BENZON SYMPOSIUM No. 51

NEUROTRANSMITTER TRANSPORTERS: BASAL FUNCTION AND DRUG TARGETS

AUGUST 9-12, 2004, COPENHAGEN, DENMARK

Organizing committee:
Ulrik Gether, Orla Miller Larsson, P. Høngaard Andersen, Arne Scousboe
Povl Krogsgaard-Larsen and Sven Frøkjær

Abstracts - MONDAY, August 9, 2004

Monoamine Transporters: beyond reuptake
Caron MG, Duke University Medical Center, Durham, USA
Neurotransmitter transporters play a fundamental role in the regulation of neuronal activity by limiting the diffusion and action of neurotransmitters in the extracellular space. Genetic inactivation of plasma membrane and vesicular transporters in the mouse has revealed a key role of these proteins in the maintenance of the homeostasis of presynaptic neurochemistry and physiology. We have used these genetic animal models to probe novel mechanisms of neuronal responses to monoamines as well as plasticity to drugs of abuse. Mice lacking either the dopamine (DAT-KO), norepinephrine (NET-KO) or vesicular monoamine transporter (VMAT2+/), which recapitulate pharmacological models of "behavioral sensitization" associated with exposure to psychostimulants have been used in comparaison to pharmacologically sensitized and wild type mice to profile the expression of 36,000 genes/EST by microarray analysis. Of six commonly up- and down-regulated genes in the striatum, one of the more interesting changes is a decrease of 50% in the transcript for PSD-95 Mice lacking PSD-95 (PSD-95-GK) recapitulate the molecular, cellular and behavioral phenotypes of the original mouse models suggesting that modulation of PSD-95 might contribute not only to learning and memory but also to drug-related plasticity. In DAT-KO and amphetamine treated mice, we have demonstrated that neuronal and behavioral responses to dopamine are exerted, at least in part, via a lithium-sensitive signaling cascade involving concomittant inactivation of Akt/PKB and activation of GSK-3. The biochemical changes are not affected by activation of the cAMP pathway but are effectively reversed by inhibition of dopamine synthesis, D2 receptor blockade, or lithium. Pharmacological inhibition or genetic inactivation of GSK-3 significantly reduces the dopamine-dependent locomotor behaviors in mice. These findings support a role for the Akt/GSK-3 signaling pathway as an important mediator of dopamine and lithium actions in vivo. Pharmacological modulation of this signaling pathway might be relevant to the management of dopamine–related disorders.

Biophysical probing of biogenic amine transporters
Gether U, Molecular Neuropharmacology Group, Department of Pharmacology, University of Copenhagen, Copenhagen, Denmark
The long-term goal of our research is to understand the molecular and cellular mechanisms governing the activity and availability of Na^+/Cl^- dependent biogenic amine transporters in the synaptic membrane and how this is modulated by endogenous substrate as well as by cocaine, amphetamine and related psychostimulants. In particular, we have aimed at implementing a series of different biophysical approaches in our studies. Using fluorescent cocaine analogues we have been able both to characterize the biophysical properties of the cocaine binding crevice in the biogenic amine transporters and with a novel series of analogs to visualize trafficking of the dopamine transporter (DAT) directly in living cells. Moreover, by application of a fluorescence polarization assay we have obtained insight into the structural basis for the interaction between the DAT and the PDZ domain containing protein PICK1. In conjunction with analysis of a series of C-terminal DAT mutations, these studies have challenged the paradigm that PDZ domain interactions are critical for endoplasmic reticulum (ER) export and surface targeting of the DAT. Rather we propose a role of PDZ domain interactions for microdomain association and possibly transporter phosphorylation. Recently, we have furthermore implemented the use of confocal single molecule fluorescence spectroscopy. This has allowed direct assessment membrane mobility and microdomain association of DAT tagged with YFP (yellow fluorescent protein). Our data suggest both lipid rafts and cytoskeleton association of DAT in neuronal cells. Finally, we are currently applying confocal single molecule fluorescence spectroscopy to study the stoichiometry of oligomerization of this class of transporters.

Deciphering a role for oligomerization in neurotransmitter transporters

Sitte HH, Just H, Korkhov VM, Farhan H, Seidel S & Freissmuth M, Institute of Pharmacology, Medical University of Vienna, Vienna, Austria

The most prominent role that neurotransmitter:sodium symporters (NSS) play in synaptic transmission is the recapture of previously released neurotransmitters from the synaptic cleft. Apart from this well-characterized inward transport mode, NSS mediate charge movements and are moreover capable to transport in the reverse direction. Constitutive organization in an oligomeric quaternary structure seems to be the rule for the NSS family members; however, it has been repeatedly shown that the glycine transporter exists in monomeric form. We have examined two NSS members (including the transporters for serotonin and GABA) to address (i) the individual characteristics of putative contact sites, (ii) the contribution of oligomerization to surface expression and (iii) the functional implication of oligomer formation.

Several different oligomerization domains have been reported in NSS, they act to mediate the protein-protein interaction in symmetric or asymmetric fashion. Only properly oligomerized NSS pass the rigid quality control mechanisms of the endoplasmic reticulum. Subsequently, NSS-oligomers recruit components of the COPII-complex and enter the secretory pathway to reach the cell surface. We probed the functional implication of oligomerization by generating a concatemeric fusion protein consisting of the transporters for serotonin and GABA. We propose that oligomerization serves carrier-mediated substrate efflux and is therefore a prerequisite for the action of drugs of abuse such as ecstasy and other amphetamine derivatives.

Glycine transporter 2: structure, function and subcellular localization

Aragón C, Centro de Biología Molecular "Severo Ochoa" Facultad de Ciencias, Universidad Autónoma de Madrid, Madrid, Spain

The inhibitory action of glycine neurotransmitter in spinal cord and brain stem of vertebrates is terminated by reuptake through sodium-driven plasma membrane glycine transporters. Our research interest is focused on the study of the structural basis of the function and regulation of the neuronal glycine transporter GLYT2. In this report, the function of its second intracellular loop (IL2) has been examined. IL2 contains charged and polar residues, which are strictly conserved among the subfamily of Na^+-and Cl^--coupled amino acid neurotransmitter transporters, and some of them fulfill the consensus sequence for PKC phosphorylation. We show that positions T419, S420, and mainly K422 are conformationally connected with the substrate-binding site and their substitutions to acidic residues abolish the GLYT2 response to 4α-phorbol 12 myristate 13-acetate (PMA). This establishes a new structural basis of PMA action on this glycine transporter. In addition, we have studied other aspect of GLYT2 regulation by subcellular redistribution mechanisms. Since we have recently proven the existence of a SNARE-mediated and Ca^{2+}-dependent regulatory mechanism that controls the surface expression of GLYT2, we became interested in the intracellular localization of the transporter. GLYT2 is detected in small vesicles. We performed a qualitative and quantitative characterization of these vesicles by using several experimental approaches. A highly pure preparation of synaptic vesicles from rat brainstem was obtained, and the presence of several marker proteins quantified through immunoblot and densitometric analysis, showing that GLYT2 is more abundant in the vesicles than other plasma membrane transporters. The immunogold labelled preparation visualized by electron microscopy contained clear vesicles of around 50 nm of diameter where synaptophysin, synaptobrevin and other synaptic vesicle proteins colocalize with GLYT2. Immunoisolation experiments also support GLYT2 presence in synaptic vesicles. However, the electron microscopy quantification indicated that very few of the GLYT2-containing vesicles include the vesicular GABA/glycine transporter (VIAAT). The nature of the GLYT2-containing vesicles will be discussed.

Oligomerization of the dopamine transporter: cocaine analogs alter the conformation of the dimer interface

Javitch JA, Columbia University, Center for Molecular Recognition, Department of Pharmacology, New York, NY, USA

Cross-linking of Cys306 at the extracellular end of the sixth transmembrane segment (TM6) has identified TM6 as part of the dimerization interface of the dopamine transporter (DAT). The motif GVXXGVXXA occurs at the intracellular end of TM6 in DAT and is found in a number of other neurotransmitter transporters. This sequence was originally found at the dimerization interface in glycophorin A, and it promotes dimerization in model systems. Mutation of either glycine disrupted DAT expression and function. Thus, the intracellular end of TM6, like the extracellular end, is likely to be part of the dimerization interface.

We have now explored the possibility that DAT exists as a higher order oligomer in the plasma membrane. Cysteine cross-linking of wild type DAT with copper or mercury resulted in bands on SDS-PAGE consistent with dimer, trimer, and tetramer, suggesting that DAT forms a tetramer in the plasma membrane. A cysteine depleted DAT (CD-DAT) into which only Cys243 or Cys306 was reintroduced was cross-linked to dimer, suggesting that these endogenous cysteines in TM4 and TM6, respectively, were cross-linked at a symmetrical dimer interface. Reintroduction of both Cys243 and Cys306 into CD-DAT led to a pattern of cross-linking indistinguishable from that of wild type, with dimer, trimer, and tetramer bands. This indicated that the TM4 interface and the TM6 interface are distinct and further suggested that DAT may exist in the plasma membrane as a dimer of dimers, with two

symmetrical homodimer interfaces. The cocaine analog MFZ 2-12 and other DAT inhibitors, including benztropine and mazindol, protected Cys243 against cross-linking. In contrast, two substrates of DAT, dopamine and tyramine, did not significantly impact cross-linking. We propose that the impairment of cross-linking produced by the inhibitors results from a conformational change at the TM4 interface, further demonstrating that these compounds are not neutral blockers but by themselves have effects on the structure of the transporter.

Structural and Functional Probing of EmrE, a Bacterial Multidrug Transporter

Schuldiner S, Institute of Life Sciences- Hebrew University of Jerusalem, Jerusalem, Israel

The Small Multidrug Resistance (SMR) family of transporters is widespread in bacteria and is responsible for resistance to toxic aromatic cations by proton-linked efflux. EmrE is an SMR from *Escherichia coli* that has been the subject of intensive investigation over the last decade. EmrE is classified as a multidrug transporter because its substrates include a wide variety of cationic aromatic hydrocarbons of varying size, structure and charge. It catalyses the electrogenic efflux of monovalent substrate molecules and the electroneutral efflux of divalent ones in exchange for two protons through a hydrophobic pathway in the protein. The amino acid sequence of EmrE is predicted to form four transmembrane α-helices. Negative-dominance studies, cross-linking, ligand binding and hetero-oligomer formation, all confirm that EmrE functions as an oligomer. We have determined the three-dimensional structure to 6.5 Å resolution of the *Escherichia coli* multidrug transporter EmrE by electron cryo-microscopy of two-dimensional crystals. The structure of EmrE consists of a bundle of 8 helices with one substrate molecule bound near the centre. The substrate binding chamber is formed from 6 helices and is accessible both from the cytoplasm and laterally from the lipid bilayer.

EmrE has only one membrane embedded charged residue, Glu-14, which is conserved in more than hundred homologous proteins. Glu-14 is part of the binding domain of substrates and protons. As shown by a variety of criteria, including direct observation of substrate induced proton release, deprotonation of Glu14 is necessary to allow substrate binding. The mechanistic implications of this finding will be discussed.

1. Gutman, N., Steiner-Mordoch, S. and Schuldiner, S. (2003) *J. Biol. Chem.* 278, 16082-16087.
2. Ubarretxena-Belandia, I., Baldwin, J.M., Schuldiner, S & Tate, C.G (2003). EMBO J., 22, 6175-6181.
3. Elbaz, Y., Steiner-Mordoch, S., Danieli, T. and Schuldiner, S. (2004) *Proc. Natl. Acad. Sci. USA,* 101, 1519-1524.
4. Gottschalk, K., Soskine, M., Schuldiner, S. and Kessler, H. (2004) *Biophys. J*., in press.
5. Soskine, M., Adam, Y. and Schuldiner, S. (2004) *J. Biol. Chem.,* 279, 9951 – 9955

The Biochemistry and Biophysics of OxlT, a Member of the Major Facilitator Superfamily

Maloney PC, Johns Hopkins Medical School, Department of Physiology, Baltimore, Maryland, USA

OxlT, a member of the Major Facilitator Superfamily, is a 12-helix antiporter that functions to exchange external oxalate (-OOC-COO-) for internal formate (HCOO-), the product of intracellular oxalate decarboxylation. Because this exchange carries negative charge inward, and because decarboxylation consumes a cytosolic proton, the result is to establish an inwardly directed proton-motive force; comparable schemes for generation of a proton-motive force are now known to be widely spread among bacteria, based on decarboxylation as well as on other patterns of metabolic activity.

An analysis of OxlT topology suggests that helices 2 and 11 may contain ligand-binding sites, and this inference is strengthened by trials using cysteine substitution variants as targets for both cysteine-directed probes. For example, helix 11 contains the only charged residue (K355) in the hydrophobic sector, and loss of function in the K355C derivative suggests this positive center is essential. We now conclude that K355 plays a role in OxlT function rather than assembly, since oxalate transport is restored by exposing the pre-assembled single cysteine variant, K355C, to a cysteine-specific reagent (MTSEA) that generates a new positive center as R–S-S-C-C-NH_3^+; mass spectrometry confirms the expected increased mass (76 daltons) under these conditions. Other work, using disulfide traps to infer proximity document that helices 2 and 11 lie close to each other, and electron crystallography (with S. Subramaniam, NCI, NIH) shows a structure (6.5 Å resolution) of the substrate-bound form in which helices 2 and 11 are in close juxtaposition. Further, an OxlT homology model, based on the X-ray structure of the open conformation of related antiporter (GlpT), indicates that K355 on helix 11 is in direct contact with the hydrophilic transport pathway. Thus, both biochemical and biophysical approaches now contribute to understanding structure/function relationships in this membrane transport protein.

The Passion of the Permease

Kaback HR, Howard Hughes Medical Institute/University of California, Los Angeles, CA, USA

Membrane transport proteins transduce free energy stored in electrochemical ion gradients into a concentration gradient and are a major class of membrane proteins, many of which play important roles in human health and disease. Recently, the x-ray structure of the Escherichia coli lactose permease (LacY), an intensively studied member of the Major Facilitator Superfamily of transport proteins (1), was solved at 3.5 Å (2). LacY is composed of N- and C-terminal domains, each with six transmembrane helices, symmetrically positioned within the molecule. The structure

represents the inward-facing conformation, as evidenced by a large internal hydrophilic cavity open to the cytoplasmic side. The structure with a bound lactose homolog reveals the sugar-binding site in the cavity, and residues involved in proton translocation are delineated. A mechanism for translocation across the membrane is proposed in which the sugar binding site has alternating accessibility to either side of the membrane.

1. H. R. Kaback, M. Sahin-Tóth, A. B. Weinglass, *Nature Rev Mol Cell Biol* 2, 610-622 (2001)
2. J. Abramson et al., *Science* 301, 610-615 (2003)

Poster No. I-1

The proton-coupled peptide transporter PepT1 is a functional multimer

Panitsas K-E, Boyd CAR & Meredith D, Department of Human Anatomy & Genetics, University of Oxford, UK

Proton-coupled peptide transporters have been shown to be expressed in the brain (PepT1 by Fei *et al.* 1994 Nature 368, 563-6; PepT2 by Doring *et al.* 1998 J Clin Invest 101, 2761-7). Here we invetigate whether PepT1 acts as a functional monomer or multimer.

Uptake studies were performed as previously reported (Meredith *et al*. 2000 Eur J Biochem 267, 3723). The epitope tag FLAG (YKDDDDK) was inserted by PCR to measure PepT1 expression levels by luminometry (Konstas *et al*. 2001 Pflugers Arch 442, 752). PepT1-FLAG showed normal wild-type expression and function, whereas a W294F-PepT1 mutant was expressed at the membrane but was not functional. Co-expression of W294F-PepT1 in an increasing ratio up to 1:4 with constant PepT1-FLAG resulted in a reduced D-Phe-L-Gln uptake while the expression levels of PepT1-FLAG remained constant, implying that PepT1 functions as a multimer. Modeling as a homomultimer gave a Hill plot stoichiometry of 4.2±1.8, and plotting normalized data as fractional uptake versus the mole fraction of the mutant could not distinguish between the tetramer and the pentamer model with two PepT1-FLAG copies as the minimal functional requirement. However, the possibility that PepT1 is a heteromultimer with an as yet unidentified endogenous *Xenopus* protein cannot be excluded.

In conclusion, this study shows that PepT1 functions as a multimer, a finding that will be important for structure-function modeling of this protein and potentially for the second isoform PepT2.

Poster No. I-2

Conformationally sensitive cysteine mutants in intracellular loop 3 of the dopamine transporter

Dehnes Y, Hastrup H & Javitch JA. Center for Molecular Recognition, Columbia University, New York, NY, USA

The dopamine transporter (DAT) is responsible for the inactivation of released dopamine through its reuptake at the plasma membrane. DAT is also the major molecular target of several psychoactive drugs, including cocaine. We have previously shown that Cys342 of the intracellular loop 3 (IL3) is conformationally sensitive (Ferrer et al., 1998; Chen et al., 2000). In an effort to investigate the properties and putative roles of the residues throughout IL3, we made a background DAT construct in which we mutated all intra- and extracellular cysteines (X7C). Twenty cysteine mutants in IL3 from Phe332 to Ser351 in the X7C background were stably expressed in EM4 cells. The affinity of most of the mutants for binding the tritiated cocaine analog [^{3}H]MFZ 2-12 was similar to that of X7C. However, the binding properties of several mutants (F332C, S334C, Y335C and D345C) were severely altered, with affinities 100-1000 fold lower than X7C. A similar pattern of apparent affinity changes was seen for cocaine- and mazindol binding, as well as for tyramine uptake. Interestingly, in S334C the presence of 10 μM Zn^{2+} stimulated uptake and dramatically lowered the apparent K_M for tyramine. Similar effects of Zn^{2+} were observed with Y335A, K264A, D345A, and D436A (Loland CJ et al., 2002; Loland CJ et al., 2004). Further, the methanethiosulfonate (MTS) derivatives (-EA, -ET and –ES), dramatically inhibited [^{3}H]MFZ 2-12 binding in membrane preparations of a number of the IL3 Cys mutants (F332C, S333C, S334C, Y335C, N336C, N340C, M342C, D345C and T349C). Cocaine and dopamine protected S333C, N336C, M342C and T349C from reaction with the MTS reagents. The protection was seen at 4 °C as well as RT. In summary, many positions in IL3 appear to be conformationally sensitive, as illustrated by the change in MTS-reactivity upon cocaine and dopamine binding, and the Zn^{2+}dependent reversal of a mutation-induced shift of the conformational equilibrium in the transporters.

Poster No. I-3

Cloning and pharmacological characterization of the chicken serotonin transporter

Elfving B, Department of Biological Psychiatry, Psychiatric University Hospital of Aarhus, Århus, Denmark

The serotonin transporter (SERT) belongs to a family of sodium-chloride dependent transporters responsible for uptake of amino acids and biogenic amines from extracellular spaces. SERT represents the main pharmacological target in the treatment of several clinical conditions, including depression and anxiety. Selective serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants (TCAs) are the most predominantly prescribed drugs in the treatment of depression. In addition to antidepressants also psychostimulants, like cocaine and amphetamines, are important SERT antagonists.

Although considerable efforts the mapping of residues defining the antagonist binding pocket, as well as the substrate permeation pathway, is still incomplete.

In the present study we report the cloning and characterization of chicken SERT (gSERT). Although uptake kinetics was very similar to human SERT (hSERT) the pharmacological profiles differed considerably for the two species. We find that gSERT is capable of discriminating between different SSRIs; the potency of S-citalopram and paroxetine is reduced more than 40-fold. A cross-species chimera strategy was undertaken and followed by species-scanning mutagenesis. Differences in pharmacological profiles were tracked to amino acid residues 169, 172, and 586 in hSERT. Structure-activity studies on structurally related compounds indicated that species divergences in drug sensitivity between hSERT and gSERT were arising from differences in coordination or recognition of an important aminomethyl pharmacophoric substructure, which is shared by all high-affinity antidepressants. We suggest that A169 and I172 at hSERT are important residues in sensing the N-methylation state of SERT antagonists.

Poster No. I-4

The role of Sec24 in export of GAT-1 from the endoplasmic reticulum

Farhan H, Korkhov VM, Paulitschke V, Freissmuth M & Sitte HH, Institute of Pharmacology, Medical University of Vienna, Vienna, Austria

In recent years it has become increasingly appreciated that membrane proteins use intracellular domains to mediate export from the endoplasmic reticulum (ER). We recently found out that the proximal half of GAT1 C-terminus was responsible for ER export. In this study we further charcterized this segment.

Using C-terminal truncation mutants of GAT1 we narrowed down the ER export signal to three hydrophobic residues ($V^{569}MI^{571}$). When we replace these residues by serines (GAT1-SSS), the transporter is retained in the ER, further supporting the notion that $V^{569}MI^{571}$ represents an ER export motif. Replacement of any of the hydrophobic residues by serine led to a reduction of surface expression which was due to a slower ER export rate. Sec24 is a component of the COPII coat hat is known to function as a cargo receptor for export from the ER. Using FRET microscopy we showed that YFP-Sec24D interacts with the C-terminus of GAT1. This observation is based on: (i) Sec24D does not show a FRET signal with GAT1 lacking the C-terminal 37 amino acids (GAT1-Δ37), while there is an interaction with GAT1-Δ27 (ii) Sec24D interacts with an oligomerization deficient GAT1 mutant that otherwise has an intact C-terminus. Interestingly, GAT1-SSS was still able to interact with Sec24D. While the export motif is not conserved in NSS family members, the proximally located segment is highly conserved ($R^{566}L^{567}$). A GAT1 truncation mutant that still contains the $R^{566}L^{567}$ (GAT1-Δ32) motif exhibited a robust FRET signal with Sec24D. Mutation of the RL motif led to a complete loss of the FRET signal. We conclude that ER export of GAT1 is dependent on Sec24D, which binds to the $R^{566}L^{567}$ motif but not to the ER export motif ($V^{569}MI^{571}$). Binding of Sec24D is not dependent on the oligomeric state of GAT1 but this conformation maybe important to promote COPII coat assembly.

