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The effect of NAPQI on neurotransmitter transporters

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I dedicate my thesis to my wonderful aunt Gülay Ertop whom I lost during my thesis project. You will live in my heart forever.

ABSTRACT

Paracetamol (Acetaminophen) is in use as an analgesic drug for decades, although its mechanism of action is not understood yet. Among various theories, one states that the toxic metabolite of Paracetamol NAPQI (N-acetyl-p-benzoquinone imine) can modify TRP-channels, leading to analgesia via this pathway. NAPQI is a cysteine-modifying reagent that, under therapeutic concentrations of paracetamol, binds to the sulphur group of glutathione and is excreted. When the glutathione stores are depleted, NAPQI can irreversibly bind cysteine residues of other proteins, rendering them non-functional. This can possibly result in life-threatening liver failure.
The analgesic action of paracetamol also seems to include the serotonergic system. Serotonin and norepinephrine can blunt pain sensation via descending neurons. The serotonin transporter (SERT), which is essential for the reuptake of serotonin, has been reported to be susceptible to cysteine modifying reagents.

Therefore, we investigated a possible effect of NAPQI on SERT by performing radioligand uptake assays in cells stably expressing wildtpe SERT. Furthermore, we generated alanine mutants of cysteines in SERT to investigate the mechanism of NAPQI.
Our results show that the metabolite NAPQI is capable of inhibiting the uptake of serotonin. Interestingly, the double mutant C109A/C357A was inhibited by NAPQI similar to wildtype. Furthermore, the inhibition of SERT was clearly reversible and competing with serotonin.
The transporter for γ-aminbutyric acid, GAT, was tested for a similar effect by NAPQI. Results show that, unlike SERT, GAT was not inhibited.

We conclude that NAPQI inhibits SERT in a specific manner, and, unlike its action on TRP channels, it does so in a reversible and competitive manner. Blockade of SERT could increase the analgesic effect of descending antinociceptive neurons, providing an additional explanation for the analgesic mechanism of paracetamol.

ZUSAMMENFASSUNG

Paracetamol (Acetaminophen) ist ein seit Jahrzehnten gebräuchliches Schmerzmittel, obwohl dessen Wirkmechanismus nicht aufgeklärt ist. Laut eine vieler Theorien kann NAPQI (N-Acetyl-p-Benzoquinonimin), der toxische Metabolit des Paracetamol, TRP-Kanäle modifizieren und so zu einer analgetischen Wirkung führen. NAPQI ist eine Cystein-modifizierende Substanz, die bei therapeutischen Konzentrationen von Paracetamol nach Bindung an die Schwefelgruppe von Glutathion ausgeschieden wird. Bei Erschöpfung des Glutathion-Speichers kann NAPQI irreversibel an Cystein-Seitenketten von anderen Proteinen binden, die daraufhin ihre Funktion verlieren. Dieses Ereignis kann möglicherweise zu einem Leberversagen führen.
Die analgetische Wirkung des Paracetamol könnte das serotonerge System der Schmerzempfindung auch mit einschließen. Serotonin und Norepinephrin können die Schmerzempfindung mit Hilfe efferenter Neuronen abstumpfen. Der Serotonintransporter SERT ist für die Wiederaufnahme von Serotonin zuständig und empfindlich auf Cystein-modifizierende Substanzen.
Aufgrund dieser Datenlage wurde mit Hilfe von Radioliganden-Aufnahme Assays eine mögliche Wirkung von NAPQI auf SERT in Zellen mit Wildtyp-SERT untersucht. Zusätzlich wurden Alanin-Mutanten einiger Cysteine des SERT erstellt, um die Wirkung des NAPQI durch die Cysteine festzustellen.

Die Ergebnisse dieser Arbeit zeigen, dass der Metabolit NAPQI die Aufnahme von Serotonin hemmen kann. Die Doppelmutante C109A/C357A wurde von NAPQI wie bei Wildtyp inhbiert. Wir fanden zusätzlich heraus, dass die Hemmung von SERT deutlich reversibel und kompetitiv mit Serotonin war. Als Kontrolle wurde der Transporter für γ-Amino-Buttersäure (GABA) auch nach einer möglichen Wirkung des NAPQI überprüft, wobei hier eine irreversible Hemmung festgestellt wurde.

Mit Hilfe dieser Arbeit haben wir eine spezifische Hemmung von SERT durch NAPQI gezeigt, die im Gegensatz zu TRP Kanälen reversibel und kompetitiv ist. Diese Hemmung könnte die analgetische Wirkung auf antinozizeptive Neuronen erhöhen und so auf einen zusätzlichen Wirkmechanismus von Paracetamol hindeuten.

1. INTRODUCTION

1.1. Neurotransmitters and Neurotransmitter Transporters

Before the mid-nineteenth century, scientists assumed that neurons communicate by electrical signalling. The work of Alan Hodgkin and Andrew Huxley in 1952 (Hodgkin & Huxley 1952) confirmed that action potentials are transferred through the nerve cells by ion permeation. Otto Loewi identified the transmission via Vagustoff and Henry Dale identified that the Vagustoff to be Acetylcholine (Dale and Dudley, 1929). Loewi and Dale won the 1936 Medicine and Physiology Nobel Award for this important discovery.

Neurotransmitters are small chemical compounds also known as chemical messengers, which enable neurotransmission. They transmit signals from one nerve cell (neuron) to another "target" neuron.

Major neurotransmitters include:

- Amino acids such as glutamate, D-serine, γ-aminobutyric acid (GABA), glycine
- Peptides such as somatostatin, opioid peptides
- Monoamines such as serotonin (SER, 5-HT), dopamine (DA), norepinephrine (noradrenaline; NE, NA), epinephrine (adrenaline), histamine.

Neurotransmitters are classified into "inhibitory" or "excitatory" depending on their effect on the target cell: if they hyperpolarize the target cell, they are inhibitory (they increase the threshold for the cell to fire an action potential), whereas if they depolarize it, they are excitatory. The ion selectivity of the corresponding receptors at the postsynaptic membrane determines whether the cell is hyperpolarized or depolarized. Neurotransmission, also called synaptic transmission, is the process by which neurotransmitters are released by a neuron (the presynaptic neuron), and bind to and activate the receptors of another neuron (the postsynaptic neuron). Neurotransmission is essential for the process of communication between two neurons.

The typical course of a neurotransmission is as follows: when an action potential of an excited neuron reaches the synapse, it leads to depolarization of the presynaptic membrane, followed by influx of Ca^{2+} into the intracellular compartment. The rise of Ca^{2+} induces neurotransmitter-filled vesicles to fuse with the presynaptic membrane, releasing their content into the synaptic cleft (Südhof 2012). The released neurotransmitters bind to neurotransmitter receptors at the postsynaptic membrane (membrane of the target cell), leading to the depolarisation or hyperpolarisation of the cell. (Vanhatalo & Soinila 1998).

Receptor-mediated responses may result from the influx of ions via ionotropic receptors or lead to activation of intracellular signalling cascades through G protein-coupled receptors (metabotropic receptors).

1.2. Structure and function of neurotransmitter transporters

After release of neurotransmitters and receptor activation, neurotransmitters in excess have to be actively removed from the synaptic cleft by either enzymatic processing or reuptake by transporters which are high-affinity integral membrane proteins located on the presynaptic membrane (Masson et al. 1999).

Transporters for amino acids (e.g. SLC1, SLC7, SLC17), nucleotides (e.g. SLC28, SLC35), sugars (e.g. SLC2, SLC50, SLC37), and drugs (e.g. SLC47), play an important role in maintaining crucial processes within and between cells via controlling uptake and efflux (Colas et al., 2016) . The solute carrier

family 6 (SLC6) includes transporters for the inhibitory neurotransmitters such as GABA and glycine, proteinogenic amino acids, the metabolic compound creatine, the osmolytes taurine and betaine, as well as transporters for serotonin, dopamine and norepinephrine (Beuming et al. 2006). In the human genome, the solute carrier family 6 (SLC6) consists of 20 members (Chen et al. 2004; Bröer 2006). SLC6 transporters are not only located on neurons and glia, but also in many non-neural tissues. Furthermore, plenty of family members are expressed excessively in the kidney.

In the SLC6 family, approximately half of the transporters use the electrochemical potential of extracellular Na^+ ions as an energy source in order to transport their substrate across membrane (Table 1). Extracellular chloride ions are also co-transported, but the exact role of chloride in the transport process has not yet been fully determined. The bacterial orthologue LeuT has been first crystallized and is known to be chloride-independent (Zomot et al. 2007).

One interesting phenomenon exists in the literature regarding sodium/chloride-coupled neurotransmitter transporters: they can operate in two directions. As compared with other substances, amphetamines are well known to induce reverse transport, which has been recorded for many transporters (Roux & Supplisson 2000). By virtue of the low concentration of sodium ions in the cytoplasm compared to the extracellular space, reverse transport is per se a rare event. Nevertheless, in the event of large amounts of substrates transported, the resulting ion conductivity becomes much larger and reverse transport is quite likely to occur (Sitte & Freissmuth, 2010). For example, presence of releasing substrate, such as amphetamines, trigger reverse transport. For this effect, it seems that both the N-terminus and the oligomerization are necessary. Truncation experiments of the N terminus showed that the amphetamine-induced reverse transport in SERT was abrogated when a certain N-terminal stretch was missing (Sucic et al., 2010). This was also found in in DAT (Seidel et al. 2005). On N-terminal serines, PKC- and amphetamine-stimulated phosphorylation of DAT might to contribute to this mechanism by changing the conformation of DAT affirmative for reverse transport (Cervinski et al., 2005).

1.3. Monoamine transporters and psychostimulant action

Monoamine transporters belong to the SLC6 gene family and are responsible for the uptake of biogenic amines. This subfamily consists of the serotonin transporter (SERT, *SLC6A4*), the dopamine transporter (DAT, *SLC6A3*) and the norepinephrine transporter (NET, *SLC6A2*).

Human gene name	Protein name	Predominant substrates	Transport type/coupling ions	Predominant tissue distribution	Link to disease
SLC6A1	GAT1	GABA	C/Na+, Cl-	GABAergic neurons in central and peripheral nervous system, some non-neural tissues	Epilepsy, schizophrenia
SLC6A2	NET	norepinephrine, dopamine	C/Na+, Cl-	Central and peripheral nervous system, adrenal gland, placenta	Depression, orthostatic intolerance, anorexia nervosa, cardiovascular diseases
SLC6A3	DAT	dopamine	C/Na+, Cl-	Brain (dopaminergic neurons)	Parkinsonism, substance abuse, ADHD
SLC6A4	SERT	serotonin	C/Na+, Cl-, K+	Central and peripheral nervous system, epithelial cells, platelets	Anxiety, depression, autism, substance abuse

***Table 1**: Features of the members of the solute carrier 6 (SLC6) gene family. Figure adapted by* (Chen et al., 2004).

Monoamine transporters share a predicted structure (Kristensen et al. 2011) which includes more than 600 amino acids and twelve hydrophobic membrane-spanning segments (Blakely et al. 1991). The transport of substrate generally requires 1 sodium (Na^+) and 1 chloride (Cl^-) ion (symport) against 1 potassium (K^+) ion (antiport) (Pacholczyk et al. 1991; Guastella et al. 1990; Ramamoorthy et al. 1993).

Monoamine signalling is regulated by a different set of macromolecules, which includes biosynthetic enzymes, secretory proteins, pre- and postsynaptic receptors, transporters and ion channels.
They play a modulatory role in neurotransmission and are associated with various physiological functions such as regulation of movement, mood, attention and sleep. Disturbances in the regulation and function of the monoamines result in pathological conditions like sleep disturbances, Parkinson's disease and childhood parkinsonism, depression and other mood-related disorders (Kurian et al., 2011; Ng et al., 2015)
Monoamine transporters are pharmacological targets of antidepressants and psychostimulant drugs (Jayanthi & Ramamoorthy 2005). There is a large number of published studies describing that selective monoamine transporter inhibitors (e.g., paroxetine, mazindol, imipramine, desipramine, nisoxetine) are beneficial in the treatment of numerous brain-releated disorders such as anxiety disorders, mood disorders, schizophrenia, personality disorders, eating disorders, Parkinson's disease, Alzheimer's disease, depression, addiction and abuse, ADHD (attention deficit hyperactivity disorder), cocaine dependence, stroke, obesity, chronic pain, migraine, epilepsy, narcolepsy and multiple sclerosis (Sasaki-Adams & Kelley 2001; Zhou, Zhang, et al. 2003; Zhou, Kläß, et al. 2003; Smith et al. 1999; Zhang et al. 2002; Fleishaker 2000; Wong et al. 2000; Van Moffaert & Dierick 1999; Plewnia et al. 2002; Versiani et al. 2002).

These psychostimulant drugs not only inhibit the monoamine uptake via their transporters, but are also taken up by the transporters, leading to a reverse transport of monoamine transporters via a non-exocytotic mechanism (Sulzer et al. 2005; Sitte & Freissmuth 2010). Some of those substances (e.g. D-amphetamine,) are still largely used in the treatment of attention deficit hyperactivity disorder (ADHD) and prescribed for weight control and narcolepsy (Olfson et al., 2013) . In the central and peripheral nervous system, each monoamine controls different physiological and behavioural functions. Serotonin regulates aggression, mood, motivation, appetite, sleep and sexual activity. In addition, changes in the serotonin signalling have been linked to mental illnesses related to these biological processes (Coccaro 1989; Owens & Nemeroff 1994). Besides this, serotonin also has important peripheral actions, which include mediation of vasoconstriction, placental and gastrointestinal functions. Dysregulation of dopamine transmission is associated with attention/hyperactivity disorder (ADHD), addiction, schizophrenia, Tourette's syndrome and Parkinson's disease (Bannon et al. 1995). Mood, attention, arousal, stress-responsiveness and affective disorders are controlled by norepinephrine (Klimek et al., 1997; Leonard, 1997; Ressler & Nemeroff, 1999; Schildkraut, 1965). Selective serotonin reuptake inhibitors (SSRIs) such as escitalopram and paroxetine (Wong et al. 1995) and selective norepinephrine reuptake inhibitors (SNRI) such as reboxetine (Andersen et al. 2009) are successfully used in treatment of depressive disorder; methylphenidate is used in the treatment of ADHD; DARIs (dopamine reuptake inhibitors) are highlighted in the treatment of obesity.

Regulation of transporters can take place via phosphorylation-dependent and -independent post-translational modifications. Post-translational modifications can

a. alter intrinsic transport activity,
b. modify transporter turnover,
c. modulate exocytic fusion of transporter
d. modulate sequestration of transporter from the plasma membrane via regulating endocytic machinery pathways.

In addition, transporter regulation can also happen through their association with other interacting proteins via phosphorylation-dependent or -independent pathways (Eriksen et al. 2010).

1.3.1. Serotonin transporter

The Na^+/Cl^--dependent serotonin transporter (SERT) plays a role in the uptake of synaptic 5-HT. This is the main process of terminating serotonergic neurotransmission. A single gene, SLC6A4, encoding SERT (Ramamoorthy et al. 1993) is responsible for extracellular 5-HT clearance (Blakely et al. 1991; Hoffman et al. 1991; Lesch et al. 1993). Human SERT (hSERT) consists of 630 amino acids.

