5-HT_3 receptors and emesis

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Nausea and vomiting. Nausea and vomiting is a major problem which occurs during and after the treatment with certain drugs like anesthetics, opioid-analgesics or cytostatics. Postoperative nausea and vomiting (PONV) is called the "big" little problem. As a consequence, the choice of the anesthetic drug(s) plays a role in preventing PONV [Apfel et al., Anesthesiology, 1999; Eberhart et al., Eur J Anaesthesiol, 1999]. Nausea and vomiting can be mediated *via* peripheral and/or central nervous pathways. Within the peripheral nervous system, vagal afferents, which are stimulated by 5-HT released from enterochromaffin cells, are of importance. The chemoreceptor trigger zone of the area postrema is another important center for the control of emesis. It is located at the boarder of the central nervous system and can be easily stimulated by drugs which do not enter the brain. In contrast, other centers which modulate emesis are located within the brain, e.g. the nucleus tractus solitarius.

5-HT_3 receptors. In contrast to other neurotransmitter receptors which are involved in the modulation of emesis (including nicotinic ACh, $dopamine_2$, $histamine_1$, neurokinin, opioid, and cannabinoid receptors), 5-HT_3 receptors are located in all emesis-controlling centers mentioned above. It is known that 5-HT_3 receptor antagonists successfully suppress nausea and vomiting [Tramer et al., Br Med J, 1997]. They can be used as antiemetics during therapy with cytostatics [Marty, Eur J Cancer, 1990] and are also useful against PONV [Rodrigo et al., Anaesth Intensive Care, 1994]. 5-HT_3 receptors are ligand-gated ion channels which trigger fast postsynaptic transmission, like glycine-, nicotinic ACh and $GABA_A$ receptors. The 5-HT_{3A} receptor is composed of five identical A-subunits which guarantee a constant stoichiometry. This is of advantage for the study of molecular mechanisms. In recent studies, the molecular mechanisms of (anti)emetic drugs at this receptor have been studied [Barann, et al., Naunyn Schmiedebergs Arch Pharmacol, 2000; Barann et al., Neuropharmacology, 2000; Barann et al., Br J Pharmacol, 2002; Walkembach et al., Br J Pharmacol, 2005; Barann et al., Eur J Pharamacol, 2006]. For this purpose, excised outside-out patches of HEK293 cells, stably transfected with the human 5-HT_{3A} receptor cDNA were formed (voltage-clamp mode) and fast solution exchange systems were used. In addition, radioligand binding studies and [^{3}H]5-HT uptake measurements (study of the 5-HT transporter) were performed supplementally. It was found that besides direct interactions of (anti)emetics with 5-HT_3 receptors, the drugs may trigger indirect processes leading to changes of the free 5-HT concentration. Such mechanisms may include the changes in 5-HT metabolism, 5-HT uptake and 5-HT release.

Results. As can be seen in Table 1, nearly all *antiemetic* drugs studied and some anesthetics with low emetogenic incidence, inhibited 5-HT_{3A} receptors at clinical free plasma concentrations. The possible mechanisms underlying the inhibition are diverse and include allosteric modulatory sites [Barann et al., Br J Pharmacol, 2002], competitive antagonism [Walkembach et al., Br J Pharmacol, 2005], changes in desensitization kinetics, [Barann et al., Neuropharmacology, 2000] and block of the channel pore [Barann et al., Naunyn Schmiedebergs Arch Pharmacol, 2000; Schneider et al., in: Molecular and Basic Mechanisms of Anesthesia, Ed. Urban & Barann, 2002].

In contrast for most *emetic* drugs at clinical plasma concentrations, we found no inhibition. Some of these drugs induced weak potentiation (apomorphine [which showed intrinsic activity], ergotamine and morphine [which both slowed down desensitization]

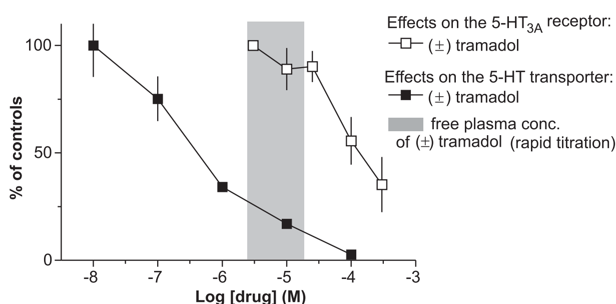

Fig. 1. Effects of (±) tramadol on the human 5-HT transporter ([^{3}H]5-HT uptake) and the human 5HT$_{3A}$ receptor (patch clamp). The data are presented as the means of 3–5 experiments ± SD

and halothane [which accelerated the activation]; (unpublished data).

An *indirect* mechanism at 5-HT$_3$ receptors might be valid for tramadol, an atypical analgesic which, besides its low affinity for μ-opioid receptors, inhibits the reuptake transporters for norepinephrine and 5-HT. It was found that tramadol, but not its active metabolite O-demethyltramadol, suppressed the human 5-HT transporter by 80% at plasma concentrations which are obtained after rapid titration (Fig. 1.). At this concentration range, the human 5-HT$_{3A}$ receptor was practically unaffected by tramadol (inhibition less than 6%) [Barann et al., Eur J Pharamacol, 2006]. As a consequence, it is possible that the 5-HT$_{3A}$ receptor is still sensitive against any raise of 5-HT levels induced by this drug. The same indirect mechanism appears not to be valid for morphine, since recent unpublished experiments suggest that this opioid does not inhibit the 5-HT transporter.

Applying fast solution exchange systems on the excised patches, it is possible to record processes with

Tab. 1. Effects (A, M, P, D) of (anti) emetics at human 5-HT$_{3A}$ receptors

Drug	Plasma concentration (μM)	IC$_{50}$ (μM)	Effect (A–D)	Inhibition in plasma (%)
anti-emetics:				
Cannabinoids (7)	0.02–0.2	0.03–0.3	M	10–50
Ondansetron	0.2	0.0003	AC	100
Metoclopramide	0.1–0.2	0.060	AC	75
Propofol	1	14	P, D (?)	< 15
Tropisetron	0.2	0.0003	AC	100
emetics:				
Apomorphine	0.0001	0.4	AA, D	0 + partial Agonist*
Ergotamine	0.001	> 30	D	0*
Halothane	200	n.d.	AA (?)	0*
Tramadol	7	200	(indirect, transporter inhib.)	5**
Morphine	0.15	0.3	AC, M (?)	0–30*
O-Demethyltramadol	1.0	100	–	0
„neutral" drugs (low incidence of emesis):				
Methohexital	1.1	125	P	0
Pentobarbital	78	120	P	30
Sevofluran	300	430	P	30
Thiopental	9	78	P	< 10

The data suggest that emetogenic drugs, in contrast to antiemetic drugs, produce no inhibition of human 5-HT$_{3A}$ receptors at clinical (free) plasma concentrations. Some emetic drugs (apomorphine, halothane, ergotamine, morphine, unpublished data) resulted in potentiation (*) and the emetic analgesic tramadol may activate 5-HT$_3$ receptors indirectly *via* a 5-HT transporter inhibition (**).
Possible mechanisms:
AA, AC = agonist binding site (activation or competitive antagonism), M = allosteric, modulatory binding site (e.g. cannabinoids), P = channel pore, D = desensitization

time constants well below 10 ms (e.g. receptor activation or channel block). The measured signals (peak currents) might then occur within 20 ms. However, taking these peak currents as control values (functional endpoints) is often not suitable for the detection of a mechanism like competition at the agonist binding site, since binding and especially unbinding of a competitor might take seconds or even minutes. We observed such problem with metoclopramide, which was characterized as a noncompetitive antagonist using fast patch-clamp experiments, contrasting our radioligand binding results [Walkembach et al., Br J Pharmacol, 2005; Walkembach et al., in: Basic and Systematic Mechanisms of Anesthesia, Ed. Mashimo, Ogli, Uchida, International Congress Series, 2005].

Conclusions. Our results suggest that:

(1) Clinical plasma concentrations of antiemetics and low-emetogenic anesthetics inhibit human 5-HT_{3A} receptors.
(2) Clinical concentrations of emetic drugs do not inhibit the function of human 5-HT_{3A} receptors. Some emetogenic drugs facilitate the function of 5-HT_{3A} receptors.
(3) Indirect mechanisms, affecting 5-HT_3 receptors by changes in the 5-HT concentration might underlie (anti)emesis.
(4) The decision, which molecular mechanism is valid for a drug-receptor interaction, is dependent on the choice of a functional endpoint that considers the kinetics of this mechanism.

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Norepinephrine transporter knockout-induced gene regulation

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The norepinephrine transporter (NET) located in the plasma membrane of noradrenergic neurons is responsible for the rapid re-uptake of released norepinephrine (NE). This enables fine tuning of noradrenergic neurotransmission and may prevent rapid desensitization of pre- and postsynaptic adrenoceptors. The NET, together with the dopamine transporter (DAT) and the serotonin transporter (SERT), belongs to the family of Na^+- and Cl^--dependent monoamine transporters (MATs). Among these MATs, the human NET (hNET) was the first cloned transporter [Pacholczyk et al., Nature, 1991]. The hNET protein consists of 617 amino acids and is characterized by 12 transmembrane domains. The hNET gene, which has been localized to chromosome 16q12.2 [Brüss et al., Hum Genet, 1993], consists of 14 coding exons and spans about 45 kb [Pörzgen et al., Biochem Biophys Res Commun, 1995]. The NET is a target for psychostimulants (e.g. cocaine and amphetamine) and it is the primary target for clinically important antidepressants such as the tricyclic antidepressants desipramine or nortriptyline or the selective norepinephrine re-uptake inhibitor (SNRI) reboxetine [Bönisch & Brüss, Handb Exp Pharmacol, in press].

