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Minireview

The Mitochondrial Benzodiazepine Receptor: Evidence for Association with the Voltage-Dependent Anion Channel (VDAC)[1]

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Specific, high-affinity receptors for numerous drugs have recently been localized to mitochondrial membrane proteins. This review discusses the association of the mitochondrial receptor for benzodiazepines (mBzR) with the voltage-dependent anion channel (VDAC), indicating a possible auxiliary role for VDAC as a putative drug binding protein. The proposed subunit composition of the purified mBzR complex isolated from rat kidney mitochondria includes VDAC, which functions as a recognition site for benzodiazepines (e.g., flunitrazepam), the adenine nucleotide carrier (ADC), and an 18 kDa outer membrane protein identified by covalent labelling with the mBzR antagonists isoquinoline carboxamides (e.g., PK14105).

KEY WORDS: Mitochondria; receptor; benzodiazepine; isoquinoline carboxamide; anion channel.

INTRODUCTION

The selective, high-affinity interaction of small, biological molecules with their cellular receptors is the initial step in mediating many dynamic physiological processes. The actions of hormones, metabolites, and toxins, and the therapeutically relevant effects of some drugs occur via specific cell membrane receptors (Cuatrecasas and Hollenberg, 1976). The use of radiolabelled ligands in receptor binding experiments is a well-documented experimental approach which permits the identification, localization, and pharmacological characterization of specific receptors (Snyder, 1986).

One of the most interesting trends in the area of molecular pharmacology is the identification of novel radioligand binding sites on the membranes of mitochondria for numerous drugs and small molecules. Mitochondrial "secondary receptors" or "alloreceptors" can be distinguished from the primary, clinically relevant binding site both pharmacologically and physiologically.

Mitochondrial receptors for benzodiazepines, a class of drugs used therapeutically as anxiolytics, muscle relaxants, and anticonvulsants, have been identified (Snyder *et al.*, 1990), characterized, and most recently purified (McEnery *et al.*, 1989a,b, 1991a,b). Benzodiazepines are not unique, however, in their ability to target mitochondrial proteins with high affinity and specificity. For example, 1,4-dihydropyridines and phenylalkylamines, which are known to bind with high affinity to the L-type calcium channel (Belleman *et al.*, 1981; Ferry *et al.*, 1984: Curtis and Catterall, 1985) and the multidrug resistance protein (see review, Zernig, 1990; Safa *et al.*, 1987), also bind to a mitochondrial receptor (Zernig *et al.*, 1990; Ballestra *et al.*, 1990) which has been tentatively identified as the anion channel of the mitochondrial

[1]Abbreviations and chemical names: Ro5-4864: 7-chloro-1,3-dihydro-1-methyl-5-(*p*-chlorophenyl)-2*H*-1,4-benzodiazepin-2-one; Ro15-1788: ethyl 8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-α]-[1,4]benzodiazepine-3-carboxylate; AHN-086: (1-(2-isothiocyanatoethyl-7-chloro-1,3-dihydro-5-(4-chlorophenyl)-2*H*-1,4-benzodiazepin-2-one hydrochloride); PK11195: 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-isoquinoline-3-carboxamide; PK14105: 1-(2-fluoro-5-nitrophenyl)-3-isoquinolinecarboxylic acid.

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inner membrane (Zernig *et al.*, 1990). The immunosuppressant peptide cyclosporin A, which binds to a soluble protein, cyclophilin, found originally in lymphoid cells (Harding and Handschumacher, 1988; Handschumacher *et al.*, 1984), has been shown to be a potent inhibitor of the calcium-dependent "permeability transient channel" of mitochondria (Szabo and Zoratti, 1991; Crompton *et al.*, 1988; Novgorodov *et al.*, 1990). The hormone triiodothyronine, which binds to a nuclear receptor (Sterling, 1979), has been demonstrated to bind to a mitochondrial membrane protein (Sterling *et al.*, 1978). Also, the synthetic estrogen diethylstilbestrol (DES) has been shown to inhibit the purified proton-motive F_1F_0-ATPase of the mitochondrial inner membrane (McEnery and Pedersen, 1986) by interacting with the F_0 proton channel (McEnery, *et al.*, 1989).