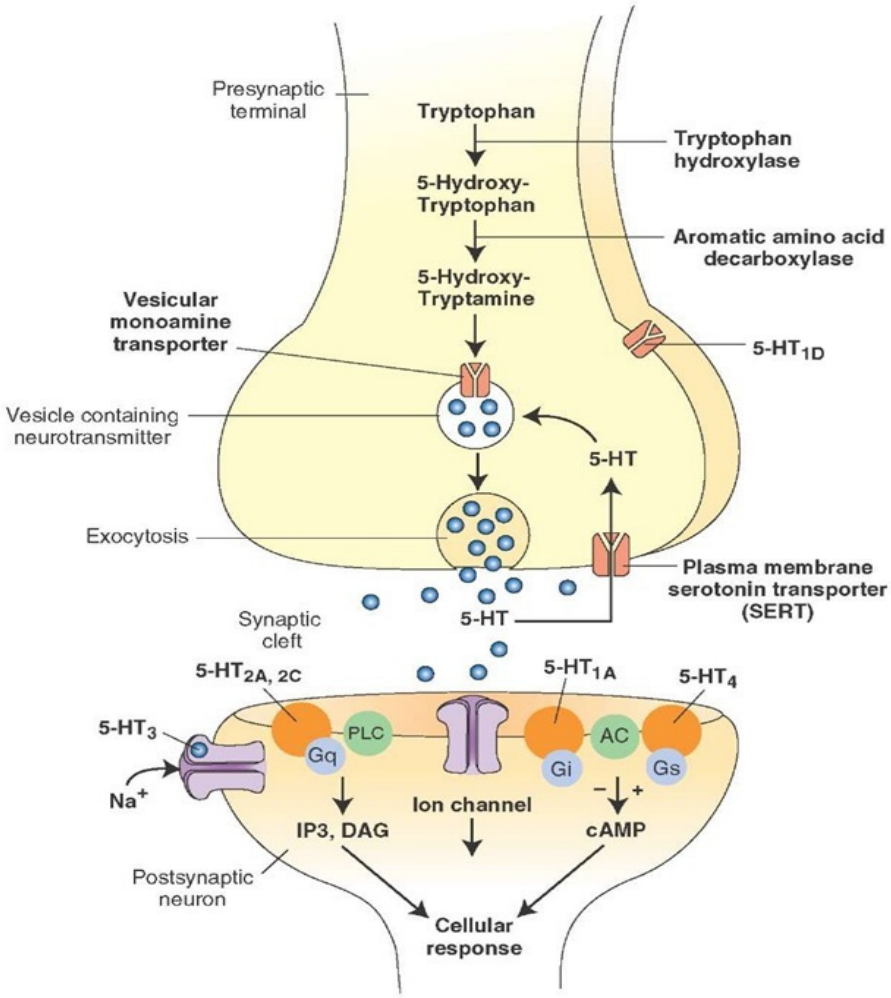


Figure 1: Synthesis and release of serotonin**.** cAMP = cyclic adenosine monophosphate; AC = adenylyl cyclase; DAG = diacylglycerol; G_i,G_q,G_s = different G-proteins; IP_3 = inositol triphosphate; 5-HT_{1A}, 5-HT_{1D}, 5-HT_{2A}, 5-$HT_{2C,}$ 5-HT_3, 5-HT_4 = different 5-HT receptors (figure from What-When-How.com.

Besides serotonergic neurons, SERT is expressed in peripheral tissue (Lesch et al. 1993; Rudnick 1977) including specialized cells of the gut (Gordon & Barnes 2003), placenta (Balkovetz et al. 1989), lung (Paczkowski et al. 1996), blood lymphocytes (Faraj et al. 1994; Gordon & Barnes 2003) and also platelets (Jayanthi et al. 2005; Carneiro & Blakely 2006; Carneiro et al. 2008).

Additionally, findings of modified SERT expression in various types of psychopathology point out the importance of SERT in maintaining normal brain function (Murphy et al. 2004). SERT also interacts with psychostimulants such as cocaine, which are inhibitors, while others like amphetamine derivatives including fenfluramine and 3,4-methylenedioxymethampetamine (MDMA or “Ecstasy”) are alternative substrates for SERT (Rudnick & Wall 1992).

Serotonin is one of the key components in the relay of pain, which is possible by descending serotonergic pathways. Especially in the periphery, it enhances the inflammatory signals and contributes hyperalgesia, the excessive sensation (Sommer, 2010).

1.3.2. Dopamine transporter

In and around the synapse, the concentration of dopamine (DA) is reduced by reuptake. The dopamine transporter (DAT) is a main regulator of dopamine neurotransmission and is located in chromosome 5p15.3 (Giros et al. 1992), encoding 620 amino acids.

DAT is mainly expressed in dopaminergic neurons (as shown in figure 2) and predominantly present in the brain. DAT is also found in the periphery, including lymphocytes (Amenta et al. 2001). DAT knockout mice exhibit hyperactivity, dwarfism, cognitive deficits, sleep dysregulation, alterations in gut motility, skeletal abnormalities and stereotypical behaviors (Gainetdinov & Caron 2003; Giros et al. 1996).

Furthermore, the dopamine transporter is the main molecular target for several psychostimulants such as cocaine, amphetamine and methamphetamine (Ritz et al. 1987; Kuhar et al. 1991).

The basis of DAT for normal DA clearance and signalling states that DAT dysfunction may apply to various brain disorders associated with dysregulation of DA transmission such as schizophrenia, affective disorders and addiction. Human neuroimaging and genetic studies demonstrate modified DAT availability or function in attention deficit hyperactivity disorder (ADHD). Two ADHD medications, methylphenidate and amphetamine target DAT (Logan et al. 2007; Volkow et al. 2001). Abnormal DAT regulation and function correlated with ADHD in which DAT coding variant A559V was identified (Mazei-Robison & Blakely 2005; Mazei-Robison et al. 2008).

Decreased mesocorticolimbic DA transmission is one of the identified symptoms of depression (Nestler & Carlezon 2006). Some medications with proven antidepressant effects in humans (e.g., nomifensine, amineptine) are DA uptake inhibitors.

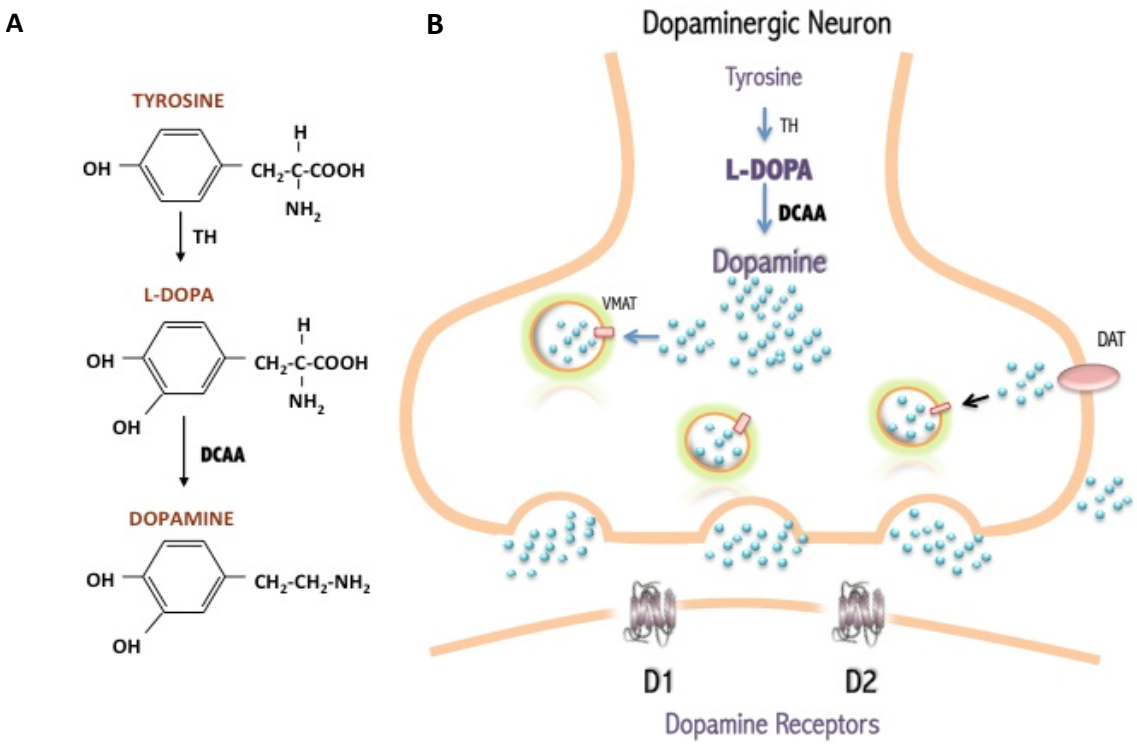


Figure 2: A, Pathway of biosynthesis of dopamine.; B, The fate of dopamine in a typical dopaminergic neuron after biosynthesis. Dopamine is stored in intracellular vesicles after uptake through the vesicular monoamine transporters (VMAT). After release, dopamine binds to dopamine receptors D1 and D2. Reuptake of excess dopamine is ensured with the dopamine transporter (DAT). Figure adapted from intechopen website.

1.3.3. Norepinephrine transporter

Norepinephrine (NE), also known as noradrenaline, is the main neurotransmitter of the sympathetic nervous system (Zhou 2004; Schroeter et al. 2000).

It is taken up by the norepinephrine transporter (NET) which is encoded by the SLC6A2 gene in humans (Pacholczyk et al. 1991) and is located in chromosome 16q13-q21 (16q12.2) (Brüss et al. 1993).

It has been demonstrated that NET is selectively expressed on NE nerve terminals which enable temporal and spatial control of the activity of NE (Foote et al. 1983; Moore & Bloom 1979). Several studies have demonstrated that NET is also expressed in peripheral tissue (e.g., adrenal glands, placenta, vas deferens (Jayanthi et al. 2002; Schroeter et al. 2000; Sung et al. 2003). NE is also a target for psychostimulants such as cocaine and amphetamines (Jayanthi et al. 2002; Pacholczyk et al. 1991). Previous studies have found out that the modified NET function is associated with mood, cardiovascular disorders and attention (Esler et al. 2006; Haenisch et al. 2009; Hahn et al. 2008; Kim et al. 2006; Rumantir et al. 2000; Shannon et al. 2000). In addition to that, NE plays an essential role in human physiology and pathology such as mood and sleep regulation (Young & Landsberg 1998). Therefore, NET is used in the treatment of mood and cognitive disorders (Blier 2001; Bönisch & Brüss 2006).

Moreover, norepinephrine signalling is controlled by NET, thereby, a crucial homeostatic mechanism and its dysregulation via psychostimulants is thought to provide the neurochemical and behavioral effects of psychostimulants (Dipace et al. 2007) and chronic stress (Miner et al. 2006).

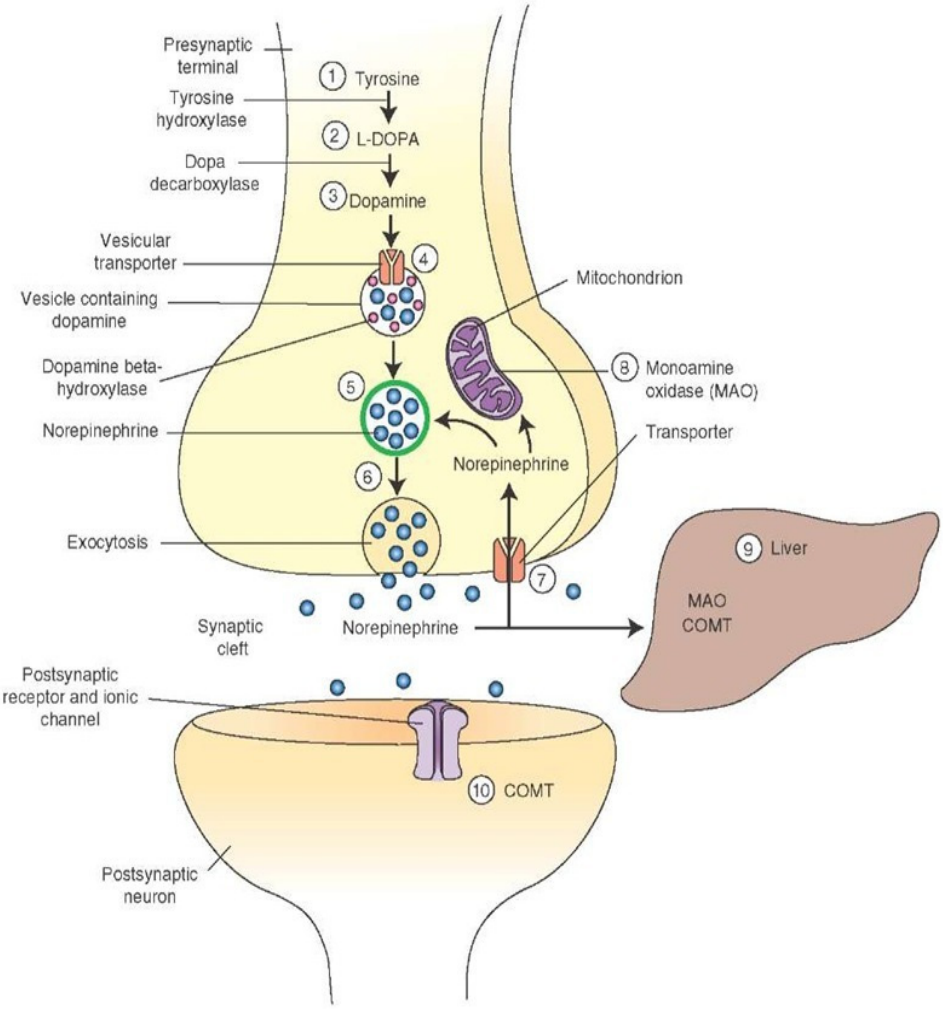


Figure 3: Synthesis and release of norepinephrine. COMT = catechol-O-methyltransferase (figure from what-when-how website)

1.4. γ-Aminobutyric acid (GABA) transporter

Gamma-aminobutyric acid (GABA) transporters are also members of the SLC6 family (Liu et al. 1993). GABA is stored in small vesicles and they are transported through the vesicular GABA transporter (VGAT). By way of fusion of the small vesicles to the presynaptic membrane, GABA is released into the synaptic cleft, where it can bind to $GABA_A$ or $GABA_B$ receptors (fig. 4). Afterwards, in surrounding glia cells or in the pre-synapse, where it is stored in vesicles or degraded via Succinic semialdehyde dehydrogenase (SSADH) and GABA-transaminase (GABA-T), it is taken up again quickly by GABA transporters, (Owens & Kriegstein 2002).

GABA is converted from glutamate by glutamic acid decarboxylase. It is the major inhibitory neurotransmitter in the mammalian central nervous system (Krnjević, 2004; Danbolt, 2001), whereas glutamate is the major excitatory neurotransmitter. Balance of GABAergic and

glutamatergic signals is crucial in the functioning of the central nerve system. Therefore, an inequality of these two neurotransmitter systems is presumably involved in many if not all central nerve system disorders to some scope, making the GABAergic system an applicable target for CNS drugs (Olsen 2002). Manipulation of the GABAergic neurotransmission is generally related to some disorders such as insomnia (Ebert et al. 2006), epilepsy (Galanopoulou 2010; Macdonald et al. 2010), and anxiety disorders (Kalueff & Nutt 2007). In addition to this, there is proof of involvement of this receptor in schizophrenia and some other neuropsychiatric disorders (Lewis et al. 2008; Möhler 2009; Vinkers et al. 2010). It is involved, directly or indirectly, in the most aspects of normal brain function such as memory, cognition, and learning, as well as in the development in of tolerance and addiction (Enoch 2008; Tan et al. 2010). Moreover, it may also prove beneficial to target GABA receptors in the treatment of neuropathic pain (Munro et al. 2009; Zeilhofer et al. 2009).

