All roads lead to Rome, but which one is the best?

The direction of Autism Spectrum Disorder drug development

By Louise Witteman 3158241. Supervision: Prof. Peter Burbach

May-June 2013

Universiteit Utrecht

Inhoud

Summary for the laymen

About 1% of the population is affected by Autism Spectrum Disorder (ASD). The main symptoms of ASD are poor communication, poor social interactions and abnormal behavior. The last decade, the general conception about ASD switched from being a developmental disorder to being a treatable disorder later in life. This change in conception provided a great boost to ASD-drug development. The last decade, knowledge about underlying mechanisms of ASD greatly increased and currently many clinical trials are up and running. But how far are we exactly on our way to treat ASD and what can we expect in the future?

Since the number of people diagnosed with ASD is increasing, more and more researchers are getting interested in finding ways to treat ASD. Currently, different pathways are discovered that play a role in the development of ASD. These pathways provide drugable targets, which means that we are on our way to develop effective drugs that target the core mechanisms that cause ASD.

Interestingly, most of the pathways and genetic alterations that have been found in ASD-patients play a role in protein synthesis at the synapses of our brain. Synapses are contact sites between brain cells where communication takes place. This communication is crucial for the brain to executive its functions, such as learning and memory. Miscommunication between cells, due to alterations in protein synthesis, can cause ASD.

Currently, drugs have been developed and tested in animals with promising results. These drugs are now also tested in humans. Although positive results have been booked in ASD-patients that were treated by the new drugs, there are still serious issues to overcome. For instance, drugs might be affective in just a small population of ASD-patients, since there is a big variety in the cause of ASD among patients. Moreover, long term side effects need to be established and there are question marks about what these drugs will eventually mean for the quality of life for ASD-patients. Positive results in research is mainly based on clinical findings (severity of the disorder etc.), whereas they do not take into account the actual improvement on the quality of life for the patient. For example, when a child is suffering from highly severe ASD and treatment lowers this to severe ASD, researchers will probably be excited with this outcome. However, the child might still not be able to attend school activities and is still severely affected.

Nevertheless, big steps have been made forward the last decade in our understanding of ASD. Moreover, many different drugs, targeting different pathways are tested in humans at this moment. Taken together, it seems that we are in the middle of a revolution in the ASD-drug development field. For the first time, core pathways that cause ASD are targeted. These findings might also be applicable to other disorders of which we initially thought that they were untreatable.

Summary

About 1% of the population is affected by Autism Spectrum Disorder (ASD). The main symptoms of ASD are poor communication, poor social interactions and abnormal behavior. The last decade, the general conception about ASD switched from being a developmental disorder to being a treatable disorder at later ages. This change in conception provided a great boost to ASD research and drug development.

Several important pathways of ASD have now been elucidated. These pathways are mainly involved in protein synthesis at spines, important for axonal-dendritic contact. In ASD, the regulation of protein synthesis is changed. Since protein synthesis is highly important for information processing and communication between neurons, altered protein synthesis directly affects synaptic plasticity.

When synaptic plasticity is disturbed, miscommunication between neurons and within neuronal networks occurs. Synaptic plasticity is crucial, among many other functions, for learning and memory. Alterations in these processes can therefore cause cognitive decline and many neurological disorders, such as ASD.

Recently, insights in these pathways paved the way for drug development. For instance, mGluR5 is believed to be overactive in ASD. This causes excessive protein synthesis and enhanced LTD at dendritic spines. Other pathways that are involved in the regulation of dendritic protein synthesis are downstream targets of mGluR5, such as the mTOR- and the Ras-ERK-pathway. In addition, the endocannabinoid system (ECS) is also found to control protein synthesis.

Moreover, the ECS regulates the release of neurotransmitters at the synapses. When the ECS does not function well, contacts can get too weak or too strong. Again, this leads to abnormal communication between neurons.

Another important aspect for proper communication is the ratio between excitatory and inhibitory (E/I-ratio) synapses. This ratio is highly dependent on protein synthesis.

These different pathways that are involved in protein synthesis which are thought to be altered in ASD form interesting targets for drugs. Nowadays, there are many different researches going on in order to boost drug development for ASD. Currently, the main focus seems to be on mGluR5-inhibitors as possible ASD-treatment and several mGluR5-inhibitors are now tested in clinical trials. However, clinical trials with drugs that interfere with other targets are running or in preparation as well.

The future direction of ASD drug development is hard to predict. Even though positive results have been booked so far by clinical trials, there are still issues to overcome. For instance, long term treatment might cause serious side effects, due to the involvement of the before mentioned pathways in peripheral cells as well. Moreover, the pathways that are elucidated are mainly based on research to fragile X. Although it is likely that these disorders show a great overlap, there is also chance that they show important pathological differences that still need to be elucidated. In addition, little or no research has been done that uses quality of life as an outcome measure. Therefore, it might be questionable how useful the treatment is for patients.

Last but not least, the direction of drug development and how fast this will go, not only in ASD, is strongly dependent on money issues, sharing knowledge and politics.

Abstract

Autism Spectrum Disorder (ASD) is a mental disorder that affects roughly 1% of the population. The main symptoms of ASD are poor communication, poor social interactions and abnormal behavior such as repetitive behavior.

Recently, research to drug development for ASD has received a great amount of interest, due to increasing knowledge about proteins and pathways involved in ASD. These pathways mainly include protein synthesis regulation systems, such as the mGluR5-pathway and the endocannabinoid system. Protein synthesis is highly important during brain development for cell migration and synaptogenesis, but also in the mature brain to maintain the balance between excitatory and inhibitory synapses and to make synaptic plasticity possible. These processes are all highly important for cell communication and thereby brain functioning.

The insight that processes in the mature brain also contribute to ASD provide the possibility to treat ASD at later ages, whereas the initial conception about ASD was that it is a developmental disorder that only could be treated at a very young age. This highly increased the interest of many researchers into ASD drug development.

Currently, there are clinical trials running with drugs that target the core pathways of ASD. Although the results are promising, one should take into account that many of these drugs also target peripheral cells and therefore may cause side effects on the long run. Therefore, the future of the direction of drug development remains uncertain. In addition, money issues, politics and sharing knowledge may be important issues to consider regarding to drug development.

Introduction

Autism Spectrum Disorder (ASD) is a mental disorder that affects roughly 1% of the population. The main symptoms of ASD are poor communication, poor social interactions and abnormal behavior such as repetitive behavior [1].

Currently there are two main strategies to treat ASD-patients: medication and education. Both strategies mainly focus on improving the quality of life, by improving children's independency, their communication skills, their personal responsibility and by decreasing the maladaptive behavior as much as possible [2,3]. For instance, most drugs that are available to treat ASD are addressing the stereotypic behaviors of ASD-patients. However, they are not specific for ASD and come with serious side effects. These side effects range from gaining weight to extrapyramidal effects such as uncontrolled movements. Moreover, there is not much or no strong evidence for the beneficial effects of most drugs and little is known about proper doses that should be prescribed [2,4]. In short, available treatment methods do not effectively treat ASD, nor do they affect the pathogenic mechanisms underlying ASD.

In order to maximize the effect of current treatments, researchers are focusing on methods to diagnose ASD as early as possible. The eventual goal is to start treatment at a very young age. The younger the child is, the easier it will be to correct symptoms by proper teaching methods and behavioral strategies. Moreover, further mal-development will be limited when treatment starts at a very young age. So the earlier the treatment starts, the better the outcome will be.

Therefore, researchers are focusing on the many genes that were found to be involved in ASD. They are trying to link these genetic mutations to fMRI-studies in order to clarify what goes wrong during development. This increases the chance of early diagnosis, which results in an early start of education or medication [1]. This urge to diagnose ASD as early as possible is a consequence of ASD being considered a developmental disorder, meaning that the cognitive deficits one might experience during adulthood are caused by alterations during brain development [5].

However, this general conception about ASD being a developmental disorder has recently changed. Many animal studies indicate that cognitive impairments are not just the result of alterations during development. In fact, processes that contribute to ASD might start in development (such as alterations of cell migration), but continue to affect the mature brain during adulthood. Or mutations in certain genes that affect development, might also have consequences in the mature brain since these proteins will still be expressed in the adult brain. These altered proteins contribute significantly to cognitive impairments during adulthood. This means that therapeutically targeting these altered proteins could result in major cognitive improvements, even when treatment starts at a later age. In animal models it has already been demonstrated that treatment starting after development can indeed still reverse cognitive disability [5,6].

However, there is still a great lack of knowledge about the exact mechanisms that cause ASD. Exploring molecular pathways that are changed in ASD-adolescents or adults is now translated into the discovery of drugable targets that could improve the symptoms even when treatment starts at a later age.

Developing drugs that will target the pathogenesis of ASD is difficult due to the variety of symptoms, a continuously expanding list of candidate genes that are causal to ASD, the big variety among patients and because many patients suffer from comorbidities. Altogether, this makes it very hard to develop proper animal models and thus to conduct research on ASD. Moreover, typical ASD symptoms such as communication deficits are difficult to measure in animals. In addition, mutations that have been linked to ASD are often rare. Although these mutations might increase our knowledge about ASD, they do not provide general therapeutic targets directly [3,7–9].

Still, much progression has been made the last decade. Large samples of DNA from autistic patients have been studied and it is now clear that ASD is highly genetic. In human as well as animal studies, many different genes have been found to play a possible role in ASD and they do show similarities: they often code for proteins important for synaptic formation, the organization of excitatory and inhibitory synapses or act like scaffolds in the synaptic density [7,8,10]. Together with post-mortem studies of ASD patients, in which an increase in dendritic spine density was found, these results suggest that ASD may be caused by alterations in synapses [11] .

Important insights in ASD pathology and possible methods to reverse this are coming from findings in Fragile X Syndrome, neurofibromatosis, Tuberosis Sclerosis and other disorders that result in cognitive decline (as in ASD). These disorders show great similarities with ASD and often suffer from comorbidity with ASD. Since these disorders might share the pathogenesis, results from these studies and animal models from these fields have been considered as relevant models for ASD as well [5,12].

The aim of my thesis is to provide an overview of the direction in which drug development for ASD research is going to. I will do this by explaining the core deficits in ASD, how these deficits can be targeted, and what clinical trials (and patent applications) are currently running. Moreover, I will elaborate on the usability and potential benefits and downsides of drugs that are currently investigated. At last I will discuss how the direction of drug development is influenced and provide food for thought on why we conduct research: do we all have the same goals?

Chapter I: Pathogenesis

What goes wrong during and after development, that leads to ASD? As I already described briefly in the introduction, ASD is characterized by synaptic pathology, a synaptic disease so to say. Post mortem studies of ASD patients revealed an increase in spine density in specific brain areas, deviating spine morphologies [7,11] and, moreover, studying samples of ASD patient-DNA elucidated rare mutations in many different genes that are important for synaptic functioning [8].

Synapses are the contact sites of the brain that make it possible to transfer information from one cell to another. Axonal presynaptic sites contact postsynaptic sites located on dendrites of other neurons. Dendrites contain a tremendous amount of small protrusions, called spines. These spines contain specific regions, postsynaptic densities (PSD), that form the actual contact points with the presynapse. Dendritic spines can either be excitatory or inhibitory and they come in different shapes and sizes. Their morphology changes continuously and responds to presynaptic input and activity. In short, spine morphology is highly associated with the function they perform [13].