Poster No. I-5

Tetrameric structure of the dopamine transporter: cysteine cross-linking reveals a cocaine-sensitive symmetrical dimer interface in the fourth transmembrane segment

Hastrup H, Beuming T, Weinstein H & Javitch JA, Department of Neuropharmacology, The Panum Institute, Copenhagen University, Copenhagen, Denmark

Cross-linking of Cys306 has identified the sixth transmembrane segment (TM6) as part of the dimerization interface of the dopamine transporter (DAT). Recently we identified an additional symmetrical interface involving residues in TM4. Reintroduction of the endogenous Cys243 and Cys306 into the Cys-depleted DAT (CD-DAT) led to cross-linking into dimer, trimer and tetramer, suggesting that DAT forms a tetramer in the plasma membrane. Here we have mutated to Cys, one at a time, residues Gly234 to Leu255 in the TM4 region and we have stably expressed these mutants in HEK293 cells. In a background of CD-DAT, treatment with $HgCl_2$ of P235C, P236C, W238C, Q239C, T241C, A242C, A243C, V245C, and L246C produced cross-linked dimer. P235C, R237C, W238C and Q239C were cross-linked using the bifunctional reagent bis-EA. A structural model positions residues Pro235 to Trp256 in an α-helix, with residues Pro235 to Trp238 accessible from the aqueous milieu at the extracellular end of the transmembrane segment. MFZ 2-12 and other DAT inhibitors protected P236C, Q239C, C243 and L246C against cross-linking with $HgCl_2$, suggesting that helix rotations at the interface causes a separation of these residues that form a narrow stripe on the interacting α-helices. Molecular dynamics simulations of helix-interaction models were used to rationalize the cross-linking pattern for TM4, and suggested a distortion of the helix around residue Thr241.

Poster No. I-6

Homophilic dimerization of transmembrane domain 2 via an interplay of hydrophobic interactions and hydrogen bonding mediates oligomerization of GABA transporter-1

Korkhov VM, Farhan H, Freissmuth M & Sitte HH, Institute of Pharmacology, Medical University of Vienna, Vienna, Austria

Neurotransmitter:sodium symporters (NSS) - GAT1, SERT, DAT, Glyt etc. - mediate rapid clearance of neurotransmitter molecules from the synaptic cleft. These proteins have been shown to exist in oligomeric forms. Several motifs have been identified within the transmembrane region of these transporters (including TM2, TM4,

TM6 and TM12) that represent contact sites. We have shown previously that the leucine heptad repeat of TM2 takes a crucial part in GAT1 surface expression and oligomer formation. Here we address the molecular determinants in TM2 of GAT1 that drive this association. We show that mutations of Tyr^{86} (to alanine) and Glu^{101} are detrimental to transporter oligomerization, escape from intracellular compartments and insertion into plasma membrane. Subtsrate translocation by Tyr^{86}Ala-GAT1 is unchanged relative to wild-type GAT1. Interestingly, the properties of Tyr^{86}Phe mutant are largely unchanged; thus the phenyl ring of Tyr^{86} is presumably involved in dimerization. Mutations Glu^{101}Ala/Asp/Gln confer intercellular retention of the transporter. While uptake by the alanine or the glutamine mutant is not measurable, Glu^{101}Asp-GAT1 is active with a K_M close to that of the wild-type protein. The mutant transporters do not retain intact wild type GAT1 inside the cell, which is indicative of lack of interaction. A β-lactamase fragment complementation supports this finding. Finally, FRET microscopy revealed a homophilic interaction between TM2-segments. We propose a model whereby TM2-TM2 association involving hydrophobic contacts of leucine heptad repeats and Tyr^{86}, and hydrogen bonds between Glu^{101} residues drive GAT1 oligomerization.

Poster No. I-8

Investigation of the complex ligand recognition pattern of the PICK1 PDZ domain

Madsen K[1], Beuming T[2], Chang V[2], Weinstein H[2] & Gether U[1], [1]Neuropharmacology, Institute of Pharmacology, The Panum Institute, University of Copenhagen, Denmark, [2]Department of Physiology and Biophysics, Weill Medical College, CORNELL University , NY, USA

The 48kDa protein PICK1 interacts via its single PDZ domain with the monoamine transporters, the AMPA receptor, receptors tyrosine kinases and several other transmembrane proteins as well as with PKCα. The specificity of PDZ interactions is largely determined by the C-terminal residue (P0) and the third residue from the C-terminus (P-2). The P0 residue of the ligand is hydrophobic and docks into a hydrophobic pocket in the PDZ domain (S0). The character of the P-2 interaction with the αB1 position in the PDZ domain constitutes the basis for classification of PDZ interactions. Class I interactions are characterized by a serine or a threonine in the peptide making a hydrogen bond with a conserved histidine in the αB1 position in the PDZ domain. In contrast, Class II interactions are characterized by a hydrophobic residue in the P-2 position of the peptide docking into a second hydrophobic pocket in the PDZ domain (S-2). The PDZ domain of PICK1 is one of only a few PDZ domains that can bind both class I and class II sequences. Moreover, the specificity for the C-terminal residue (P0) is very diffuse. Despite this apparent promiscuity of the PICK1 PDZ domain, it readily excludes other closely related PDZ binding sequences. We have established a quantitative assay based on fluorescence polarization that allows determination of affinities of peptides for PICK1. We show that PICK1 binds the dopamine transporter class II sequence with an affinity of 1.3μM, whereas the PKCα class I sequence is bound with an affinity of 14μM. The class I sequence presented by the unrelated β_2 receptor is bound very weakly (K_d ~0.5mM). Substituting the αB1 lysine in PICK1 to a histidine to mimic a class I PDZ domain switches the specificity with respect to the DAT and PKCα peptides, whereas the β_2 peptide is still bound very weakly. Changing the αB1 lysine in PICK1 to a valine to mimic a class II PDZ domain, does not change the specificity, suggesting that the αB1 lysine in PICK1 serves as a class II hydrophobic residue. We also show that PICK1 prefers the classical P0 residues in the order V>I>L.

Poster No. I-9

Electrogenic properties of intracellular dopamine transporter mutants with altered Zn^{2+} sensitivity

Meinild A-K, The Neuropharmacology Group, The Panum Institute, University of Copenhagen, Copenhagen, Denmark

The physiological role of the dopamine transporter (DAT) is termination of dopaminergic synaptic transmission in the brain. This is achieved by removal of dopamine (DA) from the synaptic cleft by electrogenic Na^+/Cl^--dependent symport. We have analyzed transporter-associated currents in three hDAT mutants in which three intracellular residues (Lys264, Tyr335 and Asp345) one at a time were mutated to alanines. We have shown previously that when expressed in COS cells these mutants display reduced DA-uptake rate and an inverted response to Zn^{2+}. Thus, in contrast to the inhibitory effect of Zn^{2+} on wild type DAT, micromolar concentrations Zn^{2+} rescued mutant DA uptake. Similar results were obtained when the mutants were expressed in *Xenopus leavis* oocytes. By application of the two-electrode voltage clamp method we observed in all three mutations that Zn^{2+} alone elicited current at hyperpolarizing potentials. Ion substitution experiments suggested that the current was carried by Cl^-. Moreover, we found that all three mutants were characterized by a large tonic Na^+-leak. This leak conductance was unaffected by substrates but could be blocked by cocaine at high concentrations (1 mM). We propose that Lys264, Tyr335 and Asp345, which are situated in the second and third intracellular loop of the DAT, are part of an intracellular 'gating' domain that plays a key role in regulating uncoupled conductances through the transporter.

Poster No. I-10

A single point-mutation converts the proton-coupled peptide transporter PepT1 into a facilitated transporter with an embedded non-specific cation channel

Meredith D, Department of Human Anatomy & Genetics, University of Oxford, UK

Proton-coupled peptide transporters have been shown to be expressed in the brain (PepT1 by Fei et al 1994 Nature 368, 563-6; and PepT2 by Doring et al 1998 J Clin Invest 101, 2761-7). Here a conserved arginine (R282) in TM7 of PepT1 is shown by site-directed mutagenesis to be a key residue for protein function. Substitution of R282 with a glutamate residue (R282E-PepT1) gave a functional protein in *Xenopus* oocytes whose transport rate was independent of the extracellular pH and could not accumulate substrate above equilibrium. The binding affinity of the mutant transport protein was unchanged from the wild-type. Thus R282E-PepT1 appears to have been changed from a proton-driven to a facilitated peptide transporter. In addition, peptide transport by R282E-PepT1 still induced a depolarisation of membrane potential, and more detailed study by two-electrode voltage clamping revealed that it behaved as a peptide-gated non-selective cation channel, with the ion selectivity series Li>Na>N-methyl-D-glucamine at pH 7.4. There was also a proton conductance (comparing pH_{out} 7.4 and 8.4), and at pH_{out} 5.5 the predominant conductance was for potassium ions. Therefore, it can be concluded that changing R282 to a glutamate not only uncouples the wild-type co-transport of protons and peptides, but also creates a peptide-gated cation channel in the protein. This is conceptually similar to the neuronal glutamate transporters (EAAT family), and suggests how such transporters may have evolved.

Poster No. I-11

Second-Site Suppressor Mutation Analysis of a Bacterial Homologue of Mammalian Sodium-Coupled Neurotransmitter Transporters

Pinto W & Rudnick G, Department of Pharmacology, Yale University, New Haven, USA

Our laboratory has recently characterized a bacterial tryptophan transporter (TnaT) of the NSS family, which is homologous to mammalian monoamine transporters. Second-site suppressor mutation analysis is being used to identify amino-acid residues that may be in close proximity in the tertiary structure of TnaT.

The *tnaT* gene from *Symbiobacterium thermophilum* was expressed in a variety of *E. coli* strains including CY15222, a tryptophan auxotroph with endogenous tryptophan transporters inactivated. Several deleterious mutations in transmembrane domains (TM) 1, 5, 7 and 10 of TnaT were generated at conserved positions, based on previous data from monoamine transporters. A functionally inactive (<3% of wildtype transport) TnaT mutant, E403G (TM10), was expressed in CY15222 bacteria and used to identify second-site suppressor mutations. We have developed a minimal growth media containing limiting tryptophan, which can selectively enrich cultures of CY15222 for rare cells expressing functional TnaT in an excess of cells expressing inactive TnaT mutants. The CY15222 strain also has β-galactosidase fused in frame to the tryptophan-induced catabolic enzyme, tryptophanase. This permits discrimination of rare second-site suppressor mutants which can transport tryptophan, as blue colonies on selective agar plates containing X-gal. A second-site suppressor mutant retaining the primary mutation (E403G) was identified as a blue colony and found to exhibit nearly 89% of wild-type [^{3}H]-tryptophan transport activity. Current studies are underway to identify and characterize the mutation and to determine its proximity to Glu-403 by cross-linking. Second-site suppressor analysis in TnaT may potentially provide important structural information for transporters of the NSS family.

Poster No. I-12

Chemical cleavage of the purified serotonin transporter: Evidence for distinct conformational rearrangements of accessible cleavage sites upon binding of serotonin, citalopram and the cocaine analogue RTI-55

Rasmussen SGF, Adkins EM & Gether U, Molecular Neuropharmacology Group, Department of Pharmacology, The Panum Institute, University of Copenhagen, Copenhagen, Denmark

The serotonin transporter (SERT) belongs together with the closely related transporters for norepinephrine (NET) and dopamine (DAT) to the family of Na^+/Cl^- -dependent neurotransmitter transporters. The SERT, DAT and NET have gained much attention as targets for the action of several psychoactive compounds including cocaine, amphetamine and antidepressants. Structurally they are characterized by the presence of 12 transmembrane segments; however, in the absence of high-resolution structural information little is known about their tertiary and quaternary structure. Although several conformationally active positions have been identified in the transporter molecule in recent years, the molecular mechanics of the transport process and how it is blocked by inhibitors remain poorly understood. To define and compare the structural changes that occur in response to binding of substrate and inhibitors we have employed a 'chemical cleavage' strategy in which we take advantage of the ability of Cu^{2+}-phenanthroline to cleave the peptide backbone in the presence of ascorbate and H_2O_2.

Following purification the SERT is treated with Cu^{2+}-phenanthroline and ascorbate/H_2O_2 for 30 min at 30°C and the resulting peptide fragments is separated by SDS/PAGE and visualized by Western blotting. Immunodetection using the transporters N-terminal FLAG epitope reveal fragments of 17-19kDa, ~23kDa, ~33 kDa and ~38 kDa. Binding of 5-HT, RTI-55, and citalopram to the SERT prior to the cleavage reaction results in an altered cleavage

pattern. With 5-HT bound to the transporter the ~33 kDa fragment increase in intensity, while the intensity of the ~38 kDa fragment increases when citalopram is bound. The intensity of the ~17-19 kDa fragment decreases when 5-HT, RTI-55, and citalopram is bound to the SERT. The intensity of ~23kDa fragment remains unchanged with or without ligands bound.

The distinct cleavage profiles suggest different ligand stabilized conformational states of the transporter where specific sites is either exposed or shielded from chemical cleavage. To the best of our knowledge this is the first report describing distinct conformational rearrangement in SERT in response to binding of antidepressants as compared to cocaine-like compounds and the substrate serotonin.

Poster No. I-13

Mapping the Transport Pathway of the Dopamine Transporter by the Substituted-Cysteine Accessibility Method: the second transmembrane segment

Sen N, Center for Molecular Recognition, Columbia University, New York, NY, USA

The dopamine transporter (DAT) mediates the sodium-dependent translocation of dopamine across the plasma membrane.

Inhibition of DAT is a major mechanism of action of cocaine and other psychostimulants. Although substantial efforts have been focused on defining residues in DAT involved in cocaine recognition, it is difficult to differentiate direct and indirect effects of mutations, and the location of the binding site is unclear. Here, in an attempt to determine whether TM2 contributes to the binding site and/or transport pathway of DAT, we mutated to cysteine, one at a time, each of the residues from Phe98 to Gln122 in and flanking TM2, in an appropriate DAT background construct. We expressed the mutants stably in HEK 293 cells and measured surface expression using an impermeant, lysine-reactive biotinylation reagent. A number of TM2 cysteine mutants, including F98C, G110C, P112C, E117C, did not express at the cell surface. These residues are highly conserved in related neurotransmitter transporters, suggesting that they play an important structural role and are critical for proper folding. For the remaining mutants, although the level of expression varied, the apparent affinity for tyramine uptake was similar to that of the background construct for each of the mutants except M106C and Y115C, which had higher apparent affinity for tyramine uptake. The inhibitory potency of cocaine was similar in all the mutants tested. For the constructs that expressed on the cell surface, we tested whether the substituted cysteines were water accessible based both on the functional effects of reaction of several charged methanethiosulfonate (MTS) derivatives, MTSEA, MTSES, MTSET, as well as on the biochemical determination of reaction of biotin-cap MTSEA. Based on these criteria, none of the cysteines in TM2 was water-accessible. Given these results, it is unlikely that TM2 lines a water-accessible binding site or the transport pathway.

Based on recent findings in DAT and the GABA transporter that TM2 may form an oligomeric interface, we attempted to cross-link all the cysteine mutants that expressed at the cell surface. M106C, V107C and I108C were inefficiently crosslinked by treatment with $HgCl_2$, and, as we observed at the symmetrical TM4 interface, this cross-linking was inhibited by the presence of the cocaine analogue MFZ 2-12. These findings are consistent with TM2 being located near to a symmetrical dimer interface, but based on the weak cross-linking compared to our results in TM4 and TM6 and the very few residues in TM2 that were cross-linked, it seems unlikely that TM2 forms an extensive tightly packed symmetrical dimer interface. Moreover, the lack of cross-linking of cysteines substituted for Leu99, Leu113 and Leu120, 3 of the residues that along with Met106 form a “leucine-zipper like” motif in TM2, raises some doubt as to a direct role for this motif in symmetrical TM2 dimerization.

Poster No. I-15

Three-dimensional structure of the bacterial multidrug transporter EmrE determined by electron cryo-microscopy

Ubarretxena-Belandia I, Baldwin JM, Butler PJG, Schuldiner S[1] & Tate CG, MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK, [1]Institute of Life Sciences, Givat Ram, Hebrew University, Jerusalem, Israel

EmrE is a bacterial multidrug transporter of the Small Multidrug Resistance family, which extrudes large hydrophobic cations such as tetraphenylphosphonium (TPP^+) out of the cell by a proton antiport mechanism. It is an extremely unusual transporter because it is contains only 110 amino acid residues forming 4 transmembrane domains, and it functions as an oligomer. We have studied EmrE using a number of biophysical techniques and substrate binding measurements. Binding measurements were performed on EmrE purified in dodecylmaltoside (DDM) to determine the stoichiometry of TPP^+ binding: the data showed that one TPP^+ molecule bound per EmrE dimer. Equilibrium analytical ultracentrifugation analysis of the purified EmrE showed that it was a dimer with a dissociation constant of 2.5 μM. Reconstitution of purified EmrE at low lipid:protein ratios in either the presence or the absence of TPP^+ produced well ordered 2-dimensional crystals. Electron cryo-microscopy was used to collect images of frozen hydrated EmrE crystals and the three-dimensional structure of substrate-bound EmrE was determined to 7.5 Å resolution. The structure of EmrE consists of a bundle of 8 transmembrane α-helices with one substrate molecule bound near the centre, in the same position determined from the projection difference images. The substrate binding

chamber is formed from 6 helices and is accessible both from the aqueous phase and laterally from the lipid bilayer. The most remarkable feature of the structure of EmrE is that it is an asymmetric homo-dimer.

Poster No. I-16

The aqueous accessibility in the external half of transmembrane domain I of the GABA transporter GAT-1 is modulated by its ligands

Yonggang Zhou, Hebrew University Hadassah Medical School, Jerusalem, Israel

The sodium- and chloride-dependent γ-aminobutyric acid (GABA) transporter GAT-1 is the first identified member of a family of transporters, which maintain low synaptic neurotransmitter levels and thereby enable efficient synaptic transmission. In order to obtain evidence for the idea that the highly conserved transmembrane domain I (TMD I) participates in the permeation pathway, we have determined the impact of impermeant methanethiosulfonate reagents on cysteine residues engineered into this domain. As a background the essentially insensitive but fully active C74A mutant has been used. Transport activity of mutants with a cysteine introduced cytoplasmic to glycine-63 is largely unaffected and is resistant to the impermeant MTS reagents. Conversely, transport activity in mutants extracellular to glycine-63 is strongly impacted. Nevertheless, transport activity could be measured in all but three mutants-G65C, N66C and R69C. In each of the six active cysteine mutants the activity is highly sensitive to the impermeant MTS reagents. This sensitivity is potentiated by sodium in L64C, F70C and Y72C, but is protected in V67C and P71C. GABA protects in L64C, W68C, F70C and P71C. The non-transportable GABA analogue SKF100330A also protects in L64C, W68C and P71C as well as V67C, but strikingly potentiates inhibition in F70C. Although cysteine substitution in this region may have perturbed the native structure of GAT-1, our observations, taken together with the recently published accessibility study on the related serotonin transporter [Henry L.K., et al (2003) J. Biol. Chem. **278**, 37052-37063], suggest that the extra-cellular part of TMD I is conformationally sensitive, lines the permeation pathway and forms a more extended structure than expected from a membrane-embedded α-helix.

BENZON SYMPOSIUM No. 51
NEUROTRANSMITTER TRANSPORTERS: BASAL FUNCTION AND DRUG TARGETS
AUGUST 9-12, 2004, COPENHAGEN, DENMARK

Organizing committee:
Ulrik Gether, Orla Miller Larsson, P. Høngaard Andersen, Arne Scousboe
Povl Krogsgaard-Larsen and Sven Frøkjær

Abstracts - TUESDAY, August 10, 2004

Ion conductances in neurotransmitter transporters
Amara SG, Department of Neurobiology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA
Neurotransmitter transporters present on the plasma membrane contribute to the clearance and recycling of neurotransmitters and can have a profound impact on the extent of receptor activation during neuronal signaling. Our major research efforts have focused on the structure, regulation and cellular physiology of two families of sodium-dependent neurotransmitter transporters: the biogenic amine and the excitatory amino acid carriers. The dopamine, norepinephrine and serotonin transporters (DAT, NET and SERT) are well-established targets for addictive drugs including cocaine and amphetamines, and for therapeutic antidepressants. Using whole-cell and perforated patch clamp recordings we have shown that substrates of the DAT, such as dopamine and amphetamine, increase the firing activity of dopamine neurons in culture independent of D2 autoreceptor activation. The change in firing rate appears to be regulated directly through the activation of a DAT-mediated anion conductance that can be blocked by cocaine and other DAT-inhibitors. Unlike receptor-mediated anion conductances that are generally inhibitory, the DAT-associated anion current depolarizes dopamine neurons and increases their firing rate. This new functional property suggests that, in addition to removing dopamine from the extracellular space, DAT has the capacity to modulate neuronal excitability and neurotrans-mitter release. Although they are members of a structurally distinct carrier family, excitatory amino acid transporters (EAATs) also possess a ligand-gated chloride channel activity that can regulate neuronal excitability. Structure-function studies support the notion that the binding sites for substrates, inhibitors, and co-transported ions, as well as the transport pathway, are formed from multiple domains which can undergo conformational changes during the transport cycle. This lecture will consider some of the novel aspects of neurotransmitter transporter function and will present the results of molecular biological, electrophysiological and cell biological approaches aimed at defining the relationships between neurotransmitter transporter structure, substrate transport, and ion permeation.

Molecular Characterization of the Substrate Binding Pocket of Glutamate Transporters
Kanner BI, Borre L, Brocke L & Grunewald M, Department of Biochemistry, Hebrew University – Hadassah Medical School, Jerusalem, Israel
Glutamate transporters are essential for terminating synaptic excitation and for maintaining extracellular glutamate concentrations below neurotoxic levels. These transporters also mediate a thermodynamically uncoupled chloride flux, activated by two of the molecules they transport — sodium and glutamate. Five eukaryotic glutamate transporters have been cloned and identified. They exhibit ~50% identity and this homology is even greater at the carboxyl terminal half, which is predicted to have an unusual topology. Determination of the topology shows that the carboxyl terminal part contains several transmembrane domains separated by two oppositely oriented reentrant loops. In these structural elements, we have identified several conserved amino acid residues which play crucial roles in the interaction with the transporter substrates sodium, potassium and glutamate. Moreover, the two reentrant loops come into close proximity of each other as evidenced by paired cysteine mutagenesis. Investigations of the relationship between the coupled and uncoupled fluxes show that although both are sodium dependent, the conformation gating the anion-conductance is different than that during substrate translocation. Recent evidence indicates that the substrate-binding site and one of the gates, or a residue controlling the gating process, are in close physical proximity.