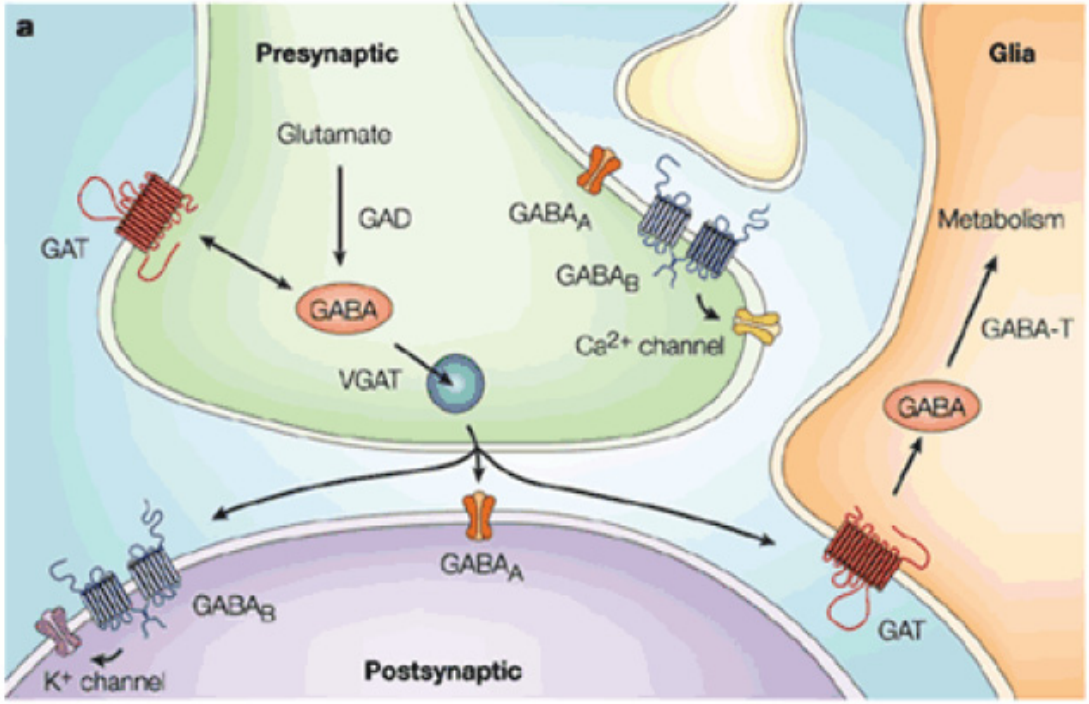


Figure 4: The structure of GABAergic synapse. GABA is synthesized from glutamate by the glutamic acid decarboxylase GAD, and stored in vesicles via transport through the vesicular GABA transporter (VGAT). Upon the depolarisation of the neuron, GABA is released itno the synaptic cleft where it activates the GABA receptors $GABA_A$ and $GABA_B$. Subsequently, it is taken up via the GABA transporter (GAT) into either glia cells or the presynaptic neuron. They are either restored in vesicles or degraded via the transaminase GABA-T to be further metabolised. Figure from Owens and Kriegstein, 2002.

1.5. Psychostimulant action of cocaine on monoamine transporters

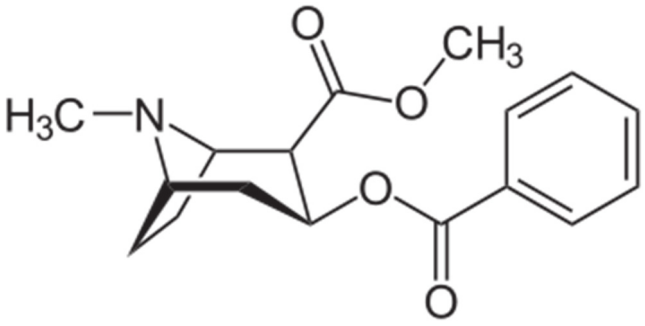


Figure 5: Structure of Cocaine

Cocaine (benzoylmethylecgonine, fig.5) is a tropane alkaloid obtained from the Andean plant *Erythroxylum coca*. It was used by indigenous people in South America for a long time and became popular all around the world by way of Sigmund Freud's reports in 1884 and introduction of Coca Cola in 1886 (Sulzer et al. 2005). Its usage as a local anaesthetic by Freud's suggestions and Koller's demonstration widely led to the discovery of its toxic effects (Y.A. et al., 2001).
In 1910, Alfred Fröhlich and Otto Loewi from the Institute of Pharmacology of Vienna were among the first who tried to shed light on the pharmacological role of cocaine (Sulzer et al. 2005).
Moreover, drugs of abuse such as cocaine and amphetamines target monoamine transporters. For DAT, NET and SERT, cocaine acts as a non-selective inhibitor with a relatively high affinity (Eshleman et al. 1999). It increases the synaptic concentration of all monoamines by stimulating the release and inhibiting the reuptake, triggering the cocaine reward especially through the increase of dopamine (Rothman et al., 2001)
For various monoamine transporters several cocaine analogues have been developed with both higher affinity and improved selectivity to treat cocaine addiction, of which some are still debated (Newman & Kulkarni 2002; Dutta et al. 2003; Loland et al. 2008).

1.6. Paracetamol

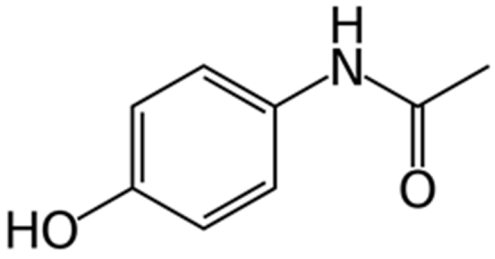


Figure 6. Chemical structure of paracetamol

Paracetamol (Fig.6), also known as Acetaminophen in North America, derives from its chemical name para-acetylaminophenol (APAP) and acts antipyretic and analgesic. APAP intoxication is the most common cause of death in the United States (Bronstein et al. 2009).
This substance was first synthesised in Germany in 1878 by Morse and clinically used by Von Mering as an antipyretic in 1887 after the search for a less toxic compound than acetanilide, the only antipyretic and analgesic medication in that time (Bertolini et al. 2006).

In 1948 Brodie and Axelrod found paracetamol to be the major metabolite for the analgesic action of acetanilide (Brodie & Axelrod 1948). Since then, it has become one of the most commonly used analgesic and antipyretic drug (Anker & Smilkstein 1994; Meredith & Goulding 1980).

1.6.1. Pharmacokinetics or metabolism of acetaminophen

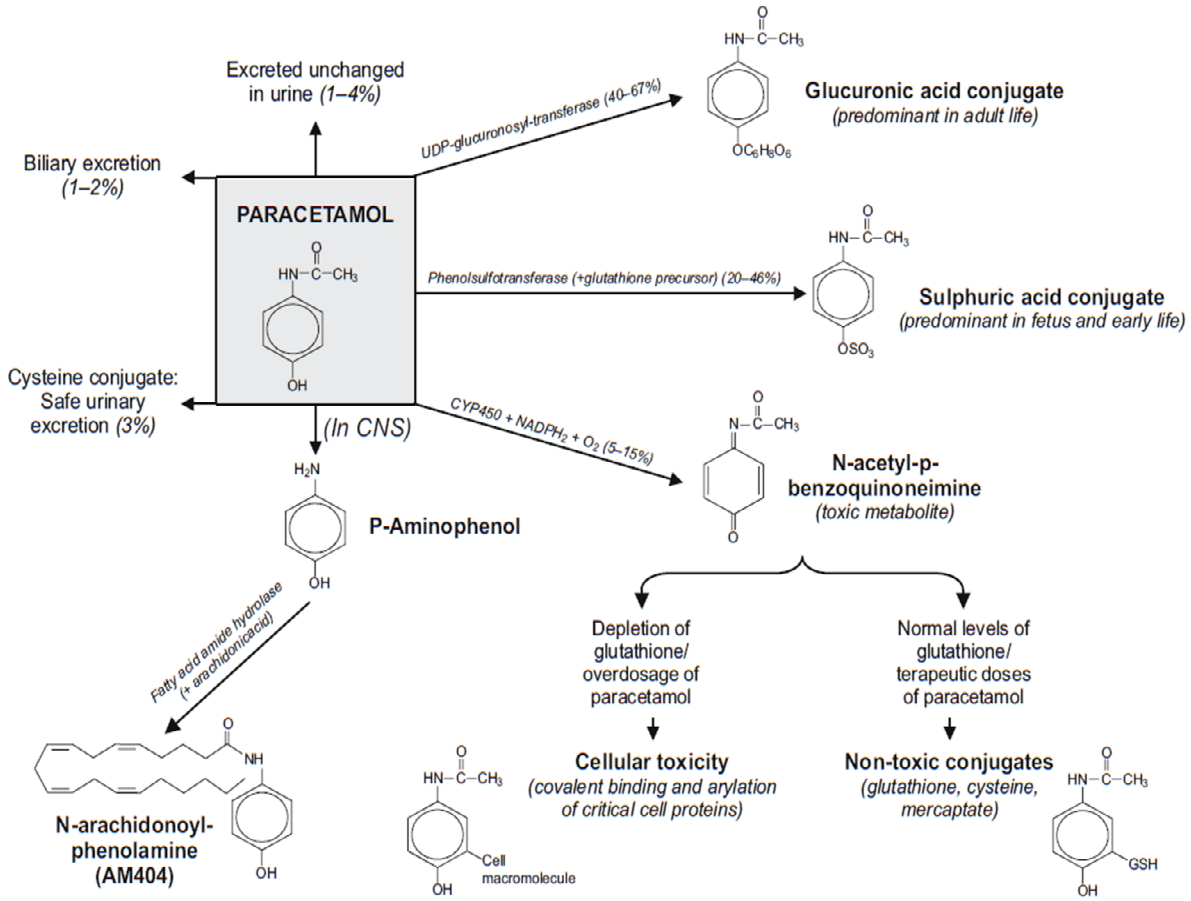


Figure 7: Metabolism of Paracetamol (figure from CNS Drug Reviews)

The main organ for the metabolism of paracetamol is the liver which eliminates approximately 25% of the therapeutic dose of paracetamol by first pass metabolism (Clements et al. 1978). In adults, APAP is safe at the therapeutic doses. Approximately 90% of paracetamol is conjugated with glucuronide (40-67%), sulphate (20-46%) and cysteine (3%) to inactive and harmless metabolites (fig. 7) (Bertolini et al. 2006). Around 5% is eliminated unaltered. The remaining 5% of APAP is oxidized via the cytochrome P450 (CYP450) (including CYP1A2, CYP3A4, and mainly CYP2E1) enzymes to the electrophilic and toxic metabolite N-acetyl-p-benzoquinoneimine, NAPQI (Nelson 1990). NAPQI is inactivated enzymatically via glutathione-S-transferases or non-enzymatically by binding glutathione, resulting in non-toxic cysteine or mercaptate conjugates, which are then eliminated (Miller et al. 1976).

The typical dose of paracetamol for the desired analgesic and antipyretic effects is 650-1000 mg for adults, which can be taken every 4 hours, up to a suggested maximum daily dose of 4 g. At therapeutic doses of acetaminophen, NAPQI is detoxified by glutathione and is excreted after

glucuronidation as the mercapturic acid into urine (Miller et al. 1976; Potter et al. 1985; James et al. 2003). After an overdose, glutathione is depleted and the increasing amount of NAPQI covalently binds to cysteine residues on proteins as 3-(cysteine-S-yl)-acetaminophen (APAP-CYS) (Streeter et al. 1984). When glutathione stores are depleted below a critical rate (almost 30% of normal stores) free NAPQI can attack nucleophilic sites on essential cellular macromolecules, inducing a series of events that may lead to cell death (Mitchell et al. 1973b) (fig. 7).

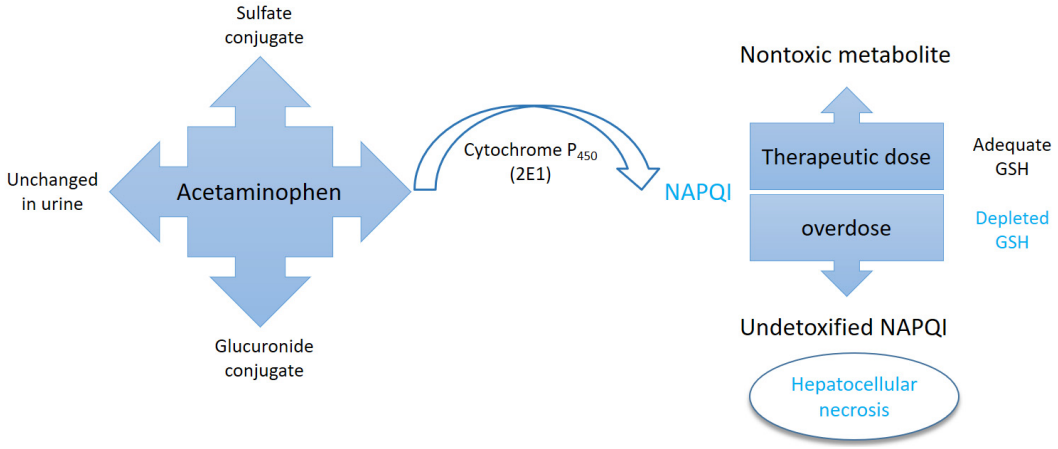


Figure 8: Toxicity mechanism of Acetaminophen

Hepatotoxicity involves factors such as increased frequency of paracetamol dosing, elongated of excessive dosing, increased capacity for P450 activation to NAPQI, decreased glutathione existence, or decreased sulfation and glucuronidation capacity. Another important factor of increased risk is chronic ethanol abuse (3 or more alcoholic drinks per day). Although, the combination of hepatic enzyme induction and glutathione depletion appears to increase paracetamol toxicity in chronic alcoholism, acute alcohol ingestion reduces toxic metabolic activation based on competitive inhibition and depletion of cytosolic NADPH (nicotinamide adenine dinucleotide phosphate) and therefore plays a preventive role in hepatotoxicity (Draganov et al. 2000; Teschke et al. 1979; Zimmerman & Maddrey 1995; Prescott 2000; Thummel et al. 1988; Tredger et al. 1985).

It was observed that simultaneous use of paracetamol and alcohol may increase the CYP2E1-mediated metabolism of paracetamol to NAPQI. In non-alcoholics, NAPQI is detoxified by conjugation with glutathione. In contrast, in alcoholics, the combination of CYP2E1 induction and glutathione depletion produce NAPQI accumulation (Draganov et al. 2000).

Some drugs such as carbamazepine, phenobarbital, phenytoin, primidone, and rifampicin in particular also induce cytochrome P450 enzymes (Miners et al. 1984) and may thus increase the formation of NAPQI.

1.6.2. Acetaminophen mechanism of toxicity

Although paracetamol is one of the safest analgesics when taken at the suggested therapeutic dose (Jackson et al, 1984), approximately 200 humans die every year of fulminant hepatic failure from APAP overdose (Rowden et al. 2006)

Nevertheless, paracetamol has been in clinical use for over 30 years and it is still unclear how the exact mechanism of acetaminophen toxicity happens (Shannon et al. 2009). Present literature implies N-acetyl-para-benzoquinone-imine (NAPQI) as the primary metabolite responsible for hepatotoxicity. The liver is the target organ for APAP toxicity because this is where it is detoxified.

Although NAPQI is toxic, it can be neutralized by conjugation with glutathione (GSH) (Jaeschke & Bajt 2006). However, in case of an overdose of acetaminophen, the generation of NAPQI exceeds the detoxification capacity of GSH, which leads to covalent binding of the sulfhydryl group to cysteines of cellular proteins (Cohen et al. 1997; Bond 2009).

Furthermore, N-Acetyl cysteine (NAC) is an acetylated cysteine residue that provides L-cysteine, the amino acid required for the biosynthesis of glutathione after being deacetylated in the liver (Viña et al. 1978). NAC is an important antidote used to treat overdoses of acetaminophen by leading to an increase in glutathione (GSH) levels, which protect against endogenously generated reactive oxygen species and reactive species such as NAPQI (Bessems & Vermeulen 2001).

Animal experiments have shown that the administration of high dose of acetaminophen to mice causes to centrilobular necrosis leading to death. Post administration of NAC effectively protects mice against this acetaminophen-induced hepatotoxicity (Williamson et al. 1982; Nagasawa et al. 1984; Roberts et al. 1987)

Recently, NAPQI has been shown to act on transient receptor potential (TRP) channels which are a family of non-selective cation permeable channels, permeable to calcium ions (Hofmann et al. 2000; Inoue 2005; Nilius & Voets 2005; Venkatachalam & Montell 2007).

TRPA1 is a unique sensor of noxious stimuli and a probable drug target for analgesics (Story et al. 2003; Bautista et al. 2006; Kwan et al. 2006; Jordt et al. 2004; Bandell et al. 2004).

Chemical activation of TRPA1 produces pain, and causes hyperreactivity and irritation in skin and visceral organs (Baraldi et al. 2010; Holzer 2011; Viana 2011). However, there are symptoms that indicate that TRPA1 is present in the spinal cord, and its role in spinal processing of nociceptive input is not certain (Kim et al. 2010).

The findings that TRPA1 is activated by electrophilic compounds (Jordt et al. 2004; Bandell et al. 2004) gave rise to speculate that compounds such as NAPQI and p-benzoquinone (p-BQ), p-AP and APAP indirectly activate TRPA1 on primary sensory neurons. The configuration of these electrophilic compounds in vivo is catalysed by a couple of enzymes, containing COX, cytochrome P450 (CYP450) monoxygenases, peroxidases, many of which are existing in the central nervous system (Chen et al. 2008; Dahlin et al. 1984; Prescott et al. 1981; Pascoe et al. 1988; Shinoda & Aoyama 2007).

