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The noradrenaline transporter as site of action for the anti-Parkinson drug amantadine

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ABSTRACT

Amantadine is an established antiparkinsonian drug with a still unclear molecular site of action. *In vivo* studies on rodents, *in vitro* studies on tissue of rodents as well as binding studies on *post mortem* human tissue implicate monoamine transporters and NMDA receptors. In order to re-examine its action at human variants of these proteins on intact cells we established cells stably expressing the human NR1/2A NMDA-receptor, noradrenaline transporter (NAT) or dopamine transporter (DAT) and tested the activity of amantadine in patch-clamp, uptake, release, and cytotoxicity experiments. Amantadine was less potent in blockade of NMDA-induced inward currents than in blockade of noradrenaline uptake and in induction of inward currents in NAT expressing cells. It was 30 times more potent in blocking uptake in NAT- than in DAT cells. Amantadine induced NAT-mediated release at concentrations of 10–100 μM in superfusion experiments and blocked NAT-mediated cytotoxicity of the parkinsonism inducing neurotoxin 1-methyl-4-phenyl-pyridinium (MPP^+) at concentrations of 30–300 μM, whereas 300–1000 μM amantadine was necessary to block NMDA-receptor mediated cytotoxicity. Similar to amphetamine, amantadine was inactive at α_{2A}-adrenergic receptors and induced reverse noradrenaline transport by NAT albeit with smaller effect size. Thus, amantadine acted as "amphetamine-like releaser" with selectivity for the noradrenergic system. These findings and differences with memantine, which had been reported as less efficient antiparkinsonian drug than amantadine but in our hands was significantly more potent at the NMDA-receptor, suggest contributions from a noradrenergic mechanism in the antiparkinsonian action of amantadine.

1. Introduction

Since a case report on improvements of parkinsonian symptoms while receiving amantadine for influenza prophylaxis and subsequent controlled trials reporting efficacy in the treatment of Parkinson's disease (Parkes et al., 1970; Schwab et al., 1969) amantadine is an established antiparkinsonian agent. It is especially useful as an adjuvant to levodopa where it markedly improved motor response complications (Rajput et al., 1998; Sawada et al., 2010; Thomas et al., 2004; Verhagen et al., 1998) and as a drug which is available for intravenous administration (Adler et al., 1997; Ruzicka et al., 2000). Several modes of action have been proposed from *in vitro* experiments. Amantadine was shown to inhibit neuronal uptake of dopamine and noradrenaline into rat brain homogenates (Fletcher and Redfern, 1970), slices (Heikkila and Cohen, 1972) and synaptosomes (Herblin, 1972; Thornburg and Moore, 1973). It weakly released dopamine and noradrenaline from nerve endings isolated from rat brain (Haacke et al., 1977) and augmented the release of dopamine at high doses in the striatum of rats (Papeschi, 1974; Scatton et al., 1970). An action mediated by post-synaptic dopamine receptors appeared unlikely since the IC_{50} in displacing the dopamine receptor ligand [^{3}H]N-n-propylnorapomporphine in striatal membranes was reported to be about 1 mM (Dunn et al., 1986). More recently, an inhibitory action of amantadine at NMDA receptors has been shown; it competed with [^{3}H]MK-801 binding in membrane homogenates of post-mortem human frontal cortex (Kornhuber et al., 1991), antagonized inward current responses to NMDA in freshly dissociated rat hippocampal and striatal neurons (Parsons et al., 1996), inhibited the NMDA-evoked [^{3}H]ACh release in slices of the rabbit caudate nucleus (Lupp et al., 1992), reduced NMDA receptor-mediated neurotoxicity in cultures of rat retinal ganglion cell neurons (Chen et al., 1992) and rat neuron-enriched cerebrocortical cultures (Lustig et al., 1992). Thus, experimental evidence to date

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reflects a summary of effects obtained in various species including binding data from homogenized post-mortem brain.

In Parkinson's disease, in addition to dopamine neurons, brainstem noradrenergic nuclei show signs of degeneration, and noradrenergic drugs may modify parkinsonian symptoms. Besides the beneficial effects of α-2 adrenoreceptor antagonists in levodopa-induced dyskinesia (Bezard et al., 1999; Rascol et al., 2001; Savola et al., 2003) and the improvement of freezing gait by the synthetic noradrenaline precursor L-threo-3,4-dihydroxyphenylserine (Narabayashi et al., 1987; Tohgi et al., 1993), noradrenaline uptake inhibitors were used in Parkinson's disease, not only in treatment of depression, but also of motor function (Jankovic, 2009; Laitinen, 1969; Marsh et al., 2009). On the other hand, amantadine displayed antidepressant action in various studies (for review (Huber et al., 1999)).

The above findings motivated us to re-examine the pharmacology of amantadine at the noradrenaline transporter (NAT), dopamine transporter (DAT) and the NMDA-receptor by expressing the human recombinant proteins in cell culture. The pharmacology of amantadine at the human transporters was not studied before, and studying the human proteins seemed advisable considering differences in substrate and inhibitor affinities between rodent and human versions reported previously (Giros et al., 1992; Paczkowski et al., 1999). In cAMP accumulation, monoamine uptake and release, patch-clamp and cytotoxicity experiments only effects on the transporters and receptors in living cells were investigated.

2. Methods

2.1. Cell culture and molecular biology

Human embryonic kidney 293 cells were grown in minimum essential medium with Earle's salts 1-glutamine, 10% heat-inactivated fetal bovine serum and 50 mg/l gentamicin on 60 or 100 mm tissue culture dishes (Falcon) at 37 °C and 5% CO_2/95% air. The conditions for cell culture and transfection of cells stably expressing the porcine α_{2A}-adrenoceptor fused at the C-terminus to the amino terminus of wild type $G\alpha_{i-1}$ were described previously (Kudlacek et al., 2002). For stable expression of human DAT and NAT in HEK cells the expression vector pRc/CMV was used as described previously (Pifl et al., 2004b). For patch-clamp experiments and for all experiments on NMDA-receptor expressing cells the tetracycline-regulated expression system called T-REx™ was used which allowed expression of the proteins in an inducible manner (Invitrogen GmbH, Lofer, Austria). T-REx™ cells stably expressing the tetracycline repressor protein were stably transfected with the respective transporter cDNA using the expression plasmid pcDNA4/TO and a calcium phosphate method as described previously (Pifl et al., 1996). Cell clones expressing the transporter were selected with 0.3 g/l zeocin in the presence of 5 mg/l blasticidin. For expression of the human NR1/2A NMDA receptor the cDNA of the hNR1 subunit (received in bluescript from Dr. Shigetada Nakanishi, Osaka) was subcloned into pcDNA4/TO. Two million cells 293/T-REx™ cells were plated into 100-mm diameter cell culture dishes one day before transfection. At the day of transfection, the medium was first changed and six to 7 h later, 1 μg of NR1/pCDNA4/TO and 5 μg hNR2A/pcDNAI (received from Dr. Antonio Ferrer-Montiel, Elche) in 450 μl of H_2O were mixed with 50 μl of 2.5M $CaCl_2$ and the further transfection procedure was as described (Pifl et al., 1996). One day after transfection, plates were split 1:4 to 1:8, and on the next day selection of cells was started by adding zeocin at 0.3 g/l and blasticidin at 5 mg/l. After two weeks, single clones were transferred with Gilson pipette tips into 48 well plates containing medium and selecting antibiotics. Six days later each of the cell clones was split after trypsinisation into of a 24-well and a 48-well plate, one well each. On the next day tetracycline was added (0.1 mg/l) for induction of the NMDA receptor in the 48-well plate. Cell clones dying in the 48-well plate after adding tetracycline (0.1 mg/l) for induction of the NMDA receptor were further grown up from the corresponding 24-well plate, and receptor expression was verified by cell death following induction with tetracycline and its prevention with 200 μM memantine.