The Chloride Permeation Pathway of a Glutamate Transporter and its Proximity to the Glutamate Translocation Domain

Vandenberg RJ, Ryan RM & Mitrovic AD, Institute for Biomedical Research, University of Sydney, Sydney, NSW, 2006, Australia

Excitatory amino acid transporters (EAATs) regulate glutamate concentrations in the brain to maintain normal excitatory synaptic transmission. A widely accepted view of transporters is that they consist of a pore with alternating access to the intracellular and extracellular solutions, which serves to couple ion movement to the movement of substrate. However, recent observations that EAATs, and also a number of other neurotransmitter transporters, can also function as ligand-gated chloride channels have blurred the distinctions between transporters and ion channels. Here we show that mutations in the second transmembrane domain (TM2) of EAAT1 alter anion permeation properties without affecting glutamate transport and that a number of TM2 residues are accessible to the external aqueous solution. Furthermore, we demonstrate that the extracellular edge of TM2 is in close proximity to a membrane-associated domain that influences glutamate transport. Finally, we have identified a residue at the intracellular edge of TM2 that may form a gate for the chloride channel. These results suggest that TM2 forms part of the chloride permeation pathway and may also indirectly influence glutamate translocation through the transporter. This study will provide the foundation for beginning to understand how transporters can function as both a transporter and a chloride channel.

Cellular distribution and function of glutamate transporters

Danbolt NC[1], Attwell D[3], Dehnes Y[1], Furness DN[2], Gundersen V, Hamann M[3], Holmseth S[1], Qureshi A[1], Rossi D[3] & Ullensvang K[1], [1]Center of Molecular Biology and Neuroscience & Department of Anatomy, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway, [2]MacKay Institute of Communication and Neuroscience, Keele University, Keele, England, [3]Department of Physiology, University College London, Gower Street, London, England

Glutamate transporters (EAAT1-5) maintain low resting levels of extracellular glutamate and protect neurons against excitotoxicity. EAAT2 is the major subtype in the entire CNS with the exception of the cerebellum, retina, circumventricular organs and inner ear where EAAT1 is dominant. Both EAAT1 and EAAT2 are expressed in astroglial plasma membranes in the mature and normal CNS apart from retina where EAAT2 is neuronal. The concentrations of EAAT1 and EAAT2 in the cerebellum and in the hippocampus, respectively, are as high as one percent of total tissue protein. Although most of the EAAT2 protein is astroglial, a small fraction of it is expressed in nerve terminals. Here we show by quantitative electron microscopical immunocytochemistry of D-aspartate-like immunoreactivity that this tiny fraction of EAAT2 is responsible for about 3/4 of the measured D-aspartate uptake in "synaptosomal" preparations (which in fact also contain glial fragments) and for about half of the uptake into hippocampal slice preparations. We have also determined the total tissue concentrations of the EAAT1, 3 and 4 and present an updated map of the distribution of EAAT-type transporters around hippocampal and cerebellar synapses. During the production of antibodies to EAAT3, unexpected cross-reactivities were observed. These observations are reminders that immunocytochemistry requires extensive specificity controls.

Poster No. II-1

Inhibiting efflux, but not influx, through the human serotonin transporter; antagonising MDMA and other amphetamines *in vitro* and *in vivo*

Sinning S, Sitte HH, Iwamoto H, Ramamoorthy S, Wegener G, DeFelice LJ & Wiborg O, Department of Biological Psychiatry, Psychiatric University Hospital of Aarhus, Århus, Denmark

A group of compounds resembling the inhibitor imipramine were surprisingly found to elicit an apparent stimulation of serotonin uptake by the human serotonin transporter (hSERT). The effect of a specific compound from this group of activators, IDB-N, was studied in detail to characterise the precise method of action. IDB-N was not found to influence surface expression of hSERT, nor was any significant effect on the activity of monoamine oxidases or vesicular monoamine transporters observed. *In vitro* uptake assays using COS-1 cells expressing hSERT showed that IDB-N was found to predominantly stimulate uptake when extracellular concentrations of serotonin (5-HT) were low and when long incubation periods were applied. No effect of IDB-N was observed in a standard K_M-V_{MAX}-assay, where incubation times are short. These results could be a consequence of IDB-N altering efflux, and we were able to show that efflux induced by the amphetamines 3,4-methylene-dioxymethamphetamine (MDMA) or para-chloroamphetamine (PCA) can be inhibited by IDB-N in hSERT expressing HEK-293 cells superfused with either MPP^+ or 5-HT. Electrophysiological experiments on Xenopus oocytes support the observation that IDB-N specifically inhibits efflux through hSERT without affecting influx. In principle, inhibition of amphetamine induced efflux of neurotransmitter mediated by a transporter without concurrent inhibition of uptake could be used as a method of antagonising amphetamines in the amphetamine toxicated brain. This would allow for a normalisation of neurotransmitter levels via reuptake by the neurotransmitter transporters. Indeed, we were able to show by microdialysis on awake freely moving Sprague-Dawley rats, that the MDMA induced release of 5-HT is blunted by IDB-N.

Poster No. II-2

Transporter proteins involved in the recycling of the transmitter glutamate

Chaudhry FA, Boulland JL, Qureshi T, Solbu TT, Jenstad M, Bredahl MKL, Gammelsaeter R, Edwards RH & Storm-Mathisen J, Institute of Basic Medical Sciences & Centre for Molecular Biology and Neuroscience, University of Oslo, Norway

Sustained synaptic transmission requires replenishment of the transmitters in the synaptic vesicles. It is generally believed that the amino acid glutamine is the precursor for the fast transmitters glutamate and GABA. We have characterized a family of glutamine transporters involved in the recycling of glutamine. The Na+ dependent translocation of glutamine by SN1 is coupled to countertransport of H+. Thus, the electroneutral transport generates shallow gradients enabling the protein to work in both directions depending on the concentration gradients. The homologues SAT1/ATA1/SA2 and SAT2/ATA2/SA1 have lost such coupling to H+, and are thus able to generate much higher concentration gradients energized by the electrochemical gradient of Na+. SAT1 and SAT2 are enriched on a subset of nerve terminals. We find specific targeting of SN1 to glial processes surrounding some of these synapses. Thus, our data suggest that these transporters are involved in the glutamate/GABA-glutamine recycling, and that due to differentiated expression of these transporters a functional diversity exists among synapses. A different family of proteins, VGLUT1-3, transporting glutamate into the synaptic vesicles, has been identified on the vesicular membranes, transporting glutamate into the synaptic vesicles. We detect high levels of VGLUT2 expression during early development. Transient developmental expression of VGLUT3 has been detected in a variety of cells, including non-glutamatergic ones. VGLUT3 co-expresses with the vesicular transporter proteins for monoamines (VMAT2), acetylcholine (VAChT) and GABA (VGAT) in select neuronal populations at specific developmental stages. Our data suggest complex modulatory roles of glutamate.

Poster No. II-3

VGLUT Expression Levels as a Source of Variability in Quantal Size

Wojcik SM[1], Rhee JS[2], Herzog E[1], Sigler A[2], Jahn R[2], Takamori S[2], Brose N[1], Rosenmund C[2], [1]Max Planck Institute for Experimental Medicine and [2]Max Planck Institute for Biophysical Chemistry, Goettingen, Germany

Quantal neurotransmitter release at excitatory synapses depends on glutamate import into synaptic vesicles by vesicular glutamate transporters (VGLUTs). Of the three known transporters, VGLUT1 and VGLUT2 are expressed prominently in the adult brain, but during the first two weeks of postnatal development VGLUT2 expression predominates. We generated VGLUT1 knockout mice by targeted gene deletion and analyzed glutamatergic transmission. Deletion of VGLUT1 in mice causes lethality in the third postnatal week, coincident with a developmental phase of strong VGLUT1 up-regulation. Glutamatergic neurotransmission is drastically reduced in autaptic neurons from VGLUT1 deficient mice, with a specific reduction in quantal size. The remaining activity correlates with the expression of VGLUT2. This reduction in glutamatergic neurotransmission can be rescued and enhanced with overexpression of VGLUT1, resulting in quantal events exceeding wild-type size. Thus, the expression level of VGLUTs determines the amount of glutamate that is loaded into vesicles and released, which suggest a role for the number of transporter molecules in the control of maximal vesicle filling levels.

Poster No. II-4

Expression of vesicular glutamate transporters, VGLUT1 and VGLUT2, in cholinergic spinal motoneurons

Herzog E[1], Landry M[2], Buhler E[3], Bouali-Benazzouz R[2], Legay C[4], Henderson CE[3], Nagy F[2], Dreyfus P[5], Giros B[1] & El Mestikawy S[1], [1]INSERM U513, Créteil, France, [2] INSERM EPI99-14, Bordeaux, France, [3] INSERM U623, Marseille, France, [4] CNRS URA 295, Paris, France, [5] INSERM EI 0011, Créteil, France

Recently, three vesicular glutamate transporters, named VGLUT1, -2 and –3 were identified. These transporters are responsible for the vesicular storage of glutamate prior to its regulated release at the synapse. VGLUTs are invaluable markers for glutamatergic neurons and nerve terminals in the brain. Mammalian spinal motoneurons are cholinergic neurons that have long been suspected to also use glutamate as neurotransmitter. Here, we report that VGLUT1 and VGLUT2 are expressed in rat spinal motoneurons. Both proteins are present in somato-dendritic compartments as well as in axon terminals in primary cultures of immunopurified motoneurons and in sections of spinal cord from adult rat. However, neither VGLUT1 nor VGLUT2 is targeted to the neuromuscular junctions. After intracellular injection of biocytin in motoneurons, VGLUT2 is observed in anterogradely labeled terminals contacting Renshaw inhibitory interneurons. These VGLUT2- and VGLUT1-positive terminals do not express VAChT, the vesicular acetylcholine transporter.

The present study establishes for the first time that mammalian spinal motoneurons express VGLUT1&2, and that they are targeted to non-cholinergic colaterals contacting Renshaw cells. These findings open new perspectives for the study of the pathophysiology of motor pathways.

The transport of glutamate into synaptic vesicles

Fremeau RT Jr, Kam K, Qureshi T, Storm-Mathisen J, Chaudhry FA, Nicoll RA & Edwards RH, Anatomical Institute, University of Oslo, Norway, and Departments of Neurology and Physiology, UCSF School of Medicine, SF, CA, USA

Exocytotic release of the principal excitatory neurotransmitter glutamate depends on its transport into synaptic vesicles, but the proteins responsible for this activity have remained elusive until recently. Originally discovered as Na^+-dependent inorganic phosphate transporters, the vesicular glutamate transporters (VGLUTs) reside on synaptic vesicles and accumulate glutamate with the properties previously described in native synaptic vesicles. Since the VGLUTs also appear to mediate a chloride conductance, they may encode three distinct activities, each with important consequences for transmitter release. Phosphate uptake could activate the enzyme known as phosphate-activated glutaminase which produces glutamate. The chloride conductance may influence the H^+ electrochemical gradient across the vesicle membrane. To understand the physiological role of the VGLUTs, we have recently disrupted the gene encoding VGLUT1 in mice.

Heterogeneity of glutamatergic neurons in the central nervous systems revealed by the distribution of vesicular glutamate transporter subtypes

Giros B, Gras C, Gilchrist J, Kashani A, Bournaud M, Herzog E & El Mestikawy S, INSERM U513 "Neurobiology and Psychiatry" Faculté de Médecine, CRÉTEIL, France

The amino-acid glutamate, in addition to its key role in metabolism, is used as an excitatory neurotransmitter by more than 40% of neurons in the central nervous system. Glutamatergic transmission is involved in most, if not all, superior brain functions (motor, sensory, cognitive, autonomic regulation etc). The implication of excitatory glutamatergic transmission in many neurological or psychiatric diseases is established (Amyotrophic Lateral Sclerosis, Alzheimer's disease, ischemia, epilepsy…) or suspected (Parkinson's disease, schizophrenia, autism). Recently, three vesicular glutamate transporters (VGLUT1-3) were identified which are distantly related to the large family of Na+-dependent inorganic phosphate transporters. In contrast with their structural and functional similarities, VGLUTs have complementary distributions in the rat brain. VGLUT1 is expressed by glutamatergic neurons of the cerebral and cerebellar cortices, the hippocampus and the thalamus. VGLUT2 is present in excitatory neurons throughout the diencephalon and the brainstem. Thus all acknowledged glutamatergic terminals of the brain utilize VGLUT1 or VGLUT2. In contrast, VGLUT3 has a very restricted and completely unexpected localization in the brain. VGLUT3 is found in all cholinergic interneurons of basal ganglia (caudate-putamen and accumbens), in some GABAergic/CCKergic basket cells in the hippocampus, in GABAergic neurons from interpeduncular nucleus and in serotonergic neurons in the raphe nucleus. Thus VGLUT3 is often present in locally projecting neurons. The strategic localization of VGLUT3 suggests that sub-populations of cholinergic, GABAergic and serotoninergic terminals have the potential to release simultaneously their cognate transmitter and glutamate.

VGLUTs represent unprecedented landmarks to define three distinct populations of excitatory glutamatergic neurons that can no longer be considered as a homogeneous population. Thanks to the discovery of VGLUT1-3 research efforts will help to better understand the rationale for the heterogeneity of excitatory neurons and their functional consequences for the normal and pathological central nervous system.

Poster No. II-5

Role of Chloride in Cystine Transport by System x_c^-

Coleman RR, Teusink MJ, Andersen J. & Chase LA, Biology and Chemistry Departments, Hope College, Holland, MI, USA

System x_c^- is a member of the family of heteromeric amino acid transporters and is expressed in glia, fibroblasts and macrophages. It is a Na^+-independent, anionic amino acid transporter that mediates the direct exchange of extracellular cystine for intracellular glutamate. This transporter has been demonstrated to play a significant role in 1) supplying cells with precursors for glutathione synthesis (Bannai and Tateishi, 1986) and 2) the regulation of extracellular levels of glutamate and dopamine in the rat striatum (Baker et al., 2002). In this study, we have used radioligand uptake assays and steady state kinetics to investigate the chloride dependence of System x_c^- natively expressed in cultured human glioma cells (U138 MG). In the complete absence of extracellular chloride, or in the presence of anion inhibitors, e.g. 9-anthracene carboxylic acid, System x_c^- does not transport cystine or glutamate. Using a Hill analysis, we have demonstrated that transport of one molecule of cystine requires at least 2 chloride ions. In addition, we have examined the dependence of the V_{max} of cystine transport on chloride concentration (20 mm – 140 mM). Such analysis demonstrated that the V_{max} of cystine transport is independent of chloride concentration, suggesting that the chloride ions must bind to System x_c^- before cystine. Finally, the $K_{0.5}$ for chloride is dependent on the cystine concentration and the K_m for cystine transport is dependent on the chloride concentration. The mutual dependence of the $K_{0.5}$ values suggests that cystine and chloride are transported simultaneously by System x_c^-.

Poster No. II-6

Transport of L-[^{14}C]cystine by HEK_{GLAST}, HEK_{GLT1} and HEK_{EAAC1} cell lines

Hayes D, Weisßner M[1], Rauen T[2] & McBean GJ, Department of Biochemistry, Conway Institute, University College Dublin, Ireland, [1]ETH Zürich, Switzerland; [2]Institut für Biochemie, Universität Münster, Germany

Transport of the di-amino acid L-cystine is essential for synthesis of the major cellular antioxidant, glutathione. Here, we have characterised for the first time the sodium-dependent transport of L-cystine by individual members of the glutamate transporter family over-expressed in HEK cells.

Sodium-dependent uptake of L-[^{14}C]cystine was temperature dependent, linear between 1-20 min and showed at least a 4-fold increase over transport into HEK293 cells. The K_m values were 106 ±18 µM (GLAST), 21 ± 10 µM (GLT1) and 100 ± 19 µM (EAAC1). The maximum rate of transport (V_{max}) at 25^{o}C was significantly greater in HEK_{EAAC1} cells (116 ± 9 pmol/min/mg protein) than in either HEK_{GLAST} (80 ± 6 pmol/min/mg protein) or HEK_{GLT1} cells (60 ± 7 pmol/min/mg protein).

Transport of L-[^{14}C]cystine was potently inhibited by L-glutamate , with IC_{50} values of 90 µM (GLAST), 0.85 µM (GLT1) and 1.53 µM (EAAC1). Reduction of L-[^{14}C]cystine to L-[^{14}C]cysteine with 1 mM cysteinylglycine caused a 2.5-fold increase in transport into HEK_{GLT1} cells, and a 4-fold increase in transport into HEK_{EAAC1} cells. However, most of the augmented transport into HEK_{GLT1} cells was not GLT1-mediated, whereas almost all the increased uptake in HEK_{EAAC1} cells was via EAAC1.

It is concluded that all three high affinity glutamate transporters actively take up cystine, but that L-cysteine is a preferential substrate for the EAAC1 transporters. Thus, the transport of cystine depends both on the extracellular redox state, and on the concentration of amino acids, such as glutamate.

Poster No. II-7

The determination of the concentrations of the glutamate transporter EAAT3 (EAAC) in brain tissue and unexpected cross-reactivities of antibodies directed to EAAT3

Holmseth S[1], Dehnes Y[1], Bjørnsen LP[1], Furness DN[2], Bergles D[3] & Danbolt NC[1],[1]Centre for Molecular Biology and Neuroscience, Departments of Anatomy, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway. [2]MacKay Institute of Communication and Neuroscience, Keele University, Keele, Staffs, England. [3]Department of Neuroscience, Johns Hopkins School of Medicine, Baltimore, USA

The central nervous system (CNS) expresses five different glutamate transporter proteins (EAAT1-5). EAAT1 and EAAT2 are predominantly astroglial proteins and are essential for maintaining low resting levels of extracellular glutamate and for protecting neurons against excitotoxicity. The roles of the other three transporters remain elusive. EAAT4 and EAAT5 are predominantly expressed in the cerebellar Purkinje cells and in the retina, respectively, while EAAT3 is expressed in neurons throughout the CNS. Here we have produced a variety of antibodies to EAAT3 and subjected them to extensive specificity testing. Several of them recognized non-EAAT3 proteins in addition to or in stead of EAAT3. One particular stretch of the EAAT3-sequence gave rise to antibodies recognizing tubulin in spite of the absence of primary sequence homology. This illustrates the importance of proper specificity testing of antibodies. The best antibodies were used to immunoisolate EAAT3 protein and to determine the concentrations of EAAT3 protein in absolute terms (µg/mg tissue) in the major CNS regions of young adult rats by quantitative immunoblotting using known amounts of the pure protein as standards. The total concentration of EAAT3 is about 100-fold lower than that of EAAT2.

Poster No. II-8

Pharmacological characterization of human excitatory amino acid transporters in a fluorescence-based membrane potential assay

Jensen AJ & Bräuner-Osborne H, Department of Medicinal Chemistry, The Danish University of Pharmaceutical Sciences, Copenhagen, Denmark

We have expressed the human excitatory amino acid transporters EAAT1, EAAT2 and EAAT3 stably in HEK293 cells and characterized the transporters pharmacologically in a conventional [^{3}H]-D-aspartate uptake assay and in a fluorescence-based membrane potential assay, the FLIPR Membrane Potential (FMP) assay. The K_m and K_i values obtained for 12 standard EAAT ligands at EAAT1, EAAT2 and EAAT3 in the FMP assay correlated well with the K_i values obtained in the [^{3}H]-D-aspartate assay (r^2 values of 0.92, 0.92 and 0.95, respectively). Furthermore, the pharmacological characteristics of the cell lines in the FMP assay were in good agreement with previous findings in electrophysiology studies of the transporters. The FMP assay was capable of distinguishing between substrates and non-substrate inhibitors and to discriminate between "full" and "partial" substrates at the transporters. Taking advantage of the prolific nature of the FMP assay, interactions of the EAATs with substrates and inhibitors were studied in some detail.

This is the first report of a high throughput screening assay for EAATs. We propose that the assay will be of great use in future studies of the transporters. Although conventional electrophysiology set-ups might be superior in terms of studying sophisticated kinetic aspects of the uptake process, the FMP assay enables the collection of considerable amounts of highly reproducible data with relatively little labor. Furthermore, considering that the number of EAAT

ligands presently available is limited, and that almost all of these are characterized by low potency and a low degree of subtype selectivity, screening of compound libraries at the EAAT cell lines in the FMP assay could help identify structurally and pharmacologically novel ligands for the transporters.

Poster No. II-9

Differentiated expression of SAT2/ATA2/SA1 in the central nervous system

Jenstad M & Chaudhry FA, Institute of Basic Medical Sciences and Centre for Molecular Biology and Neuroscience, University of Oslo, Oslo, Norway

Glutamine is the most abundant free amino acid in plasma, cerebrospinal fluid and brain extracellular space. It plays a critical role in nitrogen metabolism, detoxification of ammonia and production of urea, and in the formation of amino acids, amino sugars, purines and pyrimidines. In the CNS, circumstantial evidence has suggested that the transmitter glutamate recycles through an indirect mechanism that involves transport into glia, conversion to glutamine, release of the glutamine from glia, transport into neurons and conversion back to glutamate before repackaging into synaptic vesicles. A novel family of amino acid transporters capable of glutamine transport in heterologous expression systems has been identified. SAT2/ATA2/SA1 translocates glutamine by coupling the movement of glutamine to Na+ transport down its electrochemical gradient. Ubiquitous mRNA for SAT2 has been detected in rat by northern blotting and in situ hybridization. We have generated specific antibodies to a GST-fusion protein containing the N-terminal of SAT2. We detect SAT2 expression throughout the central nervous system. However, SAT2 is enriched in a subset of nerve cells including pyramidal cells in the hippocampus and cortex. Our data suggest the involvement of SAT2 in N-homeostasis and in the supply of glutamine as a precursor for the synthesis of glutamate in a subset of nerve cells.

Poster No. II-10

Quantitative localization of glutamate transporters at the calyces of Held in the medial nucleus of the trapezoid body

Lehre KP, Hansen M, Puente N & Osen K, Department of Anatomy, Institute of Basic Medical Sciences, University of Oslo, POB 1105 Blindern, N-0317 Oslo, Norway

The synapses of the large synaptic nerve terminals called the calyces of Held are unique in that it is possible to record simultaneously from both the pre- and postsynaptic elements. This has led to considerable interest in these synapses as models for investigation of glutamatergic synaptic transmission. Because glutamate transporters modulate transmitter diffusion, detailed data on the localizations and concentrations of the transporters are essential for mathematical models of synaptic transmission. In this study we provide the first immunocytochemical data on the localization of glutamate transporters at the calyces of Held. To obtain quantitative data on the density of transporters in the cell membranes, we have employed post-embedding electron microscopy comparing the immunoreactivities to other brain subregions where we previously have determined the transporter membrane densities. Our results indicate that the quantitatively dominating glutamate transporter at the calyces of Held in young adult rats is the GLT/EAAT2 subtype, localized in the glial cell membranes surrounding the nerve terminals.

Poster No. II-11

Differential modulation of glutamate transporters by docosahexaenoic acid

Berry C, Hayes D, Murphy A, Weisßner M[1], Rauen T[2] & McBean GJ, Department of Biochemistry, Conway Institute, University College Dublin, Ireland, [1]ETH Zürich, Switzerland; [2]Institut für Biochemie, Universität Münster, Germany

We have investigated the effect of *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA) on the activity of GLT1, GLAST and EAAC1 individually expressed in human embryonic kidney (HEK) cells.