The purpose of this review is to discuss the role of the voltage-dependent anion channel (VDAC) of the mitochondrial outer membrane as a functional component of the purified mitochondrial benzodiazepine receptor (mBzR). This work has been reported at a recent meeting (McEnery *et al.*, 1991a). It is interesting to consider the possibility that the presence of alloreceptors in mitochondria reflect highly conserved binding domains which function in a manner similar to, although distinct from, the nonmitochondrial receptor. For example, the central benzodiazepine receptor is associated with a chloride ion channel coupled to the $GABA_A$-receptor, while VDAC, a component of the peripheral (or mitochondrial) receptor, is a voltage-dependent ion channel which serves as a major port of metabolite flux between the cytoplasm and the inner mitochondrial membrane.

PHARMACOLOGY AND SUBCELLULAR DISTRIBUTION OF THE MITOCHONDRIAL BENZODIAZEPINE RECEPTOR (mBzR)

The benzodiazepine receptor present in peripheral tissues such as kidney, adrenal, testis, and ovary can be distinguished from the central benzodiazepine receptor by many criteria. In neurons, the benzodiazepine receptor is associated with the $GABA_A$-receptor localized to the plasma membrane (Mohler and Okada, 1977; Squires and Braestrup, 1977; Tallman *et al.*, 1980) of presynaptic terminals. In contrast, in peripheral tissues, the benzodiazepine receptor is localized primarily to the outer membrane of mitochondria (Anholt *et al.*, 1986; Basile and Skolnick, 1986), although nonmitochondrial binding sites have been identified in red blood cells (Olsen *et al.*, 1988). The receptor was first identified in non-neuronal tissues by Braestrup and Squires (1977) who employed [^{3}H]-diazepam (Fig. 1) as the diagnostic radioligand. This non-neuronal (hence the term "peripheral") binding site was first detected in kidney, and then later observed in most other tissues including neurons. It was subsequently determined that receptors for benzodiazepines could be classified into two distinct types based on their binding specificity (Skowronski *et al.*, 1987). Clonazepam and Ro15-1788 were very potent at the therapeutically relevant central site, whereas Ro5-4864 (4′-chlorodiazepam) potently displaced diazepam from the peripheral site. Flunitrazepam and diazepam have similar potencies for both types of binding sites (Regan *et al.*, 1981). Of the benzodiazepines, flunitrazepam has the property of acting as a photoaffinity ligand. Flunitrazepam covalently labels the 53 kDa subunits of the GABA-receptor (Mamalaki *et al.*, 1987) and in peripheral tissues, a 30–32 kDa protein (Snyder *et al.*, 1987). AHN-086, an isothiocyanato derivative of Ro5-4864, has been used recently as a covalent probe for benzodiazepine binding sites (Lueddens *et al.*, 1986). AHN-086 has been shown to label a 30–32 kDa protein in rat pineal gland (McCabe *et al.*, 1989). These results will be discussed in greater detail in a following section.

In addition to the benzodiazepines, the isoquinoline carboxamides PK11195 and PK14105 (Doble *et al.*, 1987b) are noncompetitive antagonists of benzodiazepine binding to the peripheral receptor. Moreover, PK14105 has been used as a photoaffinity ligand which modifies an 18 kDa protein (Doble *et al.*, 1987a). During longer exposure times, PK14105 has also been shown to be incorporated into a 30–32 kDa protein (Riond *et al.*, 1989). The photolabelled, denatured 18 kDa PK14105 binding protein has been isolated, cloned, and sequenced from rat (Antkiewicz-Michaluk *et al.*, 1988; Sprengel *et al.*, 1989) and bovine adrenal gland (Parola and Laird, 1991; Parola *et al.*, 1991). The cDNA encoded for a novel membrane protein and there are no reports of an associated enzymatic activity. The drug-dependent selective labelling of either an 18 kDa or a 30–32 kDa protein indicated a possible multisubunit composition of the intact receptor.