TRPV1 activators are capable of producing spinal analgesia (Eimerl & Papir-Kricheli 1987) and TRPV1 is coexpressed with TRPA1 on a subpopulation of nociceptive sensory neurons (Fernandes et al., 2012) . This led David Anderson and coworkers to investigate whether the analgesic effect of APAP could include the activation of TRPA1 expressed on the central terminals of primary afferent neurons. For this reason, they investigated the role of TRPA1 in the antinociceptive effect of systemic and intrathecal administration of APAP and identified whether its metabolites such as p-BQ and NAPQI, produce antinociception via interacting with TRPA1 in the spinal cord. Indeed, results showed that NAPQI and p-BQ are potent activators of TRPA1. This indicates that there is a new role of TRPA1 in synaptic signalling and that spinal TRPA1 activation through a non-antinociceptive mechanism promotes the analgesic activity of acetaminophen (Andersson et al. 2011).

AIMS OF THE THESIS

NAPQI binds to the sulfhydryl group of glutathione at therapeutical doses of paracetamol. Once the glutathione stores of the body are emptied, it covalently binds to cysteine residues of proteins, leading to 3-(Cysteine-S-yl) adducts (Mitchell et al. 1973b; Jollow et al. 1973). The cysteine-modifying agent 2-(aminoethyl)-methanethiosulfonate hydrobromide (MTSEA) was reported to inhibit the function of SERT (Androutsellis-Theotokis et al., 2001), and cysteine mutants protected the transporter from MTSEA inhibition. Accordingly, we wondered whether NAPQI can similarly affect the function of the transporter and whether the effects can be prevented by mutations in cysteine residues of SERT.

Hence, the aims of this thesis hence were following:

1. Does NAPQI affect wildtype SERT in its uptake? If so, how does this change with cysteine mutants thereof?
2. If yes, is the effect
   - time- or concentration-dependent?
   - competitive or non-competitive?
3. Does NAPQI similarly affect other transporters such as the GABA transporter GAT?
4. Can we recapitulate the effects of MTSEA on SERT?

2. MATERIAL AND METHODS

2.1. Cloning

2.1.1. Generating competent E. coli bacteria

Chemical competent E. coli bacteria were prepared as following: 10 ml LB medium (containing 10µg/ml tetracycline) was inoculated with a bacterial E. coli colony (XL10) from a tetracycline added agar plate. That preculture was incubated at 37°C overnight under continuous agitation, and 2ml of that was transferred into an autoclaved Erlenmeyer-flask including 200 ml prewarmed LB. The flask was incubated at 37°C until OD550 of 0.5 was reached, which indicates exponential growth of bacteria. Once the cells had reached this phase, they were placed them on ice and kept them there for 10 minutes. The bacteria were harvested by centrifugation in sterile centrifugation for 15 minutes (4°C 1500g). The supernatant was poured off and then supernatant and resuspended in 20 ml of ice-cold TSS buffer (Trypton 1%, Yeast Extract 0.5%, NaCl 100mM, Polyethylenglycol(PEG) 10%, DMSO 5%, MgCl2 50 mM, pH 6.5). Afterwards, 5 ml glycerol was added and 50µl of competent bacteria were distributed in sterile Eppendorf-tubes, immediately frozen in liquid nitrogen and stored at -80°C.

2.1.2. QuikChange site-directed mutagenesis

Mutant transporters were generated by the QuikChange Site-Directed Mutagenesis kit from Agilent Technologies (Santa Clara, CA). The QuikChange Site-Directed Mutagenesis kit was used to make point mutations by replacing amino acids. The mutagenesis was performed by using two synthetic oligonucleotide complementary primers. Table 2 shows the amount of each component used for the PCR reaction.

Reagents	Volume
10× reaction buffer [(100mM KCl, 100mM(NH4)2SO4, 200mM Tris-HCl (pH 8.8), 20mM MgSO4, 1%Triton® X-100, 1mg/ml nuclease-free BSA)]	2,5 µl
dsDNA template	5-50 ng
Quick Solution	0,75 µl
dNTP mix	0,5 µl
Primer	10 µl
PfuTurbo DNA polymerase (2.5 U/µl)	1 µl
ddH2O	Add 25 µl

Table 2: Quantities used for site-directed mutagenesis for each reaction. This procedure utilizes a double-stranded DNA (dsDNA) vector within localized gene of interest and two complementary primers containing the desired mutation.

A plasmid containing the gene of interest was used as a template. Both strands of the plasmid were amplified introducing the desired mutation by PCR (see table 3 for the PCR reaction).

Temperature (°C)	Time	Cycles
95	2 min	1x
95	20 sec	20x
58	20 sec	
68	4 min 30 sec	
72	5 min	1x
4	Endless	1x

Table 3: Quickchange PCR protocol

After the PCR reaction, the samples were treated with the endonuclease DpnI for 30 minutes at 37°C. The DpnI endonuclease which uses 5'-Gm6ATC-3' as a target sequence is specific for methylated and hemimethylated DNA. This enzyme was used to digest only the parental methylated and hemimethylated DNA template. As DNA from PCR reactions is not methylated, it is resistant to this enzyme. Therefore, only mutated DNA should stay unharmed.
Next, these mutated plasmids were transformed into competent bacteria and then they were grown over night on LB agar dishes which contained selected antibiotics appropriately to the antibiotic resistance gene on the plasmid.

2.1.3. Transformation

Transformation is a useful method to bring the desired plasmid into the bacterial cells in order to express the gene of interest. Plasmids were transformed into competent bacteria XL10. 100µl of competent bacteria cells (as described in section 8.1) were thawed on ice. After that 5µl of the DNA was added and gently mixed by flicking the bottom of the tube a few times. DNA/competent cell mixture was kept on ice for 20 minutes for incubation then a heat shock each transformation tube was given a heat shock of 42°C for 45 seconds. The time can be altered depending on the cell type (45 second is usually ideal, but this differs depending on the competent cells you are using). Following this, the tubes were put back on ice for 2 minutes. That procedure allowed plasmid DNA to enter the competent bacteria. 1 ml of LB (Lysogeny Broth) medium was added. Transformed bacteria were incubated for one hour at 37°C. During the incubation period bacteria expressed the plasmid-encoded gene for antibiotic resistance. Next, the bacteria were centrifuged for 5 minutes at 10,000 rpm (MiniSpin by Eppendorf), the supernatant was poured off and the pellet was resuspended gently. Then all of the transformation was spread on an LB agar plate containing the appropriate antibiotic. Lastly, bacteria were grown overnight in an incubator.
To obtain the best chance of getting single colonies, 50µL of transformation was added on one plate and the rest on a second plate (a method used in place of centrifuge).

2.1.4. DNA/Plasmid isolation

DNA was purified from liquid bacteria cultures (2ml), using NucleoSpin® Plasmid QuickPure Kit (Marcherey&Nagel) according to the manufacturer's protocol. After bacteria were lysed, both chromosomal DNA and protein were denaturated and precipitated, while the small bacterial DNA plasmids stayed in the solution and bound to a column in order to elute.
For qualitative analysis DNA was isolated from 2ml overnight LB cultures (Mini prep), using the NucleoSpin® Plasmid QuickPure Kit (Marcherey&Nagel) following the manufacturer's protocol. After this, lysation of bacteria, proteins and genomic DNA is precipitated and the plasmid of interest was bound to a column followed by elution later on.
DNAs obtained via "mini preps" were used for sequencing and diagnostic restrictions digest. In order to perform Midi Prep, proper plasmids were transformed and larger amounts of plasmid were isolated using 250 ml LB cultures.

2.2. Cell culture

2.2.1. Cultivation

Products are required for cell culture (Media, Trypsin, serum and antibiotics) were purchased from Sigma-Aldrich, (St. Louis, MO), cell culture dishes were from Sarstedt (Germany).
HEK293 cells (The human embryonic kidney 293 cell line was used) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich) supplemented with High Glucose (4.5 g/L) containing 10% (w/v) fetal calf serum (Serum is an extremely complex solution of albumins, globulins, growth inhibitors, and growth promoters) and 1% Penicillin/Streptomycin (10,000 units penicillin/10mg/streptomycin/ml) on polystyrene culture dishes. Maintenance of stable cell lines: 50 µg/ml G418 was added to DMEM. Cells were kept in an incubator (CO_2 Incubator CB, Binder) at 37°C in 5% CO_2 conditions. CO_2 was needed to control pH because cell physiology is highly sensitive to pH variations.
For this cultivation, the cells were incubated in an incubator overnight, then the medium was aspirated by use of a pump. In order to get rid of excess medium and dead unattached cells and cell debris, the cells were washed with 1x phosphate buffered saline (PBS; 1 mM $MgCl_2$; 0, 1 mM $CaCl_2$). In order to detach the cells from the culture dish, adherent cells had to be trypsinized, which is why 1ml of Trypsin/EDTA (0.5g trypsin/0.2g EDTA; Sigma-Aldrich) was added. The cells were observed under the microscope (generally for after about 3 to 5 minutes). When the cells pulled away from each other and rounded up, trypsin was neutralized by adding 8 ml of prewarmed cultivation medium. The dissociated cells were transferred into a sterile 50 ml centrifuge tube (Greiner) and harvested by centrifugation for 5 minutes at 1000 rpm. The neutralized dissociation solutions were aspirated from the cell pellet and the cells were resuspended in 8 mL fresh, pre-warmed growth medium.

2.2.2. Cell counting/seeding

As mentioned in section 3.2.1, cells were detached by adding Trypsin/EDTA solution (1ml per 10 ml cultivation medium), harvested by centrifugation and resuspended in fresh cultivation medium.
It was ensured that, the cell suspension to be counted was well mixed by either gentle agitation of the falcon tube or vortex. Before the cells had a chance to settle at all, cells were taken out of

approximately 10 to 15µl of the cell suspension and added to the object slide which consisted of 9 large squares which are subdivided into 16 smaller squares each.
Cells were counted by moving the hemocytometer to the next set of 16 corner squares and in this way counting was continued until all 16 corners were counted by using a hand tally counter. The average cell count was determined and multiplied by 10,000 (104). The final value being the number of cells per ml in the original cell suspension.

2.2.3. Transfection

JetPrime® DNA transfection (Polyplus transfection)

In this study, we used jetPRIME® as a transfection reagent which is well known for use in common experiments. HEK293 cells were seeded at 2.5x106 cells per 10cm dish 24h before transfection. The number of seeded cells depends on the cell type. Furthermore, cell confluence should be around 50% to 70% on the day of transfection to have high transfection efficiency. Appropriated amount of jetPrime® transfection reagent and DNA were added into the cells respectively and incubated for 4h. The medium of cells was changed 4h post-transfection and kept in an incubator overnight. The next day, the transfection efficiency was verified under the microscope and the cells were cultured for the two days following.

Generation of stable cell lines

The antibiotic G418 was used to select the cells that had integrated the plasmid coding for a selection marker and the gene of interest into their genome. The first step in this process was that cells were selected by 250µg/ml G418 for 10 days and the selection medium was changed several times to remove the dead cells. When resistant clones appeared, clones were picked and reseeded to facilitate growth. New cell lines were tested for functional expression of SERT by fluorescent microscopy as well as functional assays. Once generated and cultivated, to avoid the exclusion of the plasmid after a long cultivation period, cells that stably expressed the constructs were propagated in the medium containing 50µg/ml G418 per 10 ml.

2.3. Pharmacological Assays

2.3.1. Uptake assay

In order to test the functional activity of the serotonin transporter, a pharmacologically relevant uptake assay was performed using tritiated substances. Until recently, radioactively labeled compounds were used to measure serotonin, norepinephrine and dopamine transporter uptake. Following the standard protocol, transfected cells or stable cell lines (hSERT expression was induced by addition of tetracycline (TET/antibiotic) the day before) were seeded on Poly-D-Lysine (PDL purchased by Sigma-Aldrich) coated 48well cultivation dishes. 1x105 cells per well were seeded in a final volume of 500µl standard cultivation medium and incubated overnight at 37°C and 5% CO2.
The following day the cultivation medium was removed by aspiration and cells were covered with 500µl pre-warmed to Krebs-HEPES Buffer pH 7.3 at room temperature. Then cells were incubated at room temperature with 100µl of the uptake solution which contained a fixed concentration of the tritiated substance and different amounts of the unlabeled substance. In order to determine non-

specific uptake, cells were pre-incubated with 10µM paroxetine (for hSERT) for 5 min following incubation with [³H] 5HT for 1 min at room temperature. And also in order to get specific uptake, Km and Vmax values were determined via unlabeled substrate which was used at increasing concentrations to dilute the specific activity of [³H] 5HT. Thus, 0,2-100µM [³H] 5HT (containing 0.2µM [³H]) was added and incubated for 1min at RT.

Incubation time was dependent on the predicted transport rate of each transporter. After the incubation period, cells were washed with 500µl ice cold KHB (in order to stopped uptake) and immediately cells were lysed by adding 500µl 1% (w/v) SDS and the cell suspension was transferred to scintillation-vials and 2 ml of the scintillation cocktail (Rotisint® eco plus LSC-Universalcocktail; Roth) was added and counted in a beta counter. The scintillation cocktail absorbs emitted energy produced by the disintegration of the radioisotopes and re-emits it as flashlight. These signals can be detected and measured in a -counter (TRI-CARD 2300 RT (Packard)).

Data were analyzed by GraphPadPrism5.0 software. Vmax and Km values are calculated by non-linear regression curve fitting.

The Km value or Michaelis-Menten constant is described as the substrate concentration at half-maximum enzyme velocity (Vmax/2). To characterize enzyme reactions, the Km value defines half of the substrate binding sites to be occupied, which is in regard to the concentration of the substrate needed for performing a catalytic reaction (Berg et al. 2007). The reaction mechanism can be simply described as

$E + S \leftrightarrow [ES] \leftrightarrow P + E$

where the concentrations of the enzyme E and of the substrate S are considered to transition to an intermediate complex of enzyme and substrate ES, which, through the activity of the enzyme, gives rise to the product P and the free enzyme (REFERENCE).

These kinetic parameters also describe the affinity of a substrate to a transporter. Therefore, a similar reaction can be applied, consisting of the outward-facing (To) and the inward conformation (Ti) of the transporter, resulting in:

$To + S \leftrightarrow [TS] \leftrightarrow S + Ti$

Here, TS represents all intermediate conformations of the transporter from the bound state with substrate until the transition into the inside of the cell (REFERENCE).

2.3.2. Inhibition assay

The main purpose of this method is to analyze the potency of a putative antagonist to inhibit the transport of a substrate. It is characterized by the IC50 value that is defined as the concentration of the antagonist needed for 50% of inhibition of substrate transport.

Substrate concentration was kept constant and blockers were used at increasing concentrations according to expected IC50 values. After incubation reaction was stopped by washing the cells with ice cold uptake buffer, lysed by 1%SDS and cell suspension was transferred to scintillation vials and 2 ml of liquid scintillation cocktail was added and counted in a beta counter. In order to evaluate the data, GraphPadPrism® software was used. “% Uptake relative to control” values were plotted against logarithmic concentrations of NAPQI and analyzed by “One site competition” to calculate IC50 and Ki values.