Recently, Caron and co-workers [Xu et al., Nature, 2000] described mice with targeted disruption of the NET gene. These NET-knockout (NET-KO) mice show profound alterations in NE homeostasis. In spite of an increased activity of tyrosine hydroxylase, brain NE level was by up to 70% lower than in wild-type (WT) mice, and the clearance of the released NE was much slower, indicating that diffusion becomes the main mechanism for NE clearance in NET-KO mice [Xu et al., Nature, 2000]; this shows the importance

of the NET in maintaining a physiologically high intraneuronal NE content and in preventing NE volume transmission by escaping from the synaptic cleft to reach more distant brain areas. Xu et al. [Nature, 2000] also showed that NET-KO mice behaved like WT mice treated with antidepressants. Thus, these NET-KO mice may provide an interesting animal model to examine the reason for the 2–3 week delay in the antidepressant effect of antidepressant drugs (e.g. reboxetine) and to explore changes in the expression of genes potentially involved in antidepressant action. This delay may potentially be due to changes in the expression of a) adrenoceptors (e.g. down-regulation of β-adrenoceptors), b) neuropeptides and/or their receptors or c) neurotrophins and/or their receptors. Newly developed neuropeptide receptor ligands are presently under study as potential antidepressant drugs [Holmes et al., Trends Pharmacol Sci, 2003]. According to the "neurotrophic hypothesis of depression" [Castren, Curr Opin Pharmacol, 2004; Duman, Neuromolecular Med, 2004], antidepressants are thought to increase neurotrophic effects in the central nervous system (CNS).

We examined the NET-KO-induced CNS changes in mRNA and/or protein expression of α- and β-adrenoceptors as well as mRNA expression changes of neuropeptide Y (NPY), galanin (GAL) and their G-protein coupled receptors (NPY1-R, NPY2-R, NPY5-R, and GAL1-R and GAL3-R), and of the neurotrophins BDNF (brain-derived neurotrophic factor), NT3 (neurotrophin 3) and NT4/5 and their tyrosine kinase receptors (TrKA-R, TrKB-R, TrKC-R and P75-R).

In autoradiographic studies, we showed a decreased binding of the β-adrenergic ligand [^{3}H]CGP12177 in the cerebral cortex of NET-KO mice, indicating down-regulation of β-adrenoceptors similar to that obtained with antidepressant treatment; furthermore, binding of [^{3}H]prazosin to α_1-adrenoceptors in the cerebral cortex of NET-KO mice was also decreased [Dziedzicka-Wasylewska et al., Neuropsychopharmacology, in press]. The latter finding is in accordance with data of Xu et al. [Nat Neurosci, 2000] who found a decreased expression of α_1-adrenoceptors in the hippocampus. By means of quantitative real-time PCR [Gilsbach et al., Biotechniques, 2006], we revealed that mRNAs encoding the α_{2A}- and the α_{2C}-adrenoceptor were up-regulated in the brainstem, and that α_{2C}-adrenoceptor mRNA was also elevated in the hippocampus and striatum of NET-KO mice. These results were confirmed at the protein level by means of quantitative autoradiography using the selective α_2-adrenoceptor antagonist [^{3}H]RX821002, namely we found increased densities of α_2-adrenoceptors in several brain regions of NET-KO mice such as the hippocampus, striatum and amygdala [Gilsbach et al., J Neurochem, 2006]. The up-regulation of CNS α_2-adrenoceptors was further confirmed by a functional "*in vivo*" test, in which the α_2-adrenoceptor agonist clonidine caused a significantly greater reduction of locomotor activity in NET-KO mice than in WT-mice [Gilsbach et al., J Neurochem, 2006].

Using qPCR, a comparison between WT and NET-KO mice revealed also NET-KO-induced changes in the expression of mRNAs encoding neuropeptides or neurotrophins and their receptors in some of the examined brain regions (olfactory bulb, cortex, cerebellum, brain stem, hippocampus, striatum and hypothalamus). For the neuropeptides and their receptors, we observed no significant change in NPY expression in any brain region, also no change in NPY1-R expression, a tendency to up-regulation of NPY2-R in the brain stem and hippocampus, and a tendency to down-regulation of NPY5-R in the hypothalamus. Neurotrophins and their receptors showed the following NET-KO-induced changes: no change in BDNF in any brain region, a tendency for down-regulation of NT3 in the cerebellum, a tendency to up-regulation of NGF in the cortex, and a tendency to up-regulation of NT4/5 in the cerebellum and striatum. Furthermore, among the neurotrophin receptors, TrKA-R was not changed, TrKB-R tended to be down-regulated in olfactory bulb and cerebellum, and moderately up-regulated in the cortex, whereas TrKC-R tended to be decreased in most brain regions (except in the hypothalamus); P75-R, the common receptor for all four neurotrophins, tended to be higher expressed in the hippocampus of NET-KO mice. All above described changes in gene expression were less than two-fold and must, therefore, be regarded as not yet significant. While knockout of the NET induced a decrease in mRNA expression of GAL, GAL1-R and GAL3-R in the cerebellum, the mRNA of GAL3-R tended to be increased in the striatum.

It remains to be shown whether changes at the protein level are more pronounced. These results reveal that knock-out of the NET differentially affected the mRNA expression of genes of the neuropeptide and neurotrophin system of the CNS.

Molecular pharmacology of 5-HT$_3$ receptors

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Serotonin (5-HT) mediates its multiple effects *via* at least seven families of 5-HT receptors (named 5-HT$_1$ to 5-HT$_7$ receptors) comprising an increasing number of members. With the exception of the 5-HT$_3$ receptor family, all other 5-HT receptors are G protein-coupled receptors. 5-HT$_3$ receptors belong to the superfamily of Cys-loop ligand-gated ion channels which are homo- or heteromerically composed of five subunits. The human 5-HT$_{3A}$ receptor cDNA was first cloned from human amygdala, and it encodes a protein of 478 amino acids [Miyake et al., Mol Pharmacol, 1995]. This integral membrane protein contains four transmembrane domains (TM) and a large extracellular N-terminal ligand-binding domain. The 5-HT$_{3A}$ receptor subunit is able to form functional homomeric ion channels, whereas the 5-HT$_{3B}$ subunit [Davies et al., Nature, 1999] and the recently identified C, D and E subunits [Niesler et al., Gene, 2003] are not able to form functional homomeric receptors but may influence the receptor properties when co-expressed with the A subunit [Niesler and Brüss unpublished observations].

5-HT$_3$ receptors are expressed in the periphery and in the central nervous system (CNS) as well as in several neuroblastoma cell lines. In the CNS, 5-HT$_3$ receptors are found in the hippocampus, amygdala and the area postrema (chemoreceptor trigger zone). In the periphery 5-HT$_3$ receptors are predominantly expressed on neurons of the enteric nervous system. When activated by 5-HT, 5-HT$_3$ receptors which are permeable to Na^+, Ca^{2+} and K^+ mediate a fast excitation of the neuron. 5-HT$_3$ receptors are known to be involved in the mediation of emesis, pain and may participate in the pathogenesis of psychiatric disorders and gut disorders such as irritable bowel syndrome. 5-HT$_3$ receptor antagonists (e.g. ondansetron, tropisetron) are clinically used to manage emesis and nausea induced by radiation or chemotherapy in cancer patients. The usefulness of alosetron in patients suffering from irritable bowel syndrome has been reported but severe side effects (intestinal ischemia) had hampered this indication.