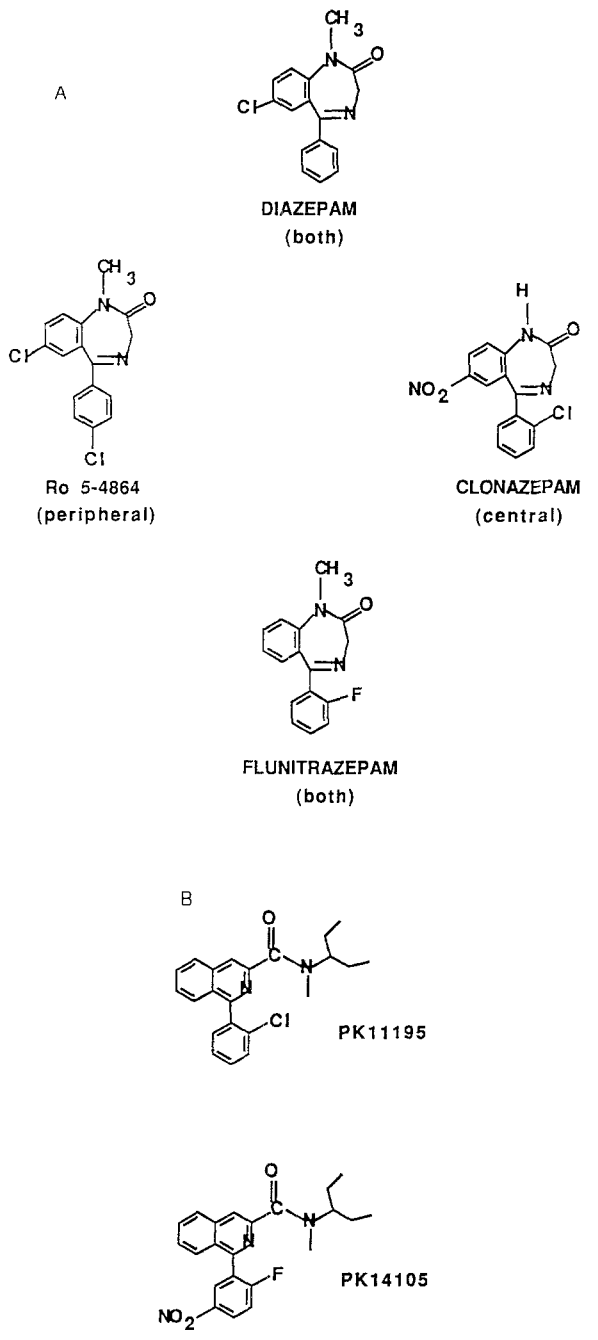

Fig. 1. The structure of the ligands employed in the characterization of the mitochondrial benzodiazepine receptor. The mitochondrial benzodiazepine receptor (mBzR) is characterized by its ability to bind benzodiazepines (panel A) and isoquinoline carboxamides (panel B) with high affinity and specificity. The benzodiazepine Ro5-4864 is specific for the mitochondrial (peripheral) site, whereas diazepam (Valium) and flunitrazepam interact with both the peripheral and the central site. Clonazepam and Ro15-1788 (not shown) are specific for the central benzodiazepine receptor. The isoquinoline carboxamides PK11195 and PK14105 bind to only the peripheral site.

CELLULAR EFFECTS OF LIGANDS FOR THE mBzR

The question that must be asked in studying alloreceptors is whether these proteins are merely drug-binding proteins with no associated physiological function, or whether they represent bona fide cellular receptors. There are numerous reports of mBzR ligands as modulators of cellular processes, for example: induction of *c-fos* (Curran and Morgan, 1985), modulation of cardiac channels (Mestre *et al.*, 1984), and chemotaxis (Ruff *et al.*, 1985). There are several recent review which address the pleiotropic effects of mBzR ligands (Snyder and Verma, 1989; Krueger, 1991). In many of these studies involving intact cells, it has been noted that benzodiazepine ligands function as agonists while isoquinoline carboxamide ligands serve as antagonists. The direct interaction of mBzR ligands on isolated mitochondria resulted in an inhibition of mitochondrial respiration (Hirsch *et al.*, 1989a; Moreno-Sanchez *et al.*, 1991b), calcium efflux (Moreno-Sanchez *et al.*, 1991a), steroid transport, and coupled steroidogenesis (Papadopoulos *et al.*, 1990; Krueger and Papadopoulos, 1990; Larcher *et al.*, 1989). In most cases both classes of mBzR ligands give rise to similar effects, with a drug specificity compatible with the pharmacology of the ligand binding site. For example, Hirsch *et al.* (1989a) reported that nanomolar concentrations of Ro5-4864 and PK11195 lead to a decrease in the respiratory control ratio.