Alterations in spine morphology are crucial for executing brain functions, such as learning and memory formation. In general, enlargement of spines for a longer period of time is associated with strengthening of the synaptic contacts, called long term potentiation (LTP), whereas weakening the synaptic contacts, or long term depression (LTD), is associated with shrinkage of spines. Both types of plasticity are activity-dependent [14].

Causal to this activity-dependent memory formation is local mRNA translation at the dendrites. Proper communication between neurons and within neuronal circuits requires, among others, protein synthesis and a balance between excitatory and inhibitory synapses (the E/I-ratio). The latter is crucial for proper brain development and maintenance of neuronal circuits within the mature brain. Without proper protein synthesis, neuronal circuits might get disturbed leading to neurological disorders such as ASD [14,15]. Evidence for uncontrolled protein synthesis at the synapses of ASD patients, is nicely reviewed by Ebert and Greenberg [16].

The idea of synaptic alterations in ASD is strengthened by genetic findings. For instance, rare mutations were found in genes that code for cell adhesion and scaffolding proteins (such as Neuroligins and Neurexins), genes that play a role in signal transduction and the control of protein synthesis and genes that are important regulators of the balance between excitatory and inhibitory synapses [17]. In short, these genes are all important for proper communication between the pre- and postsynapse. Although mutations may be rare, they can be causal to ASD [6].

In this chapter, I will elaborate on the link between protein synthesis, synaptic communication and communication within neuronal circuits and how changes in protein synthesis might contribute to ASD

mGLuR5

First, I will go into more detail on the regulation of protein synthesis at dendritic sites during memory formation. As described in the introduction of this chapter, both LTP and LTD are activity-dependent. In ASD, activity-dependent LTD is believed to be enhanced [18].

Activity-dependent LTD can be initiated by the activation of the group I metabotropic receptors, mGluR1 and mGluR5, located at excitatory dendritic postsynaptic sites. Active mGluR5 activates a

cascade that leads to local protein synthesis at the dendrite, required for LTD, see figure 1. In ASD, protein synthesis is believed to be strongly enhanced, one reason being overactivation of mGluR5, which leads to enhanced LTD [18–20].

Normally, activated mGluR5 leads to phosphorylation and thereby activation of Ras/MEK/ERK and the mTOR-pathway through PI3K activation at dendritic sites of the hippocampus [21].

Upon activation of the mTOR-pathway, downstream targets such as eIF4E, a cap-binding protein, IE-binding protein (IE-BP) and S70-kinase get activated [22,23], see figure 1. These proteins contribute to the control of postsynaptic protein synthesis, which is crucial for a proper synaptic response. Malfunctioning of this control system can cause miscommunication between neurons that results in autistic features [22,24].

Another pathway that is activated by mGluR5 is the Ras/MEK/ERK-pathway. ERK phosphorylation is demonstrated to be increased in FSX-mouse models [25] as well as in tuberosis sclerosis (TSC)-mouse models [26], although the latter findings are not always consistent [27]. The increase of ERK-phosphorylation leads to enhanced mGluR5-dependent LTD which, in the TSC-mice, could be reduced by MEK inhibition [26]. This indicates that interference with Ras/MEK/ERK could provide possible drug targets.

In addition, the Ras/MEK/ERK-pathway has been linked to spine morphology [28]. The MEK/ERK pathway is suggested to activate PAK [29]. PAK is an important regulator of actin dynamics and thereby spine morphology, see figure 1. This raised the idea of inhibiting PAK in order to control spine morphology, and thereby function. A genetic approach that inhibited PAK in mice successfully decreased spine densities and improved behavior [30], making PAK an interesting protein to target by drugs to treat ASD.

Besides ERK and mTOR-activation, active mGluR5 is important for the trafficking of FMRP-granules. These granules transport FMRP and Fmr1-mRNA to the dendrites [31]. FMRP can bind to different mRNAs and thereby preventing their translation [32,33]. Interestingly, FMRP also targets and thereby negatively regulates mGluR5-activity and expression, [34,35] providing a negative feedback loop, see figure 1.

FMRP is especially known from research on Fragile X Syndrome (FSX). FSX is caused by mutations in the Fmr1-gene that causes silencing of the gene [27]. As described previously, FSX and ASD show a great overlap indicating a shared pathology. Recently it was demonstrated that FMRP targets many proteins that have been linked to ASD [35] supporting the idea that similar mechanisms underlie FSX and ASD. Since FMRP is silenced in Fragile X, that likely shares pathology with ASD, one can imagine that this has big consequences for local protein synthesis at the dendrites and affects synaptic plasticity.

The Endocannabinoid System

Besides mGluR5 and FMRP, the endocannabinoid system (ECS) is also involved in the regulation of protein synthesis and synaptic functioning. This system is important for emotional processing and (fear)learning and memory formation. Moreover, recent evidence demonstrates that the ECS is involved in ASD. For instance, it was demonstrated that the ECS is altered in a VPA-rat autistic model

[36]. In addition, research indicates that rare mutations in Neuroligins, that are causal to ASD, can impair the tonic signaling of the ECS [37].

The ECS contains two important cannabinoid (CB) receptors: CB1R and CB2R. Two endogenous substrates of these receptors are arachidonolyl ethanolamine (AEA) and 2-arachinodonyl glycerol (2-AG). Both endocannabinoids are locally produced when required [38].

The way in which the ECS affects synaptic communication is by retrograde endocannabinoid (CB) signaling. Endogenous CBs are produced and released at the postsynapse upon presynaptic activation. For instance, presynaptic activation and excitatory neurotransmitter release will activate postsynaptic mGluR5. Upon mGluR5 activation, 2-AG is produced and released at the postsynapse [39–41]. Since mGluR5 is believed to be overactivated in ASD, this raises the possibility of excessive 2-AG production and the involvement of the ECS in ASD.

2-AG in turn binds to and activates presynaptic CB1Rs. Activation of CB1R results in a decrease of neurotransmitter release at the presynaptic site. This holds true for both GABA and glutamate. The ECS thus functions as a negative feedback loop, see figure 1 [41,42], thereby providing a regulation mechanisms that controls the amount of neurotransmitter release and thus synaptic communication.

As described earlier, the ECS itself also plays an important role in protein synthesis. This was demonstrated by the application of THC to mice, an exogenous CB that stimulates CB1R. This caused long term memory deficits. This cognitive impairment was correlated with excessive protein synthesis in the hippocampus, measured as increased phosphorylation of p70S6K. Moreover, blocking protein synthesis in combination with THC-application did not result in memory deficiencies, revealing that CB1R is involved in the regulation of protein synthesis. The increase in protein synthesis was a result of an increased mTOR-activity upon THC-application. This provides evidence for the interaction between the ECS and the mTOR-pathway [43].

The cognitive deficits and activation of the mTOR-pathway by CB1R were mainly caused by CB1Rs located on GABAergic interneurons and to a lesser extent on glutamatergic neurons. This led to an imbalance between E/I. The authors suggest that this imbalance results in the activation of NMDARs and thereby activate the mTOR-pathway [43], see figure 1. Although THC is an exogenous CB, these results still hint towards an interaction between the ECS, mTOR, and excessive protein synthesis which could disturb the axonal-dendritic communication. It would be interesting to test whether 2-AG application has the same effect as THC application to demonstrate whether endogenous CBs have the same effect.

Besides protein synthesis, CB1R plays an important role in LTD induction. Binding of postsynaptically released endocannabinoids to CB1R triggers CB1R-dependent LTD [44]. It shares mechanisms with mGluR5-dependent LTD in which CB1R probably acts downstream of mGluR5 [45]. In sum, the ECS is an important regulator of protein synthesis and is involved in synaptic plasticity and cognitive functioning. Together with the findings described here, the ECS is a plausible candidate to play a role in ASD development.

The E/I-ratio; GABAergic switch

Besides protein synthesis, a constant balance between E/I is required for correct synaptic communication within neuronal circuits. At early developmental stages, GABAergic excitatory signaling is very important for the development, maturation and survival of neurons [46].

Normally, GABAergic signaling is excitatory at the early stage of brain development, but it becomes inhibitory over time. This depends on the expression and thereby presence of ionchannels. At early developmental stages the transporter NKCC1 is expressed. NKCC1 transports Cl^- into the cell. Later, GABAergic signaling leads to the expression of KCC2, which exports Cl^- out of the cell, making GABAergic signaling inhibitory [47].

Interestingly, it was recently demonstrated that Neuroligin2 (NL2) plays a major role in the regulation of KCC2-expression. Therefore, NL2 also plays an important role in the decision whether GABAergic signaling is excitatory or inhibitory. Downregulation of Neuroligin2 in cortical mouse neurons was correlated with less expression of KCC2 [48]. When the expression level of KCC2 is not sufficient, Cl^- will accumulate in the cell and GABAergic signaling remains excitatory [49].

It turned out that NL2 is not only important for the switch from excitatory to inhibitory, but also plays a role in mature neurons to ensure that GABAergic signaling stays inhibitory. NL2 is reported to not just affect KCC2 expression levels on GABAergic synapses but also to affect the levels of excitation of glutamatergic spines. This proposes a key function for NL2 in the E/I-ratio [48]. Alterations in NL2 thus have a great impact on neuronal network functioning, although the exact mechanism remains to be elucidated.

Since it was previously discovered that FMRP interacts with NL2, it seems plausible that NL2 is involved in the pathology of Fragile X syndrome and ASD. However, it could not directly be observed that a lack of FMRP increased NL2 protein levels [50]. It would be interesting to repeat the experiment of NL2 expression and how this affects KCC2 expression, but this time explore what happens if NL2 is overexpressed instead of downregulated. This could possibly mimic the pathology in FSX/ASD, since FMRP interacts with NL2 and thus might results in overexpression of NL2 in FSX-mouse models.

Due to the influence of NL2 on KCC2 expression, alterations in expression levels of NL2 might have serious consequences for the GABAergic switch from excitatory to inhibitory signaling. This has a big impact on neuronal migration, synaptogenesis and the development of neuronal circuits. Together with the finding that FMRP interacts with NL2 and that altered NL2-expression is correlated with changes in social behavior in mice and rats [24,50–52] , this indicates that NL2 might be an important factor in ASD-development.

Besides NL2, its presynaptic binding partner Neurexin1 is found to be mutated in several ASD-patients. This mutation weakens the communication between neurons and thus increases the chance of miscommunication and disrupted neuronal circuits [53], again supporting the importance of NL2 functioning in ASD. Investigating the mechanism through which NL2 regulates KCC2 expression and influences the GABAergic switch might reveal new drugable targets to treat ASD.

The GABAergic switch is also affected when, for instance, iontransporters or GABA-receptors get mutated. These mutations might have a great influence on the E/I-ratio, since Cl^- could accumulate inside the cells [49]. Interestingly, mutations in GABA-receptors have recently been reported in

humans suffering from ASD [54]. This could result in higher excitable brains in ASD-patients, which is in line with the hypothesis of Rubenstein and Merzenich [55] that states that (some forms of) ASD is caused by an increased excitability of the brain. This might be an interesting thought developing new therapeutic interventions.

The E/I-ratio; glutamatergic signaling

Besides GABAergic signaling, glutamatergic signaling is also important for the E/I-ratio. Normally, mGluR5 activation stimulates AMPAR-internalization, see figure 1. AMPARs are important glutamate-receptors at excitatory dendritic synapses [56]. However, in cultured hippocampal neurons, the absence of FMRP leads to a higher rate of AMPAR internalization. Thereby the amount of AMPARs at the cell surface of the PSD is decreased. In other words, the cells become less sensitive to glutamate en thus less excitable by presynaptic glutamate release, disturbing the information processing [57].