2.2. Determination of cAMP formation

Receptor-dependent inhibition of cAMP formation in stable HEK293 cells was assessed as described previously (Bofill-Cardona et al., 2000). Cells were seeded in poly-D-lysine-coated 6-well plates (2.5×10^5 cells/well) and 1 day later labeled with 2,8-[^{3}H]adenine (1 μCi/well) overnight, incubated in 1 ml PBS containing 100 μM of the cAMP-specific phosphodiesterase-IV inhibitor Ro-201724, stimulated with forskolin (25 μM), and receptor-dependent inhibition at RT was determined after a 25 min incubation with α_2 adrenoceptor agonist UK 14,304 at the indicated concentrations in the absence or presence of 0.1 μM yohimbine or of 10–1000 μM amantadine. After termination by cell lysis, [^{3}H]cAMP was isolated by sequential chromatography on Dowex AG 50W-X4 and neutral alumina.

2.3. Patch clamp experiments

About $6–9 \times 10^4$ cells were split into poly-D-lysine-coated 35 mm tissue culture dishes. Patch-clamp recordings were performed 3–4 days later, 18 h (NMDA-receptor) or 2–3 days (transporters) after induction of proteins by adding tetracycline. The bath solution for experiments on NMDA-receptor expressing cells was as reported by (Monyer et al., 1992) and consisted of (mmol/l): 150 NaCl; 5.4 KCl; 1.8 $CaCl_2$; 5 HEPES; pH 7.2, NaOH. The final osmolarity was 275 mOsm/l. Patch pipettes were filled with (mmol/l): 140 CsCl; 1 $MgCl_2$; 10 EGTA; 10 HEPES; pH 7.2, CsOH, with an osmolarity of 303 mOsm/l. The bath solution for experiments on NAT or DAT expressing cells consisted of (mmol/l): 4 Tris–HCl; 6.25 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); 120 NaCl; 5KCl; 1.2 $CaCl_2$; 1.2 $MgSO_4$; 130 NaCl; 34 D-glucose and 0.5 ascorbic acid; pH 7.2. The final osmolarity was 300mOsm/l. Patch pipettes were filled with (mmol/l): 130 KCl; 0.1 $CaCl_2$; 2 $MgCl_2$; 1.1 EGTA; 9 HEPES; 0.65 TRIS; pH 7.2, with an osmolarity of 270 mOsm/l. Patch electrodes were pulled from borosilicate glass capillaries (GB150F-8P, Science Products, Hofhem Germany) with a programmable Brown-Flaming micropipette puller (P-97; Sutter Instruments Co., USA) were heat-polished to a final tip resistance of 3–6 MΩ. Recordings were performed in the whole-cell configuration of the patch-clamp technique using an Axopatch 200B patch clamp amplifier and the pClamp data acquisition system (Axon Instruments, Foster City, CA, U.S.A.) at ambient temperature (25 ± 2 °C) and clamping the cells to the holding potential of −60 to −80 mV. Test drugs were applied with a PTR-2000/DAD-12 drug application device (ALA Scientific Instruments Inc., Westbury, NY), which allows a complete exchange of solutions surrounding the cells under investigation within <100 ms; the cells were continuously superfused with bathing solution. IC_{50} estimates for the inhibition of NMDA-induced inward currents were obtained by fitting the data of individual experiments to the equation $f = \min + (\max - \min)/(1 + x/IC_{50})$. Current in the presence of drug (f) was expressed as % of the current induced by 100 μM NMDA. Parameters estimated by the fitting procedure were current minimum ("min" = maximal inhibition) and the IC_{50} value, i.e. the drug concentration necessary for 50% of the maximum inhibition. "max" denotes the inward current in the absence of drugs, x the molar concentration of the tested drug.

2.4. Uptake of monoamines

Cells were seeded in poly-D-lysine-coated 24-well plates (1×10^5 cells/well) and, 1 day later, uptake was performed at 37 °C for 2.5 min as described previously (Pifl et al., 1996). The uptake buffer consisted of (mmol/l): 4 Tris–HCl; 6.25 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); 120 NaCl; 5 KCl; 1.2 $CaCl_2$; 1.2 $MgSO_4$; 5 D-glucose; 0.5 ascorbic acid; pH 7.1. Uptake was started by addition of (−)[7-^{3}H]noradrenaline or [7-^{3}H]dopamine (New England Nuclear GmbH, Vienna) at a final concentration of 0.1 or 0.3 μM, respectively. Inhibition by desipramine, cocaine and amphetaminue was determined by testing 5-6 different concentrations of the respective substance in 3–8 independent experiments. Data from each individual experiment were fitted to the equation $f = \min + (\max - \min)/(1 + x^{Hillslope}/IC_{50}^{Hillslope})$, "min" being non-specific uptake, "max" the uptake in the absence of inhibiting drug, x the molar concentration of the inhibiting drug, and IC_{50} the drug concentration that inhibits 50% of specific uptake.

2.5. Assay of reverse transport

Cells were seeded onto poly-D-lysine-coated 5-mm-diameter glass cover slips in 96-well tissue culture plates (3×10^4 cells/well) and loaded with 0.5 μM 1-methyl [^{3}H]-4-phenyl-pyridinium (MPP^+, 2 Ci/mmol, New England Nuclear GmbH, Vienna) at 37 °C for 20 min. Coverslips were transferred to small chambers and superfused with the same buffer as used in uptake experiments (25 °C, 1.0 ml/min) as described recently (Pifl et al., 2004b). Amantadine and amphetamine were included in the superfusion buffer as indicated in the Figure legend. The radioactivity released during consecutive 4 min fractions was expressed as percentage of the total radioactivity present in the cells at the beginning of each fraction.