5-HT$_3$ receptors have been and will be intensively investigated because of their potential pathophysiological and therapeutic implications. Thus, in the last ten years upon cloning of the human 5-HT$_{3A}$ receptor cDNA a number of new efforts concerning this receptor type have been made. The gene encoding the human 5-HT$_{3A}$ receptor has been assigned to chromosome 11q23.1-q23.2 [Uetz et al., FEBS Lett, 1994; Weiss et al., Genomics, 1995]. This gene has been completely amplified and sequenced by exon to exon polymerase chain reaction and the coding region which is interrupted by eight introns was found to be stretched over about 14.5 kilobases [Brüss et al., Neuropharmacology, 2000]. Knowledge of the genomic sequence facilitated the investigation of alternative splicing and naturally occurring variations (SNPs) in this gene which may be of pathophysiological relevance. By RT-PCR from various human tissues, a short truncated (h5-HT$_{3AT}$) and a long (h5-HT$_{3AL}$) splice variant of the human 5-HT$_{3A}$ (h5-HT$_{3A}$) receptor subunit were identified. The deduced protein of the short isoform consists of 238 amino acids (aa) with a single transmembrane domain (TM1). Compared to the known 5-HT$_{3A}$ receptor, the long isoform contains 32 additional amino acids in the extracellular loop between TM2 and TM3. Both splice variants are co-expressed together with the 5-HT$_{3A}$ subunit in the amygdala and hippocampus, whereas in the placenta

only the short variant is co-expressed. These splice variants, when expressed in transfected human embryonic kidney (HEK) 293 cells, are not able to form functional homomeric receptors, but modify the 5-HT response at heteromeric h5-HT_{3A} receptors. Co-expression of the short variant considerably decelerates the desensitization of the 5-HT_3 receptor; thus, heteromeric assemblies of h5-HT_{3A} and the h5-HT_{3AT} subunit exhibit 5-HT-induced cation fluxes which are much larger than those through homomeric h-HT_{3A} receptors. In contrast, heteromeric complexes containing the h5-HT_{3AL} subunit display reduced cation fluxes [Brüss et al., Naunyn Schmiedebergs Arch Pharmacol, 2000]. By screening the 5-HT_{3A} receptor gene in a large group of psychiatric patients, a missense mutation in exon 9 of a schizophrenic patient at a conserved position (Pro^{391}Arg), and a second missense mutation (Arg^{344}His) was detected in another schizophrenic patient, but not in any of the controls. These variations could not be associated with schizophrenia or bipolar affective disorder [Niesler et al., Pharmacogenetics, 2001], but may be of functional importance. The substitution of proline 391, an alpha-imino acid, by arginine in the second intracellular loop of the protein may affect the conformation of the receptor. Investigation of the pharmacological and functional properties of the Pro^{391}Arg variant by radioligand binding and patch clamping in stably transfected HEK293 cells exhibited no changes in the receptor density and the affinities for nine representative ligands (five agonists and four antagonists). The potencies and efficacies of three 5-HT_3 receptor agonists in inducing currents through the ion channel and the potencies of two 5-HT_3 receptor antagonists in blocking 5-HT-evoked currents did not differ between wild-type and variant receptors. In addition, there were no differences in the desensitization kinetics of both receptor isoforms [Kurzwelly et al., Pharmacogenetics, 2004]. Since the patch clamp technique is very time consuming for analysis of multiple ion channel isoforms and multiple receptor ligands, a new technique for a rapid and high-throughput investigation of 5-HT_3 receptors has been developed in our laboratory. This technique is based on the calcium permeability of 5-HT_3 receptors and quantifies 5-HT_3 receptor-mediated calcium influx by fast (0.1 s) optical measurement of calcium-induced aequorin bioluminescence in 96-well plates. In addition to radioligand binding, this new technique was applied for the functional investigation of the Arg^{344}His variant of the human 5-HT_{3A} receptor in comparison to the wild-type receptor [Combrink et al., Naunyn Schmiedebergs Arch Pharmacol, 2005].

Interestingly, it was recently found that 5-HT_{3A} receptors were susceptible to stereoselective inhibition by cannabinoids which act at an allosteric modulatory site of this receptor with potencies comparable to those at cannabinoid receptors. [Barann et al., Br J Pharmacol, 2002]. These cannabinoid effects at 5-HT_3 receptors could also be demonstrated *in vivo* in the rat by measurement of the inhibition of 5-HT_3 agonist-induced (*meta*-chloro-phenylbiguanide) von Bezold-Jarisch reflex [Godlewski et al., Br J Pharmacol, 2003]. Thus, cannabinoids and newly developed related drugs may be valuable in therapeutic attenuation of 5-HT_3 receptor-mediated effects. Since cannabinoids also concentration-dependently inhibit calcium-influx *via* 5-HT_{3A} receptors in the above-mentioned fast aequorin assay (unpublished results), it should be possible to identify the allosteric modulatory site at this receptor by application of site-directed mutagenesis and/or chimeric receptors generated from the human 5-HT_{3A} and the structurally related nicotinic acetylcholine alpha-7 subunit. In addition, it is tempting to speculate whether the other 5-HT_3 receptor subunits (B, C, D, E) modulate, mask or enhance these cannabinoid-mediated effects in various tissues, expressing different subunit compositions of 5-HT_3 receptors. The newly developed calcium imaging technique will help to find answers to a plenty of open and interesting questions concerning the complex physiology of 5-HT_3 receptors.

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NMDA receptors and cell death

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NMDA receptors are ligand-gated ion channels that are activated by the most ubiquitous excitatory neurotransmitter glutamate and provide upon activation very high Ca^{2+} currents. Under physiological conditions they are not only involved in neurotransmission but are also important for neuronal plasticity. Under pathological conditions such as cerebral ischemia, they are a major source of increased intracellular Ca^{2+} concentrations that can lead to excitotoxic cell death. Depending on the quantity of Ca^{2+} entry, cell death may occur instantly or programmed cell death may develop with some delay.

As many membrane proteins, NMDA receptor ion channels are linked to the actin cytoskeleton. The link is indispensable to yield high Ca^{2+} currents. After Ca^{2+} influx, the binding to actin filaments gets lost because F-actin is Ca^{2+}-dependently severed by gelsolin and other actin-binding proteins. This effect can be measured as the loss of conductance and is called rundown. We have studied the effect of retarded actin polymerization/depolymerization dynamics on NMDA receptor-mediated effects. We used gelsolin-null mice as a genetic model for slowed actin depolymerization dynamics and cytochalasin D to pharmacologically mimic the effect of gelsolin.

Although the vast majority of NMDA receptors are localized on dendrites, they are also located on presynaptic varicosities of many neurons [Fink et al. Naunyn Schmiedebergs Arch Pharmacol, 1996]. Activation of these presynaptic NMDA receptors either by NMDA or the endogenous agonist L-glutamate causes neurotransmitter release which can be used to monitor *in vitro* the function of these NMDA receptors.

Neocortical brain slices were prepared from gelsolin-null mice or the corresponding wild-type mice. After incubation with [^{3}H]noradrenaline, the slices were superfused with Mg^{2+}-free Krebs-Henseleit buffer. Exocytotic release of [^{3}H]noradrenaline was stimulated by addition of NMDA for 2 min. Cytochalasin D was present during incubation, the other drugs during superfusion.

Primary neuronal cell cultures were prepared from embryonal cerebral cortex (E16-E18) of gelsolin-null mice or corresponding wild-type mice or rats. Neurons were maintained in serum-free medium until they were used for experiments *in vitro* on day 8–10.

In addition to the modifications of actin remodeling, we studied whether statins which have been proven in clinical studies to reduce stroke incidence and stroke outcome would have an effect on NMDA-induced neurotransmitter release and NMDA receptor-mediated excitotoxicity. In case of NMDA-induced [^{3}H]noradrenaline release, slices were prepared from mice treated for 60 days with atorvastatin before sacrifice. Neuronal cell cultures used for excitotoxicity studies were treated for 4 days with 1 µM atorvastatin before glutamate was applied.

Semiquantitative RT-PCR, immunoprecipitation and Western blotting were conducted according to standard protocols. Primer sequences used for PCR were for NR2A: 5'-TTA TTG GGA GAT GTC CCT CG-3' (sense) and 5'-CAC GTC TAT TGC TGC AGG AA-3' (antisense); NR2B: 5'-ATC AGT GCT TGC TTC ACG G-3' (sense) and 5'-GGG TTG GAC TGG TTC CCT AT-3' (antisense); NR2C: 5'-CAG CCC AGA CAG CAT GTC T-3' (sense) and 5'-ACC CCA CTG TCC CTG TAG C-3' (antisense); NR2D: 5'-CGA TGG CGT CTG GAA TGG-3' (sense) and 5'-CTG GCA AGA AAG ATG ACC GC-3' (antisense) [Freeman et al., MGI Direct Data Submission, 1998].

In brain slices prepared from gelsolin-null mice, the NMDA-induced [^{3}H]noradrenaline release was by about 40% higher than in controls. This effect was in-

dependent of the NMDA concentration applied for stimulation. The modulatory effect of presynaptic α_2-receptors remained unaffected, i.e. clonidine caused an inhibition by ~80% in slices prepared from gelsolin-null mice as well as in slices from wild-type control mice. Rauwolscine caused an increase in either genotype. When actin filaments were disrupted pharmacologically *in vitro* by exposure to cytochalasin D the NMDA-induced [^{3}H]noradrenaline release in control slices was reduced. In slices from gelsolin-null mice, the NMDA-induced [^{3}H]noradrenaline release was reduced to the level observed in untreated wild-type slices indicating that cytochalasin D functionally compensated for the loss of gelsolin in gelsolin-null mice.

Primary cultured cortical neurons were exposed to glutamate to induce excitotoxic cell death and to staurosporine or AF64A to induce apoptotic cell death. The excitotoxic as well as the delayed apoptotic cell death were much more pronounced in gelsolin-null neurons. While the increased sensitivity to glutamate/Ca^{2+}-excitotoxicity of gelsolin-null neurons was consistent with the expectation; it was most intriguing to investigate the antiapoptotic effect of gelsolin. Again, cytochalasin D was used to enhance depolymerization of actin filaments and to study whether the antiapoptotic effects of gelsolin were mediated by its effect on the actin cytoskeleton. In fact, cytochalasin D reduced the degree of apoptotic cell death after AF64A in gelsolin-null neurons to the level observed in untreated wild-type neurons indicating that actin remodeling by gelsolin protects from apoptotic cell death. During apoptosis gelsolin is cleaved by caspase-3 [Harms et al., Mol Cell Neurosci, 2004] which may augment its antiapoptotic properties because cleaved gelsolin has increased F-actin severing activity [Kothakota et al., Science, 1997].

