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ORIGINAL RESEARCH

Atomoxetine affects transcription/translation of the NMDA receptor and the norepinephrine transporter in the rat brain – an in vivo study

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Abstract: Attention-deficit/hyperactivity disorder (ADHD) is the most frequently diagnosed neurodevelopmental disorder. The norepinephrine transporter (NET) inhibitor atomoxetine, the first nonstimulant drug licensed for ADHD treatment, also acts as an *N*-methyl-*D*-aspartate receptor (NMDAR) antagonist. The compound's effects on gene expression and protein levels of NET and NMDAR subunits (1, 2A, and 2B) are unknown. Therefore, adolescent Sprague Dawley rats were treated with atomoxetine (3 mg/kg, intraperitoneal injection [ip]) or saline (0.9%, ip) for 21 consecutive days on postnatal days (PND) 21–41. In humans, atomoxetine's earliest clinical therapeutic effects emerge after 2–3 weeks. Material from prefrontal cortex, striatum (STR), mesencephalon (MES), and hippocampus (HC) was analyzed either directly after treatment (PND 42) or 2 months after termination of treatment (PND 101) to assess the compound's long-term effects. In rat brains analyzed immediately after treatment, protein analysis exhibited decreased levels of the NET in HC, and NMDAR subunit 2B in both STR and HC; the transcript levels were unaltered. In rat brains probed 2 months after final atomoxetine exposure, messenger RNA analysis also revealed significantly reduced levels of genes coding for NMDAR subunits in MES and STR. NMDAR protein levels were reduced in STR and HC. Furthermore, the levels of two SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins, synaptophysin and synaptosomal-associated protein 25, were also significantly altered in both treatment groups. This in vivo study detected atomoxetine's effects beyond NET inhibition. Taken together, these data reveal that atomoxetine seems to decrease glutamatergic transmission in a brain region-specific manner. Long-term data show that the compound's impact is not due to an acute pharmacological effect but lasts or even amplifies after a drug-free period of 2 months, leading to altered development of synaptic composition. These alterations might contribute to atomoxetine's clinical effects in the treatment of ADHD, a neurodevelopmental disorder in which synaptic processes and especially a dysregulated glutamatergic metabolism seem to be involved.

Keywords: attention-deficit hyperactivity disorder (ADHD), neurodevelopment, atomoxetine, in vivo study, altered gene expression, *N*-methyl-*D*-aspartate receptor

Introduction

The pathogenesis of attention-deficit/hyperactivity disorder (ADHD), the most frequently diagnosed neuropsychiatric disorder in childhood with a prevalence of 5%–6%,[1] and the mechanisms of its psychopharmacological treatments are still far from being completely understood. Distinctly increased hyperactivity, pronounced inattention, and impulsivity not appropriate for age are the three cardinal symptoms of ADHD.[2,3] ADHD often persists into adulthood; 1%–4% of adults seem to remain affected.[4] It

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is hypothesized that dysfunctional norepinephrinergic (NE) and dopaminergic signal processing within various brain areas, especially in the cortico-striatal-thalamic-cortical loop, give rise to an imbalanced state of arousal. This, in consequence, presumably leads to the noted ADHD symptoms.[5] To date, psychostimulants such as methylphenidate or amphetamine salts represent the first-line treatment to attenuate ADHD core symptoms.[6] Nonetheless, about 10%–20% of the patients show only limited benefit from stimulants,[7] whose major effects seem to depend on the inhibition of the striatal presynaptic dopamine transporter.[8,9]

A growing body of evidence indicates that alterations of the monoaminergic system alone are not the only primary pathophysiological cause for ADHD symptoms; dysfunctions of the glutamatergic neurotransmitter system also seem to play a major role in the pathophysiology of ADHD.[10,11] Glutamate is the main excitatory neurotransmitter in the mammalian brain, and over 60% of all synapses are glutamatergic.[12] The glutamate receptors include the G-protein-coupled metabotropic glutamate receptors and the ionotropic cation-permeable receptor-channels: the kainate receptors, the AMPAR (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors), and the NMDAR (*N*-methyl-*D*-aspartate receptors) with seven different subunits: NR1, NR2A–D and NR3A, and B.[13] The receptor subunits and their isoforms have unique biophysical properties and display a defined regional and developmental expression pattern. The NR2 subunits play a crucial role during brain development, controlling synaptic plasticity and memory function.[14] In the rat brain, NR2B-containing receptors contribute to long-term potentiation (LTP) during adolescence, whereas NR1/NR2A receptors mediate LTP in adults.[15] *Grin2B* (NR2B) is predominantly expressed during early developmental stages, and its expression is downregulated around the third postnatal week, whereas the subunit NR2A first appears around birth, dramatically increases thereafter, and peaks 3 weeks after birth.[16] NMDARs are not limited to the postsynaptic membrane. They were also identified at extra-, peri-, and presynaptic sites,[17–20] where they display diverse physiological roles, eg, signaling through extrasynaptic NMDARs, which mainly contain the NR2B subunit, have inhibitory effects.[21] Recently, dysfunctions of the NMDARs have been linked to ADHD.[22] LTP is strongly mediated by the NMDARs containing the subunit 2A. The spontaneously hypertensive rat (SHR), the best-characterized ADHD animal model, produces NMDARs more frequently containing NR2B than NR2A.[22] Consequently, AMPAR insertion into the postsynaptic membrane is suppressed, which reduces LTP and synaptic plasticity.[23,24] Although glutamate has a predominant role in synaptic plasticity, learning and memory, pathological concentrations are highly excitotoxic and lead to neuronal cell death.[12] Lou, in a 1996 study, already linked excessive glutamate release in the striatum (STR) to the onset of ADHD.[25]

Atomoxetine, a selective NE transporter (NET) antagonist,[26–28] is the first nonstimulant compound licensed for the treatment of ADHD in children, adolescents, and adults.[29] Current knowledge about atomoxetine's cellular mechanisms of action is still limited. After oral application, increased intrasynaptic NE levels are detectable within hours in the nonhuman primate brain as shown by positron emission tomography (PET) with (S,S)-[18F] FMeNER-D2, a ligand to the NET.[30] A second PET study with the same ligand indicates that NET is occupied within 15 minutes after intravenous application of atomoxetine at intracerebral concentrations as low as 16 ng/mL measured in the thalamus. The authors conclude that clinical doses of atomoxetine occupy NET almost completely within 15 minutes.[31] Atomoxetine's earliest therapeutic effects, however, only occur after 2–3 weeks of treatment.[32] Therefore, it seems to be very unlikely that the therapeutic effects are solely due to the NET inhibition. In addition, the recommended therapeutic plasma level is 200–1,000 ng/mL,[33] a concentration presumably leading to much higher brain levels than measured in the above mentioned PET study.[34]

Few in vivo or in vitro studies have been conducted to investigate atomoxetine's cellular and neurochemical effects.[35–37] Furthermore, until now no study has characterized its long-term biological effects. The aim of the present study was to ascertain atomoxetine's further cellular action beyond the inhibition of NET. Previously, we could show that atomoxetine acts as an NMDAR antagonist in clinically relevant doses in vitro.[38] Therefore, in the present study, we addressed the issue of whether atomoxetine also alters transcript and protein levels of the NMDAR subunits (NR1, NR2A, and NR2B) and NET. Additionally, we analyzed immediate and long-term effects of atomoxetine on the expression and protein levels of the mentioned NMDAR subunits and NET in the male adolescent brain.

