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GLUTAMATE TRANSPORTERS IN PANCREAS AND EPITHELIA: PHYSIOLOGICAL ROLES AND DYNAMIC REGULATION

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GENERAL ABSTRACT

Glutamate is the main excitatory neurotransmitter of the mammalian nervous system and is involved in neuronal plasticity, memory and learning.
Emerging evidences suggest that glutamate is also present in peripheral tissues, where it plays a role in both cellular homeostasis and in autocrine/paracrine communication as an extracellular signalling molecule [Hedigerand Welbourne, 1999; Nedergaard *et al.*, 2002].
The extracellular glutamate concentration is tightly controlled by high affinity glutamate transporters, whose expression and modulation in the peripheral tissues have been poorly investigated. In this work we analyse the high affinity glutamate transporters EAAC1 and GLT1 in epithelia and endocrine pancreas, respectively.

EAAC1 was cloned from rabbit intestine [Kanai and Hediger, 1992] and its expression and function have been well characterised in absorptive epithelia, such as intestine and kidney, where it represents the major transporter for the dicarboxylic amino acids [Peghini *et al.*, 1997]. Less is known about the molecular mechanisms that regulate its surface expression and activity.
During the past few years, it has become clear that the activity of these transporters can be rapidly regulated by redistribution of proteins to and from the plasma membrane: a process that can be controlled by dynamic protein-protein interactions.
Therefore, the research presented in the chapter II focuses on the molecular mechanisms which regulate EAAC1 trafficking in epithelial cells. EAAC1 has a conserved sequence present in the C-terminal domain of EAAC1, that mediates interactions with class I PDZ proteins. In the past years, we demonstrated that the PDZ-target sequence and PDZ proteins are responsible for the retention and stability of EAAC1 at the plasma membrane [D'Amico *et al.*, 2010].
The aim of the present work is to verify whether this PDZ-target sequence is also important for the transporter's biosynthetic delivery.
Our data indicate that PDZ interactions occur early in the biosynthetic pathways and are involved in the ER-to-Golgi trafficking, as well as in Post-Golgi trafficking of EAAC1. Removal of the PDZ motif delays rather than prevents the ER export and the plasma membrane delivery of the transporter, thus indicating that PDZ interactions facilitate the ER-Golgi trafficking. Possibly, PDZ-interactions favour the transporters homo-oligomerization, a process required for the efficient ER export of EAAC1. Alternatively, PDZ domain-proteins

may couple EAAC1 with protein complexes required for the efficient fusion of carrier vesicles to the appropriate target membranes. Further studies will be needed to identify the PDZ protein/s involved in the EAAC1 biosynthetic delivery.

On the other hand, the presence of glutamate as an intercellular signal mediator in endocrine pancreas is well established [Moriyama and Hayashi, 2003]. In the Central Nervous System (CNS), glutamate may cause cell death by excitotoxicity, that is physiologically prevented by glutamate clearance systems [Choi *et al.*, 1988]. The effect of glutamate on islet viability, the expression of glutamate transporters and their physiological roles in the endocrine pancreas are still unclear.

Thus, in the III chapter we examine the effects of glutamate and the function of sodium dependent glutamate transporters in clonal β-cells and in human isolated islets of Langerhans. We demonstrate that exposure to elevated glutamate concentrations induces a significant cytotoxic effect in pancreatic β-cells, due to the prolonged activation of ionotropic glutamate receptors. We provide evidence that the key regulator of the extracellular glutamate clearance in the islet is the glial glutamate transporter 1 (GLT1/EAAT2). GLT1 is the only high affinity glutamate transporter expressed in the islets and localizes to the β-cell plasma membrane. Finally, as diabetes is characterized by selective beta-cell death, and our data indicate that GLT1 controls beta-cell survival, we investigate the expression of GLT1 in type 2 diabetes mellitus (T2DM) patients. We show an altered GLT1 localisation in pancreases from T2DM patients, suggesting a decreased glutamate clearance ability in these subjects.

In chapter IV, is reported the experience at University of Texas Health Science Center at San Antonio (UTHSCSA), USA. The aim of this project is to find interactors of IAPP, a protein involved in diabetes, by means of Yeast Two Hybrid Screening, a technique which allows the identification of direct protein-protein interactions. In prospect, this technique will be useful to find proteins that are associated with glutamate transporters and that potentially regulate their expression and function.

CHAPTER I: General Introduction

Glutamate has been traditionally characterised as an excitatory neurotransmitter in the mammalian nervous system. An increasing number of evidences suggests that glutamate can also play a significant role in peripheral tissues, where it can be a central metabolite for cellular homeostasis as well as an extracellular signal mediator.

1 GLUTAMATE IN THE CENTRAL NERVOUS SYSTEM

In the Central Nervous System (CNS), glutamate is the major excitatory neurotransmitter, and it controls several important functions, such as neuronal plasticity in cognitive tasks, memory and learning [Fonnum 1984; Ottersen and Storm-Mathisen, 1984; Collingridge and Lester, 1989; Headley and Grillner, 1990].

Glutamate signalling between cells involves several distinct phase of activity:

1-Presynaptic neurotransmitter release

2-Receptor activation

3-Neurotransmitter uptake.

In a pre-synaptic cell, glutamate is stored into vesicles, ready for release in response to a stimulus. Following vesicular exocytosis, the transmitter diffuses across the synaptic cleft and binds to post-synaptic receptors, that are specifically positioned by membrane clustering proteins. Receptors activation induces cascades of intracellular activity in the post-synaptic cell. Finally the transmitter is taken up by transporters, which control temporally and spatially the glutamate-mediated signalling and allow the transmitter recycling.

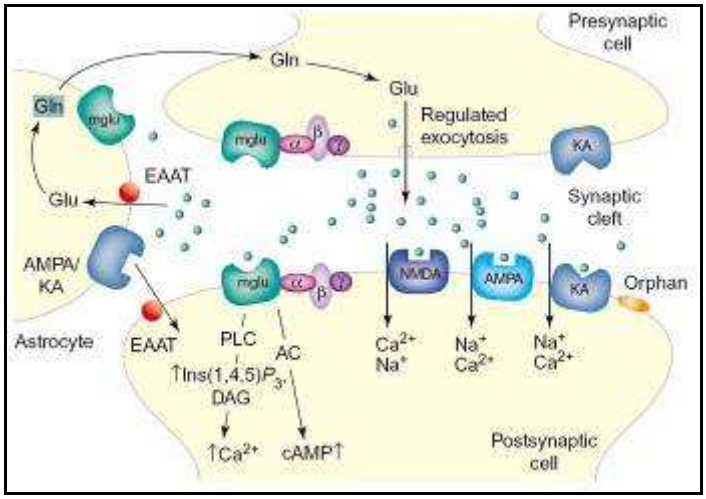

FIG. 1: Glutamate Synapse
[Skerry and Genever, 2001]

1.1 Presynaptic neurotransmitter release

The cytosolic concentration of glutamate is 5 mM in neurons and around 2-3 mM in astrocytes [Fitzpatrick *et al.*, 1988; Osen *et al.*, 1995].

As the blood brain barrier has a very low permeability to glutamate, most of the glutamate present in the brain is synthesised de novo. Essentially, all glutamate in the brain is synthesised by transamination of α-ketoglutarate, in both neurons and glia. In addition, in glial cells is expressed glutamine synthetase, an enzyme that converts glutamate to glutamine, before it is released into the extracellular space [Fonnum, 1993].

Then, glutamine is taken up by neurons and converted to glutamate before being packaged into synaptic vesicles. Glutamate uptake into the secretory granules is driven by vesicular glutamate transporters and represents the rate limiting step for glutamate release. These transporters localise to the vesicle membrane and their transport activity depends on electrochemical proton gradient across the vesicle membrane generated by H+ ATPase [Maycox *et al.* 1988].

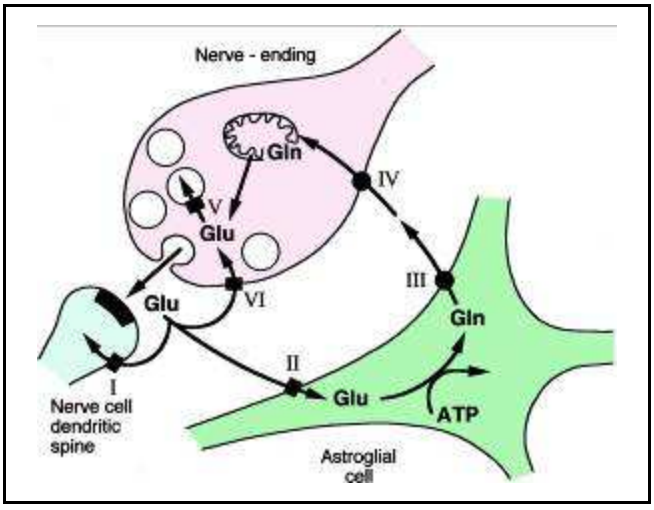

FIG. 2: Glutamine-Glutamate cycle in CNS. Glutamate released from the nerve terminal by exocytosis is taken up by glutamate transporters present presynaptically (VI), postsynaptically (I) and extrasynaptically in astroglial cells (II). Astroglia converts glutamate into glutamine. Glutamine is subsequently released from the glial cells by means of glutamine transporter (III) and taken up by neurons by another glutamine transporter (IV). Neurons convert glutamine back to glutamate and load synaptic vesicles (V) [Danbolt, 2001].

1.2 Receptor activation

There are two broad categories of glutamate receptors, the ion channel-forming or "ionotropic" receptors and the "metabotropic" receptors, coupled to GTP binding proteins (G proteins). The ionotropic receptors are further classified into three populations: those activated by N-methyl-D-Aspartate (NMDA), those that respond to Kainic acid (KA), and those sensitive to a-amino-3hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). Glutamate binding to these receptors promotes channel opening and the entry of cations (Na, K, Ca) into

the cell, that induces membrane depolarisation. Ionotropic glutamate receptors are produced by the homo- and/or heteromeric combination of different subunits, whose composition determines the selective conductances.

Metabotropic glutamate receptors have seven membrane spanning domains and can be expressed in both pre- and post-synaptic membranes. These receptors are subdivided into three groups based on sequence information and intracellular effectors systems. Group I receptors (mGluI and mGlu3) are coupled to Phospholipase C (PLC) and lead to activation of Protein Kinase C (PKC) and to an increase in intracellular calcium concentration. Group II receptors (mGlu2 and mGlu5) and Group III receptors (mGlu4, mGlu6, mGlu7, mGlu8) initiate the inhibitory cAMP cascade, through adenilate cyclases (AC).

The intracellular signals generated by activation of the ion channels and the effects of the receptors on PLC and AC enzymes are determined by the different intracellular environments in which these receptors operate [Michaelis, 1998].

1.3 Neurotransmitter uptake

Responsible for most of the glutamate uptake in the brain, high affinity plasma membrane glutamate transporters fulfil a crucial role in glutamatergic neurotransmission by removing glutamate from the synaptic cleft.

Glutamate transporters are transmembrane glycoproteins, facing the extracellular environment and thus well positioned to regulate extracellular Excitatory Amino Acids (EAA) concentrations.

These transporters maintain the glutamate gradient 10000 fold higher in intracellular (3-10 mM) than extracellular (0,3- 10 μM) compartments, using the energy available from the transmembrane membrane potential and ion gradients to drive their neurotransmitter substrate against concentration gradient [Auger and Attwell, 2000]. The transport stoichiometry is such that the inward movement of one glutamate, three sodium and one proton is coupled to the outward transport of one potassium ion [Danbolt, 2001; Barbour *et al.,* 1988]. Thus, the transport is electrogenic, because it causes a net transport of positive charge inside the cell.

Recent data indicate the mechanism for transport cycle, that comprises several steps (Fig. 3). Under steady state conditions the empty glutamate carrier is in outward-facing mode, with the binding sites exposed to the extracellular side. After interaction with 1 molecule of substrate$^-$ /H^+ and 3 Na^+, the loaded transporter responds with a conformational change, that induces closure of an external gate (HP2 domain, Fig. 4), leading to an occluded state. Another

conformational change determines the opening of the internal gate (HP1 domain, Fig. 4), resulting in the inward facing configuration, with substrate release in the cytoplasm. Thereafter, K+ binds to the carrier inside and promotes the relocation of the empty carrier [Boudker *et al.*, 2007]. If the transporter does not bind the K^+, it can operate in exchange mode, binding again sodium and glutamate from the intracellular side [Zerangue and Kavanaugh, 1996b].

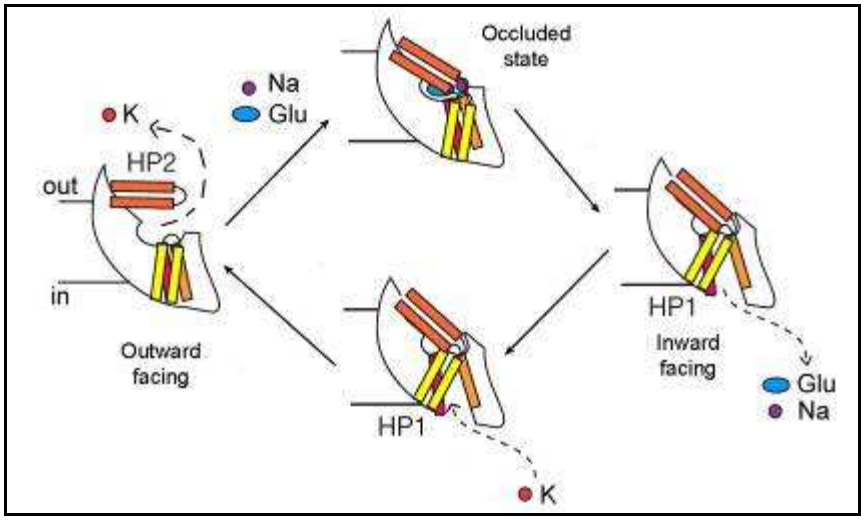

FIG. 3: Glutamate transporters' transport cycle [Boudker *et al.* 2007].

The cycling time of the human glutamate transporter has been estimated to be approximately 50 ms [Wadiche *et al.* 1995], which is significantly slower than the rate of synaptic glutamate release. Thus, the emerging view is that when carriers are present at a high density, they may serve as glutamate binding sites, thus limiting neurotransmitter actions on receptors [Seal and Amara, 1999].

Glutamate binding rapidly reduces the extracellular glutamate concentration and slows down the diffusion of the neurotransmitter away from the release site. However, this mechanism could lead to a paradoxical effect: given that the bond is reversible, glutamate may re-enter the cleft, upon unbinding from the transporters [Rusakov and Kullmann, 1998].

The traditional view is that transporters are important for neurotransmitter clearance and glutamate recycling after vesicular release.

However, the presence of EAATs in non-glutamatergic systems suggests that they could have also metabolic functions. Indeed, in GABAergic neurons, glutamate uptake can provide the substrate for the intracellular production of GABA or in Dopaminergic neurons transporters may carry cysteine, a fundamental building block for glutathione [Seal and Amara 1999].

There is also evidence that transporters may be more important for prevention of glutamate spillover onto extrasynaptic receptors and crosstalk to neighbouring neurons, rather then simply controlling the duration of the EPSCs (Excitatory Post Synaptic Currents) [Overstreet *et al.*, 1999; Rusakov *et al.*, 1999].

Astrocytes and neurons express high affinity, sodium dependent excitatory amino-acid transporters, belonging to SLC1 family. They are classified in five different subtypes, including: glutamate aspartate transporter (GLAST/EAAT1), glutamate transporter 1 (GLT1/EAAT2), excitatory amino acid carrier (EAAC1/EAAT3), EAAT4 and EAAT5.
Family members share 40% to 65% of sequence identity and a common structure, as hypothesized from the crystallised prokaryotic analogous [Yernool *et al.*, 2004]. In figure 4 is shown the transporter structure, with intracellular N and C terminal tails, 8 transmembrane loops and two re-entrant hairpin loops (HP1 and HP2), that partially span the phospholipidic bilayer. The sodium and substrate binding sites are defined by the 8th transmembrane domain and the two re-entrant loops.

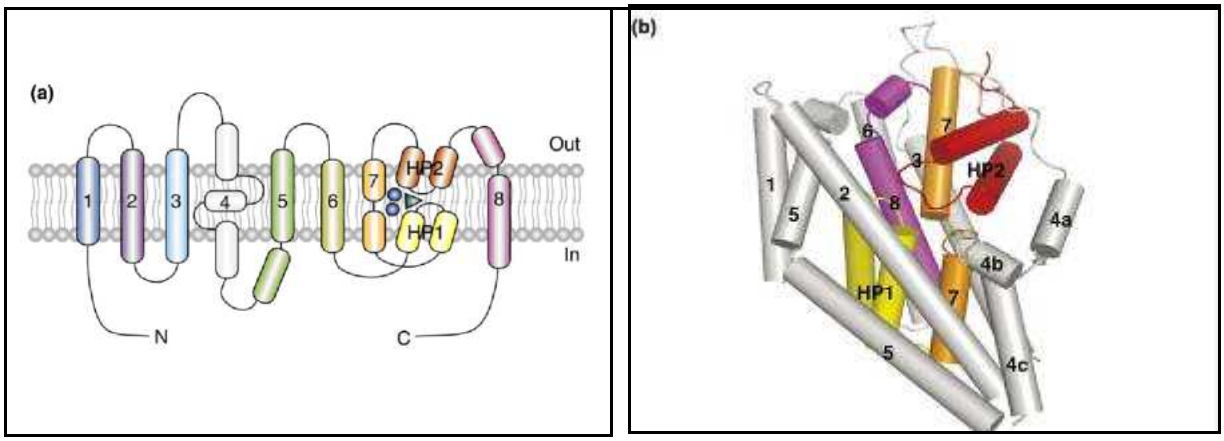

FIG. 4: (a) Sketch representation of the hypothetical membrane spanning domains in high affinity glutamate transporters. (b) Tridimensional representation of predicted domains. [Torres and Amara, 2007]

These glutamate transporters display heterologous regional and cellular expression profiles [Nakayama *et al.*, 1996].
The two astrocytic glutamate transporters GLAST and GLT1 display a 65% identity of the amino acid sequences and are present in the same cells. Indeed, both GLAST and GLT1 localise to astrocytes, with a predominant expression of GLAST in Bergmann glia in the cerebellum and GLT1 in astrocytes in the cortex and forebrain [Rothstein *et al.*, 1994]. More recent studies indicate these two glutamate transporters are expressed in a subgroup of

neurons. These transporters are believed to sequester the majority of extracellular glutamate released from neurons. More detailed studies have uncovered that GLT1 and GLAST expressions are not restricted to the central nervous system alone (see below).

EAAC1 localise to neurons throughout the CNS and is particular abundant in glutamatergic neurons of the cortex (layers II-IV), striatum and hippocampus (pyramidal cells of the CA2-CA4 regions), and in the granular layer of cerebellum (purkinje cells) [Bar-Peled *et al.*, 1997]. Interestingly, among all the SLC1 members, EAAC1 is the transporter mainly expressed and characterized in peripheral tissues [Kanai and Hediger, 1992, Mukainaka *et al.*, 1995].

EAAC1 is also able to transport L-Cysteine, with an affinity constant of 190 μM and a maximal rate flux similar to that of glutamate. Cysteine not only competes with L-glutamate uptake but also causes transporter-mediated release of transmitter by heteroexchange, leading to elevated extracellular glutamate concentrations [Zerangue and Kavanaugh 1996].

It has been hypothesised that following oligomeric assembly, EAAC1 can also operate in the chloride channel mode [Eskandari *et al.*, 2000].

EAAT4 is predominantly expressed in cerebellar Purkinje cells on postsynaptic dendritic spines [Nagao *et al.*, 1997]. It shows also an uncoupled chloride conductance associated with substrate transport.

EAAT5 is primarily expressed in the retina [Arriza *et al.*,1997]; it is associated with rod photoreceptors and some bipolar cells and exhibits a high chloride conductance, that may participate in visual processing.

1.3.1 Glutamate mediated excitotoxicity

Glutamate can cause neuronal cell death due to its excessive accumulation and subsequent overstimulation of ionotropic receptors. The mechanism described involves cell swelling and disintegration [Choi *et al.*, 1988].

The extracellular glutamate concentration has been estimated around 1-2 μM, but it can increase in many pathological conditions that are characterised by defect in the blood brain barrier and/or by cellular damage, such as stroke, trauma, multiple sclerosis and meningitis [Auger and Attwell, 2000]. Also astrocytes can release glutamate in response to molecules such as prostaglandin E, ATP, glutamate and bradikinin [Parpura *et al.*, 1994; Bezzi *et al.*, 1998; Jeremic *et al.*, 2001]. Finally, sodium-dependent glutamate transporters can release glutamate when they operate in reverse mode and also the cysteine-glutamate exchanger xc- can contribute to this release [Barbour *et al.*, 1988; Rossi *et al.*, 2000; Warr *et al.*, 1999].

Glutamate transporters are important to prevent neurotoxicity mediated by glutamate. Glial but not neuronal glutamate transporters are critical to maintain extracellular glutamate below toxic levels [Kanai and Hediger 2004].

Consistent with the role of GLT1 in neuroprotection, GLT1 knock out mice revealed selective neuronal degeneration in the hippocampal CA1 region [Tanaka *et al.*, 1997]; GLAST knock out mice develop normally but they exhibited motor dis-coordination during difficult tasks, consistent with abnormality in the cerebellum, where it is mainly expressed [Watase *et al.*, 1998].

On the other hand, knock out mice for the neuronal transporter EAAC1 develop normally and have a regular motor coordination, showing no apparent neurological disorders, but a decreased spontaneous locomotory activity **[**Peghini *et al.*, 1997].

Interestingly, a reduction in the expression and function of glutamate transporters has been detected in several neurodegenerative diseases, thus indicating that they may be involved in the onset and progress of neurodegeneration.

2 GLUTAMATE IN PERIPHERAL TISSUES

An increasing number of evidence suggests that glutamate serves for important functions also outside the CNS. In particular, glutamate can play a dual role in peripheral tissues: the first in cellular homeostasis and the second as an extracellular signal mediator, in autocrine and paracrine systems. Consequently, also the glutamate transporter function can be extended to the regulation of cellular metabolism and organ homeostasis as well as to cell signalling within non-excitable cells. Accordingly, the localisation of GLAST, GLT1 and EAAC1 in peripheral tissues is not only associated with nerve terminals, suggesting that they fulfil additional roles in these organs.

2.1 Cellular and organ homeostasis

Glutamate outside the CNS is present at millimolar concentrations in the cytosol of most cell types. At cellular level, glutamate contributes to the production of several important molecules, including proteins, nucleic acids, glutathione, polyamines and aminosugars. In addition, it has important roles as an intermediate metabolite (figure 5) [Newsholme *et al.*, 2003].