2.6. Cell viability

Cells were seeded in 96 well plates (NR1/2A cells: $1,5 \times 10^4$; NAT and DAT cells: 5×10^3 cells/well). One day later proteins were induced by tetracycline at a final concentration of 0.1 mg/l and the cells were exposed to the drugs as indicated. One day (NR1/2A cells) or three days later (NAT or DAT cells) the number of living cells was estimated by measuring acid phosphatase activity according to (Connolly et al., 1986) as described (Pifl et al., 2004a). Cells were washed with PBS and incubated with 0.1 M sodium acetate (pH 5.5) containing 0.1% Triton X-100 and 10 mM p-nitrophenyl phosphate (Fluka) at 37 °C for 2 h.

After stopping the reaction by addition of 10 ml 1 M NaOH to each well absorbance was measured at 405 nm using a microplate reader. For each treatment

a quadruplicate of wells was determined in at least 4 independent experiments. Statistical significance was determined by paired Student's *t*-test followed by Bonferroni correction comparing the effect of amantadine with the effect of vehicle-treated cells induced by tetracycline and, in case of NAT and DAT cells, treated by MPP^+.

3. Results

3.1. Amantadine had no affinity for α_2 adrenoceptors

Amantadine in concentrations up to 1 mM did not modify the concentration-dependent inhibition of forskolin-stimulated cAMP accumulation by the α_2 adrenoceptor agonist UK 14,304 in HEK293 cells stably expressing the porcine α_{2A}-adrenoceptor fused at the C-terminus to the amino terminus of wild type $G\alpha_{i-1}$, whereas 0.1 μM of the prototypical α_2 adrenoceptor antagonist yohimbine shifted the concentration-response curve of UK 14,304 more than 100-fold (Fig. 1). In addition to this lack of antagonistic action at the α_2 adrenoceptor, amantadine (0.1–1 mM) did not change the accumulation of cAMP in the absence of UK 14,304, which ruled out any agonistic activity at this receptor as well (data not shown).

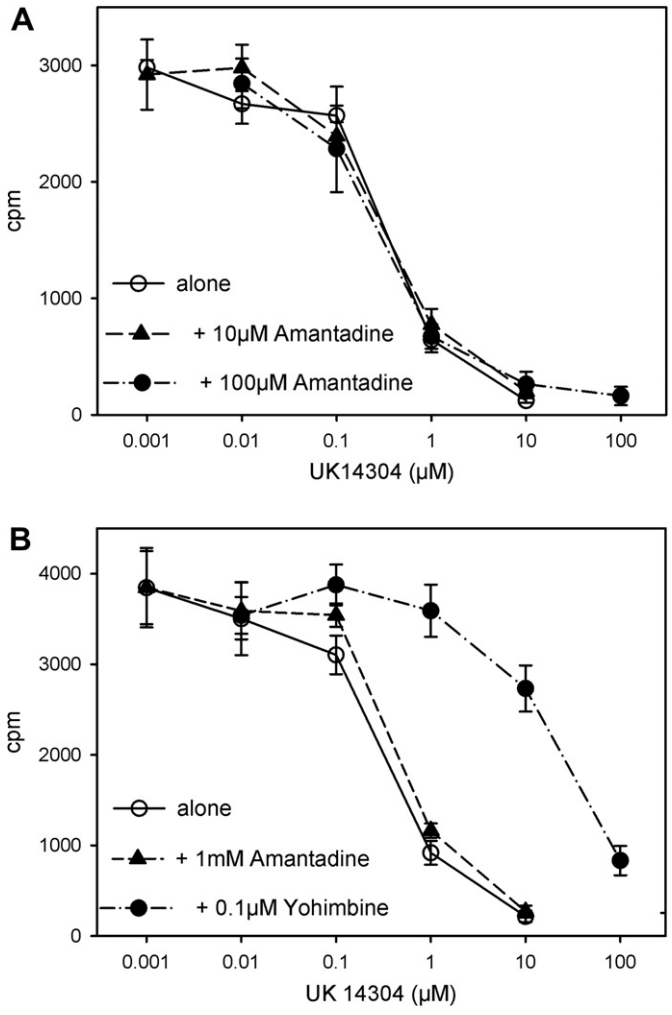

Fig. 1. Concentration-dependence of the α_2 adrenoceptor agonist UK14304 effect on forskolin-stimulated cAMP accumulation. HEK293 cells stably expressing α_{2A}-adrenoceptor expressing were labeled with 2,8-[^{3}H]adenine and exposed to UK14304 at the concentration indicated in the absence (open circles) or (A) presence of amantadine (10 μM, triangles; 100 μM, closed circles), (B) amantadine (1 mM, triangles) or yohimbine (0.1 μM, closed circles). After a 25 min incubation [^{3}H]cAMP was isolated as described under Methods. Symbols represent mean values ± SEM of 3 independent experiments, each in duplicates.

3.2. Amantadine blocked NMDA-induced currents at concentrations beyond 30 μM

NMDA at the concentration of 100 μM induced an inward current in HEK293 cells expressing the human NR1/NR2A receptor. Amantadine blocked this inward current at a concentration of 30 μM by 8.5 ± 1.4% ($n = 13$), at 100 μM by 32 ± 7% ($n = 15$; $p < 0.5$ *vs.* 30 μM) and at 1 mM by 72 ± 8% ($n = 12$; $p < 0.5$ *vs.* 100 μM) (Fig. 2A, B, C). By contrast, ketamine blocked the NMDA-induced inward current at the concentration of 3 μM by 28 ± 3% ($n = 6$), at 10 μM by 68 ± 4% ($n = 7$; $p < 0.5$ *vs.* 3 μM) and at 30 μM by 83 ± 7% ($n = 9$) (Fig. 2D, E, F). An estimate of the potency as described in Methods gave an IC_{50} of 197 ± 93 μM ($n = 5$) for amantadine and 5.6 ± 0.2 μM ($n = 3$) for ketamine.

3.3. Amantadine blocked the NAT more potently than the DAT

Amantadine blocked noradrenaline uptake by HEK293 cells expressing the human NAT in a concentration-dependent manner; the IC_{50} of blockade was 41 ± 4 μM (Fig. 3). Thirty times higher concentrations of amantadine were necessary to block dopamine uptake by HEK293 cells expressing the human DAT (IC_{50} 1.22 ± 0.08 mM). To validate the uptake assays standard inhibitors were investigated under our experimental conditions. For desipramine the IC_{50} values were 0.005 ± 0.002 μM ($n = 3$) and 14.0 ± 1.2 μM ($n = 3$), for cocaine 1.13 ± 0.27 μM ($n = 4$) and 0.89 ± 0.08 μM ($n = 8$) and for amphetamine 0.27 ± 0.02 μM ($n = 4$) and 0.87 ± 0.16 μM ($n = 7$) at NAT and DAT expressing cells, respectively.