2.4. Materials

2.4.1. Standard solutions/buffers

Phosphata buffered saline (PBS)
2.7 mM KCl, 1.5 mM KH2PO4, 137 mM NaCl, 4.3 mM Na2HPO4 ×2H2O, (pH 7.3)

TSS buffer
Trypton 1%, Yeast Extract 0.5%, NaCl 100 mM, Polyethylenglycol (PEG) 10%, DMSO 5%, MgCl2 50 mM, pH 6.5

Dulbecco's modified eagle's medium (DMEM; Sigma Aldrich)
Supplemented with High Glucose (4.5 g/L) containing 10% (w/v) fetal calf serum (Serum is an extremely complex solution of albumins, globulins, growth inhibitors, and growth promoters) and 1% Pen Strep (10,000 units penicillin/10 mg/streptomycin/ml)

Krebs-HEPES buffer (KHB)
25 mM HEPES; 120 mM NaCl; 5 mM KCl (Merck); 1,2 mM CaCl2 (Merck); 1,2 mM MgSO4 (Merck); 5 mM D-glucose (Roth) (pH 7,3)

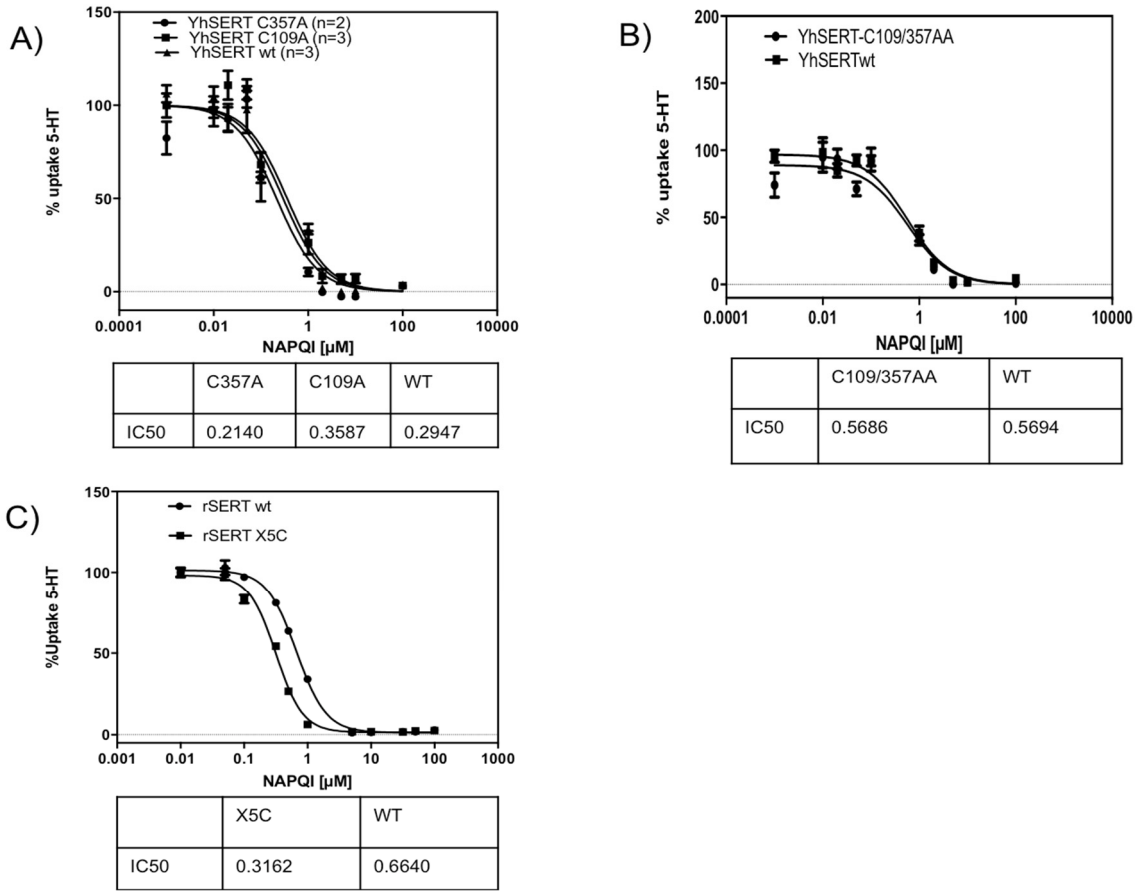
Lysogeny Broth medium (LB)
NaCl2 10%, Peptone 10%, Yeast extract 5%, ddH2O appr. Vol.

5×Poly-D-lysine
25 mg Poly-D-lysine (sigma), ddH2O ad. 100 ml

3. RESULTS

3.1. Evaluation of the inhibitory capacity of NAPQI on different mutated serotonin transporters

Serotonin transporter (SERT) comprises a single reactive external cysteine residue at position 109 (Chen et al. 1997) and seven predicted cytoplasmic cysteine residues. Therefore, I performed three inhibition uptake assay with alanine mutants of the cysteine residues C109 and C357 and a double alanine mutant thereof. In addition, I used the quintuple cysteine mutant of the rat serotonin transporter C15A/C21A/C109A/C357I/C622A that was formerly described by the lab of Gary Rudnick for the sensitivity to another cysteine-modifying agent (Androutsellis-Theotokis et al. 2001). Figure 8 shows that neither mutation of single cysteine residues nor the multiple mutants had any effect on inhibitory potency of NAPQI.



	C357A	C109A	WT
IC50	0.2140	0.3587	0.2947

	C109/357AA	WT
IC50	0.5686	0.5694

	X5C	WT
IC50	0.3162	0.6640

Figure 8: Evaluation of inhibition capacity of NAPQI on different mutated serotonin transporters. Inhibition of [^{3}H] 5-HT uptake by NAPQI was determined in HEK293 cells transiently transfected with wild type and mutant cDNAs respectively using jetPrime (as described under Materials and Methods). A) Single mutant C357A, C109A B) double mutant C109/C357AA and C) mutant rat SERT. Cells were incubated with 0.1µM [3H] 5-HT and increasing concentration of NAPQI and background was measured at 10µM paroxetine. Data, plotted according to the sigmoidal model. Mean and SEM from a representative experiment run in triplicate are shown.

3.2. Time dependent uptake inhibition assay

SERT-expressing cells were pre-incubated with 1µM NAPQI for various times. Uptake inhibition was then performed in the presence or absence of NAPQI. Even without preincubation NAPQI reduced the uptake of 5-HT by 70% (Fig.10 0min/UPT+Nap). Preincubation for longer periods up to 30 min did only result in a slightly higher reduction of uptake (max 80%). Covalent modification of a protein would result in an effect, which could be observed even if the modifying drug is removed. When we exchanged the preincubation buffer with buffer containing no NAPQI, uptake was immediately back to almost 100%. This clearly indicates that NAPQI does not covalently modify the transporter but rather inhibits uptake in a reversible manner.

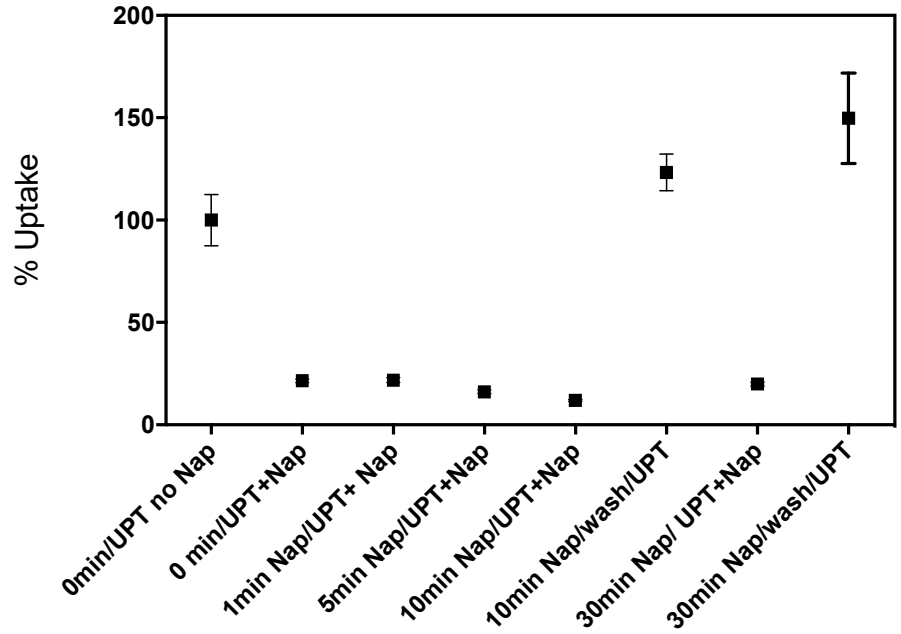


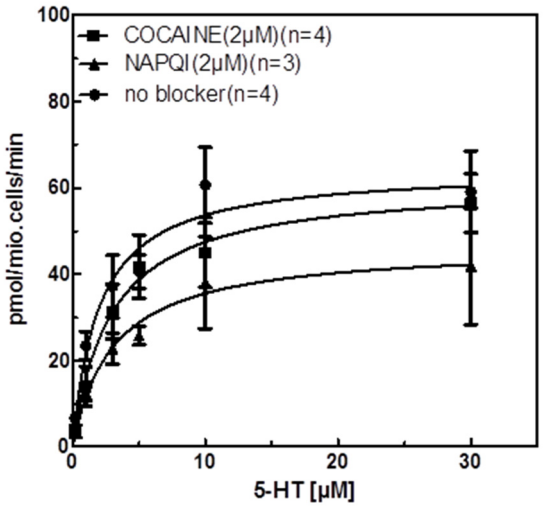
Figure 10: Time dependent uptake inhibition assay. Assay was performed in transiently transfected HEK293 cells and cells were exposed to 1µM NAPQI for different time points (0/1/5/10/30 minutes) and various washing steps. Uptake incubation was performed in the presence or absence of NAPQI. Samples were plotted % versus time [min]

3.3. NAPQI is a competitive inhibitor of uptake of SERT

Our hypothesis was that NAPQI binds to transporters irreversibly and modifies them, inhibiting the function of the transporter. However, the previous results showed that NAPQI binds to the transporters reversibly. Thereby, we wondered whether NAPQI inhibits the transporters in a competitive or non-competitive manner. In order to answer this question, we performed an uptake assay using increasing concentrations of substrate in the absence or presence of cocaine or NAPQI at indicated concentrations. To investigate whether NAPQI is a competitive inhibitor, we used cocaine because binding studies of cocaine, imipramine, and the SSRIs paroxetine and citalopram imply the presence of more than one binding site on the SERT (REF). Cocaine, imipramine and citalopram act as competitive inhibitors of 5-HT reuptake (Sur et al. 1998).

If the uptake reaches the same Vmax (maximum velocity) in the presence and absence of the inhibitor, while the value of Km (Michaelis-Menten constant) increases, this is a sign for competitive

enzyme inhibition. If Vmax is decreased while Km is not altered this would point to a non-competitive inhibition. In order to check if NAPQI is competitive inhibitor, saturation uptake was performed. As expected, cocaine inhibits the uptake of 5-HT in a competitive way. NAPQI interestingly shifted the Km value to the right but also reduced Vmax (Fig. 11).



	5-HT (n=4)	COCAINE (2µM) (n=4)	NAPQI (2µM) (n=3)
Vmax	60.35	59.91	50.81
Km	1.59	3.256	3.363

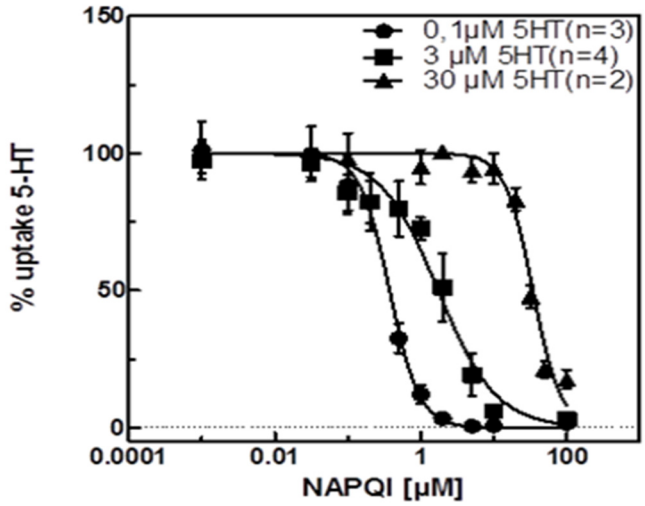
Figure 11: Saturation uptake of serotonin in the presence or absence of cocaine. Uptake experiments were performed using increasing concentrations of substrate (0.2, 1, 3, 5, 10, 30, 100µM of [3H] 5-HT) in the absence or presence of cocaine or NAPQI, each 2 µM. Cells were transfected with wild-type SERT. Cells were incubated with 0.2 µM [^{3}H] 5-HT, in the presence or absence of 10µM paroxetine to determine nonspecific uptake. Table below shows calculated Vmax and Km for each fit.

3.4. Increase of serotonin shifts the inhibition curve for NAPQI

The results in figure 11 point towards a mixed competitive inhibition. In order to investigate this in detail, we performed the experiments in a different way where we increased substrate concentrations and tested for a potential shift in the IC50 values of NAPQI.

In the Cheng-Prusoff Equation "Ki= IC50 ÷ (1+[S] / Km)" Ki is the inhibition constant for a drug where 50% of receptors are occupied, while the IC50 value defines the experimentally found value where 50 % of the enzymatic reaction (uptake) is inhibited. [S] defines the concentration of the substrate (5-HT) used while Km is the affinity of the ligand. This means that IC50 is dependent on Km and the ligand concentration.

As IC50 = Ki * (1+[S]/Km) and in most experiments the ligand is used below its Km value, this infers that IC50~Ki. If the concentration of the ligand is now increased, IC50 will increase as Km and Ki are constant values. The Km value for 5-HT was determined as 1.6 µM (Fig 12).



	0.1 µM 5-HT (n=3)	3 µM 5-HT (n=4)	30 µM 5-HT (n=2)
IC50	0.3729	1.732	34.25

Figure 12: Uptake inhibition experiments in the presence of increasing serotonin concentrations. Experiments were performed using 0, 1µM, 1µM and 10µM serotonin on SERT with increasing concentrations of NAPQI. Background was measured at 10µM paroxetine. Data, plotted according to the hyperbolic model, are shown as means of a representative experiment carried out in triplicates. Table below indicates the calculated IC50 values for each 5-HT concentration.

3.5. Effects of NAPQI on other transporters: GABA

Gamma-aminobutyric acid (GABA) and glutamate are the major inhibitory and excitatory neurotransmitters in the mammalian central nervous system (Krnjević 2004; Danbolt 2001). The reuptake transporter especially for GABA is target of various therapeutically used drugs. We wanted to test wether NAPQI inhibits uptake of not only monoamines but also of other neurotransmitters. As the exact mechanism of paracetamol is not identified yet, involvement of the GABAergic system could still be a possible explanation, as targeting of GABA receptors shows benefits in the treatment of neuropathic pain (Munro et al. 2009; Zeilhofer et al. 2009). However, even at a high concentration of NAPQI (30µM) for increasing time periods no reduction of GABA uptake could be observed (fig. 13).

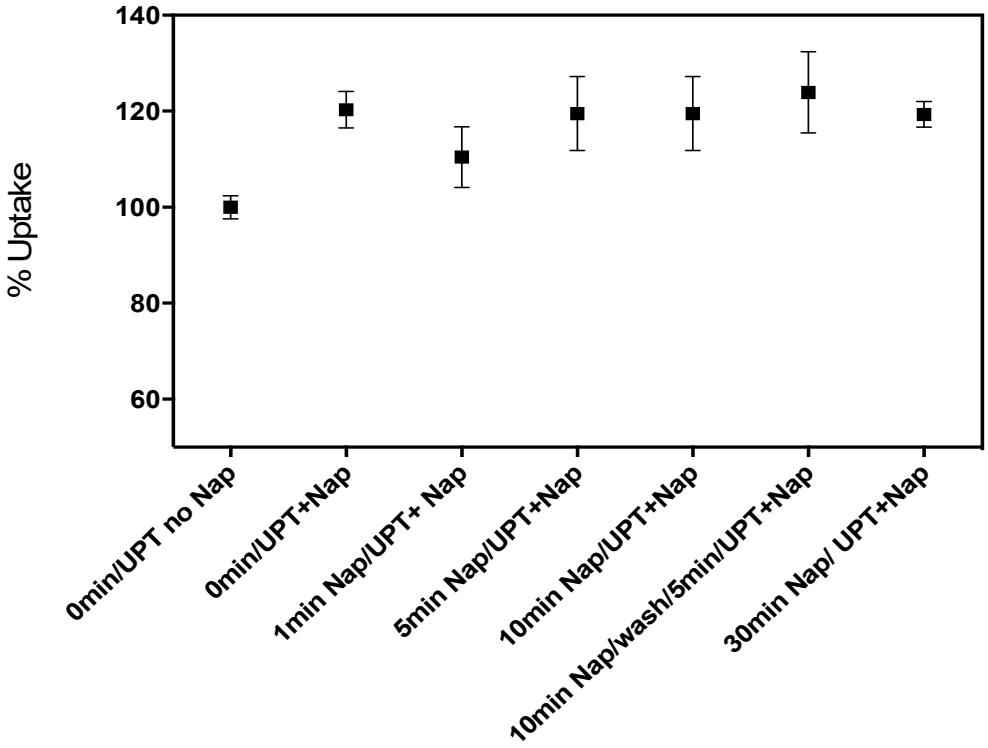


Figure 13: Uptake inhibition of the GABA transporter. Time-dependent uptake inhibition of HEK293 cells stably expressing rat GAT at different time points (0/1/5/10/30 minutes) and a washing step (wash). Uptake inhibition was performed in the absence or presence of 30 µM NAPQI (NAP). 10 µM Tiagabine was used to block GAT. Samples were plotted as % uptake versus time in minutes.