Prefrontal cortex (PFC) and STR, as part of the cortico-striatal-thalamic-cortical circuit, and mesencephalon (MES) and hippocampus (HC), as parts of the limbic system and crucial for learning and memory,[22] were investigated separately. Doing so allowed us to detect possible brain region-specific effects.

Materials and methods

Animal housing

Crl:CD(SD) rats (Charles River Laboratories:Cesarean derived [Sprague Dawley]) for breeding were obtained from Charles River Laboratories (Wilmington, MA, USA) and housed in groups of two under controlled temperature (21°C±2°C), humidity (60%–65%), and a 12:12 hour light-dark cycle. Food and water were available ad libitum. Pregnant rats were housed separately, and pups were separated from the dams at postnatal day (PND) 21.

Treatment procedures

Male adolescent rats were treated from PND 21–42 and either analyzed immediately or housed for another 2 months off-drug and analyzed thereafter. The two groups were named "early treatment group" and "late treatment group", respectively.

Atomoxetine hydrochloride (Sigma-Aldrich, St Louis, MO, USA) was dissolved in 0.9% saline (Fresenius Kabi AG, Bad Homburg, Germany) and was administered by intraperitoneal (ip) injection into rats (n=7–8) at a dose of 3 mg/kg daily dose. Control animals (n=7–8) were age-matched to atomoxetine treated rats (PND 21 days) and received 0.9% saline. Solutions were sterile filtered (0.2 µm). All animal experiments were approved by the Committee for Animal Experimentation of the University of Ulm and the regional administrative authority (Registration Number 944). All procedures were carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC).

Extraction of brain tissue

At the end of the treatment period, male adolescent rats were anesthetized with carbon dioxide and the brain was removed. The brain hemispheres were sagittally separated and the STR, MES, PFC, and HC were resected by microdissection. Brain maps from *The Rat Brain: In Stereotactic Coordinates*.[39] were used for orientation. All samples were snap-frozen in liquid nitrogen and stored at –80°C. All preparations were performed in cold sterile Hank's Balanced Salt Solution (HBSS; PAA Laboratories GmbH, Pasching, Austria).

Extraction of total RNA/quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Isolation of total RNA from frozen brain tissue was performed using the RNeasy kit (QIAGEN, Venlo, the Netherlands) as described by the manufacturer. The total RNA was eluted in 60 µL RNase-free water. For the reverse transcription (RT)-mediated PCR studies, first strand synthesis and qRT-PCR measurements were carried out in a one-step, single-tube format using the QIAGEN QuantiFast SYBR Green RT-PCR kit. The qRT-PCR analysis was performed using the Rotor-Gene® real-time PCR machine (QIAGEN). For each measurement, as much as 1 µL of undiluted total RNA was measured. RT conditions were as follows: 10 minutes at 50°C, 5 minutes at 95°C, followed by 40 cycles of PCR: 10 seconds at 95°C for denaturation, 30 seconds at 60°C for annealing and elongation. All measurements were run in duplicates. To ascertain primer specificity, melting curves were driven from 60°C ramping to 95°C in 1°C steps, while fluorescence was recorded continuously. The RT and PCR reactions were carried out using QIAGEN QuantiTect Primer Assay oligonucleotides amplifying the following transcripts: *solute carrier family 6 member 2* (*Slc6a2*, QT00184604); *glutamate receptor ionotropic N-methyl-D-aspartate 1* (*Grin1*, QT00182287); *glutamate receptor ionotropic N-methyl-D-aspartate 2A* (*Grin2A*, QT0037928); *glutamate receptor ionotropic N-methyl-D-aspartate 2B* (*Grin2B*, QT00184793); *synaptophysin* (*Syp*, QT02338056); and *synaptosomal-associated protein 25 kDa* (*Snap25*, QT00196413).

Analysis of qRT-PCR data

Cycle threshold for each run was set to 0.01. Transcript levels of the transcript of interest were normalized to messenger RNA (mRNA) levels of the *60 S ribosomal protein L13a* (*RPL13A*), which was calculated to be the most robustly expressed housekeeping gene among six tested genes using a geometric averaging method (geNorm).[40,41] Other genes tested include *tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide, glyceraldehyde-3-phosphate dehydrogenase, cyclophilin A, beta-actin, hydroxymethylbilane synthase*, *RPL13A*. Cycle threshold values (Ct_v) were inserted in the following formula:

$$VC_{TG} = 10\text{^} (Ct_v/-\text{slope}) \quad (1)$$

$$VC_{HKG} = 10\text{^} (Ct_v/-\text{slope}) \quad (2)$$

where VC_{TG} is virtual concentration of the target gene (TG) transcripts, and VC_{HKG} is virtual concentration of the housekeeping gene (HKG) transcripts. To normalize the expression of the TG to the HKG, ratios were calculated as,

$$RC_{TG} = VC_{TG}/VC_{HKG} \quad (3)$$

where RC_{TG} is relative concentration of the TG compared to the expression of the HKG. All oligonucleotides used in

the qRT-PCR were purchased from QIAGEN and display ~100% efficiency (personal communication with QIAGEN). Therefore, the value of the "−slope" in the above mentioned formula was set to −3.33, which represents 100% efficiency.

Preparation of tissue lysates

Frozen brain tissues were lysed in 100 µL (STR, PFC) to 200 µL (MES, HC) of sodium dodecyl sulfate (SDS) sample buffer (37.8% glycerol, 6% SDS [2x crist], 2.27% Tris, 2% bromophenol blue solution [10 mg/mL], 0.15% dithioerythritol, pH 6.9). Crude lysates were passed through QIAGEN QIAshredder spin columns for 2 minutes at 16,060 rcf. Samples were denatured for 30 minutes at 70°C and stored at −80°C.