It appears to be a paradox, but since a previous experiment showed an increase in AMPAR internalization due to a lack of FMRP, could not be blocked by chemical inhibition of mGluR5, it could be indicated that there must be spontaneous activation of mGluR-5 that is responsible for the AMPAR internalization [58]. This supports the idea of increased excitability at synapses in ASD. One way or the other, these effects do not only affect one synapse, but disturb the entire neuronal circuit. This imbalance might also explain why epilepsy occurs more frequently in autistic patients than in the general (hospital) population [57].

To sum up, ASD is caused by altered protein synthesis at dendritic sites. This is a consequence, of many different pathways that control or regulate protein synthesis. These changes in protein synthesis result in abnormal communication between neurons and within neuronal circuits as it amplifies LTD and disturbs the E/I-ratio. This abnormal communication causes problems at axonal-dendritic information processing that leads to neurological disorders, such as ASD.

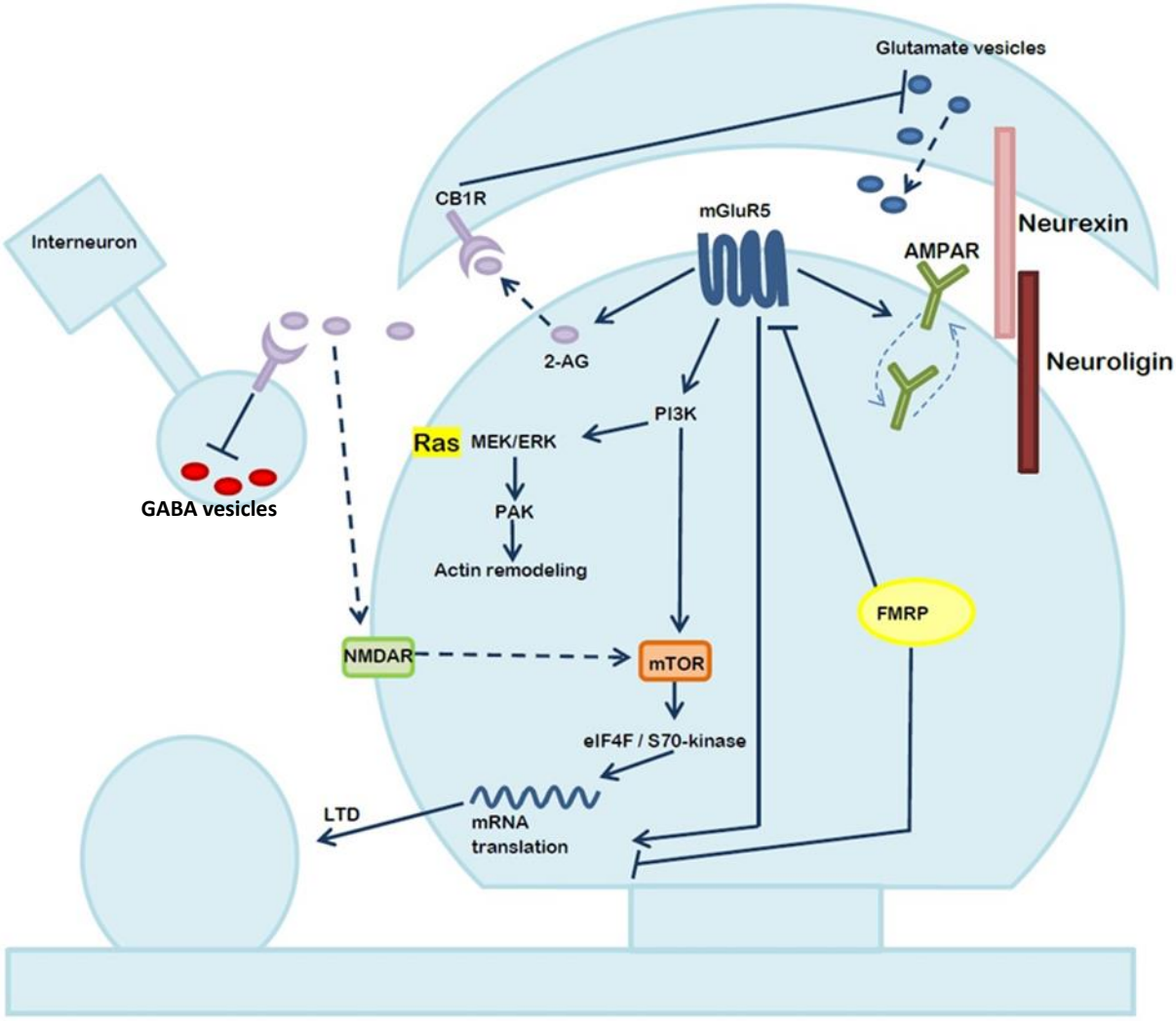

Figure 1. Pathways involved in ASD that cause altered protein synthesis. In ASD, many different pathways have been found to play a possible role in ASD-development. However, these different pathways seem to converge on protein synthesis at dendritic spines. Protein synthesis is important for synaptic communication and plasticity. Altered protein synthesis might therefore lead to abnormal communication and thereby disrupting neuronal circuits that cause ASD. Important factors in dendritic protein synthesis are mGluR5, that regulates AMPAR internalization and activates several pathways important for protein synthesis, such as the mTOR, Ras/ERK and the production of endocannabinoids. Neurexin and Neuroligin are depicted, since they may contribute to the GABAergic switch. This switch plays a major role during development (required for synaptogenesis, maturation of neurons and neuronal migration) as well as during adulthood (to maintain inhibitory GABAergic signaling).

Chapter II: Interference with core pathways

The molecular pathways as described in 'Pathology' are schematically depicted in figure 2. Basically, the figure shows several options to interfere with altered protein synthesis or the consequences of altered protein synthesis to target molecular pathways underlying ASD. In short, based on current knowledge, drug development for ASD can target (1) the overactive mGluR5 (and the pathways that are activated by mGluR5), (2) the ECS, (3) the GABAergic switch and/or (4) the imbalanced E/I-ratio, see figure 2. Here, I will shortly describe preclinical findings that support that these core molecular pathways can be targeted by drugs. Targeting these pathways will make drugs to treat ASD more specific and might therefore be more effective than the currently available treatments.

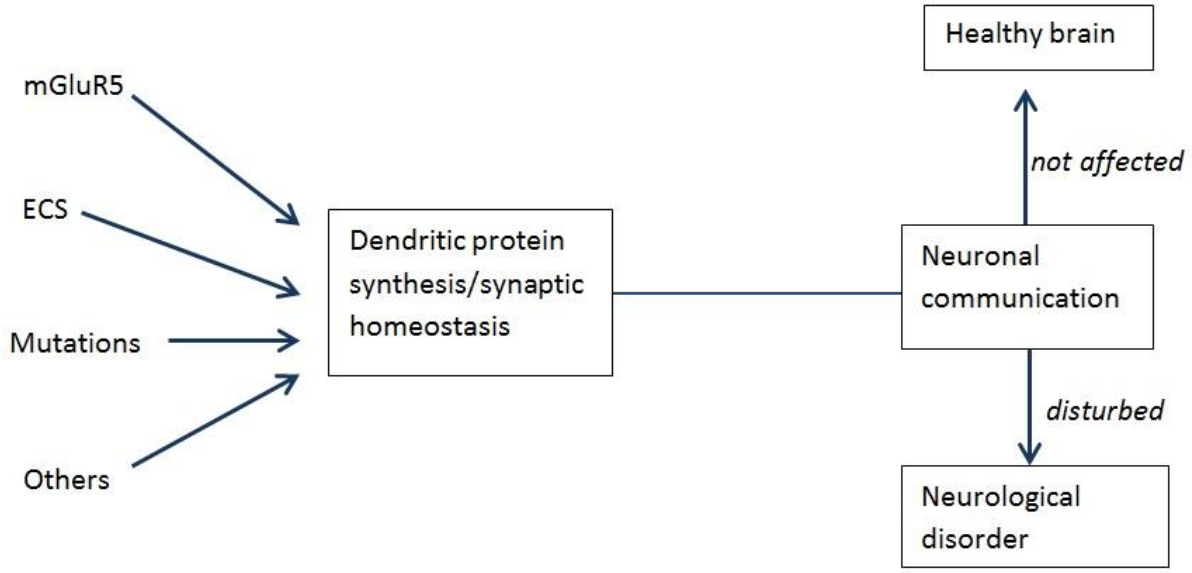

Figure 2. Schematically depicted pathways that affect synaptic homeostasis. When this homeostasis is out of balance, this might cause ASD. The pathways involved in protein synthesis and homeostasis therefore provide drugable targets to treat ASD. For synaptic homeostasis, it is also important that the E/I-ratio remains balanced. Mutations in Neuroligins might play a crucial role in this. Besides the pathways depicted here, possibly many other, yet unknown, pathways are involved.

mGluR5

The effects of overactive mGluR5 could be prevented by either inhibition of mGluR5-activity or by downregulation of its downstream effectors, such as the mTOR-pathway or ERK-activity. Especially mGluR5-antagonists have received a lot of attention the last decade.

One of the first substances that was discovered as an mGluR5-antagonist is ((2-methyl-6-(phenylethynyl)-pyridine) MPEP. MPEP has been demonstrated to be an effective mGluR5-antagonist that could reduce characteristic autistic behavior in mice [59]. However, this compound cannot be used in men due to its toxic effects in humans and its short half-life [60].

Since MPEP cannot be used in humans, the search for other compounds went on. Less than a decade ago, a screening showed that fenobam is an mGluR5-antagonist. It has a high affinity for the receptor and binds specifically to mGluR5 but not to other mGluRs [61]. Fenobam binds at the same

place on mGluR5 as MPEP, but with a higher specificity and it has a longer half-life. Moreover, fenobam enters the brain rapidly after oral administration in mice [62].

Experiments showed that fenobam inhibited mGluR5-activity, had positive effects on spine morphology- and density [63] and increased cognition in FSX-mice [64]. In addition, fenobam has previously been tested in a clinical trial to treat anxiety [65]. Since this trial already showed that the drug is fairly safe in humans, fenobam could be a potential drug to treat autism. However, it should be noted that contrary findings on cognition were found in rats; they performed worse on distinct learning tasks[66]. Therefore this might require extra attention when it comes to clinical trials.

Another mGluR5-antagonist that reversed altered spine morphology, some social behavior in FSX-mouse models and rescued prepulse inhibition, an important FSX-feature, is AFQ056 [67,68]. This compound too was used in clinical trials before, for Parkinson's disease. Although sometimes dizziness was reported, the authors concluded that the drug was pretty well tolerated [69].

Even though the before mentioned compounds are promising, the interest in developing new, better or different mGluR5-inhibitors continues. This was demonstrated by the recent discovery of 2-chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy)phenyl)-1Himidazol-4-yl)ethynyl)pyridine (CTEP). This allosteric inverse agonist of mGluR5 is highly specific for mGluR5. It is able (in mice) to quickly enter the brain and compared to, for instance fenobam, it has a very long half-life. Moreover, the required oral intake dose is much lower and it has a slower clearance than fenobam [25].

In FSX-mice models, CTEP restored the enhanced protein synthesis, learning deficits, the increased susceptibility to audiogenic seizures, hyperactivity and the increased dendritic spine density. In addition, treatment of mice with CTEP reduced the increased mGluR5-dependent LTD as was measured in acute hippocampal. Interestingly, CTEP was able to reduce audiogenic seizure susceptibility in mice with different genetic backgrounds [70].