Exposure of HEK_{GLT1} and HEK_{EAAC1} cells to DHA increased the rate of uptake of D-[^{3}H]aspartate uptake by over 55% of control. In contrast, exposure of HEK_{GLAST} cells to DHA caused almost 40% inhibition of D-[^{3}H]aspartate transport. Maximal inhibition of transport in HEK_{GLAST} cells was observed after 10 min pre-incubation with DHA, whereas stimulation of uptake in HEK_{GLT1} and HEK_{EAAC1}cells was greatest after 20-40 min pre-incubation with DHA.

Removal of extracellular calcium increased the inhibitory potential of DHA in HEK_{GLAST} cells. In contrast, in the absence of extracellular calcium, the stimulatory effect of DHA on D-[^{3}H]aspartate uptake in HEK_{GLT1} cells was abolished, and significant inhibition of the transport process by DHA was observed. The CaM kinase II inhibitor, KN-93, had no effect on DHA-mediated inhibition of transport into HEK_{GLAST} cells, but did abolish the stimulatory effect of DHA on transport mediated by GLT1.

We conclude that DHA differentially modulates glutamate transporter subtypes by different mechanisms and timecourses. In the case of GLT1, DHA appears to stimulate D-[^{3}H]aspartate uptake by a mechanism requiring extracellular calcium and involving CaM kinaseII. In contrast, the inhibitory effect of DHA on GLAST does not require extracellular calcium, and does not involve CaM kinaseII.

Poster No. II-12

Glutamate transporters: Differential localization and function

Rauen T[1], Balani P[1], Mim C[2], Pow D[3] & Grewer C[2], [1]Westfälische-Wilhelms-Universität Münster, Münster, Germany, [2]University of Miami, Miami, USA, [3]University of Queensland, Brisbane, Australia

Five different subtypes of glutamate transporters have been cloned to date, and all of these subtypes appear to be expressed in the mammalian retina. Here, we compared the localization, the kinetics, the pharmacology, and the electrophysiological properties of the major glutamate transporter subtypes including two splicing variants of GLT1a. These transporters are differentially expressed in the retina: GLAST1 is localized only in glial cells; while GLT1a, GLT1v, GLT1c, EAAC1 and EAAT5 are expressed in neuronal cells. Heterologously expressed transporters exhibited under steady-state conditions no significant subtype differences in their substrate affinity, but demonstrated a unique pharmacological profile, suggesting structural differences in their binding sites. To investigate differences between the subtypes at the level of ion binding and translocation steps, laser-induced photolysis of αCNB-caged L-glutamate was used. L-glutamate-induced transient currents showed no differences between the kinetics of GLT1a- and GLT1v-mediated pre-steady-state currents. However, comparing the steady-state cycle time of the highly anion-conducting EAAT4 with that of GLAST1, GLT1 and EAAC1, indicated that the EAAT4 cycle time constants are about 5-10 times larger. This suggests that EAAT4, in contrast to the other subtypes, is a high-affinity, low-capacity transporter. The transient kinetic experiments in combination with the differential localization of glutamate transporters (pre-, post-, extra-synaptic and glial) shed new light onto their complex function in regulating the glutamate concentration in the retina and in the brain. Our results suggest that rapid binding of glutamate to synaptic transporter binding sites, that act as a glutamate buffer, could provide a discriminating mechanism to temporally control the activation of AMPA and NMDA receptors by glutamate.

CG and TR are grateful for financial support from the Deutsche Forschungsgemeinschaft

Poster No. II-13

A Neuronal Glutamate Transporter Mutant Impaired in Substrate Translocation Exhibits an Altered Anion Conductance Selectivity

Rosental N, Hebrew University Hadassah Medical School, Jerusalem, Israel

The eukaryotic glutamate transporters mediate, besides sodium - and potassium–coupled electrogenic glutamate transport, an uncoupled sodium – and glutamate dependent anion – conductance. It has been proposed that glutamate bound to the transporter directly gates anion permeation (Wadiche et al (1995) Neuron 15 721-728).

Here we describe a mutant from the neuronal transporter EAAC-1 whose properties are compatible with this idea. Mutation of the asparagine residue from the conserved motif NMDGT, located in transmembrane domain 7, to glutamine (N366Q) results in a reduced apparent affinity of both sodium and substrate as monitored by the transport current. In contrast to wild type EAAC-1, where the I_{max} values with the substrates L-aspartate, D-aspartate and glutamate were indistinguishable, in N366Q, the I_{max} for L-glutamate and D-aspartate were only 13 ±6% and 27±5% of that for L-aspartate. Interestingly, in the N366Q the selectivity of the substrate induced anion conductance selectivity was changed such that NO_3^- was around 3.5 fold less permeable than ClO_4^-, whereas in the will type both anions had similar permeability. These observations suggest that the altered substrate interaction in the mutant leads to a selectivity change of the anion conductance gated by substrate.

Poster No. II-14

Characterization of Novel L-*Threo*-β-benzyloxyaspartate Derivatives, Potent Blockers of the Glutamate Transporters

Shimamoto K[1], Sakai R[2], Takaoka K[1], Yumoto N[3], Nakajima T[1], Amara SG[4] & Shigeri Y[3], [1]Suntory Institute for Bioorganic Research, [2]Kitasato University School of Fisheries Sciences, [3]National Institute of Advanced Industrial Science and Technology (Japan), [4]University of Pittsburgh School of Medicine (USA)

Non-transportable blockers of the glutamate transporters are important tools for investigating mechanisms of synaptic transmission. We characterized novel L-*threo*-β-benzyloxyaspartate (L-TBOA) analogs possessing a substituent on its benzene ring. The analogs significantly inhibited labeled glutamate uptake, the most potent of which was (2*S*, 3*S*)-3-{3-[4-(trifluoromethyl)benzoylamino]benzyloxy}- aspartate (TFB-TBOA). In an uptake assay using cells transiently expressing excitatory amino acid transporters (EAATs), the IC_{50} values of TFB-TBOA for EAAT1, EAAT2, and EAAT3 were 22 nM, 17 nM, and 300 nM, respectively. TFB-TBOA was significantly more potent at inhibiting EAAT1 and EAAT2 compared with L-TBOA (IC_{50} values for EAATs1-3 were 33 μM, 6.2 μM, and 15 μM, respectively). Electrophysiological analyses revealed that TBOA analogs block the transport-associated currents in all five EAAT subtypes and also block leak currents in EAAT5. The kinetics of TFB-TBOA differed from the kinetics of L-TBOA probably due to the strong binding affinity. Notably, TFB-TBOA is highly selective for EAATs. Moreover, intracerebroventricular administration of the TBOA analogs induced severe convulsive behaviors in mice, probably due to the accumulation of glutamate. Taken together, novel TBOA analogs, especially TFB-TBOA, should serve as useful tools for elucidating the physiological roles of the glutamate transporters.

Poster No. III-1

Homology modeling of human vesicular glutamate transporters

Almqvist J[&], Hovmöller S[&] & Da-Neng Wang[*], [&]Department of Structural Chemistry, Arrhenius Laboratory, Stockholm University, Sweden, [*]Skirball Institute of Biomolecular Medicine and Department of Cell Biology, New York University School of Medicine, New York, NY, USA

VGLUT1 and VGLUT2 are transport proteins that import glutamate into synaptic vesicles at presynaptic nerve terminals of excitatory neural cells. The two proteins are highly homologous (sharing 76% sequence identity) and differ in regional expression in the mammalian CNS. Although their 3D structures have not yet been determined, hydropathy analysis of several such vesicular neurotransmitter transporters predicts 12 putative transmembrane segments. Flanking these are roughly 60 residues in the sequence of both the N- and C-terminal part of VGLUT1 and VGLUT2 that are predicted to be disordered in the structure of the two proteins. However, for the predicted transmembrane region, possible structural templates exist. Recently, structures of two bacterial transport proteins were solved to high resolution: the glycerol-3-phosphate transporter (GlpT) and the lactose permease (LacY) with which VGLUT1 and VGLUT2 share 18-20% sequence identity. While the target/template sequence identity is low (which is expected for analogues or distant homologues), several sequential features indicate structural similarity between these different transporters. We therefore constructed full-atom molecular models of VGLUT1 and VGLUT2 by homology modeling using parts of the bacterial transport proteins' structures as templates. Based on these structure models, we can speculate on possible mechanisms for the substrate translocation process.

Poster No. III-2

Localization of VGLUT3, the vesicular glutamate transporter type 3, in the rat brain

Gilchrist J[1], Herzog E[1], Gras C[1], Muzerelle A[2], Ravassard P[3], Gaspar P[2,] Giros B[1] & El Mestikawy S[1], [1]INSERM U513, [2]INSERM U106, [3]CNRS UMR 7091, France

We have previously reported the cloning and characterization of a third and unexpected subtype of vesicular glutamate transporter (VGLUT3). In this study, we use specific nucleic probes and antisera to describe the detailed regional and cellular distribution of the VGLUT3 transcript and protein in the rat brain.

VGLUT3 transcripts are found in the caudate-putamen, olfactory tubercles, hippocampus, interpeduncular nucleus, and raphe nuclei, as well as in a few scattered neurons in the cerebral cortex. The distribution of the VGLUT3 protein is largely overlapping with that of the transcript, consistent with an expression in locally projecting neurons. However, in some regions (like the subtantia nigra pars compacta), the protein is found but not the mRNA, suggesting the existence of longer VGLUT3-positive projections. In a previous study, VGLUT3 was found to be expressed in cholinergic neurons of the striatum and serotoninergic neurons of the raphe nuclei. Here, we observe VGLUT3 in a subpopulation GABAergic neurons in the hippocampus and interpeduncular nucleus. These data further hint to an unconventional role of VGLUT3 as a modulator of synaptic transmission in neurons having another biochemical phenotype.

Poster No. III-3

Functional characterization of the third isoform of the vesicular glutamate transporter VGLUT3

[1,2]Gras C, [1]Herzog E , [1]Giros B, [2]Gasnier B & [1]El Mestikawy S, [1]Faculte de Medecine, Institut National de la Sante et de la Recherche Medicale (INSERM) Unite 513, Creteil Cedex, France, [2]Institut de Biologie Physico-Chimique, Centre National de la Recherche Scientifique (CNRS) Unité Propre de Recherche 1929, Paris, France

Recently, two vesicular glutamate transporters (VGLUT1 and VGLUT2) have been identified. Together, VGLUT1 and VGLUT2 operate at most central glutamatergic synapses. In this study, we characterized a third vesicular glutamate transporter (VGLUT3), highly homologous to VGLUT1 and VGLUT2.

Vesicles obtained from BON cells stably expressing VGLUT3 accumulate [^{3}H]glutamate. This uptake can be inhibited by CCCP (an H^+ ionophore), meaning that it is driven by a transmembrane H^+ electrochemical gradient. Furthermore, use of nigericin and valinomycin reveals that vesicular uptake of glutamate by VGLUT3 is dependent principally on the electrical component of this H^+ gradient. Analysis of the substrate selectivity of VGLUT3 shows that L-glutamate, but not L-aspartate, nor ACh or GABA, inhibits [^{3}H]glutamate uptake. VGLUT3 activity is strongly inhibited by Evans Blue, a competitive inhibitor of glutamate uptake into synaptic vesicles. The VGLUT3-mediated uptake follows a Michaelis-Menten kinetic with a mean Km value of 0,52mM.

In conclusion, VGLUT3 accumulates L-glutamate with bioenergetic and pharmacological characteristics similar to those displayed by brain synaptic vesicles and by the type-1 and type-2 isoforms in heterologous systems.

Poster No. III-4

Localization of vesicular glutamate transporters in the normal and pathological human brain

Kashani A[1], Hirsch E[2], Giros B[1] & El Mestikawy S[1], INSERM U513, [2] INSERM U289, France

Glutamate is the main excitatory neurotransmitter in the brain. Recently, three vesicular glutamate transporters (VGLUT1-3) were identified. All three carriers are distantly related to the large family of Na^+-dependent inorganic phosphate transporters and share a high degree of structural and functional homology. In rodents, VGLUT1 is

expressed by glutamatergic neurons of the cerebral and cerebellar cortices, the hippocampus and the thalamus. VGLUT2 is found in excitatory neurons throughout the diencephalon and the brainstem. VGLUT1&2 have largely complementary distributions with a partial overlap in few brain areas. Finally, the third isoform (VGLUT3) is found in some subpopulations of cholinergic, serotonergic and GABAergic neurons. Thus the three vesicular glutamate transporters unravel three anatomically and functionally distinct systems of glutamatergic neurons.

We have developed subtype-specific antiserums and successfully mapped VGLUT1 and VGLUT2 in the human brain. Furthermore, using western blotting and immunoautoradiography we have observed significant modifications of VGLUT1 and VGLUT2 expression in basal ganglia and frontal and temporal cortices of patients suffering of Idiopathic Parkinson's disease (IPD). For example, in the putamen and in the frontal cortex a 20% increase and 50% decrease (repectively) of VGLUT1 are observed. The status of VGLUT3 in IPD is under current investigation. Thus, in addition to the well known dopaminergic lesion, IPD is associated with severe modifications of glutamatergic inervation.

BENZON SYMPOSIUM No. 51

NEUROTRANSMITTER TRANSPORTERS: BASAL FUNCTION AND DRUG TARGETS

AUGUST 9-12, 2004, COPENHAGEN, DENMARK

Organizing committee:
Ulrik Gether, Orla Miller Larsson, P. Høngaard Andersen, Arne Scousboe
Povl Krogsgaard-Larsen and Sven Frøkjær

Abstracts - WEDNESDAY, August 11, 2004

Molecules in motion: Multiple mechanisms that regulate the GABA transporter GAT1
Quick MW, Department of Biological Sciences, University of Southern California, Los Angeles, California, USA
At particular synapses in the CNS, GABA transporters have been shown to control the time course and/or the amplitude of inhibitory post-synaptic currents, and to prevent spillover of GABA to neighboring synapses. Thus, regulating GABA transporter function may have important physiological consequences. In general, such regulation could occur by changing the number of functional transporters on the plasma membrane or altering the rate at which GABA is sequestered. Our recent work has focused on the role of direct phosphorylation and protein-protein interactions in governing these processes in the rat brain GABA transporter GAT1. In terms of transporter subcellular redistribution, we are presently testing a model in which GAT1 is directly phosphorylated by C-kinase or tyrosine kinase in a mutually exclusive manner, and that the relative abundance of these states determines in part the relative subcellular distribution of the transporter by affecting its rate of internalization. In terms of unitary transport properties, we are presently testing a model in which the SNARE protein syntaxin 1A inhibits transport at a step after substrate binding through a series of direct inter-protein and intra-protein interactions. We speculate that control of transport rates occurs through regulation of the fourth intracellular domain of the transporter, which acts as a barrier to transport, similar to inactivation or regulatory domains of other carriers and ion channels.

The resurfacing of the presynaptic choline transporter
Blakely RD, Vanderbilt University, Suite 7140 MRBIII, Vanderbilt, Nashville, TN, USA
The hemicholinium-sensitive choline transporter (CHT) has been known for decades as an activity enriched in cholinergic terminals of the CNS and PNS. Choline uptake is thought to be rate limiting for acetylcholine production, particularly during high rates of stimulation. Activity-dependent alterations in choline uptake have been described, suggesting that CHT regulation may be an important aspect of the plasticity of cholinergic neurons. Recently, we reported the cloning of mouse and human CHTs and the development of CHT specific antibodies suitable for synaptic localization and regulation studies. These efforts demonstrate that CHT protein is enriched in presynaptic terminals as expected. Unexpectedly, the bulk of CHT protein is localized cytoplasmically, associated with small 30-40 nm diameter vesicles that can be shown to contain the vesicular acetylcholine transporter (VACHT) as well as acetylcholine. Activity-dependent recruitment of CHT to the plasma membrane is evident following depolarization, paralleling increases in choline uptake. These studies reveal that CHTs are mobilized from a subpool of cholinergic synaptic vesicles, possibly the Reserve Pool (RP), with distinct kinetics and sensitivities to depolarization/second messengers as the Readily Releasible Pool (RRP). To evaluate these concepts in vivo and to assess the physiologic significance of CHT in a whole animal context, we have disrupted the murine CHT locus by gene targeting approaches. CHT -/- animals demonstrate a complete loss of HC-3 sensitive acetylcholine synthesis and demonstrate a time-dependent loss of cholinergic signaling in muscle. Newborn CHT -/- animals exhibit anoxia and paralysis and die within an hour after birth. CHT +/- animals are grossly normal but do exhibit pharmacologic sensitivities in keeping with a diminished reserve capacity for CHT expression. CHT +/- mice also exhibit normal levels of choline transport activity despite a 50% loss of protein, revealing a posttranslational mechanism engaged to sustain choline uptake and ACh production at wildtype levels. These studies demonstrate new paradigms for the study of CHT regulation and underscore the relevance of CHT as a candidate gene for disorders associated with disrupted cholinergic function including dementias, myasthenias and heart disease.

Protein-protein interactions in glutamate transporter assembly and trafficking

Robinson MB, González MI, Kalandadz A, Fournier KM, Sheldon A, Susarla S & Krizman E, Departments of Pediatrics and Pharmacology, Children's Hospital of Philadelphia/University of Pennsylvania, Philadelphia, Pennsylvania, USA

A family of Na^+-dependent transporters limit both excitatory synaptic transmission and the toxic potential of glutamate. Different members of this family are selectively enriched in glial processes that sheath the synapse, while others are expressed on post-synaptic spines at excitatory synapses and/or by inhibitory neurons. These transporters are thought to exist as homomultimeric assemblies on the plasma membrane. This presentation will cover two aspects of transporter trafficking. First, our recent identification of conserved motifs required for endoplasmic reticulum retention and proper maturation of transporters will be described. In the second part of this presentation, our studies of the regulation of the number of transporters at the plasma membrane will be discussed. Published evidence that the number of transporters at the plasma membrane can be rapidly (within min) regulated by a variety of signaling molecules will be summarized. In recent studies, we have found that one of the neuronal transporters (EAAC1/EAAT3) appears to recycle at the plasma membrane with a half-life of 5-7 min; both delivery of transporter to the plasma membrane and removal of transporter may be independently regulated. We have identified three proteins that interact with the transporters and may be important for assembly and/or regulated trafficking. We have also been working to identify signaling molecules and transporter domains/motifs that may be required for these effects. In summary, as is true for many receptors and other neurotransmitter transporters, glutamate transporters are dynamically regulated; it is possible that this regulation may have implications for the plasticity of synaptic signaling.

Regulation and dysregulation of glutamate transporters by structure, lozalication and targeting

Rothstein JD, Johns Hopkins University, Department of Neurology, Baltimore, MD, USA

Glutamate is the principal excitatory neurotransmitter in the nervous system. Inactivation of synaptic glutamate is principally handled by the GLT1/EAAT2 glutamate transporter. In spite of its critical importance in normal and abnormal synaptic activity, no practical pharmaceutical can positively modulate this protein. Using a blinded screen of 1040 FDA approved drugs and nutritionals, we discovered that multiple beta-lactam antibiotics are potent stimulators of GLT1/EAAT2. Furthermore, this action appears to be mediated thru activation of the genetic promoter for GLT1/EAAT2. Data on activation of EAAT3 and EAAT4 genes will be presented. To further identify additional transporter activators, EAAT1, EAAT2 and EAAT4 BAC-ds-Red (or eGFP) mice were generated. beta Lactams and various semi-synthetic derivatives are potent antibiotics that act to inhibit bacterial synthetic pathway. When delivered to animals, the lactam ceftriaxone increased brain expression and biochemical activity of GLT1/EAAT2. Glutamate transporters are important in normally preventing glutamate neurotoxicity. Ceftriaxone was neuroprotective in vitro when used in paradigms of ischemic injury and motor neuron degeneration, both based in part on glutamate toxicity. When used in an animal model of the fatal disease amyotrophic lateral sclerosis (ALS), the drug delayed loss of strength and increased mouse survival. Thus these studies provide a new class of potential neurotherapeutics that act to modulate the expression of glutamate neurotransmitter transporters via gene activation.

Poster No. IV-1

Regulation of the Dopamine Transporter by Interacting Proteins

Torres GE, Duke University, Durham, NC, USA

In neurons, the classical biogenic amines dopamine, norepinephrine, and serotonin act as neurotransmitters controlling a large variety of functions including locomotion, autonomic function, hormone secretion, and the complex behaviors associated with affect, emotion, and reward. A key step that determines the intensity and duration of monoamine signaling at synapses is the reuptake of the released transmitter back to nerve terminals by plasma membrane transporters. These proteins are also high-affinity targets for cocaine and amphetamines, drugs that are highly addictive and, thus represent major abused substances worldwide. Clinically, biogenic amine transporters are molecular targets for therapeutic agents used in the treatment of psychiatric disorders, such as attention-deficit hyperactivity disorder (ADHD), obsessive-compulsive disorder (OCD), depression, and others. These agents bind to biogenic amine transporters, disrupt transporter function, and thereby prolong the intensity and duration of biogenic amines in the brain. Despite the importance of biogenic amine transporters in controlling brain function, very little information is available regarding the cellular and molecular regulation of these proteins. This information is essential to understand their contribution to abnormal brain function. Given the physiological roles that monoamine transporters play in the regulation of monoamine transmission, recent investigations have focused on the mechanisms that regulate the localization and function of these transporters. These studies have challenged the original view that monoamine transporters at presynaptic terminals were relatively static proteins. Indeed, over the past few years, an increasing number of monoamine transporter interacting proteins have been identified. These interactions might be important for clustering, compartmentalization, trafficking, and regulation of transporter function. Hence, these new regulatory mechanisms might have important implications as novel targets for therapeutic approaches. Here, I will describe the approaches used in our laboratory to identify transporter interacting proteins and summarize the main functional aspects of our findings.