Determination of protein content/coomassie staining

Protein concentrations of brain lysates were assayed via the amido black staining method.[42] A calculated 15 µg of each sample collected from one brain region was subjected to the same 10% SDS-polyacrylamide gel electrophoresis. Equal loading was confirmed using total protein stains (GelCode Blue Stain Reagent; Thermo Fisher Scientific, Waltham, MA, USA).[43]

Immunoblotting

A quantity of 15 µg of protein per sample was subjected to 10% SDS-polyacrylamide gel electrophoresis and separated as appropriate in buffer solution (960 mM glycine, 17 mM SDS, 125 mM Tris). Proteins were electrophoretically transferred (90 V for 105 minutes at 4°C) onto a methanol activated polyvinylidene fluoride membrane using a tank blot transfer system (Bio-Rad Laboratories, Hercules, CA, USA) flooded with buffer solution (195 mM glycine, 25 mM Tris, 20% methanol, 0.1% SDS). Blotted membranes were blocked in 5% fat-free milk dissolved in Tris buffered saline (150 mM NaCl, 50 mM Tris, pH 7.6) supplemented with 0.05% Tween 20 (TBST) for 1.5 hours. Membranes were incubated with primary antibodies diluted in blocking solution for 1–1.5 hours. The following primary antibodies were used: rabbit polyclonal antibody against the NET (SAB2102224; Sigma-Aldrich) at a 1:1,000 dilution,[44] rabbit polyclonal antibody against the NMDAR subunit NR1 (SAB4300405; Sigma-Aldrich) at a dilution of 1:1,000, rabbit polyclonal antibody against the NMDAR subunit NR2A (M-264; Sigma-Aldrich) at 1:1,000,[45] rabbit polyclonal antibody against the NMDAR subunit NR2B (AP08705PU-N; Acris Antibodies GmbH, Herford, Germany) at 1:1,000, mouse monoclonal antibody against synaptophysin (Syp) (S 5768; Sigma-Aldrich) at 1:1,000,[46] and rabbit polyclonal antibody against the synaptosomal-associated protein 25 kDa (Snap25) (ab41455; Abcam, Cambridge, UK) 1:2,000.[47] Membranes were washed for 30 minutes with three changes of 0.2% TBST and were incubated in blocking solution supplemented with the following horseradish peroxidase-conjugated secondary antibodies: swine anti-rabbit 1:1,000 (Dako, Glostrup, Denmark) and goat anti-mouse 1:10,000 (DIANOVA GmbH, Hamburg, Germany) for 1 hour, with three 10-minute subsequent wash steps using 0.2% TBST. Blots were exposed to Pierce ECL Western blot detection reagent (Thermo Fisher Scientific). Photosensitive films (GE Healthcare Europe GmbH, Freiburg, Germany) were exposed to horseradish peroxidase-driven light emission. All steps were performed at room temperature.

Quantification of Western blots

Films and coomassie stained SDS gels were digitized by scanning without modifying picture properties eg, gain, color, or contrast (HP MP260; Canon, Tokyo, Japan). Densitometry was performed using ImageJ software (available from the US National Institutes of Health) to measure mean grey values of immunoblot signals and coomassie-stained polyacrylamide gels loaded with 5 µg of protein. Background areas flanking the respective signals were measured, and the mean background values were subtracted from mean grey values obtained by measuring signals. The background adjusted values of proteins of interest were normalized to the signal intensity of the respective total protein coomassie stainings.

Statistical analysis

Statistical significance of qRT-PCR values, as well as those from the densitometric measurements of Western blots, were analyzed using a two-tailed Wilcoxon–Mann–Whitney test (SPSS software version 17.0; IBM Corporation, Armonk, NY, USA). Statistical significance was accepted at $P<0.05$, and $P<0.1$ was considered indicative for possible trends.

Results

Effects of atomoxetine on body weight

The body weight of the animals was monitored daily during the treatment period to exclude any anorexic effect of atomoxetine.[48] The adolescent atomoxetine-treated rats displayed no altered weight progression compared to their saline treated littermates (Figure S1).

Effects of atomoxetine on NMDA receptor and NET

During the 21-day treatment period (PND 21–42), which is equivalent to childhood and adolescence in humans, atomoxetine altered both mRNA and protein doses of NET and NMDAR subunits 1, 2A, and 2B compared to controls. Detected effects were not evenly distributed throughout the brain, but rather, were identified in distinct brain regions (STR, MES, and HC). Major differences in the brains of rats analyzed immediately after the final atomoxetine application (early treatment group) and those which were housed for 2 more months off-drug (late treatment group) were identified. The PFCs in both groups were not affected by the compound. The results of both mRNA and protein analyses are summarized in Figure 1.

Slc6a2/NET

Since atomoxetine has a very high affinity to NET (dissociation constant [Kd] =0.29 nM),[49] we addressed the question as to whether *Slc6a2* (NET gene) expression and/or its protein amounts are affected after in vivo atomoxetine exposure over a period of 21 days. Measurements of transcript amounts of *Slc6a2* revealed no significant alterations in both early and late treatment groups compared to controls (Figure 2A). In contrast, immunoblotting analysis of samples derived from the hippocampus of both treatment groups displayed reduced NET levels compared to saline controls. More specifically, in the HC of the early treatment group norepinephrine transporter signals were markedly reduced by 32%±3.5% (P=0.0005). In hippocampal samples of rats assigned to the late treatment group, NET levels were also found to be reduced by trend (Figures 1, 2B and C).

Effects of atomoxetine on the NMDAR subunits

Previously, it has been hypothesized that the onset of ADHD might be linked to a hyperglutamatergic state.[11] Several magnetic resonance spectroscopy (MRS) studies revealed dysregulations in the glutamatergic system and effects of ADHD drugs on glutamate metabolism.[50–53] In line with these findings, our group recently demonstrated (in vitro) atomoxetine's antagonistic property at the NMDAR.[36] Therefore, we addressed the issue of whether atomoxetine affects the expression and/or the protein levels of the three