Both classes of ligands have been reported to stimulate cholesterol transfer from the outer mitochondrial membrane to the inner membrane coupled to pregnenolone production (Papadopoulos *et al.*, 1990; Krueger and Papadopoulos, 1990). There are some exceptions to this general trend. Whereas PK11195 and other mBzR ligands have been shown to stimulate cholesterol translocation in adrenocortical cells (Papadopoulos *et al.*, 1990), only the benzodiazepine flunitrazepam was reported to have an inhibitory effect on hormone-activated steroidogenesis in cultured steroidogenic cell lines (Papadopoulos *et al.*, 1991). It is of interest to note that the isoquinoline carboxamide ligands were apparently without effect on tropic-hormone-activated steroidogenesis. One possible interpretation of these studies is that under some conditions, benzodiazepines are able to modulate and apparently uncouple the complex series of sequential steps in steroidogenesis. The site of direct uncoupling may involve the obligatory transfer of the

steroid substrate from one subunit (presumably the 30–32 kDa benzodiazepine recognition component) to the next (the 18 kDa component). A second explanation for the effect of mBzR ligands on steroidogenesis may be an indirect uncoupling mediated via alterations in cytoplasmic calcium levels via release from mitochondrial stores (Moreno-Sanchez *et al.*, 1991a). Fluctuations in cellular calcium has been reported to stimulate steroidogenesis in adrenal mitochondria (Capponi *et al.*, 1988; Koritz, 1986).

The role of mBzR in mediating the transfer of metabolites across the outer mitochondrial membrane has also been proposed to explain the inhibitory effect of porphyrins on the mBzR receptor (Verma *et al.*, 1987; Baker and Fanestil, 1991). Heme synthesis is known to involve a spatially defined series of catalytic events which traverse the outer and inner mitochondrial membranes. It is interesting to note that the most potent inhibitors of mBzR binding are those porphyrins which are putative substrates for the mitochondrial porphyrin transporting machinery (Verma and Snyder, 1988).

PURIFICATION OF THE MITOCHONDRIAL BENZODIAZEPINE RECEPTOR

My strategy for elucidating the identity and function of the mBzR has focused on the purification of the active receptor complex. There have been numerous reports which indicate that the binding or recognition sites for benzodiazepines differ from those of the isoquinoline carboxamides, perhaps indicating a receptor composed of nonidentical subunits. The use of protein-modifying reagents such as diethylpyrocarbonate (Skowronski, *et al.*, 1987), eosin-5-maleimide, and mercuric chloride (McEnery *et al.*, 1990, 1991b) have demonstrated a differential effect on the binding of the two classes of ligands, with the benzodiazepine-binding component being the more labile site. Also, the apparent targets of benzodiazepine (flunitrazepam) and isoquinoline carboxamide (PK14105) photolabelling covalently modify two distinct mitochondrial proteins. Recently, the cloned 18 kDa PK14105 binding site has been expressed in transformed kidney cells which contained endogenous mBzR (Sprengel *et al.*, 1989). The increase in the number of mBzR binding sites (B_{max}) after transfection with the clone was minimal, and under these conditions, one cannot discount the possibility that the expressed 18 kDa protein was associating with endogenous mBzR subunits.

The solubilization of the active form of the mBzR from rat kidney mitochondrial membranes was accomplished only with the non-ionic detergent dodecyl-β-D-maltoside (DM), a detergent which had been used by other investigators to solubilize other labile, multisubunit membrane proteins (Peterson *et al.*, 1988). This purification procedure is in sharp contrast to the conventional method(s) for purifying VDAC for purposes of studying its channel properties (De Pinto *et al.*, 1989). The DM-soluble fraction was chromatographed on a hydroxyapatite column in the presence of DM with the peak of binding activity eluted at very high concentrations of potassium phosphate (1.2 M). Upon analysis by SDS-PAGE, the peak of binding activity, which is 60 to 100-fold enriched compared to starting mitochondrial membranes, is observed to contain three proteins with apparent molecular weights of 32, 30, and 18 kDa (McEnery *et al.*, 1991b). These proteins migrate as a single peak with an apparent molecular weight of 50–70 kDa on a Superose 12B sizing column consistent with the subunits migrating as an intact complex. The 32 kDa protein is covalently modified by flunitrazepam, while the 18 kDa protein is modified by PK14105 in the purified receptor preparation. Based upon these data, it is considered that the recognition site for benzodiazepines resides on the 32 kDa protein, while the 18 kDa protein functions as the isoquinoline carboxamide recognition site.