Poster No. IV-2

Constitutive and PKC-regulated catecholamine transporter internalization is mediated by two distinct and novel endocytic motifs

Holton KL[1] & Melikian HE[1,2], [1]Brudnick Neuropsychiatric Research Institute, Department of Psychiatry and [2]Program in Neuroscience, University of Massachusetts Medical School, Worcester, MA, USA

The catecholamines norepinephrine (NE) and dopamine (DA) are associated with a wide variety of neuronal functions including movement control, mood and rewarding behaviors. Presynaptic reuptake processes tightly control extracellular NE and DA levels, and are mediated by the high affinity, Na^+/Cl^--dependent, plasma membrane transporters NET and DAT, respectively. Catecholamine signaling and homeostasis are exquisitely sensitive to the number of functional cell surface transporters, as demonstrated both by pharmacological transporter blockade with addictive psychostimulants and by genetic transporter knockdown. NET and DAT surface expression is highly plastic, and is dynamically modulated in response intracellular signaling molecule activation. For example, protein kinase C (PKC) activation sequesters NET and DAT in endosomal pools, demonstrating that membrane trafficking influences transporter surface levels. We recently established that DAT robustly traffics and that PKC-mediated DAT sequestration is achieved by modulating intrinsic DAT internalization and recycling rates. While it is clear that neurotransmitter transporters are subject to membrane trafficking, the structure/function relationships linking transporters to the cellular endocytic machinery are not well defined. Our current work reveals a single carboxy-terminal DAT domain that contains distinct motifs required for constitutive and PKC-regulated DAT endocytosis. Site-directed mutagenesis and gain-of-function experiments demonstrate that these motifs are both necessary and sufficient to drive constitutive and PKC-regulated DAT endocytosis. Interestingly, mutations in the constitutive endocytic motif do not attenuate PKC-stimulated DAT internalization, consistent with the hypothesis that constitutive and regulated transporter internalization may be governed by independent cellular mechanisms. Parallel experiments demonstrate an identical endocytic motif in NET. This motif does not conform to classic clathrin-mediated endocytic signals and is highly conserved throughout the Na^+/Cl^-–dependent neurotransmitter transporter gene family, suggesting that neurotransmitter transporters may have evolved specialized endocytic mechanisms.

Poster No. IV-3

Differential regulation of amphetamine-mediated dopamine release and binding to the dopamine transporter (DAT) by protein kinase C α and β isoforms

Johnson L'A, Guptaroy B, Lund S & Gnegy ME, Department of Pharmacology, University of Michigan, Ann Arbor, Michigan, USA

The plasmalemmal dopamine transporter, DAT, is the site of action of amphetamine (AMPH) which stimulates efflux of dopamine (DA) from the terminal into the synaptic cleft. We found that protein kinase C (PKC) activation and intracellular Ca^{2+} are required for the AMPH-stimulated efflux of DA, suggesting that classical PKCs regulate DA efflux. In this study we examined the effect of PKC isozymes on AMPH-mediated DA release in superfused rat striatal slices, differentiating the PKC isozymes through the use of selective inhibitors. The non-selective classical PKC inhibitor Go6976 (130 nM) reduced AMPH-mediated DA release from 0.39 ± 0.05 to 0.13 ± 0.05 pmole/mg ptn ($p < 0.05$,n=3). Rottlerin (10 μM), a selective inhibitor of the novel PKCδ, had no effect on AMPH-mediated DA release. The selective PKCβ inhibitor, hispidin (10 μM), reduced AMPH-mediated DA release from 0.65 ± 0.07 to 0.21 ± 0.06 pmole/mg ptn ($p<0.01$,n=3). Another selective PKCβ inhibitor, Ly379196 (100nM), reduced AMPH-mediated DA release from 5.5 ± 1.4 to 1.7 ± 0.3 ($p<0.05$, n=4) fold stimulation over basal release. However, 50μM safingol, a specific PKCα inhibitor, increased AMPH-mediated DA release from 0.11 ± 0.01 to 0.23 ± 0.01 pmole/mg ptn ($p<0.001$,n=3). None of the drugs affected baseline efflux nor [^{3}H]DA uptake. Co-immunoprecipitation studies showed that DAT and PKCβ are complexed but not DAT and PKCα. These results suggest that classical PKCs are important regulators of AMPH-mediated DA release and that selective PKC isozymes provide differential regulation of efflux through DAT. Funded by NIH grant DA11697, DA 11495 and Pharmacological Sciences Training Grant GM07767.

Poster No. IV-4

Multiple sorting signals involved in the trafficking of the vesicular acetylcholine transporter

Kim M-H & Hersh LB, Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, KY, USA

The vesicular acetylcholine transporter (VAChT) is responsible for the transfer of the neurotransmitter acetylcholine (ACh) from the cytoplasm into synaptic vesicles. The cytoplasmic tail of VAChT has been shown to contain signals that direct its sorting and trafficking. We have studied the role of clathrin associated protein complexes in VAChT sorting to synaptic-like microvesicles in PC12 cells. A fusion protein between the VAChT cytoplasmic tail and GST was used to identify VAChT-clathrin associated protein adaptin gamma (adaptor protein-1) and adaptin alpha (adaptor protein-2) complexes from a rat brain extract. *In vivo* coimmunoprecipitation was used to confirm adaptin alpha and adaptin gamma complexes. Deletion and site directed mutagenesis show that the VAChT cytoplasmic tail contains

multiple trafficking signals. These include a non-classical tyrosine motif that serves as the motif for adaptin alpha interaction, and a phosphorylation/dileucine sequence that serves as the recognition sequence for adaptin gamma. A classical tyrosine motif is also involved in VAChT trafficking, but does not interact with any of the known adapter proteins. Disruption of either the adaptin alpha of adaptin gamma signals leads to primarily cell surface VAChT. Thus there appears to be two endocytosis motifs, one involving the adaptor protein-1 binding site and the other involving the adaptor protein-2 binding site. These results suggest a complex trafficking pathway for VAChT in PC12 cells.

Pharmacology and structure-activity relations of cloned as well as neuronal and glial GABA-transporters: Functional implications

Schousboe A, Department of Pharmacology, The Danish University of Pharmaceutical Sciences, Copenhagen, Denmark

Termination of GABAergic neurotransmission is brought about by diffusion of GABA in the synaptic cleft followed by transport into the presynaptic nerve ending as well as surrounding astrocytes. This uptake is mediated by high affinity GABA transporters whose activity is coupled to the sodium gradient and the membrane potential. Hence, under prevailing physiological conditions these transporters may maintain an intra-/extracellular GABA concentration gradient of the order of 10^5. To date 4 high affinity GABA transporters have been cloned. The nomenclature may be somewhat confusing since in the mouse GAT1-4 correspond to GAT-1, BGT-1, GAT-2 and GAT-3 in rats and humans. The pharmacological properties of these transporters have been extensively studied and a number of GABA analogues of restricted conformation and exhibiting large differences with regard to lipophilicity have been synthesized. Some of these are selective for the individual transporters but so far the pharmacological diversity between neuronal and glial GABA transport does not appear to be reflected by distinct inhibitors of GAT1-4. These structure-activity relations will be reviewed in the light of information available in recent literature (1-3).

References:

1. Sarup A, Larsson OM, Bolvig T, Frølund B, Krogsgaard-Larsen P, Schousboe A. 2003. *Neurochem Int* 43, 445-451.
2. Sarup A, Larsson OM, Schousboe A. 2003. *Curr Drug Targ CNS Neurol Dis* 2, 269-277.
3. Sarup A, Larsson OM, Schousboe A. 2004. In: Strategies in Molecular Neuropharmalogy (Bräuner-Osborne H, Schousboe A, eds). Humana Press, Totowa, NJ, pp 175-190.

SSRIs: Any room for improvement?

Bøgesø KP, Medicinal Chemistry Research, H. Lundbeck A/S, Valby-Copenhagen, Denmark

SSRIs are the most widely used drugs for treatment of depressive disorders. The most prominent unmet need of SSRIs is their slow onset of action, i.e. full effect is obtained only after 6-8 weeks of treatment. The slow onset has been ascribed to initial feed-back inhibition of serotonin release by stimulation of somatodendritic 5-HT_{1A} autoreceptors. Co-treatment with the unselective 5-HT_{1A} antagonist pindolol has shown varying results in clinical trials.

We have studied a number of SSRI augmentation strategies. The 5-HT_{1A} concept described above resulted in the combined 5-HT uptake inhibitor/5-HT_{1A} antagonist Lu 36-274, which in rat microdialysis studies produced a 450-500% increase of extracellular 5-HT in frontal cortex and showed much faster onset of action than fluoxetine in vivo in a schedule-induced polydipsia model. Another interesting augmentation strategy is the combination of 5-HT uptake with 5-HT_{2C} antagonism. Combination experiments with SSRIs and 5-HT_{2C} antagonists demonstrate substantial increase in ventral hippocampal extracellular 5-HT in both acute and chronic microdialysis experiments in rats as well as enhanced effect in animal models such as the mouse light-dark box test.

However, an unexpected and highly interesting enhancement effect was discovered during the development of the S-enantiomer of citalopram, escitalopram. Although R-citalopram has low affinity for the serotonin reuptake site, we have shown in a large number of pharmacological models that it inhibits the effect of escitalopram. Further, escitalopram consistently shows faster onset of action and higher efficacy than citalopram. The mechanism behind this unique interaction will be discussed.

Poster No. IV-5

Targeting of the proton-coupled glutamine transporter, SN1, suggests roles in synaptic functions and pH homeostasis

Solbu TT, Boulland JL, Bredahl MKL, Zahid W, Storm-Mathisen J & Chaudhry FA, Institute of Basic Medical Sciences, and Centre for Molecular Biology and Neuroscience, University of Oslo, Oslo, Norway

Nerve cells specifically target proteins, important for signaling and for the generation of transmitters, to the synapses. Thus we find high concentrations of receptor proteins in the post-synaptic membrane and proteins involved in vesicle fusion concentrated in the terminals. The fast transmitters glutamate and GABA are generally believed to be recycled through uptake into the surrounding glia, and subsequently conversion to glutamine, which is then transported back to the nerve terminals. We have earlier demonstrated targeting of glutamate transporters to glial processes adjacent to

synapses. Recently, several glutamine transporters have been molecularly characterized. The translocation of glutamine through the bi-directional system N transporter, SN1, is coupled to protons. Moreover, SN1 is exclusively expressed in glia and concentrated in the vicinity of especially GABAergic terminals, indicating a role in the supply of glutamine to the nerve terminals. In the kidney, most of the filtrated glutamine is reabsorbed by glutamine transporters expressed at the apical membranes of the proximal tubuli. Interestingly, the subcellular localization of SN1 indicates a role in the supply of glutamine from the blood to the tubuli for the production and secretion of NH_4^+. In addition, we find SN1 in intracellular vesicle-like structures suggesting regulated membrane trafficking of SN1. During chronic metabolic acidosis, SN1 is induced concomitantly with an increase in the expression level and activity of phosphate-activated glutaminase (PAG), a key enzyme in the catabolism of glutamine. Our data on the differentiated expression of SN1 in polarized cells in the kidney and synaptic areas in the CNS suggests roles in the regulation of brain transmitters and of body pH.

Poster No. IV-6

A Regulated Association of the LIM Domain Protein Hic-5 with the Antidepressant-Sensitive Serotonin Transporter (SERT) in Native Tissues

Carneiro AMD & Blakely RD, Vanderbilt University, Nashville, TN, USA

In the central nervous system, presynaptic SERT proteins constitute the majormode of inactiva tion of serotonin (5-HT) following transmitter release. SERTs are also expressed by several nonneuronal cell populations including colonic epithelial cells, pulmonary smooth muscle cells and platelets. SERT activity and/or surface trafficking are subject to rapid regulation following kinase activation/phosphatase inhibition, though precise mechanisms remain ill defined. One possibility includes the alteration of physical associations between transporters and interacting proteins through either direct phosphorylation of SERT and/or the modulation of transporter-linked scaffolding systems. In an effort to identify and characterize SERT-interacting partners subject to kinase regulation, we have investigated the interactions of the native SERT with the multiple LIM domain protein Hic-5, previously established to interact with SERT proteins through yeast 2-hybrid studies (Carneiro et al., 2002). Using platelet extracts, we observe that SERT specific antisera co-immunoprecipitate SERT and Hic-5, interactions not evident using nonimmune serum. Further studies of the platelet SERT:Hic-5 complex reveal both phorbol ester and 5-HT modulated associations. Studies are underway to map sites of SERT:Hic-5 interactions, to define mechanisms by which signaling pathways 5-HT and psychostimulants influence complex stability, and to define additional partners whose localization to SERTs could be Hic-5 dependent. Clarifying these transporter protein associations may provide insights into pathways altered in mood disorders and offer new targets for therapeutics.

Supported by T32 MH65215 (A.M.C.) and DA07390 (R.D.B.)

Poster No. IV-7

Mutation of putative phosphorylation sites in hSERT and PMA regulation

Larsen MB, Fjorback AW, Ramamoorthy S & Wiborg O, Biological Psychiatry, Psychiatric University Hospital, Aarhus, Denmark

The human serotonin transporter (hSERT) is internalized in response to treatment with the phorbol ester PMA, leading to reduction in serotonin uptake. PMA treatment leads to an increase in hSERT phosphorylation and concomitant down regulation of serotonin (5-HT) uptake. We questioned whether direct phosphorylation of the transporter protein is the trigger for internalization and functional down regulation of SERT. To address this question we generated serotonin transporter mutants with 15 putative phosphorylation sites mutated to alanine residues. Putative phosphorylation sites were identified using the Netphos prediction tool. The predicted phosphosites were found mainly in the N-terminal tail containing 11 sites; one site is located in intracellular loop 2, one in intracellular loop 3 and two are located in the C-terminal end of SERT. We constructed three different hSERT mutants; one with mutation of the N-terminal putative phosphosites, one mutant with deletion of the phosphosites in the loops and C-terminal and one mutant with deletion of all the predicted putative phosphosites. These constructs were transfected into HEK-293 cells and analyzed with respect to 5-HT uptake capacity. All constructs supported active 5-HT transport. In order to investigate the effect of PCK, we incubated the transfected HEK-293 cells with PMA prior to the 5-HT uptake assay. However, all three mutants were inhibited to the same extend as the WT hSERT transporter, indicating that these sites are not the ones that are phosphorylated on PKC activation or that the phosphorylation of hSERT is not the trigger for the observed down regulation. Further studies are underway to examine whether the mutated hSERT constructs are subjected to phosphorylation. This will be investigated by incubation of the transfected HEK-293 cells with ^{32}P-ortophosphate following stimulation with PMA. The mutated transporters will be immunoprecipitated and analyzed to identify the phosphosites in hSERT. This will confirm if the phosphorylation act as a direct trigger for internalization or if is secondary to the internalization.

Poster No. IV-8

Investigating the molecular basis for constitutive internalization and degradation of the dopamine transporter

Fog J, Bjerggaard C & Gether U, Molecular Neuropharmacology Group, Department of Pharmacology, The Panum Institute, University of Copenhagen, Copenhagen, Denmark

Recently, we have shown that although ER export and targeting of the dopamine transporter (DAT) to the cell surface is critically dependent on discrete epitopes in the distal C-terminus, these events do not require canonical PDZ domain interactions with proteins such as PICK1. To clarify the actual role of PDZ domain interactions for DAT function we have expressed the wild type DAT and a number of C-terminal mutants either alone or together with PICK1 in HEK293, N2A neuroblastoma and PC12 cells. By employment of a surface strip-biotinylation assay we observed in all three cell lines that DAT does not reside statically in the plasma membrane but undergoes rapid constitutive internalization. Addition of a single alanine residue to the C-terminus of the DAT (+Ala), which disrupts its ability to form canonical PDZ domain interactions, caused a marked increase in this constitutive internalization. Preliminary data furthermore suggested that overexpression of PICK1 together with wild type DAT decreased constitutive internalization whereas overexpression together with the +Ala mutant had no effect. These data suggest that PDZ domain interaction with proteins such as PICK1 serve to stabilize the DAT in the plasma membrane by inhibiting its constitutive internalization. However, stimulation with amphetamine, or activation of protein kinase C (PKC) with phorbol esters, increased intracellular accumulation of both the DAT and +Ala. Additionally, we observed by confocal microscopy analysis that PICK1 is co-internalized with DAT upon stimulation with phorbol esters. Thus, although PDZ interaction might stabilize DAT at the cell surface they do not prevent regulation of surface expression by PKC and amphetamine. In parallel to these studies we are currently exploring the function of several putative DAT interacting proteins identified either by yeast two-hybrid screens or proteomics based approaches.

Poster No. IV-9

Crucial role of the second intracellular loop of GLYT2 in glycine transport and regulation

Fornés A, Aragón C, & López-Corcuera B, Centro de Biología Molecular "Severo Ochoa" Facultad de Ciencias, Universidad Autónoma de Madrid, Madrid, Spain

Na^+ and Cl^--coupled glycine transporters control the availability of glycine neurotransmitter in the synaptic cleft of inhibitory glycinergic pathways. In this study, we have investigated the involvement of the second intracellular loop of the neuronal glycine transporter 2 (GLYT2) on the protein conformational equilibrium and the regulation by 4α-phorbol 12 myristate 13-acetate (PMA). By substituting several charged (K415, K418 and K422) and polar (T419 and S420) residues for different amino acids and monitoring plasma membrane expression and kinetic behaviour, we found that residue K422 is crucial for glycine transport. The introduction of a negative charge in 422, and to a lower extent in neighbouring N-terminal residues, dramatically increases transporter voltage dependence as assessed by response to high potassium depolarising conditions. In addition, [2-(trimethylammonium)ethyl] methanethiosulfonate (MTSET) accessibility revealed a conformational connection between K422 and the glycine binding/permeation site. Finally, we show that the mutation of positions T419, S420, and mainly K422 to acidic residues abolishes the PMA-induced inhibition of transport activity and the plasma membrane transporter internalization. Our results establish a new structural basis for the action of PMA on GLYT2 and suggest a complex nature of the PMA action on this glycine transporter.

Poster No. IV-10

EAAT4 trafficking in Purkinje neurons

Gincel D & Rothstein J, Johns Hopkins University, Baltimore, Maryland, USA

Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system and is removed from the synaptic cleft by excitatory amino acid transporters (EAATs). Although EAAT4 is the major glutamate transporter in purkinje cells in the cerebellum, its involvement in Purkinje cell death and cerebellar apoptosis derived diseases have not been studied. Our goal is to study the regulation of EAAT4 trafficking in purkinje neurons using organotypic slices and dissociated Purkinje cell culture. We transfected organotypic cerebellar slices with GFP-tagged EAAT4 and followed EAAT4 trafficking over time. In addition, we also transfected slices with EAAT4 lacking the c terminal, an area that is responsible for interaction with GTRAPs and other proteins. Following EAAT4 expression and trafficking pattern would enable us to study EAAT4 and other glutamate transporter involvement in synaptic plasticity.

Poster No. IV-11

Regulation of the Neuronal Glutamate Transporter EAAC1 by Lipid Rafts/Caveolae

González MI, Susarla BTS, Fournier K & Robinson MB, Departments of Pediatrics and Pharmacology, Children's Hospital of Philadelphia, University of Pennsylvania, Philadelphia, PA, USA

Glutamate transporters are an important mechanism for the removal of glutamate from the extracellular space. Failure of these transporters produces an excessive accumulation of glutamate that may trigger excitotoxicity and cell death. The amount of glutamate uptake depends on the number of transporter molecules expressed at the cell membrane. Recently, we have found that the turnover rate of EAAC1 at the cell membrane is about 5-7 minutes. This suggests

that the final number of transporters expressed at the cell surface results from the equilibrium of the number of transporters removed and inserted from the cell membrane. In the present study, we investigated the potential role of lipid rafts/caveolae in the regulation of EAAC1 cell surface expression. Preincubation of C6 glioma cells with methyl-β-cyclodextrin (MβCD), a drug that disrupts lipid rafts/caveolae, increased EAAC1-mediated uptake but did not modify glycine uptake. In addition to MβCD, filipin and nystatin (drugs that also disrupt lipid rafts/caveolae), increased EAAC1 cell surface expression. In cultured neurons, all three drugs increased EAAC1 cell surface expression and filipin reduced the endocytosis of the transporter. Using dis-continuous sucrose gradient centrifugation we found that EAAC1 immunoreactivity is enriched in the lipid rafts/caveolae associated fractions, suggesting that EAAC1 is located in caveolae. Caveolins are the major structural proteins of caveolae. Using coimmunoprecipitation studies we found that EAAC1 interacts with caveolin-1 and caveolin-2. These data suggest that internalization of EAAC1 may be mediated by a lipid rafts/caveolae dependent mechanism. They also suggest that regulation of EAAC1 cell surface expression and activity may require an interaction between EAAC1 and caveolins.

Poster No. IV-12

Functional interaction between the serotonin transporter and ionotropic glutamate receptors

Hansen, KB, H. Lundbeck A/S, Valby-Copenhagen, Denmark

Ionotropic glutamate receptors (iGluRs) are ligand-gated ion channels that mediate the majority of excitatory synaptic transmission in the CNS. The iGluRs have been classified based on their pharmacological and physiological properties as AMPA receptors, kainate receptors, and NMDA receptors.

In the present study, we demonstrate functional interaction between SERT and the AMPA receptor subtype GluR1 resulting in ~38% potentiation of the current. The interaction was investigated using two-electrode voltage-clamp in *Xenopus* oocytes co-expressing SERT and GluR1. The potentiation had fast kinetics and occurred only when SERT and GluR1 were co-activated. The observed response upon co-activation was 1.48±0.08 normalized to the GluR1 response alone, which is significantly potentiated compared to the predicted response of 1.07±0.04 obtained by adding the individual responses from SERT and GluR1 alone. SSRIs were able to abolish the potentiation verifying that expression of SERT is necessary for the potentiation.

Different iGluR subtypes were tested and AMPA receptor subtypes GluR1-3 interacted functionally with SERT, however kainate subtypes GluR5-6 and the chimera GluR6NT-R3, with the extracellular N-terminal domain from GluR6, showed no functional interaction. Mutants of SERT with deletions in the intracellular termini were still able to potentiate GluR1. In addition, SERT-DAT chimeras with the intracellular termini from DAT and the extracellular and transmembrane domains from SERT were also able to potentiate GluR1, whereas DAT alone was not. These results indicate that the intracellular C-terminus of GluR1 and the intracellular tails of SERT are not involved in the interaction. Finally, SERT and GluR1 could be co-immunoprecipitated in both HEK-293 cells and rat brain indicating that SERT and GluR1 interact physically.

In conclusion, the results demonstrate functional interaction between SERT and AMPA receptors by direct protein-protein interaction between the transmembrane or extracellular domains.