Transamination of ketoglutarate, an intermediary of the citric acid cycle, produces glutamate, whereas glutamine is synthesised by incorporation of an ammonium ion into glutamate. The two enzymes that catalyse these reactions, glutamate dehydrogenase and glutamine synthetase, are present in almost all life forms [Nedergaard *et al.*, 2002].

Glutamate and glutamine act as nitrogen exchanging factors, and they are involved in the acid-base homeostasis, both at cellular and at organism level.

The normal arterial glutamate concentration is 10-30 μM, which is similar to the Km of the high affinity transporter, thus indicating that the activity of the transporters may be determinant for glutamate/glutamine inter- and intra-organ fluxes.

The source of circulating L-glutamate is not dietary intake but the liver, where glutamate transporters facing the sinusoidal capillaries release glutamate produced from glutamine in a reaction catalysed by the phosphate dependent glutaminase (PDG). Glutamate generated by these upstream periportal hepatocytes is in part captured by glutamate transporter activity (maybe GLT1) in the downstream perivenous hepatocytes, coupled with glutamine synthesis and release, supporting an inter-organ glutamine flux.

EAAC1 present in muscles and lung takes up glutamate coupled to glutamine synthesis, reinforcing the interorgan glutamine flux.

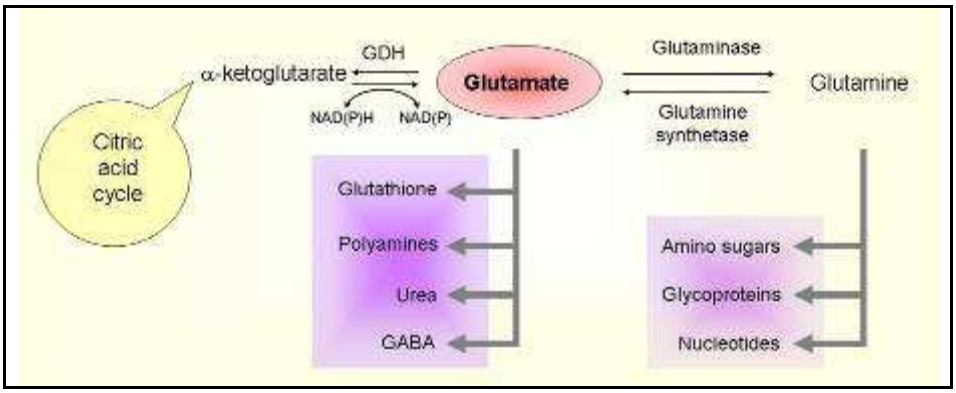

FIG. 5: Role of glutamate as a key metabolite [Nedergaard *et al.*, 2002].

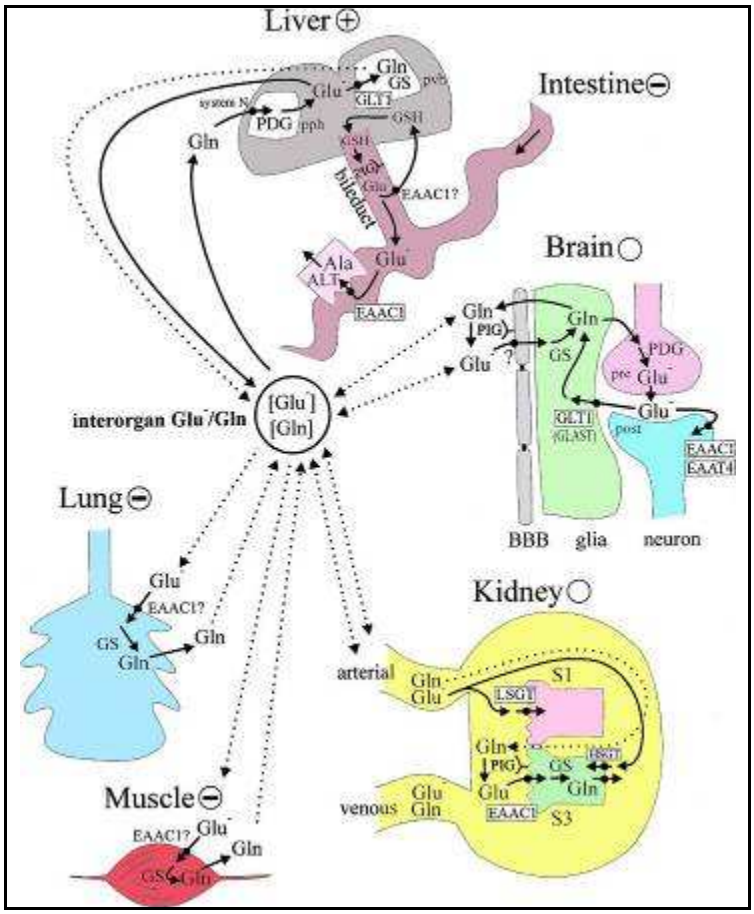

FIG. 6: Interorgan and intraorgan glutamate fluxes. Organs showing net glutamate release or uptake are shown as open circles with + or -, respectively. Organs with high unidirectional uptake but not removal are shown as empty open circles. Liver heterogeneity is presented by periportal (pph) or perivenous (pvh) hepatocytes and in renal tubule, proximal convoluted (S1) and straight (S3) are shown.
Double-arrowed dotted lines represent bidirectional fluxes.
Gln: glutamine
GSH: glutathione
PIG: Phosphate independent but bicarbonate dependent glutaminase
PDG: Phosphate dependent glutaminase
GS: Glutamine synthetase
ALT: Alanine aminotransaminase
BBB: Blood Brain Barrier
LSGT: Low affinity transporter
HSGT: High affinity transporter

[Hediger and Welbourne, 1999]

In renal tubules, glutamate transport and glutamine hydrolysis are physically and functionally coupled. Low and high affinity sodium dependent glutamate transporters are present in the different membrane domains of epithelial cells: the high affinity EAAC1 is distributed along

the apical brush border, increasing in density from early to late proximal tubule, whereas another transporter subtype is present on the basal membrane.

In the renal lumen, the distribution of the apical membrane EAAC1 overlaps the expression of Phosphate independent, but bicarbonate dependent glutaminase (PIG), that catalyses glutamine hydrolysis to glutamate. Together with γ-glutamil-transferase, PIG and EAAC1 form a functional unit that not only regulates glutamine metabolism, but it also controls paracellular permeability.

Moreover, it has been hypothesised that this functional unit produces three intracellular signals: (I) changes in intracellular glutamate concentration; (II) Localised increase in ADP, which stimulates glycolysis and ATP production; (III) intracellular rise in H+ concentration, resulting from the transport of glutamate and protons into the cell [Welbourne and Matthews 1999].

GLAST is expressed in basolateral membranes of the transitional epithelium in kidney, where it may be involved in glutamate homeostasis. Its role is likely related to the uptake of glutamate from the blood side into the epithelial cells for sustenance. Together with the glutamate transporter EAAC1, GLAST likely contributes to the inter-organ glutamine flux.

In kidney, these transporters cooperate for the reabsorption of most amino acids that are filtered at the glomeruli, thus leaving only small amounts excreted in the final urine.

The first third of the proximal convoluted tubule is responsible for the reabsorption of >90% of filtered glutamate by a low-affinity high-capacity transporter, that has not been cloned yet. EAAC1 will participate in the final recovery of di-carboxylic amino acids that may escape the early part of the tubule [Shayakul *et al.,* 1997].

Moreover, an old study supports a role of EAAs in hepatic and renal gluconeogenesis [Heitmann and Bergman, 1981].

2.2 Glutamate signalling in peripheral tissues

Recent studies indicate that in non-neuronal tissues, cytosolic glutamate represents also a potential transmitter pool with signalling functions. Indeed it has been suggested that glutamate may act in autocrine and paracrine systems, such as pancreas, bone, stomach, intestine, liver, lung and kidney [Skerry and Genever, 2001; Danbolt, 2001; Rzeski *et al.*, 2001].

In general, peripheral cells are not specialized to transmit electrical signals, thus rapid membrane currents evoked by glutamate might be less effective than long term effects on intracellular messengers.

To fulfil signalling functions, peripheral tissues should express the necessary machinery for glutamate signal transduction, as happens in the CNS: vesicles storage systems, functional receptors and plasma membrane transporters.

2.2.1 Glutamate storage into synaptic vesicles

The detection of VGLUTs in non-neuronal peripheral tissues has been used to demonstrate the presence of peripheral glutamatergic paracrine/autocrine systems [Moriyama and Omote, 2008].

These vesicle transporters are widely distributed in Peripheral nervous system (PNS), such as in the retina [Fremeau *et al.*, 2002; Mimura *et al.*, 2002], trigeminal ganglion [Li *et al.*, 2003], dorsal root ganglia (DRG) [Olivera *et al.*, 2003; Li *et al.*, 2003], intraganglionic laminar endings (IGLEs) of esophagus [Rabb *et al.*, 2003] and enteric neuron [Tong *et al.*, 2001].

Interestingly, their expression is also extended in various non neuronal tissues such as bone cells, pancreatic islet cells and pineal cells [Skerry and Genever, 2001].

It was found that pineal SLMVs contain both VGLUT1 and 2, as revealed by biochemical, immunohistochemical and electronmicroscopical evidence [Morimoto *et al.*, 2003].

The same approaches indicated the presence of both VGLUT1 and 2 in glucagon-containing secretory granules of α cells, suggesting that L-glutamate is co-secreted with glucagon upon low glucose stimulation [Hayashi *et al.*, 2003]. Vesicular transporters are also expressed in F cells, and are associated with pancreatic polypeptide-containing secretory granules, suggesting that F cells are also glutamatergic, and that pancreatic polypeptides and L-glutamate are co-secreted [Hayashi *et al.*, 2003b]. By contrast, neither VGLUT1, 2 nor 3 are expressed in β and δ cells, thus these cells can not be considered glutamatergic cells [Moriyama and Hayashi 2003].

In the gastrointestinal tract, L cells, that secrete glucagon-like peptide 1 (GLP-1) and polypeptide YY (PYY), express VGLUT2 in secretory granules [Hayashi *et al.*, 2003]. The stomach mucosa, especially the antrum and pylorus regions, contains VGLUT2.

Also in bone vesicular glutamate transporters have been identified, but there are some discrepancies regarding the isoform expressed in osteoblasts, as some groups have detected VGLUT1 and other VGLUT2 [Hayashi *et al.*, 2003; Hinoi *et al.*, 2002; Redecker *et al.*, 2003].

2.2.2 Glutamate receptors

Glutamate receptors (GluRs) are widely expressed outside the CNS by cells that have not been traditionally associated with glutamate mediated signalling.

Some of the peripheral glutamate receptors have been cloned and sequenced and seem to be identical to those involved in neurotransmission [Gill and Pulido 2001]. However, it is likely that rapid excitatory currents are less important in non-excitable cells, whereas long term effects on membrane potential, cytosolic calcium levels and second messenger system might be more relevant.

Moreover, the glutamate concentration outside the brain is higher than its receptor affinity. It has been hypothesised that post-translational modifications, such as glycosylation or phosphorilation, might reduce the affinity of glutamate receptors in peripheral tissues, compared with receptors in the CNS [Gill and Pulido 2001].

Studies of the distribution of radiolabelled glutamate receptor binding agents revealed significant binding in the spleen, bone, pancreas, lung, heart, liver, kidney and intestine [Samnick *et al.,* 1998; Nasstrom *et al.,* 1993].

The table below summarizes the glutamate receptors subtypes found in peripheral tissues, together with the proposed function in the particular environments.

Tissue/Cell Type	Glutamate receptors expressed				Proposed function
	NMDA	AMPA	Kainate	Metabotropic	
Astrocytes	-	+	+	+	Neuron-Astrocytes communication
Cerebral Endothelium	+	+	-	+	Unknown
Osteoblasts	+	+	-	+	Modulate differentiation and bone-forming activity
Osteoclasts	+	-	-	-	Modulate differentiation and resorption
Megakaryocytes	+	-	-	-	Modulate differentiation and maturation
Platelets	+	-	-	-	Regulate adhesiveness
Keratinocytes	+	+	-	+	Regulate differentiation
Melanocytes	-	-	-	+	Control proliferation and cytotoxicity
Merkel cells	?	?	?	-	Mechanosensation
Pancreas	+	+	+	+	Regulate hormone secretion
Lung	+	-	-	-	Provoke edematous lung injury?
Ileum	+	-	-	+	Modulate contractility
Hepatocytes	-	-	-	+	Protect from hypoxic injury
Heart	+	+	+	+	Modulate cardiac function
Thymus	-	-	-	+	Unknown
Kidney	+	-	-	-	Cytoprotective
Adrenal gland	+	-	-	-	Unknown
Taste buds	?	-	?	+	Sense umami taste

TABLE 1: Tissue distribution and function of glutamate receptors [Skerry and Genever, 2001].

The localisation of GluRs to specific structures in peripheral tissues hints at their importance in the control of cellular homeostatic mechanisms, cell differentiation, excitability, cell injury and metabolism [Rockhold *et al.,* 1989; Said, 1999; Said *et al.,* 1996]. Their presence in several endocrine organs, such as pancreas, pituitary, testis, and kidney, implicates these receptors in the regulation of hormone secretion [Inagaki *et al.*, 1995].

In the stomach, glutamate modulates histamine-induced acid secretion in rats and glutamate is also able to mediate contractility in the gastric fundus, jejunum, ileum and large intestine [Tsai *et al.,* 1999, Shannon and Sawyer, 1989].

Expression of various glutamate receptors has been reported in testes, suggesting the occurrence of glutamatergic system [Gill *et al.,* 2000; Storto *et al.,* 2001]. Indeed, GluR5 and KA1, the functional units of kainate receptors, are both expressed and present in the inner acrosomal membrane, where glutamate is also stored, suggesting an intracrine signalling [Re, 2002].

Pinealocytes express metabotropic glutamate receptor types 3 and 5, and AMPA type ionotropic receptors [Moriyama *et al.*, 2000; Yatsushiro *et al.*, 2000]. Stimulation of mGluR3 causes inhibition of melatonin synthesis through an inhibitory cAMP cascade.

Ionotropic and metabotropic glutamate receptors have been found in lingual tissues and are responsible for sensing the umami taste, the taste deriving from monosodium glutamate [Monastyrskaia *et al.*, 1999; Sako and Yamamoto, 1999].

Glutamate receptors can also act as mediators of injury or inflammation [Said 1999; Said *et al.*, 1996; Lipton and Rosenberg, 1994] and peripheral tissues containing GluRs are also potential target sites for excitotoxicity.

In general, sustained activation of peripheral NMDA and AMPA receptors does not mediate excitotoxic cell death. A probable explanation is that both the depolarization and the increase in intracellular calcium levels in response to glutamate may be only transient in these tissues. However, it has been reported a case of peripheral excitotoxicity in the lung [Said *et al.,* 1996].

In the lung, receptor expression has been demonstrated and glutamate has been shown to induce excitotoxic changes that are inhibited by receptor antagonists. Furthermore, NMDA alters hypoxic pulmonary vasoconstriction in isolated perfused rat lung [Said *et al.*, 1996].

Also in the liver, mGlu5 receptors are expressed by hepatocytes and may be involved in hypoxic injury [Storto *et al.*, 2000].

2.2.3 Glutamate transporters

Glutamate levels in blood and other fluids are tightly controlled around 30-80 µM [Meldrum, 2000]. Most organs, including the kidneys, intestine, lungs, muscles and liver, express highly efficient glutamate transport systems. The high capacity of the peripheral glutamate transporters is exemplified by the observation that continuous intravenous infusion of glutamate causes only a transient elevation of the plasma concentration of the amino acid [Hanley and Varelas, 1999].

Amino acid transporters have been involved in protein synthesis, regulation of cellular metabolism, production of metabolic energy, cell growth, cell volume regulation, nerve transmission, and absorption of amino acids from the lumen in polarised epithelia.

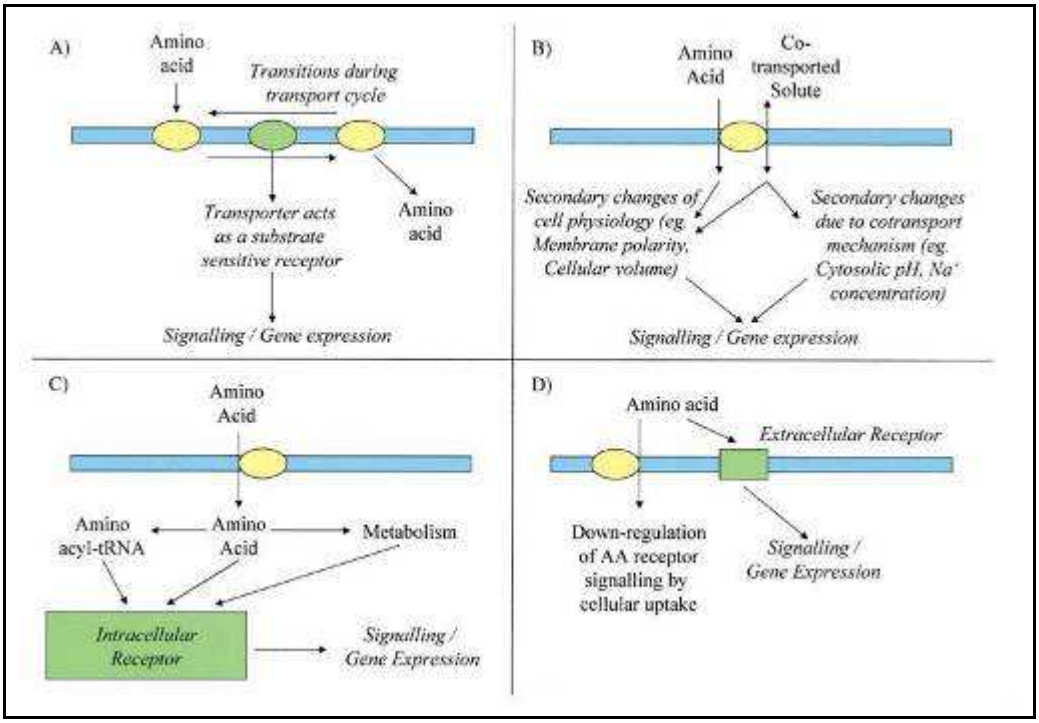

FIG. 7: Potential mechanisms of signal transduction mediated by amino acid transporters [Hyde *et al.*, 2003].

In addition to a traditional role in amino acid transport, transporters may function as amino acid sensors. Direct and indirect mechanisms have been hypothesised for the sensing of amino acids levels (Fig. 7). Transporters may undergo conformational changes (Fig. 7A) or their activity may change pH or membrane potential (Fig. 7B). They may also regulate external amino acid concentration and consequent receptor activation (Fig. 7D). Alternatively, they may control amino acid access to intracellular receptors mechanism (Fig. 7 C) [Hyde at al., 2003; Kwon, 2004].

Sodium dependent glutamate uptake systems have been identified in the following tissues:

Intestine:

In intestine, dietary proteins are absorbed in the form of small di-tri-peptides and individual amino acids, resulting from enzymatic breakdown. Thus, glutamate transporters serve for intestinal absorption of acidic amino acids, a sort of "nutritional uptake".

EAAC1 was first cloned from rabbit intestine [Kanai and Hediger 1992]. Its highest expression is in small intestine mucosa, but it is also present in neurons of the enteric nervous system, where it can cooperate in signal transmission.

Kidney:

Most amino acids, including EAA, are filtered by the kidney and reabsorbed via specialised transport system in the tubuli, so that most of the filtered amino acids are recovered from the final urine. The amino acids reabsorption is mainly mediated by Na/K coupled amino acid transporters, situated on the apical membrane and basolateral membrane of the epithelial cells [Kanai *et al.*, 2000].

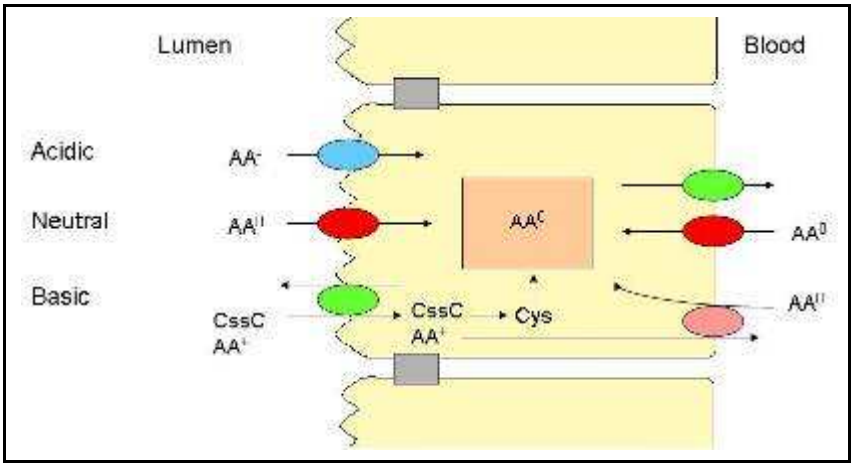

FIG. 8: Trans-epithelial transport of amino acids in the renal tubule [Kanai *et al.*, 2000]

For EAAs uptake, EAAC1 is present in the apical membrane of proximal tubules, in particular in S2-S3 segments and to a less extent in S1, suggesting that an additional glutamate transporter probably exists in the kidney [Kanai and Hediger 2003].

EAAC1 is an important regulator of kidney metabolism, acid-base balance and osmolar homeostasis. The importance of EAAC1 for the functioning of the kidney is evident from

studies with EAAC1 knock out mice: they develop dicarboxilic aminoaciduria, an increased EAA renal excretion, consisting in 1400 fold for glutamate and 10 fold for aspartate. All other amino acids are excreted in comparable amounts and other renal amino acid transporters do not compensate for the loss of EAAC1 function [Peghini *et al.*, 1997].

GLAST mRNA and protein were found in the transitional surface epithelium of the renal pelvis and in the *macula densa* portion of the distal convoluted tubules.

GLT1 mRNA has also been detected in the kidney, but no significant expression of GLT1 protein was observed [Berger and Hediger, 2006] .

Pancreas:

Hormone secretion in the endocrine pancreas is not a simple response to glucose and other nutrients: the endocrine cells express several receptors for neurotransmitters, including acetylcholine, GABA and glutamate [Molnar *et al.*, 1995; Weaver *et al.*, 1996, Liu *et al.*, 1997].

The role of glutamate in the pancreas was first suggested in 1992, in studies using a perfused pancreas model where insulin secretion was abolished by infusion of AMPA receptor antagonist [Bertrand *et al.*, 1992].