BIOCHEMICAL AND IMMUNOLOGICAL EVIDENCE FOR THE ROLE OF VDAC AS A FUNCTIONAL COMPONENT OF THE MITOCHONDRIAL BENZODIAZEPINE RECEPTOR

There is very good experimental evidence that the major 32 kDa protein photolabelled by flunitrazepam in rat kidney mitochondria is VDAC. Antisera raised against the receptor complex reacted with only the native and flunitrazepam-labelled 32 kDa protein (anti-32 kDa) by Western blot analysis. This protein was subsequently identified as the voltage-dependent anion channel (VDAC) of the outer mitochondrial membrane by numerous criteria. The 32 kDa protein present in the purified mBzR preparation was labelled with [^{14}C]-dicylcohexylcarbodiimide ([^{14}C]-DCCD), a carboxylate-modifying reagent known to covalently label VDAC (Nakashima *et al.*, 1986; De Pinto *et al.*, 1985). In crude mitochondrial membranes, the anti-

32 kDa antiserum reacted with all 5–6 isoforms of the [^{14}C]-DCCD labelled proteins which are resolvable by two-dimensional electrophoresis (McEnery *et al.*, 1991a,b). The anti-32 kDa antiserum also reacted with VDAC purified by standard procedures (De Pinto *et al.*, 1989). This indicates that the anti-32 kDa antiserum may identify a common epitope found in all VDAC molecules. Antibodies obtained from Dr. Hartmut Wohlrab (Boston Biomedical Institute) which react with the 30 kDa adenine nucleotide carrier (ADC) of the mitochondrial inner membrane (Rasmussen and Wohlrab, 1986) reacted with the 30 kDa protein of the purified mBzR complex. A working model for the minimal subunit composition of the intact mBzR includes a role for VDAC, the ADC, and the 18 kDa PK14105 binding protein. Based on Western blot analysis using subunit-specific antisera and [^{125}I]-protein A, it has been determined that VDAC and the 18 kDa protein are present in stoichiometric amounts in the highly enriched hydroxyapatite fraction (unpublished observation).

Further evidence for the role of VDAC as a component of the mBzR comes from a comparison of autogradiographic studies, which localize radioligand binding sites, with immunohistochemistry. The mBzR has been localized autoradiographically to olfactory nerves and glia in neuronal tissues (Anholt *et al.*, 1984) and the posterior lobe of the pituitary, the cortex (especially the zona glomerulosa) of the adrenal gland, and the interstitial tissue of the testis (De Souza *et al.*, 1985). In preliminary experiments employing the anti-32 kDa (anti-VDAC) antiserum, VDAC co-localizes to areas of greatest ligand binding in all peripheral tissues examined. Another group employing antibodies against the 18 kDa PK14105 binding protein (Moynagh *et al.*, 1991) have reported similar patterns of co-localization of ligand binding with immunoreactivity in brain sections.

What evidence is there for the role of VDAC in receptor function? The effect of flunitrazepam on hormone-stimulated steroidogenesis (Papadopoulos *et al.*, 1991) validates the use of this ligand as an important antagonist of the mBzR and directs attention toward the role of the VDAC as the benzodiazepine-binding component of the receptor. Numerous attempts to separate VDAC from either the ADC or 18 kDa mBzR subunit have resulted in loss of ligand binding. Specific reagents which inhibit VDAC channel function [e.g., concanavalin A-Sepharose (Colombini, 1980) and Konig's polyanion (Colombini *et al.*, 1987)] have been used to potently abolish ligand binding (McEnery *et al.*, 1991a; Hirsch *et al.*, 1989b). Scatchard analysis of [^{3}H]-PK11195 and [^{3}H]-Ro5-4864 binding sites in intact mitochondria indicates a receptor density of 3.0 pmol/mg and 2.75 pmol/mg, respectively, in rat kidney mitochondria (Awad and Gavish, 1987), indicating a stoichiometric relationship between the benzodiazepine and isoquinoline carboxamide recognition sites. Assuming a molecular weight of the mBzR of 70 kDa, the mBzR would account for 0.1–0.2% of total mitochondrial protein, or approximately 2% of the outer membrane. The density of the mBzR is very similar to the calculated abundance of VDAC in mammalian mitochondria.