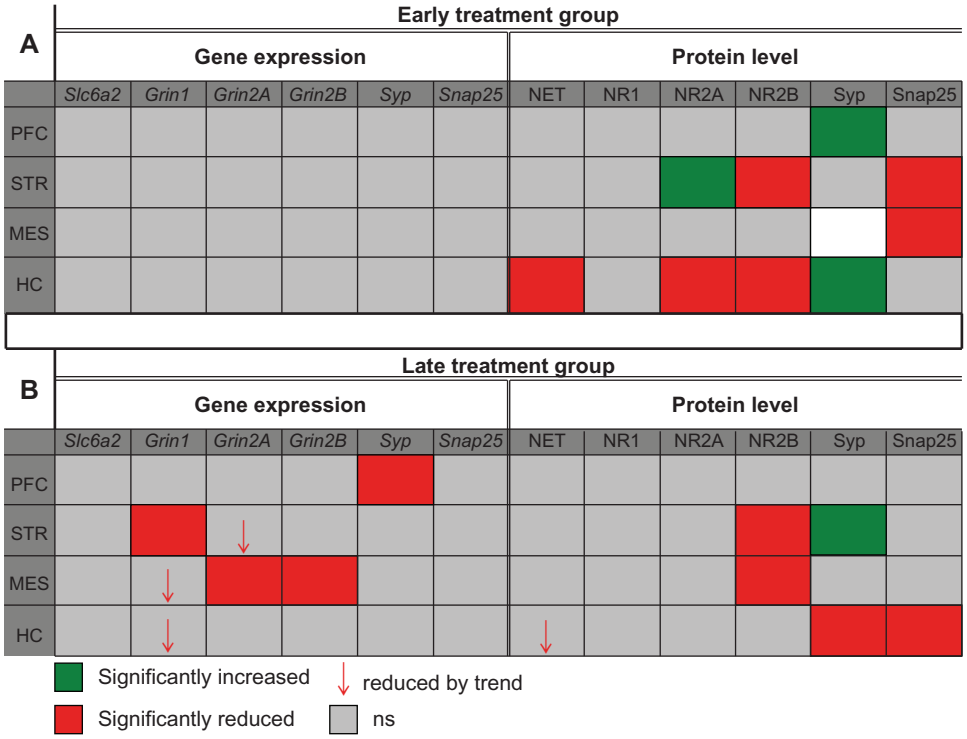

Figure 1 Summary of the mRNA and protein analysis.

Notes: Summary of results analyzing the early treatment group (PND 21–42) (**A**); summary of results analyzing the late treatment group (treatment from PND 21–42, analysis at PND 101) (**B**). Statistical analysis was performed using a two-tailed Wilcoxon–Mann–Whitney test. Significance was accepted at $P<0.05$; n=7–8.

Abbreviations: *Grin*, *glutamate receptor ionotropic N-methyl-D-aspartate*; HC, hippocampus; MES, mesencephalon; NET, norepinephrine transporter; NR, *N*-methyl-*D*-aspartate receptor subunit; PFC, prefrontal cortex; PND, postnatal day; *Slc6a2*, *solute carrier family 6 member 2; Snap25, synaptosomal-associated protein 25 kDa*; STR, striatum; *Syp, synaptophysin*; mRNA, messenger RNA.

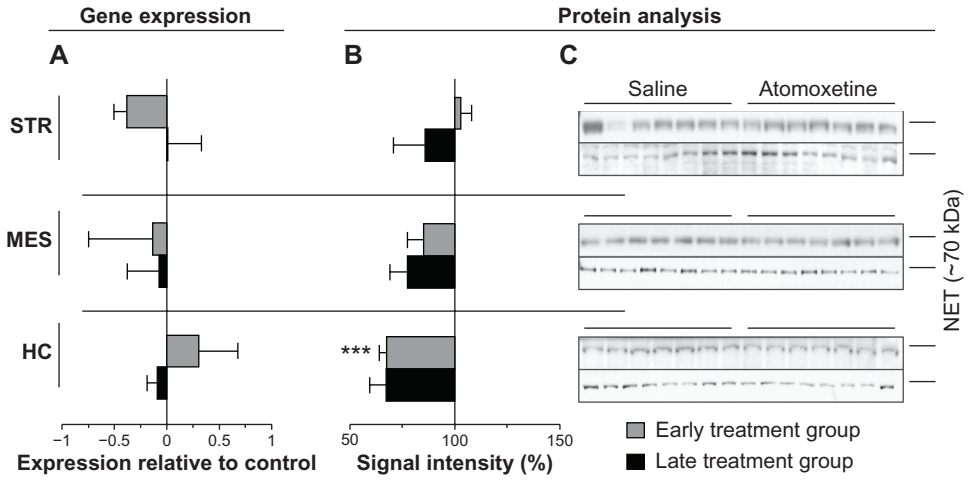

Figure 2 Atomoxetine induced changes on *Slc6a2* messenger (m)RNA and NET levels.

Notes: Male adolescent rats were treated for 21 days (postnatal days 21–41) with atomoxetine hydrochloride (3 mg/kg, intraperitoneal [ip]) or saline (0.9%, ip). The striatum (STR), mesencephalon (MES), and hippocampus (HC) of the early treatment group were analyzed 24 hours after the last ip application, whereas the brains of rats assigned to the late treatment group were probed after a treatment-free period of 2 months. (**A**) *Solute carrier family 6, member 2* (*Slc6a2*) expression was probed in the STR, MES, and HC. Bar diagrams depict mean values of quantitative reverse transcription polymerase chain reaction (qRT-PCR) measurement. (**B**) Bar diagrams depict mean values obtained by densitometric quantification of the (**C**) respective Western blots detecting the norepinephrine transporter (NET) at a molecular weight of ~70 kDa. Controls were male age-matched saline-treated rats. All data are presented as ± standard error of the mean; early treatment group n=7; late treatment group n=8; ***$P<0.001$.

main NMDAR subunits, NR1 (*Grin1*), NR2A (*Grin2A*), and NR2B (*Grin2B*) in vivo. Atomoxetine exposure for 21 days reduced the expression and protein amount of the NMDAR subunits in the rat brain compared to controls.

Grin1/NR1

In the STR of the late treatment group, atomoxetine significantly reduced both mRNA and protein levels of NR1 by 13%±4.2% (P=0.0498) and 36%±6.7% (P=0.0069), respectively (Figure 3). We also identified reduction of *Grin1* mRNA species in MES and HC of the late treatment group. However, statistical analysis of atomoxetine's impact on *Grin1* expression in MES and HC revealed only a reduction by trend (Figure 3A). The assayed samples collected from rats assigned to the early treatment group did not elicit significant changes after atomoxetine exposure (Figures 1 and 3).