It has been hypothesised that glutamate may act as an intracellular messenger in beta cells [Maechler and Wollheim, 1999]. If true, glutamate transporters may be directly involved in the modulation of insulin release.

A glutamate transporter similar to GLT1 has been cloned from pancreas [Manfras *et al.*, 1994], however cellular localisation of this transporter is still unclear. The only finding in isolated islets incubated with D-aspartate, showed sodium dependent accumulation almost exclusively in cells at the islet periphery, supposed to be alpha cells [Weaver *et al.*, 1998].

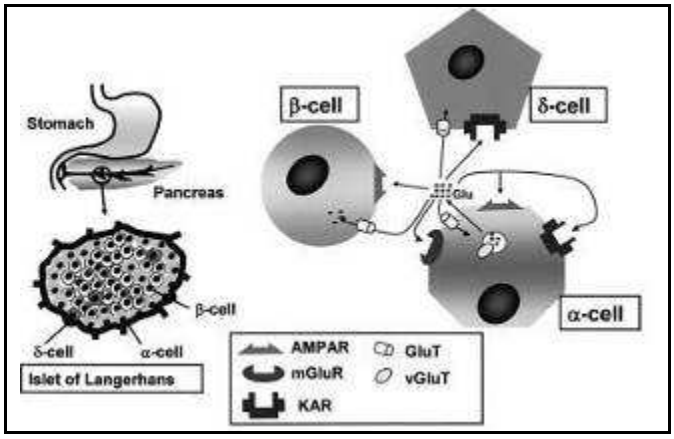

FIG. 9: Glutamate signalling in pancreas. In the islet of Langerhans, functional GluRs, which could modulate secretion of hormones, are identified in α cells [AMPA receptors, KA receptors and mGluRs), in β cells (AMPA receptors), and in δ cells (KA receptors), respectively. High affinity Glu transporters and VGLUTs are also expressed in the islet.

[Hinoi *et al.*, 2002]

Heart:

Amino acids, and in particular glutamate, aspartate, glutamine and alanine, play important roles in the metabolism of heart myocytes. During ischemia, glutamate infusion improves the mechanical function of the myocardium.

GLAST and EAAC1 mRNA have been detected in the heart [Nakayama *et al.*, 1996].

Placenta:

This important tissue has a primary role in the life of a foetus, secreting hormones and providing nutrients and oxygen.

GLAST, GLT1 and EAAC1 have been identified in the placenta, but the interesting aspect is that while placenta supplies the foetus with most of the amino acids, it removes glutamate from the foetal circulation [Schneider *et al.*, 1979]. Since the blood brain barrier is incomplete in foetus, glutamate removal may be important for normal brain development and can provide a protection mechanism.

Mammary Gland: Only GLAST and GLT1 are expressed in the lactating mammary gland [Martinez-Lopez *et al.*, 1998].

Bone:

Glutamate may be one of the endogenous paracrine/autocrine factors used for intercellular communication in bone cells. NMDA receptors appear to be involved in the process of bone formation [Dobson and Skerry, 2000].

Osteoblasts and osteoclasts within the bone cortex express the GLAST glutamate transporter, whereas mononuclear marrow cells express the GLT1 [Espinosa *et al.*, 1999].

Skin:

Keratinocyte differentiation might be regulated by glutamate communication pathway. There is also evidence that other non-keratinized cells that reside within the epidermis, as melanocytes, also use glutamate as a signalling molecule [Frati *et al.*, 2000]. It has been proposed that a sub-epidermal source of glutamate might be involved.

A high level of expression of glutamate transporters has been demonstrated in basal epidermal keratinocytes, and also dermal fibroblasts express functional glutamate transporters, GLAST and GLT1 [Cooper *et al.*, 1998].

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AIM OF THE THESIS

Glutamate is known as an excitatory neurotransmitter in the mammalian nervous system. Emerging evidence suggests that glutamate is also present in peripheral tissues, where it can play a dual role: the first in cellular homeostasis and the second as an extracellular signal mediator, in autocrine and paracrine systems. Relatively little attention has been paid to the functional expression of molecules required for glutamate signalling (receptors, plasma membrane and vesicular transporters) in peripheral non-neuronal tissues.
Given the crucial roles of glutamate transporters in the different peripheral tissues, it can be significant to analyse their functional expression in those environments.
In particular, in this work we analyse the two high affinity glutamate transporters EAAC1 and GLT1.

EAAC1 is present in the renal tubule, where it mediates acidic amino acid reabsorption and regulates acid-base homeostasis. Recent studies indicate that protein-protein interactions and intracellular trafficking represent an important mechanism to regulate transporter activity at the plasma membrane. Therefore, our studies focus on EAAC1 trafficking in epithelial cells (Chapter II).

On the other hand, the role of glutamate as an intercellular signal mediator in endocrine pancreas is established, while the effect of glutamate on islet viability, the expression of glutamate transporters and their physiological functions in the endocrine pancreas are still unclear. In this study we examine the effects of glutamate and the function of the sodium dependent glutamate transporter GLT1 in islet cell models and in isolated islets of Langerhans (Chapter III).

In chapter IV, is reported the experience at University of Texas Health Science Center at San Antonio (UTHSCSA), USA. The goal of this project is to find out interactors of IAPP, a protein involved in diabetes, by means of Yeast Two Hybrid Screening, that allows the identification of direct protein-protein interactions. In prospect, this technique will be a tool to find proteins associated with glutamate transporters.

CHAPTER II: Role of PDZ-mediated interactions in EAAC1 biosynthetic delivery in epithelial cells

1 ABSTRACT

EAAC1 is a high affinity glutamate transporter expressed in neurons and in epithelia. In the Central Nervous System (CNS), EAAC1 controls synaptic transmission and it is involved in long term potentiation (LTP) in memory and learning. In absorptive epithelia, in particular in intestine and kidney, EAAC1 is the major transporter for the dicarboxylic amino acid uptake, as shown by the fact that EAAC1 was first cloned from rabbit intestine [Kanai and Hediger, 1992] and EAAC1 knock out mice develop dicarboxylic aminoaciduria, a disease characterized by increased glutamate and aspartate excretion in urines [Peghini *et al.*, 1997].

In order to be functional, the transporter must be expressed at the plasma membrane, thus alterations of its delivery to or retention in the plasma membrane may be involved in aminoaciduria.

Aim of this work is to study trafficking of the high affinity glutamate transporter EAAC1/EAAT3 in epithelial cells.

Studies performed in the recent years indicate that the surface expression of EAAC1 is controlled by associated proteins that remain to be investigated.

We have focused our attention on a conserved sequence present in the C-terminal domain of EAAC1, that mediates interaction with class I PDZ proteins. PDZ-based interactions and the PDZ protein network ensure that the membrane proteins are correctly delivered to and efficiently retained at the cell surface.

In the past years, we demonstrated that the PDZ-target sequence and therefore PDZ proteins are responsible for the retention and stability of EAAC1 at the plasma membrane. Indeed, removal of this sequence causes the transporter relocalisation from the surface to endocytic compartments [D'Amico *et al.*, 2010].

In this work, we aim at demonstrating whether this PDZ-target sequence is important also for biosynthetic delivery of the transporter.

Our data indicate that PDZ interactions occur early in the biosynthetic pathways and are involved in the ER-to-Golgi trafficking, as well as in Post-Golgi trafficking of EAAC1. Removal of the PDZ motif delays rather than prevents the ER export of the transporter, thus indicating that PDZ interactions facilitate the ER-Golgi trafficking. Possibly, PDZ-interactions favour the transporters homo-oligomerization, a process required for the efficient

ER export of EAAC1. Alternatively, PDZ domain-proteins may couple EAAC1 with protein complexes required for the efficient fusion of carrier vesicles to the appropriate target membrane.

2 INTRODUCTION

2.1 Epithelia

A primary function of epithelial cells is to provide tissue compartmentalization, determining an interface with the external environment, while supporting specialized functions internally.

To establish defined boundaries, cells that cover the external surface and line internal compartments must form barriers to prevent unrestricted exchange of materials.

The plasma membranes of these cells effectively prevent most hydrophilic solutes from crossing the boundary, but also the paracellular pathway between cells is sealed by the junctional complexes. Tight junctions seal the paracellular pathway, the adherens junctions and desmosomes provide the strong bonds necessary to maintain cellular proximity and allow tight junction assembly. Adherens junctions are also critical for epithelial polarization and differentiation. Gap junctions are cell-cell contacts that provide cellular communication.

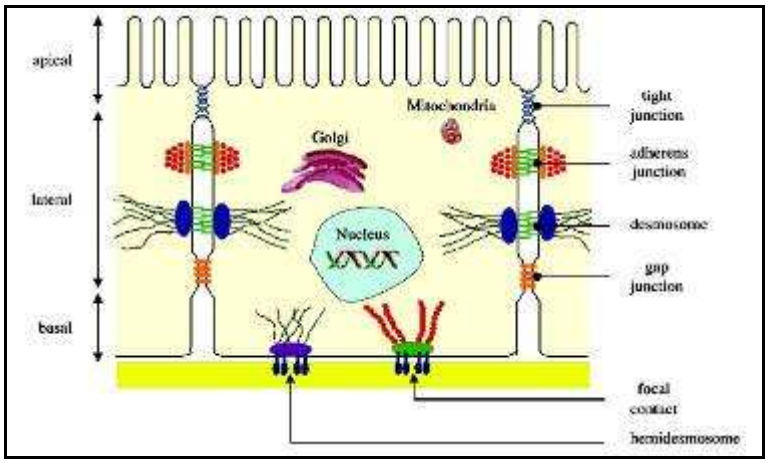


FIG. 1: Schematic representation of an epithelium. The apical, lateral and basal domains are indicated. The junctional complexes are shown.

The deriving absolute barrier would prevent any communication with the external environment, at least via the paracellular pathway. For this reason, the boundaries formed by epithelia are essential for life, but this tissue must be also selectively permeable to ensure the correct composition of, and transport between, distinct compartments.

This is particularly true in the absorptive epithelia, such as intestine and kidney, where the epithelial barrier supports molecule transport while preventing microbial contamination of the surrounding tissues.

2.2 Epithelia Polarity and Trafficking

2.2.1 Absorptive epithelia: Intestine and Kidney

The major function of small intestine epithelium is the absorption of dietary nutrients, water, electrolytes and vitamins, while in the kidney the re-absorption of those substances regulates and maintains body fluid composition.

Intestinal absorption or renal re-absorption implies the movement of a solute from the intestinal/renal lumen to the blood, passing through the epithelium. This transcellular transport is characterised by active or passive movement of solutes and water through cells by means of the specific transmembrane transport proteins. To ensure directional transport of nutrients across the epithelia, these systems are expressed and targeted to different membrane compartments in epithelial cells. As a consequence, these cells exhibit cell-surface polarity, which means that their plasma membrane is divided into specialized regions, apical and basolateral, that are exposed to different environments and which have a characteristic protein and lipid composition.

The maintenance of tissue polarity and regulation of plasma membrane stability of these transporters are fundamental, and are achieved by the highly elaborated cell machinery.

2.2.2 Epithelia polarity

In the past years, an increasing number of disease have been shown to involve defective targeting and trafficking of proteins. These diseases can be grouped under the name of "sorting disorders" and they result from abnormal delivery of functional important proteins to the cell surface [Brown and Breton, 2000].

The plasma membrane of epithelial cells is divided into subdomains of distinct molecular composition: the apical, basal-lateral, and junctional membrane domains (Fig. 1). The apical domain of epithelial cells forms a continuous plane at the cell apex, that is specialised with microvilli. The apical domain is usually in contact with the external surface of an organism, such as developing urine in renal tubule, luminal content in the intestine and secretory material in exocrine glands. In contrast, the basolateral surface faces the internal surface of the organism, being in contact with the blood circulation and adjacent cells.

Each domain has specific protein complexes composed of distinct transmembrane, membrane-associated and cytosolic compartments. These protein complexes mediate the adhesive properties of cells, the formation of the paracellular barrier, and ensure the vectorial solute transport that occurs in absorptive epithelia.

The structural and functional polarity of epithelial cells relays on the selective insertion of proteins and lipids into distinct plasma membrane domains. Proteins are specifically targeted to distinct plasma membrane domains by mechanisms that include an interaction between signals within the protein itself (sorting signal) and a cellular sorting machinery which recognizes the sorting motif. Formation of these protein complexes is determined in large part by interactions of modular protein-binding domains.

2.2.3 Vesicular transport

The basic mechanism to transfer proteins between organelles is mediated by carrier vesicles that continually bud from one membrane and fuse with other and is driven by protein-protein interactions.

Each vesicle transport reaction can be divided into four essential steps, that include vesicle budding, transport, tethering and fusion [Bonifacino and Glick, 2004].

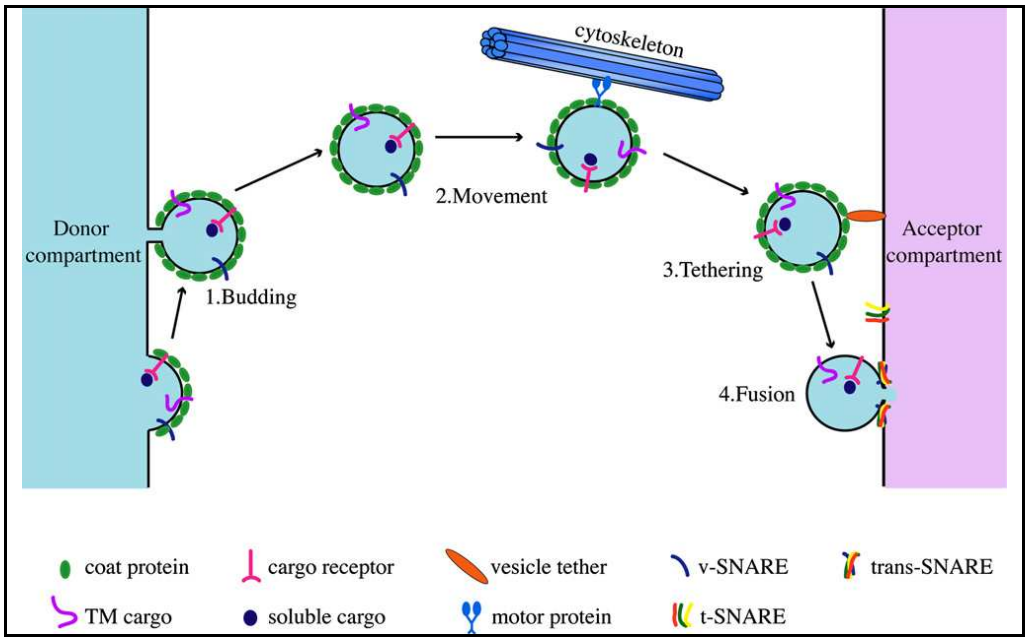


FIG. 2: The Four Essential Steps in Vesicle Transport. (1) *Budding*: coat proteins are recruited onto the donor membrane to induce the formation of a vesicle. Cargo and SNAREs are incorporated into the budding vesicle by binding to coat subunits. (2) *Movement*: the vesicle moves toward the acceptor compartment by diffusion or with the aid of a cytoskeletal track. (3) *Tethering*: tethering factors work in conjunction with Rab GTPases to tether the vesicle to their acceptor membrane. (4) *Fusion*: the vesicle-associated SNARE and the SNARE on the acceptor membrane assemble into a four-helix bundle (trans-SNARE complex), which drives membrane fusion and the delivery of cargo. [Cai *et al*., 2007]

A fundamental element of membrane traffic is vesicle formation, or budding, that is initiated by the selection and concentration of cargo proteins within a plasma membrane subdomain.

This occurs through interactions between sorting determinants on the cargo protein and cytosolic coat components that direct cargo to the forming vesicles [Aridor *et al.*, 1996].

Protein coats are dynamic structures that are recruited from the cytosol onto donor membrane by small GTPases and they deform flat membranes into round buds, which leads to the release of the coated vesicle.

Vesicular transport within the early secretory pathways is mediated by two types of non-clathrin coated vesicles: COP-I and COP-II [Allan and Balch 1999]. COP-I primary acts from the Golgi to the endoplasmic reticulum (ER) and between Golgi cisternae. COP-II mediates trafficking from the ER to Golgi [Barlowe *et al.*, 1994; Letourneur *et al.*, 1994; Waters *et al.*, 1991].

Clathrin coated vesicles, the first identified [Pears, 1975], are mainly derived from the plasma membrane or the Trans-Golgi network (TGN) and are transported to endosomes [Owen *et al.*, 2004]. The main adaptors are proteins of AP2 complex.

Coat recruitment is coupled with the acquisition of SNAREs (SNAP- Soluble N-ethylmaleimide sensitive-factor Attachment Protein- Receptors) proteins that direct the vesicles to their target membranes [Söllner *et al.*, 1993].

After budding, cargo vesicles are transported to their final destination by diffusion or by motor-mediated transport along a cytoskeletal track.

In particular, vesicles interact with microtubules, by means of the so-called "microtubule motors", dynein and kinesin families [Schroer and Sheetz, 1991]. It has been shown that microtubule disruption perturbs the delivery of proteins to the cell surface, and causes a marked shift in the distribution of many membrane proteins from their usual surface location onto scattered intracellular vesicles [Brown and Stow, 1996]. In the absence of an intact microtubule network, also the exocytic step of the recycling pathway is inhibited, thereby causing the same phenotype. Thus, microtubule are involved both in the initial delivery of newly synthesised proteins to the cell surface and in the continued maintenance of specific cell surface domains. Moreover, not only apical targeting is achieved by microtubules, as it is now clear that also basolateral protein trafficking can also involve microtubules [Brown *et al.*, 1991; Lafont *et al.*, 1994] .

Also the actin cytoskeleton may serve as mechanical element that drives and guides vesicles movement within the cells [Rogers and Gelfand, 1998]. In addition, the actin cytoskeleton may directly concur to cell polarity by anchoring proteins in specific plasma membrane domain [Hammerton *et al.*, 1991]. This interaction is usually mediated by cross-linking and anchoring proteins such as ankirin and fodrin [Nelson and Hammerton, 1989].

The last step in vesicle-mediated transport is the recognition and fusion of the vesicle with its target membrane, that involves the so-called tethering factors and SNAREs.

Acting upstream of the SNAREs, tethering factors mediate the first point of contact and the specificity of vesicle targeting, interacting and working together with coat proteins. Tethering is a term used to describe the initial interaction between a vesicle and its target membrane. Tethering factors may couple the recognition of a vesicle to the process of vesicle uncoating and then bring the vesicles in closer contact with its target compartment [Malsam *et al.*, 2005].

Almost all tethering factors fall into two broad categories: long putative coiled coil proteins and multi-subunit complexes. To date, eight conserved complexes have been identified as crucial for exocytic and endocytic trafficking events (Fig. 3) [Cai *et al.*, 2007].

Tethers can be also Rab effectors and Rab exchange factors. Rabs are small GTPases of the Ras superfamily that continuously cycle between the cytosol and membranes. Rabs act at multiple stages of the exocytic and endocytic pathway, and in their GTP-bound form they appear to facilitate the recruitment of tethers to specific locations.

Tethering factors may actively promote SNARE-mediated membrane fusion by stimulating the formation of trans SNARE complexes [Shorter *et al.*, 2002].

SNAREs, a family of membrane proteins that are related to three different neuronal proteins: sinaptobrevin, syntaxin and SNAP-25. Specific membrane associated SNAREs form a sort of lock and key that is activated by selective recognition among these proteins. A SNARE on a transport vesicle (v-SNARE) pairs with its cognate SNARE-binding partner (t-SNARE) on the appropriate target membrane [Rothman, 1994; Sollner *et al.*, 1993].

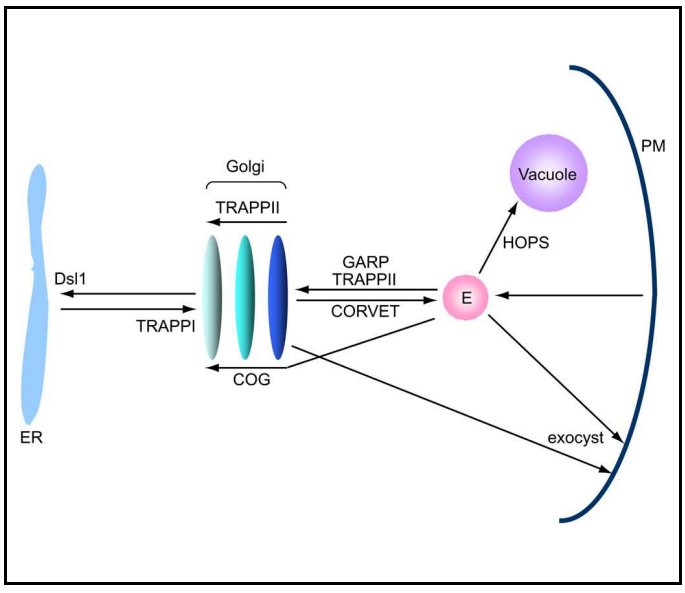


FIG. 3: Tethering complexes that act in the secretory and endocytic pathways. Protein complexes that play a role in vesicle tethering are indicated. *TRAPPI* acts in ER-to-Golgi traffic, while *Dsl 1* operate in the reverse transport. *TRAPPII* has been implicated in transport events that take place within the Golgi, and from the endosome to the late Golgi. *COG* has been proposed to tether vesicles from the endosome to the cis-Golgi. *GARP/VFT* complex has been implicated in traffic from the endosome to the trans-Golgi. The *CORVET* complex may be required for tethering events between the trans-Golgi and endosome. *HOPS* functions between the endosome and vacuole and in homotypic vacuole fusion. The *exocyst* complex binds to post-Golgi and recycling vesicles at the plasma membrane. [Cai *et al.*, 2007]

2.2.4 *Protein delivery to the plasma membrane*

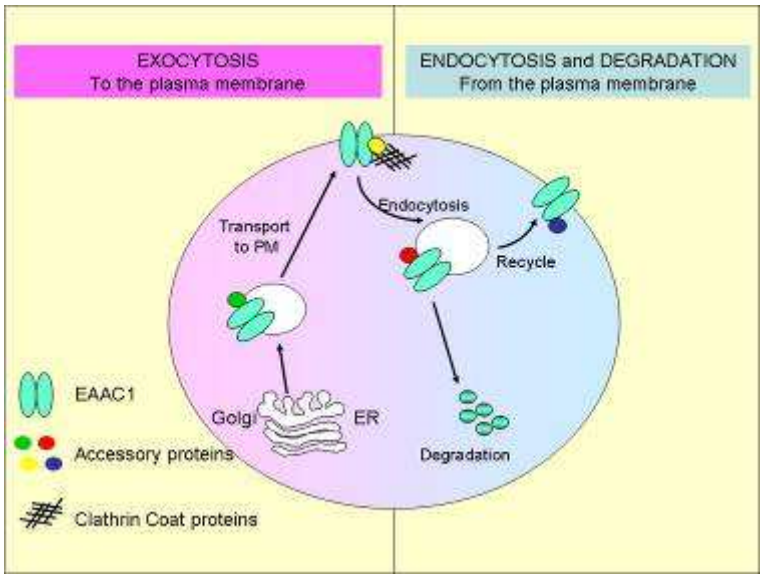


FIG. 4: Schematic representation of protein trafficking. Protein density at the plasma membrane is the result of dynamic movement to and from the plasma membrane.