Chronic treatment of FSX-mice with CTEP did not alter body weight, temperature, or general fitness. Nor did it result in side effects such as altered locomotor activity. The only possible negative side effect that was observed was a small reduction in grip strength of the mice. The authors concluded that chronic treatment with CTEP is well tolerated by the animals [70].

In short, inhibition of mGluR5-activity has been extensively researched during the last few years. Inhibition of mGluR5-activity in mouse models has been proven to be a promising therapy. Inhibition of mGluR5 not only reduced excessive protein synthesis, but has also been demonstrated to improve social behavior and repetitive behavior, increase memory performance and decrease spine density [59,63,67,71]. Although the findings of improved social behavior were not always consistent [72] and sometimes mGluR5 inhibition decreased the total distance that is traveled by the mice [67] which could hint towards some form of sedation, it is still worthwhile to test mGluR5-inhibitors in clinical trials due to the many positive effects that were found, in order to test whether these inhibitors could improve autistic-features in humans.

mTOR

Another possibility to oppose the effects of overactive mGluR5 is targeting its downstream effectors, such as mTOR. This option has been explored in mice.

Treatment of adult tuberosis-sclerosis (TSC) mouse models with rapamycin, a well-known mTOR-inhibitor that is used to prevent organ rejection after transplantation by suppressing the immune system, improved social interacting behavior and decreased S6K-phosphorylation, an indirect measure of protein synthesis [73].

Also morphological changes observed in PTEN-deficiency mice (used as an ASD-model) were reversed by rapamycin treatment [74]. In addition a review by Ehninger and Silva described that rapamycin improved cognition, social interaction and altered brain morphologies and decreased epileptic seizures [75].

Ras/MEK/ERK-pathway

Another downstream target of mGluR5 is the Ras/MEK/ERK-pathway. This pathway has been demonstrated to be a possible drug target as well. Recently, lovastatin was tested in mice. Lovastatin, which belongs to the farnesyltransferase inhibitors which are used to reduce the risk on a stroke, prevents recruitment of Ras to the cell membrane. This prevents Ras from getting activated [76].

Lovastatin was administered to FSX-models and WTs. It was demonstrated (in both WT and mutants) that the amount of active Ras-ERK and thus protein synthesis was reduced, without affecting the amount of Ras and ERK. In addition, lovastatin suppressed the mGluR5-dependent LTD to normal levels in FSX-models, without affecting LTD in WTs. The reduction of LTD resulted in lower audiogenic seizure susceptibility and severity [77].

The required dose to achieve these beneficial effects was dependent on the genetic background of the mice (in contrast to CTEP that was effective in mice independent of their genetic background). Importantly, lovastatin can be orally administered to humans as the compound lovastatin lactone. The previously described findings could be reproduced using this drug as well, indicating promising results for lovastatin to treat ASD or FSX in humans [77].

Another interesting finding is that CTEP was also able to reduce the activity of the Ras/MEK/ERK pathway, indicating that CTEP acts on different pathways important to ASD [25].

PAK-inhibition

As described previously, PAK also acts downstream of mGluR5. Recently a compound that inhibits PAK, FRAX486, has been discovered.

FRAX486 was discovered by a high-throughput screen and was found to be highly specific for group I PAKs. These PAKs are enriched in the brain. FRAX486 is a small molecule that can enter the brain at sufficient concentration to inhibit PAK (in mice) and its concentration in the brain starts to decrease after 24 hours, meaning that this small molecule has a long half-life. Still, daily injections in mice did not result in accumulation of the substance in the brain.

FRAX486 was able to reduce the typical increased apical dendrite spine density of the temporal cortex in FSX-mouse models. Interestingly, on a behavioral level it was demonstrated that this decrease in spine density went along with reduced seizure susceptibility, reduced hyperactive and repetitive behavior. The latter is especially important since this is one of the core symptoms of ASD [78].

The Endocannabinoid System

The second system that is depicted in figure 2 and provides possibilities to interfere with, regarding ASD is the ECS. The CB1R, as described in Chapter I, is involved in the regulation of protein synthesis and could therefore play an important role in ASD. The consequences of CB1R expression levels on autistic features have been tested in mice.

Genetic knockdown of the CB1R in Fmr1-knockout mice resulted in less prominent FSX features. For instance, mGluR5 was less active and the sensitivity to audiogenic seizures and cognitive deficits was reduced. These results demonstrate the importance of CB1R in FSX and imply that CB1R signaling is overactive in FSX [79].

Besides genetic manipulation, administration of the CB1R inhibitor rimonabant to Fmr1-knockout mice has been shown to decrease phosphorylation, and thereby activation of Akt as well as p70S6K. This indicates that rimonabant lowers protein synthesis. In addition, spine density was reduced to normal levels and the formation of mature spines was stimulated [79].

Interestingly, the Fmr1-knockouts showed impaired novel object recognition and memory consolidation. This could be enhanced by treatment with either rimonabant or MTEP (another mGluR5-inhibtor). A combination of the two showed even further enhancement. This indicates that as well as the mGluR5-pathway as the ECS are contributing to the cognitive deficits observed in FSX-models [79]. Therefore a combination of drugs, that will target multiple molecular pathways, might be even more effective in treating ASD.

E/I-ratio

As described in chapter I, mutations may also lead to altered protein expression and thereby disturb the E/I-ratio. This imbalance might (partly) cause ASD, since the E/I-ratio is crucial for normal communication between nerve cells. An imbalance between E and I means that some contacts are getting too strong, while others get too weak, or that nerve cells are overreacting in response to a signal or not respond while they should. The general idea of an increased E/I-ratio in ASD, meaning that the balance is hanging over towards excitation provides two possible targets. This ratio can either be caused by too little GABAergic signaling or too much glutamatergic signaling. It therefore seems that stimulating GABAergic signaling or inhibiting glutamatergic signaling might be effective in ASD-treatment.

In order to test whether an increase in GABAergic signaling might reduce ASD-like pathogenesis, arbaclofen, also called STX209, was applied to FSX-mice. Arbaclofen is a GABA-B receptor agonist. Treatment with arbaclofen improved several pathological conditions at the dendritic spine level: the rate of AMPAR-internalization decreased, excessive protein synthesis was decreased and also the increase spine densities were restored back to normal [80].

Interestingly, the effect of this drug has also been tested on behavior. It was demonstrated that arbaclofen positively affects repetitive behavior in FSX-models. However, it also affected these types of behavior in WT mice and, moreover, impaired motor coordination and decreased locomotor activity in either WT as FSX-models was observed, which could hint towards sedating properties [80].

The positive effect of enhancing GABAergic signaling was also demonstrated by a reduction of audiogenic seizures in FSX-mouse models [81], improved social behavior and fear-conditioning in Dravet-Syndrome-models [82] and improvements in neurological dependent misbehaviors [83].

Interfering with the pathway of NL2 that regulates the GABAergic switch might be another option. Although the pathway is not elucidated yet, it is possible to interfere with the effect of mutated NL2. For instance, mutations in NL2 might result in accumulation of Cl^- inside the cell. This can be prevented by application of bumetanide, a diuretic that prevents accumulation of Cl^- inside the cell. Thereby, bumetanide causes GABAergic signaling to become inhibitory. Testing bumetanide in rats showed that the accumulation of Cl^- inside the cell was reversed by bumetanide application [84]. To the best of my knowledge, bumetanide has not been tested in ASD-, or other relevant, mouse models with regard to autistic features.

Instead of targeting GABAergic signaling, one could also target glutamatergic signaling to restore the balance. Treatment of an autistic mouse model, that suffered from NMDAR-hypofunction, with an NMDAR-agonist improved social interactions and restored the NMDA/AMPA-ratio. Moreover, enhancement of glutamatergic sensitivity of the mGluR5 also restored this ratio. In addition, the excitability of cells was decreased and social interactions increased [85].

Notably, since restoring the balance between excitatory and inhibitory signals in the brain is also the goal of many anti-epileptics, many of these drugs might already be available.

Chapter III: Clinical Trials

Many drugs that are discovered or developed will not make it to the drug-market. Whether a drug will make it or not is highly dependent on the Food and Drug Administration (FDA). First, I will line out which path is generally followed in drug development, see table 1. This will help to understand how far the research and drug development is in ASD and FSX-research field.

First, animal tests are done in order to demonstrate the mechanism of action of a certain drug. This is called preclinical testing and should show that the drug is safe and has a great potential to be beneficial in humans. Considering these results, it will be decided whether it is plausible to continue drug development or whether it will be a waste of money.

Approval of the FDA and a local institutional review board (IRB) at this stage means that clinical trials can be started up. Needless to say that a clinical trial has to be very well prepared and that all the participants have to be very well informed about the potential risks they are taking.

Clinical trials usually consist of three phases. The first phase serves to get insight in the most frequently occurring side effects and the metabolism of the drug in humans. Therefore, phase one is almost ever conducted with healthy volunteers. After a drug is considered to be (fairly) safe for human use, the drug can continue to phase two.

In phase two the effectiveness of the drug on a certain disorder or disease is investigated. Most of the time this phase consist of a placebo-controlled trial to make sure that beneficial effects are a consequence of the drug. Short term side effects can also still be observed.

When the prior phases support beneficial effects in patients, the last phase can be entered. Phase three is meant to test different dosages, different populations and interactions with other medicines. This last phase thus requires a large test sample and is highly expensive.

When all this information from clinical trials adds up to a safe and effective drug, the sponsor of the drug has to convince the FDA that this drug is worth to get onto the market. Altogether, clinical trials take several years (www.fda.gov, May 2013).

Clinical Phase	**Preclinical**	**Phase I**	**Phase II**	**Phase III**
Purpose	• Mechanism of action • Potential benefit in humans • Safety	• Side effects • Pharmacokinetics	• Effectiveness • Side effects	• Define dosage • Chemical interactions
Subjects	Animals	Healthy volunteers	Patients	Heterogeneous group of people

Table 1. Overview of the different clinical trial phases and their main purposes, based on www.fda.gov.

In this chapter I will provide an overview on the currently running clinical trials for drugs to treat ASD. I will only describe those trials that target the molecular pathways as described in Chapter I. Many research that will be described however, is conducted on FSX patients. Due to the overlap of both disorders and the suggested similarities in pathology as described in the previous chapter, it is worth to look at drug development of Fragile X in order to get insight in future ASD treatment.

mGluR5-antagonists

Due to the effect of overactive mGluR5 on protein synthesis in different systems, the possibility of inhibition of mGluR5 to reduce autistic features has been extensively researched over the last decade. Already more than 30 years ago, the compound fenobam was tested in a clinical trial for anxiety. This trial demonstrated that fenobam is fairly safe to use in humans, although insomnia and drowsiness were reported a couple of times. Still, there was no reason for severe safety concerns [65]. Although the mechanism of action was not clear at that time, we now know that fenobam inhibits mGluR5-acitivity. This makes fenobam a plausible drug to treat ASD and it entered clinical trials for FSX a couple years ago.

The results of the first open label pilot in FSX-subjects were reported in 2009. The study included 12 adult FSX-subjects. The pharmacokinetics were demonstrated to be dose-dependent, although highly variable among patients. Importantly, no clinical abnormalities or significant side effects were observed [86].

These results were encouraging and a randomized blind placebo controlled trial is now in preparation to check for the pharmacokinetic properties and possible side effects of oral administered fenobam in healthy subjects. The study is planned to start in May 2013 and should be finished in 2015 (www.clinicaltrials.gov, May 2013).