3.4. Amantadine induced inward currents in NAT expressing cells

Blockade of monoamine uptake can be induced by pure uptake inhibition or by an amphetamine-like releasing effect. In order to elucidate the type of interaction of amantadine with the NAT, we investigated the action of amantadine on currents in NAT expressing cells by patch-clamp experiments in the whole-cell configuration. In these cells, 10 μM noradrenaline induced an inward current, which was blocked by the presence of 30 μM cocaine (Fig. 4A, B). Amantadine, at the concentration of 10 μM, induced an inward current which was about 20% of that induced by 30 μM noradrenaline (Fig. 4B). The magnitude of inward current did not increase when amantadine was superfused at 100 μM (Fig. 4C) and the inward current induced by 10 μM noradrenaline was attenuated in the presence of 30 μM amantadine (Fig. 4D). Noradrenaline and dopamine also induced inward currents in DAT expressing cells (Fig. 5), a carrier-mediated effect because it was blocked by 30 μM cocaine (shown for 10 μM NA in Fig. 5A). Amantadine, in concentrations up to 30 μM, was not able to induce inward currents in DAT expressing cells and weakly inhibited the effect of 10 μM noradrenaline (Fig. 5B, C).

3.5. Amantadine induced NAT-mediated release

Since the patch-clamp experiments suggested an amphetamine-like activity of amantadine, we tested the drug in superfusion experiments on HEK293 cells expressing the human NAT and preloaded with the metabolically inert transporter substrate [^{3}H]MPP^+. Amantadine induced tritium efflux in a concentration-dependent manner, with a maximum effect at 100 μM which was about 40% of the release induced by 10 μM amphetamine (Fig. 6A). The effects of both, amphetamine and amantadine, were carrier-mediated, because the release was blocked by 10 μM of the transporter blocker mazindol (Fig. 6B, C).

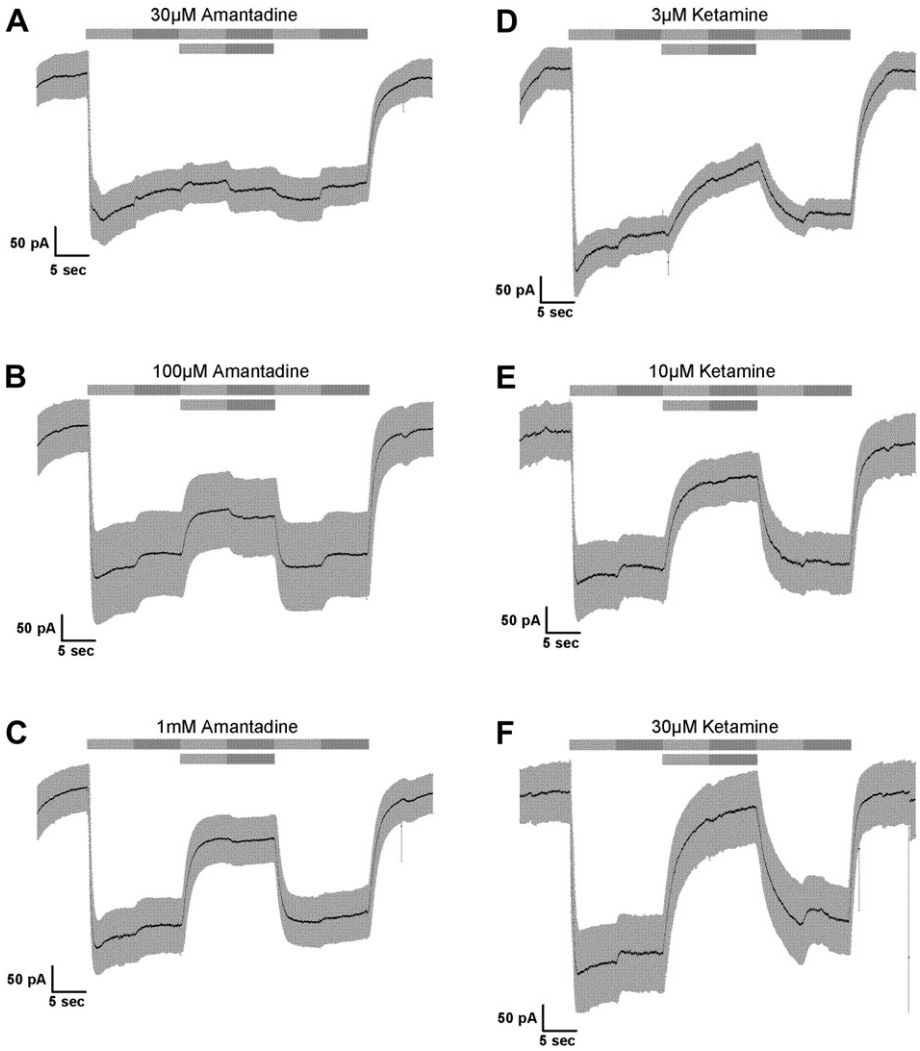

Fig. 2. Effect of amantadine or ketamine on inward currents induced by NMDA in HEK293 cells stably expressing the NR1/2A receptor. Cells were voltage-clamped at a holding potential of −60 mV and superfused for 30 s with 100 μM NMDA (upper bar) in the absence or presence (lower bar) of amantadine (30 μM, A; 100 μM, B; 1000 μM, C) or ketamine (3 μM, D; 10 μM, E; 30 μM, F). Light/dark grey in bars indicates change to different superfusion syringes as a control for potential artifacts of switches. Shown are mean values of current traces ± SEM (grey area) of 12–15 (A–C) or 6–9 (D–F) cells.

3.6. Amantadine blocked NAT-mediated cytotoxicity more potently than NMDA- and DAT-mediated cytoxicity

In order to investigate the long term activity of amantadine on the receptor or transporters cytotoxity assays were established.

In cells expressing the human the NR1/2A under the control of the T-REx™ system, cytotoxicity was induced by treatment with tetracycline for 24 h eliciting synthesis of the functional NMDA-receptors which confer a detrimental effect presumably due to constant influx of calcium. Cytotoxic effects of drugs unrelated to the receptor were determined by exposing the cells to the drugs in the absence of tetracycline and were not observed for up to 0.3 mM of amantadine or ketamine and up to 0.1 mM of memantine (open symbols in Fig. 7A). Ketamine and memantine blocked tetracycline induced cytotoxicity concentration-dependently at concentrations of 10 μM and above, whereas amantadine was only active at concentration of 0.3–1 mM (closed symbols in Fig. 7A).