The correct surface expression of membrane proteins is a multistep process [Mostov *et al.*, 2003] involving:

1- Protein folding and maturation

2-Export from the ER

3-Golgi processing

4-Biosynthetic sorting from the Trans-Golgi Network to the correct plasma membrane domain (apical or basolateral)

5- Anchoring and/or retention at the plasma membrane

1) Protein folding and maturation

Protein folding and maturation are essential to ensure efficient protein transport through the secretory pathway. In eukaryotic cells, newly synthesised transmembrane proteins are inserted co-translationally into the endoplasmic reticulum (ER), with the assistance of molecular chaperons and folding enzymes [Nickel and Wieland, 1998; Nishikawa *et al.*, 2005]. These enzymes interact with the nascent polypeptide chains and facilitate its folding, oligomerization, maturation and post-translational modifications, important for the correct protein trafficking.

Incorrectly folded or incorrectly oligomerized proteins are potentially cytotoxic, and are removed by a quality control system [Bychkova and Ptitsyn, 1995; Kim and Arvan, 1998]. When mis-folded proteins accumulate in the ER, prolonged ER chaperon binding reduces the

concentration of free chaperones, that represents a key step in ER quality control. To cope with the resulting ER stress, cells induce the transcription of gene products that facilitate the processing of aberrant proteins and that attenuate protein translation. If protein repair by ER chaperons is unsuccessful, aberrant proteins are cleared from the ER by a mechanism termed ER associated degradation (ERAD) [Brodsky and McCracken 1997; McCracken and Brodsky, 2003; Tsai and Rapoport, 2002]. In this process, aberrant proteins are translocated to the cytosol and degraded by the ubiquitin-proteasome system. A single point mutation can have a dramatic effect on this folding process, as observed for the cystic fibrosis transmembrane regulator (CFTR) deleted in position 508 [Brown *et al.*, 1998; Qu *et al.*, 1997].

In ER compartments, occurs the first modification of proteins after synthesis: the addition of mannose-6-phosphate group [Sly and Fischer, 1982]. This sugar residue binds to specific mannose-6-phosphate receptors in the Golgi cisternae and causes these proteins to be selected for packaging into specific vesicles directed to lysosomes.

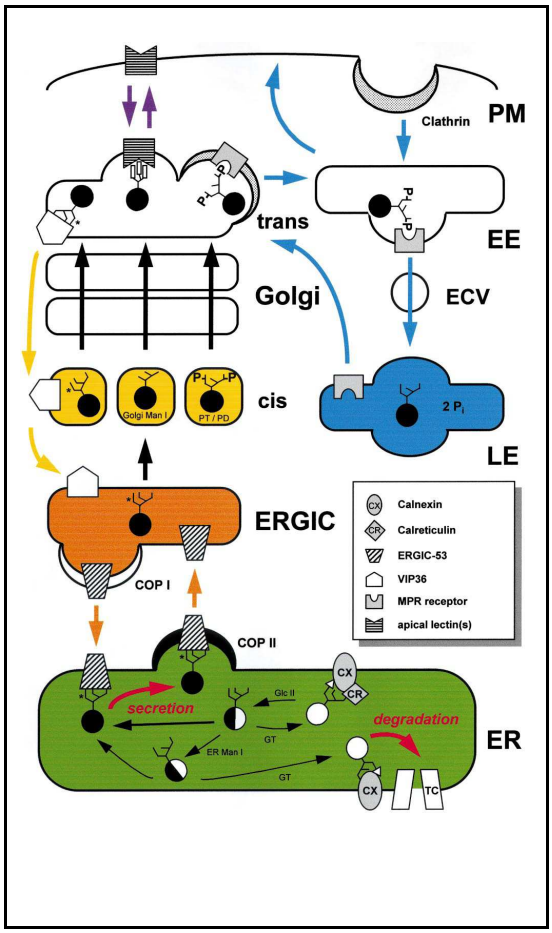


FIG. 5: Lectin-mediated glycoprotein transport in the secretory pathway. After synthesis and N-glycosylation, glycoproteins bind to the calnexin and/or calreticulin. Then, the glycoproteins are arranged by glucosidase II (GlcII) and, if still incompletely folded (white), they are reglycosylated by GT and re-directed to another cycle of quality control.
After prolonged residence in the ER, mannosidase I (ER Man I) removes one mannose residue. Incompletely folded and reglycosylated glycoproteins are targeted to the cytosol and degraded by the proteasome.
Correctly folded proteins (black) are transport-competent and some of them (*) may bind to ERGIC-53, which recruits them to COPII buds and thereby facilitates transport to the ERGIC.
In the cis-Golgi, glycoproteins are either trimmed to Man5 prior to reglucosylation by Golgi glycosyltransferases, or tagged with the lysosomal signal Man-6-P by sequential action of phosphotransferase (PT) and phosphodiesterase (PD).
Some glycoproteins escape cis-Golgi trimming but may be recognized by VIP36 and recycled to the cis-Golgi for another attempt. Proteins carrying Man-6-P residues are recognized by MPRs in the trans-Golgi and sorted to endosomes via clathrin-coated vesicles.
N-Glycans also serve as signals for Golgi exit and apical targeting in epithelial cells.

ECV, endosomal carrier vesicles; EE, early endosome; LE, late endosome; TC, translocation channel.
[Hauri *et al.*, 2000]

2)Export from the ER

Correctly folded proteins are then ready for the export from the ER. For the ER-to-Golgi transport and recycle between the ER and the cis face of the Golgi stack, SNARE proteins are also required [Wooding and Pelham, 1998; Banfield *et al.*, 1995; Rowe *et al.*, 1998].

At the level of the ER, proteins destined for export are thought to become concentrated into departing transporter vesicles, while residents of the ER are excluded [Balch *et al.*, 1994], indicating the existence of a machinery involved in active packaging of cargo molecules. The selection of cargo molecules at this step could be mediated by p24 proteins.

Cargo might interact with coat components either directly or indirectly via cargo-coat receptors. Among these, ERGIC-53 has been described as a mannose-specific lectin that constitutively cycles through the early secretory pathway, implicating a role of sorting of mannose-containing cargo molecules.

Simultaneously, retrograde protein transport occurs from the Golgi to ER [Tsai *et al.*, 2002]. It is not clear if this retrograde transport involves the intermediate compartment or if proteins can be transported directly to the ER.

The traffic from the ER is mediated by protein-protein interactions, that involve adaptor proteins and sequences on the cargo protein. The VMI-motif of GAT1 provides an example of a cargo-based motif that specifies export from the ERGIC (ER to Golgi Intermediate Compartment) [Farhan *et al.*, 2008].

3)Golgi processing

During their transport to the surface, cargo proteins pass through the various cysternae of the Golgi apparatus. In mammalian cells, multiple Golgi stacks are linked together to form a reticular structure that is actively maintained around the centrosome by interactions with microtubules and accessory proteins [Rios and Bornens., 2003]. The cis and trans side of these stacks are associated with networks of tubular structures: the cis-Golgi network or endoplasmic reticulum-Golgi intermediate compartment (ERGIC) and the trans Golgi network (TGN) respectively, representing the cargo entry and exit points [Allan and Balch, 1999].

As cargo moves through the Golgi stack, it is modified by Golgi-associated processing enzymes, that ensure correct post-translational modification of proteins and promote efficient sorting and trafficking of secretory proteins.

The most common form of post-translational processing is glycosylation [Wang *et al.*, 1994]. Vertebrates and especially mammals, have evolved a highly complex glycan repertoire, that represents the molecular base for interspecies recognition systems.

In mammals, the enzymatic process of glycosylation utilizes nine monosaccarides, that are provided by conserved biosynthetic reactions and are produced predominantly in the secretory pathway of the cell [Ohtsubo and Marth, 2006]. Within the Golgi apparatus, glycans become increasingly oligomeric and branched as they transit to the latter portion of the secretory system; the final proteins are then delivered to the cell surface and extracellular compartments.

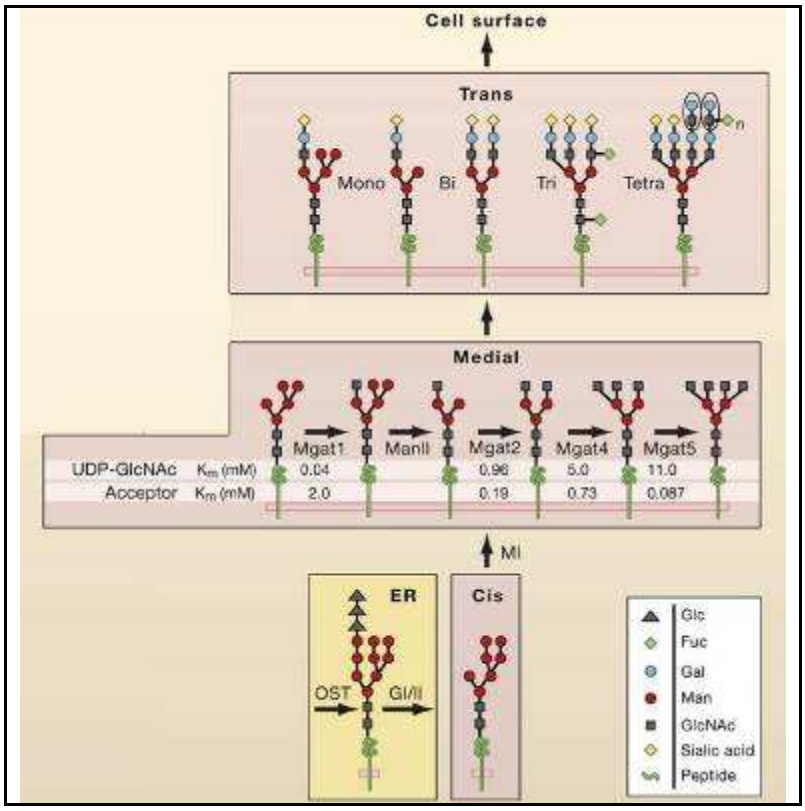


FIG. 6: Glycosylation pattern along the biosynthetic pathway. Different glycans composition is achieved gradually and is characteristic of each cellular compartment.

[Dennis *et al.*, 2009]

There are several types of glycosylation, whose classification depends on target amino acids. N-glycans are linked to asparagine residues of the protein, in the Asn-X-Ser/Thr motif. O-glycans are attached to a subset of serines and threonines [Schachter, 2000; Yan and Lennarz, 2005].

Glycans produced in the secretory pathway participate in multiple mechanisms of cellular regulation. The influence of glycans on protein-protein interactions encompasses a large number of cellular functions that span from nascent protein folding and intracellular

trafficking to roles in extracellular compartments where cell-cell communication is modulated.

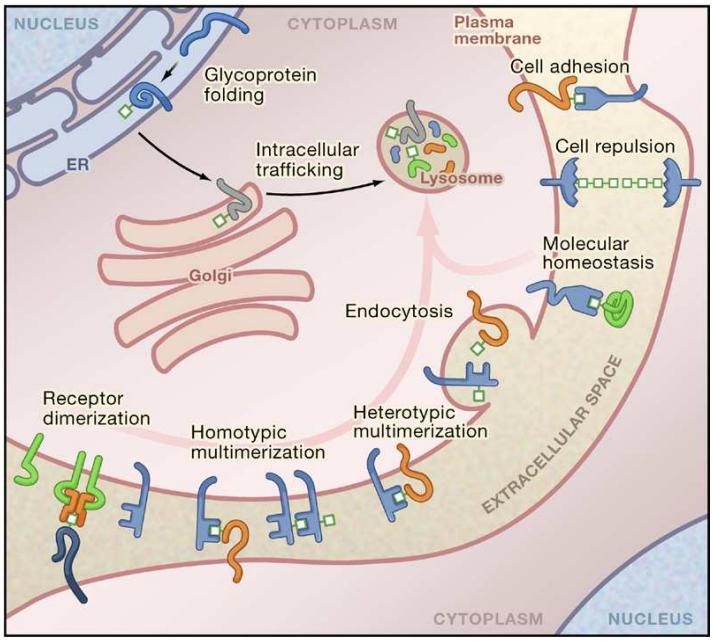


FIG. 7: Glycan functions. Glycans function is not limited to intracellular traffic. It serves for several cellular functions, that are represented here [Ohtsubo and Marth, 2006].

4)Biosynthetic sorting from the Trans-Golgi Network to the correct plasma membrane domain (apical or basolateral)

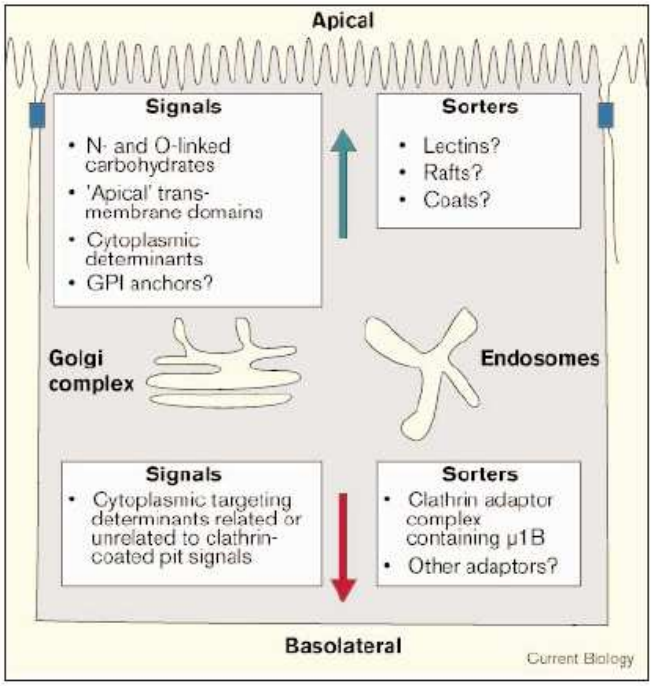


FIG. 8: Targeting of membrane proteins in polarised cells. The different types of targeting signals that mediate apical or basolateral transport are indicated.

Following post-translational modifications, the trans side of the Golgi stack serves as a key sorting station, directing cargo to multiple intracellular and extracellular destinations. Indeed, the stack in the trans cysternae are sorted into constitutive secretory vesicles that fuse with the plasma membrane. In epithelial cells, at the level of TGN, apical or basolateral proteins are sorted into different vesicles that fuse with the respective apical or basolateral domain [Brown and Breton, 2000].

In the table below, the main sorting signals are summarised:

Table 1 | Sorting signals and the elements of the sorting machinery

Signals	Examples	Elements of the sorting machinery
Apical		
Lipid rafts	HA, PLAP, GPI-anchored proteins	VIP17/MAL, galectin-4, FAPP2, annexin-13b, annexin-2, kinesin, KIFC3
Glycosylation	Clusterin (gp80), H,K-ATPase β-subunit, P75NTR, LPH, SI, glycoprotein g114	Lipid rafts, galectin-3, kinesin, KIF5B
Cytoplasmic sequences	Rhodopsin, megalin, receptor guanylate cyclase, M_2 muscarinic receptors, ATP/B, copper-ATPase, NKCC2, PMCA2	Dynein light-chain Tctex
Transmembrane sequence	H,K-ATPase α-subunit	
PDZ motifs	CFTR, Na^+/H^+ exchanger, NaPiII	NHERF, NaPi-Cap2
Basolateral		
Cytoplasmic YXXΦ (Φ-bulky hydrophobic) motifs	LDL receptor, VSV-G, pIgR, hTfnR (human transferrin receptor)), TGN38, AQP4	AP-1, AP-4, exocyst
Cytoplasmic [DE]XXXL[LI] motifs	E-cadherin, sulfate/bicarbonate/oxalate anion exchanger sat-1, MCAM, NKCC1 CD147, MHCII, furin	AP-1, exocyst
PDZ motif	Kir 2.3, GABA transporter, BGT-1, GABA transporter, GAT-2, Syndecan-1, α5β-1 integrin	Syntrophin, Lin-7/CASK, PSD-93, SAP97, Cdc42
Non-canonical sequences	Transferrin, NCAM, TGF-β receptor	

Apical sorting determinants and their cellular adaptors or receptors are still unclear and remain to be identified. Until know, an heterogeneous population of apical sorting signals have been described (Fig. 8).

Among them, is the lipid anchoring glycosyl-phosphatidylinositol bridge (GPI), a post-translational modification in the ER, which is the first apical sorting signal that was described [Lisanti *et al.*, 1988; Brown *et al.*, 1989]. Other determinants for proper apical delivery are the presence of N- or O-glycans [Le Bivic *et al.*, 1993; Yeaman *et al.*, 1997; Benting *et al.*, 1999; Alfalah *et al.*, 1999; Jacob, *et al.*, 2000].

Specialised lipid rich membrane segments, called glycolipid rafts, appear to be involved in this process [Harder et a., 1998]. Some polypeptides of the annexin family also participate in apical transport of raft-associated membrane proteins. Recent data have highlighted the involvement of a new lectin family for apical trafficking, the galectins. In enterocytes,

interaction between galectin-4 and sulfatides plays a role in the organisation of lipid rafts for efficient apical surface delivery [Danielsen and van Deurs, 1997]. On the contrary, galectin-3 in MDCK cells seems to target apical vesicle in a raft-independent mechanism [Delacour *et al.*, 2006].

Apical sorting signals that mediate the incorporation into membrane microdomains have also been identified in the transmembrane domains of proteins. The length and the primary amino acid sequence of the transmembrane domain seem to be important for rafts association and lipid based sorting [Munro, 1995; Nezil and Bloom, 1992].

In addition, apical sorting signals of different nature have been identified in the cytosolic tails of rhodopsin [Chuang and Sung, 1998], megalin [Marzolo, *et al.*, 2003; Takeda *et al.*, 2003] and receptor guanylyl cyclases [Hodson *et al.*, 2006]. There is still a significant number of apical proteins with unknown apical sorting information, and therefore other signals variants hidden in proteins may also play a role in apical protein delivery.

Basolateral signals are localised in the cytoplasmic domain of basolateral proteins and consist of tyrosine or dileucine motifs, which are often found in the vicinity of stretches of amino acids.

5) <u>Anchoring and/or retention at the plasma membrane</u>

The fine modelling of plasma membrane domains is achieved by protein-protein interactions, that may increase the residence time of a specific protein at the plasma membrane. Many of these ancillary proteins act as a bridge between the transmembrane protein and the cytoskeleton, creating a stable complex at the plasma membrane. This interactions may prevent plasma membrane protein internalisation in endocytic or degradative compartments.

It has been established that receptor mediated endocytosis and sorting in endosomal-lysosomal system occur through coated areas of the membranes. Protein coats are mainly composed of the structural protein clathrin, of adaptor proteins (AP) and other accessory factors. The recognition of specific sorting motifs in the intracellular tails of membrane proteins by these adaptor proteins activates the internalisation processes via protein interactions. The signals are similar to that of basolateral targeting, as they contain tyrosine-based sequences and di-leucine motifs.

2.3 PDZ Domain-containing proteins

Essentially, protein trafficking along the biosynthetic route strongly relies on the coordinated cooperation of proteins. At molecular level the interactions are driven by structurally conserved domains that specifically recognise peptide ligands in other proteins [Pawson *et al.*, 2002]. Among these, in the recent years, PDZ domains have been described and involved in several step of the biosynthetic pathway.

The most abundant protein-protein interaction domains that contribute to tethering proteins to the cytoskeleton and to the trafficking machinery are PDZ domains [Ponting 1997; Rongo, 2001], contained in the so-called PDZ proteins.

PDZ domains are modular domains of 90 amino acids . They were originally identified as conserved sequence elements within the post synaptic density protein PSD95, the Drosophila tumour suppressor dlg-A, and the tight junction protein ZO-1 [Pawson and Scott, 1997]. PDZ domains are often found in proteins with other known interaction domains or signalling domains.

The primary function of PDZ domain is to recognise and bind specific amino acid sequences localized at the COOH terminus of target proteins. Depending on the target sequence, PDZ domains are traditionally classified as class I, class II and class III domains, which recognise the consensus sequences listed in the table below [Brone and Eggermont, 2005]:

PDZ domain	Consensus sequence
Class I	X-S/T-X-Φ
Class II	X- Φ-X-Φ
Class III	E-D-X-W-C/S

Cristallographic studies demonstrate that the PDZ domain is composed of six β strands forming two opposing antiparallel sheets flanked by two alpha helices. The COOH terminus of the interacting protein lies within an elongated surface groove as an antiparallel beta strand and the hydrophobic side chain inserts into an hydrophobic cavity at the end of the groove [Doyle *et al.*, 1996].

PDZ proteins often contain multiple protein-protein interaction domains that can cooperate to bind more than one target ligand. They have also been demonstrated to bind directly to other PDZ proteins, forming homomeric and heteromeric complexes [Brenman *et al.*, 1996].

On the basis of their domains, PDZ proteins can be divided into three major groups:

1-PDZ-only proteins: containing one or more PDZ domains

2-Membrane associated guanylate kinase (MAGUK) domain-containing proteins

3-PDZ proteins that contain other protein interacting domains, e.g. SH3, PH and L27 domains in addition to their PDZ domains.

PDZ interactions are not limited to transmembrane proteins, but also include a large number of different cytosolic proteins comprising components of signal transduction pathways and the cytoskeleton (Fig. 9).

The interaction of PDZ domains with binding partners can be directly regulated by posttranslational modification triggered by receptor activation. Both intracellular and extracellular signals can control the phosphorilation of residues within the C-terminal target sequences of interacting partner or within the PDZ domain itself.

Thus, it seems likely that regulated association of PDZ-containing proteins may represent a general mechanism for regulating the temporal and spatial assembly of complexes.