After treatment with atorvastatin, the NMDA-induced [^{3}H]noradrenaline release was increased as compared to placebo-treated controls whereas basal efflux remained unaffected. Excitotoxic cell death in cultured neurons was assessed 24 h after glutamate (50 μM) exposure and determined by LDH release measurement; it was reduced by >70% [Bösel et al., J Neurochem, 2005]. Since this effect was preceded by reduced Ca^{2+} influx, we studied whether atorvastatin modified the expression of NMDA receptor subunits. However, the amounts of mRNA transcripts of NR2A, 2B, 2C or 2D were not different in statin-treated neurons as compared to control neurons. In order to study protein expression of membrane-bound NMDA receptors, we immunoprecipitated the NR2B subunit, which confers the highest Ca^{2+} currents to the NMDA receptor, with the PSD95 protein but we did not observe any differences.

It is concluded that rapid remodeling of the actin cytoskeleton by e.g. gelsolin is an antiexcitotoxic and antiapoptotic mechanism. Statin treatment increases NMDA-induced noradrenaline release and reduces Ca^{2+} excitotoxicity but the underlying mechanisms need to be further elucidated.

Molecular pharmacology of $P2Y_{12}$-receptors

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The $P2Y_{12}$-receptor plays an important physiological and pathophysiological role in ADP-induced platelet aggregation [Hollopeter et al., Nature, 2001; Takasaki et al., Mol Pharmacol, 2001; Zhang et al., J Biol Chem, 2001]. In addition, the receptor mediates an inhibition of action potential-evoked influx of calcium through N-type calcium channels in neuronal cells [Kulick & von Kügelgen, J Pharmacol Exp Ther, 2002; Simon et al., J Biol Chem, 2002] and is likely to be involved in neuromodulation in the postganglionic

sympathetic nervous system inhibiting the action potential-evoked release of noradrenaline and sympathetic cotransmitters [Queiroz et al., J Pharmacol Exp Ther, 2003]. Pretreatment of cells with pertussis toxin blocks the effect of receptor activation, demonstrating the coupling of the receptor to Gi-proteins [Hollopeter et al., Nature, 2001; Kulick & von Kügelgen, J Pharmacol Exp Ther, 2002]. ADP and its analogue 2-methylthio-ADP act as potent agonists at native as well as at recombinant $P2Y_{12}$-receptors. The platelet $P2Y_{12}$-receptor is the target for the active metabolites of the thienopyridine compounds clopidogrel and prasugrel, which are used to block platelet aggregation in the prevention of thrombotic diseases such as myocardial infarction or stroke [Savi et al., Biochem Biophys Res Commun, 2001; Hasegawa et al., Thromb Haemost, 2005]. Cangrelor (AR-C69931MX) is a highly potent (affinity constant of about 0.3 nM) and competitive antagonist at this receptor. However, cangrelor is not subtype-selective; it also blocks $P2Y_{13}$-receptors in the same concentration range [von Kügelgen, Pharmacol Ther, 2006]. Information about the molecular structure of the $P2Y_{12}$-receptor and the amino acid residues involved in ligand binding is limited. A patient with a congenital bleeding disorder has been shown to carry a polymorphism of the $P2Y_{12}$-receptor with a change of arginine 256 to glutamine (Arg256Gln) at one allele and a change of arginine 265 to tryptophan (Arg265Trp) at the other allele. When expressed in CHO K1-cells, the Arg256Gln and the Arg265Trp receptor constructs failed to mediate responses to stimulation by ADP [Cattaneo et al., Proc Natl Acad Sci USA, 2003], indicating that the amino acid residues Arg256 and Arg265 are important for ligand binding or receptor function.

In the present study, we searched for additional residues which are important for the structure or the function of the $P2Y_{12}$-receptor protein. The sequence encoding the human $P2Y_{12}$-receptor was cloned from human brain mRNA into the pcDNA3.1 expression vector. Site-directed mutations were introduced using mutagenic primers and standard PCR techniques. Wild type and mutant receptors were then expressed in human 1321N1 astrocytoma cells or CHO K1 cells which were cultured in the presence of the selection antibiotic G418 (800 mg/l). Expression of the receptors was monitored by direct immunofluorescence staining using a fluorochrome-labeled antibody directed against a receptor epitope. The immunofluorescence staining revealed that almost all analyzed mutant receptors were expressed at similar or slightly higher levels when compared to those of the wild type $P2Y_{12}$-receptor (with the exception of Cys17Ala/Cys97Ala-mutant receptors which showed a lower expression). Changes in receptor function were then assessed by measuring the agonist-induced inhibition of adenylate cyclase activity. Cellular cAMP production was increased by the addition of isoproterenol (1321N1 astrocytoma cells) or forskolin (CHO K1 cells).

ADP (0.1 to 100 µM) and the P2Y-receptor agonist 2-methylthio-ADP (1 pM to 1 µM) were used to activate the receptors. In cells expressing wild type receptors, the agonists caused a concentration-dependent inhibition of cellular cAMP production (the half-maximal concentration of 2-methylthio-ADP was about 1 nM; that of ADP about 3 µM; maximal responses: inhibition of the cellular cAMP levels by about 50%). Four extracellular cysteine residues have been proposed to form disulfide bridges between extracellular receptor domains and to represent the sites of action of the active metabolites of clopidogrel [Savi et al., Biochem Biophys Res Commun, 2001]. An important role of extracellular cysteine residues for receptor function has previously also been shown for the human $P2Y_1$-receptor [Hoffmann et al., J Biol Chem, 1999]. In a first series of experiments, we, therefore, analyzed receptor constructs with cysteine residues replaced by alanine. In astrocytoma cells expressing recombinant receptors in which both the cysteine residue Cys17 in the extracellular N-terminus as well as the cysteine residue Cys270 in the third extracellular loop were replaced by alanine residues (Cys17Ala/Cys270Ala), 2-methylthio-ADP caused an inhibition in adenylate cyclase activity with a reduced maximal response (maximal inhibition by about 10%). The same was true for cells expressing mutant receptors in which Cys97 (located near the exofacial end of transmembrane region 3, TM3) as well as Cys175 (in the extracellular loop 2) were replaced by alanine residues (Cys97Ala/Cys175Ala; maximal inhibition by about 10%). In contrast, in cells expressing Cys17Ala/Cys97Ala-, Cys17Ala/Cys175Ala-, Cys97 Ala/Cys270Ala- or Cys175Ala/Cys270Ala-mutant receptors, any inhibitory action of 2-methylthio-ADP was lost. These results demonstrate the presence of two disulfide bridges between first, Cys17 and Cys270 (connecting the N-terminus to the third extracellular loop) and second, Cys97 and Cys175 (connecting the exofacial end of TM3 to the extracellular

loop 2). Moreover, the results suggest that the presence of only one disulfide bridge reduces the maximal response to receptor activation. Hence, the results are compatible with the view that active metabolites of clopidogrel or prasugrel interact with extracellular cysteine residues of the $P2Y_{12}$-receptor.

Next, we searched for amino acid residues in TM6 and TM7 possibly involved in ligand binding. Replacement of arginine 256 in the upper third of TM6 by the acidic residue aspartic acid (Arg256Asp), combined replacement of histidine 253 and arginine 256 by alanine (His253Ala/Arg256Ala), replacement of tyrosine 259 on top of TM6 by aspartic acid (Tyr259Asp) and replacement of lysine 280 in the upper third of TM7 by alanine (Lys280Ala) abolished the responses to stimulation by 2-methylthio-ADP. In contrast, in cells expressing Arg256Lys mutant receptors (replacement of the basic residue arginine at position 256 by the basic residue lysine) as well as in cells expressing Arg256Ala mutant receptors, stimulation by 2-methylthio-ADP caused an inhibition in adenylate cyclase activity. The Arg256Lys-construct showed no obvious difference when compared with the wild type receptor (if there was a change, then 2-methylthio-ADP appeared to be slightly more potent, i.e., there was a small shift of the concentration-response curve to the left). The maximal response to stimulation of the Arg256Ala mutant was reduced (maximal inhibition by about 29%); the respective half-maximal concentration of 2-methylthio-ADP was increased in these experiments with the Arg256Ala mutant (to about 10 nM). The results indicate the involvement of the basic residue histidine 253 in TM6 of the $P2Y_{12}$-receptor, of the basic residue arginine 256 in TM6, of the polar residue tyrosine 259 on top of TM6 and of the basic residue lysine 280 in TM7 in ligand recognition. A similar role in receptor function has been proposed for basic amino acid residues in the upper thirds of TM6 and TM7 of the $P2Y_1$-receptor [Jiang et al., Mol Pharmacol, 1997]. Hence, the ADP-sensitive $P2Y_1$- and $P2Y_{12}$-receptors appear to share important structural properties despite a relatively low similarity in average amino acid composition (identity of residues of only 18.8%).