Effects of drugs on NAT- or DAT-mediated cytotoxicity were determined in cells expressing the human NAT or DAT under the control of the T-REx™ system by exposing the cells to 1 μM MPP^+ and tetracycline and measuring cell viability 72 h later (closed symbols in Fig. 7B,C); cytoxicity is due to the transporter-mediated accumulation of the neurotoxin MPP^+. Potential cytotoxic effects of the drugs themselves were measured in the absence of MPP^+ (open symbols in Fig. 7B, C).

In NAT expressing cells the NAT inhibitor desipramine blocked cytotoxicity in a concentration-dependent manner starting at 0.1 μM, whereas the DAT inhibitor GBR 12909 was only weakly protective at 3 μM. Amantadine blocked NAT-mediated MPP^+-cytoxicity concentration-dependently starting with significant effects at 30 μM. Viability of NAT expressing cells in the absence of MPP^+ was decreased by 300 μM amantadine to 80% of control-treated cells (open triangles in Fig. 7B) and viability in the presence of MPP^+ was preserved by 300 μM amantadine to the same level (closed triangles in Fig. 7B). Ketamine and memantine were not protective in concentrations up to 300 and 100 μM, respectively, concentrations at which these drugs already displayed a weak toxic effect in the absence of MPP^+.

In DAT expressing cells, the DAT inhibitor GBR 12909 blocked MPP^+-induced cytotoxicity at 0.03 μM and above, restoring viability concentration-dependently up to control levels (closed symbols in Fig. 7C). Amantadine displayed considerable cytotoxic

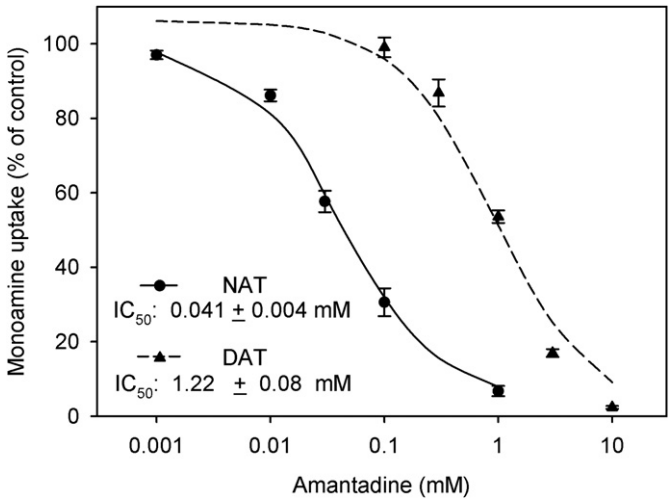

Fig. 3. Inhibition of uptake by amantadine in cells expressing the human NAT or DAT. Concentration-inhibition curves for its effect on [^{3}H]-noradrenaline uptake in HEK293 cells stably expressing the human NAT (circles) or on [^{3}H]-dopamine uptake in HEK293 cells stably expressing the human DAT (triangles). Specific activity of the radiotracers amounted to about 0.375 Ci/mmol noradrenaline or dopamine. Cells were incubated in 24-well plates for 2.5 min at 37 °C with tritiated monoamines in the absence (control) or presence of amantadine at the concentrations indicated, and uptake was determined as described under Methods. Symbols represent means ± SEM of 3–5 independent experiments, each in duplicates. The data of each experiment were fitted by nonlinear regression, and the means of the IC_{50} values ± SEM are given in the Figure.

effects in the absence of MPP^+ at concentrations of 300 μM and 1 mM, lowering viability to 76 and 56%, respectively (open symbols in Fig. 7C). In the presence of MPP^+, amantadine had a weak but significant protective effect at 100 μM and restored viability to 62% of control at 1 mM (closed symbols in Fig. 7C).

4. Discussion

In this study, we provide evidence that *in vitro* on intact, living cells amantadine has higher potency at the human NAT than at the human NMDA-receptor: (1) in patch-clamp experiments 10 μM amantadine induced an inward current in NAT-expressing cells, whereas 30 μM amantadine was inactive in blocking the inward current induced by 100 μM NMDA in NMDA-receptor expressing cells; (2) in cytotoxicity experiments ≥ 30 μM amantadine significantly blocked NAT-mediated cytotoxicity of MPP^+, whereas 300 μM amantadine were necessary to block NMDA-receptor mediated cytotoxicity.

We only examined cells expressing the human NR1/2A receptor, but this receptor is abundant in the human striatum and highly expressed in medium spiny neurons (Kosinski et al., 1998; Kuppenbender et al., 2000), the main striatal projections neurons within the basal ganglia circuit and driven by striatal NR2A subunits under parkinsonian conditions (Fantin et al., 2008). On the other hand, only low levels of NR1/2A receptor were found in human globus pallidus which receives in its internal segment a glutamatergic input from the subthalamic nucleus which is overactive in Parkinson's disease and seems to be enriched in NR1/2D receptors (Kosinski et al., 1998); still, differences in potency of amantadine reported at rat NR1A/2A and NR1A/2D receptors were less than 3-fold (Parsons et al., 1999), so at least equipotency of amantadine at the NAT and the NR1A/2D receptor can be expected from our findings. The potency of amantadine at the human NR1/2A on NMDA-induced currents (estimated $IC_{50} = 197$ μM) was considerably lower than the values reported for cultured cortical neurones from foetal rats or rat NR1a/2A receptors