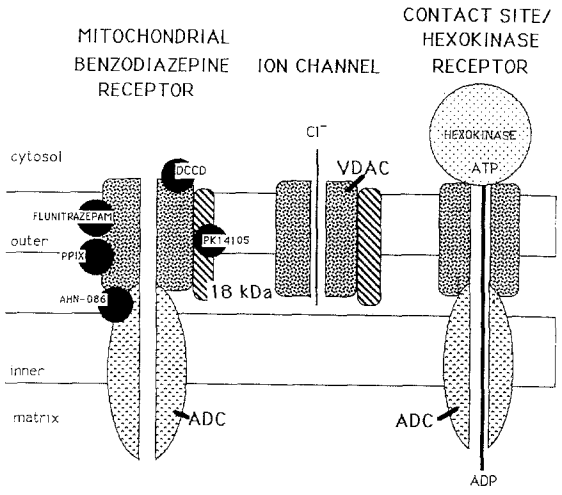

Fig. 2. The mitochondrial benzodiazepine receptor (mBzR) and the role of the voltage-dependent anion channel (VDAC). VDAC is reported to have at least three properties in addition to its role as an ion channel. As depicted, VDAC functions as a component of the mitochondrial contact site, the putative location for metabolite transport from matrix to cytosol. In this capacity, VDAC is believed to interact with the adenine nucleotide carrier (ADC) of the mitochondrial inner membrane and also with an undefined calcium transport protein (Kottke *et al.*, 1988; Sandri *et al.*, 1988). VDAC also functions as a receptor for hexokinase (see text). Lastly, VDAC is proposed to be associated with the mitochondrial benzodiazepine receptor which, like the contact site, is comprised of VDAC, the ADC, and, in the case of the intact mBzR, an 18 kDa protein. Based on covalent labelling of the intact mBzR complex (depicted diagramatically in this figure) it has been determined that VDAC is the target for [^{14}C]-DCCD and the benzodiazepines [^{3}H]-flunitrazepam and [^{3}H]-AHN-086, which is also incorporated into the ADC. Protoporphyrin IX (PPIX) has also been demonstrated to inhibit ligand binding to the mBzR. The 18 kDa protein is the apparent initial recognition site for benzodiazepine antagonists, e.g., [^{3}H]-PK14105. Upon longer incubation times with [^{3}H]PK14105, the drug is observed to label VDAC, implying a close association between VDAC and the 18 kDa protein. It is proposed that all three proteins comprise the active receptor.

PERSPECTIVES AND CONCLUSIONS

In addition to functioning as an ion channel, VDAC has been reported to interact directly with other proteins (Fig. 2). VDAC has been demonstrated to be localized to the junction between mitochondrial outer and inner membranes (termed "contact sites") (Weiler *et al.*, 1985; Ohlendieck *et al.*, 1986; Sandri *et al.*, 1988). The coupling of nucleotide transport from the mitochondrial matrix to the cytoplasm is believed to involve the close association of ADC with VDAC (Benz *et al.*, 1988). Also, VDAC has the unique property of functioning as a reversible intracellular receptor for hexokinase (Felgner *et al.*, 1979; Parry and Pedersen, 1983) and creatine kinase (Font *et al.*, 1987). Furthermore, it has been suggested that the ternary complex of VDAC, hexokinase, and the ADC allows for the kinetically advantageous access of hexokinase for matrix ATP (Aurora and Pedersen, 1988). As shown in Fig. 2, it is proposed that a similar ternary complex may exist for VDAC with the 18-kDa PK14105 binding protein and the ADC. As there are known isoforms for the ADC (Neckelmann *et al.*, 1987) and VDAC (M. Forte, personal communication), at this time it is premature to identify the exact molecular properties of the mitochondrial carriers which are associated with the mBzR.

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