As described in chapter II, AFQ056 is another promising mGluR5-inhibitor in the light of ASD drug development. Like fenobam, AFQ056 already went through clinical trials for another disorder: Parkinson's. In a double blind, placebo controlled trial, all AFQ056-treated patients reported some negative side effects. The most common one was dizziness[69]. Still the authors concluded that the drug was pretty well tolerated. Taken together, these findings support a potential role for AFQ056 in FSX/autism-treatment due to its effect on the core pathways and due to its safety.

Whether AFQ056 could also have beneficial effects in humans suffering from FSX was tested a few years ago. 30 FSX patients attended in a clinical trial that focused on behavioral changes measured by the Aberrant Behavior Checklist–Community Edition (ABC-C). This is a measure for repetitive behavior, inappropriate speech and hyperactivity. Although at first no effect of AFQ056 was measured, it turned out that this was a consequence of the amount of FMRP that patients had in their blood. Patients with a fully methylated Fmr1-promotor did not have any FMRP-mRNA in their peripheral blood, while partly methylation resulted in measurable FMRP-mRNA. Analyzing the effect of AFQ056 on the ABC-C score showed that treatment was especially effective in patients who completely lacked Fmr1-mRNA [87].

Another compound, acamprosate, also entered clinical trials. For a long time, acamprosate was known to protect cells from neurotoxicity through affecting NMDAR-activation. However, a study on alcohol abuse revealed that acamprosate in fact inhibits mGluR5-activity rather than affecting NMDAR-activity [88].

In clinical trials, this drug has been shown to greatly improve social behavior and communication, some of the main characteristics in autism, in three FSX-subjects (this is the total number of patients who enrolled this trial). They were treated with acamprosate for 16 to 28 weeks. Improvements were measured by the Clinical Global Impressions-Improvement (CGI-I) scale. Moreover, the drug was very well tolerated with one participant reporting no side effects at all and two participants reporting emesis, nausea and sedation. The latter effects could be reduced by lowering the dose [89].

These results paved the way for an additional trial. Recently, the results of a prospective open-label study in which 12 FSX-subjects (ranging from 5 till 17 years of age) were treated for ten weeks with acamprosate were published. Nine of these subjects showed to be '(very) much improved' based on the CGI-I scale. Also for secondary measures as hyperactivity and different social behaviors, they improved after ten weeks. Moreover, the use of acamprosate was well tolerated since only some mild side effects were experienced [90].

Interestingly, the drug is not only tested in FSX-patients, but is also enrolling a clinical trial phase 3 for autistic patients. ASD subjects will receive acamprosate for ten weeks after it will be measured whether they improved on the ABC-subscale of social withdrawal. Also CGI-I will be scored (www.clinicaltrials.gov, May 2013).

Ras/MEK/ERK-pathway

As shown in figure 1, excessive mGluR5-activation leads to enhanced activity of the Ras/MEK/ERK-pathway, which in the end results in increased protein synthesis. This pathway has recently been proven to be a possible therapeutic target.

The FRAXA-foundation is currently recruiting FSX-subjects to participate in an open label study tot test lovastatin. Some patients are already treated with lovastatin for one month. Still, data is not available yet (www.fraxa.org).

Moreover, the promising PAK-inhibitor Frax486 will be tested in clinical trials as well, but no experiments have started yet according to the FRAXA-foundation (www.fraxa.org).

Endocannabinoid system

As was demonstrated in mice, rimonabant is able to reduce autistic features. Rimonabant has been tested in many clinical trials on obesity, diabetes, schizophrenia and substance dependency. However, no results of clinical trial have been reported that tests the effects of rimonabant in ASD or FSX yet (www.clinicaltrials.gov, May 2013).

The balance between excitation and inhibition

Due to positive preclinical results, arbaclofen has been tested in humans suffering from FSX. Post hoc analyses of data revealed improved social behavior due to treatment with arbaclofen. This was demonstrated in a randomized placebo-controlled trial in which 64 participants were included. The primary outcome measure, the irritability subscale of the Aberrant Behavior Checklist (ABC-I), did not show significant differences. While, on the other hand, parents reported improvement in problematic behaviors of their child. As already was demonstrated in mice, arbaclofen had some sedating properties in humans as well. However, this could be prevented by lowering the dose [91].

Arbaclofen also runs in clinical trial phase 3 for ASD (www.clinicaltrials.gov, May 2013). Notably, it was reported by FRAXA last month, that the sponsor of Arbaclofen (Seaside Therapeutics) does not continue the clinical trials due to disappointing results (www.fraxa.org, June 2013).

Another compound to restore the E/I-balance is riluzole. Riluzole is known as a drug prescribed for ALS (http://www.nlm.nih.gov, May 2013) but has now been linked to autism. The compound blocks sodium channels and thereby prevents the release of glutamate over GABA at presynaptic sites [92].

Due to its limiting effect on glutamatergic signaling, it is interesting to see the effect of riluzole in autistic or FSX-patients. A first, small trial with the compound included three autistic patients (in the range from 15-20 years of age). They were treated for a certain period of time (ranging from four months to two years), depending on how they reacted to the drug. They all suffered from repetitive behavior with one of them suffering so badly from these behaviors that he could not attend any school activities. Moreover, two subjects showed severe aggressive behavior and suffered from self-injuries.

The effect of the drug was measured by the CGI-Severity score. Before the beginning of the treatment, they were all scored as 'markedly ill'. After treatment with riluzole, they were scored as 'moderately ill', demonstrating an improvement. Moreover, regarding to repetitive and aggressive behavior, all subjects were scored as 'much' or even 'very much' improved.

Negative side effects were only reported by one case. He reported anemia. However, this was not serious enough to stop treatment. No other side effects were reported [93]. Although this trial is of course too small to draw any conclusions and does not include any controls, the results are promising. Especially the chronic treatment strategy adds to the validity of the experiment. Taken together, these first results stimulate further exploration of riluzole.

Interestingly, the diuretic bumetanide also entered clinical trials to treat ASD. Due to the importance of Cl^- in whether GABA is excitatory or inhibitory and due to the possibility that this switch is disturbed in autism, the effects of this diuretic were tested in children diagnosed with infantile autistic syndrome (IAS).

The effect of bumetanide was tested at five IAS-children. They were treated with the diuretic twice a day for a period of three months. Three months after treatment the scores on childhood autism rating scale (CARS), ABC, Regulation Disorders Evaluation Grid (RDEG) and restricted repetitive behavior (RRB), to score autistic symptoms, behavioral changes, activity and repetitive and restrictive behavior respectively, were improved in all five children. Moreover, no side effects were found. The two somewhat older children did respond less to the treatment. However, since the number of participants is so small, no conclusions can be drawn yet [49].

Still, due to the highly positive results, a randomized controlled trial was performed next. This time, 54 participants were tested in a double blind, placebo-controlled trial. The group was quite heterogeneous. They were treated for three months. Patients were regularly checked for side effects, and tested for CARS, CGI and autism diagnostic observation study (ADOS). ADOS assesses social interactions, communication and play-behavior.

Treatment with bumetanide strongly reduced the autistic symptoms of children compared with placebo-treatment. Moreover, one month after finishing the treatment, the symptoms elevated

again. In addition, the scores on ADOS were improved especially in less severe autistic children. However, they did not reach significance for the total group of bumetanide treated children compared to placebo-treated children. There were very few side effects. Hypokalemia was reported, but could be treated by a potassium-containing syrup. In sum, long term treatment with this diuretic improved the autistic children's behavior with little, mild side effects [94].

Recently, it was reported that bumetanide was also tested in a boy who suffers from FSX and shows autistic features. This boy was treated with bumetanide for three months. CARS, ADOS, ABC, RDEG and RRB scores all improved. Mild hypokalemia was observed, but treated successfully with potassium-containing syrup. No other side effects were observed [95].

In sum, there are many drugs that target core pathways of ASD and show positive results in humans. Table 2 provides an overview of different compounds that might be used to treat ASD and how effective they have been proven for certain outcome measures. The amount of Pubmed hits provides an indication of the interest in certain strategies. As can be concluded, mGluR5-inhibition receives the most attention.

Drug target	Lead compounds	Safety/ concerns	Clinical trial phase	Outcome measure	Effectiveness (based on outcome measure)	Sponsors	Pubmed hits FSX / ASD
mGluR5	Fenobam	Safe	Phase I (FSX)	N/A	N/A	Neuropharm LTD, FRAXA	92 / 44
	AFQ056	Fairly safe/ dizziness	Phase II (FSX)	ABC-C	Effective	Novartis Pharma	
	Acamprosate	Safe	Phase III (FSX/ASD)	CGI-I ABC	Effective	Indiana University, NIH, NIMH	
	CTEP	Safe	Preclinical	N/A	N/A	N/A	
	R04917523	N/A	N/A	N/A	N/A	Hoffman-La Roche	
Ras-MEK/ERK	Lovastatin	N/A	Phase I (FSX)	N/A	N/A	FRAXA	20 / 18
PAK	Frax486	N/A	Will start Phase I	N/A	N/A	FRAXA	10 / 5
E/I-ratio (GABAergic and NMDAR-signaling is used to search in Pubmed)	Bumetanide	Safe	Phase II (ASD)	CARS ABS RDEG RRB ADOS CGI	Effective	Brest Public Hospital	9 + 4 / 27 + 5
	Riluzole	Fairly safe	Phase II (ASD)	CGI	Fairly effective	NIH, Indiana University, NIMH	
	Arbaclofen*	Fairly safe/ sedation	Phase III (ASD)	ABC-I	Not effective	Seaside Therapeutics	
ECS	Rimonabant	N/A	N/A	N/A	N/A	N/A	3 / 6

Table 2. Overview of lead compounds in ASD drug development. Greatest interest is shown in mGluR5-inhibitors. Most of the drugs that have been tested in humans are (fairly) safe so far. Most drugs show positive results for the outcome measures used.

*was recently reported to be retracted from further development

Chapter IV: Patent Applications

In order to get insight in which compounds are the most promising and what the future directions of drug development will be, I have done research to patent applications. However, entering this world is one thing, but gaining the information you need is another.

Since searching on my own did not provide the results I was hoping for, I contacted several people in the field to ask for help. Dr. Will Spooren, one of the authors that I have come across to write my thesis and Section head of Behavioral Pharmacology and Pre-Clinical Imaging at Hoffman-la Roche, reported that they hire professionals to gain information about patents. These specialists are expensive and thus not reachable for myself. Dr. Spooren added that he assumes the list of relevant patents is very small (Dr. Will Spooren, June 2013, Section head of Behavioral Pharmacology and Pre-Clinical Imaging at Hoffman-la Roche).

The latter was supported by Oscar Schoots, Managing Director at Utrecht University. He explained me that there are many claims on one compound. Not all the claims, if any, will get patented and moreover, most of the time patent applications are not granted yet. On the internet, it is hard to distinguish between granted and ungranted patents. In addition, filed patents are not available for the public for the first 18 months. Therefore, information is never up to date (Dr. Oscar Schoots MBA, May 2013, Managing Director at Utrecht University).

At the FRAXA-foundation, they seem to have more insight in patent request due to their own applications. The Medical Director, Dr. Michael Tranfaglia, at FRAXA reports that there are several patents filed due to FRAXA's research. He mentions that the most important ones are mGluR5-inhibitors and PAK-inhibitors. However, detailed information about which compounds are running for patent applications at what pharmaceutical companies is unknown. In addition, he assumes that there are many more patents running, but no information is provided (also not to FRAXA) until licensing agreements are formed (Dr. Michael Tranfaglia, June 2013, Medical Director and Chief Scientific Officer at FRAXA).