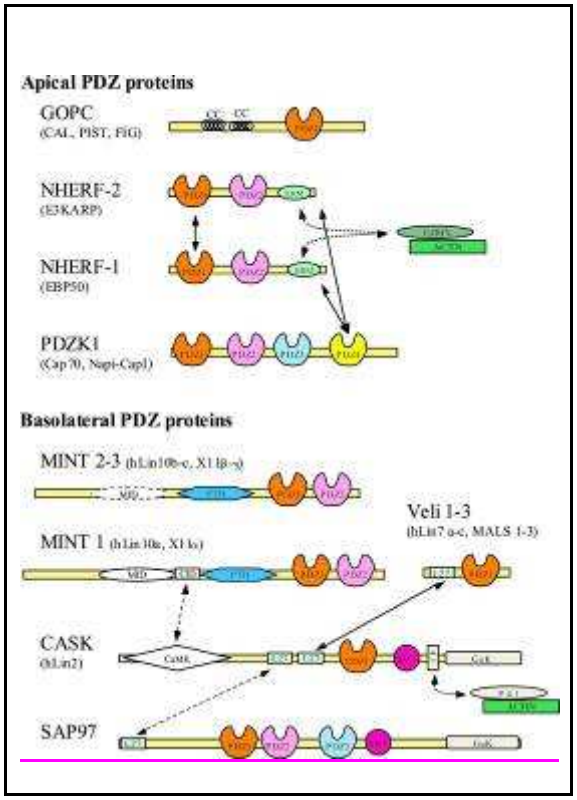


FIG. 9: Schematic structure of PDZ domain-containing proteins that play a role in the polarized expression of membrane proteins. Alternative names of the PDZ proteins are indicated in brackets. Possible interactions between PDZ proteins in epithelial cells (solid lines) and neurons (dashed lines) are indicated. Possible binding to the actin cytoskeleton is indicated by dotted lines.

4.1, protein 4.1-interacting domain or Hook domain; CAL, CFTR-associated ligand; CaMK, calmodulin kinase II-like domain; CAP70, CFTR accessory protein-70; CASK, calcium/calmodulin-dependent serine protein kinase; CID, CASK-interacting domain; CC, coiled-coil domain; E3KARP, NHE3 kinase A regulatory protein ; EBP50, ezrin-radixinmoesin-binding phosphoprotein-50; ERM, ezrin-radixinmoesin-binding domain; FIG, Fused in glioblastoma; GOPC, Golgi-associated PDZ and coiled-coil motif-containing protein; GuK, guanylate kinase-like domain; L27, L27 domain; MALS, mammalian Lin-7; Mint, Munc18-1-interacting protein; NaPi-Cap1, Na^+/phosphate cotransporter-CFTR accessory protein 1; NHERF, Na^+/H^+exchanger regulatory factor; P4.1, protein 4.1; PDZ, PDZ domain; PDZK1, PDZ containing 1; PIST, PDZ domain protein interacting specifically with TC10; PTB, phosphotyrosine-binding domain; SAP97, synapse-associated protein 97; SH3, SH3 domain; Veli, vertebrate Lin-7.

[Brone and Eggermont, 2005]

2.3.1 Role of PDZ domains in cell polarity and protein trafficking

PDZ domains have been involved in several processes that may be relevant to protein delivery to polarized domains.

- *ER export:* PDZ proteins have been involved in the early events of assembly, processing and delivery of interacting partner. The interaction of SAP97 (a PDZ protein) with NMDA receptors occurs in the endoplasmic reticulum and is critical for the delivery of receptor complexes to synapses [Sans *et al.*, 2001; Hayashi *et al.*, 2000; Shi *et al.*, 2001; Prybylowski *et al.*, 2002; Sprengel *et al.*, 1998; Mori *et al.*, 1998]. It has been proposed that in neuronal processes NMDA receptors are retained in the ER, unless an interaction with PDZ proteins occurs, thus allowing receptor transport to the plasma membrane.
- *Selective plasma membrane targeting:* Each PDZ protein is generally restricted to specific subcellular domains, such as synapses, cell-cell contacts, or the apical/basolateral cell surface [Fanning and Anderson, 1999]. This leads to the speculation that PDZ domains are necessary for the biosynthetic targeting and polarised expression of membrane proteins. An example is the GAT2 transporter, that has a sequence of 22 amino acids at the C terminus required for the transporter's basolateral distribution. Whereas the GAT3 C-terminal sequence contains a PDZ consensus sequence, necessary for its apical targeting [Muth *et al.*, 1998].
- *Retention and stabilisation in specific subdomains*: PDZ network ensures that the membrane proteins are efficiently delivered and retained at the cell surface. As PDZ proteins contain actin binding domains, they appear to act as a bridge between PDZ binding proteins and actin cytoskeleton, therefore anchoring target proteins to the cell surface. For example, actin confines GAT1 to the plasma membrane via ezrin, and this interaction is mediated through the PDZ-interacting domain of GAT1 [Imoukhuede *et al.*, 2009]
- *Organization of functional domains*. PDZ proteins have been involved also in tethering or clustering functionally interdependent proteins in specific cell surface domains, thereby increasing the efficiency of their functions. Indeed, the structural features of PDZ domains allow them to mediate more than one specific protein-protein interaction. The ability to cluster transmembrane receptors and channels has great functional significance. Receptor clustering is a well-established prerequisite for

receptor activation and might also provide a mechanism to coordinate activity of different transmembrane receptors and ion channels.

An example is provided by the PDZ protein NHERF (Na^+/H^+exchanger regulatory factor), that has two PDZ domains and binds directly to both the β2-adrenergic receptor and NHE-3. It has been demonstrated that activation of β2-adrenergic receptor can alter the activity of NHE and that NHERF is required for this regulation [Hall *et al.,* 1998]. In addition, the PDZ protein NHERF both binds to the cystic fibrosis transmembrane regulator (CFTR) chloride channel and the Na+/H+ exchanger on the apical plasma membrane [Short *et al.,* 1998].

2.4 Regulation of EAAC1 surface expression, function and trafficking

2.4.1 Intestinal and renal transport systems

In kidney and intestine, an important category of polarized proteins are amino acids transporters. Indeed, to perform a directional transepithelial solute transport, membrane transporters need to be targeted selectively to either the apical or basolateral membrane domain.

Amino acid transporters are important for organism physiology as they can influence normal cell function, can regulate synaptic signalling, neuronal excitability and pH homeostasis in several tissues [Bröer, 2008].

Definitely, amino acids transporters expressed in absorptive epithelia are critical for the homeostasis of plasma amino acid levels and for the supply of amino acids to all tissues, where they serve as building blocks for protein synthesis, as precursors for a wide variety of bioactive molecules, and as energy metabolites. This is illustrated by a number of inherited disorders affecting amino acid transport in epithelial cells, such as cystinuria, lysinuric protein intolerance, Hartnup disorder, iminoglycinuria, dicarboxylic aminoaciduria, and some other less well-described disturbances of amino acid transport.

Table 1. Apical amino acid transporters

System	cDNA	SLC	Amino Acid Substrates	Disorder	Mechanism	Tissue
B^0	B^0AT1 B^0AT2	SLC6A19 SLC6A15	AA^0 P,L,V,I,M	Hartnup	Na^+-Sp	Ki,SI Ki,Br
$b^{0,+}$	rBAT/ $b^{0,+}AT$	SLC3A1/ SLC7A9	R,K,O,cystine	Cystinuria Cystinuria	AA^+/AA^0- Ap	Ki,SI Ki,SI
Gly	XT2	SLC6A18	G	Iminoglycinuria (candidate)	n.d.	Ki
IMINO	IMINO	SLC6A20	P, HO-P	Iminoglycinuria (candidate)	Na^+,Cl^--Sp	Ki,SI,Br Ki,SI,Br
PAT (Imino acid)	PAT1	SLC36A1	P,G,A GABA, β-A	Iminoglycinuria (candidate)	H^+-Sp	Ki,Lu,Ht
	PAT2	SLC36A2	P,G,A	Iminoglycinuria (candidate)	H+-Sp	
X^-_{AG}	EAAT3	SLC1A1	E,D	Dicarboxylic	Na^+,H^+-Sp K^+-Ap	Ki,SI,Br

Summary of the major apical amino acid transporters found in kidney and intestine. AA°, neutral amino acids: HO-P, hydroxyproline; β-A, beta-Alanine; O, Ornitine; All the other amino acids are listed in one-letter code; Br, Brain; Ht, Heart; Ki, Kidney; Lu, Lung; SI, small intestine. [Broer, 2008b]

Based on functional studies in kidney and intestine and the amino acid profile in the urine of individuals with different aminoacidurias, five transport activities were proposed [Milne, 1964; Scriver *et al.*, 1976; Thier and Alpers, 1969; Young JA, Freedman, 1971].

1) the "neutral system" or "methionine preferring system" transporting all neutral amino acids;

2) the "basic system" transporting cationic amino acids together with cystine;

3) the "acidic system" transporting glutamate and aspartate;

4) the "iminoglycine system" transporting proline, hydroxyproline, and glycine;

5) the β-amino acid system.

2.4.2 EAAC1

We have focused our study on the glutamate transporter excitatory amino acid carrier 1 (EAAC1/ EAAT3) [Royle *et al.*, 2003].

EAAC1 is largely distributed in absorptive epithelial tissues such as kidney and intestine. EAAC1 was first cloned from rabbit intestine [Kanai and Hediger 1992] and EAAC1 mRNA was found in the small intestine where its amount increases after a high protein diet in animals [Erickson *et al.*, 1995]. Moreover, there is evidence of the presence of EAAC1 protein in enteric nervous system [Liu *et al.*, 1997]. In rat kidney, the transporter is predominantly located in the brush border membrane of proximal tubule S2 and S3 segments [Shayakul *et al.*, 1997]. In this location, the primary function of EAAC1 transporter is the final re-

absorption from the glomerular filtrate of glutamate and aspartate that escaped the early part of the proximal tubule [Burch *et al.*, 1978; Silbernagl, 1983]. The EAAC1-mediated absorption is highly effective since more than 90% of the filtered dicarboxylic amino acids are recovered from the final urine [Hediger, 1999].

EAAC1, located in the apical membrane of proximal tubules epithelia, is functionally and physically coupled to the phosphate-independent but bicarbonate-dependent glutaminase (PIG) and regulates both glutamine metabolism and paracellular permeability [Welbourne & Matthews, 1999] (Fig. 10).

EAAC1 has also been involved in glutathione homeostasis as it can transport cysteine, whose abundance is the rate limiting step in glutathione synthesis [Zerangue *et al.*, 1996; Aoyama *et al.*, 2006].

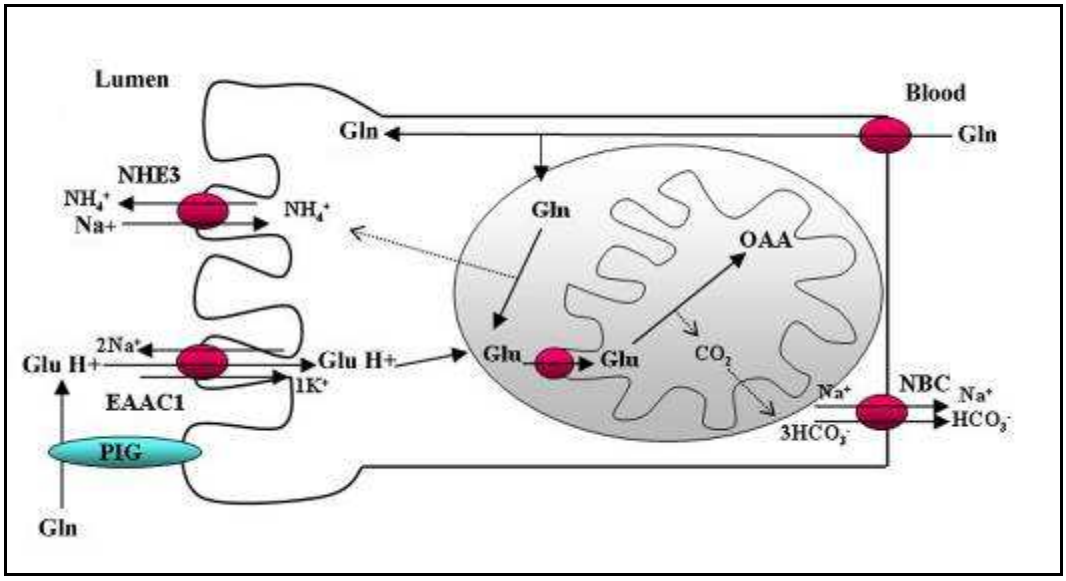


FIG. 10: Metabolic acidosis and glutamate metabolism in rat kidney proximal tubule cells. Image modified from Hediger, 1999.

Alterations in the human EAAC1 transporter activity are associated with dicarboxylic aminoaciduria, an inborn error of glutamate/aspartate transport across epithelial cells of kidney and intestine, characterized by an excessive excretion of glutamate and aspartate. The importance of EAAC1 in kidney function is confirmed by mice models deficient in EAAC1 that develop dicarboxylic aminoaciduria without neurological or cognitive abnormalities [Peghini *et al.*, 1997].

In order to be functional, EAAC1 must localize to the apical plasma membrane of epithelial cells [Beckman and Quick, 1998].

A hierarchy of trafficking processes, coordinated by several distinct signals, and interacting proteins must control EAAC1 density at the plasma membrane. However, our understanding of the molecular mechanisms which govern EAAC1 delivery and dynamics at the cell surface is at the beginning.

Plasma membrane delivery of EAAC1 is a complex mechanism that requires homo-oligomerization and efficient export of newly synthesized transporters from the Endoplasmic Reticulum (ER), a process which is regulated by interactions with accessory proteins, like GTRAPs (Glutamate transporter associated proteins). These proteins are structurally related to the Ras Superfamily, but they lack a GTP-binding consensus motif. In the last years, it has been demonstrated that GTRAP 3-18 delays the ER exit of EAAC1 by interaction with its C-terminus [Lin *et al.,* 2001; Haase *et al.,* 2001; Ruggiero *et al.,* 2008].
The EAAC1 targeting to polarized domains of epithelia and neurons relays on selective sorting into transport carriers by a not-yet identified sorter complex, and subsequent delivery to the plasma membrane [Cheng *et al.,* 2002].
The surface density of EAAC1 is also controlled by rapid constitutive cycling between the plasma membrane and intracellular compartments, with the proportions at the cell surface and in endosomal compartments depending on the relative rates of transporter insertion or removal from the plasma membrane [Fournier *et al.,* 2004; Gonzalez *et al.,* 2007]. Interestingly, this mechanism has been found to be involved in long term potentiation and fear conditioning [Levenson *et al.,* 2002; Pita Almenar *et al.,* 2006] and may also play an important role in epithelia homeostasis. Several proteins may play a role in this process, in particular proteins involved in the fusion of vesicles to the plasma membrane such as SNAPs (soluble *N*-ethylmaleimide–sensitive attachment proteins) and SNAP receptors (SNAREs) [Deken *et al.,* 2000; Quick, 2003; Sung *et al.,* 2003]. Indeed, there is evidence that synthaxin 1A and SNAP23 are required for the trafficking of EAAC1 [Fournier and Robinson, 2006; Yu *et al.,* 2006; Zhu *et al.,* 2005].
In addition, it is known that EAAC1 interacts with PICK-1 (protein interacting with C-kinase) [Gonzalez and Robinson 2003]; that may recruit PKC or other signalling molecules into a complex with the transporter.

In the past years, we have shown that in the extreme cytoplasmic C-terminus of EAAC1 there is a consensus sequence for class I PDZ domain-binding ligands (X-S/T-X-Φ, where X is any amino acid and Φ a hydrophobic amino acid) (Figure 11A).

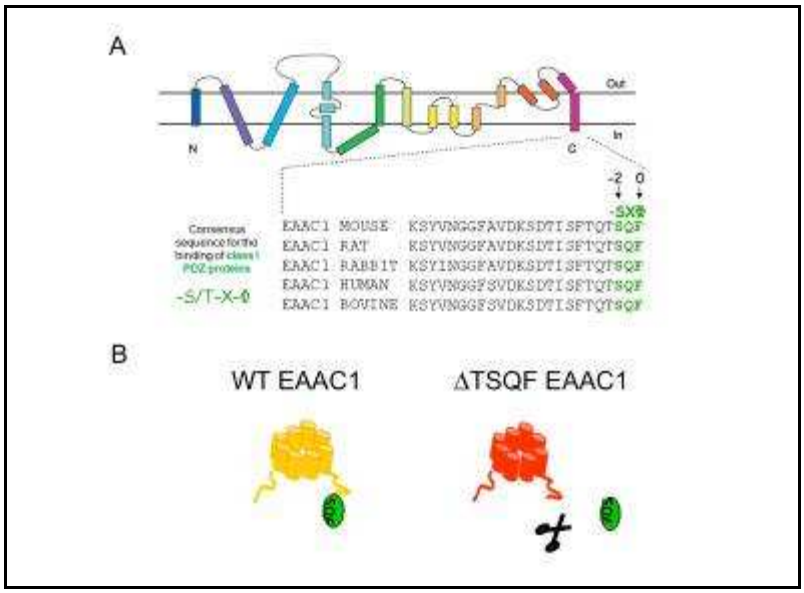


Fig. 11: EAAC1 has a conserved class I PDZ target motif. A) The COOH-terminal amino acids of the glutamate transporter EAAC1 from the various species, obtained from the National Center for Biotechnology Information databases. The putative PDZ target motif "SQF" is highlighted in green. B) Schematic representation of the wild type (WT) and deleted (ΔTSQF) transporters used in the project.

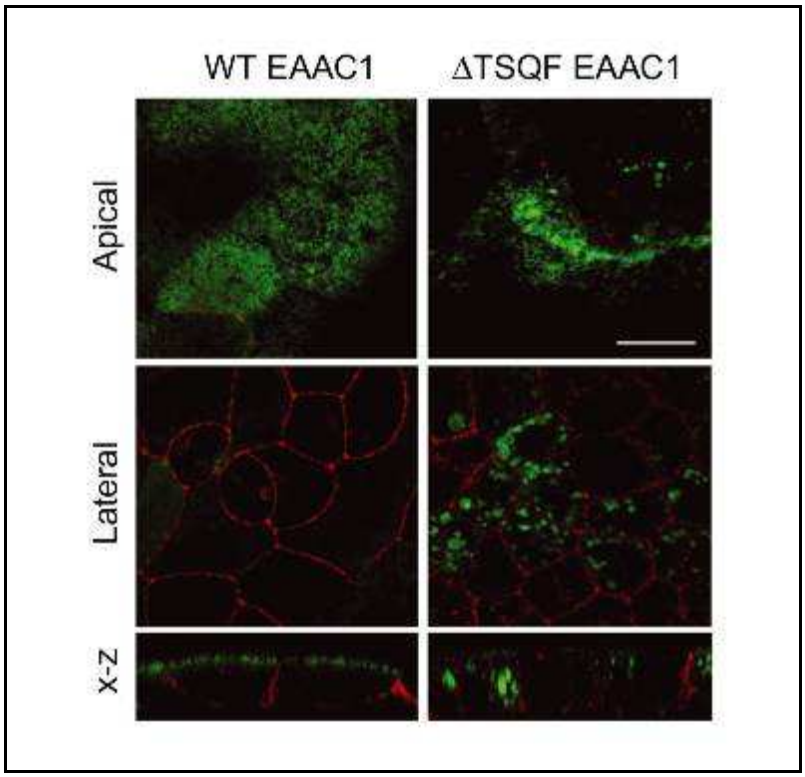


Fig. 12: Deletion of the PDZ target motif decreases the surface expression of the EAAC1 transporter in MDCK cells. MDCK clones expressing the indicated constructs (green) were grown to confluence on transwell filters, fixed, stained with anti-β-catenin (red) antibody and analysed using confocal microscope. Apical, Lateral and x-z scanning are shown. Scale bar: 20 μm.

This sequence would potentially provide docking sites for proteins involved in the targeting, scaffolding and clustering, but its role in transporter function and localisation was not formally proved [D'Amico *et al.*, 2010].

To understand its function, a truncated mutant lacking the PDZ target motif (ΔTSQF EAAC1) and fused to GFP was generated (Fig. 11B). Its distribution was analysed in the Madin Darby Canine Kidney (MDCK) epithelial cell line, a line that endogenously expressed EAAC1 [Shayakul *et al.*, 1997] and therefore all proteins involved in its regulation. Differently from the wild-type transporter, the ΔTSQF EAAC1 mutant prevalently localised in intracellular vesicles (Fig. 12), which have been identified as endocytic and degradative compartments.

Thus, these data suggested that PDZ target sequence plays an important role in transporter trafficking.

We previously demonstrated that the PDZ-target sequence was required to prevent the transporter endocytosis and degradation.

The aim of the present project is to understand whether the class I PDZ binding domain could also affect the biosynthetic delivery of EAAC1 in MDCK cells. We demonstrate that this motif increases EAAC1 trafficking efficiency at different steps of the biosynthetic pathway.

3 RESULTS

3.1 Removal of the PDZ target sequence impairs EAAC1 membrane stability in MDCK cells.

In the previous studies, we demonstrated that the removal of the PDZ target motif SQF increased the transporter's internalisation in endocytic compartments. To confirm these findings, we measured the cell surface resident time of GFP-wild-type and GFP-ΔTSQF transporters (Fig. 1) by means of time-lapse TIRF imaging, after the trafficking of newly synthesized proteins to the plasma membrane had been blocked by Brefeldin A (BFA) incubation.

Total internal reflection fluorescence microscopy (TIRFM) allows the selective excitation of GFP-labelled transporters located in or immediately below the plasma membrane (100 nm above the glass coverslip) and the exclusion of signals arising from structures in the cell interior [Axelrod, 2001].

BFA is a fungal metabolite that is used to accumulate newly synthesised transporters in the ER, thus blocking both the apical and basolateral delivery of membrane proteins in MDCK cells [Lippincott-Schwartz *et al.*, 1989; Low *et al.*, 1992; Wagner *et al.*, 1994]. This strategy allowed us to analyse the membrane stability of EAAC1, isolating the endocytic pathway without the contribution of the biosynthetic delivery. Under these conditions, if the GFP transporter is internalized, the fluorescence signal recorded by TIRFM should progressively decrease, because the transporter distance from the plasma membrane increases. Figure 1 shows representative image sequences of the wild-type and ΔTSQF EAAC1 transporters, together with the averaged fluorescence intensity curves. The total (punctate and diffuse) fluorescence intensity of the wild type GFP-transporter remained almost constant during the 3-min recording, but that of the ΔTSQF mutant markedly decreased. Quantification of the fluorescence changes indicated a 15.92 ± 0.31% decrease in the total EGFP-EAAC1 ΔTSQF signal after 3 min, but only a 2.02 ± 0.03% decrease in that of the wild-type transporter ($p < 0.05$), thus indicating that the PDZ target sequence controls the transporter resident time in the plasma membrane and prevents its internalization.