4. DISCUSSION

4.1. Evaluation of inhibition capacity of NAPQI on different mutated serotonin transporters

As mentioned before, NAPQI binds to the Sulfhydryl group of glutathione under therapeutical doses of Paracetamol. In case of taking overdose of Paracetamol, glutathione stores are emptied and thus NAPQI binds sulfhydryl groups on cysteine residues of other proteins (Mitchell et al. 1973a; Jollow et al. 1973). Binding of NAPQI to TRPA1 showed functional alterations of the channel (Andersson et al. 2011). As we found out that NAPQI inhibits SERT the most logical explanation would have been a similar covalent modification. The serotonin transporter consists of a single reactive external cysteine residue at position 109 and 7 predicted cytoplasmic cysteines (Chen et al. 1997). Furthermore, some cysteine residues have been indicated to be important for expression of SERT (Sur et al. 1997). Previous experiments by Jae-Won Yang (institute of pharmacology) have shown using mass spectrometry that at least a rather C-terminally located cysteine (C622) can be modified by NAPQI (data not shown). As it was shown before, deletion of the last 16 amino acids of SERT does not affect its function (El Kasaby et al 2010). Therefore, the modification of cysteine 622 will most likely not be responsible for the inhibition of SERT by NAPQI. Based on this, we performed uptake inhibition experiments to investigate inhibition effect of NAPQI on serotonin transporter mutants.
However, as shown in Fig.8, inhibition effect of NAPQI still continued although the cysteine at position 109 were not present. According to Andreas Androutsellis's publication, his outcomes

indicated that when 2 cysteine residues were mutated at position 109 and 357, in contrast to wild type SERT, the mutants are resistant against the inhibition effect of the cocaine analogue MTSEA (2-(aminoethyl)methanethiosulfonate hydrobromide) (Androutsellis-Theotokis et al. 2001). Therefore, we mutated two cysteine residues at position C109 and C357 and after that 5 cysteines from rat SERT which were shown to react with other cysteine-reactive compounds. On the contrary, as shown in Fig.8, our mutated cysteine residues did not affect the susceptibility of SERT to NAPQI. Therefore, we can conclude that modification of these residues is not the mechanism underlying the inhibition of SERT by NAPQI.

4.2. Time dependent uptake inhibition assay

To find out whether or not NAPQI covalently binds to SERT, we performed uptake inhibition experiment in the presence or absence of NAPQI. When we incubated our cells with NAPQI and then washed it away, the transport capacity came back immediately (Fig 10). Taken this result, together with the fact that removing accessible cysteine residues by mutagenesis did not change the inhibitory profile of NAPQI, one can clearly state that NAPQI inhibits the 5-HT uptake in a reversible way. A similar effect of NAPQI was observed for the uptake of dopamine via DAT while the uptake of MPP+ via the norepinephrine transporter NET was not altered at all (data not shown). These experiments were performed parallel to my thesis by other colleagues at the institute of pharmacology.

4.3. NAPQI is a competitive inhibitor of SERT

However, we explored that NAPQI reversibly inhibits SERT, we performed the saturation uptake of serotonin in the presence or absence of an inhibitor. In a competitive inhibition, the competitive inhibitor shows similarity to the substrate and it binds to the active site of the protein and substrate and inhibitor cannot bind to the active site at the same time. Therefore, the substrate is prevented from binding to the same active site of enzyme. Competitive inhibition is characterised by competitive inhibitors that can be overcome by an increasing the amount of substrate. Although competitive inhibitor could change the Km value, an enzyme will have the same Vmax as in the presence or absence of an inhibitor (Berg et al. 2002).

In order to examine whether or not NAPQI is a competitive inhibitor we used cocaine. Because cocaine, imipramine and citalopram behave like competitive inhibitors of 5-HT reuptake (Sur et al. 1998).

As shown in figure 11, Vmax of serotonin in the presence and absence of cocaine and NAPQI were similar: in the presence of NAPQI, Km of serotonin was 2-fold increased and shifted to the right like cocaine. This could point towards a mixed competitive inhibition.

4.4. Increasing of serotonin shifts the inhibition curve for NAPQI

In accordance with competitive inhibition, and because an inhibitor can act like a substrate and bind to the active site of the enzyme, it prevents binding of the substrate, and more substrate is used to compete with inhibitor. Thus, the concentration of a drug which displaces 50% of the specific binding of the substrate (IC50) may vary between experiments by experimental conditions like substrate or inhibitor concentration. Further, via using Cheng-Prusoff equation (Cheng & Prusoff 1973), the IC50 value is converted to the inhibition constant (Ki) and this quantitative measure shows how much of drug or inhibitor is needed to inhibit the transporter. Due to this reason, we

performed uptake inhibition experiment to see whether or not serotonin and NAPQI compete with each other. The experimental data extracted from figure 12 show that the expected values are roughly correct for 0.1 and 3µM. The experimental IC50 determined at 30µM 5-HT is too high. This could have been because of the concentrations of NAPQI not being higher than 100µM. This was because of its limitation of solubility and the data points being inaccurate in this region. Slight reduction of 5-HT might help to circumvent this problem and would be a possibility to check, but was not performed during my thesis.

When we calculate our IC50 values using Cheng-Prusoff equation which was shown in Result section, it indicates that, in case of increasing concentrations of serotonin, IC50 value of serotonin becomes larger, although the concentration of NAPQI was in a micromolar range. Thus, these results again demonstrate that NAPQI is a competitive inhibitor.

We conclude that NAPQI, the toxic metabolite of Paracetamol, is capable of blocking SERT in a reversible competitive way. Blockage of SERT would increase the analgesic effect of descending antinociceptive neurons, providing a new perspective for the analgesic mechanism of Paracetamol.

Therefore, we propose the inhibition of SERT as a putative mechanism for the analgesic action of paracetamol. Although paracetamol is in use as an analgesic drug for decades, the precise mechanism of its action remains unclear. One explanation points to the serotonergic system to be involved in this mechanism (Karandikar et al., 2016; Tjølsen et al., 1991; Pini et al., 1996). Descending serotonergic neurons are known to play an important role in the inhibition of pain sensation. Given the possibility that paracetamol at therapeutical doses can be transformed to NAPQI in the nervous system as proposed by Andersson et al (Andersson et al. 2011) it could reach local concentrations high enough to block 5-HT reuptake into synapses. This would result in an elevated and prolonged 5-HT concentration in the synaptic cleft, thereby increasing the antinociceptive action of 5-HT. A similar effect is the reason why antidepressants show antinociceptive properties (Mochizucki 2004; Richeimer et al. 1997; Micó et al. 2006). Although it has to be proven that paracetamol really increases 5-HT concentrations in the nervous system and therefore NAPQI might be the active component responsible for the antinociceptive action, the experiments performed within this thesis might provide the first experimental data to explain the mode of action of paracetamol.

4.5. Effects of NAPQI on the GABA transporter

GABA is an inhibitory neurotransmitter and localized both presynaptically in primary afferents and postsynaptically in dorsal horn interneurons in the mammalian spinal cord (Malcangio & Bowery 1996).

The GABAergic system plays an outstanding role in presynaptic inhibition of primary afferents, therefore influencing sensory transmission, nociception, and motor activity on both pre- and postsynaptic levels (Barker & Nicoll 1972; Polc 1982; Cattaert et al. 1992; Stuart & Redman 1992).

Due to these features of GABA, we wanted to investigate whether NAPQI has an effect on this transporter. Our uptake inhibition experiments indicate that NAPQI has no effect on the GABA transporter (GAT). Human GAT has 38% homology with human SERT (uniprot alignment) and, compared to SERT, has less cysteine and histidine residues in the extracellular and intracellular loops.

5. LIST OF FIGURES

Figure number	Source[1]
Figure 1	http://what-when-how.com/neuroscience/neurotransmitters-the-neuron-part-4/
Figure 2	http://www.intechopen.com/books/a-synopsis-of-parkinson-s-disease/pathophysiology-of-l-dopa-induced-dyskinesia-changes-in-d1-d3-receptors-and-their-signaling-pathway
Figure 3	http://what-when-how.com/neuroscience/neurotransmitters-the-neuron-part-4/
Figure 4	Owens and Kriegstein, 2002
Figure 5	https://en.wikipedia.org/wiki/Cocaine
Figure 6	Self-designed
Figure 7	CNS Drug Reviews, Vol.12, No.3-4, 2006
Figure 8	https://en.wikipedia.org/wiki/Paracetamol

[1] as of July 2019

6. REFERENCES

Amenta, F. et al., 2001. Identification of dopamine plasma membrane and vesicular transporters in human peripheral blood lymphocytes. Journal of Neuroimmunology, 117(1-2), pp.133–142.

Andersen, J. et al., 2009. Recent advances in the understanding of the interaction of antidepressant drugs with serotonin and norepinephrine transporters. Chemical communications (Cambridge, England), pp.3677–3692.

Andersson, D.A. et al., 2011. TRPA1 mediates spinal antinociception induced by acetaminophen and the cannabinoid Δ 9 -tetrahydrocannabiorcol.

Androutsellis-Theotokis, A., Ghassemi, F. & Rudnick, G., 2001. A Conformationally Sensitive Residue on the Cytoplasmic Surface of Serotonin Transporter. Journal of Biological Chemistry, 276(49), pp.45933–45938.

Anker, A.L. & Smilkstein, M.J., 1994. Acetaminophen: Concepts and controversies. Emergency Medicine Clinics of North America.Vol.12(2)()(pp 335-349), 1994., (2), pp.335–349.

Balkovetz, D.F. et al., 1989. Evidence for an imipramine-sensitive serotonin transporter in human placental brush-border membrane. Journal of Biological Chemistry, 264(4), pp.2195–2198.

Bandell, M. et al., 2004. Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. Neuron, 41(6), pp.849–857.

Bannon, M., Sacchetti, P. & Granneman, J.G., 1995. The Dopamine Transporter: Potential involvement in Neuropsyhiatric Disorders. In F. Bloom & D. Kupfer, eds. Psychopharmacology. pp. 179–188.

Baraldi, G., Preti, D. & Geppetti, P., 2010. Transient Receptor Potential Ankyrin 1 (TRPA1) Channel as Emerging Target for Novel Analgesics and Anti-Inflammatory Agents. Medical Chemistry, 53, pp.5085–5107.

Barker, J.L. & Nicoll, R.A., 1972. Gamma-aminobutyric acid: role in primary afferent depolarization. Science, 176(38), pp.1043–1045. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=4338197.

Bautista, D.M. et al., 2006. TRPA1 Mediates the Inflammatory Actions of Environmental Irritants and Proalgesic Agents. Cell, 124(6), pp.1269–1282.

Berg, J.M., Tymoczko, J.L. & Stryer, L., 2002. Biochemistry. W H Freeman, New York., pp.320–323. Available at: papers2://publication/uuid/7EB6183C-F1A3-4903-A72F-B1A659CECF68.

Berg, J.M., Tymoczko, J.L. & Stryer, L., 2007. Stryer Biochemie, Available at: http://books.google.com/books?id=jQKGAAAACAAJ.

Bertolini, A. et al., 2006. Paracetamol: New vistas of an old drug. CNS Drug Reviews, 12(3-4), pp.250–275.

Bessems, J.G. & Vermeulen, N.P., 2001. Paracetamol (acetaminophen)-induced toxicity: molecular and biochemical mechanisms, analogues and protective approaches. Critical reviews in toxicology, 31(1), pp.55–138. Available at: http://www.tandfonline.com/doi/full/10.1080/20014091111677\nhttp://www.ncbi.nlm.nih.gov/pubmed/11215692.

Beuming, T. et al., 2006. A comprehensive structure-based alignment of prokaryotic and eukaryotic neurotransmitter/Na+ symporters (NSS) aids in the use of the LeuT structure to probe NSS structure and function. Molecular pharmacology, 70(5), pp.1630–42. Available at: http://molpharm.aspetjournals.org/cgi/doi/10.1124/mol.106.026120\nhttp://www.ncbi.nlm.nih.gov/pubmed/16880288.

Björkman, R. et al., 1994. Acetaminophen blocks spinal hyperalgesia induced by NMDA and substance P. Pain, 57(3), pp.259–264.

Blakely, R.D. et al., 1991. Cloning and expression of a functional serotonin transporter from rat brain. Nature, 354(6348), pp.66–70. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1944572.

Blier, P., 2001. Crosstalk between the norepinephrine and serotonin systems and its role in the antidepressant response. Journal of Psychiatry and Neuroscience, 26(SUPPL.).

Bond, G.R., 2009. Acetaminophen protein adducts: a review. Clinical toxicology (Philadelphia, Pa.), 47(1), pp.2–7. Available at: http://dx.doi.org/10.1080/15563650801941831.

Bönisch, H. & Brüss, M., 2006. The norepinephrine transporter in physiology and disease. Handbook of Experimental Pharmacology, 175, pp.485–524.

Brodie, B. & Axelrod, J., 1948. The fate of acetanilide in man. Journal of Pharmacology and Experimental Therapeutics, pp.29–38.

Bronstein, A. et al., 2009. 2008 Annual Report of the American Association of Poison Control Centers' National Poison Data System (NPDS): 26th Annual Report. Clinical toxicology (Philadelphia, Pa.), pp.911–1084.

Bröer, S., 2006. The SLC6 orphans are forming a family of amino acid transporters. Neurochemistry International, 48(6-7), pp.559–567.

Brüss, M. et al., 1993. Chromosomal mapping of the human gene for the tricyclic antidepressant-sensitive noradrenaline transporter. Human genetics, 91(3), pp.278–80. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8478011.

Carneiro, A.M.D. et al., 2008. Interactions between integrin αIIbβ3 and the serotonin transporter regulate serotonin transport and platelet aggregation in mice and humans. Journal of Clinical Investigation, 118(4), pp.1544–1552.

Carneiro, A.M.D. & Blakely, R.D., 2006. Serotonin-, protein kinase C-, and Hic-5-associated redistribution of the platelet serotonin transporter. Journal of Biological Chemistry, 281(34), pp.24769–24780.

Cattaert, D., el Manira, a & Clarac, F., 1992. Direct evidence for presynaptic inhibitory mechanisms in crayfish sensory afferents. Journal of neurophysiology, 67(3), pp.610–24. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1578247.

Cervinski, M.A., Foster, J.D. & Vaughan, R.A., 2005. Psychoactive substrates stimulate dopamine transporter phosphorylation and down-regulation by cocaine-sensitive and protein kinase C-dependent mechanisms. Journal of Biological Chemistry, 280(49), pp.40442–40449.

Chen, C. et al., 2008. Identification of novel toxicity-associated metabolites by metabolomics and mass isotopomer analysis of acetaminophen metabolism in wild-type and Cyp2e1-null mice. Journal of Biological Chemistry, 283(8), pp.4543–4559.

Chen, J.G., Liu-Chen, S. & Rudnick, G., 1997. External cysteine residues in the serotonin transporter. Biochemistry, 36(6), pp.1479–1486.

Chen, N.H., Reith, M.E.A. & Quick, M.W., 2004. Synaptic uptake and beyond: The sodium- and chloride-dependent neurotransmitter transporter family SLC6. Pflugers Archiv European Journal of Physiology, 447(5), pp.519–531.

Cheng, Y. & Prusoff, W.H., 1973. Relation between the inhibiton constant (Ki) and the concentration of inhibitor which causes 50 percent inhibiton (IC50) of an enzymatic reaction. Biochemical Pharmacology, 22(23), pp.3099–3108. Available at: http://linkinghub.elsevier.com/retrieve/pii/0006295273901962.

