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Sodium/Chloride-Dependent Transporters: Elucidation of Their Properties Using the Dopamine Transporter

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The mechanisms controlling the intensity and duration of synaptic transmission are numerous. Once an action potential reaches a nerve terminal, the stored neurotransmitters are released in a quantum fashion into the synaptic cleft. At that point, neurotransmitters can act on post-synaptic receptors to elicit an action on the post-synaptic cell or act at so-called auto-receptors that are located on the presynaptic side and which often regulate the further release of the neurotransmitter. Whereas the action of the neurotransmitter receptors is regulated by desensitization phenomenon, the major mechanism by which the intensity and duration of neurotransmitter action is presumably regulated by either its degradation or its removal from the synaptic cleft. In the central nervous system, specialized proteins located in the plasma membrane of presynaptic terminals function to rapidly remove neurotransmitters from the synaptic cleft in a sodium chloride-dependent fashion. These proteins have been referred to as uptake sites or neurotransmitter transporters. Once taken up by the plasma membrane transporters, neurotransmitters are repackaged into secretory vesicles by distinct transporters which depend on a proton gradient.

The study of reuptake mechanisms goes back some thirty years ago with the identification of the reuptake processes in brain slices. Over this period of time reuptake mechanisms have been characterized for most known neurotransmitters in the brain. Over the last several years the interest in these neurotransmitter transporters has increased due to the realization that in addition to their involvement in termination of synaptic transmission, they represent the endogenous target for several psychostimulants as well as antidepressant drugs. In addition, many of these neurotransmitter transporters function as neuronal gates for various neurotoxins. Therefore,

understanding the functioning and regulation of these transporters has become an important issue of neurobiology.

The first clue to the elucidation of this family of proteins was brought about several years ago when Barech Kannerin Israel purified a GABA transporter from brain and used the amino acid sequence to clone a cDNA encoding this protein. It was found that this protein contained roughly 700 amino acids with 12 putative transmembrane domains connected by various extracellular and intracellular loops. In such a model the amino and C terminal of the protein would be intracellular. Soon thereafter, Susan Amara's lab at Yale University reported the elucidation of the structure of the human noradrenaline transporter (NET) using expression cloning in COS cells. It was rapidly realized that the norepinephrine transporter and the GABA transporter obviously belonged to the same family of proteins since significant amino acid sequences were found between these two proteins and both of these proteins appeared to have 12 putative transmembrane domains.

The realization that these two neurotransmitter proteins belong to a family of related proteins provided the key to the elucidation of the structure of several of these neurotransmitter transporters. Using the polymerase chain reaction Randy Blakeley of Emory University in collaboration with our laboratory amplified a series of 10-12 distinct PCR fragments which obviously encoded related members of this family. In order to identify which of these PCR partial fragments might represent transporters of interest, we used in situ hybridization in rat brain to determine the expression pattern of the various PCR fragments. One of these PCR fragments was found to specifically be expressed in the raphe nucleus of the brain and further characterization of this fragment by the cloning of a full length cDNA revealed that this cDNA encoded a transporter for the neurotransmitter serotonin. In a similar fashion a transporter for the amino acid proline was isolated and characterized in our laboratory. As our interest was more specifically into the dopamine transporter (DAT), we repeated the PCR amplification starting from mRNA isolated from the rat's substantia nigra. A single major PCR fragment was isolated from this exercise and used to clone a full length cDNA encoding a rat dopamine transporter. This transporter was later used to isolate the human homolog of the dopamine transporter. Both of these dopamine

transporters shared a high degree of sequence homology. Both of them contained 12 putative transmembrane domains with several consensus phosphorylation sites on putative intracellular loops as well as the amino and C terminal tails of the protein. Extensive pharmacological characterization of the cloned rat and human transporters revealed that the two transporters were highly similar in their pharmacological characteristics but that the heterologous expression of the transporter into neuronally derived cells appeared to recapitulate more precisely the properties of the neurotransmitter transporter as studied in brain preparations.

Since it has been postulated that the neurotoxin MPTP is specifically taken up in dopaminergic cells by the intermediary of the dopamine transporter, we studied whether the expression of the dopamine transporters in host cells would render these cells susceptible to the cytotoxicity of MPTP. Quite interestingly it was found that after three or six days of treatment cells expressing a high level of the dopamine transporter, were much more susceptible to the cytotoxic effect of MPTP as opposed to control cells or to cells expressing very low concentrations of the transporter.

The cloning of the cDNA for several neurotransmitter transporters has opened the way to try to elucidate the various properties of these interesting proteins. Studies in our laboratory as well as several others have revealed quite interestingly that for the neurotransmitters dopamine, norepinephrine and serotonin unlike the situation which exists with the heterogencity of G protein-coupled receptors for these molecules, there appears to be unique gene products which encode distinct dopamine, norepinephrine and serotonin transporters but so far no subtypes have been identified. In addition, studies looking at the association of these various transporter genes with several pathophysiological situations has revealed an absence of linkage with any of these diseases.