In sum, patent requests are highly protected and it is hard to get insight in these. This protection is required due to the high costs that go hand in hand with drug development and the need to receive a return on investment in order to make the circle round: only when money is made, money can be invested in drug development.

Chapter V: Discussion

The findings of my research indicate that mGluR5-inhibitors receive the most attention in drug development. Also PAK-inhibitors are promising compounds due to the running patent applications. The interest in these compounds is high at fundamental research level as well as at later stages of drug development. Patent applications are likely running for these drugs, meaning that industries are willing to invest in these drugs and thus are confident about the effects and usability of the compounds. If these drugs reach the market, the core pathways that cause ASD will be targeted for the first time.

At first, this sounds like groundbreaking news for ASD-patients. However, during my search to the direction of drug development in ASD, I discovered three invisible boundaries that could influence the direction of drug development. These boundaries are less optimistic for drug development than the groundbreaking findings do suggest.

First, I would like to discuss the usability of the different drugs described in this thesis. Next, I will elaborate on the boundaries I discovered during my literature research and describe how these boundaries may affect drug development.

Usability of the drugs

mGluR5-inhibitors

According to my findings, mGluR5-inhibitors receive great attention among researchers and, since there are patent applications running for mGluR5-inhibitors, provide promising compounds to reach the drug market. The idea is that inhibition of mGluR5-activity would reduce excessive protein synthesis at dendritic spines and thereby restore the communication between nerve cells.

Due to the involvement of mGluR5 in different aspects as I described in my thesis, mGluR5 seems a promising and possibly a very effective drug. It not only targets enhanced mGluR-dependent LTD, but is also involved in the production, and thereby activation, of 2-AG in the ECS. Moreover, targeting mGluR5 will affect mTOR and ERK-pathways at the same time. Therefore, I believe that mGluR5 has a major impact on the symptoms of ASD. However, this great impact also leads to strong question marks with the eventual usability of these mGluR5-inhibitors for ASD-patients over time.

Due to the involvement of mGluR5 in many different pathways that cause ASD, its effects on different pathways might get indistinct. This becomes a problem when side effects occur, since it will not be clear what the exact problem is.

Moreover, mGluR5 expression is not restricted to the brain but is expressed in many peripheral cell types as well, [96,97] and its association with skin cancer development [97] term treatment might have serious consequences which are not yet explored.

mTOR-inhibitors

The well-known mTOR inhibitor rapamycin was demonstrated to have positive effects on autistic patients. Moreover, the drug is used already in humans that underwent organ transplantation. However, the negative aspect of rapamycin is that it suppresses the immune system. Therefore, long term treatment increases the susceptibility of patients to opportunistic infections.

Moreover, patients that underwent renal transplantation and were treated with rapamycin suffered from a wide range of side effects. Ranging from skin disorders, coughs and infectious diseases to intestinal pneumonitis and thrombocytopenia [98,99]. So even though positive results were booked in autistic patients, it is highly questionable whether long term treatment with rapamycin will increase the quality of life of these patients.

Affecting the ECS

Since CB1R is important for protein synthesis, it would be interesting to target this receptor to treat ASD. However, due to its expression in peripheral tissues as well [100] it might be worthwhile to first explore the additional risk on side effects in organs before starting long term treatment.

Moreover, the compound rimonabant that is currently tested as a drug to treat ASD already demonstrated to cause serious side effects when it was used to treat obesity. Side effects included neurological disorders such as depression and was retracted from the drug market by the FDA [101].

Although rimonabant might not be the perfect candidate for long term treatment, I do think that there are still possibilities for drug development in the ECS. Mainly because the effect of NL2 has been recently discovered, but the exact pathway is not clarified yet. Conducting fundamental research to understand this pathway may reveal new, drugable targets that may not have these negative side effects over time.

The E/I-ratio

So far, the diuretic bumetanide seems to be a safe and highly effective drug. Although hypokalemia can occur, this side effect can be treated fairly easy. Another benefit is that this drug is already available on the market (http://www.nlm.nih.gov, June 2013)

Other drugs that restore the E/I-balance often act on GABAergic or glutamatergic signaling (such as arbaclofen that acts on GABA-receptors). Negative about these drugs is that they target multiple brain areas. However, not every brain area contributes in the same way to ASD and therefore, successful treatment of one area might have opposite effects in the other.

In short, the common problem I foresee is that the drugs are not specific enough. The drugs will target multiple body parts or brain areas, thereby inevitable target well working systems. This may cause serious side effects over a longer period of time. Interestingly, there are mGluR5 positive allosteric modulators used in experiments, that are specific for synaptic mechanisms [27]. Exploring the possibilities of developing drugs that target a system in a specific area (either within neurons or in brain areas) might greatly reduce long term side effects.

As long as drugs are not highly specific I would suggest using several drugs at the same time, each acting on different systems. Perhaps the same effects will be reached by using two or three drugs at a low dose instead of using a high dose of one drug. Lowering dosages might be a possibility to reduce side effects, as has been demonstrated in several clinical studies (as discussed in chapter III).

Also important to note is that many findings for ASD are coming from research to FSX. Although it is likely that these two disorders share pathology, there are probably also differences. In addition, it might be questionable whether certain alterations (such as increased excitability of the brain) are representative for ASD, which core symptoms are poor social interaction, poor communication and altered behavior.

Moreover, pathways are suggested to be involved in ASD due to findings of rare, causal mutations that have been found in ASD-patients. Since the found mutations are rare, there is a serious chance that not every ASD is caused by the same mutations or disturbances in pathways. Therefore, developed drugs might be effective in just a small part of the autistic population. As is very nicely reviewed by Delorme et al., serious problems that researchers will come across is that research today is focusing on the core pathways that are involved in ASD. However, targeting these core pathway does not necessarily mean that the quality of life for patients increases [102]. A clear example of this was the research to arbaclofen. Initially, no significant improvements were found for the outcome measure ABS-I. Still, parents did report improvement of problematic behaviors of their child [91]. This demonstrates the discrepancy between promising results at the bench- and bedside.

To go back to the results of my thesis, mGluR5-inhibitors receive the most attention in drug development, although there are risks attached to its usage. Also PAK-inhibitors are promising compounds due to the patent application. The interest in these compounds is high at fundamental research as well as at later stages in drug development. It is likely that there are patent applications running for these drugs, meaning that industries are willing to investigate in these drugs and thus are confident about the effects and usability. Strikingly, although clinical results for bumetanide are highly positive and the drug is already available, I did not find researchers other than Ben-Ari that are investigating the possibilities of this diuretic.

Invisible boundaries

At first, the fact that research is done to the core pathways of ASD sounds like groundbreaking news to ASD-patients. However, during my search to the direction of drug development in ASD, I discovered three invisible boundaries that could strongly influence this direction. These boundaries could limit drug development and are thus have a negative effect on drug development in contrast to what the groundbreaking findings do suggest.

The first striking boundary I discovered is the difference in urge and willingness to publish data between fundamental research, clinical trials and patent requests. Whereas fundamental findings cannot wait to be shared by the authors, outcomes of clinical trials are much harder to find and patent requests are not public for the first 18 months at all.

The urge to publish at the fundamental research level has two sides: on the one hand researchers get highly motivated to work as efficiently as possible due to competition between the groups. This requires faster, more creative and more critical research which is beneficial for development. On the other hand, the urge to publish may turn into blindness for results. Researchers might choose for the fastest way to get positive data in order to publish it. This leads to biased outcomes. Thereby, some findings might easily get neglected and others might get overrated.

For instance, knockout of FMRP alters spine morphology in cultured hippocampal neurons of BL6-mice, but has a different effect on neurons against a FVB genetic background. The authors conclude that the findings on FVB-neurons were in contrast with previous findings and therefore continued their experiments with BL6-neurons [68].

Nevertheless, these results did show that a lack of FMRP has a less prominent effect on spine morphology in FVB-neurons than in BL6-neurons. Moreover, the required dose of lovastatin to reduce seizure susceptibility also differed between BL6 and FVB-mice [77]. This thus raises my

questions: how do we exclude that knockout of FMRP might have different effects against different genetic backgrounds? How can we neglect findings without explaining them? And based on what other findings do the authors conclude that their current experimental setup is less reliable compared to previously used experiments?

Fortunately the effect of genetic backgrounds has been tested in mice. Experiments demonstrated that mice lacking FMRP also show different behavioral effects, dependent on their genetic background. Therefore, one might argue that FSX-mouse models not only show the effect of FMRP but of the genetic background as a whole [103].

Logically, researchers choose the path which is most promising. But if two strains show different results, how do we decide which one is the most representative model for humans? And why are we not interested in the underlying mechanism of these differences in order to predict what will work best in humans? I think it would be a benefit to be more critical in this phase. Investigate differences between animal models and develop criteria for proper animal models, will contribute to safer and better predictable drug effects in humans.

The second type of boundary that I came across, are the invisible boundaries between different research areas. Although there are many different research areas, the human body is still working as one organism in which the different 'research areas' are integrated and function dependently on each other. Therefore, I think we should be careful with mGluR5- and mTOR-inhibitors. These compounds might have beneficial effects in brains and show positive results regarding to ASD-symptoms, but side effects that occur by long term treatment are still not clear. These side effects may be serious, due to the involvement of mGluR5 and mTOR in important pathways throughout our body.

Moreover, even within the brain there are distinct researchers focusing on different brain parts or use different techniques. Recently, an attempt has been made to stimulate the sharing of knowledge between different research groups. In order to increase the knowledge and thereby fasten drug development for ASD, a collaboration between academic centers, industries and patients, called the European Autism Interventions- a Multicenter Study for Developing New Medicine (EU-AIMS), has come into existence.

The EU-AIMS aims to develop new tools in order to discover new drugs to treat ASD. Moreover, they want to facilitate the translation from bench to bedside by providing a research network and by developing expert clinical sites. The EU-AIMS therefore created several different 'workpackages'. These all focus on their own project, namely animal studies, finding new biomarkers and clinical trials. Their aim is to collaborate in order to speed up drug development. In order to do this, they will provide a database that is assessable to a broad range of researchers. To this end it will be possible to use pre-existing knowledge, share knowledge and help each other in order to support and accelerate findings in ASD research (Murphy & Spooren, 2012) (www.eu-aims.eu, May 2013).

Although the initiative sounds promising, sharing data is still difficult due to the unsolved puzzle on how to develop software that is able to process and manage this data. Today, the Software SimBioMS is one of the most important software to run, organize and link large amounts of data and is especially developed to stimulate collaborations [104]. However, in order to use one software

system, different disciplines should have one way to communicate, while they actually all use their own formats and languages [105]. This still forms an obstacle that has to be overcome in order to effectively share data and use it (Dr. Ugis Sarkans, Technical Team Leader at EU-AIMS, 15th May, 2013)

The third boundary I would like to discuss is the fact that medical professionals are obligated by law to provide any medical help whenever possible and how this can be in conflict with politics, financials, research and drug development. To illustrate my point I will use Pompe´s disease as an example.

Pompe´s disease is very rare. The costs of drug development and its production should therefore be covered by a very small market and thus, the price per individual is much higher than for more general disorders. Recently, Dutch politicians debated whether the extremely high costs for treatment of these patients should be covered by our social health care system or not (www.volkskrant.nl). Not covering these costs means that patients have two options: spending 400.000 to 700.000 euros a year on their treatment or deteriorate until they will not be able to breath anymore (depending on the severity of their illness).