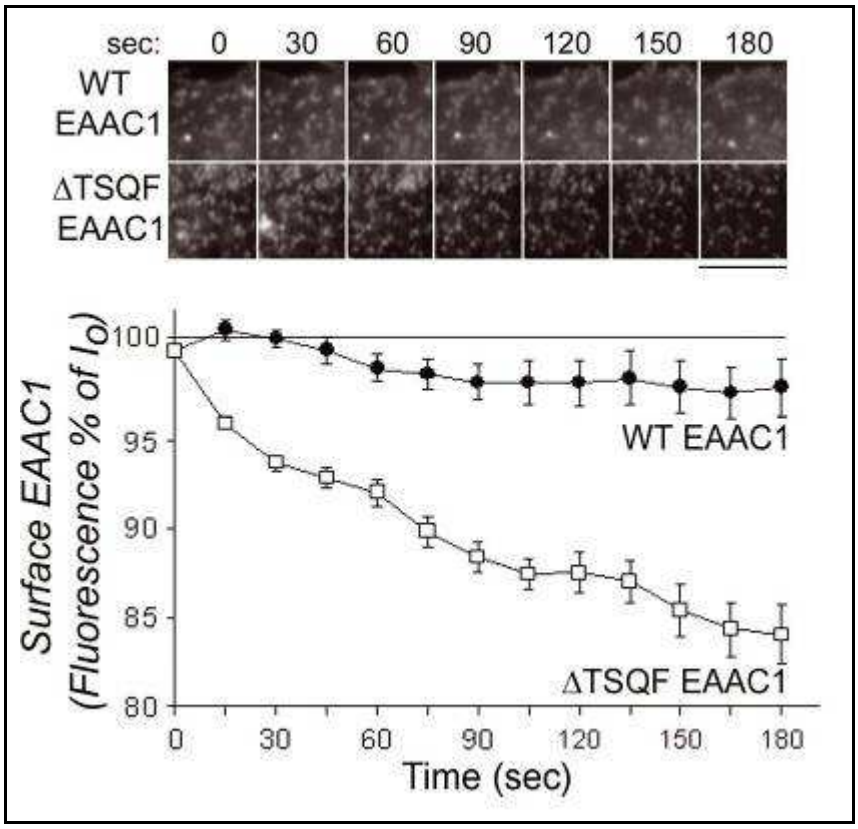


Fig. 1: Removal of the PDZ interacting motif increases the transporter internalization. Time-lapse imaging of EAAC1 constructs by means of TIRF microscopy. The clones were incubated with 5 µg/mL BFA for 30 min, before TIRF microscopy in the continuous presence of BFA. Representative image sequences are shown in the upper panel; the time of the individual frames is indicated. Bar 5 µm. Averaged fluorescence intensity curves for wild-type (circles) and ΔTSQF (squares) EAAC1, showing the percentage of proteins detected at the cell surface by TIRFM, are reported in the lower panel. Data are expressed as the percentage of initial average intensity (I0) and plotted against time (n = 15).

3.2 Removal of the PDZ target motif delays the surface targeting of EAAC1

We then focused on a possible involvement of PDZ interactions in the biosynthetic pathway (Fig. 2). We first used a functional assay to study the transporter surface delivery. Three hours after transient transfection, MDCK cells were incubated for 12 hours with 5 µg/ml BFA to accumulate a cohort of newly synthesised transporters in the ER (Fig. 2A). The block can be removed after extensive washing, thus allowing the synchronised delivery of transporter to the plasma membrane, where it can be monitored by uptake experiments (Fig. 2B). Indeed, D-aspartate does not permeate the plasma membrane and it is taken up only by transporters at the plasma membrane [Seal and Amara, 1999].

Uptake measurements obtained in the continuous presence of BFA were not significantly different from GFP-transfected cells, thus indicating that BFA treatment successfully entrapped transporters within the cells. After BFA wash-out, the wild type transporter was rapidly targeted to the plasma membrane: its surface activity increased over time and reached the maximal value within 30 minutes chase (290±23% over GFP-transfected cells). Similarly, the ΔTSQF transporter reached the cell surface but its activity was lower than wild type transporter already at early time points after BFA wash-out (11.81±0.61% less than wild type

at 5 and 10 min chase), suggesting a slower surface appearance of the transporter lacking the PDZ interacting sequence (ΔTSQF). By 20 minutes' chase, the truncated transporter activity dramatically decreased of 39.4±1.9% relative to wild type transporter ($p<0.05$), confirming also the defect in membrane stability and in endocytic pathway.

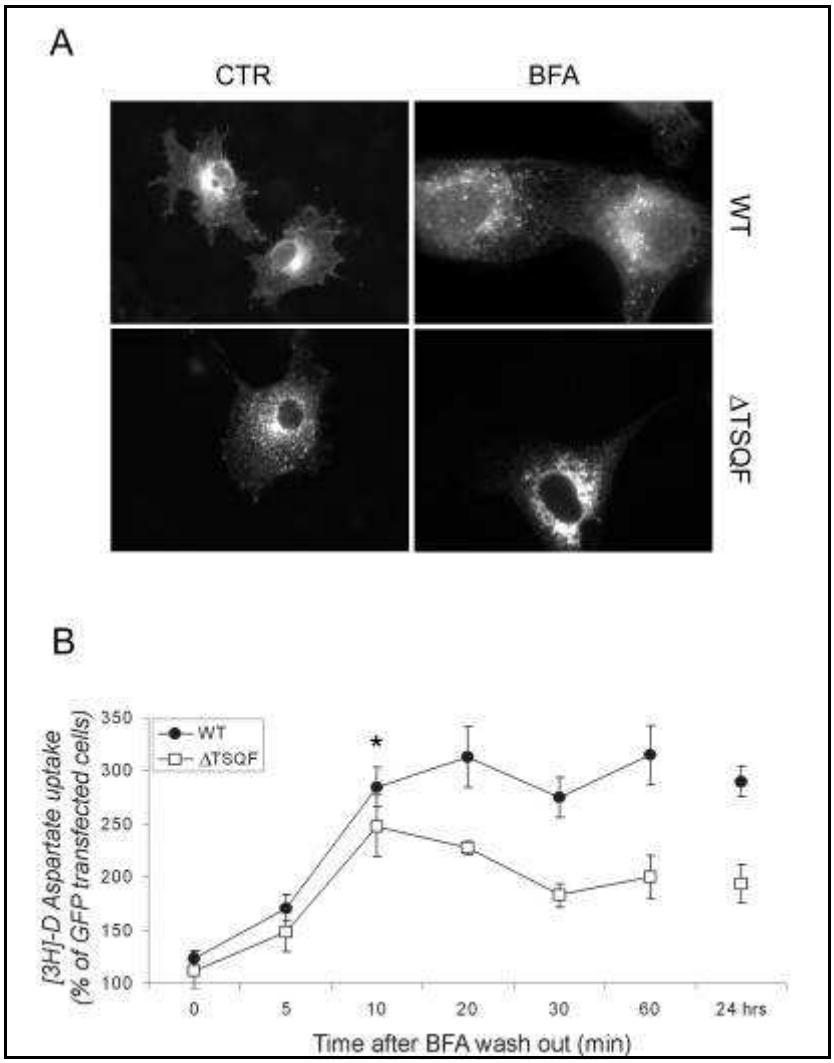


Fig. 2: Plasma membrane delivery is impaired in deleted transporter. A) Three hours after transient transfection of wild-type and ΔTSQF mutant, MDCK cells were incubated with 5 μg/mL Brefeldin A (BFA) for 12 h to accumulate proteins in a single compartment. Immunofluorescence confirmed efficacy of the treatment. B) After BFA wash-out, the surface appearance of the transporter was evaluated by means of [3H]-D-aspartate uptake experiments. Data (mean values ± SEM) are expressed as a percentage of GFP-transfected cells at t0 of chase (n = 5, in triplicate) (*$p < 0.05$).

These data indicate that the mutant EAAC1 reaches the plasma membrane, although inefficiently, but can not accumulate, suggesting both defects in delivery and in the retention of mutant transporter at the plasma membrane.

3.3 PDZ interactions modulate the ER-to-Golgi trafficking

To understand at which step PDZ interactions control the EAAC1 delivery, we took advantage of the glycosylation state of EAAC1 (Fig. 3).

EAAC1 is a N-glycosylated protein and the normal processing of the N-linked oligosaccharides to the complex state occurs within 45 minutes [Yang and Kilberg, 2002].

The N-linked oligosaccharides are processed to the complex state by resident Golgi enzymes, preceding surface delivery. Processing of the high mannose type N-linked glycans (core EAAC1, arrow) to complex type oligosaccharides (mature EAAC1, arrowhead) in the cis/medial Golgi region is reflected by a decrease in the electrophoretic mobility, providing a method to monitor the transporter trafficking in the early biosynthetic pathway [Pfeffer and Aivazian, 2004].

Therefore, the state of oligosaccharide may be used as an indicator of the extent of ER-to-Golgi trafficking [Yang and Kilberg, 2002]. In particular, the presence of a high mannose oligosaccharide indicates that the protein has not left the ER membrane after translation.

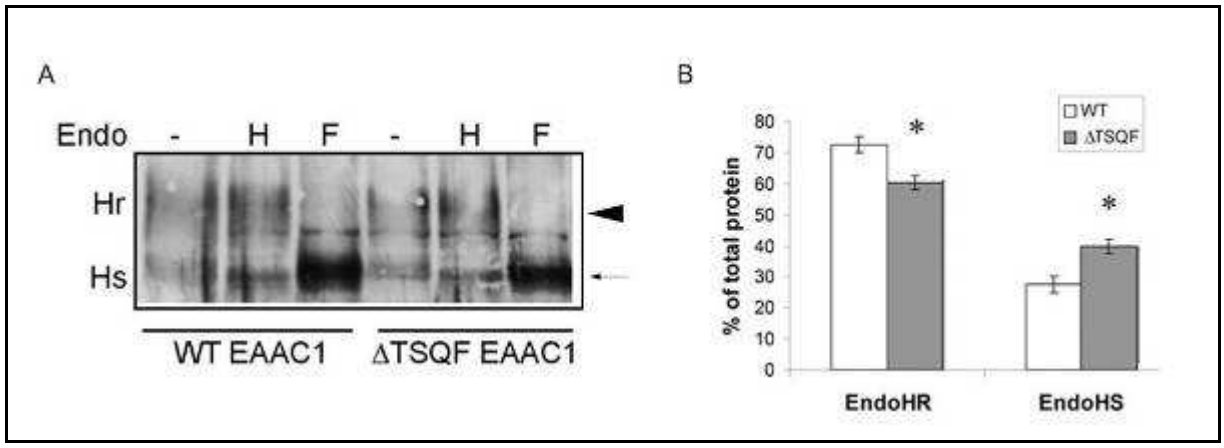


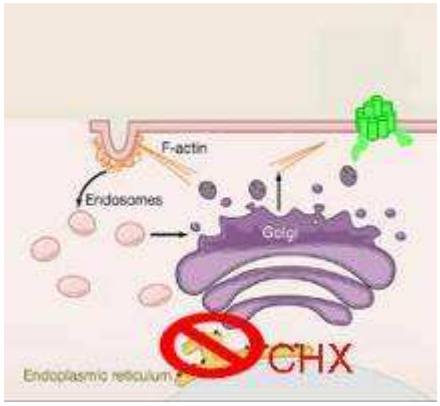
Fig. 3: The removal of the PDZ interaction motif delays the ER-to-Golgi traffic of EAAC1. The ER-to-Golgi trafficking of the indicated EAAC1 constructs was followed by monitoring their glycosylation pattern and sensitivity to Endoglycosidase H digestion. 500 µg of total protein extracts were incubated with Endoglycosidase H (H) or PNGaseF (F) for 5 hrs, before processing for immunoblotting. A) A representative immunoblot is shown. The arrowhead and arrow indicate the complex-glycosylated (Hr, mature and resistant to EndoH digestion) and the high-mannose (Hs, core and sensitive to Endo H digestion) forms, respectively. B) Quantitative analysis is reported. Data (mean value ± SEM) are expressed as a percentage of total EAAC1 protein (n = 3) [$^{*}p < 0.05$: ΔTSQF mutants versus wild type (WT)].

To identify the core form of the transporter, protein samples were treated with Endoglycosidase H (Endo H), that selectively recognises and digests high mannose N-linked oligosaccharides present in the ER. Samples were also digested with N-Glycosidase F (PNGase F), that completely removed all glycans present in the protein. Then proteins were separated on SDS-PAGE and immunoblotted with an antibody against GFP. A representative blot is shown in figure 3A and the band quantification is reported in figure 3B. The removal of PDZ target motif, slightly increased the transporter fraction sensitive to EndoH digestion and decreased the complex glycosylated EAAC1 population in the cell lysate, thus indicating an effect of PDZ proteins on the ER-to-Golgi trafficking. From ER, the transporter could be

either processed by biosynthetic enzymes and progress through the biosynthetic pathway, or could be addressed to degradation compartments.

3.4 PDZ interactions affect the biochemical turnover of the truncated transporter

The biochemical turnover of the high mannose form was determined by means of Cycloheximide (CHX) chases (Fig. 4). As Cycloheximide (CHX) inhibits protein translation in eukaryotes, it allowed us to follow the fate of the previously synthesised EAAC1 high mannose form, excluding the appearance and contribution of newly synthesised transporters. We incubated EAAC1 expressing clones with 100 µg/ml CHX for the indicated times and then cells were lysed and proteins were separated on SDS-PAGE (Fig. 4).



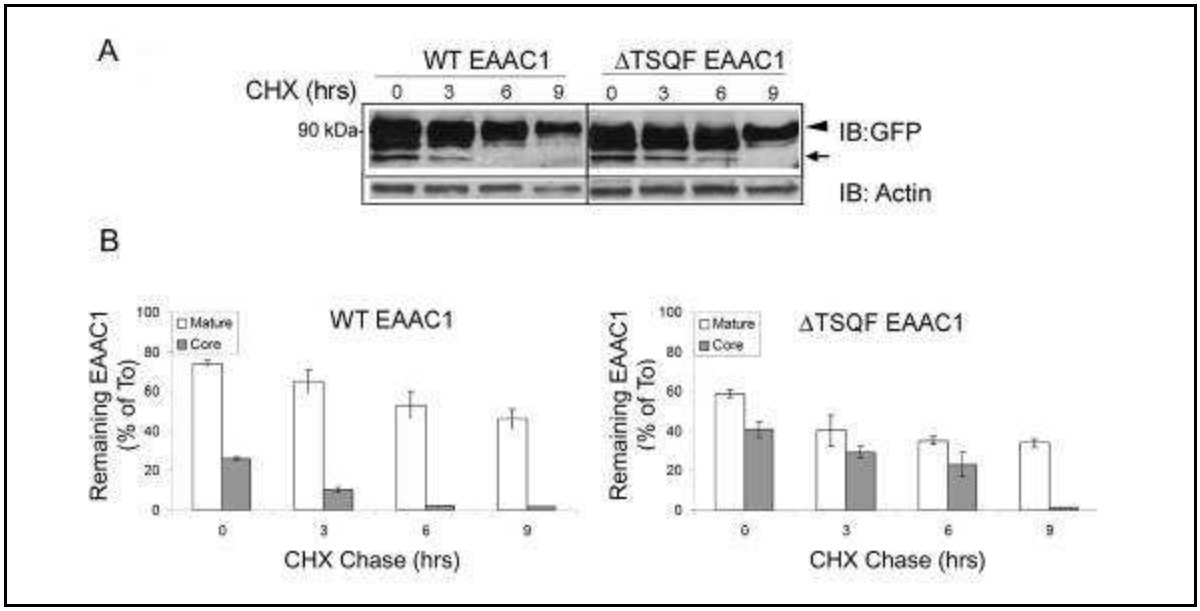


Fig. 4: ΔTSQF mutant shows different maturation and turnover compared to wild type (WT). The turnover of WT and ΔTSQF core- and mature-forms was monitored by their disappearance in the presence of 100 µg/ml CHX, by means of SDS–PAGE and immunoblotting. Equal amounts of cell lysates were loaded and blotted against GFP/Actin. A) A representative immunoblot is shown. B) Quantitative analyses are shown for both the constructs. Data (mean value ± SEM) were corrected for actin content and were expressed as a percentage of total protein (core + mature forms) at t_0 of chase (n = 3).

In these experimental conditions, if the delivery of the protein is not blocked in the ER, the disappearance of the ER form should be accompanied by a concomitant increase of the mature form.

Without CHX chases (0), the amount of the ΔTSQF core form is higher than that of the wild type transporter, confirming the previous results.

However, after CHX chases, the high mannose form (core) of the ΔTSQF transporter was able to proceed in the following compartments, as indicated by the progressive decrease of its core form, although at a slower rate than wild type. Indeed, ΔTSQF EAAC1 became fully glycosylated at longer times (9 hours) than wild type transporter (6 hours).

This data indicate that the removal of the PDZ target sequence delayed, but did not prevent, the ER-to-Golgi progression of ΔTSQF EAAC1.

CHX experiments suggested also a different turnover rate between the fully glycosylated mature form of the wild type and the ΔTSQF transporters, with the latter disappearing faster than wild type. To confirm this hypothesis, we measured the half life of mature EAAC1 constructs by means of metabolic pulse-chase experiments. Transporters were metabolically labelled for 3 hours with [35S]methionine and [35S]cysteine, and then chased for 6 hours to eliminate the ER form. Then they were immuno-precipitated and separated by SDS–PAGE (Fig. 5). The rate of degradation was measured by following the disappearance of the higher mature band. We found that deletion of the PDZ target sequence significantly decreased the half life of mature EAAC1, thus suggesting that PDZ interactions affect also the post-Golgi trafficking of EAAC1.

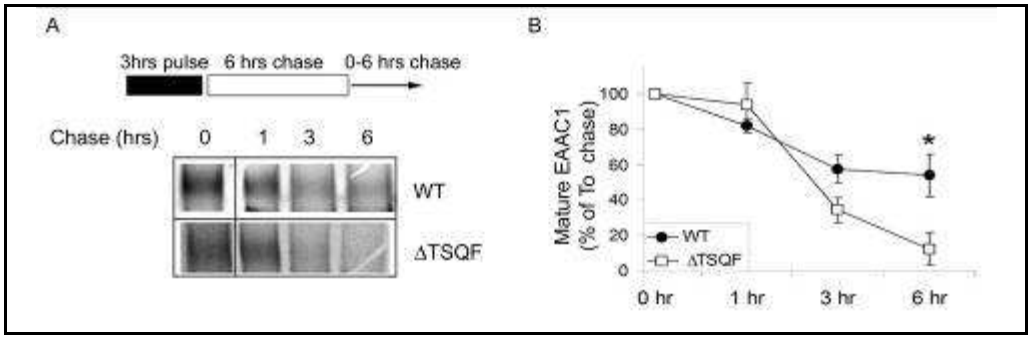


Fig. 5: The half life of the complex glycosylated form is decreased for the ΔTSQF mutant. Metabolically labelled WT and truncated transporters were chased at 37°C, as indicated in the scheme, immunoprecipitated, and analysed on 10% SDS–PAGE. A) The figure shows one out of two experiments. B) Data are expressed as a percentage of total protein at t0 of chase. The quantisation reflects the average of duplicate experiments, (*$p < 0.05$).

3.5 The truncated transporter accumulates in post-Golgi compartments

We then followed the post-Golgi fate of newly synthesized transporters with a morphological approach (Fig. 6).

Three hours after transient transfection, both the wild type and truncated transporters were detected in a tubuloreticular network typical of ER, no punctuated pattern was evident. The subsequent incubation for two hours at 20°C [Griffiths and Simons, 1986] caused the accumulation of both constructs in juxtanuclear structures reminiscent of the Golgi complex (Fig. 6). When samples were warmed for 15 minutes at 31°C, the normal protein trafficking was restored, and both constructs were found in vesicle-like structures emerging from the perinuclear region. However, by thirty minutes chase, the wild type transporter was clearly detected at the cell surface, whereas truncated EAAC1 accumulated in vesicular structures inside the cells. Because also the ΔTSQF was detected at the cell surface in this condition (Fig. 6A, inset), our data are compatible with a defect in both the surface delivery and the endocytic retrieval of EAAC1.

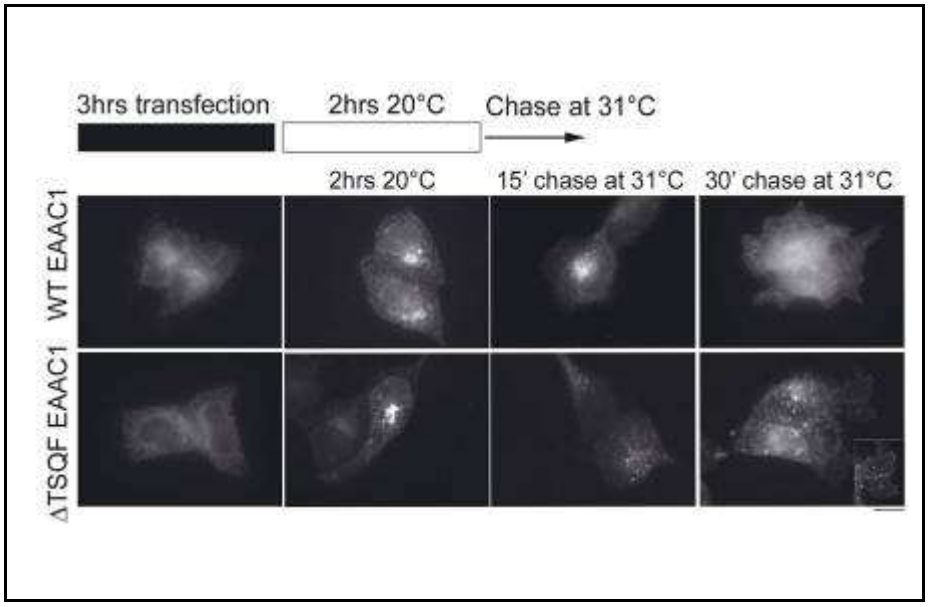


Fig. 6: Removal of the PDZ target sequence causes the accumulation of EAAC1 in post-Golgi compartments. Immunolocalization of newly synthesized wild-type and ΔTSQF transporters. Three hours after transient transfection, MDCK cells were incubated for 2 h at 20◦C (to block proteins in the TGN) and then shifted at 31◦C for 15 and 30 min before imaging. Scale bar: 10 μm.

We have already demonstrated that truncated EAAC1 accumulated in vesicles of the endocytic compartments [D'Amico *et al.*, 2010], we now performed double immunofluorescence experiments to verify whether truncated EAAC1 accumulated also along the biosynthetic pathway (Fig. 7).