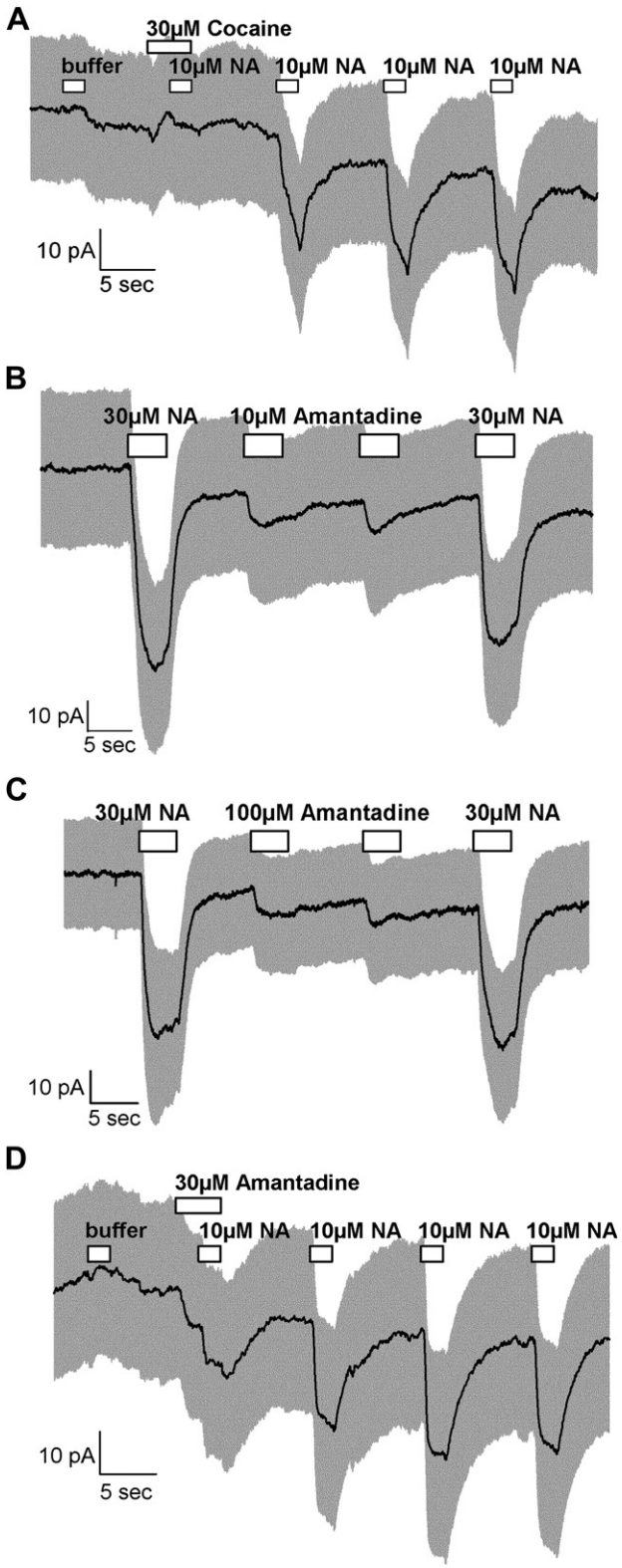

Fig. 4. Effect of amantadine in whole-cell patch-clamp recordings of HEK293 cells stably expressing the human NAT. Cells were voltage-clamped at a holding potential of −80 mV and superfused for 2 (A, D) or 4 (B, C) seconds with the drugs (NA, noradrenaline) indicated. In interaction experiments cocaine (A) or amantadine (D) were already present in a pre-run of 2 s. Shown are mean values of current traces ± SEM (grey area) of 14–19 cells.

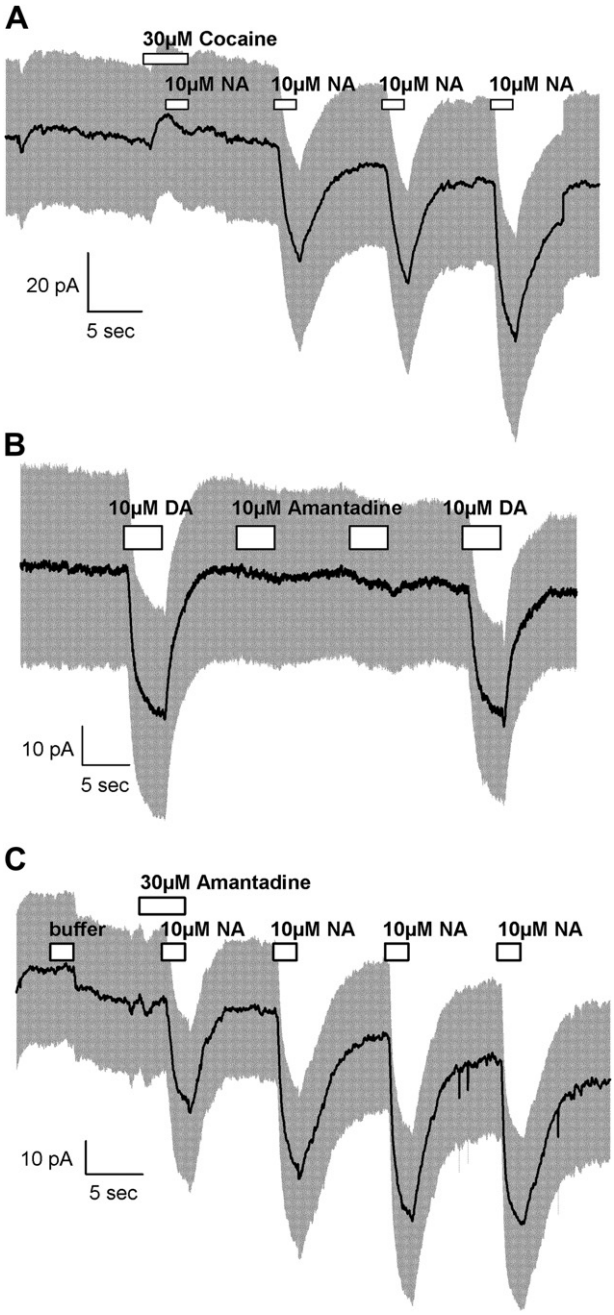

Fig. 5. Effect of amantadine in whole-cell patch-clamp recordings of HEK293 cells stably expressing the human DAT. Cells were voltage-clamped at a holding potential of −80 mV and superfused for 2 (A, C) or 4 (B) seconds with the drugs (NA, noradrenaline; DA, dopamine) indicated. In interaction experiments cocaine (A) or amantadine (C) were already present in a pre-run of 2 s. Shown are mean values of current traces ± SEM (grey area) of 7–12 cells.