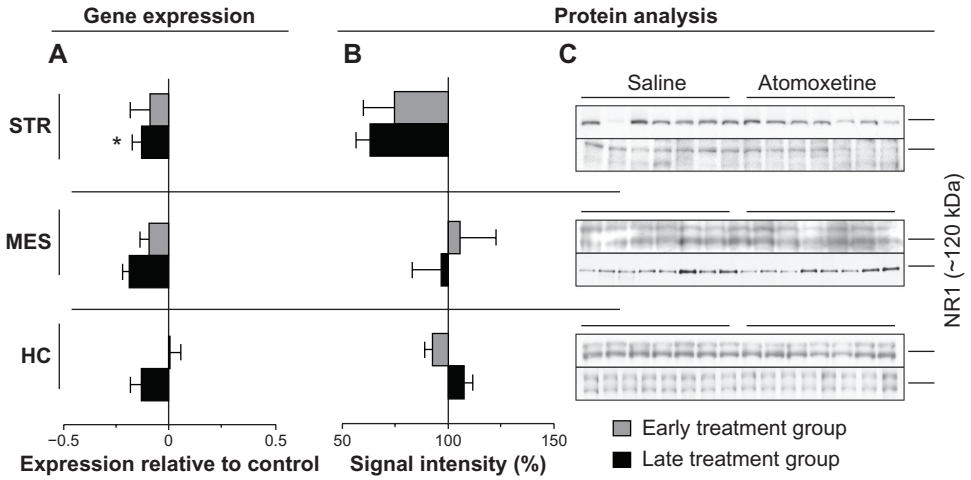

Figure 3 Atomoxetine induced changes on *Grin1* messenger (m)RNA and NR1 levels.

Notes: Male adolescent rats were treated for 21 days (postnatal days 21–41) with atomoxetine hydrochloride (3 mg/kg, intraperitoneal [ip]) or saline (0.9%, ip). The striatum (STR), mesencephalon (MES), and hippocampus (HC) of the early treatment group were analyzed 24 hours after the last ip application, whereas the brains of rats assigned to the late treatment group were probed after a treatment-free period of 2 months. (**A**) *Glutamate receptor ionotropic N-methyl-D-aspartate 1 (Grin1)* expression was probed in the STR, MES, and HC. Bar diagrams depict mean values of quantitative reverse transcription polymerase chain reaction (qRT-PCR) measurement. (**B**) Bar diagrams depict mean values obtained by densitometric quantification of the (**C**) respective Western blots detecting the glutamate receptor ionotropic *N*-methyl-*D*-aspartate 1 (NR1) at a molecular weight of ~120 kDa. Controls were male age-matched saline-treated rats. All data are presented as ± standard error of the mean; early treatment group n=6–7; late treatment group n=7–8; *$P<0.05$.

Grin2A/NR2A

The qRT-PCR measurements of total RNA isolated from brain regions of the late treatment group, elicited 34%±3.2% lower *Grin2A* transcript in MES (P=0.0069) and 16%±4.6% reduced levels of striatal *Grin2A* (P=0.0829) (Figure 4A). However, the treatment displayed no effect on NR2A protein levels (Figure 4B and C). Furthermore, atomoxetine exposure affected neither *Grin2A* expression nor NR2A protein levels in the tested brain regions of the early treatment group (Figures 1 and 4).

Grin2B/NR2B

Atomoxetine displayed profound impact on *Grin2B* expression in the late treatment group. The compound markedly reduced *Grin2B* mRNA levels by 50%±3.7% in the MES (P=0.0206) (Figure 5A), whereas mesencephalic NR2B immunoblot signals remained unaltered (Figure 5B and C). Additionally, we identified significantly reduced NR2B protein levels in the STR (P=0.0011) in both early (33%±8.2% reduction) and late treatment (66%±3.7% reduction) groups (Figure 5B and C). Furthermore, hippocampal NR2B amounts in rats assigned to the early treatment group were also found to be lower (38%±8.1% reduction) compared to saline controls (Figure 5B and C). In contrast, atomoxetine did not affect expression of the probed genes in the analyzed brain regions of animals assigned to the early treatment group.

Provoked alterations in synaptic composition – preliminary findings

To test the hypothesis concluded from the above mentioned results, if the detected changes in the glutamatergic system – mainly decreased levels of NMDAR – would lead to further alterations in the synaptic composition, we investigated the levels of two SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins, Syp and Snap 25. Both were significantly altered in both treatment groups (Figures 1, 6, and 7).

Discussion

In the present study, we assessed the question of immediate and long-term impact of a chronic 3-week application of atomoxetine on the NE and glutamatergic system. The assessment was conducted immediately after the 21 days of exposure (early treatment group) and 2 months later (late treatment group).

The administered atomoxetine ip dosage of 3 mg/kg per day is approximately twice the maximum of the recommended daily dosage in humans. However, considering the much faster metabolism in rats, the applied dosage is well within the therapeutic range. Sprague Dawley rats exposed to this particular atomoxetine dosage for 3 weeks displayed profound changes in both NET and NMDAR subunit transcriptional and translational processes. The effects on mRNA and protein levels of NET and the NMDAR subunits differed across the analyzed

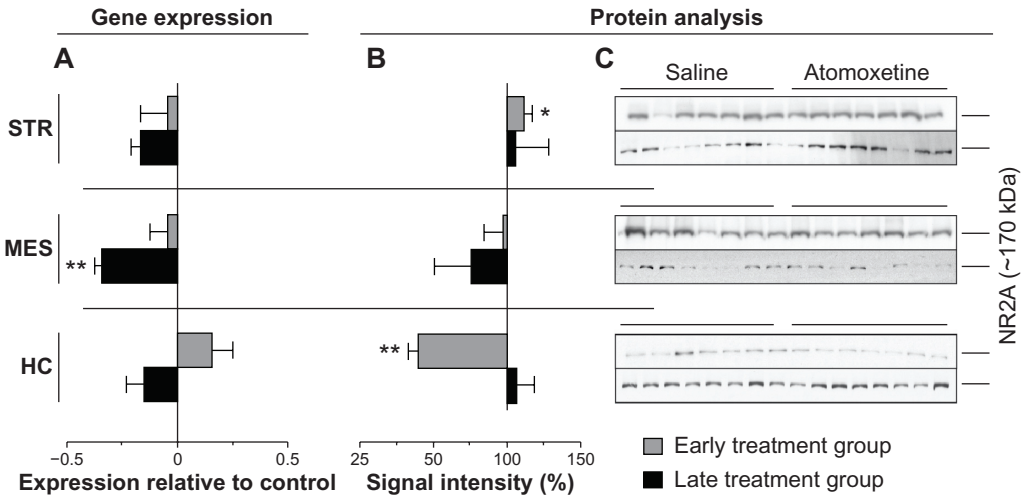

Figure 4 Atomoxetine induced changes on *Grin2A* messenger (m)RNA and NR2A levels.