Poster No. IV-13

Characterization and mapping of an allosteric citalopram-binding site at the serotonin transporter

Larsen MB[a], Chen F[a], Neubauer HA[a], Sánchez C[b], Plenge P[c] & Wiborg O[a], a: Laboratory of Molecular Neurobiology, Department of Biological Psychiatry, Aarhus Psychiatric University Hospital, Risskov, Denmark. b: Neuropharmacological Research, H. Lundbeck A/S, Copenhagen, Denmark. c: Laboratory of Neuropsychiatry, Department of Pharmacology, Rigshospitalet, Copenhagen, Denmark

It has previously been shown that the dissociation of various ligands from the serotonin transporter (SERT) can be modulated by several drugs. In the present study we characterize the dissociation of [^{3}H]-S-citalopram from the cloned SERT in greater detail. We confirm the dissociation of [^{3}H]-S-citalopram to be modulated by different drugs to a varying degree. The allosteric modulation of dissociation is independent of temperature as well as the presence of sodium ions in the dissociation buffer. Dissociation of [^{3}H]-S-citalopram from a complex with the SERT double mutant N208Q/N217Q, which is unable to homo-oligomerize, is retarded with an efficiency similar to that observed for the wild type, indicating that the allosteric mechanism is mediated within a single subunit. A species scanning mutagenesis study comparing human and bovine SERT revealed that methionine 180, tyrosine 495 and serine 513 are important in mediating the allosteric effect as well as contributing to high-affinity binding at the primary site.

We suggest the two distinct binding sites to be partially overlapping and located within the same subunit and that the allosteric effect is mediated by a steric trapping of the ligand at the primary binding site.

Poster No. IV-14

Partitioning of the serotonin transporter into cholesterol-enriched lipid microdomains modulates transport of serotonin

Magnani F, Williams C, & Haase J, Department of Biochemistry, Trinity College, Dublin, Ireland

The serotonin transporter (SERT) is an integral membrane protein responsible for the clearance of serotonin from the synaptic cleft following the release of the neurotransmitter. SERT plays a prominent role in the regulation of serotoninergic neurotransmission and is a molecular target for multiple antidepressants as well as substances of abuse. Here we show that SERT associates with cholesterol-enriched lipid rafts in both a heterologous expression system and rat brain, and that the inclusion of the transporter into lipid microdomains is critical for serotonin uptake activity. SERT is present in a subpopulation of lipid rafts, which is soluble in Triton X-100, but insoluble in other non-ionic detergents such as Brij 58. Desegregation of lipid rafts upon depletion of cellular cholesterol results in a decrease of serotonin transport capacity (V_{max}), due to the reduction of turnover number of serotonin transport. Our data suggest that the association of SERT with lipid rafts may represent a mechanism for regulating the transporter activity, and consequently serotoninergic signalling in the central nervous system, through the modulation of the cholesterol content in the cell membrane. Furthermore, SERT-containing rafts are detected in both intracellular and cell surface fractions, suggesting that raft association may be important for trafficking and targeting of SERT.

Poster No. IV-15

Functional expression cloning identifies a MAP kinase phosphatase as modulator of dopamine transporter function

Mortensen OV, Prasad BM, Larsen MB & Amara SG, Department of Neurobiology, University of Pittsburgh, Pittsburgh, USA

The biogenic monoamine transporters are the primary mechanism for clearance of their respective neurotransmitter from the extracellular space and serve as important regulators of signal amplitude and duration at synapses.

We have found that modulation of transporter number by activating protein kinase C (PKC) and thereby stimulating trafficking of carriers to and from the cell surface is a process that seems to vary with the expression system used. To identify molecules responsible for these differences we have successfully used functional co-expression cloning in *Xenopus* oocytes. This led to the identification of a MAP kinase phosphatase, MKP3, as a modulator of PKC induced internalization of transporters and other membrane proteins. Surprisingly conventional MAP kinase families are not involved in the PMA-stimulated internalization, as MAP kinase inhibitors had no effect on internalization, nor did the activity state of different MAP kinases correlate with the PMA-induced down regulation. These results suggest that MAP kinase phophatases modulate a novel signaling transduction pathway. To identify other proteins involved in the trafficking of neurotransmitter transporters we are currently using substrate-trapping methods to isolate target substrates of MKP3.

(This work was supported by Howard Hughes Medical Institute and the Alfred Benzon Foundation)

Poster No. IV-16

Identification and characterisation of serotonin transporter-interacting proteins

Müller H[1,2,3], Wiborg W[3] & Haase J[1,2], [1] Department of Biochemistry, Trinity College, Dublin 2, Ireland, [2] Department of Biochemistry, Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland [3] Department of Biological Psychiatry, Psychiatric University Hospital, Skovagervej 2, 8240 Risskov, Denmark

The serotonin transporter (SERT) is an integral membrane protein that belongs to the family of Na^+/Cl^- dependent neurotransmitter transporters. SERT is responsible for controlling the magnitude and duration of serotonergic neurotransmission by clearing released serotonin from the synaptic cleft. SERT represents a molecular target for clinically effective antidepressants, as well as for drugs of abuse. The effects of SERT modulation following administration of these agents suggest that SERT may be tightly regulated. Recent studies indicate that SERT is regulated by interacting with other proteins. Here, we used the yeast two-hybrid system to search for proteins, which interact with the N- and C-termini of human SERT. Several interacting candidates were identified; one of these encodes a protein of unknown function. The interaction between the N-terminus of SERT and this novel protein was demonstrated to be specific in GST-pulldown experiments using lysates from transfected HEK293 cells. This protein also showed specific binding to the N-terminus of the human dopamine transporter. [^{3}H]-5-HT transport assays performed on HEK293 cells co-transfected with human SERT and the novel SERT-interacting protein revealed a dramatic decrease in 5-HT uptake, suggesting a functional regulation of SERT. Furthermore, we have been investigating the mechanism responsible for the 5-HT transport inhibition. In addition, an antibody against this novel protein has been developed and proven to be specific and highly sensitive.

Poster No. IV-17

Differential Localization of Vesicular Glutamate Transporters 1 and 2 in the Rat Striatum

Raju DV[1] & Smith Y[1,2], [1]Yerkes National Primate Research Center, [2]Neurology, Emory University, Atlanta GA, USA

Because of its multifarious functions, glutamate is ubiquitously distributed in the CNS, making difficult the identification of cells that use glutamate as neurotransmitter or metabolic agent based solely on glutamate localization. The recent cloning of vesicular glutamate transporters (vGluTs), specifically vGluT1 and vGluT2, provide new tools to selectively label glutamatergic terminals. Although they appear to display a complementary distribution in the rat CNS, little is known about the exact sources of axon terminals expressing either transporter in the basal ganglia. To further characterize this issue, we undertook an electron microscopic immunolocalization study of vGluT1 and vGluT2 in the rat striatum. Both transporters were expressed exclusively in terminals forming asymmetric synapses onto either spines or dendritic shafts. However, the proportion of axo-spinous and axo-dendritic synapses formed by immunoreactive terminals differed significantly among vGluT1- and vGluT2-labeled boutons. While more than 95% of vGluT1-immunoreactive terminals formed axo-spinous synapses, about 20% of vGluT2-expressing terminals formed axo-dendritic synapses. In light of previous studies showing that corticostriatal afferents synapse predominantly onto spines and thalamostriatal afferents mainly contact dendrites, these observations raise the possibility that both vGluT1 and vGluT2 maybe expressed in corticostriatal afferents, while thalamostriatal afferents primarily use vGluT2. Electron microscopic colocalization studies revealed that a minority (1%) of terminals coexpress vGluT1 and vGluT2 in the striatum, suggesting that terminals endowed with these transporters largely arise from different neuronal populations. Tract tracing studies to determine the exact source of vGluT1- and vGluT2-expressing afferents are currently in progress.

Poster No. IV-18

Characterization of intracellular trafficking vesicles of GLYT2

Rodenstein L, Aragón C, & López-Corcuera B, Centro de Biología Molecular "Severo Ochoa" Facultad de Ciencias, Universidad Autónoma de Madrid, Spain

The neuronal glycine transporter GLYT2 is a plasma membrane protein that removes glycine neurotransmitter from the synaptic cleft allowing the termination of the glycinergic signal. GLYT2 undergoes a regulated intracellular trafficking, increasing plasma membrane transporter levels during depolarisation-induced exocytosis in a calcium-dependent manner. Consequently, GLYT2 is present in small intracellular vesicles. The goal of the present report is the qualitative and quantitative characterization of the intracellular vesicles containing GLYT2. For this purpose, different approaches have been used. A highly pure preparation of synaptic vesicles from rat brainstem synaptosomes was obtained, and the presence of several marker proteins was quantified through immunoblot and densitometric analysis. The preparation, strongly enriched in synaptic vesicle proteins and mainly devoid of plasma membrane contaminants, contains GLYT2 more abundantly than other plasma membrane transporters such as the GABA transporter GAT1 or the glucose transporter GLUT3. Quantitative immunogold labelling and electron microscopy of the purified vesicles demonstrate that GLYT2 is localized in clear vesicles of around 50 nm of diameter where synaptophysin, synaptobrevin and other synaptic vesicle proteins colocalize. However, very few of the GLYT2-containing vesicles include the vesicular GABA/glycine transporter (VIAAT). Immunoisolation experiments also support the synaptic-like nature of the GLYT2-containing vesicles.

Poster No. IV-19

Identification of proteins that interact with glycine transporter GlyT2

Scholze, P, Max-Planck-Institute for Brain Research, Frankfurt/Main, Germany

Glycine serves as the major inhibitory neurotransmitter in brainstem and spinal cord by binding and activating glycine receptors. In addition, glycine acts as a co-agonist at ionotropic glutamate NMDA (N-methyl-D-aspartic acid) receptors throughout the central nervous system. At both receptors, the concentration of glycine in the synaptic cleft must be tightly regulated. The primary means of modulating extracellular levels of glycine is by re-uptake through high-affinity glycine transporters. To date, two different sodium and chloride dependent glycine transporters have been identified: GlyT1, which is mainly located in the plasma membrane of glial cells, and GlyT2, which is primarily found in neurons.

In the past years it has become increasingly clear that ion channels as well as neurotransmitter transporters do not exist simply as isolated macromolecules at the pre- or postsynaptic membrane in vivo, but are tightly associated with anchoring or regulatory proteins. Therefore, the aim of the current project was to identify proteins interacting with intracellular regions of the glycine transporter GlyT2.

We performed a yeast two-hybrid screen of a rat brain cDNA-library using the intracellular carboxyterminal tail of GlyT2 as bait and identified the PDZ domain protein syntenin-1 as an intracellular binding partner of this transporter. In pull-down experiments, the interaction between GlyT2 and syntenin-1 was found to involve the C-terminal amino acid residues of GlyT2 and the PDZ2 domain of syntenin-1. Syntenin-1 is widely expressed in brain and colocalizes with GlyT2 in brainstem sections. Furthermore, syntenin-1 binds syntaxin 1A, which is known to regulate the plasma

membrane insertion of GlyT2. Thus, syntenin-1 may be an in vivo binding partner of GlyT2 that regulates its trafficking and/or presynaptic localization in glycinergic neurons.

Poster No. V-1

The link between transient and transport currents in the GABA cotransporter rGAT1 is preserved in low chloride

Bossi E, Soragna A, Pisani R, Giovannardi S, Fesce D & Peres A, Laboratory of cellular and Molecular Physiology, DBSF, University of Insubria, Varese, Italy

We have recently proposed a simple three-state kinetic scheme for rGAT1, in which the presteady-state current in absence of GABA, I_{pre} (due to an intramembrane charge movement) is simply linked to the transmembrane transport-associated current, I_{tr} (Peres et al., 2004 NIPS, 19:80-84). This interpretation suggests a GABA-induced conversion of the capacitive-like behaviour of I_{pre} to the resistive-like behaviour of I_{tr}. At saturating GABA, I_{tr} is given by the product of the amount of displaced charge (Q_{in}, obtained from the integration of the I_{pre} transients) by the relaxation rate of I_{pre} (r), *i.e.:* $I_{tr}(V) = Q_{in}(V) \cdot r(V)$. We have extended this interpretation, analysing the effects of low external Cl^- on the electrophysiological properties of rGAT1 expressed in Xenopus oocytes. In presence of saturating GABA and in low Cl^-, I_{tr} is reduced at moderate potentials, while it is increased at more negative voltages; correspondingly, the Q_{in} vs V curve is negatively shifted, and the rate r becomes faster at moderate potential, remaining substantially unaltered at more negative voltages. Despite the diversity and complexity of these effects, whose bases remain to be clarified, the relationship introduced above maintains its validity. Our model also predicts an inverse correlation between charge relaxation rate and apparent GABA affinity (Fesce et al. 2002 J.Physiol. 545:739-750). While this prediction is fully verified in different Na^+ concentrations, in low Cl^- the increase in relaxation rate is accompanied by an increase in apparent GABA affinity, suggesting an additional positive effect of low Cl^- on the GABA binding rate. In conclusion, the results in low Cl^- reinforce the hypothesis that substrate binding to rGAT1 converts the intramembrane charge movement into the transmembrane current that goes together with GABA uptake, without strongly altering either the amount of charge moved or the migration rate.

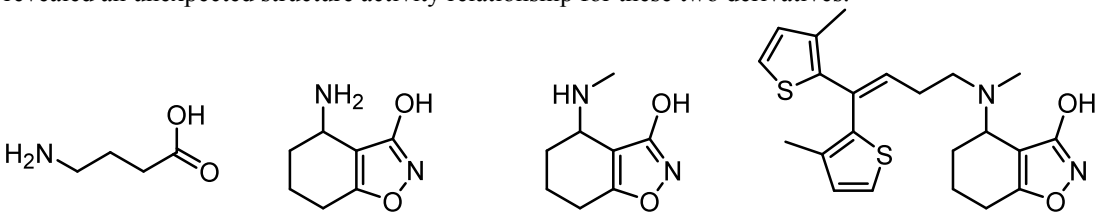
Poster No. V-2

Selective inhibitors of GABA uptake: Lipophilic diaromatic derivatives of *exo*-THPO

Clausen, RP, Department of Medicinal Chemistry, Danish University of Pharmaceutical Sciences, Copenhagen, Denmark

We present the pharmacology of a series of lipophilic diaromatic derivatives of *exo*-THPO as potent inhibitors of GABA uptake. All compounds inhibited uptake into rat brain synaptosomal preparations and several derivatives maintained the increased inhibitory effects at GABA uptake into cortical glia cell cultures compared to neuronal cell cultures, as already seen with *exo*-THPO and in particular N-*Me*-*exo*-THPO.

Surprisingly compound **1** in the series turned out to have a new and different pharmacological profile at the cloned transporter subtypes GAT1-4 as compared to similar structures. The separate synthesis of the enantiomers of **1** revealed an unexpected structure activity relationship for these two derivatives.



GABA *exo*-THPO *N*-Me-*exo*-THPO **1**

Since a majority of the compounds penetrate the blood brain barrier, as judged by their anticonvulsive effects *in vivo*, several of the compounds are potential pharmacological tools in investigating the significance of inhibition of glial uptake or inhibition of uptake mediated by transporter subtypes using compound **1**. Comprehensive *in vivo* characterisation employing compound **1** is now ongoing.

Poster No. V-3

Novel *Seco*ergoline Derivatives Inhibit Both GABA and Glutamate Uptake in Rat Brain Homogenates: Synthesis, In Vitro Pharmacology and Modelling

Héja L, Kovács I, Szárics É, Incze M, Temesváriné-Major E, Dörnyei G, Peredy-Kajtár M, Gács-Baitz E, Szántay C & Kardos J, Chemical Research Center, Hungarian Academy of Sciences, Budapest, Hungary

Here we report on the inhibitory effects of a series of novel *seco*ergoline derivatives on [^{3}H]GABA and [^{3}H]D-aspartate uptake in plasma membrane vesicle suspensions isolated from the rat cerebral cortex. Three of twelwe secoergoline derivatives (**8**, **9** and **11**) containing bioisosteric sequences of GABA and Glu inhibited both GABA and

Glu uptake whereby they appeared to be equipotent inhibitors with IC_{50} values between 270-1100 μM. In the presence of GABA and Glu transport-specific non-transportable inhibitors, inhibition of GABA and Glu transport by **8**, **9** and **11** proceeded in two phases. The two phases of inhibition were characterised by IC_{50} values between 4-180 nM and 360-1020 μM and different selectivity sequences. These findings may indicate the existence of some mechanism possibly mediated by a previously unrecognised GABA-Glu transporter.

Derivatives with the *cis*, but not the *trans* configuration (**8** *vs.* **7** and **11** *vs.* **12**) of bulky ester groups showed significant inhibitory effect. The *cis-trans* selectivity can be explained by docking these *seco*ergolines in a three-dimensional model of the second and third transmembrane helices of GABA transporter type 1. Based on modelling studies, three residues (Asn-137, Ser-133 and Thr-89) have been identified (besides the previously recognized Tyr-140), which could possibly be involved in ligand binding.

Poster No. V-4

Development of a Pharmacophore Model for the GABA Transporter GAT1

Høg S, Clausen RP, Brehm L, Frølund B & Greenwood JR, Department of Medicinal, The Danish University of Pharmaceutical Sciences, Copenhagen, Denmark

In order to investigate the structural requirements of the GABA transporter GAT1[1] a pharmacophore model for GAT1 has been developed. On the basis of available structure-activity data, a consistent alignment of GABA uptake inhibitors was developed, indicating a common binding mode at GAT1 of the GABA uptake inhibitors included in this study.

The initial alignment was based upon low energy conformers of seven structurally diverse GABA uptake inhibitors. The conformational space of the GABA uptake inhibitors was searched employing MM3* and the GB/SA solvation model as implemented in MacroModel 8.1.[2] By fitting known ligands to the initial alignment, information about the space available for ligands in the binding pocket (receptor-excluded volume) of GAT1 as well as some areas of the binding pocket not available for ligands (receptor-essential volumes) has been obtained.

On the basis of the developed pharmacophore model, we have been able to explain the GABA uptake inhibitory activity of a series of previously tested compounds,

[1]Borden L A. Transporter Heterogeneity: Pharmacology and cellular localization. *Neurochem Int* 1996; 29; 335-356

Poster No. V-5

Pharmacological properties of glycine transport in the rat retina

Salceda, R, Departamento de Neurociencias. Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, México D.F., México

The high affinity glycine transport is the primary means for inactivating synaptic glycine. Two different transport genes, named Glyt-1 and Glyt-2 have been cloned. Immunohistochemical studies indicated the occurrence of Glyt-1 in the retina , but there is no evidence for the expression of Glyt-2 transporter. We performed a pharmacological characterization of glycine transport in the rat retina at different postnatal ages.

^{3}H-glycine uptake increased linearly during the two first weaks of postnatal age reaching maximum values at 12 days age, then decreased to the adult values at 18 days age. A high affinity transport system with an apparent Km of 200 and 100 μM in the adult and immature retina, respectively was found. Glycine uptake was Na^+-dependent at all ages studied. The sodium Hill coefficient uptake in the adult retina was 1.8. Glycine accumulation in the retina was insensitive to staurosporine and phorbol esters and slightly inhibited by okadaic acid. The glycine analogue sarcosine at 1.0 mM concentration reduced 40% glycine uptake in both adult and immature retina.Besides,100μM of amoxapine, a selective Glyt-2 blocker, inhibited glycine uptake by 40%.

In addition of the occurrence of Glyt-1 in the retina ,our results provide evidence of the presence of Glyt-2 and / or another isoform of the glycine transporter. A possible role of glycine transporter in retina development is suggested.

Poster No. V-6

Pharmacological characterization of GABA transporter and their heterogeneous functions: Implications of astrocytic GABA transport and protein-protein interactions

Sarup A, Larsson OM & Schousboe A, Department of Pharmacology, The Danish University of Pharmaceutical Sciences, Copenhagen, Denmark

The GABAergic inhibitory tone in the central nervous system is pivotal for controlling excitatory neurotransmission. The concerted action of GABA transporters (GAT1-4) fine-tunes the extracellular GABA concentration at synaptic and extra-synaptic loci. Neuronal GAT1-mediated GABA transport has been extensively characterized and is target for the anti-epileptic drug tiagabine. In contrast, the functional importance of GAT2-4 and astrocytic GABA uptake as modulators and inactivation agents of the GABAergic neurotransmission has been much less explored. Recent studies have demonstrated that selective inhibition of astrocytic GABA transport provide potent anticonvulsant efficacy. Moreover, a novel selective lipophillic *N*-methyl-*exo*-THPO analog with potent inhibitory effect on two different GATs display an unexpected *in vivo* pharmacological profile. Two lines of investigations in our current research aim at a further characterization of these differential physiological roles of the GAT subtypes. Rat thalamus neurons *in*

vivo lack GAT1 protein expression whereas both GAT1 and GAT4 are robustly expressed by thalamic astrocytes. We have investigated the GABA transport kinetics and inhibitory effect of *N*-methyl-*exo*-THPO on GABA transport in primary cultures of thalamic astrocytes. The IC_{50} for *N*-methyl-*exo*-THPO in this preperation is 39 μM. The K_m for the saturable GABA transport was 37 μM. The observation that two GABA transporter subtypes are expressed by thalamic astrocytes *in vivo*, raises the possibility that GAT subtypes might be co-regulated and even biophysically interact. The latter is investigated by Confocal Förster Energy Resonance Transfer and Fluorescence lifetime imaging using fusion proteins of GFP^2/EYFP with GAT subtypes.

BENZON SYMPOSIUM No. 51

NEUROTRANSMITTER TRANSPORTERS: BASAL FUNCTION AND DRUG TARGETS

AUGUST 9-12, 2004, COPENHAGEN, DENMARK

Organizing committee:
Ulrik Gether, Orla Miller Larsson, P. Høngaard Andersen, Arne Scousboe
Povl Krogsgaard-Larsen and Sven Frøkjær

Abstracts - THURSDAY, August 12, 2004

Physiological characterization and disease phenotypes of glycine transporter knockout mice
Betz H, Max-Planck-Institute for Brain Research, Frankfurt, Germany
Glycine is a major inhibitory neurotransmitter in the mammalian CNS. Upon Ca^{2+}-triggered release from glycinergic nerve terminals, glycine causes postsynaptic inhibition by binding to strychnine-sensitive glycine receptors (GlyRs). Glycinergic neurotransmission is terminated by the reuptake of glycine into glycinergic nerve terminals and neighbouring glial cells. This reuptake process is mediated by specific Na^+/Cl^--dependent glycine transporters, GlyT1 and GlyT2.

We have analyzed the structure and physiological roles of these membrane protein by using molecular approaches and by generating transporter deficient mice. Our data are consistent with the glial transporter GlyT1 catalyzing the removal of glycine from the synaptic cleft, whereas GlyT2 is required for the reuptake and reloading of glycine into synaptic vesicles. Both GlyT1 and GlyT2 are essential for CNS function, as revealed by the lethal disease phenotypes of the respective knockout mice. GlyT1 deficient mice suffer from severe over-inhibition and phenotypically mimick human glycine encephalopathy, whereas GlyT2 deletion causes over-excitation with persistent convulsions as seen in severe forms of hyperekplexia.