Presynaptic autoreceptors and heteroreceptors modulating transmitter release are targets for novel therapeutic agents

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The discovery that the cytoplasmic membrane of presynaptic nerve terminals possesses receptors that modulate the release of neurotransmitters was made more than 30 years ago. This new concept represents a clear departure from the traditional view that neuronal communication was unidirectional, i.e. from the nerve terminal to the postsynaptic receptor, because the transfer of information *via* presynaptic receptors occurs in the opposite direction: from the synaptic cleft to the nerve terminals which release the neurotransmitter.

The term autoreceptor was employed to describe the presynaptic receptors which were acted upon by the endogenous transmitter of the neuron, triggering a regulatory feedback loop through which the transmitter can modulate its own release. The presynaptic inhibitory terminal autoreceptors were first described in peripheral noradrenergic neurons: these presynaptic autoreceptors were soon established to correspond to a novel subtype of adrenoceptor, the alpha-2 adrenoceptor, which was shown to possess different pharmacological properties than the alpha-1 adrenoceptor .

The evidence for the existence of presynaptic terminal autoreceptors that inhibit the release of neurotransmitters was based on the following findings: 1) the calcium-dependent release of the neurotransmitter elicited by action potentials, was inhibited by receptor

agonists; 2) antagonists blocked the effects of the agonists; 3) antagonists, on their own, enhanced the stimulation-evoked release of the transmitter, particularly at low and intermediate frequencies of nerve stimulation; 4) the interaction between agonists and antagonists that modulate transmitter release was of a competitive nature. Evidence for this autoregulation of neuronal chemical signaling by presynaptic inhibitory autoreceptors was obtained under *in vitro* and *in vivo* experimental conditions both in the peripheral and in the central nervous systems.

In addition to the presynaptic alpha-2 adrenoceptors modulating noradrenaline release through a negative feedback mechanism, presynaptic terminal autoreceptors now recognized include those for dopamine (D2/D3), acetylcholine (M-2), histamine (H-3), serotonin (5-HT_{1D} in humans and 5-HT_{1B} in rodents), GABA (GABA-B) as well as for excitatory amino acid transmitters.

Presynaptic terminal facilitative autoreceptors exist for the modulation of acetylcholine release (nicotinic receptor subtype), and also for noradrenaline (beta-2 subtype).

Most neurons possess autoreceptors located not only on presynaptic terminals but also on their somata and dendrites, where they modulate the firing rate of the neuron. Activation of these inhibitory somatodendritic autoreceptors by agonists reduces the firing rate of the neuron, while antagonists block the effects of the agonists.

The term presynaptic heteroreceptors was introduced to identify a second category of presynaptic receptors that modulate transmitter release, in this case as a response to chemical signals present in the synaptic cleft, other than the neuron's own transmitter. These presynaptic heteroreceptors are sensitive to co-transmitter neuropeptides, to transmitters released from adjacent terminals, or to other chemicals that are locally produced or blood borne, that either inhibit or facilitate the release of a neurotransmitter. For example, noradrenaline nerve terminals possess facilitative angiotensin-2 receptors and presynaptic inhibitory opiate receptors. Acetylcholine, serotonin and glutamate nerve terminals possess alpha-2 presynaptic inhibitory heteroreceptors.

Presynaptic release-modulating receptors represent suitable targets for pharmacological intervention by exogenous compounds acting as agonists, partial agonists or antagonists. Such compounds may be of therapeutic value by influencing transmitter release presynaptically and having fewer side effects than the well-established approach of using agonist or antagonist drugs to stimulate or block postsynaptic receptors. Three marketed drugs act – at least partly – by selective stimulation or blockade of presynaptic release-modulating receptors: 1) the antidepressant mirtazapine, antagonist of alpha-2 adrenoceptors modulating the release of noradrenaline and serotonin; 2) the recently (2002) FDA-approved drug for the treatment of schizophrenia, aripiprazole, a central dopamine autoreceptor partial agonist. Aripiprazole does not elevate prolactin levels as most antipsychotics do; 3) sumatryptan and second generation tryptans for the treatment of migraine. These compounds are selective 5-HT_{1D} agonists which inhibit presynaptically the release of substance P and CGRP. These examples may only represent the beginning of a new generation of innovative drugs with useful therapeutic properties and improved ratios of efficacy versus side effects.

Atypical β-adrenoceptors

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The term atypical β-adrenoceptors designates receptors which are resistant to classical β_1-/β_2-adrenoceptor antagonists like propranolol. For many years, this term included β_3-adrenoceptors, which were cloned in 1989, and cardiostimulant atypical β-adrenoceptors, which have not been cloned so far. This latter receptor, known also as "atypical β-adrenoceptor" or "putative β_4-adrenoceptor" is now termed the "low-affinity state of β_1-adrenoceptor" [Alexander et al., Br J Pharmacol, 2004].

We decided to determine the type of atypical β-adrenoceptors and their role in the regulation of the cardiovascular function in pithed and vagotomized rats. This model offers the opportunity to study drug effects on the peripheral cardiovascular system without interference with reflex loops implicating the central nervous system. For comparison, a β_3-adrenoceptor-mediated thermogenesis in the brown adipose tissue was recorded simultaneously. According to Kaumann and Molenaar [Naunyn Schmiedebergs Arch Pharmacol, 1997], a β_3-adrenoceptor-mediated effect should be (i) mimicked by selective β_3-adrenoceptor agonists (e.g. CL 316243), (ii) mimicked by non-conventional partial agonists (e.g. CGP 12177 and cyanopindolol; i.e. drugs which block β_1- and/or β_2-adrenoceptors at concentrations much lower than those required to activate atypical/β_3-adrenoceptors); (iii) resistant to blockade by antagonists possessing only high affinity for β_1- and β_2-adrenoceptors and (iv) antagonized by β_3-adrenoceptor antagonists (e.g. SR 59230A).

We found [Malinowska and Schlicker, Br J Pharmacol, 1996, 1997] that heart rate (HR) was not affected by CL 316243 but was dose-dependently increased by CGP 12177 and cyanopindolol by about 40 and 30% of the basal values (pED_{50} values 8.0 and 7.3, respectively). CL 316243, CGP 12177 and cyanopindolol increased temperature in the brown adipose tissue at pED_{50} values of 8.6, 7.4 and 6.3, respectively. The cardiostimulatory effects of CGP 12177 and cyanopindolol were not influenced by the combined administration of CGP 20712 and ICI 118551 at 0.1 µmol/kg each (selective β_1- and β_2-adrenoceptor antagonists, respectively) but they were diminished by 10 µmol/kg of the non-selective β-adrenoceptor antagonist bupranolol, of CGP 20712A (known to block the low-affinity state of β_1-adrenoceptors at high doses) and of SR 59230A. The thermogenic action of CGP 12177 was not modified by ICI 118551 and CGP 20712 but was reduced by bupranolol and SR 59230A (10 µmol/kg each). Thus, there is a difference with respect to the rank orders of antagonistic potencies for cardiostimulation (CGP 20712 ≥ SR 59230A ≥ bupranolol > ICI 118551) and thermogenesis (SR 59230A = bupranolol > CGP 20712 > ICI 118551).

We have concluded that the positive chronotropic effects of CGP 12177 and cyanopindolol are mediated by atypical β-adrenoceptors, the current name of which is the "low-affinity state of β_1-adrenoceptor". They are atypical β-adrenoceptors since (1) they are activated by non-conventional partial agonists and (2) they are resistant to classical β_1- and β_2-adrenoceptor antagonists. They are different from β_3-adrenoceptors since (1) they are not activated by relevant doses of β_3-adrenoceptor agonists and (2) they are blocked by a high dose of CGP 20712, which does not modulate responses stimulated by β_3-adrenoceptors. The view that the cardiostimulatory effect of CGP 12177 is indeed receptor-mediated was confirmed further by the fact that it was inhibited by (–)- but not by (+)-bupranolol at 10 µmol/kg [Malinowska et al., Br J Pharmacol, 2003].

An activation of the low-affinity state of β_1-adrenoceptors induces not only positive chronotropic but

also positive inotropic and lusitropic effects [Zakrzeska et al., Br J Pharmacol, 2005]. Thus, in experiments performed in pithed rats, we noticed that CGP 12177 (0.1–100 nmol/kg) and cyanopindolol (1–1000 nmol/kg) produced a strong and dose-dependent increase in myocardial contractility as reflected by the enhancement of the left ventricular systolic pressure (LVSP) by about 30 and 20% of the basal values and the rate of intraventricular pressure rise ($+dP/dt^{-1}_{max}$) by about 120 and 60% of the basal values, respectively. In addition, the rate of ventricular relaxation, reflected by the intraventricular pressure decline ($–dP/dt^{-1}_{max}$), was increased by about 80 and 50% of the basal values, respectively. All cardiostimulant effects of CGP 12177 were reduced by bupranolol and CGP 20712 (10 µmol/kg each). The positive chronotropic effects of both non-conventional partial agonists remained almost unchanged for 30 min after their administration, whereas their positive inotropic action (both LVSP and $+dP/dt^{-1}_{max}$) decreased by about 50% over the period of 30 min.