Choi, S.S., Lee, J.K. & Suh, H.W., 2001. Antinociceptive profiles of aspirin and acetaminophen in formalin, substance P and glutamate pain models. Brain Research, 921(1-2), pp.233–239.

Christensen, A.P. & Corey, D.P., 2007. TRP channels in mechanosensation: direct or indirect activation? Nature reviews. Neuroscience, 8(7), pp.510–521.

Clements, J.A. et al., 1978. Kinetics of acetaminophen absorption and gastric emptying in man. Clinical pharmacology and therapeutics, 24(4), pp.420–31. Available at: http://www.ncbi.nlm.nih.gov/pubmed/688732.

Coccaro, E.F., 1989. Central serotonin and impulsive aggression. In British Journal of Psychiatry. pp. 52–62.

Cohen, S.D. et al., 1997. Selective protein covalent binding and target organ toxicity. Toxicology and Applied Pharmacology, 143(1), pp.1–12.

Colas, C., P.M.U. Ung, and A. Schlessinger. 2016. SLC transporters: Structure, function, and drug discovery. *Medchemcomm*. doi:10.1039/c6md00005c.

Corey, D.P. et al., 2004. TRPA1 is a candidate for the mechanosensitive transduction channel of vertebrate hair cells. Nature, 432(7018), pp.723–730.

Dahlin, D.C. et al., 1984. N-acetyl-p-benzoquinone imine: a cytochrome P-450-mediated oxidation product of acetaminophen. Proceedings of the National Academy of Sciences of the United States of America, 81(5), pp.1327–31. Available at:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=344826&tool=pmcentrez&rendertype=abstract.

Danbolt, N.C., 2001. Glutamate uptake. Progress in neurobiology, 65(1), pp.1–105.

Dipace, C. et al., 2007. Amphetamine induces a calcium/calmodulin-dependent protein kinase II-dependent reduction in norepinephrine transporter surface expression linked to changes in syntaxin 1A/transporter complexes. Molecular pharmacology, 71(1), pp.230–239.

Draganov, P. et al., 2000. Alcohol-acetaminophen syndrome. Even moderate social drinkers are at risk. Postgraduate medicine, 107(1), pp.189–195.

Dutta, A.K. et al., 2003. Dopamine transporter as target for drug development of cocaine dependence medications. European Journal of Pharmacology, 479(1-3), pp.93–106. Available at: http://www.sciencedirect.com/science/article/pii/S0014299903023136.

Ebert, B., Wafford, K.A. & Deacon, S., 2006. Treating insomnia: Current and investigational pharmacological approaches. Pharmacology and Therapeutics, 112(3), pp.612–629.

Eimerl, D. & Papir-Kricheli, D., 1987. Epidural capsaicin produces prolonged segmental analgesia in the rat. Experimental Neurology, 97(1), pp.169–178.

Enoch, M.A., 2008. The role of GABAA receptors in the development of alcoholism. Pharmacology Biochemistry and Behavior, 90(1), pp.95–104.

Eriksen, J., Jørgensen, T.N. & Gether, U., 2010. Regulation of dopamine transporter function by protein-protein interactions: New discoveries and methodological challenges. Journal of Neurochemistry, 113(1), pp.27–41.

Eshleman, a J. et al., 1999. Characteristics of drug interactions with recombinant biogenic amine transporters expressed in the same cell type. The Journal of pharmacology and experimental therapeutics, 289(2), pp.877–885.

Esler, M. et al., 2006. The neuronal noradrenaline transporter, anxiety and cardiovascular disease. Journal of psychopharmacology (Oxford, England), 20(4 Suppl), pp.60–66.

Faraj, B.A., Olkowski, Z.L. & Jackson, R.T., 1994. Expression of a high-affinity serotonin transporter in human lymphocytes. International Journal of Immunopharmacology, 16(7), pp.561–567.

Fernandes, E.S., M.A. Fernandes, and J.E. Keeble. 2012. The functions of TRPA1 and TRPV1: Moving away from sensory nerves. *Br. J. Pharmacol.* doi:10.1111/j.1476-5381.2012.01851.x.

Fleishaker, J.C., 2000. Clinical pharmacokinetics of reboxetine, a selective norepinephrine reuptake inhibitor for the treatment of patients with depression. Clinical pharmacokinetics, 39(6), pp.413–27. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11192474.

Flower, R.J. & Vane, J.R., 1972. Inhibition of prostaglandin synthetase in brain explains the anti-pyretic activity of paracetamol (4-acetamidophenol). Nature, 240(5381), pp.410–411.

Foote, S., Bloom, F. & Aston-Jones, G., 1983. Nucleus Locus Ceruleus: New Evidence of Anatomical and Physiological Specificity. Physiological Reviews, 63, pp.844–914.

Gainetdinov, R.R. & Caron, M.G., 2003. Monoamine transporters: from genes to behavior. Annu Rev Pharmacol Toxicol, 43, pp.261–284. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12359863.

Gainetdinov, R.R., Sotnikova, T.D. & Caron, M.G., 2002. Monoamine transporter pharmacology and mutant mice. Trends in Pharmacological Sciences, 23(8), pp.367–373.

Galanopoulou, A.S., 2010. Mutations affecting GABAergic signaling in seizures and epilepsy. Pflugers Archiv European Journal of Physiology, 460(2), pp.505–523.

Giros, B. et al., 1992. Cloning, pharmacological characterization, and chromosome assignment of the human dopamine transporter. Mol Pharmacol, 42(3), pp.383–390. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1406597.

Giros, B. et al., 1996. Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. Nature, 379(6566), pp.606–612. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8628395\nhttp://www.ufrsdv.u-bordeaux2.fr/siteIML/Maste2biosante/Master2biosantecours/articles/Pharmaco comp-Giros.pdf.

Gordon, J. & Barnes, N.M., 2003. Lymphocytes transport serotonin and dopamine: Agony or ecstasy? Trends in Immunology, 24(8), pp.438–443.

Greengard, P., 2001. The neurobiology of slow synaptic transmission. Science, 294(5544), pp.1024–1030. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11691979\nhttp://science.sciencemag.org/content/sci/294/5544/1024.full.pdf.

Guastella, J. et al., 1990. Cloning and expression of a rat brain GABA transporter. Science, 249(4974), pp.1303–1306. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1975955.

Haenisch, B. et al., 2009. Association of major depression with rare functional variants in norepinephrine transporter and serotonin1A receptor genes. American Journal of Medical Genetics, Part B: Neuropsychiatric Genetics, 150(7), pp.1013–1016.

Hahn, M.K. et al., 2008. Multivariate permutation analysis associates multiple polymorphisms with subphenotypes of major depression. Genes, Brain and Behavior, 7(4), pp.487–495.

Hodgkin, a L. & Huxley, a F., 1952. A quantitative description of membrane current and its applicaiton to conduction and excitation in nerve. J Physiol, 117(4), pp.500–544. Available at: papers3://publication/uuid/6AB86D34-792D-424B-806B-374D9A02786F.

Hoffman, B.J., Mezey, E. & Brownstein, M.J., 1991. Cloning of a serotonin transporter affected by antidepressants. Science, 254(5031), pp.579–580.

Hofmann, T. et al., 2000. Transient receptor potential channels as molecular substrates of receptor-mediated cation entry. Journal of Molecular Medicine, 78(1), pp.14–25.

Holzer, P., 2011. Transient receptor potential (TRP) channels as drug targets for diseases of the digestive system. Pharmacology and Therapeutics, 131, pp.142–170.

Inoue, R., 2005. TRP channels as a newly emerging non-voltage-gated CA2+ entry channel superfamily. Current pharmaceutical design, 11(15), pp.1899–914. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15974967.

Jaeschke, H. & Bajt, M.L., 2006. Intracellular signaling mechanisms of acetaminophen-induced liver cell death. Toxicological sciences : an official journal of the Society of Toxicology, 89(1), pp.31–41. Available at: http://toxsci.oxfordjournals.org/content/89/1/31.full.

James, L.P., Mayeux, P.R. & Hinson, J.A., 2003. Acetaminophen-induced hepatotoxicity. Drug Metabolism and Disposition, 31(12), pp.1499–1506.

Jayanthi, L.D. et al., 2005. Evidence for biphasic effects of protein kinase C on serotonin transporter function, endocytosis, and phosphorylation. Molecular pharmacology, 67(6), pp.2077–2087.

Jayanthi, L.D. & Ramamoorthy, S., 2005. Regulation of monoamine transporters: influence of psychostimulants and therapeutic antidepressants. The AAPS journal, 7(3), pp.E728–E738.

Jayanthi, L.D., Vargas, G. & DeFelice, L.J., 2002. Characterization of cocaine and antidepressant-sensitive norepinephrine transporters in rat placental trophoblasts. British journal of pharmacology, 135(8), pp.1927–34. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1573321&tool=pmcentrez&rendertype=abstract.

Jollow, D.J. et al., 1973. Acetaminophen-induced hepatic necrosis. II. Role of covalent binding in vivo. The Journal of Pharmacology and Experimental Therapeutics, 187(1), pp.195–202.

Jordt, S.-E. et al., 2004. Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. Nature, 427(6971), pp.260–265. Available at: http://www.nature.com/nature/journal/v427/n6971/full/nature02282.html\nhttp://www.nature.com/nature/journal/v427/n6971/pdf/nature02282.pdf.

Kalueff, A. V. & Nutt, D.J., 2007. Role of GABA in anxiety and depression. Depression and Anxiety, 24(7), pp.495–517.

Karandikar, Y., P. Belsare, and A. Panditrao. 2016. Effect of drugs modulating serotonergic system on the analgesic action of paracetamol in mice. *Indian J. Pharmacol.* doi:10.4103/0253-7613.182874.

Kim, C.-H. et al., 2006. A polymorphism in the norepinephrine transporter gene alters promoter activity and is associated with attention-deficit hyperactivity disorder. Proceedings of the National Academy of Sciences, 103(50), pp.19164–19169. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1748193&tool=pmcentrez&rendertype=abstract.

Kim, Y.S. et al., 2010. Expression of transient receptor potential ankyrin 1 (TRPA1) in the rat trigeminal sensory afferents and spinal dorsal horn. The Journal of comparative neurology, 518(5), pp.687–98. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20034057.

Kristensen, A.S. et al., 2011. SLC6 neurotransmitter transporters: structure, function, and regulation. Pharmacological reviews, 63(3), pp.585–640. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21752877.

Krnjević, K., 2004. How does a little acronym become a big transmitter? Biochemical Pharmacology, 68(8), pp.1549–1555.

Kuhar, M.J., Ritz, M.C. & Boja, J.W., 1991. The dopamine hypothesis of the reinforcing properties of cocaine. Trends in Neurosciences, 14(7), pp.299–302.

Kurian, M.A., P. Gissen, M. Smith, S.J.R. Heales, and P.T. Clayton. 2011. The monoamine neurotransmitter disorders: An expanding range of neurological syndromes. *Lancet Neurol.* doi:10.1016/S1474-4422(11)70141-7.

Kwan, K.Y. et al., 2006. TRPA1 Contributes to Cold, Mechanical, and Chemical Nociception but Is Not Essential for Hair-Cell Transduction. Neuron, 50(2), pp.277–289.

Lesch, K.P. et al., 1993. Isolation of a cDNA encoding the human brain serotonin transporter. J Neural Transm Gen Sect, 91(1), pp.67–72.

Lewis, D.A. et al., 2008. Subunit-selective modulation of GABA type A receptor neurotransmission and cognition in schizophrenia. American Journal of Psychiatry, 165(12), pp.1585–1593.

Liu, Q.R. et al., 1993. Molecular characterization of four pharmacologically distinct gamma-aminobutyric acid transporters in mouse brain [corrected]. The Journal of biological chemistry, 268(28), pp.2106–2112.

Logan, J. et al., 2007. Imaging the norepinephrine transporter in humans with (S,S)-[11C]O-methyl reboxetine and PET: problems and progress. Nuclear Medicine and Biology, 34(6), pp.667–679.

Loland, C.J. et al., 2008. Relationship between conformational changes in the dopamine transporter and cocaine-like subjective effects of uptake inhibitors. Molecular pharmacology, 73(3), pp.813–823.

Macdonald, R.L., Kang, J.-Q. & Gallagher, M.J., 2010. Mutations in GABAA receptor subunits associated with genetic epilepsies. The Journal of physiology, 588(Pt 11), pp.1861–9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2901974&tool=pmcentrez&rendertype=abstract.

Malcangio, M. & Bowery, N.G., 1996. GABA and its receptors in the spinal cord. Trends Pharmacol Sci, 17(12), pp.457–462. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9014500.

Masson, J. et al., 1999. Neurotransmitter transporters in the central nervous system. Pharmacological reviews, 51(3), pp.439–64. Available at:

http://www.sciencedirect.com/science/article/pii/S0065280602290025\nhttp://www.ncbi.nlm.nih.gov/pubmed/10471414.

Mazei-Robison, M.S. et al., 2008. Anomalous dopamine release associated with a human dopamine transporter coding variant. The Journal of neuroscience : the official journal of the Society for Neuroscience, 28(28), pp.7040–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18614672\nhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2573963.

Mazei-Robison, M.S. & Blakely, R.D., 2005. Expression studies of naturally occurring human dopamine transporter variants identifies a novel state of transporter inactivation associated with Val382Ala. Neuropharmacology, 49(6), pp.737–749.

Meredith, T.J. & Goulding, R., 1980. Paracetamol. Postgraduate medical journal, 56(July), pp.459–473.

Micó, J.A. et al., 2006. Antidepressants and pain. Trends in Pharmacological Sciences, 27(7), pp.348–354.

Miller, R., Roberts, R. & Fischer, L., 1976. Acetaminophen elimination kinetics in neonates, children, and adults. Clinical pharmacology and therapeutics, 19, pp.284–294.

Miner, L.H. et al., 2006. Chronic stress increases the plasmalemmal distribution of the norepinephrine transporter and the coexpression of tyrosine hydroxylase in norepinephrine axons in the prefrontal cortex. J Neurosci, 26(5), pp.1571–1578. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16452680.

Miners, J.O., Attwood, J. & Birkett, D.J., 1984. Determinants of acetaminophen metabolism: effect of inducers and inhibitors of drug metabolism on acetaminophen's metabolic pathways. Clinical pharmacology and therapeutics, 35(4), pp.480–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6705446.

Miranda, H.F. & Pinardi, G., 2004. Isobolographic analysis of the antinociceptive interactions of clonidine with nonsteroidal anti-inflammatory drugs. Pharmacological Research, 50(3), pp.273–278.

Mitchell, J.R. et al., 1973a. Acetaminophen induced hepatic necrosis. IV. Protective role of glutathione. Journal of Pharmacology and Experimental Therapeutics, 187, pp.211–217. Available at: http://www.scopus.com/inward/record.url?eid=2-s2.0-0015733053&partnerID=40&md5=8f18180291657075826cc12620e9d785.

Mitchell, J.R. et al., 1973b. ACETAMINOPHEN-INDUCED HEPATIC NECROSIS. IV. PROTECTIVE ROLE OF GLUTATHIONE. Journal of Pharmacology and Experimental Therapeutics, 187(1), pp.211–217. Available at: http://jpet.aspetjournals.org/content/187/1/211.abstract.

Mochizucki, D., 2004. Serotonin and noradrenaline reuptake inhibitors in animal models of pain. Human Psychopharmacology, 19(SUPPL. 1).

Van Moffaert, M. & Dierick, M., 1999. Noradrenaline (norepinephrine) and depression: Role in aetiology and therapeutic implications. CNS Drugs, 12(4), pp.293–305.

Moore, R. & Bloom, F., 1979. Central catecholamine neuron systems: anatomy and physiology of the norepinephrine and epinephrine systems. Annual Review of Neuroscience, 2, pp.113–68.

Möhler, H., 2009. Role of GABAA receptors in cognition. Biochemical Society transactions, 37, pp.1328–1333.

Munro, G., Ahring, P.K. & Mirza, N.R., 2009. Developing analgesics by enhancing spinal inhibition after injury: GABAA receptor subtypes as novel targets. Trends in Pharmacological Sciences, 30(9), pp.453–459.

Murphy, D.L. et al., 2004. Serotonin transporter: gene, genetic disorders, and pharmacogenetics. Mol Interv, 4(2), pp.109–123. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15087484.