References:
J. Gomeza, S. Huelsmann, K. Ohno, V. Eulenburg, K. Szoeke, D. Richter and H. Betz. *Neuron* 40 (2003) 785--796.
J. Gomeza, K. Ohno, W. Armsen, B. Laube and H. Betz. *Neuron* 40 (2003) 797-806.

Dendritic release of dopamine by mid-brain dopaminergic neurons: reverse transport or exocytosis?
Mintz I, Northwestern University Feinberg School of Medicine, Chicago, USA
Two mechanisms have been proposed to mediate the dendritic release of dopamine in mid-brain nuclei, reverse transport of dopamine or Ca-dependent exocytosis. To distinguish between these two possibilities, we have used two-photon imaging of Ca or Na indicators (Oregon Green 488 or SBFI), in rat parasagittal brain slices, and monitored the Ca signals or Na signals in dendrites of Substantia Nigra dopaminergic neurons during the dendritic release of dopamine (DA). Release was triggered by stimulation of these cells subthalamic (STN) input, and recorded as a D2-mediated inhibition (D2-IPSP) in the whole-cell patch-clamp configuration. As a control for detection of Na signals during reverse transport, we monitored the changes in cytosolic Na produced by bath application of veratridine.

In cells silenced with bias current injection, STN stimulation triggered local nimodipine-sensitive Ca signals, which suggest gating of L-type Ca channels by the subthalamic synapse. In conditions that optimize detection of D2-IPSPs (after blockade of glutamate ionotropic receptors with CNQX (10 μM) and D-APV (25 μM) and reduction of STN-EPSPs to less than 0.5 mV), the dendritic release of DA occurred without any measurable increase in the cell excitability (membrane depolarization or increased spike frequency), or changes in dendritic Ca or Na concentration.

These observations suggest that dopamine release can occur without the large increases in dendritic Ca, that follow activation of high-threshold Ca channels or internal Ca release. They are equally hard to reconcile with DA reverse transport, which, in the classic model of a 2:1:1 cotransport of Na, Cl and dopamine, is driven by membrane depolarization and large (> 50 mM) increases in cytosolic Na concentration.

(Supported by R01 NS36795).

Glutamate transporter in disease

Trotti D, Massachusetts General Hospital, Harvard Medical School, Charlestown – Boston, USA

Impairment and loss of the glutamate transporter GLT1 (a.k.a. EAAT2) has been reported in both sporadic and familial cases of amyotrophic lateral sclerosis (ALS) as well as in rodent models of the disease. Caspase-3 (cp-3) is pathologically activated in transgenic SOD1 mice model of ALS and its activation has been described both in neurons and astrocytes in the spinal cord. GLT1 is one of five high affinity glutamate transporters and it is responsible for the reuptake of more than 90% of the released glutamate. GLT1 has a predominant glial localization and has one cp-3 putative site in its cytoplasmic, C-terminal domain (-DTID-S). As motor neurons depend on GLT1 uptake in astrocytes to avoid excitotoxicity, it is possible that in ALS GLT1 becomes a substrate for cp-3 cleavage and that the resulting impairment of the transporter leads to excitotoxic damage of the motor neurons. In the present study, we investigated whether the glutamate transporter GLT1 could be a substrate for activated cp-3. We found that GLT1 is cleaved *in vitro* by active cp-3 and that the cleavage occurs at the consensus site at the aspartate residue in position 505. Other caspases such as caspase-7/8 or 6 do not cleave GLT1. GLT1 cleavage appears to be selective as the other mayor glial glutamate transporter GLAST is insensitive to cp-3 activation. Functionally, cp-3 activation causes a time-dependent impairment of GLT1. *Xenopus* oocytes expressing human GLT1 (EAAT2) and injected with the active form of cp-3 showed a progressive inhibition of GLT1-mediated uptake current and uptake of substrate (~60% within 30 min), paralleled by a loss of GLT1 immunoreactivity. Cp-3 cleavage of GLT1 occurs also in ALS SOD1-G93A mice and SOD1-H46R rats. In these animal models of ALS, the time-course of appearance of the cp-3-derived GLT1 fragments paralleled the time-course of cp-3 activation during the disease progression. In conclusion, we have demonstrated that cp-3 cleaves and inactivated the glutamate transporter GLT1. This event occurs *in vivo* in SOD1 transgenic animal models of ALS, and likely contributes to the excitotoxic damage to motor neurons in ALS.

Modulation of GABA and glutamate transporter function: Implications for the treatment of epilepsy and other CNS disorders

White HS, University of Utah Dept. Pharmacology & Toxicologym Salt Lake City, Utah, USA

Within the central nervous system (CNS) the amino acids glutamate and gamma amino butyric acid (GABA) modulate excitatory and inhibitory neurotransmission, respectively. The concentration of both neurotransmitters within the synaptic and extrasynaptic space is tightly regulated by neuronal and glial transporter proteins. To date, five different high-affinity glutamate transporters (i.e., GLT1, GLAST, EAAC1, EAAT4 and EAAT5) and three different high-affinity (GAT1-3) and one low-affinity (BGT1) GABA transporters have been cloned. Increased release or reduced uptake of glutamate has been associated with excessive excitation and neurotoxicity. In addition, prolonged elevation of synaptic glutamate contributes to neuronal cell loss and hyperexcitability in a number of neurological disorders including epilepsy, amyotropic lateral sclerosis and neuronal loss associated with focal and global hypoxia / ischemia. As such, strategies that either decrease neuronal release or enhance reuptake of glutamate would be expected to protect nervous system tissue from excitatory mediated neurotoxicity and attenuate glutamate-mediated hyperexcitability.

The approval and subsequent registration of the selective GAT1 inhibitor tiagabine as an antiepileptic drug for the treatment of partial epilepsy clearly demonstrates the functional importance of GABA transporters as therapeutic targets. GAT1 is localized to both the neuronal and glial compartment of the brain. Experimental findings with neuronal and glial specific GAT1 inhibitors suggest that there may be distinct advantages to selectively inhibiting glial GABA transporters (White et al., JPET. 302:636-644, 2002). Although no clear functional role for the other GABA transporters has been established, the finding that they display regional and cell type specific localization suggests that they might play important roles within the CNS. The therapeutic opportunities for drugs that modulate glutamate and GABA transporter function will be reviewed and discussed.

Poster No. VI-1

Localization of Photoaffinity Labeling Sites of Inhibitors at the Human Dopamine Transporter

Justice JB[1], Wirtz S[1], Pham A[1], Jackson T[1], Lever JR[2], Zou M[3], Newman AH[3], Parnas L[4] & Vaughan RA[4], [1]Department of Chemistry, Emory University, Atlanta, Georgia, USA, [2]Department of Radiology, University of Missouri-Columbia, Columbia, Missouri, USA, [3]Medicinal Chemistry Section, National Institute on Drug Abuse – Intramural Research Program, Baltimore Maryland, USA, [4]Department of Biochemistry and Molecular Biology, University of North Dakota, Grand Forks, North Dakota, USA

The covalent sites of attachment at the human dopamine transporter (hDAT) of radiolabeled photoaffinity ligands such as ^{125}I MFZ 2-24, a tropane based irreversible cocaine analog, have been investigated. Following labeling of membrane preparations, the hDAT is solubilized and separated on a 7.5% SDS PAGE gel. The radioactive band at 80 kDa is excised and digested by trypsin, chymotrypsin, or cyanogen bromide and run on a 16.5% SDS PAGE gel. The radiolabeled peptides are extracted and separated by HPLC. The radioactive fractions are subjected to further digestion (thermolysin, Pro C, cyanogen bromide) and reanalyzed by HPLC. CNBr digest of ^{125}I MFZ 2-24 labeled hDAT produced a single small peptide, very few of which occur in a CNBR digest of hDAT. The HPLC retention time is

consistent with the CNBr peptide PLFYM in TMD2. Additional digests, including chymotryptic digestion of the CNBr peptide and tryptic digests of hDAT followed by CNBr, further supported this interpretation.

Poster No. VI-7

Signal Transduction by Glutamate Transporters: Role of Caspase-Dependent Truncation of Cytoplasmic Domains

Rodriguez-Kern A, Gegelashvili M, Zhang J, Sung L & Gegelashvili G, Department of Pharmacology, DUPS, Copenhagen, Denmark, and BioSignal, Torrance, USA

Malfunctioning or aberrant expression of high-affinity glutamate transporters amplifies the excitotoxic component in neurodegeneration. In neural cells, glutamate transporters EAAC1 and GLT1 at their cytoplasmic C-terminal domains are cleaved by caspases that are activated by low sublethal doses of some pro-apoptotic agents (e.g. Alzheimer's amyloid beta peptide, glutamate receptor ligands). Such a proteolytic modification of EAAC1, a first reported case among the neurotransmitter carriers, alters the cell surface targeting of this transporter, as well as triggers downstream signaling events that affect MAP kinase phosphorylation and, unexpectedly, induce the expression of another glutamate transporter, EAAT4. Such an unusual signaling by glutamate transporters depends on functional modification of their C-terminal domains and is partially mediated by peptides that are produced by the caspase-dependent cleavage. These soluble peptides contain several short sequences or conserved motifs that may functionally interact with other cytoplasmic or nuclear signaling complexes. For example, the truncation of EAAC1 produces peptides that contain PDZ domain-binding motif, as well as a stretch of amino acid residues present in 82-FIP, a novel RNA- binding protein that also interacts with FMRP (fragile X mental retardation protein). In conclusion, glutamate transporters, that represent novel targets for caspases, exhibit unusual signal-transducing properties that could possibly affect neuronal function in some neurological and cognitive disorders.

Poster No. VI-3

Do mice still like cocaine when their dopamine transporter is resistant to cocaine inhibition?

Chen R, Han D & Gu H, Ohio State University, Departments of Pharmacology and Psychology, Ohio, USA

Cocaine's addictive and rewarding effects have been proposed to be primarily mediated by its blockade of the dopamine transporter (DAT). However, the deletion of the DAT gene in mice does not abolish cocaine-induced conditioned place preference (CPP) and cocaine self-administration, which are commonly used to measure cocaine's rewarding effects. The likely functional compensation for lacking the DAT gene in the knockout mice may have confounded the precise role of the DAT in the cocaine-rewarding pathway. Therefore, a novel approach was employed to engineer a mouse line carrying a functional mutant DAT with cocaine resistance. By using species scanning mutagenesis, we have identified F105 in transmembrane domain II of DAT as an important residue for high affinity cocaine binding. Further random mutagenesis around F105 gave us a triple mutant with 50 folds more resistance to cocaine than the wild type when expressed in cultured cells, whereas the DAT activity from the mutants retains 70% of that from the wild type. A knock-in mouse line has been generated with the wild type DAT replaced by the cocaine resistant mutant. We are now in the process of producing enough homozygous mutant mice for experiments. In the next several months, we will analyze the cocaine effects in biochemical and behavioral tests. The responses of these mutant mice to cocaine will provide more precise information on the role of DAT in cocaine rewarding and other cocaine induced effects.

Poster No. VI-4

Antipsychotic potential of GlyT-1 inhibitors

Didriksen M, Hertel P, Mørk A & Arnt J, H. Lundbeck A/S, Valby-Copenhagen, Denmark

Reduced NMDA receptor-mediated glutamatergic neurotransmission is assumed to be an important feature contributing to the schizophrenic symptoms. In contrast to dopaminergic agonists, non-competitive NMDA receptor antagonists induce a syndrome indistinguishable from schizophrenia including positive and negative symptoms and cognitive deficits. It is hypothesised that augmentation of the NMDA receptor function will have antipsychotic potential. Activation of the NMDA receptor complex by compounds acting at the glutamate-binding site has been plagued by severe side effects. However, stimulating the glycine co-agonist site may be a suitable approach. In the present experiments the effect of the glycine transport type-1 (GlyT-1) inhibitor NFPS ({[3-(biphenyl-4-yloxy)-3-(4-fluoro-phenyl)-propyl]-methyl-amino}-acetic acid) on the extracellular level of glycine, augmentation of the NMDA response and effect in models related to psychosis was investigated. NFPS dose-dependently increased the extracellular level of glycine in the ventral hippocampus of freely moving rats. Systemically administered NFPS enhanced dopamine output in the nucleus accumbens elicited by local VTA injection of NMDA indicating an enhanced NMDA receptor-mediated neurotransmission *In Vivo*. NFPS did not antagonise PCP-induced hyperactivity in mice whereas amphetamine-induced hyperactivity in rats was dose-dependently reversed. The cognitive impairment in rats induced by PCP in the Morris' Water Maze was fully reversed by NFPS. In conclusion, inhibition of the GlyT-1 may have beneficial effect on psychotic symptoms as well as cognitive deficits.

Serotonin transporter: Structure, function and regulation

Rudnick R, Department of Pharmacology, Yale University School of Medicine, New Haven, CT, USA

A rare coding mutant of serotonin transporter (SERT), in which Ile-425 is replaced with valine, has been found in patients with several psychiatric disorders including obsessive-compulsive disorder (OCD), anorexia nervosa, and Asperger's syndrome. The severity of these disorders appears to correlate with the combined presence of I425V and the long form of the 5HTT gene promoter which leads to increased SERT expression levels. Expression of SERT I425V in HeLa and COS-7 cells in culture demonstrated increased transport by this mutant relative to wild type SERT. Agents that stimulate cGMP-dependent protein kinase increase the activity of wild type SERT but not the I425V mutant. Agents that inhibit the kinase or cGMP synthesis decrease the activity of I425V but not wild type. These data are consistent with the proposal that SERT is phosphorylated by cGMP-dependent protein kinase and that the phosphorylated form is more stable in molecules bearing the I425V mutation. Mutations at putative phosphorylation sites alter the kinetics of transport and the response to kinase activation in a manner that is consistent with this proposal.

Genetic variation and transporter pharmacology

Madras, BK, Harvard Medical School, NEPRC, Southborough, MA, USA

Attention deficit hyperactivity disorder (ADHD) affects approximately 4% of children and may persist into adulthood. Although the pathophysiology of ADHD is poorly understood, the central role of dopamine and norepinephrine in anti-hyperactivity medications implicates brain catecholamine systems in ADHD. The dopamine transporter (DAT), an important regulator of extracellular dopamine, is a principal target of anti-hyperactivity medications (e.g. methylphenidate, d-amphetamine) in brain. At the molecular level, the 3'-untranslated region of the dopamine transporter gene varies in length due to a polymorphic variable number tandem repeat (VNTR) polymorphism. The repeat region varies from 3 to 11 copies but alleles with 10 copies of the 40-base repeat unit have been associated with ADHD. The findings account for a small fraction of the variance, but an association has been reported in a majority of studies. A link between polymorphisms in the dopamine transporter gene in ADHD and regulation of dopamine transporter density would be supported if abnormal levels of the dopamine transporter are expressed in brain of subjects with ADHD. The density of the dopamine transporter, measured by Single Photon Emission Computed Tomography (SPECT) or Positron Emission Tomography (PET), was elevated in some, but not all studies. The association between DAT density and the 10/10 repeat was furthermore inconsistent, suggesting the need to broaden the search for single nucleotide polymorphisms also present in the 3'-UTR of the DAT gene. To expand the focus of ADHD further, we investigated the potential contribution of trace amines and trace amine receptors to ADHD. The trace amine phenylethylamine (PEA) reportedly is reduced in urine of children with ADHD and PEA levels can be elevated with anti-hyperactivity medications. (PEA is robustly transported by the dopamine and norepinephrine transporter and methylphenidate is a potent inhibitor of PEA transport. Furthermore, PEA is a potent agonist at the trace amine receptor subtype 1. Based on these data, we postulate that, as a substrate for monoamine transporters, PEA may contribute to the therapeutic efficacy of anti-hyperactivity medications. Support: DA06303, DA15305, RR00168

Poster No. VI-5

Histological and Immunological Analysis of GlyT2 Deficient Mice

Armsen A, Max-Planck-Institute for Brain Research, Frankfurt, Germany

The glycine transporter (GlyT) 2 belongs to the family of Na^+-Cl^--dependent neurotransmitter transporters and is localized in the axon terminals of glycinergic neurons. To unreveal its function, our lab has generated mice deficient in GlyT2. These mice are normal at birth but during the second postnatal week develop a lethal neuromotor deficiency that resembles severe forms of human hyperekplexia (hereditary startle disease) and is characterized by spasticity, tremor, and an inability to right. These behavioral and additional electrophysiological results are consistent with GlyT2 having a crucial function in efficient transmitter loading of synaptic vesicles during postnatal life.

In order to detect possible developmental abnormalities in GlyT2 -/- mice that could explain their lethal phenotype, a general histological analysis was performed. Different forms of human hyperekplexia are known to be caused by mutations in glycine receptor (GlyR) structural genes. Since the neurological symptoms seen in GlyT2 -/- mice coincide with the postnatal replacement of the neonatal GlyRα2 by the adult GlyRα1 subunit, we examined GlyRα1 expression during postnatal development of the mutant mice. To this end, spinal cord sections were stained using the antibody mAb 2a that specifically detects the GlyRα1 subunit, and mAb 4b that labels all GlyRα subunits. Our results indicate unaltered expression levels and distributions of GlyRα subunits in GlyT2 deficient mice throughout postnatal development. Hence, the GlyT2 -/- phenotype cannot be explained by an impaired switch in GlyRα subunit isoform expression.

In addition, we examined a possible contribution of the GABAergic system to the phenotype of GlyT2 -/- mice. Both immunocytochemical and uptake data will be presented.

Poster No. VI-6

Phenotypical analysis of Glycine transporter 1 knockout mice

Eulenburg V, Max-Planck-Institute for Brain Research, Frankfurt, Germany

The extracellular concentration of glycine in the CNS is regulated by two different Na+ Cl- dependent neurotransmitter transporter GlyT1 and GlyT2.

GlyT1 is widely expressed in glial cells of the hippocampus, cortex and cerebellum, as well as brain stem and spinal cord, whereas GlyT2 is found predominantly in brain stem and spinal cord neurons and concentrated in the plasma membrane of axonal boutons directly apposed to glycine receptors. Consistent with these localizations, GlyT2 has been proposed to provide the principal uptake mechanism at inhibitory glycinergic synapses, whereas GlyT1 is thought to regulate glycine levels at the glycine co-agonist binding site of NMDA receptors.

To investigate the *in-vivo* role of the GlyT1 transporter more directly we generated knockout mice using a classical gene targeting approach. The GlyT1 knockout mouse die in the first 24 h after birth, showing a strong hypotonic phenotype accompanied by respiratory depression and an inability to suckle. Biochemical and immunhistochemical data failed reveal any morphological differences. Electrophysiological recordings from the hypoglossal motoneurons from GlyT1 -/- mice revealed a strong activation of strychnine sensitive glycine receptors caused by accumulation of glycine in the synaptic cleft. Taken together these data prove that in the neonatal animal GlyT1 plays an essential role for the regulation of glycine concentrations in the cerebrospinal fluid in brainstem / spinal cord.

Poster No. VI-8

Downregulation of the Astrocytic Glutamate Transporters EAAT1 and EAAT2 in Wernicke's Encephalopathy

Hazell AS, [*]Danbolt NS & [†]Sheedy D, Department of Medicine, University of Montreal, Canada; [*]Department of Anatomy, University of Oslo, Oslo, Norway, [†]Department of Pathology, University of Sydney, Australia

Recent studies have described a loss of the astrocytic glutamate transporters GLAST and GLT-1 in thiamine deficiency, a model of Wernicke's encephalopathy (WE) (Hazell et al., 2001: *J. Neurochem.* **78,** 560-568) in which severe nutritional status and chronic alcoholism lead to focal lesions in brain regions that include the thalamus and cerebral cortex. We have now studied the human analogues of these transporters, EAAT1 and EAAT2 respectively, in order to evaluate their role in the sequelae of events underlying the pathophysiology of this disorder. Samples of frontal cortex were obtained at autopsy from 5 individuals with neuropathologically confirmed WE and 5 age-matched controls. Tissues were received from the NSW Tissue Resource Centre, supported by the University of Sydney, Neuroscience Institute of Schizophrenia and Allied Disorders, National Institutes of Alcohol Abuse and Alcoholism and NSW Department of Health. Western blotting revealed a 71% loss of EAAT2 in WE cases relative to controls and a 62% reduction of EAAT1. Loss of both transporter sites was confirmed by immunohistochemical methods. These changes were accompanied by an 81% decrease in astrocyte-specific GFAP content but were not due to a loss of astrocytes since glial cell numbers were increased in this brain region. While actin levels were unchanged, α-internexin and synaptophysin content were decreased by 67% and 52% respectively, suggesting a considerable loss of both axons and synaptic terminals in this brain region. Our findings indicate that alterations of glutamatergic neurotransmission mediated by a loss of EAAT1 and EAAT2 transporters may play an important role in damage to the frontal cortex of WE patients. [Funded by the Canadian Institutes of Health Research]

Poster No. VI-9

Zn^{2+} inhibits glycine transport by glycine transporter subtype 1b:

Ju P, Aubrey KA & Wandenberg RJ, Department of Pharmacology, Institute for Biomedical Research, University of Sydney, Sydneu, Australia

In the central nervous system, glycine is a coagonist with glutamate at the NMDA subtype of glutamate receptors and also an agonist at inhibitory, strychnine sensitive glycine receptors. The GLYT1 subtypes of glycine transporters are responsible for regulation of glycine at excitatory synapses whereas a combination of GLYT1 and GLYT2 subtypes of glycine transporters are used at inhibitory glycinergic synapses. Zn^{2+} is stored in synaptic vesicles with glutamate in the brain and is believed to play a role in modulation of excitatory neurotransmission. In this study we have investigated the actions of Zn^{2+} on the glycine transporters, GLYT1b and GLYT2a expressed in *Xenopus laevis* oocytes and demonstrate that Zn^{2+} is a non-competitive inhibitor of GLYT1 but has no effect on GLYT2. We have also investigated the molecular basis for these differences and the relationship between the Zn^{2+} and the proton binding sites on GLYT1. Using site-directed mutagenesis, we identified two histidine residues, His243 in the large second extracellular look (ECL2) and His410 in the fourth extracellular loop (ECL4), as two coordinates in the Zn^{2+} binding site of GLYT1b. In addition, our study suggests that the molecular determinants of proton regulation of GLYT1b are localized to the two histidine residues of ECL4. The ability of Zn^{2+} and protons to regulate the rate of glycine transport by interacting with residues situated in ECL4 of GLYT1b suggests that this region may influence the substrate translocation mechanism.