Notes: Male adolescent rats were treated for 21 days (postnatal days 21–41) with atomoxetine hydrochloride (3 mg/kg, intraperitoneal [ip]) or saline (0.9%, ip). The striatum (STR), mesencephalon (MES), and hippocampus (HC) of the early treatment group were analyzed 24 hours after the last ip application, whereas the brains of rats assigned to the late treatment group were probed after a treatment-free period of 2 months. (**A**) *Glutamate receptor ionotropic N-methyl-D-aspartate 2A (Grin2A)* expression was probed in the STR, MES, and HC. Bar diagrams depict mean values of quantitative reverse transcription polymerase chain reaction (qRT-PCR) measurement. (**B**) Bar diagrams depict mean values obtained by densitometric quantification of the (**C**) respective Western blots detecting the glutamate receptor ionotropic *N*-methyl-*D*-aspartate 2A (NR2A) at a molecular weight of ~170 kDa. Controls were male age-matched saline-treated rats. All data are presented as ± standard error of the mean, early treatment group n=7, late treatment group n=8; *P<0.05; **P<0.01.

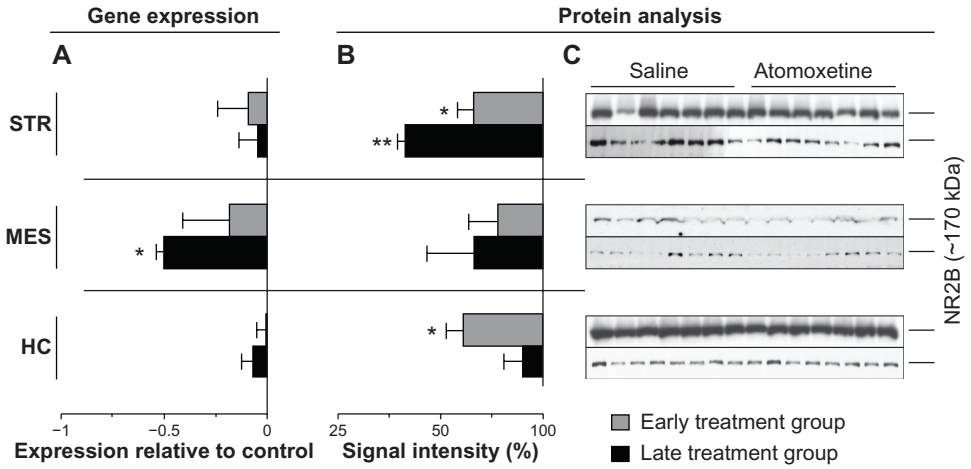

Figure 5 Atomoxetine induced changes on *Grin2B* messenger (m)RNA and NR2B levels.

Notes: Male adolescent rats were treated for 21 days (postnatal days 21–41) with atomoxetine hydrochloride (3 mg/kg, intraperitoneal [ip]) or saline (0.9%, ip). The striatum (STR), mesencephalon (MES), and hippocampus (HC) of the early treatment group were analyzed 24 hours after the last ip application, whereas the brains of rats assigned to the late treatment group were probed after a treatment-free period of 2 months. (**A**) *Glutamate receptor ionotropic N-methyl-D-aspartate 2B (Grin2B)* expression was probed in the STR, MES, and HC. Bar diagrams depict mean values of quantitative reverse transcription polymerase chain reaction (qRT-PCR) measurement. (**B**) Bar diagrams depict mean values obtained by densitometric quantification of the (**C**) respective Western blots detecting the glutamate receptor ionotropic *N*-methyl-*D*-aspartate 2B (NR2B) at a molecular weight of ~170 kDa. Controls were male age-matched saline-treated rats. All data are presented as ± standard error of the mean; early treatment group n=7; late treatment group n=8; **P*<0.05; ***P*<0.01.

treatment groups and assayed brain regions. Taking the age of the animals into account (PND 21–42), which is the treatment period equivalent to childhood and early adolescence in humans, atomoxetine displays strong impact on brain biochemistry, thus underpinning the compound's impact on cerebral development already after only 21 days of treatment.

Assaying protein levels of the reported primary biochemical target of atomoxetine, NET levels in both early and late treatment group HC samples were reduced compared to controls. However, the impact of the substance had no effect on the transporter's gene expression. As Bymaster et al[27] showed in their combined in vivo and in vitro study, atomoxetine inhibits radioligand binding in cells transfected with human NETs with binding affinity (Ki) values of 5 nM. For illustration purposes, note that recommended serum levels in humans, treated with atomoxetine, are in the range of 200–1,000 ng/mL, equivalent to 0.6-3.4 μM.[33] Presumably, brain concentrations are even higher.[34] Therefore, atomoxetine probably displays strong NE reuptake inhibitory effects, which in consequence, might have triggered

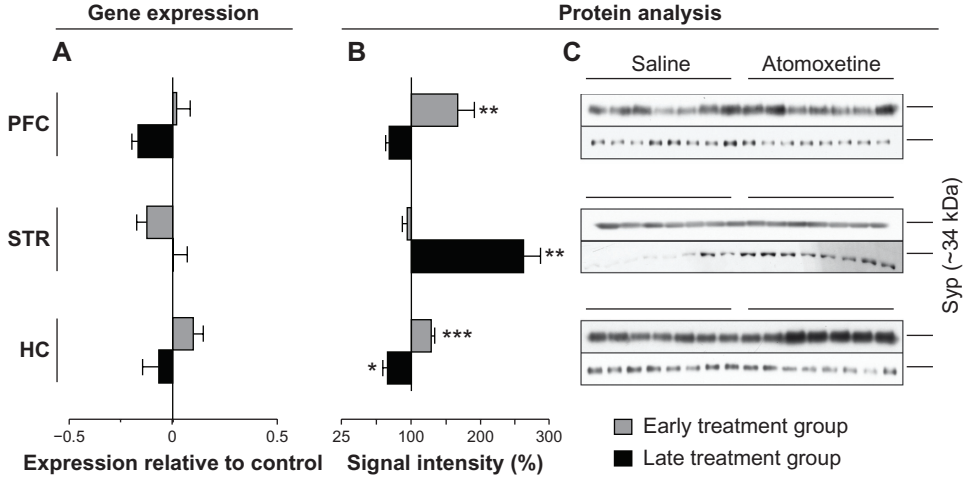

Figure 6 Atomoxetine induced changes on *Syp* messenger (m)RNA and protein levels.