For the sake of comparison, we also examined the cardiovascular effects of the selective β_1-adrenoceptor agonist prenalterol (10 nmol/kg). Qualitatively, the cardiac effects of prenalterol, CGP 12177 and cyanopindolol were very similar. They shared a positive chronotropic, inotropic and lusitropic effect. In addition, they resembled each other with respect to the time course of the three effects; thus, the positive inotropic effects are faster than the positive chronotropic effects. However, quantitatively, the effect of CGP 12177 (10 nmol/kg) amounted to only about 66% (LVSP and $+dP/dt^{-1}_{max}$), 84% ($–dP/dt^{-1}_{max}$) and 79% (HR) of the same dose of prenalterol. In addition, the cardiac effects elicited by the non-conventional partial agonists showed much slower kinetics than the corresponding effects induced by prenalterol.

In contrast to the four cardiac parameters, simultaneous determination of two vascular parameters, diastolic blood pressure (DBP) and mesenteric blood flow (MBF), led us to a quite different conclusion [Zakrzeska et al., Br J Pharmacol, 2005]. Thus, the highest doses of CGP 12177 and cyanopindolol increased DBP only by about 10% of the basal values. MBF was increased only by the highest doses of both agonists by about 31 and 13% of the basal values, respectively. None of the changes in vascular parameters elicited by the non-conventional β-adrenoceptor agonists was mediated by the low-affinity state of β_1-adrenoceptors since they were not affected by bupranolol or CGP 20712A (10 µmol/kg each).

The lack or marginal role of the low-affinity state of β_1-adrenoceptors in vessels has been also proven in experiments performed on isolated vascular preparations. On the one hand, we showed that CGP 12177 and cyanopindolol, unlike CL 316243, caused a complete (bupranolol- and CGP 20712-sensitive) relaxation of the isolated rat mesenteric artery preconstricted with phenylephrine, suggesting the possible participation of atypical β-adrenoceptors in this effect [Kozłowska et al., Br J Pharmacol, 2003]. On the other hand, more recent data obtained from experiments on the human pulmonary and the rat mesenteric arteries show that the vasorelaxant effects of both agonists result from their α_1-adrenolytic properties [Kozłowska et al., J Cardiovasc Pharmacol, 2005]. Vasorelaxant atypical β-adrenoceptors may, however, exist anyway since in the human pulmonary artery preconstricted with serotonin, cyanopindolol had a (bupranolol- and CGP 20712-sensitive) vasorelaxant effect at a concentration at which this drug does not shift to the right the concentration-response curve of serotonin for its contractile effect (unpublished results).

In conclusion, we could show for the first time that a positive chronotropic, inotropic and lusitropic effect elicited by the activation of the low-affinity state of β_1-adrenoceptors is detectable also *in vivo*. It is much more doubtful whether a stimulation of the low-affinity state of β_1-adrenoceptors also leads to vasorelaxation, both *in vitro* and *in vivo*. A potential clinical significance of the cardiostimulant low-affinity state of β_1-adrenoceptors is suggested by the fact that these receptors have been also identified in human heart, and the well-known β-adrenoceptor antagonists such as pindolol, bucindolol or alprenolol bind to the low-affinity state of β_1-adrenoceptors [Malinowska et al., Br J Pharmacol, 2003; Zakrzeska et al., 2005].

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The many faces of agmatine

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Agmatine, a cationic amine formed by decarboxylation of L-arginine by the mitochondrial enzyme arginine decarboxylase (ADC), is widely but unevenly distributed in mammalian tissues. Since the activity of mammalian ADC seems to be rather low, only a fraction of agmatine is due to endogenous enzymatic *de novo* synthesis. A substantial portion of tissue agmatine is probably absorbed from the lumen of the gut which contains a high amount of preformed agmatine. Absorption from the gut and accumulation in the tissues and cells must occur *via* a specific carrier mechanism because the compound is charged at physiologic pH and, hence, biological membranes are almost completely impermeable to the organic cation in the absence of an uptake system.

Agmatine initially attracted attention as an endogenous ligand at imidazoline receptors and α_2-adrenoceptors meeting most criteria for a neurotransmitter: it is synthesized, stored and released in the brain, inactivated by reuptake, degraded by the enzymes agmatinase and diaminooxidase, and it mediates biological actions within the central nervous system by interaction with cell-specific receptors. Independently of binding to those receptors, agmatine induces a variety

Tab. 1. The therapeutic use of agmatine

Molecular targets and cellular effects of agmatine	Potential therapeutic use
Cellular polyamine depletion, ➡ cell growth arrest, apoptosis	Cytostatic therapy of cancer Therapy of decompensated liver cirrhosis Treatment of vascular hyperplasia
Antagonism at NMDA receptors, inhibition of NO synthesis: neuroprotection after ischemic injury, anticonvulsant effects, antidepressant effects	Therapy of neurotrauma and neurogenerative diseases Seizure treatment Therapy of depression
Interaction with opioid, serotonergic and nitrergic systems and with I_1-imidazoline and NMDA receptors: antinociception, potentiation of morphine analgesia, attenuation of physical morphine dependence and withdrawal signs	Antinociceptive therapy Therapy of opiate addiction
Histamine release from enterochromaffin-like cells and mast cells: increase in gastric acid juice secretion	Prophylaxis and therapy of gastric ulcer disease
Inhibition of ligand-gated cation channels (5-HT_3 receptor, nACh receptor, K_{ATP} channel): decrease in blood pressure, stimulation of insulin secretion	Therapy of hypertension Therapy of type-2 diabetes mellitus Antiemetic therapy

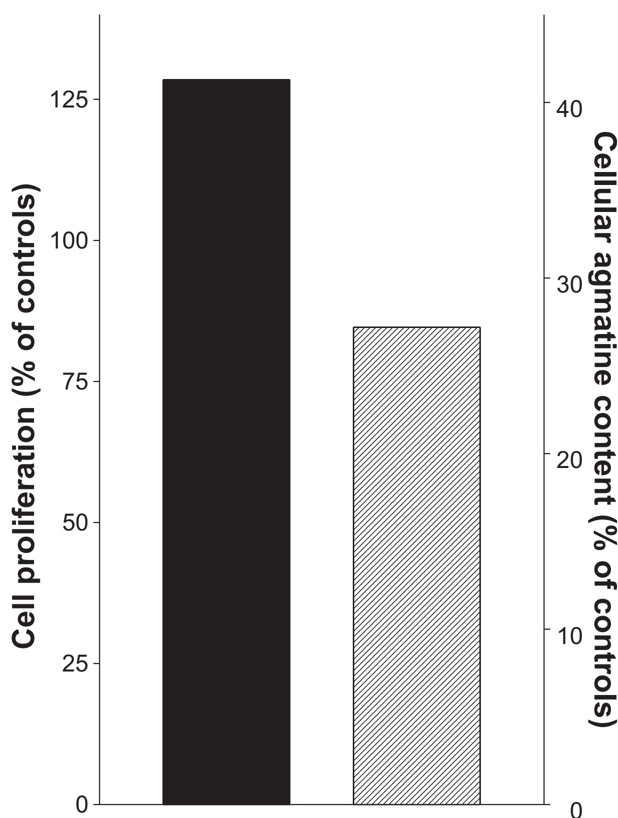

Fig. 1. Proliferation of cells (solid column) and agmatine content (hatched column) in SW480 human colon carcinoma cells 4 days after transfection with siRNA targeting human arginine decarboxylase. Cell number was estimated by measuring protein content. Left ordinate: cell proliferation expressed as percentage of proliferation of non-transfected SW480 cells. Right ordinate: cellular agmatine content expressed as percentage of that in non-transfected SW480 cells. The data are presented as the mean ± SEM of 3–7 experiments in each series

of physiological and pharmacological effects exhibiting a great therapeutic potential of the compound (Tab. 1). In particular, the antiproliferative effect of agmatine has aroused interest as a potential new alternative in treating neoplasms.

Extensive absorption of agmatine from the gastrointestinal tract, its enterohepatic circulation and pronounced accumulation in the liver and gut wall suggest a substantial involvement of agmatine in liver and gut cell homeostasis. Agmatine reduces the intracellular content of the polyamines putrescine, spermidine and spermine by reduction of the activity of the polyamine-forming enzyme ornithine decarboxylase, inhibition of polyamine uptake and stimulation of polyamine degradation by spermidine/spermine acetyltransferase and excretion. Hence, agmatine seems to be sufficiently similar to the polyamines to interfere with polyamine metabolism but is dissimilar enough to be unable to substitute functionally for missing polyamines in cell proliferation. Because of its action at multiple targets in the complex regulatory mechanisms of polyamine pathways, compensatory changes in polyamine metabolism do not occur. Since a decrease in intracellular polyamine levels is associated with a decrease in cell proliferation and changes in cell differentiation, agmatine administration to tumor cells *in vitro* results in a suppression of tumor cell proliferation and, thus, represents a promising tool for anticancer therapy.