As I previously described, medical professionals are obligated by law to provide any possible medical help when required. However, already in preclinical research it can be decided not to progress investigation of possible drugs due to the potential small market. In the case of Pompe's disease, drugs did come available and thus medical professionals are forced by law to prescribe these drugs to their patients. Despite the law and good intentions of medical professionals, insurance companies, pharmaceutics and politicians are preventing the delivery to the patients by either not taking responsibility to cover the costs (politics) or by delivering drugs at the highest price (pharmaceutical industries). Therefore, they oppose practicing the law.

Let's go back to the topic of my thesis, which leads to my second example of the influence of pharmaceutical companies and politics on drug development. Although the main stream of drug development focuses on mGluR5-inhibitors and PAK-inhibitors, other compounds, as described in my thesis, could be promising as well. One way or the other, these drugs receive less attention.

For instance, the promising results of the use of diuretics for ASD-patient are only investigated by the group of Ben-Ari. While a large amount of researchers focus on mGluR5-inhibitors, the positive results of diuretics do not stimulate additional researcher somehow. A possible explanation is that it might be less interesting because of the low costs and the wide availability of diuretics. Therefore, use of these drugs will be financially beneficial to insurance companies, but not to pharmaceutical companies. Thus, both industries could influence the drug market.

Altogether, it looks like all roads lead to Rome. What is the best road depends on person's own intentions. What is your purpose? A beautiful, fruitful journey from which we can learn? The fastest way possible to catch a big fish? Or do we want to take a route through the mountains, with great look-outs that provides us an overview of what is going on in the world which will remind us of our insignificance, but with the possibility to play a significant role in the lives of others.

Acknowledgements

I would like to start by thanking all the people who made a great contribution to my thesis.

Especially, I would like to thank Professor Peter Burbach for his supervision during my project. I would like to thank him for his time, patience and interesting insights and open-mindedness. I appreciate the time and room he provided that made my thesis my own.

Furthermore, I am greatly thankful to my parents who provided me every opportunity to finish my thesis ranging from a quiet room to work in, coffees and chitchats, to their great confidence in me.

Last but not least, I have deep respect for Piet Schat, my boyfriend. His endless patience, cheer-ups and technical support have really helped me not to give up (not only during writing my thesis, but throughout the entire Master Program). I very much look forward to enjoy our well-deserved holidays!

References

1. DiCicco-Bloom, E. *et al.* The developmental neurobiology of autism spectrum disorder. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **26**, 6897-906 (2006).

2. McPheeters, M.L. *et al.* A systematic review of medical treatments for children with autism spectrum disorders. *Pediatrics* **127**, e1312-21 (2011).

3. Myers, S.M. & Johnson, C.P. Management of children with autism spectrum disorders. *Pediatrics* **120**, 1162-82 (2007).

4. Dove, D. *et al.* Medications for adolescents and young adults with autism spectrum disorders: a systematic review. *Pediatrics* **130**, 717-26 (2012).

5. Ehninger, D., Li, W., Fox, K., Stryker, M.P. & Silva, A.J. Reversing neurodevelopmental disorders in adults. *Neuron* **60**, 950-60 (2008).

6. Spooren, W., Lindemann, L., Ghosh, A. & Santarelli, L. Synapse dysfunction in autism: a molecular medicine approach to drug discovery in neurodevelopmental disorders. *Trends in pharmacological sciences* **33**, 669-84 (2012).

7. Betancur, C., Sakurai, T. & Buxbaum, J.D. The emerging role of synaptic cell-adhesion pathways in the pathogenesis of autism spectrum disorders. *Trends in neurosciences* **32**, 402-12 (2009).

8. Sara, W. Searching for answers. *Nature* **491**, 4-6 (2012).

9. Virginia, H. Complex disorder. *Nature* **491**, 2-3 (2012).

10. Muhle, R., Trentacoste, S.V. & Rapin, I. The Genetics of Autism The online version of this article , along with updated information and services , is located on the World Wide Web at : The Genetics of Autism. (2013).

11. Penzes, P., Cahill, M.E., Jones, K. a, VanLeeuwen, J.-E. & Woolfrey, K.M. Dendritic spine pathology in neuropsychiatric disorders. *Nature neuroscience* **14**, 285-93 (2011).

12. Murphy, D. & Spooren, W. EU-AIMS: a boost to autism research. *Nature reviews. Neuroscience* **11**, 815-816 (2012).

13. Hotulainen, P. & Hoogenraad, C.C. Actin in dendritic spines: connecting dynamics to function. *The Journal of cell biology* **189**, 619-29 (2010).

14. Kasai, H., Fukuda, M., Watanabe, S., Hayashi-Takagi, A. & Noguchi, J. Structural dynamics of dendritic spines in memory and cognition. *Trends in neurosciences* **33**, 121-9 (2010).

15. Santini, E. *et al.* Exaggerated translation causes synaptic and behavioural aberrations associated with autism. *Nature* **493**, 411-5 (2013).

16. Ebert, D.H. & Greenberg, M.E. Activity-dependent neuronal signalling and autism spectrum disorder. *Nature* **493**, 327-37 (2013).

17. van Spronsen, M. & Hoogenraad, C.C. Synapse pathology in psychiatric and neurologic disease. *Current neurology and neuroscience reports* **10**, 207-14 (2010).

18. Bear, M.F., Huber, K.M. & Warren, S.T. The mGluR theory of fragile X mental retardation. *Trends in neurosciences* **27**, 370-7 (2004).

19. Huber, K.M. Role for Rapid Dendritic Protein Synthesis in Hippocampal mGluR-Dependent Long-Term Depression. *Science* **288**, 1254-1256 (2000).

20. Huber, K.M., Roder, J.C. & Bear, M.F. Chemical Induction of mGluR5- and Protein Synthesis – Dependent Long-Term Depression in Hippocampal Area Chemical Induction of mGluR5- and Protein Synthesis – Dependent Long-Term Depression in Hippocampal Area CA1. 321-325 (2013).

21. Gallagher, S.M., Daly, C. a, Bear, M.F. & Huber, K.M. Extracellular signal-regulated protein kinase activation is required for metabotropic glutamate receptor-dependent long-term depression in hippocampal area CA1. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **24**, 4859-64 (2004).

22. Banko, J.L., Hou, L., Poulin, F., Sonenberg, N. & Klann, E. Regulation of eukaryotic initiation factor 4E by converging signaling pathways during metabotropic glutamate receptor-dependent long-term depression. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **26**, 2167-73 (2006).

23. Kelleher, R.J., Govindarajan, A. & Tonegawa, S. Translational regulatory mechanisms in persistent forms of synaptic plasticity. *Neuron* **44**, 59-73 (2004).

24. Gkogkas, C.G. *et al.* Autism-related deficits via dysregulated eIF4E-dependent translational control. *Nature* **493**, 371-7 (2013).

25. Lindemann, L. *et al.* CTEP : A Novel , Potent , Long-Acting , and Orally Bioavailable Metabotropic Glutamate Receptor 5 Inhibitor □. 474-486 (2011).doi:10.1124/jpet.111.185660.

26. Chévere-Torres, I. *et al.* Metabotropic glutamate receptor-dependent long-term depression is impaired due to elevated ERK signaling in the ΔRG mouse model of tuberous sclerosis complex. *Neurobiology of disease* **45**, 1101-10 (2012).

27. Auerbach, B.D., Osterweil, E.K. & Bear, M.F. Mutations causing syndromic autism define an axis of synaptic pathophysiology. *Nature* **480**, 63-8 (2011).

28. Kumar, V., Zhang, M.-X., Swank, M.W., Kunz, J. & Wu, G.-Y. Regulation of dendritic morphogenesis by Ras-PI3K-Akt-mTOR and Ras-MAPK signaling pathways. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **25**, 11288-99 (2005).

29. Impey, S. *et al.* An activity-induced microRNA controls dendritic spine formation by regulating Rac1-PAK signaling. *Molecular and cellular neurosciences* **43**, 146-56 (2010).

30. Hayashi, M.L. *et al.* Inhibition of p21-activated kinase rescues symptoms of fragile X syndrome in mice. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 11489-94 (2007).

31. Antar, L.N., Afroz, R., Dictenberg, J.B., Carroll, R.C. & Bassell, G.J. Metabotropic glutamate receptor activation regulates fragile x mental retardation protein and FMR1 mRNA localization differentially in dendrites and at synapses. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **24**, 2648-55 (2004).

32. Ascano, M. *et al.* FMRP targets distinct mRNA sequence elements to regulate protein expression. *Nature* **492**, 382-6 (2012).

33. Niere, F., Wilkerson, J.R. & Huber, K.M. Evidence for a fragile X mental retardation protein-mediated translational switch in metabotropic glutamate receptor-triggered Arc translation and long-term depression. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **32**, 5924-36 (2012).

34. Darnell, J.C. & Klann, E. The translation of translational control by FMRP: therapeutic targets for FXS. *Nature neuroscience* **16**, (2013).

35. Darnell, J.C. *et al.* FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism. *Cell* **146**, 247-61 (2011).

36. Kerr, D.M., Downey, L., Conboy, M., Finn, D.P. & Roche, M. Alterations in the endocannabinoid system in the rat valproic acid model of autism. *Behavioural brain research* 1-9 (2013).doi:10.1016/j.bbr.2013.04.043

37. Földy, C., Malenka, R.C. & Südhof, T.C. Autism-Associated Neuroligin-3 Mutations Commonly Disrupt Tonic Endocannabinoid Signaling. *Neuron* (2013).doi:10.1016/j.neuron.2013.02.036

38. Mechoulam, R. & Parker, L. a The endocannabinoid system and the brain. *Annual review of psychology* **64**, 21-47 (2013).

39. Varma, N., Carlson, G.C., Ledent, C. & Alger, B.E. Metabotropic glutamate receptors drive the endocannabinoid system in hippocampus. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **21**, RC188 (2001).

40. Katona, I. & Freund, T.F. Endocannabinoid signaling as a synaptic circuit breaker in neurological disease. *Nature medicine* **14**, 923-30 (2008).

41. Heifets, B.D. & Castillo, P.E. Endocannabinoid signaling and long-term synaptic plasticity. *Annual review of physiology* **71**, 283-306 (2009).

42. Piet, R. *et al.* State-dependent, bidirectional modulation of neural network activity by endocannabinoids. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **31**, 16591-6 (2011).

43. Puighermanal, E. *et al.* Cannabinoid modulation of hippocampal long-term memory is mediated by mTOR signaling. *Nature neuroscience* **12**, 1152-8 (2009).

44. Kano, M., Ohno-shosaku, T., Hashimotodani, Y. & Uchigashima, M. Endocannabinoid-Mediated Control of Synaptic Transmission. 309-380 (2009).doi:10.1152/physrev.00019.2008.

45. Izumi, Y. & Zorumski, C.F. NMDA receptors, mGluR5, and endocannabinoids are involved in a cascade leading to hippocampal long-term depression. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* **37**, 609-17 (2012).

46. Jagasia, R. *et al.* GABA-cAMP response element-binding protein signaling regulates maturation and survival of newly generated neurons in the adult hippocampus. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **29**, 7966-77 (2009).

47. Ganguly, K., Schinder, a F., Wong, S.T. & Poo, M. GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition. *Cell* **105**, 521-32 (2001).

48. Sun, C., Zhang, L. & Chen, G. An unexpected role of neuroligin-2 in regulating KCC2 and GABA functional switch. *Molecular brain* **6**, 23 (2013).

49. Lemonnier, E. & Ben-Ari, Y. The diuretic bumetanide decreases autistic behaviour in five infants treated during 3 months with no side effects. *Acta paediatrica (Oslo, Norway : 1992)* **99**, 1885-8 (2010).