Notes: Male adolescent rats were treated for 21 days (postnatal days 21–41) with atomoxetine hydrochloride (3 mg/kg, intraperitoneal [ip]) or saline (0.9%, ip). The prefrontal cortex (PFC), striatum (STR), and hippocampus (HC) of the early treatment group were analyzed 24 hours after the last ip application, whereas the brains of rats assigned to the late treatment group were probed after a treatment-free period of 2 months. (**A**) *Synaptophysin (Syp)* expression was probed in the PFC, STR, and HC. Bar diagrams depict mean values of quantitative reverse transcription polymerase chain reaction (qRT-PCR) measurement. (**B**) Bar diagrams depict mean values obtained by densitometric quantification of the (**C**) respective Western blots detecting Syp (1:1000) at a molecular weight of ~34 kDa. Controls were male age-matched saline-treated rats. All data are presented as ± standard error of the mean; early treatment group n=7; late treatment group n=8; **P*<0.05; ***P*<0.01; ****P*<0.001.

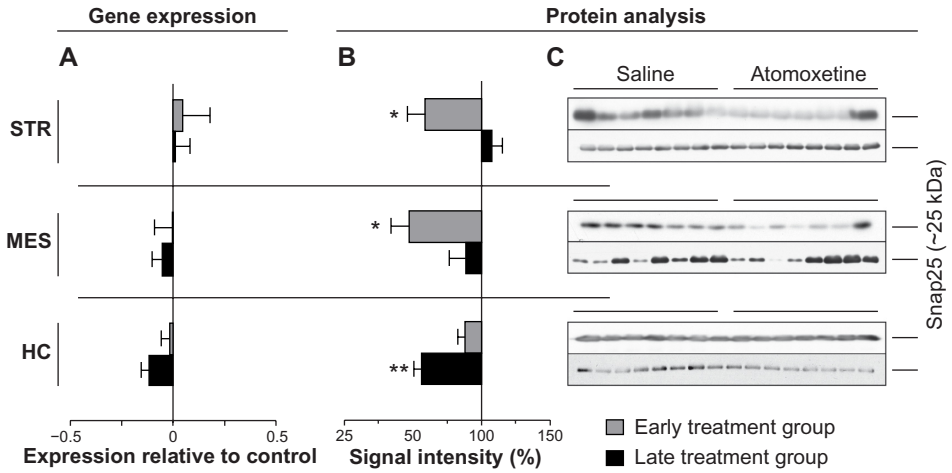

Figure 7 Atomoxetine induced changes on *Snap25* messenger (m)RNA and protein levels.
Notes: Male adolescent rats were treated for 21 days (postnatal days 21–41) with atomoxetine hydrochloride (3 mg/kg, intraperitoneal [ip]) or saline (0.9%, ip). The striatum (STR) and hippocampus (HC) of the early treatment group were analyzed 24 hours after the last ip application, whereas the brains of rats assigned to the late treatment group were probed after a treatment-free period of 2 months. (**A**) *Synaptosomal-associated protein 25 kDa (Snap25)* expression was probed in the STR, MES, and HC. Bar diagrams depict mean values of quantitative reverse transcription polymerase chain reaction (qRT-PCR) measurement. (**B**) Bar diagrams depict mean values obtained by densitometric quantification of the (**C**) respective Western blots detecting the Snap25 (1:50,000) at a molecular weight of ~25 kDa. Controls were male age-matched saline-treated rats. All data are presented as ± standard error of the mean; early treatment group n=7; late treatment group n=8; *$P<0.05$; **$P<0.01$.
Abbreviation: MES, mesencephalon.

the observed reduction of NET levels. Furthermore, altered NET levels might also be indirectly linked to atomoxetine's NMDAR antagonistic effect. As reviewed by Duguid and Smart,[54] presynaptic NMDARs affect synaptic transmission[55] by modifying neurotransmitter release via Ca^{2+} entry and influencing transmitter release of presynaptic vesicles and their fusion with the presynaptic membrane.[56,57] Pittaluga and Raiteri[58] have demonstrated increased NE release in HC modulated by presynaptic NMDARs. Atomoxetine may inhibit these presynaptic NMDARs,[23] resulting in altered transmitter release, which modulates the presynapse by affecting proteins localized to the presynapse. To strengthen our hypothesis of atomoxetine's effect at the presynapse, we additionally assayed the presynaptic vesicle marker Syp and the Snap25, known to be crucial for vesicle fusion and transmitter release into the synaptic cleft. Analysis of the two proteins revealed significant alterations in the STR and HC of both early and late treatment groups (Figures 6 and 7). Furthermore, we observed significantly reduced protein levels of hippocampal NR2B in the early treatment group and lower *Grin1* mRNA amounts in the HC of the late treatment group. Due to the nature of the techniques used, we cannot allocate the effects on the NMDAR subunits to the presynapse or postsynapse. However, our findings of altered NMDAR subunit mRNA and protein levels correlate with the changes of NET levels and altered proteins crucial for transmitter release in the HC of both treatment groups. Therefore, we hypothesize that in our in vivo study, atomoxetine treatment had a profound impact on the presynapse.

Previously, the modulatory effect of two other NMDAR antagonists, acamprosate and MK-801 (dizocilpine), on transcript and protein levels of NMDAR subunits has been demonstrated in the rat brain.[59] Rammes et al demonstrated significantly increased mRNA and protein levels in the HC and cortex after a single ip dose in adult male Wistar rats.[59] At first glance their results seem to be contrary to our findings; however, in the present study, younger rats from a different strain were probed. The two NMDAR inhibitors used by Rammes et al to block the receptor channel have diverse chemical and biochemical properties compared to atomoxetine. The chronic exposure to atomoxetine (21 days) obviously triggers diverse effects on gene expression and protein amounts of the ionotropic glutamate receptors. The discrepancies between the findings of the two studies might be due to the different age of the groups; NMDA receptors undergo prominent changes during brain development,[60,61] such as altered subunit composition, which changes their functionality[62] and modifies their pharmacological response.[14,63,64]

Notably, alterations of gene expression were only found in the late treatment group. The impact on NMDAR subunit protein levels was also evident, as they increased in the late treatment group, indicating that atomoxetine influences brain development far beyond the direct pharmacological effect. Atomoxetine seems to stimulate a change in synaptic composition that even amplifies in the course of development.

Another issue to address is the apparent discrepancy between effects on mRNA and protein levels. As we

compared the effects of atomoxetine on mRNA and protein level, we identified statistically significant alterations of both entities, but did not find congruent results. In general, mRNA and protein quantities approximately correlated. However, in a correlation study by Guo et al,[65] the expression profile of 71 genes and their respective proteins in circulating monocytes collected from 30 unrelated women were investigated. Considering the whole dataset, the authors found a striking correlation between mRNA and protein amounts, but only five genes displayed close correlation to their corresponding proteins at the study group level. Furthermore, they observed that only 9% of the protein variation was directly linked to changes in the amount of the respective mRNA species. Guo et al concluded that gene expression and protein level correlation differ between individuals.[65] Considering that in the present study, an outbred rat strain was analyzed harboring large genetic heterogeneity, our contradictory findings of effects on transcript and protein level are comprehensible. Taken together, atomoxetine seems to reduce gene expression and protein levels of NET and NMDARs, leading to further regulation of synaptic composition.