Moreover, the data from the *in vitro* experiments in cell lines are also compatible with the idea that *in vivo* a decrease in intracellular agmatine concentration might be causally related to neoplastic transformation and growth. In line with this suggestion, the agmatine concentration in neoplastic colon cancer tissue was about one half lower than in the adjacent macroscopically normal tissue. In order to substantiate this hypothesis, we have investigated in the human intestinal tumor cell line SW480 whether knock-down of ADC by RNA interference affected proliferation of SW480 cells. RNA interference targeting ADC (verified by a distinct decrease in mRNA for ADC) resulted in a significant increase in proliferation which was paralleled by a distinct decrease in the intracellular agmatine content (Fig. 1). These findings prove a regulatory role of agmatine in tumor cell proliferation which seems to be mediated by modulation of polyamine homeostasis. In this context, one can speculate whether a low content of agmatine in the chyme is associated with an increased risk for malignant transformation of intestinal and liver cells, or vice versa, whether dietary intake of bacteria producing high amounts of agmatine might be useful in the prevention of intestinal and liver tumor genesis.

Predictive medicine through genetics

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Human genetics is the study of the inherited variation within the human genome, its phenotypic consequences and the analysis of its transmission over the generations. The haploid human genome consists of 3.2×10^9 base pairs. On the average, each 1000th base pair is variable. Thus, two unrelated individuals differ by approximately 3 million base pairs. Only a part of them will have functional consequences, however. All inherited differences between people must be represented in this molecular variation. While the extent of the genetic variation is presently pinned down, it will take many years to correlate it with phenotypic consequences. This endeavor will, however, have far-reaching implications for both clinical and theoretical fields of medicine. Some major aspects will be touched: (i) The conceptual meaning of genetics in medicine, (ii) predictive genetic diagnostics in Mendelian traits, (iii) chances and limitations of predictive genetic diagnostics in multifactorial traits, (iv) pharmacogenetics and pharmacogenomics.

(i) Genetics has the advantage that it can uncover the etiology of a disease without any assumptions just by assigning a gene to a chromosomal locus. This "positional" approach will be a powerful tool in the analysis of genetically complex traits and thus help to get insights into an unknown pathophysiology. If an inherited genetic variant either in the heterozygous or in the homozygous state leads to clear phenotypic consequences, we speak of a monogenic trait. Severe enzyme defects or hemoglobinopathies may serve as classic examples. A trait has a Mendelian (dominant or recessive) mode of inheritance, because the organism lacks compensatory mechanisms. Most monogenic traits are rare in the population: their incidence depends on the complicated mechanisms of population genetics such as mutation rate and selection. Multifactorial traits such as nearly all common diseases or disease predispositions result from the simultaneous influence of several genotypes on certain functional processes leading to a disease. One has to assume that complex interactions between genotypes (epistasis) on the one hand and genotypes and environment on the other hand are the explanation for the irregular pattern with which these diseases run in families. Since a single genotype has only a rather limited effect on the phenotype, it is not easy to reliably pin down the genotype-phenotype relationship.

(ii) Presently, about 2000 different Mendelian disorders can be diagnosed by genetic methods independently of the present disease status of the patient. Diseases that are present only later in life can be diagnosed before they have become manifest. For this procedure the term predictive genetic diagnostics is used. Examples are the neurodegenerative diseases with late onset, such as Huntington's disease, the different forms of spinocerebellar ataxia, and myotonic dystrophy. For these diseases no effective treatment is available. Therefore, a predictive diagnosis may be a burden to the individual, because it leads to a "healthy patient" that may have various implications including problems with insurers or at the workplace. Therefore, genetic counseling is indispensable before predictive genetic testing is considered. Predictive genetic diagnosis is also possible for the inherited forms of cancer such as familial breast/ovary cancer, colorectal cancer, and thyroid cancer. For these tumors an efficient therapy is available if the diagnosis has been obtained early enough. Persons at risk for these tumors and particularly carriers of disease-relevant germline mutations should be included in a systemic cancer surveillance program. On the other hand, relatives in whom a familial mutation could be excluded

have no increased risk and thus can be dismissed from further surveillance.

(iii) The genetic analysis of multifactorial diseases such as diabetes mellitus, chronic inflammatory bowel disease, hypertension, epilepsy, and psychiatric disorders is still in its infancy. Several chromosomal regions where predisposing genes are located have been mapped for most of the disorders, and some genes have been identified. Nearly all of the identified genes have only a modest effect. The relative risk is in the order of 1.5 to 3. This means that for carriers of a predisposing genotype the risk to develop the disease is increased by a factor of 1.5 to 3. Presumably patients who suffer from a multifactorial disease possess a pattern of predisposing genotypes. The interaction between the genotypes of a given person and between genotypes and environment will also have an influence on the risk. Presently, predictive genetic testing has only limited power in multifactorial diseases. The situation may change once the whole predisposition pattern of a disease has been uncovered. In contrast to monogenic traits, however, the possibilities for prediction have natural limitations in multifactorial diseases. The upper limit for any prediction is the concordance rate in monozygotic twins.

(iv) For nearly 50 years, the field of pharmacogenetics has fascinated both geneticists and pharmacologists. A drug interacts with a biological target that may show genetic differences between individuals. If genetic variation of a drug target has been uncovered, it would be important for practical pharmacotherapy to predict a drug response or side effects. Classical examples of pharmacogenetics are prolonged apnea after suxamethonium application during anesthesia due to pseudocholinesterase deficiency, or mutations in the ryanodine receptor gene predisposing to malignant hyperthermia after halothan application. Impressive genetic variation has also been uncovered in the CYP genes, the products of which are intensively involved in drug metabolism, CYP2D6 being the classical example. Another classical finding is the high frequency of ALDH2 deficiency in Orientals that predisposes them to the so-called flushing effect after ethanol ingestion. The respective allele that does not exist in Europeans has a certain protective effect towards alcoholism. In contrast to expectations, progress in pharmacogenetics has been rather slow during the last decades. Presumably, most drugs interact with multiple proteins or targets. Thus, the multifactorial model will be applicable to many drug-target interactions. Recently, systematic approaches to uncover variation in genes, the products of which are potentially involved in drug action ("pharmacogenomics") were undertaken. Once a genetic variant has been uncovered it can be expressed *in vitro* and can be studied at the functional level. Genetics has opened a completely new field to pharmacological research. Once we know all proteins of the human body that interact with an ingested drug, a whole pattern of genotypes can be put together to predict the main effect and the side effects of a drug in a given person. Based on this information the physician can then select the drug and its dosage. The interplay between genetics and pharmacology expects a bright future.

Arginase and nitric oxide (NO) synthesis

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Arginase has first been described as one of the enzymes of the urea cycle, an essential hepatic pathway for detoxification of ammonia [Krebs et al., Z Physiol Chem, 1932]. In the following years, arginase was also detected in non-hepatic tissue and two isoforms of the enzyme, arginase I and II, were identified. Both enzymes have similar enzymatic properties, but differ with regard to subcellular localization and regulation

of expression [Jenkinson et al., Comp Biochem Physiol, 1996]. Whereas the role of the hepatic arginase is well characterized, the functional significance of arginase in peripheral cells is still less understood. There is increasing evidence that arginase is increased in inflamed tissue, for example in asthma bronchiale [Zimmermann et al., J Clin Invest, 2003] or exacerbated cystic fibrosis [Grasemann et al., Am J Respir Crit Care Med, 2005]. Moreover, inflammatory cells such as macrophages [Hey et al., Naunyn Schmiedebergs Arch Pharmacol, 1995] and granulocytes [Munder et al., Blood, 2005] show high levels of arginase, mainly arginase I.

The substrate of arginase, L-arginine, serves additionally as a substrate for NO synthesis. Since the inducible form of NO synthase (iNOS) is also up-regulated in inflammatory reactions, it was an interesting idea that arginase might limit the substrate availability for NO synthesis. In fact, this could be proven first for iNOS-mediated NO synthesis in alveolar macrophages (Hey et al., Br J Pharmacol, 1997) and thereafter, an indirect, pharmacological evidence was provided that arginase also limited the substrate availability for the constitutively expressed NO synthases eNOS and nNOS [Meurs et al., Br J Pharmacol, 2000; Maarsingh et al., Respir Res, 2006].

Although both arginase and iNOS are up-regulated in inflammatory processes, a detailed analysis of the effects of pro- and anti-inflammatory mediators and drugs in rat alveolar macrophages showed a complex, differential pattern of changes in both pathways. As summarized in Table 1, LPS and GM-CSF induced parallel changes of iNOS and arginase, whereas IL-4, IL-13 and TGF-β down-regulated iNOS, but up-regulated arginase. INF-γ, which was shown to be a strong inducer of iNOS, had no effects on arginase by its own, but opposed the stimulatory effect of LPS on arginase [Klasen et al., Br J Pharmacol, 2001]. The anti-inflammatory glucocorticoid dexamethasone down-regulated iNOS and arginase and opposed the various up-regulating stimuli [Klasen et al., Br J Pharmacol, 2001]. In contrast, phosphodiesterase inhibitors, which are also considered to exert anti-inflammatory effects, caused an up-regulation of iNOS and arginase [Koschorreck et al., Eur J Pharmacol, 2003; Erdly et al., Am J Physiol Lung Cell Mol Physiol, 2006]. The changes observed at the level of mRNA expression were always paralleled by corresponding effects at the level of enzyme activity.