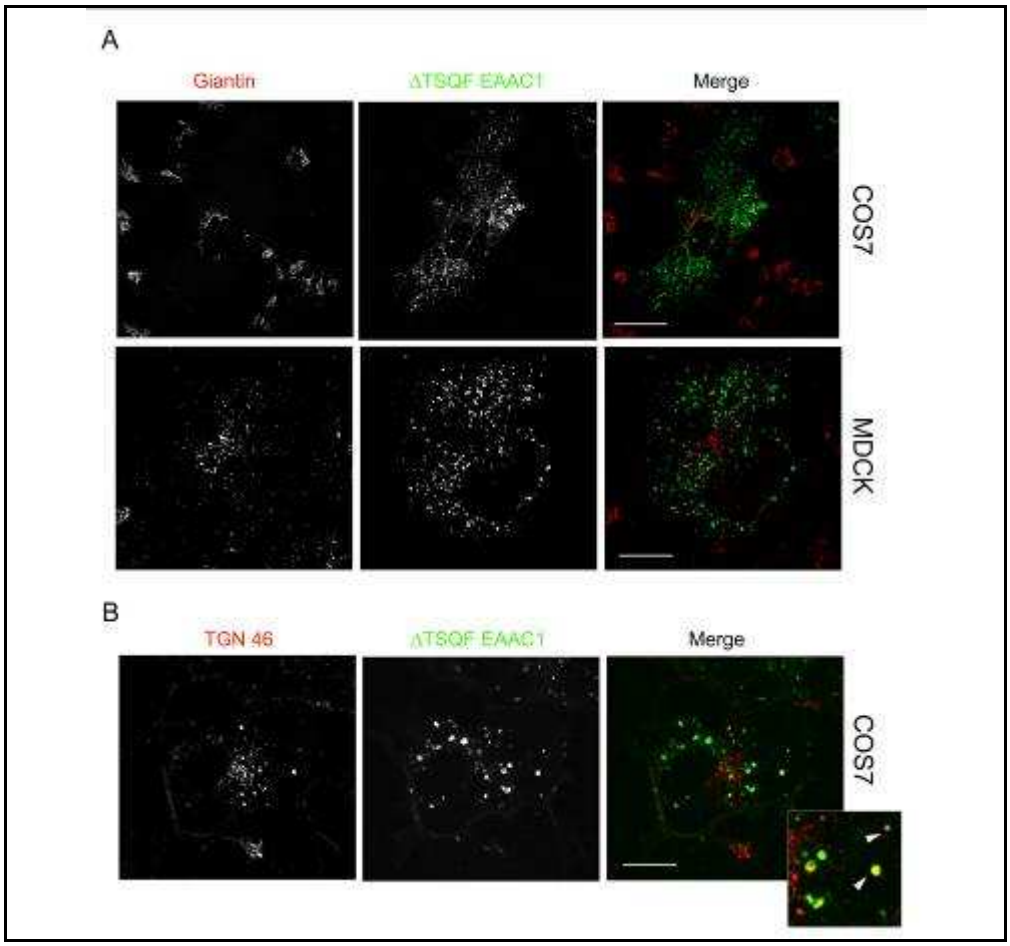


Fig. 7: The ΔTSQF transporter accumulates in post-Golgi compartments. Double immunofluorescence experiments. Non-confluent MDCK or COS7 cells expressing the ΔTSQF GFP-EAAC1 transporter (green) were stained with Giantin (red), a marker of the Golgi compartment, or TGN 46 (red), to mark the Trans Golgi Network. The figure shows both single and merged horizontal confocal sections. In the latter, the yellow/orange staining demonstrates the colocalisation of ΔTSQF EAAC1 TGN 46. In the inset is shown is a 2x magnification and arrow-heads indicate co-localisation points. Scale bar: 10 μm.

We focused our attention on Golgi and post-Golgi compartments, as during the morphological assay we detected a block at the exit from Golgi, rather than in early biosynthetic pathways. We did not detect co-localization of ΔTSQF-EAAC1 with giantin (a marker of the Golgi complex, Fig. 7A) [Seelig *et al.*, 1994] in stable clones or in transiently transfected MDCK or COS7 cells. A possible accumulation in the trans-Golgi network (TGN) was analysed in transfected COS7 cells, because the available antibody against TGN46, a marker of this compartment, did not work in MDCK cell line. Under protein delivery to the plasma membrane, the transition in this compartment is so fast that it is almost impossible to detect an accumulation of the wild type transporter protein. On the contrary the ΔTSQF mutant co-localized with TGN46 in large vesicles confined to the perinuclear region and small vesicles

at the cell periphery, further confirming a defect in the post Golgi trafficking of EAAC1 (Fig. 7B).

3.6 The ΔTSQF-Y503A double mutant confirms the involvement of PDZ interactions in biosynthetic route of EAAC1

Our results indicate that PDZ consensus sequence, and therefore PDZ interactions, are not simply involved in EAAC1 membrane stability, but they also control the efficient delivery of the transporter to the plasma membrane.

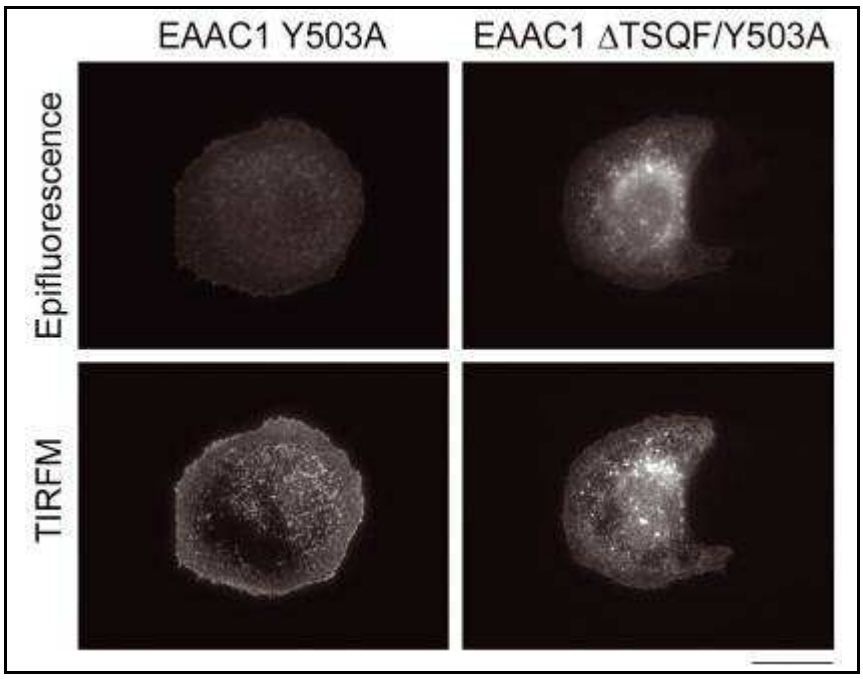


Fig. 8: Tyrosine 503 to alanine substitution increases the surface expression of both wild-type and ΔTSQF EAAC1. Distribution of Y503A and ΔTSQF/Y503A EAAC1 mutants in MDCK cells. MDCK clones were grown on glass coverslips, fixed and analysed by means of conventional or TIRF microscopy (images were collected using identical exposure times). Scale bar: 10 μm.

To confirm this possibility, we took advantage of another mutant (EAAC1 ΔTSQF-Y503A double mutant) previously generated in our laboratory. The EAAC1 ΔTSQF-Y503A double mutant carries both the deletion of PDZ-consensus sequence and a single substitution (Y503A) in the Y503 residue: a tyrosine part of a non-conventional internalisation signal, which controls the transporter's endocytosis from the cell surface (Fig. 8). In this way, any defect associated with the endocytic pathway will be repressed, presumably maintaining the phenotype linked to the biosynthetic delivery.

We first verified the surface delivery of the double mutant by means of pulse/chases experiments of BFA coupled to uptake assays with [3H]-D-aspartate. The Y503A substitution in the ΔTSQF transporter greatly prevented the uptake down regulation observed by 20 min of

chase (compare the solid with the dashed lines in the same graph). Although the ΔTSQF/Y503A double mutant almost completely restored the wild type functional behaviour, its surface activity was still lower than that of the Y503A single mutant at each time point (18.01±6.03% lower, by 20 min chase), suggesting that a fraction of the total transporter does not reach the surface, but is instead retained in intracellular compartments (Fig. 9).

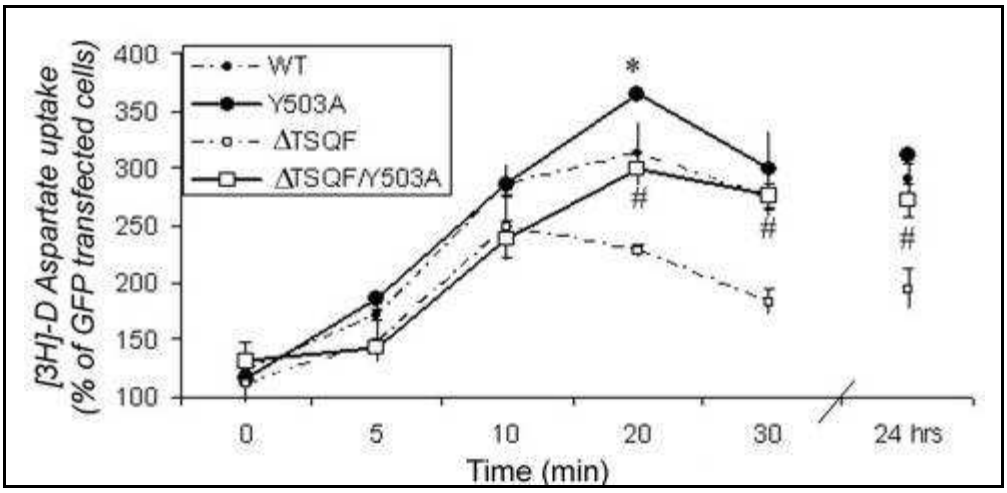


Fig. 9: The Y503A substitution rescues the internalization defect of the ΔTSQF EAAC1 but not its accumulation along the biosynthetic pathway. Cell surface delivery of ΔTSQF/Y503A (square) and Y503 mutants (circle). Three hours after transient transfection with the indicated constructs, MDCK cells were incubated with 5 µg/mL BFA for 12 h. After BFA wash-out, the surface appearance of transporters was evaluated by means of [^{3}H]-D-aspartate uptake experiments. Data (mean value ± SEM) are expressed as percentage of uptake values in GFP-transfected cells (n = 3, in triplicate). To compare the effect of the Y503A substitution, the uptake curves of wild-type (WT) or ΔTSQF EAAC1 are shown in the same graph (dashed lines, from Figure 2B) (* $p < 0.05$ versus ΔTSQF/Y503A; # versus ΔTSQF).

To verify an entrapment of transporter in intracellular compartments we measured the ER-to Golgi trafficking of the double mutant by Endoglycosydase digestion and analysis on SDS-PAGE (Fig. 10). Results confirmed that the double mutant retains the defect in the ER-to-Golgi progression, as indicated by the increased transporter fraction sensitive to EndoH digestion (10% relative to wild type) and the decreased fraction of the mature form.

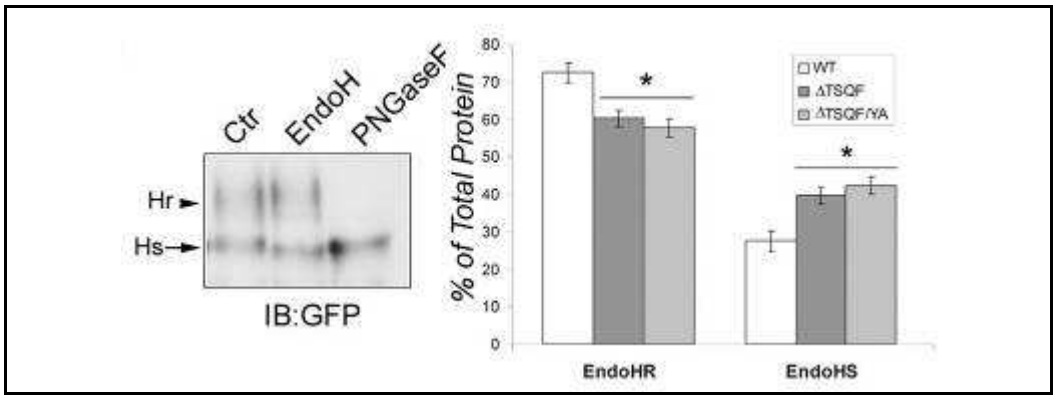


Fig. 10: ΔTSQF Y503A EAAC1 transporters maintain a delayed ER-to-Golgi maturation. Endoglycosidase digestion of the ΔTSQF/Y503A cell lysate. 500 micrograms of total extracts were incubated with Endoglycosidase H (EndoH) or N-glycosidase F (PNGaseF) for 5 hrs, before being processed for immunoblotting. A representative immunoblot and the quantitative analyses are shown. Data (mean value ± SEM) are expressed as a percentage of total EAAC1 protein ($n = 3$) ($^{*}p < 0.05$: ΔTSQF mutants versus WT). The arrowhead and arrow indicate the complex-glycosylated (Hr) and the high-mannose (Hs) forms, respectively.

Moreover, also the ΔTSQF-Y503A mutant co-localised with TGN46, the marker of the Trans Golgi Network. A pseudo-colour image reflecting the co-localisation analysis is reported in figure 11. The scale ranges from blue to red, indicating low to high co-localisation, respectively.

Taken together, these results confirm that PDZ interactions play a role not only in the endocytic trafficking of the transporter but also in the exocytic pathway.

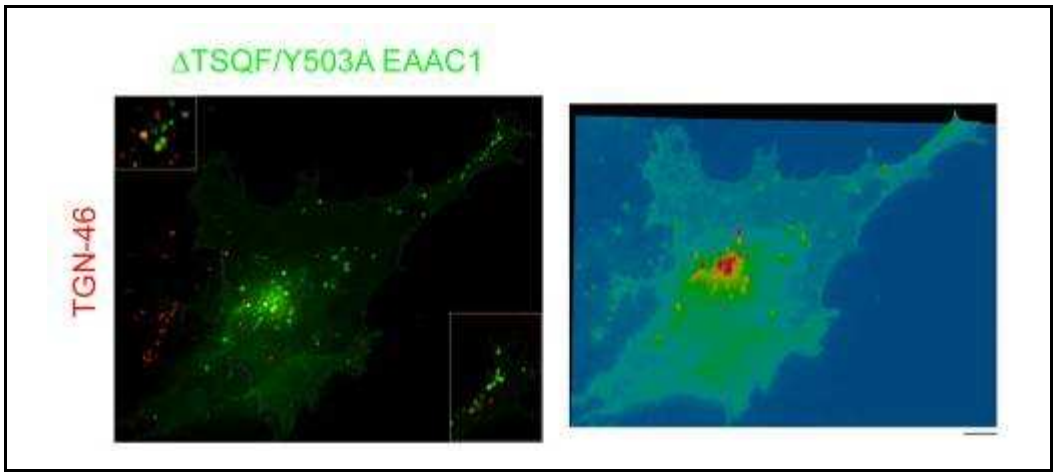


Fig. 11: The Y503A substitution does not prevent ΔTSQF EAAC1 accumulation along the late biosynthetic pathway. Double immunofluorescence staining. 24 hours after transient transfection, COS7 cells expressing the ΔTSQF/Y503A transporter (green) were fixed in paraformaldehyde and stained with TGN46 (red). Co-localisation between the two channels was calculated using the Image-ProPlus software (www.mediacy.com technical note#7; Media Cybernetics) and a 3D representation is shown. Scale bar: 10 μm..

4 DISCUSSION

In the past years, we analysed the function of a class I PDZ target sequence localized in the EAAC1 C-terminal tail. We found that it controls the transporter's surface density by increasing its surface retention and preventing its internalization in endocytic compartments.
In the present study, we investigated the involvement of the same PDZ consensus sequence in the biosynthetic pathway of EAAC1. Our data indicate also a role of PDZ interactions in the surface delivery of transporters.

4.1 PDZ interactions are required for the efficient ER-to Golgi trafficking of EAAC1.

PDZ interactions promote the transporter's plasma membrane localization, because the deletion of the C-terminal PDZ binding site dramatically decreases the EAAC1 surface expression. We provide evidence that PDZ interactions occur early in the biosynthetic pathways and are involved in the ER-to-Golgi trafficking of EAAC1.
Indeed, at the steady state, a higher fraction of the ΔTSQF mutant remains sensitive to EndoH digestion, that reveals an early form of glycosylation that occurs in the ER (Fig.3).
The ER-to-Golgi delay was confirmed by the analysis of biochemical turnover of the transporters after Cicloheximide (CHX) chases. In that experiments, the transporter neo-synthesis is blocked by CHX and the fate of the transporters synthesised before the incubation could be followed as a progressive loss of the mature and core bands on a SDS-PAGE at different time chases (Fig. 4). The WT core form disappears faster than the corresponding ΔTSQF band, suggesting that the maturation of the truncated transporter is actually slower and occurs three hours later compared to the WT. However, these experiments indicate that at longer times (9 hours) the truncated transporter can reach the mature form, thus confirming the results of surface expression of ΔTSQF transporter detected by TIRFM experiments. It is of note that the complex-glycosylated ΔTSQF increase after 9 hours of CHX chase is due to the slower maturation of the core protein and it's not related to transporter neo-synthesis, that it blocked by CHX.
On the other hand, the biochemical turnover of the complex-glycosylated form indicates that the half-life of the ΔTSQF mutant is less compared to the WT: we can estimate that it is about 6 hours for the former and 9 hours for the latter.

Interestingly, pulse and chase experiments on the biochemical turnover of the complex-glycosylated forms, indicate that removal of PDZ motif determines a faster degradation of the transporter (Fig. 5).

Most likely, the degradation step occurs after the Golgi, as suggested by the fact that the core form of ΔTSQF mutant can mature to complex glycosylated.

Removal of the PDZ motif delays rather then prevents the ER export of the transporter, thus indicating that PDZ interactions facilitate the ER-Golgi trafficking. Progression through the biosynthetic pathways requires the efficient sorting of cargo molecules into carrier vesicles and their subsequent delivery and fusion to the appropriate target membrane. Therefore, PDZ-interactions may favour the transporters homo-oligomerization, a process required for the efficient ER export of EAAC1 [Ruggiero *et al.,* 2008]. In line with this hypothesis are findings on CFTR, whose dimerization is promoted by the PDZ proteins NHERF1 and NHERF2 [Wang *et al.,* 2000].

Alternatively, PDZ domain-proteins may couple EAAC1 with protein complexes required for the fusion of carrier vesicles to the appropriate target membrane. A similar mechanism has been proposed to control the efficient synaptic delivery of NMDA receptors via the PDZ protein SAP102 and Sec8; the latter is a component of exocyst, a cytosolic complex that directs secretory vesicles to their fusion sites in the plasma membrane [Hsu *et al.,* 1999; Sans *et al.,* 2003]. Interestingly, the exocyst complex is also expressed in MDCK cells at regions of cell-cell contacts, but its accumulation in intracellular compartments, including the ER and the TGN, has also been reported in other cell types [Yeaman *et al.,* 2001].

4.2 PDZ interactions are important for the post-Golgi trafficking of EAAC1.

Our data support an involvement of PDZ interactions also in the final stages of the biosynthetic pathway, as after Golgi release the ΔTSQF and ΔTSQF/Y503A mutant accumulated in intracellular vesicles and co-localized with TGN46 in large perinuclear vesicles and in small vesicles beneath the plasma membrane (Fig. 7B and 11) thus suggesting a role of PDZ interactions in the efficient transport and fusion of carrier vesicles to the appropriate target membranes.

Interestingly, the sec complex has been shown to be required also in the final steps of the biosynthetic pathway.

A possibility is that PDZ domain-proteins may couple EAAC1 with protein complexes required for the fusion of carrier vesicles to the appropriate target membrane.
An alternative option is that PDZ proteins may function as scaffolds and link EAAC1 to motor proteins required for the transport of carrier vesicles along the microtubules.
A similar mechanism has been proposed to control the synaptic delivery of N-Methyl-D-aspartate (NMDA) receptors. Its interaction with the kinesin motor KIF17, mediated by the PDZ complex LIN2-LIN7-LIN10, ensures the efficient transport of receptors into dendrites [Setou *et al.*, 2000].

These data suggest that PDZ-mediated interactions are involved in different steps of the biosynthetic pathway, in the early ER-to-Golgi progression as well as in post-Golgi trafficking.
Given the involvement of PDZ proteins interactions at several steps of the biosynthetic/degradative pathways, it is possible that distinct PDZ proteins may interact with EAAC1.
We had identified PDZK1 as an EAAC1 interacting partner, acting at the plasma membrane as a retention protein [D'Amico *et al.*, 2010].
The future perspectives will be to identify the interacting proteins involved in EAAC1 biosynthetic delivery in physiological conditions and also to understand if cellular response to nutrients may involve changes in protein interaction and in the transporter expression at the plasma membrane.

5 MATERIAL AND METHODS

5.1 Plasmid construction

In order to express the transporters in heterologous expression systems, such as mammalian cells, we cloned the relative cDNAs in the plasmidic vectors to perform our studies. In particular, we generated EAAC1 transporter fused in-frame with the EGFP C-terminus.

The full-length coding sequence of rabbit EAAC1 (177-1782 bp, GenBankTM accession number L12411) was cloned from rabbit intestine by means of RT-PCR using oligonucleotides designed with the assistance of Primer3 software (http://primer3.sourceforge.net/) [Rozen and Skaletsky, 2000; Vanoni *et al.*, 2004].

By means of a PCR reaction carried out using the pCB6-EAAC1 [Vanoni *et al.*, 2004] as a template and the primers below, we introduced, the *BglII* and *KpnI* enzyme restriction sites at the 5' and 3', respectively.

The PCR product (bp 177-1782) was inserted in frame between the *BglII* and *KpnI* sites of digested pEGFP-C1 (Clonetech, Figure 1), creating the plasmid pEGFP-EAAC1, which expresses a protein with enhanced green fluorescent protein directly fused to the amino terminus of the EAAC1.

The EAAC1 mutants were obtained using a QuickChange site-directed mutagenesis kit using the GFP-EAAC1 wild type as a template and specific oligonucleotides (Stratagene).