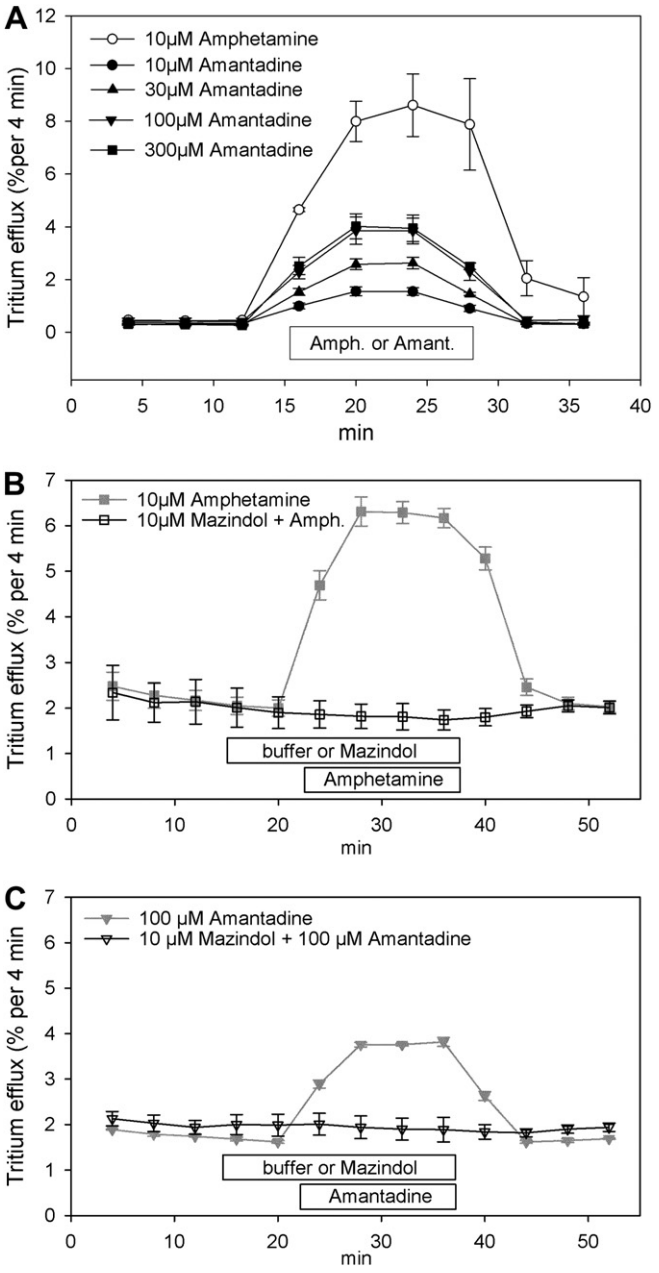

Fig. 6. Effect of amantadine on release by HEK293 cells stably expressing the human NAT. Cells grown on 5-mm-diameter coverslips were preloaded with [^{3}H]MPP$^+$ and superfused at 25 °C with standard Tris/HEPES buffer, and 4-min fractions were collected. Bars indicate fractions after exposure to 10 µM amphetamine or amantadine at the concentrations indicated (A), 10 µM mazindol and 10 µM amphetamine (B) or 10 µM mazindol and 100 µM amantadine (C). Symbols represent means ± SEM of three independent experiments.

expressed in *Xenopus* oocytes (IC_{50} = 81 and 26 µM, respectively; Parsons et al., 1999).

Amantadine blocked uptake by NAT expressing cells 30 fold more potently than uptake by DAT expressing cells. DAT blockade was shown to have antiparkinsonian effects in models of PD (Lane et al., 2005; Madras et al., 2006). However, an IC_{50} of more than 1 mM for the blockade of DAT by amantadine in our experiments makes it rather unlikely that interference with DAT function is relevant for the antiparkinsonian action of amantadine. In a study on the organic cation transporter OCT2 amantadine behaved as substrate and competitive inhibitor for OCT2; the blockade of OCT2 was expected to result in increased extracellular dopamine and therefore was suggested as antiparkinsonian mechanism (Busch et al., 1998). However, dopamine uptake and tissue content was not different between OCT2−/− and wild-type mice in a recent report making the relevance of an interaction with the OCT2 questionable (Bacq et al., 2011).

Amantadine acted at the NAT in an amphetamine-like manner inducing reverse transport, however it was less effective than amphetamine. The translocation process at the DAT or NAT is an electrogenic process by cotransport of sodium ions resulting in the

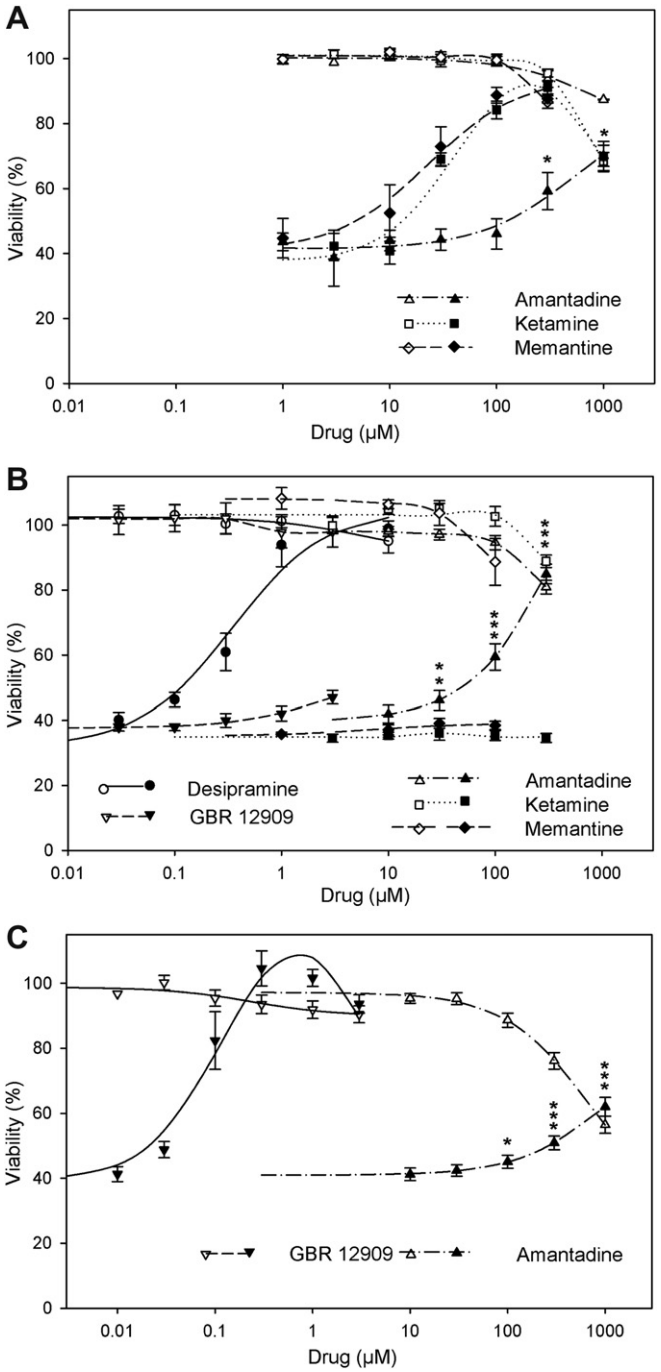

Fig. 7. Effect of amantadine on cytotoxic effects in HEK293 cells stably expressing the human NR1/2A receptor, NAT or DAT. Cells expressing the NR1/2A receptor (A), the NAT (B) or DAT (C) under the control of the T-REx™ were seeded in 96-well plates, induced by treatment with tetracycline in the absence or presence of the drugs at the concentration indicated and under inclusion of 1 μM MPP^+ in NAT or DAT expressing cells (closed symbols). Effects of the drugs unrelated to receptor or transporters (open symbols) were determined in the absence of tetracycline (A) or MPP^+ (B, C). Cell viability was determined by measuring acid phosphatase activity one (A) or three (B, C) days later. Activity was expressed as percentage of that of vehicle-treated cells. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.0001$ *vs* zero drug by paired Student's *t*-test followed by Bonferroni correction; symbols represent mean values ± SEM of 4–9 independent experiments, each in quadruplicates.