Whereas the pharmacological characterization of these transporters has been fairly well worked out, the structure-function relationships of these proteins has remained undetermined until the availability of cloned transporters. Thus it would be interesting to determine how the various structural determinants which specify ligand binding and transport, sodium dependence as well as the polarity of the transport. In an attempt to elucidate these questions we have used the approach

of building chimeric transporters between two related members of this family. The dopamine and norepinephrine transporters are among the most closely related members of the family having 78% overall identity in their amino acid sequences. Both DAT and NET effectively transport each other's substrates. NET transports dopamine and norepinephrine with similar Km values (~ 1 μM) whereas the DAT displays a ten-fold higher affinity for dopamine over norepinephrine. Despite the similarities in their transport properties, these two related transporters can be dramatically distinguished by various classes of inhibitory compounds. For example, tricyclic antidepressants have a 500-1,000-fold higher affinity for the NET than the DAT and psychostimulants such as d- and l-amphetamine display marked stereoselectivity of the dopamine transporter but not the norepinephrine transporter. Because these two transporters are structurally similar but pharmacologically distinct, there must be distinct domains within their common structural arrangements which are responsible for these differences. Thus, the chimeric transporter approach provides an excellent means for mapping various functional sites on these transporters and hopefully reveal interesting aspects of their structure-function relationships.

In order to produce functional chimeras, advantage was taken of the presence of three unique restriction sites within the structure of the DAT. These restriction sites were engineered into the norepinephrine transporter without changing the amino acid sequence. Expression of the mutated reconstituted norepinephrine transporter revealed no difference between the mutated transporter and the wild type norepinephrine transporter. This strategy provided us with four individual cassettes for each of the dopamine and norepinephrine transporter which could be interchanged by means of recombinant DNA technology to produce 14 distinct chimeric proteins. These proteins were expressed transiently in COS-7 cells and their ability to uptake either dopamine or norepinephrine as well as their ability to interact with various transport blockers were examined. Seven of these chimeric transporters displayed sufficient transport activity to be characterized reliably.

From these studies we could conclude that the amino terminal portion of these transporters along with the first and second transmembrane domains do not appear to change either the affinity

for substrate uptake or the ability of various uptake blockers to interact with these transporters. The same appeared to be true for the region comprised between transmembrane domain 3, the large extracellular loop of these transporters, as well as transmembrane domains 4 and 5. Thus, we infer from these data that these regions of the transporters might be more likely involved in functions that are common to both transporters such as the uptake mechanism itself or its dependence on sodium chloride. Quite a different picture was obtained when the last four transmembrane domains and the C terminal tail of these transporters were exchanged. In these chimeric transporters there was a change in affinity for the substrate which was similar to the affinity of the substrate of the transporter from which the last four transmembrane domains were derived. These chimeric transporters did not appreciably change the ability of blockers to interact with these transporters. Whereas the first five transmembrane domains of these transporters appear to be involved in common functions, and the last four transmembrane domains appear to specify substrate binding, the middle part, i.e., transmembrane 6, 7 and 8 of these transporters, appear to control and determine the affinity of blockers of transport. Thus, when the transmembrane domains 6, 7 and 8 of the dopamine transporter were substituted into the norepinephrine transporter background, the affinity of the tricyclic antidepressant was several thousand-fold shifted toward lower affinity suggesting that this portion of the molecule is involved in the interaction with tricyclic antidepressants.

Another unexpected but interesting finding with these chimeras in which the middle section of the dopamine transporter was inserted into the norpeinephrine transporter was a dramatic loss of affinity for cocaine. Cocaine binds with relatively similar affinity (300-400 nM) at both dopamine and norepinephrine transporters and therefore would be expected to display unaltered affinity in various chimeric transporters. However, there appears to be a selective loss of affinity which is associated with the replacement of the segments from transmembrane 5 to transmembrane 8 of the dopamine transporter into the norepinephrine transporter. These data suggest that this domain of the transporter or its interaction with other domains of the protein most likely provide a main pharmacophore for cocaine binding to these transporters.

Thus, in this work we have provided evidence that major determinants for the various functions of neurotransmitter transporters can be associated with distinct parts of these complex molecules. Whereas the C terminal part of the transporter comprising transmembrane domains 9-12 as well as the C terminal tail appears to confer substrate specificity and stereoselectivity, the central region of these transporter between transmembrane domains 5 and 8 appears to contribute to high affinity interactions with tricyclic antidepressants. In addition, based on the fact that no substantial changes in substrate uptake or blocker affinity were observed when domains comprising the amino terminal to the transmembrane domain 5 were interchanged between dopamine and norepinephrine transporters, it is inferred that these regions of the transporters must be likely involved in functions that are common to all members of this family such as their ionic dependence and substrate transport per se. Interestingly, these regions of the transporters are the most highly conserved amongst various members of the family.

Whereas normal functioning of these transporters likely requires the integrity of the whole protein, the delination of the involvement of large but discrete areas of these transporters in specific functions suggests that certain of these properties are determined by specific domains of these transporters rather than the interaction of several domains. In addition, these results provide useful information for trying to examine in greater detail the exact mechanisms or specific residues that may be involved in these functions. The discovery that in some chimeras the binding of cocaine can be virtually eliminated without interference with the uptake properties of these transporters suggests that a specific pharmacophore for cocaine binding must exist that is independent of the region that specifically interact with the substrates. Therefore, because the dopamine transporter is the proposed endogenous target of cocaine action, these findings raise the possibility that antagonists of cocaine action which might be devoid of uptake blockade activity could be developed for beneficial clinical use in the management of drug abuse.