Clinical implications

The involvement of the glutamatergic system in the pathophysiology of ADHD is recently coming more distinctly into view.[10,11] In a first MRS study, MacMaster et al[66] detected an increase in glutamatergic tone in untreated children with ADHD compared to controls. In another study, the group investigated 14 children with ADHD, medication-free, and after treatment.[52] Eleven out of 14 children were treated with psychostimulants, only three with atomoxetine. The striatal glutamate/glutamine/gamma-aminobutyric acid to creatine/phosphocreatine ratio was the only detectable change in response to medication. The ratio decreased significantly. The authors postulated that an influence on glutamate metabolism might be involved in treatment response in ADHD. Also, Wiguna et al found a decreased glutamate to creatine ratio after treatment by MRS (21 children with ADHD).[51] In this study, it was not the STR where the findings were significant but in the PFC.

The ubiquitous neurotransmitter glutamate – nearly 60% of all neuronal synapses in the brain are glutamatergic[12] – is essential as a neuronal growth factor and is pivotal during neuronal development.[67] Recently, two open-label studies with the NMDA antagonist memantine (20 mg/day) in pediatric and adult ADHD patients showed positive effects on clinical symptoms.[68] Earlier studies pointed out the pivotal role of the NR2B subunit in neuron excitability and synaptic plasticity.[69,70] Jensen et al[22] could show that hippocampal LTP in the SHR (PND 28), the best validated animal model of ADHD, was significantly reduced by an NR2B specific blocker, whereas the blocker had no effect in the age-matched control animals. The authors interpreted their results as a functional predominance of NR2B in SHR, meaning a delayed development. At PND 28, NR2A-containing NMDARs usually contribute substantially to LTP induction.[14,15]

In the present study, atomoxetine significantly reduced the protein level of NR2B in the HC of the adolescent rats. This action might contribute to the clinical effects of the compound.

Genome-wide association studies on ADHD patients pointed out that the modulation of neuronal and synaptic plasticity seems to be at least one of the essential mechanisms in ADHD pathophysiology,[71–73] even though, as reviewed by Franke et al,[74] the results of the existing genome-wide association studies showed limited overlap.

Besides dysfunctions in neuronal glutamate transmission, increasing evidence emphasizes that abnormal neuronal development, such as disturbed synaptogenesis and/or dysfunctional neuronal migration, is important in ADHD pathophysiology.[75]

In the present in vivo study, the majority of the reported effects of atomoxetine were identified in the STR and HC. Already in 1996, Lou[25] pointed out the unique anatomical characteristics of the STR, a watershed region with convergent glutamatergic afferent projections from almost the entire cortex. In ischemia-induced liberation of glutamate, this accentuated position of the STR is the cause of its particular vulnerability to lesions. Besides genetic factors, pre- and perinatal events are hypothesized to be a predominant cause for ADHD.[25] The observed cellular mechanisms of atomoxetine in this in vivo study might at least contribute to the clinical effects ameliorating ADHD core symptoms.

Limitations

This study revealed new insights of atomoxetine's biological properties at the cellular level. Nevertheless, some limitations of the study must be considered. The Sprague Dawley rats from Charles River are an outbred strain; the heterogeneity of the genetic background probably masked minor effects on gene expression and protein levels. In some cases of probed mRNA species the interindividual variation was very high. A further limitation is the total number of animals (n=7–8) per experiment, which was strongly regulated by regional administrative authorities.

Conclusion

In conclusion, the results of the present exploratory study demonstrate that atomoxetine's impact on the brain is not solely limited to the inhibition of NE reuptake. The compound alters mRNA and protein levels of the respective genes and proteins crucial for synaptic plasticity, hence influencing the neuronal homoeostasis,[76] which presumably is disturbed in ADHD. Two actions especially might be of major interest: first, the reduction of the NMDA receptor subunit NR2B in the adolescent HC since this protein seems to be elevated in the HC of the best validated animal model of ADHD, the SHR,[22] and second, the influence on *Snap25*, a novel candidate gene which has been altered in the coloboma mouse, another animal model for ADHD.[77,78]

Furthermore, we showed that atomoxetine's immediate and long-term effects on synaptic proteins differ, thus emphasizing its modulatory influence on synaptic plasticity. However, whether atomoxetine's impact can directly be linked to its NMDAR and NET antagonism or is due to a still unknown pathway has yet to be elucidated. In the present study, we were able to show immediate and long-term brain region-specific effects. Disease-specific effects must be investigated in animal models of ADHD, ie, in the above mentioned SHR.[79,80]

Disclosure

Professor, Dr Andrea G Ludolph has received research funding from UCB/Celltech, Medice, and Novartis. She was/is involved in clinical trials with Boehringer Ingelheim, Shire, and Otsuka. She discloses consulting fees and travel grants from Shire and lecture fees from Janssen and Lilly. Patrick T Udvardi, Dr Stefan Liebau, Carolin Henes, Dr Karl Föhr, Dr Jens Dreyhaupt, and Professor Tobias M Boeckers report no conflicts of interest in this work.

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Supplementary material

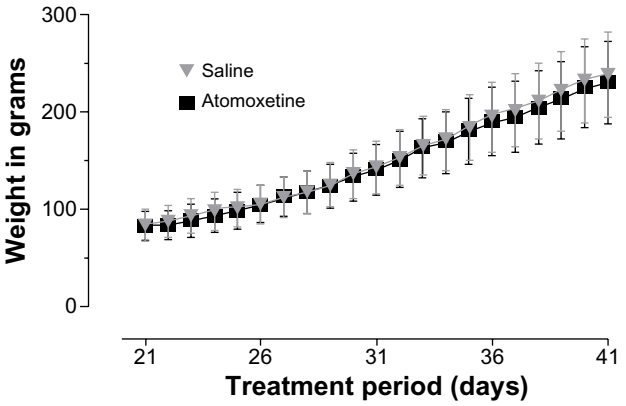

Figure S1 Weight gain of male adolescent rats during treatment period.
Notes: Sprague Dawley rats were treated once a day for 21 days (postnatal days 21–41) with atomoxetine hydrochloride (3 mg/kg intraperitoneal [ip]) or saline (0.9%, ip). The treatment period is equivalent to the period of adolescence in humans. Rats were weighed once per day before treatment. The graph depicts mean total weight of saline and atomoxetine treated rats, ± standard error of the mean; n=7.