Tab. 1. Differential regulation of arginase and iNOS mRNA expression in rat alveolar macrophages

	iNOS	Arginase I	Arginase II
Lipopolysaccharides (LPS)	↑↑↑	↑↑	↑
Interferon-gamma (INF-γ)	↑↑↑	θ–↓	↑
Interleukin-4 (IL-4)	↓↓	↑↑	(↑)
Interleukin-13 (IL-13)	↓↓	↑↑	(↑)
TGF-β	↓↓	↑–↑↑*	θ
GM-CSF	↑↑	↑↑	(↑)
Glucocorticoids	↓↓↓	↓↓↓	↓↓↓
Phosphodiesterase inhibitors	↑↑	↑↑	θ

* potentiated by low concentrations of LPS

Likewise, in airway fibroblasts the TH2 cytokines IL-4 and IL-13 caused a glucocorticoid-sensitive up-regulation of arginase I and II, but a down-regulation of iNOS [Lindemann et al., Naunyn Schmiedebergs Arch Pharmacol, 2003].

In conclusion, inflammatory stimuli lead to an up-regulation of arginase in non-hepatic cells which can cause limitation of L-arginine availability for NO synthesis. In addition, arginase might play a role in inflammation-associated remodeling processes, since it provides L-ornithine, a precursor for the synthesis of polyamines, which are regulators of cellular growth and differentiation, and of L-proline, a substrate for collagen synthesis.

Cannabinoid receptors

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Our work dedicated to cannabinoid receptors encompasses four major themes, namely (i) the identification of presynaptic cannabinoid CB_1 receptors, (ii) characterization of an endogenous tone at these receptors, (iii) examination of the behavior of cannabinoidergic mechanisms under physiological and pathological conditions and (iv) identification of allosteric effects of some cannabinoids at opioid receptors.

(i) Using superfused tissues, we could identify a series of presynaptic CB_1 receptors for the first time. These receptors inhibit noradrenaline release in the hippocampus of humans and guinea-pigs [Schlicker et al., Naunyn Schmiedebergs Arch Pharmacol, 1997] and in blood vessels of guinea-pigs [Schultheiβ et al., Naunyn Schmiedebergs Arch Pharmacol, 2005], serotonin release in the mouse brain cortex [Nakazi et al., Naunyn-Schmiedeberg's Arch Pharmacol, 2000] and dopamine release in the guinea-pig retina [Schlicker et al., Naunyn Schmiedebergs Arch Pharmacol, 1996]. Moreover, using the pithed rat model, we were able to show that the sympathetic neurons innervating the resistance vessels are equipped with inhibitory presynaptic CB_1 receptors [Malinowska et al., Naunyn Schmiedebergs Arch Pharmacol, 1997]. In the heart, the sympathetic neurons are endowed with inhibitory presynaptic CB_1 receptors, whereas such receptors are missing on the vagal neurons [Malinowska et al., Naunyn Schmiedebergs Arch Pharmacol, 2001a]. In a study on urethane-anesthetized rats, the effect of the endocannabinoid anandamide on cardiovascular parameters was examined. Anandamide elicits a drop in heart rate and blood pressure, followed by an increase in blood pressure and a more prolonged decrease in blood pressure. According to the literature, the third phase is related to the activation of CB_1 receptors. Our studies show that the first phase is mediated by the activation of vanilloid $TRPV_1$ receptors [Malinowska et al., Naunyn Schmiedebergs Arch Pharmacol, 2001b], whereas the second one involves an effect of anandamide both on the central nervous system and on the vascular wall [Kwolek et al., Br J Pharmacol, 2005].

In the above studies, we have used chemically defined ligands to prove that CB_1 receptors are involved. The most important drug in this context is rimonabant, a selective and specific CB_1 receptor antagonist or, more precisely, inverse agonist. The second approach was the use of CB_1 receptor-deficient mice (provided by A. Zimmer, University of Bielefeld). In isolated tissues of such animals, the CB_1 receptor-mediated inhibition of acetylcholine release in the hippocampus and of noradrenaline release in the vas deferens is no longer detectable. This approach is particularly valuable since there is evidence for the existence of a series of cannabinoid receptors similar but not identical to the CB_1 receptor; for their differentiation even highly selective chemical ligands may not be sufficient. The third approach is the use of antisense oligodeoxynucleotides targeting CB_1 receptor mRNA. Intracerebroventricular administration of the antisense (but not of a mismatch) oligodeoxynucleotide to rats diminished CB_1 receptor binding, the CB_1 receptor-mediated increase in ^{35}S-GTPγS binding and the CB_1 receptor-mediated inhibition of acetylcholine release in hippocampal preparations [Kathmann et al., Naunyn Schmiedebergs Arch Pharmacol, 1999].

(ii) In order to assess the relevance of a receptor, it is important to know how the parameter influenced by it will behave if this very receptor is blocked or miss-

ing. With respect to inhibitory presynaptic CB_1 receptors, one would expect that transmitter release will increase provided that these receptors are spontaneously active (subject to an "endogenous tone"). One approach was to examine whether antagonists/inverse agonists at the CB_1 receptor will increase transmitter release. Rimonabant indeed increased noradrenaline release in the guinea-pig hippocampus or dopamine release in the guinea-pig retina. There are, however, experimental models in which rimonabant was devoid of an effect, including serotonin release in the mouse brain cortex or the noradrenaline-induced increase in blood pressure and heart rate in the pithed rat, suggesting that not all presynaptic inhibitory CB_1 receptors are subject to an endogenous tone.

A second approach to detect an endogenous tone was the use of CB_1 receptor-deficient mice. Acetylcholine release in the hippocampus and noradrenaline release in the vas deferens of such animals was much higher than in the corresponding tissues of wild type mice; on the other hand, rimonabant increased transmitter release in these two locations in wild type mice [Kathmann et al., Br J Pharmacol, 2001; Schlicker et al., Br J Pharmacol, 2003]. Three alternative explanations for the increase in transmitter release in the knock-out mice could be excluded, namely that the increase in transmitter release is a general phenomenon, that it is related to a decreased function of the autoreceptor (i.e., the receptor *via* which the released transmitter inhibits its own release) and that it is the consequence of an alteration of transmitter re-uptake.

(iii) The question as to whether cannabinoidergic mechanisms undergo alterations under various physiological or pathological conditions was studied, for instance, in superfused hippocampal slices of mice aged 2–4 months (young adult) and 24–27 months (very old). The CB_1 receptor-mediated inhibition of acetylcholine release did not differ between both groups [Redmer et al., Br J Pharmacol, 2003]. If this finding can be generalized to humans, one should expect that cannabinoid receptor agonists and rimonabant do not lose their therapeutic effectiveness in old people. In another study of our group in the pithed rat [Godlewski et al., Br J Pharmacol, 2004], a septic shock was induced by lipopolysaccharide; this procedure leads to a reduction of the electrically induced increase in blood pressure without affecting the effect of exogenously added noradrenaline on blood pressure. The decrease in the electrically induced vasopressor response was counteracted by rimonabant, suggesting that endocannabinoids are formed under the conditions of septic shock, which activate presynaptic inhibitory CB_1 receptors in the resistance vessels and contribute to the fall in blood pressure. In a third project, carried out in cooperation with F.M. Leweke (University Clinic of Cologne), the impact of cannabis abuse on CB_1 and CB_2 receptor mRNA was studied in mononuclear cells of the peripheral blood. Indeed, the mRNA of either receptor, quantified by the reverse transcriptase polymerase chain reaction, was increased by about 50% in blood cells of high-frequency users of cannabis when compared to a control group. Another two parameters, i.e. sex and sleep deprivation, which were studied in parallel, did, not differ between both groups [Kathmann et al., Naunyn Schmiedebergs Arch Pharmacol, 2003]. When our own study was still in progress, Nong et al. [J Neuroimmunol, 2002] found that cannabis abuse altered CB_1 and CB_2 receptor mRNA in a qualitatively and quantitatively similar manner.

(iv) Cannabinoids and opioids interact at various functional or molecular levels. An allosteric effect of cannabinoids at μ and δ opioid receptors has been postulated by Vaysse et al. [J Pharmacol Exp Ther, 1987] and was further refined by our group using kinetic binding experiments [Kathmann et al., Naunyn Schmiedebergs Arch Pharmacol, 2006]. The dissociation of the μ opioid ligand ^{3}H-DAMGO and the δ opioid ligand ^{3}H-naltrindole was increased by Δ^9-tetrahydrocannabinol, the major psychoactive constituent of cannabis (mainly acting *via* cannabinoid receptors), and cannabidiol, occurring at very high concentrations in cannabis as well (lacking both psychoactive properties and an affinity for cannabinoid receptors), but was not affected by rimonabant. Although the effects of the two cannabinoids occurred in a very high concentration range and cannot be expected to contribute to the *in vivo* effects of these compounds, our study shows that allosteric effects of cannabinoids should be taken into account in future studies.

This has already been done in the past by Professor Dr. Dr. h.c. mult. Manfred Göthert, to whom the present article is dedicated. He and his collaborators have shown an allosteric effect of cannabinoids at the serotonin 5-HT_3 receptor: Barann et al. [Br J Pharmacol, 2002]; Godlewski et al. [Br J Pharmacol, 2003]; Przegalinski et al. [Eur J Pharmacol, 2005]. The three authors of the present article would like to thank M. Göthert for many direct and indirect contributions to their work and say Auf Wiedersehen! and Ad multos annos!