50. Dahlhaus, R. & El-Husseini, A. Altered neuroligin expression is involved in social deficits in a mouse model of the fragile X syndrome. *Behavioural brain research* **208**, 96-105 (2010).

51. Hines, R.M. *et al.* Synaptic imbalance, stereotypies, and impaired social interactions in mice with altered neuroligin 2 expression. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **28**, 6055-67 (2008).

52. Kohl, C. *et al.* Hippocampal neuroligin-2 overexpression leads to reduced aggression and inhibited novelty reactivity in rats. *PloS one* **8**, e56871 (2013).

53. Kim, H.-goo *et al.* Disruption of Neurexin 1 Associated with Autism Spectrum Disorder. 199-207 (2008).doi:10.1016/j.ajhg.2007.09.011.

54. Piton, A. *et al.* Analysis of the effects of rare variants on splicing identifies alterations in GABA(A) receptor genes in autism spectrum disorder individuals. *European journal of human genetics : EJHG* 1-8 (2012).doi:10.1038/ejhg.2012.243

55. Rubenstein, J.L.R. & Merzenich, M.M. Model of autism : increased ratio of excitation / inhibition in key neural systems. 255-267 (2003).doi:10.1046/j.1601-183X.2003.00037.x

56. Wang, Y.T. Probing the role of AMPAR endocytosis and long-term depression in behavioural sensitization: relevance to treatment of brain disorders, including drug addiction. *British journal of pharmacology* **153 Suppl** , S389-95 (2008).

57. Kohane, I.S. *et al.* The co-morbidity burden of children and young adults with autism spectrum disorders. *PloS one* **7**, e33224 (2012).

58. Nakamoto, M. *et al.* Fragile X mental retardation protein deficiency leads to excessive mGluR5-dependent internalization of AMPA receptors. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 15537-42 (2007).

59. Mehta, M.V., Gandal, M.J. & Siegel, S.J. mGluR5-antagonist mediated reversal of elevated stereotyped, repetitive behaviors in the VPA model of autism. *PloS one* **6**, e26077 (2011).

60. Anderson, J.J. *et al.* In vivo receptor occupancy of mGlu5 receptor antagonists using the novel radioligand [3H]3-methoxy-5-(pyridin-2-ylethynyl)pyridine). *European Journal of Pharmacology* **473**, 35-40 (2003).

61. Porter, R.H.P. *et al.* Fenobam : A Clinically Validated Nonbenzodiazepine Anxiolytic Is a Potent , Selective , and Noncompetitive mGlu5 Receptor Antagonist with Inverse Agonist Activity. **315**, 711-721 (2005).

62. Montana, M.C. *et al.* The Metabotropic Glutamate Receptor Subtype 5 Antagonist Fenobam Is Analgesic and Has Improved in Vivo Selectivity Compared with the Prototypical Antagonist 2-Methyl-6- (phenylethynyl) -pyridine. **330**, 834-843 (2009).

63. de Vrij, F.M.S. *et al.* Rescue of behavioral phenotype and neuronal protrusion morphology in Fmr1 KO mice. *Neurobiology of disease* **31**, 127-32 (2008).

64. Vinueza Veloz, M.F. *et al.* The effect of an mGluR5 inhibitor on procedural memory and avoidance discrimination impairments in Fmr1 KO mice. *Genes, brain, and behavior* **11**, 325-31 (2012).

65. Pecknold, J.C., McClure, D.J., Appeltauer, L., Wrzesinski, L. & Allan, T. Treatment of anxiety using fenobam (a nonbenzodiazepine) in a double-blind standard (diazepam) placebo-controlled study. *Journal of clinical psychopharmacology* **2**, 129-33 (1982).

66. Jacob, W. *et al.* The anxiolytic and analgesic properties of fenobam, a potent mGlu5 receptor antagonist, in relation to the impairment of learning. *Neuropharmacology* **57**, 97-108 (2009).

67. Gantois, I. *et al.* Chronic administration of AFQ056/Mavoglurant restores social behaviour in Fmr1 knockout mice. *Behavioural brain research* **239**, 72-9 (2013).

68. Levenga, J. *et al.* AFQ056, a new mGluR5 antagonist for treatment of fragile X syndrome. *Neurobiology of disease* **42**, 311-7 (2011).

69. Berg, D. *et al.* AFQ056 treatment of levodopa-induced dyskinesias: results of 2 randomized controlled trials. *Movement disorders : official journal of the Movement Disorder Society* **26**, 1243-50 (2011).

70. Michalon, A. *et al.* Chronic pharmacological mGlu5 inhibition corrects fragile X in adult mice. *Neuron* **74**, 49-56 (2012).

71. Pop, A.S. *et al.* Rescue of dendritic spine phenotype in Fmr1 KO mice with the mGluR5 antagonist AFQ056/Mavoglurant. *Psychopharmacology* (2012).doi:10.1007/s00213-012-2947-y

72. Silverman, J.L. *et al.* Negative allosteric modulation of the mGluR5 receptor reduces repetitive behaviors and rescues social deficits in mouse models of autism. *Science translational medicine* **4**, 131ra51 (2012).

73. Sato, A. *et al.* Rapamycin reverses impaired social interaction in mouse models of tuberous sclerosis complex. *Nature communications* **3**, 1292 (2012).

74. Zhou, J. *et al.* Pharmacological inhibition of mTORC1 suppresses anatomical, cellular, and behavioral abnormalities in neural-specific Pten knock-out mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **29**, 1773-83 (2009).

75. Ehninger, D. & Silva, A.J. Rapamycin for treating Tuberous sclerosis and Autism spectrum disorders. *Trends in molecular medicine* **17**, 78-87 (2011).

76. Kloog, Y., Cox, A.D. & Sinensky, M. Expert Opinion on Investigational Drugs Concepts in Ras-directed therapy. 2121-2140 (1999).

77. Osterweil, E.K. *et al.* Lovastatin corrects excess protein synthesis and prevents epileptogenesis in a mouse model of fragile X syndrome. *Neuron* **77**, 243-50 (2013).

78. Dolan, B.M. *et al.* Rescue of fragile X syndrome phenotypes in Fmr1 KO mice by the small-molecule PAK inhibitor FRAX486. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 5671-6 (2013).

79. Busquets-Garcia, A. *et al.* Targeting the endocannabinoid system in the treatment of fragile X syndrome. *Nature Medicine* **19**, 4-10 (2013).

80. Henderson, C. *et al.* Reversal of disease-related pathologies in the fragile X mouse model by selective activation of GABA(B) receptors with arbaclofen. *Science translational medicine* **4**, 152ra128 (2012).

81. Pacey, L.K.K., Heximer, S.P. & Hampson, D.R. Increased GABA B Receptor-Mediated Signaling Reduces the Susceptibility of Fragile X Knockout Mice to Audiogenic Seizures. **76**, 18-24 (2009).

82. Han, S. *et al.* Autistic-like behaviour in Scn1a+/- mice and rescue by enhanced GABA-mediated neurotransmission. *Nature* **489**, 385-90 (2012).

83. Gandal, M.J. *et al.* GABAB-mediated rescue of altered excitatory-inhibitory balance, gamma synchrony and behavioral deficits following constitutive NMDAR-hypofunction. *Translational psychiatry* **2**, e142 (2012).

84. Volodymyr I. Dzaha, D.M.T. NKCC1 transporter facilitates seizures in the developing brain. *Nature Medicine* **11**, 1205-1213 (2005).

85. Won, H. *et al.* Autistic-like social behaviour in Shank2-mutant mice improved by restoring NMDA receptor function. *Nature* **486**, 261-5 (2012).

86. Berry-Kravis, E. *et al.* A pilot open label, single dose trial of fenobam in adults with fragile X syndrome. *Journal of medical genetics* **46**, 266-71 (2009).

87. Jacquemont, S. *et al.* Epigenetic modification of the FMR1 gene in fragile X syndrome is associated with differential response to the mGluR5 antagonist AFQ056. *Science translational medicine* **3**, 64ra1 (2011).

88. Harris, B.R. *et al.* The neurotoxicity induced by ethanol withdrawal in mature organotypic hippocampal slices might involve cross-talk between metabotropic glutamate type 5 receptors and N-methyl-D-aspartate receptors. *Alcoholism, clinical and experimental research* **27**, 1724-35 (2003).

89. Erickson, C. a, Mullett, J.E. & McDougle, C.J. Brief report: acamprosate in fragile X syndrome. *Journal of autism and developmental disorders* **40**, 1412-6 (2010).

90. Erickson, C. a *et al.* Impact of acamprosate on behavior and brain-derived neurotrophic factor: an open-label study in youth with fragile X syndrome. *Psychopharmacology* (2013).doi:10.1007/s00213-013-3022-z

91. Berry-Kravis, E.M. *et al.* Effects of STX209 (arbaclofen) on neurobehavioral function in children and adults with fragile X syndrome: a randomized, controlled, phase 2 trial. *Science translational medicine* **4**, 152ra127 (2012).

92. Taylor, C.P. & Meldrum, B.S. Na+ channels as targets for neuroprotective drugs. *Trends in pharmacological sciences* **16**, 309-16 (1995).

93. Wink, L.K., Erickson, C. a, Stigler, K. a & McDougle, C.J. Riluzole in autistic disorder. *Journal of child and adolescent psychopharmacology* **21**, 375-9 (2011).

94. Lemonnier, E. *et al.* A randomised controlled trial of bumetanide in the treatment of autism in children. *Translational psychiatry* **2**, e202 (2012).

95. Lemonnier, E. *et al.* Treating Fragile X syndrome with the diuretic bumetanide: a case report. *Acta paediatrica (Oslo, Norway : 1992)* **102**, e288-90 (2013).

96. Bird, M.K. & Lawrence, A.J. The promiscuous mGlu5 receptor--a range of partners for therapeutic possibilities? *Trends in pharmacological sciences* **30**, 617-23 (2009).

97. Jessica, F., Teh, J.L.F. & Chen, S. PIgMent Cell & MelAnoMA transformation Glutamatergic signaling in cellular transformation. (2011).doi:10.1111/j.1755-148X.2011.00983.x

98. López, E. *et al.* Long-term mTOR inhibitors administration evokes altered calcium homeostasis and platelet dysfunction in kidney transplant patients. *Journal of cellular and molecular medicine* **17**, 636-47 (2013).

99. Morath, C. *et al.* Sirolimus in renal transplantation. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association* **22 Suppl 8**, viii61-viii65 (2007).

100. Tam, J. *et al.* Peripheral CB1 cannabinoid receptor blockade improves cardiometabolic risk in mouse models of obesity. **120**, (2010).

101. Derosa, G. & Maffioli, P. Anti-obesity drugs: a review about their effects and their safety. *Expert opinion on drug safety* **11**, 459-71 (2012).

102. Delorme, R. *et al.* Progress toward treatments for synaptic defects in autism. *Nature Medicine* **19**, 685-694 (2013).

103. Spencer, C.M. *et al.* Modifying behavioral phenotypes in Fmr1KO mice: genetic background differences reveal autistic-like responses. *Autism research : official journal of the International Society for Autism Research* **4**, 40-56 (2011).

104. Krestyaninova, M. *et al.* A System for Information Management in BioMedical Studies--SIMBioMS. *Bioinformatics (Oxford, England)* **25**, 2768-9 (2009).

105. Krestyaninova, M. & Tammisto, Y. Services Design in a Collaborative Network for Multidisciplinary Research Projects.