Similar results can be seen in the reboxetine group where a concentration of 0.43 ng/mL of drug in the blood resulted in positive staining of GLUT1 and weak staining of GLUT3 while the highest concentration measured in this group, 12.52 ng/mL, only resulted in weak staining of both GLUT1 and GLUT3.
In the sibutramine group parallel tendencies can be seen; a concentration of 2.56 ng/mL of drug in the blood gave weak staining of GLUT1 and positive staining of GLUT3, while both concentrations of 14.45 ng/mL as well as 1.27 ng/mL gave negative staining of GLUT1 and positive staining of GLUT3.

There seems not to be a logical connection between staining results and amount of drug in the blood of the rats. This might be due to the blood sample procedure, cardiac puncture, and that it might not have been performed sufficiently, as explained above. The blood samples would then be considered being incorrect.

8.3 Possible future studies

There are many theories on the development and causes of depression. This study was based on the theory where dysfunction in astroglial bioenergetics is thought to be the cause of depression. The findings of this study have however not contributed to give further evidence for this hypothesis as it could not be shown that noradrenergic stimulation would increase the amount of glucose transporters in the brain. But the hypothesis still remains very interesting and more studies are needed to explore it further. Efforts then have to be made to make sure the animals get the proper concentration of drugs in their blood until further procedures can be performed. The choice of staining method must be considered. Other methods than immunofluorescence staining should be investigated if the aim is to be able to count the amount of glucose transporters in the microscope. The choice of method for blood sampling must be considered as well.

It is beyond doubt that the astrocytes will continue to get increasing interest in the years to come as their functions and importance in brain function get more apparent.
9 CONCLUSION

The aim of the present study was to investigate if long-term noradrenergic stimulation would increase the amount of glucose transporters in the brain; it was then thought that only reboxetine, the selective noradrenergic reuptake inhibitor, would get increased amounts of glucose transporters compared to the other groups. This could not be demonstrated. In both the reboxetine group and the control group one positive slide of GLUT1 was found, and twice as many GLUT3 positive slides were found in the control group compared to the reboxetine group.

The immunofluorescence staining could not demonstrate that the levels of GLUT1 and GLUT3 were affected by the drugs used. The conditions were however not optimal, considering the lowered concentrations of drugs in the drinking water, the not optimal administration of drugs to the rats and the possible incorrect blood test results. Optimal conditions should be gained to evaluate the results properly.
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