influx of one or two positive charges for each molecule of dopamine or noradrenaline pumped into the cell. Similar to the transporter substrate noradrenaline (Galli et al., 1995), amphetamine induces inward currents in NAT-expressing cells with about the same maximum effect in the low μmolar range as noradrenaline (unpublished observation). In our study, the amantadine-induced inward-current plateaued at 10–100 μM, at a level which was about 20% of the inward current induced by noradrenaline. In agreement with the hypothesis that substrate-induced transporter currents parallels substrate-induced transporter-mediated release (Sitte et al., 1998), amantadine-induced release also plateaued well below the releasing effect of amphetamine. It is interesting to note that in superfusion experiments there was no difference between the releasing effect of 100 μM and 300 μM amantadine, whereas in the toxicity assay on NAT expressing cells 300 μM amantadine were more effective than 100 μM. Two explanations are conceivable to justify that high concentrations are necessary for cytoprotection. (i) The mechanism of cytoprotection is the blockade of the MPP^+ uptake into the cell. For induction of release however amantadine acts as transporter substrate and elicits reverse transport from the cell interior. We presume that with high concentrations transport - both forward and reverse – may be arrested which is in keeping with the limited amount of MPP^+ release induced by amantadine (at lower concentrations). (ii) When NAT expressing cells are co-incubated with amantadine and MPP^+, uptake of amantadine would be predictably outcompeted by MPP^+; MPP^+ affinity for the NAT was shown to exceed that of noradrenaline used in the uptake experiment (Pifl et al., 1996).

An antiparkinsonian action of amphetamines has been reported in the literature (Miller and Nieburg, 1973; Parkes et al., 1975; Solomon et al., 1937). Although amphetamines are well-known releasers of dopamine, and dopamine release may be part of its antiparkinsonian action, amphetamines are even more potent releasers of noradrenaline (Rothman et al., 2001) and there seems to be a considerable contribution of noradrenaline to the motor effect of low dose amphetamine (Kuczenski and Segal, 2001; Ögren et al., 1983). Our experiments on NAT-expressing HEK293 cells support a releasing action directly *via* the NAT and rule out any indirect effects of amantadine *via* NMDA receptors as found for effects of amantadine on dopamine release in microdialysis experiments in the striatum (Quack et al., 1995; Takahashi et al., 1996). Therapeutically active extracellular concentrations of amantadine were estimated to be in the low micromolar range (Kornhuber et al., 1995); it appears reasonable to speculate though that an accumulation of amantadine in noradrenergic nerve endings *via* the NAT - similarly to the accumulation of amphetamine in DAT-expressing HEK293 cells (Sitte et al., 1998) – may favour an action on noradrenergic neurons. In a previous study on the noradrenaline and dopamine releasing action of amantadine in rat tissue, 10 μM amanadine markedly increased isotope outflow from superfused iris preincubated with tritiated noradrenaline; effects on the on the dopamine-system were however less convincing because they were inferred from a potentiating effect of amantadine on electrically-induced overflow of radioactivity from neostriatal slices preincubated with tritiated dopamine (Farnebo et al., 1971). Consistent with a noradrenergic mechanism of action, amantadine (5 mg/kg i.p.) depleted noradrenaline-containing vesicles in adrenal medullary cells of rats and increased noradrenaline plasma levels of humans 1–2 h after oral administration of 100 mg (Pita and Perez, 1977; Lechin et al., 2010).

In principle, a higher potency of amantadine in blocking NAT-mediated cytotoxicity of MPP^+ than NR1/2A-mediated cytotoxicity may be due to distinct toxic mechanisms of the two toxic agents, namely MPP^+ passing the NAT and cations passing the NR1/2A receptor, mechanisms which could by distinctly affected by amantadine. However, the much weaker potency of amantadine in preserving viability of MPP^+-exposed DAT cells suggests that, in fact, different affinities to transporters and receptors are essential for these differences in potency.

It is not clear which of amantadine's pharmacological effects are responsible for its antiparkinsonian actions. Amantadine is the only drug with relevant affinity to NMDA-receptors which is established as a remedy against the motor symptoms of PD. Memantine which has clearly higher affinity to NMDA-receptors than amantadine (Parsons et al., 1995) does not appear to share the antidyskinetic actions of amantadine (Merello et al., 1999) and was cited as an antiparkinsonian agent inferior to amantadine (Danysz et al., 1997). Interestingly, in our study memantine did not protect NAT-expressing cells from MPP^+-toxicity as observed for amantadine which suggests differences between amantadine and memantine on the noradrenergic system. The lower potency of amantadine in our cytotoxicity assays on NR1/2A receptor expressing cells as compared to patch-clamp recordings can be explained by the absence of Mg^{2+} in the electrophysiological experiments, whereas cytotoxicity assays were performed in cell culture medium with Mg^{2+}-concentrations in the low millimolar range as to be expected *in vivo*. The relevance of amantadine inhibition of NMDA receptors at clinical dosage was recently challenged by the Mg^{2+}-induced potency loss of amantadine observed in two-electrode voltage-clamp recordings on oocytes expressing human NR1/2A receptors (Otton et al., 2011). NMDA receptor inhibition was perceived not to be crucial in a recent study on the neuroprotection produced by amantadine in culture models of PD (Ossola et al., 2011), a study stimulated by indirect evidence of neuroprotection in a report on amantadine treatment as an independent predictor of improved survival in PD (Uitti et al., 1996). Our finding that NAT represents a major molecular target conforms to the proposed neuroprotective effect of amantadine; pharmacological or genetic NAT blockade were reported to afford protection of dopaminergic neurons *in vivo* (Rommelfanger and Weinshenker, 2007).

In conclusion, although experiments on transfected cells obviously cannot establish amantadine's mode of action in PD, the molecular pharmacology as demonstrated in our study appears compatible with contributions from a noradrenergic mechanism.

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