Poster No. VI-10

Neurochemical evidence for the uptake and release of norepinephrine by serotonergic terminals in NET knock-out mice: implications for the action of SSRIs

Kiss JP, Zsilla G, Caron MG* & Vizi ES, Department of Pharmacology, Insitute of Experimental Medicene, Budapest, Hungary, *Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC, USA

Our aim was to investigate the functional properties of the noradrenergic system in genetically modified mice lacking the norepinephrine transporter (NET). We measured the neuronal uptake and release of [^{3}H]norepinephrine ([^{3}H]NE) from hippocampal and cortical slices of NET(-/-) knock-out (KO) and NET(+/+) wild-type (WT) mice and investigated the presynaptic α_2-adenoceptor-mediated modulation of NE release *in vitro* and *in vivo*.

The neuronal [^{3}H]NE uptake was reduced to 8.5% (hippocampus) and 25.6% (frontal cortex) of WT control in KO mice, and this residual uptake was further decreased by 80 and 100%, respectively, when a selective serotonin reuptake inhibitor (SSRI) citalopram was present during the loading. The more preserved neuronal release of [^{3}H]NE (hippocampus: 26.1%, frontal cortex: 72.2%; compared to WT) almost completely disappeared in both regions (5.9 and 4.7% of KO, respectively) in the presence of citalopram, suggesting that [^{3}H]NE was taken up and released by serotonergic varicosities. This was further supported by the finding that the release of [^{3}H]NE from hippocampal slices of KO mice was not modulated by the α2-adrenoceptor antagonist CH-38083, while the endogenous release of NE measured by microdialysis was even more efficiently enhanced by this drug in the NET deficient mice.

These data indicate that serotonergic varicosities can accumulate and release NE due to the heterologous uptake of transmitters, therefore a functional cooperation exists between the noradrenergic and serotonergic systems in the brain. Since the diffusion of NE may be spatially limited by 5-HT transporters, the SSRIs, in spite of their selectivity, might enhance not only serotonergic but also noradrenergic neurotransmission, which might contribute to their antidepressant action.

Poster No. VI-11

Hemicholinium-3 sensitive choline carriers and Alzheimer disease

Krištofiková Z, Prague Psychiatric Centre, Prague, Czech Republic

Numerous data in literature indicate marked impairment of basal forebrain cholinergic neurons in patients with Alzheimer disease. High-affinity choline transport system specifically localized on presynaptic cholinergic nerve terminals operates through hemicholinium-3 sensitive carriers as a key regulatory step in the synthesis of acetylcholine. Our previous experiments suggested alterations in the activity rather than in the number of the carriers in the hippocampus of demented patients when compared to age-matched controls. On animal models of Alzheimer disease and in experiments in vitro, nonaggregated and aggregated amyloid beta peptides eliminate the activity of hemicholinium-3 sensitive carriers, perhaps via direct binding to choline recognition site, and also inhibit the synthesis of acetylcholine in this way. Recent research suggests associations between brain cholesterol and Alzheimer disease. Although the role of cholesterol is not fully clear, it seems that cholesterol can influence the effects of amyloid beta peptides on neural membranes. The current study evaluates in vitro effects of nonaggregated and aggregated amyloid beta peptides 1-40 and 1-42 on rat hippocampal synaptosomal high-affinty choline transport and membrane fluidity. Measurements were performed on synaptosomes and membranes with altered membrane cholesterol content (depletion by cyclodextrin) or influenced via treatment of 24S-hydroxycholesterol (cerebrosterol). Conversion of cholesterol to cerebrosterol is the major pathway for elimination of brain cholesterol and the maintenance of brain cholesterol homeostasis. The experiments indicate the more pronounced effects of amyloid beta peptides and of cerebrosterol on cholesterol-depleted synaptosomes and contribute to the evaluation of mechanisms leading to Alzheimer disease.

The research was performed under GACR grant (305/03/1547)

Poster No. VI-12

Dopamine transporters Inhibitors: Correlation Between Mode of Interaction and Abuse Potential?

Loland CL[1], Katz J[2], Newman AH[2] & Gether U[1], [1]Department of Pharmacology, University of Copenhagen, Copenhagen, Denmark, [2]National Institute of Drug Abuse, NIH, Baltimore, MD, USA

Recently, we have identified a highly conserved intracellular residue in the dopamine transporter (DAT) that upon mutation to an alanine (Y335A) shows a remarkable decrease in apparent affinity for cocaine as determined in [^{3}H]dopamine uptake inhibition assays (Loland et al, PNAS, 2002). We propose that this ~100 fold decrease is not due to a direct interaction with cocaine but due to a major change in the conformational equilibrium resulting in an occluded cocaine binding crevice. We have now tested the change in apparent affinity between WT and Y335A for a series of cocaine and benztropine analogues. Several compounds displayed a change in apparent affinity resembling that of cocaine, whereas others were markedly less affected. The compounds were also administered to rats in a cocaine discrimination test. Despite minor differences in the chemical structure a remarkable correlation was observed between the change in apparent affinity and the ability of rats to recognize the compounds as cocaine: The compounds displaying a large change in apparent affinity between hDAT WT and Y335A were also recognized as cocaine in the cocaine discrimination test. In contrast, the compounds that only displayed a minor change in apparent affinity were

not recognized as cocaine by the rats. These results indicate that the abuse potential of DAT inhibitors might be predicted from their mode of interaction with the transporter. Accordingly, we propose that the conformational state of DAT promoted by a given inhibitor affects its psychostimulatory effects. Further studies will reveal the nature of the conformational states induced by cocaine-like blockers of uptake in comparison to non-cocaine-like blockers.

Poster No. VI-13

Effect of the glutamate uptake inhibitors, L-trans-pyrrolidine-2,4-dicarboxylate and DL-threo-beta-benzyloxyaspartate on neuronal damage and extracellular amino acid levels in vivo and in vitro

Camacho A, Montiel T, Sánchez AE & Massieu L, Instituto de Fisiología Celular. Universidad Nacional Autónoma de México. México, D.F., México

Glutamate extracellular concentration is highly regulated by transporter proteins due to its neurotoxic properties. Dysfunction or reverse activation of these transporters is suggested to induce the accumulation of excitatory amino acids, and neuronal damage associated with brain ischemia and hypoglycemia. We have investigated the effects of the intrahippocampal microdialysis administration of the substrate and the non-substrate glutamate transport inhibitors, L-trans-pyrrolidine-2,4-dicarboxylate (PDC) and DL-threo-beta-benzyloxyaspartate (DL-TBOA), respectively, on the extracellular levels of amino acids and neuronal damage. Administration of DL-TBOA notably increases the extracellular levels of glutamate, aspartate and glycine and severely damages the hippocampal CA1 region and dentate gyrus. PDC administration induces a more discrete elevation of excitatory amino acids and no neuronal damage. Similarly, the intrastriatal injection of DL-TBOA induced extensive damage to the striatum, while PDC had no effect. DL-TBOA toxicity was completely prevented by pretreatment with MK-801. In contrast to the in vivo results, in cultured cerebellar granule neurons 30-min exposure to PDC induces larger increases in glutamate and aspartate levels than DL-TBOA, and reduces cell survival, while DL-TBOA causes no damage. Both inhibitors significantly reduced cell survival after prolonged exposure (24 h). Partial impairment of mitochondrial metabolism induced by a subtoxic concentration of 3-nitropropionic acid, facilitates PDC toxicity but not that of DL-TBOA. Results are relevant to the knowledge of the contribution of glutamate transporters to excitotoxicity and the potential use of glutamate uptake blockers for the prevention of ischemic neuronal damage.

Supported by CONACYT 40306-M and PAPITT (UNAM) IN222503

Poster No. VI-14

Assessing the role of PDZ interactions for dopamine transporter function in vivo: generation of a knock-in mouse incapable of forming PDZ interactions with PICK-1

Nørgaard-Nielsen K, Bjerggaard C, Madsen KL & Gether U, Molecular Neuropharmacology Group, The Panum Institute, University of Copenhagen, Copenhagen, Denmark

The dopamine transporter (DAT) is responsible for the termination of dopaminergic neurotransmission at synapses by removing the transmitter from the synaptic cleft. Still, very little is known about the mechanisms governing DAT regulation and the proteins functionally associated with these regulatory processes. At the extreme C-terminus the DAT possesses a type 2 PDZ (PSD-95/Discs-large/ZO-1 homology) binding sequence shown to interact with PDZ domain proteins such as PICK1. Notably, PDZ domains bind the C-terminus of their target proteins and play a key role in 'scaffolding' and thus in assembling multi-protein complexes in cellular microdomains. Since deletion in the DAT of the PDZ binding sequence -LKV leads to retention of the transporter protein in the endoplasmatic reticulum (ER) it has been assumed that interactions with PDZ domain proteins is required for proper ER export and surface targeting; however, by employing a systematic mutagenesis approach we have previously shown that C-terminal human DAT mutants incapable of forming PDZ domain interactions could still be efficiently targeted not only to the cell surface but also into neuronal processes. Additionally, we have now generated similar results for the murine DAT, thus illustrating that the overall mechanisms underlying the regulation of both human and murine DATs presumably are the same. By applying the homologous recombination strategy we are currently generating knock-in mice of two mutant DATs incapable of forming PDZ interactions (-LLV->-AAA and -LLV->-LLVA) to assess in vivo the significance of PDZ domain interactions for DAT function.

Poster No. VI-15

Genetic polymorphism and ADHD: discovery of novel dopamine transporter inhibitors using *in silico* drug design strategy

Dormán G[1], Forró-Gulyás A[1], Ürge L[1], Darvas F[1], Sasvári-Székely M[2] & Sziráki I[3], [1]ComGenex Inc, Budapest, Hungary, [2]Semmelweis University, Institute of Med. Chemistry, Mol. Biol. and Pathobiochem., Budapest, Hungary, [3]IVAX Drug Research Institute Ltd, Budapest, Hungary

Transporter-assisted uptake of serotonin (SET), noradrenalin (NAT) and dopamine (DAT) have been drug targets for several psychiatric disorders including attention deficit and hyperactivity disorder (ADHD). The objective of this study was to discover novel inhibitors of DAT, as a specific aim of a project focusing on the role of genetic polymorphism of dopaminergic neurotransmission in ADHD. Using *in silico* drug design tools a biased compound set (35 cpds) was selected from a large discovery library (> 200, 000 cpds) synthesized at ComGenex. Based on

characteristic structural elements of known inhibitors of DAT, we first selected 116 compounds representing 13 chemical core structures by using similarity algorithm and Example Mediated Innovation for Lead evolution (EMIL) database. Then we applied Absorption-Distribution-Metabolism-Excretion-Toxicity (ADMET) filtering by MetabolExpert™ and HazardExpert™ softwares. The remaining 35 compounds were tested for DAT inhibition at 10 μM conc. using striatal synaptosomes from rat brain. Twelve compounds were active inhibitors of DAT (> 90 % inhibition), indicating that the *in silico* selection procedure was effective. Out of the 12 compounds 2 inhibited DAT also at 100 nM conc. with no or low inhibitory potency on SET or NAT. These two compounds are considered as leads for optimization and further development.

Poster No. VI-16

Distribution and pharmacology of alanine-serine-cysteine transporter 1 (asc-1) in rodent brain

Helboe L[1], Egebjerg J[1], Møller M[2], Mørk A & Thomsen C[1,*], H. Lundbeck A/S, [1]Biological Research, Valby-Copenhagen, Denmark and [2]Department of Medical Anatomy, The Panum Institute, University of Copenhagen, Copenhagen, Denmark

Functional modulation of the NMDA receptor may provide a treatment paradigm for schizophrenia. The glycine/D-serine co-agonist site may be a feasible target for positive functional modulation of the NMDA receptor which may be accomplished by elevating the endogenous levels of glycine and/or D-serine in the synaptic cleft. To this end we have cloned and characeterized the human Alanine Serine Cysteine-1 (hASC-1) transporter and compared its functional properties with the native Na^+-independent [^{3}H]D-serine transporter in rat cortical membranes. Furthermore, a polyclonal antibody against asc-1 was raised and the specificity of the antibody verified by Western blots performed on membranes prepared from HEK293 cells transiently transfected with the cloned murine asc-1. The antibody was then used to localize the transporter in the brain of two rodent species by using immunohistochemistry at the light and electron microscopical level. Asc-1-immunoreactivity (asc-1-ir) was widely distributed throughout the mouse and rat brain. Areas with high levels of asc-1-ir included hypothalamus, the medial septal area, globus pallidus, entopeduncular nucleus, cingulate and retrosplenial cortices. Moderate asc-1-ir was observed in several areas including layers III and V of the neocortex, thalamus, nucleus accumbens, caudate putamen, bed nucleus of stria terminalis, all amygdaloid nuclei, hippocampus (CA1-CA3 and hilus of the dentate gyrus), as well as several brainstem nuclei. Asc-1 ir was observed as punctuate staining consistent with varicosities matching neuronal cell bodies and dendritic fields. At the ultrastructural level, asc-1-ir was mainly confined in presynaptic terminals. Immunostaining in either glial cell bodies or perivascular sites was not observed and white matter was completely devoid of asc-1-ir. Further, the pharmacology of the Na^+-independent uptake site for [^{3}H]D-serine in rat brain synaptosomal P2 fractions was compared with the substrate specificity of the cloned human asc-1 transporter and a high degree of correlation was demonstrated. Furthermore, an inhibitor of asc-1 infused via the microdialysis probe induced increases in serine and other asc-1 substrates in rat brain. We conclude that asc-1 immunoreactivity is widespread in the brain and limited to neuronal structures and asc-1 may contribute to synaptic clearance of D-serine in brain.

Poster No. VI-17

Orphan transporters from the SLC6 family expressed in kidney and in small intestine are the sodium-dependent amino acid transport system B^0

Romeo E, Ristic Z, Dave MH, Loffing J, Warth R, Wagner CA, Camargo SRM & Verrey F, Institute of Physiology, University of Zurich, Zurich, Switzerland

Transepithelial amino acid (re)absorption from small intestine and kidney proximal tubule is driven by (a) luminal sodium-dependent co-transport system(s) named B^0 that had long resisted molecular identification. This spring, the laboratory of Stefan Broer has demonstrated that a member of the SLC6 family that is closely related to the orphan transporter XT2 functions as B^0 (B0AT1) and transports Na- (but not Cl-) dependently neutral amino acids (Broer et al., J Biol Chem, Epub March 25 2004). This transporter is thus a prime candidate for being at the origin of Hartnup disorder when mutated. Besides B0AT1 and XT2 (rat rosit) that are localized in the genome next to each other, there is another related orphan transporter, XT3, with a distinct chromosomal localization. We have analyzed in mice by real-time RT-PCR and immunocytochemistry the tissue and subcellular localization of these three gene products and of an additional related mouse transporter, XT3s1. We show that all four are localized in the brush border membrane, with differential axial gradients along the kidney proximal tubule and the small intestine. Besides, a lower expression of some of these transporters was found at the mRNA level in lung, liver, placenta, spleen and testis. The transporters XT3s1>mB0AT1>XT3>XT2 are also expressed in different area of the brain. Interestingly, OK cells that are derived from the proximal kidney tubule of opossum display a B^0-type amino acid transport but do apparently not express B0AT1, but only XT2 and XT3. This suggests that the other orphan transporters that are related to B^0AT1, are part of the sodium-dependent amino acid transport system called system B^0 that is expressed in small intestine and kidney proximal tubule.

Poster No. VI-18

Organic Cation Transporter 3 (Slc22a3) Is Implicated in Salt-Intake Regulation

Vialou V[1], Amphoux A[1], Zwart R[2], Giros B[1] & Gautron S[1], [1]Institut National de la Santé et de la Recherche Médicale U513, Faculté de Médecine, Créteil, France, and [2]Department of Molecular Genetics, The Netherlands Cancer Institute, Amsterdam, The Netherlands

Organic cation transporters (OCTs) are carrier-type permeases known to participate in general detoxification functions in peripheral tissues. Previous *in vitro* studies have suggested that OCTs ensure Uptake$_2$, a low-affinity, corticosteroid-sensitive catecholamine removal system, which was characterized initially in sympathetically innervated tissues. Although the presence of both Uptake$_2$-like transport and most OCT subtypes has also been demonstrated in the brain, the physiological role of this family of transporters in CNS remained totally unknown. In the present work, we show that the OCT3 transporter is found throughout the brain and highly expressed in regions regulating fluid exchange, including circumventricular organs such as area postrema and subfornical organ (SFO), and in other structures implicated in the sensing of changes in blood osmolarity and regulation of salt and water ingestion. OCT3/Slc22a3-deficient mice show an increase in the level of ingestion of hypertonic saline under thirst and salt appetite conditions, as well as alterations of the neural response in the SFO after sodium deprivation, as monitored by Fos immunoreactivity. This work demonstrates that the presence of OCT3 is critical for the balanced neural and behavioral responses to environmentally induced variations in osmolarity and provides for the first time physiological evidence of the importance of OCTs for CNS function.

Key words: organic cation transporter; Uptake$_2$; circumventricular organs; salt appetite; c-fos; monoamine.

Poster No. VI-19

***In Vitro* and *in vivo* characterization of antidepressant candidates**

Weikop P, Østergaard Nielsen E & Scheel-Krüger J, NeuroSearch A/S, Ballerup, Denmark

A large body of evidence indicates that depression is related to a dysfunctional activity in the prefrontal cortex and hippocampus. These structures receive powerful afferents from the serotonergic and noradrenergic neurons in the raphé nucleus and locus coeruleus, respectively, and represent major targets for action of antidepressant drugs. Current antidepressant drugs represent mainly monoamine reuptake inhibitors of various types.

Microdialysis represents a powerful tool in the discovery process for new antidepressant drugs, building a bridge between the *in vitro* reuptake inhibition in synaptosomes and the neurochemical measures of functional activity *in vivo*. In this study we have investigated the effects of mixed noradrenaline (NA) and serotonin (5-HT) reuptake inhibitors (NSRIs) on extracellular levels of NA and 5-HT in the prefrontal cortex (PFC) of anaesthetized rats. The aim was to examine the degree of correlation between *in vitro* and *in vivo* data regarding the potency of novel NSRIs to inhibit NA and 5-HT reuptake. Thus, the effects of venlafaxine on increases of 5-HT and NA levels were compared to three novel NeuroSearch compounds, labelled as NS-A, NS-B, and NS-C. These compounds represent different chemical classes of antidepressants, with varying potency for 5-HT and NA reuptake and no or only marginal effects on dopamine reuptake. The present data indicate that, at least for the selected NSRIs, a weak correlation exists between the data obtained in *in vitro*/*ex vivo* reuptake versus the *in vivo* microdialysis results aimed to evaluate the potency of the compounds to inhibit 5-HT and NA reuptake. The similar results as presented for the PFC in this study were obtained in a parallel study in the rat ventral hippocampus (Weikop et al., in preparation). It is suggested that *in vivo* data reflect, in a more relevant manner, the involvement of complex neuronal circuitry and its activation by the compounds tested. Thus, the extracellular 5-HT levels in the terminal areas are to a high degree modulated by the inhibitory 5-HT$_{1A}$ autoreceptors, as well as, stimulatory α_1-adrenoreceptors in the raphé nuclei.

Poster No. VI-20

Early loss of the glutamate transporter splice-variant GLT-1v in rat cerebral cortex following traumatic brain injury is unaffected by antioxidant treatment

Yi J-H, *Pow DV & Hazell AS, Department of Medicine, University of Montreal, Montreal, Canada; *Department of Physiology and Pharmacology, University of Queensland, Brisbane, Australia

Glutamate transporter proteins are essential for the control of interstitial glutamate levels, with an impairment of their function or levels being a major potential contributor to excitotoxicity. We have investigated the effects of lateral fluid-percussion on the levels of the glutamate transporter proteins GLT-1α, GLT-1v, GLAST, and EAAC1 in the rat in order to evaluate their pathogenetic role in this model of traumatic brain injury (TBI). Rats were exposed to TBI of moderate severity (2.0-2.5 atm) and the injured cerebral cortex studied at 6 and 24 hr post-injury. TBI resulted in a 54% decrease in GLT-1v 6 hr following the insult, which progressed to an 83% loss of the transporter after 24 hr as revealed by immunoblotting and immunohistochemical methods, and which was accompanied by neuronal loss. This down-regulation of GLT-1v was selective amongst the glutamate transporter proteins examined with no changes in GLT-1α, GLAST, or EAAC1 being observed in this brain region at either time point. Light microscopy revealed an astrocytic pattern of immunoreactivity for the novel splice variant GLT-1v. Treatment with the antioxidant N-acetylcysteine (NAC, 163mg/kg, i.p.) 5 minutes following trauma failed to reverse the down-regulation of GLT-1v at either time point, but reduced cortical injury volume to 55% of saline-treated TBI rats at 24 hr post-TBI, as assessed

by 2,3,5,-triphenyltetrazolium chloride (TTC) staining. Our results suggest that oxidative stress is not an important contributor to the early, dynamic changes in GLT-1v transporter levels in the injured cerebral cortex, but plays an important role in the pathological outcome in this region following TBI. {Supported by CIHR, Canada}

Poster No. VI-21

Glutamate transporter activity is preserved relative to cellular viability during oxidative stress in murine spinal cord cultures

Zagami CJ, Beart PM & O'Shea RD, Howard Florey Institute and Department of Pharmacology, University of Melbourne, Australia

Glutamate transporter (EAAT) dysfunction induced by oxidative stress has been implicated, along with excitotoxicity, in various neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS), a degenerative disease of motor neurons. Astrocyte function may also be involved in such diseases, as astrocytic EAATs are thought to be most important in maintaining extracellular glutamate levels. These EAATs may therefore provide an interdependent link between excitotoxicity and oxidative stress, with oxidative injury to EAATs producing excitotoxicity which in turn can produce reactive oxygen species in a vicious cycle. Here, we investigated the relationship between oxidative insults, EAAT activity and cellular viability in a murine model relevant to ALS. Dissociated spinal cord cultures were established from embryonic day 12.5 C57BL/6 mice in Neurobasal™ medium containing B27 supplement. Cells were treated at 12-13 days *in vitro* for 0.5-24h with hydrogen peroxide (300μM) and 4-hydroxynonenal (100μM). These treatments caused rapid stellation of astrocytes and the appearance of many cells, both neurons and astrocytes, that stained positive for propidium iodide, a marker of damaged cells, whilst neuritic shrinkage of motor neurons occurred more slowly. Under these conditions, cellular viability (MTT assay) decreased rapidly (maximal inhibition at 2-3h), but reductions in EAAT activity ([^{3}H]-D-aspartate uptake) occurred more slowly (maximal inhibition at >8h). Since changes in astrocyte morphology occur within 0.5-1h, they may be indicative of initial adaptive mechanisms in astrocytes which preserve EAAT function in response to oxidative injury. These adaptive mechanisms may also involve neuronal EAATs and may be due to increased trafficking of EAATs to the cell surface, or increased transporter efficiency, to maintain cellular homeostasis.