Nagasawa, H.T. et al., 1984. 2-Substituted thiazolidine-4(R)-carboxylic acids as prodrugs of L-cysteine. Protection of mice against acetaminophen hepatotoxicity. Journal of medicinal chemistry, 27(5), pp.591–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6716397.

Nelson, S., 1990. Molecular Mechanisms of the Hepatotoxicity Caused by Acetaminophen. Seminars in Liver Disease, (04), pp.267–278.

Nestler, E.J. & Carlezon, W.A., 2006. The Mesolimbic Dopamine Reward Circuit in Depression. Biological Psychiatry, 59(12), pp.1151–1159.

Newman, A.H. & Kulkarni, S., 2002. Probes for the dopamine transporter: New leads toward a cocaine-abuse therapeutic - A focus on analogues of benztropine and rimcazole. Medicinal Research Reviews, 22(5), pp.429–464.

Nilius, B. & Voets, T., 2005. TRP channels: A TR(I)P through a world of multifunctional cation channels. Pflugers Archiv European Journal of Physiology, 451(1), pp.1–10.

Ng, J., A. Papandreou, S.J. Heales, and M.A. Kurian. 2015. Monoamine neurotransmitter disorders - Clinical advances and future perspectives. *Nat. Rev. Neurol.* doi:10.1038/nrneurol.2015.172.

Olfson, M., C. Blanco, S. Wang, and L.L. Greenhill. 2013. Trends in Office-Based Treatment of Adults With Stimulants in the United States. *J. Clin. Psychiatry*. doi:10.4088/jcp.12m07975.

Olsen, R.W., 2002. GABA. In K. Davis et al., eds. Neuropsychopharmacology: The fifth Generation of progress. p. 2080.

Owens, D.F. & Kriegstein, A.R., 2002. Is there more to GABA than synaptic inhibition? Nat Rev Neurosci, 3(9), pp.715–727. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12209120.

Owens, M.J. & Nemeroff, C.B., 1994. Role of serotonin in the pathophysiology of depression: Focus on the serotonin transporter. In Clinical Chemistry. pp. 288–295.

Pacholczyk, T., Blakely, R.D. & Amara, S.G., 1991. Expression cloning of a cocaine- and antidepressant-sensitive human noradrenaline transporter. Nature, 350(6316), pp.350–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2008212.

Paczkowski, N.J., Vuocolo, H.E. & Bryan-Lluka, L.J., 1996. Conclusive evidence for distinct transporters for 5-hydroxytryptamine and noradrenaline in pulmonary endothelial cells of the rat. Naunyn-Schmiedeberg's archives of pharmacology, 353(4), pp.423–30. Available at: http://link.springer.com/article/10.1007/BF00261439\nhttp://www.ncbi.nlm.nih.gov/pubmed/8935709.

Pascoe, G.A., Calleman, C.J. & Baille, T.A., 1988. Identification of S-(2,5-dihydroxyphenyl)-cysteine and S-(2,5-dihydroxyphenyl)-N-acetyl-cysteine as urinary metabolites of acetaminophen in the mouse. Evidence for p-benzoquinone as a reactive intermediate in acetaminophen metabolism. Chemico-Biological Interactions, 68(1-2), pp.85–98.

Pickering, G. et al., 2006. Analgesic effect of acetaminophen in humans. First evidence of a central serotonergic effect. Clinical Pharmacology and Therapeutics, 79, pp.371–379.

Pini, L.A., Sandrini, M. & Vitale, G., 1996. The antinociceptive action of paracetamol is associated with changes in the serotonergic system in the rat brain. European Journal of Pharmacology, 308(1), pp.31–40.

Plewnia, C. et al., 2002. Enhancement of human cortico-motoneuronal excitability by the selective norepinephrine reuptake inhibitor reboxetine. Neuroscience Letters, 330(3), pp.231–234.

Polc, P., 1982. Enhancement of GABAergic inhibition: a mechanism of action of benzodiazepines, phenobarbital, valproate and L-cycloserine in the cat spinal cord. Electroencephalogr Clin Neurophysiol Suppl, 36, pp.188–198. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6130936.

Potter, D.W., Miller, D.W. & Hinson, J.A., 1985. Identification of acetaminophen polymerization products catalyzed by horseradish peroxidase. Journal of Biological Chemistry, 260(22), pp.12174–12180.

Prescott, L. et al., 1981. Effects of microsomal enzyme induction on paracetamol metabolism in man. British Journal of Clinical Pharmacology, 12(2), pp.149–153.

Prescott, L.F., 2000. Paracetamol, alcohol and the liver. British Journal of Clinical Pharmacology, 49(4), pp.291–301.

Ramamoorthy, S. et al., 1993. Antidepressant- and cocaine-sensitive human serotonin transporter: molecular cloning, expression, and chromosomal localization. Proceedings of the National Academy of Sciences of the United States of America, 90(6), pp.2542–6. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=46124&tool=pmcentrez&rendertype=abstract.

Richeimer, S.H. et al., 1997. Utilization patterns of tricyclic antidepressants in a multidisciplinary pain clinic: A survey. Clinical Journal of Pain, 13(4), pp.324–329. Available at: Available from Clinical Journal Of Pain in http://www.sheffieldchildrens.nhs.uk/nulj-request-form.htm.

Ritz, M.C. et al., 1987. Cocaine receptors on dopamine transporters are related to self-administration of cocaine. Science, 237(4819), pp.1219–1223.

Roberts, J. et al., 1987. Prodrugs of L-cysteine as protective agents against acetaminophen-induced hepatotoxicity. 2-(Polyhydroxyalkyl)- and 2-(polyacetoxyalkyl)thiazolidine-4(R)-carboxylic acids. Journal of Medicinal Chemistry, pp.1891–6.

Rothman, R.B., M.H. Baumann, C.M. Dersch, D. V. Romero, K.C. Rice, F.I. Carroll, and J.S. Partilla. 2001. Amphetamine-type central nervous system stimulants release norepinephrine more potently than they release dopamine and serotonin. *Synapse*. doi:10.1002/1098-2396(20010101)39:1<32::AID-SYN5>3.0.CO;2-3.

Roux, M.J. & Supplisson, S., 2000. Neuronal and Glial Glycine Transporters Have Different Stoichiometries. Neuron, 25(2), pp.373–383. Available at: http://www.cell.com/article/S0896627300809010/fulltext.

Rowden, A.K. et al., 2006. Acetaminophen poisoning. Clinics in Laboratory Medicine, 26(1), pp.49–65.

Rudnick, G., 1977. Active transport of 5-hydroxytryptamine by plasma membrane vesicles isolated from human blood platelets. The Journal of biological chemistry, 252(7), pp.2170–2174.

Rudnick, G., 2011. Cytoplasmic permeation pathway of neurotransmitter transporters. Biochemistry, 50(35), pp.7462–7475.

Rudnick, G. & Wall, S.C., 1992. The molecular mechanism of “ecstasy” [3,4-methylenedioxy-methamphetamine (MDMA)]: serotonin transporters are targets for MDMA-induced serotonin release. Proceedings of the National Academy of Sciences of the United States of America, 89(5), pp.1817–1821.

Rumantir, M.S. et al., 2000. Phenotypic evidence of faulty neuronal norepinephrine reuptake in essential hypertension. Hypertension, 36(5), pp.824–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11082150.

Sasaki-Adams, D.M. & Kelley, A.E., 2001. Serotonin-dopamine interactions in the control of conditioned reinforcement and motor behavior. Neuropsychopharmacology, 25(3), pp.440–452.

Schroeter, S. et al., 2000. Immunolocalization of the cocaine- and antidepressant-sensitive l-norepinephrine transporter. The Journal of comparative neurology, 420(2), pp.211–32.

Seidel, S. et al., 2005. Amphetamines take two to tango: an oligomer-based counter-transport model of neurotransmitter transport explores the amphetamine action. Molecular pharmacology, 67(1), pp.140–151.

Shannon, J.R. et al., 2000. Orthostatic Intolerance and Tachycardia Associated with Norepinephrine-Transporter Deficiency. New England Journal of Medicine, 342(8), pp.541–549. Available at: http://www.nejm.org/doi/abs/10.1056/NEJM200002243420803.

Shannon, M., Borron, S. & Burns, M. eds., 2009. Haddad Winchster’s Clinical Managements of Poisoning and Drug Overdose 4th ed., Philadelphia.

Shinoda, S. & Aoyama, T., 2007. Pharmacokinetics/Pharmacodynamics of Acetaminophen Analgesia in Japanese Patients with Chronic Pain. Biological and Pharmaceutical Bulletin, pp.157–161.

Sitte, H.H. & Freissmuth, M., 2010. The reverse operation of Na+Cl--coupled neurotransmitter transporters - Why amphetamines take two to tango. Journal of Neurochemistry, 112(2), pp.340–355.

Smith, M. et al., 1999. Dopaminergic agents for the treatment of cocaine abuse. Drug discovery today, 4(7), pp.322–332. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10377510.

Sommer, C. 2010. Serotonin in Pain and Pain Control.

Story, G.M. et al., 2003. ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. Cell, 112(6), pp.819–829.

Streeter, A.J. et al., 1984. The covalent binding of acetaminophen to protein. Evidence for cysteine residues as major sites of arylation in vitro. Chemico-Biological Interactions, 48(3), pp.349–366.

Stuart, G.J. & Redman, S.J., 1992. The role of GABAA and GABAB receptors in presynaptic inhibition of Ia EPSPs in cat spinal motoneurones. The Journal of Physiology, 447, pp.675–692. Available at: http://jp.physoc.org/cgi/reprint/447/1/675\npapers3://publication/uuid/B1B1E791-B47B-4DA8-AC04-AF1795F96D0B.

Sucic, S., S. Dallinger, B. Zdrazil, R. Weissensteiner, T.N. J??rgensen, M. Holy, O. Kudlacek, S. Seidel, J. Hwan Cha, U. Gether, A.H. Newman, G.F. Ecker, M. Freissmuth, and H.H. Sitte. 2010. The N terminus of monoamine transporters is a lever required for the action of amphetamines. *J. Biol. Chem.* 285:10924–10938. doi:10.1074/jbc.M109.083154.

Sucic, S. et al., 2010. The N Terminus of Monoamine Transporters Is a Lever Required for the Action of Amphetamines * □. , 285(14), pp.10924–10938.

Sulzer, D. et al., 2005. Mechanisms of neurotransmitter release by amphetamines: A review. Progress in Neurobiology, 75(6), pp.406–433.

Sung, U. et al., 2003. A regulated interaction of syntaxin 1A with the antidepressant-sensitive norepinephrine transporter establishes catecholamine clearance capacity. The Journal of neuroscience : the official journal of the Society for Neuroscience, 23(5), pp.1697–1709.

Sur, C., Betz, H. & Schloss, P., 1998. Distinct effects of imipramine on 5-hydroxytryptamine uptake mediated by the recombinant rat serotonin transporter SERT1. Journal of neurochemistry, 70(6), pp.2545–53. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9603221.

Sur, C., Schloss, P. & Betz, H., 1997. The rat serotonin transporter: identification of cysteine residues important for substrate transport. Biochemical and biophysical research communications, 241(1), pp.68–72. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9405235.

Südhof, T.C., 2012. Calcium control of neurotransmitter release. Cold Spring Harbor Perspectives in Biology, 4(1).

Tan, K.R. et al., 2010. Neural bases for addictive properties of benzodiazepines. Nature, 463(7282), pp.769–74. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2871668&tool=pmcentrez&rendertype=abstract.

Teschke, R., Stutz, G. & Strohmeyer, G., 1979. Increased paracetamol-induced hepatotoxicity after chronic alcohol consumption. Biochemical and Biophysical Research Communications, 91(1), pp.368–374.

Thummel, K.E., Slattery, J.T. & Nelson, S.D., 1988. Mechanism by which ethanol diminishes the hepatotoxicity of acetaminophen. The Journal of pharmacology and experimental therapeutics, 245(1), pp.129–36. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3361439.

Tjølsen, A., A. Lund, and K. Hole. 1991. Antinociceptive effect of paracetamol in rats is partly dependent on spinal serotonergic systems. *Eur. J. Pharmacol.* doi:10.1016/0014-2999(91)90036-P.

Tredger, J.M. et al., 1985. Effects of ethanol ingestion on the hepatotoxicity and metabolism of paracetamol in mice. Toxicology, 36(4), pp.341–352.

Vanhatalo, S. & Soinila, S., 1998. The concept of chemical neurotransmission--variations on the theme. Annals of medicine, 30(2), pp.151–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9667793.

Venkatachalam, K. & Montell, C., 2007. TRP channels. Annu Rev Biochem., 76, pp.387–417.

Versiani, M. et al., 2002. Reboxetine, a selective norepinephrine reuptake inhibitor, is an effective and well-tolerated treatment for panic disorder. Journal of Clinical Psychiatry, 63(1), pp.31–37.

Viana, F., 2011. Chemosensory properties of the trigeminal system. ACS Chemical Neuroscience, 2(1), pp.38–50.

Viña, J., Hems, R. & Krebs, H. a, 1978. Maintenance of glutathione content is isolated hepatocyctes. The Biochemical journal, 170(3), pp.627–630.

Vinkers, C.H. et al., 2010. The inhibitory GABA system as a therapeutic target for cognitive symptoms in schizophrenia: investigational agents in the pipeline. Expert opinion on investigational drugs, 19(10), pp.1217–1233.

Volkow, N.D. et al., 2001. Association of dopamine transporter reduction with psychomotor impairment in methamphetamine abusers. American Journal of Psychiatry, 158(3), pp.377–382.

Williamson, J.M., Boettcher, B. & Meister, A., 1982. Intracellular cysteine delivery system that protects against toxicity by promoting glutathione synthesis. Proceedings of the National Academy of Sciences, 79(October), pp.6246–6249.

Wong, D.T., Bymaster, F.P. & Engleman, E.A., 1995. Prozac (fluoxetine, lilly 110140), the first selective serotonin uptake inhibitor and an antidepressant drug: Twenty years since its first publication. Life Sciences, 57(5), pp.411–441.

Wong, E.H.F. et al., 2000. Reboxetine: A pharmacologically potent, selective, and specific norepinephrine reuptake inhibitor. Biological Psychiatry, 47(9), pp.818–829.

Y.A., R., B. T., and B. A. 2001. From cocaine to ropivacaine: the history of local anesthetic drugs. *Curr. Top. Med. Chem.*

Young, J.. & Landsberg, L., 1998. Catecholamines and the adrenal medulla. In J. D. Foster & J. . Wilson, eds. Williams Textbook of Endocrinology. Philadelphia, pp. 680–2.

Zeilhofer, H.U., Witschi, R. & Hösl, K., 2009. Subtype-selective GABAA receptor mimetics-novel antihyperalgesic agents? Journal of Molecular Medicine, 87(5), pp.465–469.

Zhang, A. et al., 2002. Further studies on conformationally constrained tricyclic tropane analogues and their uptake inhibition at monoamine transporter sites: Synthesis of (z)-9-(substituted arylmethylene)-7-azatricyclo[4.3.1.03,7]decanes as a Novel class of serotonin transport. Journal of Medicinal Chemistry, 45(9), pp.1930–1941.

Zhou, J., Zhang, A., et al., 2003. Biaryl analogues of conformationally constrained tricyclic tropanes as potent and selective norepinephrine reuptake inhibitors: Synthesis and evaluation of their uptake inhibition at monoamine transporter sites. Journal of Medicinal Chemistry, 46(10), pp.1997–2007.

Zhou, J., 2004. Norepinephrine transporter inhibitors and their therapeutic potential. Drugs of the Future, 29(12), pp.1235–1244.

Zhou, J., Kläß, T., et al., 2003. Synthesis and pharmacological evaluation of (Z)-9-(Heteroarylmethylene)-7-azatricyclo[4.3.1.03,7]decanes: Thiophene analogues as potent norepinephrine transporter inhibitors. Bioorganic and Medicinal Chemistry Letters, 13(20), pp.3565–3569.

Zimmerman, H.J. & Maddrey, W.C., 1995. Acetaminophen (paracetamol) hepatotoxicity with regular intake of alcohol: Analysis of instances of therapeutic misadventure. Hepatology, 22(3), pp.767–773.

Zomot, E. et al., 2007. Mechanism of chloride interaction with neurotransmitter:sodium symporters. Nature, 449(7163), pp.726–730.