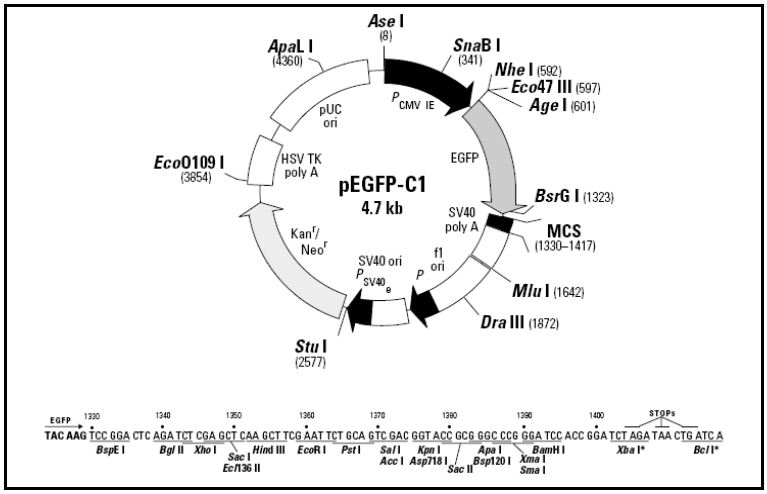


FIG. 1: pEGFP-C1 vector information

To study the contribution of the PDZ target sequence to the transporter trafficking, the GFP-EAAC1 ΔTSQF construct was created by replacing the residue T521 of EAAC1, generating a stop-codon before the conserved sequence SQF.

To repress endocytic defect, the internalisation signal YXXΦ was mutated, obtaining GFP-EAAC1 Y503A mutants [Sheldon *et al.*, 2006].
Primers were synthesized by PRIMM or NBS Biotech. The sequences of the resulting cDNAs were confirmed by DNA sequencing (PRIMM).

5.2 Cell culture

The strain II MDCK cells were cultured in MEME (Minimum Essential Medium Eagle) supplemented with 10% foetal bovine serum, 2 mM glutamine, and 100 IU/ml streptomycin/penicillin, as previously described [Perego *et al.*, 1997]. MDCK clones were grown in the same media, supplemented with 0.3 mg/ml G418 (Sigma-Aldrich).
COS7 cells were cultured in D-MEM (Dulbecco's Modified Eagle Medium) supplemented with 10% foetal calf serum, 1% glutamine and 1% streptomycin/penicillin.
Cultures were performed under standard humidified conditions of 5% CO_2 at 37° C.
All media were supplied by Sigma-Aldrich.

Transfection

Transfection was performed by means of lipofection (LipofectamineTM 2000 reagent, Invitrogen) and, 6 / 24 / 72 hours after transfection, the cells were processed for immunofluorescence, or functional and biochemical studies.
MDCK cells were plated at the indicated density in complete standard medium and the following day, they were washed with serum-free medium and transfected with a mixture of DNA and Lipofectamine reagent, as indicated in the table below:

Well	Cells	DNA	Lipofectamine Reagent	1%Gln MEME
96-well plates, each well	$5x10^3$	0.05 µg	0.12 µl	44 µl
3.5 cm Petri dishes	$3x10^5$	1 µg	3 µl	1 ml

Four hours after transfection, complete standard medium was added.

Clonal cell lines.

Stable MDCK cell clones expressing the various constructs were obtained using G418 selection (Sigma-Aldrich) [Perego *et al.*, 1997].

Briefly, cells were seeded at the density of $3x10^5$ cells in a 3.5 cm Petri dish; after 24 hours, cells were transfected with a mixture of DNA (1 µg) and LipofectamineTM (3 µl) prepared in 1 ml of serum free media (MEME supplemented only with 1% glutamine); the following day, cells were harvested and plated at low density in 10 cm Petri dishes. Clones were selected for 10 to 20 days in complete MEME supplemented with 0.6 mg/ml G418 and then harvested. At least three different clones for each construct were characterised by means of uptake experiments, western blotting and cell localisation. Clones expressing similar levels of GFP-labelled constructs were selected and further analysed.

5.3 Cell treatments

BFA

To synchronise all the neo-synthesised proteins in the exit from the ER, we used Brefeldin A (BFA) by Sigma-Aldrich. It is s a fungal metabolite which disrupts the structure and function of the Golgi apparatus, thereby creating a whole static organelle and entrapping proteins within ER [Dinter and Berger, 1998].

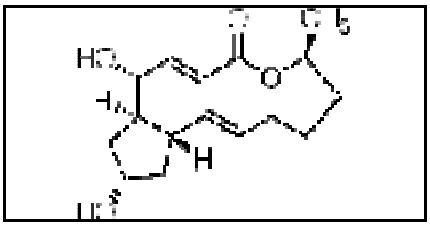


FIG. 2: The fungal metabolite Brefeldin A (BFA).
Sigma Aldrich

TIRFM imaging. 24 hours after plating, the cells were incubated with 5 µg/ml for 30 min at 37°C, and then processed for TIRFM imaging in the continuous presence of BFA.

Surface delivery and uptake experiments. $5x10^4$ cells were plated on 96 multiwells plate and transiently transfected with Lipofectamine (see above). 3 hours after transfection, the cells were treated with 5 µg/ml BFA for 12 hours. After accurate washing, clones were incubated with normal medium at 37°C for different times, as indicated, and then subjected to uptake experiments.

CHX

To inhibit protein translation and evaluate EAAC1 maturation and half-life, EAAC1 expressing MDCK cells were treated with CHX (Cicloheximide, Sigma-Aldrich) at 100 µg/ml, for different incubation times (0, 3, 6 and 9 hours). Then the medium was removed, the cell lysed, loaded on a SDS-PAGE and blotted.

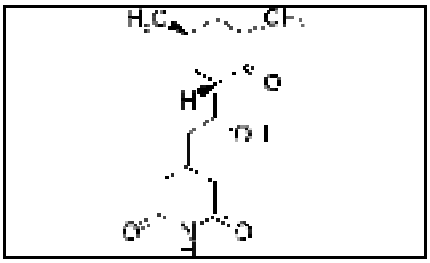


FIG. 3: Cicloheximide (CHX).
Sigma Aldrich

5.4 Antibodies and Immunocytochemistry

MDCK cells were plated on sterile glass coverslips and grown until 70% confluence in standard conditions. Then, after brief washes with PBS (150 mM NaCl, 10 mM Phosphate Buffer pH 7.4), cells were fixed in 4% paraformaldehyde or 100% methanol, depending on the primary antibody to be used, and permeabilised with 0.5% Triton X-100 (TX-100). Fixed cells were then incubated with primary antibody for 2 hours at room temperature in GDB solution (150 mM NaCl, 10 mM Phosphate Buffer pH 7.4, 0.2% Triton, 0.2% gelatine). Following incubation, cells were washed in PBS and then incubated in GDB with the appropriate fluorochrome-conjugated secondary antibodies for 1 hour at room temperature, in dark. Then, coverslips were mounted on glass slides with Phenilendiammine (1 mg/ml in Glycerol-PBS; Sigma-Aldrich) as antifade reagent and sealed with nail-polish.

Post-Golgi trafficking blockade

Two hours after MDCK transfection, cells were incubated for 2 hours at 20°C, a temperature known to inhibit post-Golgi trafficking [Griffiths and Simons, 1986]. To restore Golgi-to-membrane traffic, cells were incubated for different time (15 and 30 minutes) at 31°C and then fixed with 4% paraformaldehyde, to be imaged.

Antibodies

The following primary antibodies were used: polyclonal anti-GFP (Santa Cruz Biotechnology Inc. and Invitrogen), polyclonal anti TGN46 (Sigma), polyclonal EAAC1 (Alpha Diagnostic). Polyclonal anti-giantin was a kind gift from Dr. Manfred Renz [Seelig *et al.*, 1994].
The secondary antibodies (rhodamine-conjugated anti-mouse and anti-rabbit IgG antibodies) came from Jackson Immunoresearch (West Grove, PA, USA).

5.5 Image acquisition and analysis

The images were acquired using a Zeiss Axiovert inverted microscope equipped with a Retiga SRV CCD camera or a MRC-1024 laser-confocal scanning microscope (BioRad, Hercules, CA, USA). Z series of confocal sections were acquired separately using the FITC and rhodamine filters. The laser power and gain were adjusted to maintain the signal below saturation levels. Co-localisation between the two channels was calculated using the Image-ProPlus software (www.mediacy.com technical note#7; Media Cybernetics).

5.6 Total internal reflection fluorescence microscopy (TIRFM).

Total internal reflection fluorescence microscopy (TIRFM) allows the selective excitation of fluorochromes located in or immediately below the plasma membrane (100 nm above the glass coverslip), due to the incidence angle of the excitation light [Axelrod, 2001].

This technique is particularly useful in the study of membrane dynamics and traffic, as it could reach a high level of resolution.

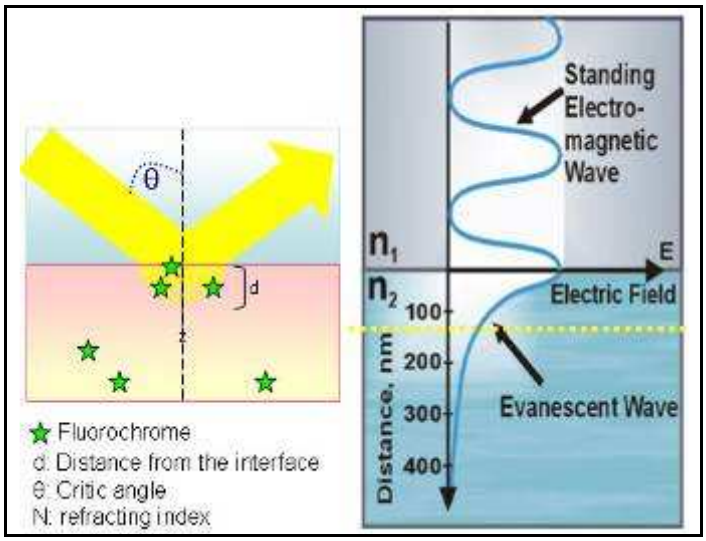


FIG. 4: TIRFM principle. The Total Internal Reflection is obtained when the sample is immersed in a media with low refracting index (n1>n2) and if the excitation light reach the sample with a critical angle. In this condition, the evanescent field is so faint that it diffuse in the sample within 100 nm but it doesn't penetrate deeper, avoiding illumination of deep fluorochromes.

Static experiments.

Non-confluent MDCK cells stably expressing the different constructs were plated onto glass coverslips. 24 hours later, cells were fixed with 4% paraformaldehyde and imaged through a TIRF microscope (Carl Zeiss Inc.) equipped with an Argon laser at 37°C using a 100 x 1.45 numerical aperture (NA) oil immersion objective. Green fluorescence was excited using the 488 nm laser line and imaged through a band-pass filter (Zeiss) onto a Retiga SRV CCD camera.

Time-lapse experiments

MDCK clones expressing the different constructs were plated onto glass coverslips at $2x10^4$ cells/cm^2 and, 24 hours after plating, single-cell imaging under TIRF illumination was carried out at four frames per minute for three minutes in the continuous presence of BFA at 37°C. Up to five cells were imaged on each coverslip in three independent experiments.

Image processing

For each recorded cell image, five 50x50-pixel regions were randomly selected within the cell, and the average fluorescence intensity and its associated standard deviation in each frame were calculated using the Image-ProPlus software (Media Cybernetics). The fluorescence intensities in the various frames were normalised to their respective initial average intensities (I_0=100%) and plotted against time. Fluorescence intensities were corrected for bleaching during the acquisition of serial images (bleaching was estimated by imaging paraformaldehyde-fixed cells). The data come from up to 15 cells for each construct.

5.7 Endo H or PNGase F treatment

To verify whether truncated EAAC1 has some defects in exit from ER, we studied the glycosylation pattern of WT, ΔTSQF and ΔTSQF Y503A transporters. Alterations in glycosylation were determined by differential glycosidase digestions, and were reflected by changes in electrophoretic mobility.

Endoglycosidase H (Endo H), cloned from *Streptomyces plicatus*, prefentially hydrolyzes N-glycans of the high mannose type [Kobata, 1979], thus identifying the early form of glycosylation. N-Glycsidase F (PNGase F), cloned from *Flavobacterium meningosepticum*, cleaves all types of asparagine-bound N-glycans, from the early to the mature complex forms [Tarentino *et al.*, 1985].

We used the reagents and the protocol supplied by the manufacturer (New England Biolabs, Beverly, MA, USA). Briefly, MDCK clones were lysed in lysis buffer (150 mM NaCl, 30 mM Tris-HCl, 1 mM $MgCl_2$, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, and 1 µg/ml aprotinin and leupeptin) and 500 µg of total protein extracts were denatured in 1x final Glycoprotein Denaturing Buffer (stock 10x) for 10 min at 100 °C. Then, samples were split into three aliquots: mock digested as control, Endo H or PNGase F digested. G5 buffer (for EndoH) or G7 buffer (for PNGaseF) were added to 1x final (stock 10X for both the buffers) and samples were subjected to digestion with 1000 units (1 µl) of Endo H (1000 U/µl, New England Biolabs) or 500 units (1 µl) PNGase F (500 U/µl, New England Biolabs). After 5

hours of incubation, the samples were precipitated, dissolved in Gel Loading buffer and analysed by either SDS-PAGE and protein blotting.

5.8 Cell lysis, SDS-PAGE and Western blotting analyses.

Cells were harvested and lysed in lysis buffer (150 mM NaCl, 30 mM Tris-HCl, 1 mM $MgCl_2$, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, and 1 µg/ml aprotinin and leupeptin). After 1 h at 4° C, lysates were centrifuged at 13000 rpm for 10 min, the extracted proteins were denaturated with sample buffer (5% SDS, 20% Glycerol, 0.3M β-mercaptoethanol, blue bromophenol) and separated by 9% SDS-PAGE (Tris-Gly/ SDS buffer: 25 mM Tris-Base, 192 mM Glycine, 0.1% SDS). Finally, proteins were transferred to a nitrocellulose membrane (Shleicher and Shull, Germany) (Transfer buffer: 25 mM Tris-Base, 192 mM Glycine, 20% Methanol). After membrane incubation with blocking buffer (2% non-fat milk, 0.1% Tween 20, 20 mM tris HCl pH 7, 150 mM NaCl), the blots were probed with the indicated primary antibodies, with peroxidase-conjugated anti-rabbit or anti-mouse IgG as secondary reagents, and visualised by ECL (Perkin-Elmer Life Science).
The X-ray films were analysed by densitometry and the results quantified using NIH Image 1.59 software.

5.9 Na^+-dependent transport activity

MDCK cells plated in 96-well plates were transfected with the different constructs (pEFGP-EAAC1 WT, ΔTSQF, ΔTSQF-Y503A, Y503A). Three hours after transfection, cells were incubated for 12 hours with BFA. The next day, we washed BFA out and we followed EAAC1 appearance at the cell surface by [^{3}H]-D-Aspartate uptake at different incubation time after BFA removal.
At each time point (0, 5, 10, 15, 20, 30, 60 minutes of chase), cells were washed twice in sodium-free solution (150 mM choline chloride, 4 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, pH 7.5) and then assessed for D-aspartate uptake (3 µCi/ml; specific activity 37 Ci/mmol, Amersham Biosciences) performed in 200 µl of uptake solution (150 mM NaCl, 4 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, pH 7.5) for 5 min at room temperature. Amino acid uptake was stopped by washing the cells twice in ice-cold sodium-free solution. The cells were dissolved in 150 µl of SDS 1% for liquid scintillation counting.
Data are presented as a percentage of GFP-transfected cells.

5.10 Metabolic labelling

Metabolic labelling was performed as described [Perego *et al.*, 1999]. Confluent MDCK clones were starved for 30 min in Dulbecco's modified Eagle's medium (DMEM) without cysteine and methionine, and metabolically labelled for 3 h with 0.1 mCi/ml of Trans35S-label (ICN Pharmaceuticals) in starving medium [Lisanti *et al.*, 1989]. The cells were washed at 4°C and chased for different times (up to 15 hours) in culture medium at 37°C. The cells were lysed, and the transporters immunoprecipitated by incubation with polyclonal anti-GFP antibody (Invitrogen, 1:1000), followed by a second incubation with protein A conjugated to Sepharose beads (Pierce) [Pietrini *et al.*, 1994]. The transporters were eluted from the protein A–beads by boiling in 0.5% SDS. The immunocomplexes were resolved on 10% SDS–PAGE and analysed by 35S emission on film.

5.11 Statistical analysis

Statistical significance between groups was determined by means of an unpaired Student's t-test; the differences were considered significant at P values of <0.05.

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CHAPTER III: Expression and pathophysiological role of GLT1 transporter in endocrine pancreas

1 ABSTRACT

Glutamate is the major excitatory neurotransmitter of the central nervous system (CNS) but it is also present in endocrine pancreas, where it is secreted by the glucagon producing α-cells and acts as signal molecule in the islets of Langerhans.

In the CNS, glutamate may cause cell death by excitotoxicity, but the effect of glutamate on islet cell function and survival is presently unknown. Moreover, although all the glutamate signalling system has been charcterized in the islets, less is known about the expression and the physiological role of glutamate transporters, that in the CNS are the main effectors of glutamate clearance.

In this study, we demonstrate that exposure to elevated glutamate concentrations induces a significant cytotoxic effect also in pancreatic β-cells, while not in α-cells. Glutamate-induced β-cell cytotoxicity is due to the prolonged activation of ionotropic glutamate receptors and leads to apoptosis. We provide evidence that the key regulator of the extracellular glutamate clearance in the islet is the glial glutamate transporter 1 (GLT1/EAAT2). GLT1 is the only high affinity glutamate transporter expressed in the islets and localizes to the β-cell plasma membrane. Its main role is to prevent glutamate-induced cytotoxicity and β-cell apoptosis.

Finally, as diabetes is characterized by an elevated beta cell death, we verified if there were some modifications in the localisation of GLT1 in type 2 diabetes mellitus (T2DM) patients. We show an altered GLT1 localisation in pancreases from T2DM patients, suggestive of a decreased glutamate clearance ability in these subjects.

In conclusion, our data demonstrate that glutamate-induced citotoxicity could represent a mechanism of β-cell death and is physiologically prevented by the activity of the glutamate transporter GLT1.

2 INTRODUCTION

2.1 Pancreas

Pancreas is a voluminous gland localised in the abdomen and implicated in the production of two different secreting products: one exocrine, containing enzymes and bicarbonate required for the digestion, and the other endocrine, involved in glucose homeostasis and metabolism. These two secreting functions are exploited by discrete structures present in the organ. The endocrine part is dispersed throughout the exocrine tissue, and it is organised small cell aggregates, called islets of Langerhans.

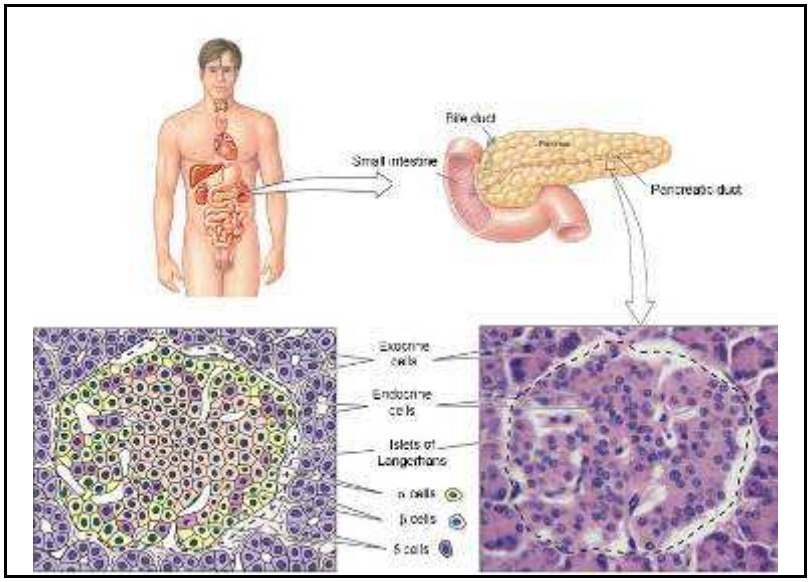

FIG. 1: Endocrine pancreas. The islet of Langerhans is shown in the histological sample. The different cell populations are represented in the scheme, a part from F cells.

[Silverthorn, 2000]

The islets of Langerhans, a miniature organ for regulation of blood glucose level, are composed of four major types of endocrine cells that secrete different hormones to regulate blood glucose levels and other aspects of metabolism. The four types of islet cells are listed in the table below, with their secreting products.

CELL	HORMONE
Alpha (α)	Glucagon
Beta (β)	Insulin
Delta (δ)	Somatostatin
F	Pancreatic Polypeptide

TABLE 1: Islet endocrine cells and their secreting products.

In the morphology of the islets, the beta cells are the most numerous and the alpha cells are larger and less numerous. Moreover, depending on the specie, it has been reported a characteristic distribution of this two cell types in the islet: in mice, beta cells are concentrated in the centre of the islet, while alpha cells are located at the periphery of the islet.

The islets are richly innervated by parasympathetic and sympathetic neurons. Several different neurotransmitters such as acetylcholine, noradrenaline, and neuropeptides are secreted from these nerve endings and regulate the secretion of hormones.

2.1.1 Insulin secretion

Pancreatic islets monitor and respond to many dietary nutrients, releasing hormones in order to meet the needs of the organism.

In particular, beta cells release insulin, a peptide hormone present in their secretory vesicles.

The primary stimulus for insulin secretion is the increase in plasma glucose concentration.

Another well known characteristic of insulin secretion is its bifasic kinetic, an essential feature that is shared between humans, rats and mice (Fig. 2). The response to glucose begins after a lag period of 1 to 2 min and is characterized by a stimulation of release that peaks after 3 to 4 min, and then declines rapidly to a nadir at 8 min. The initial spike of release is defined as the first phase. Then, the second phase is characterized by a gradually increasing rate of release to a plateau after a further 25 to 30 min.

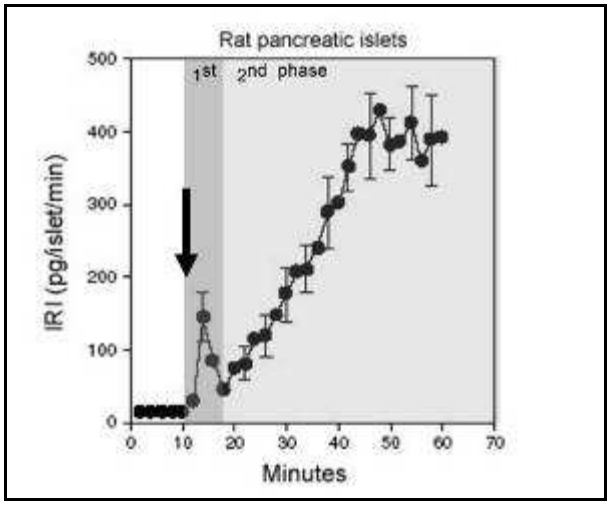

FIG. 2: Bifasic insulin release in rat islets.
The change in glucose concentration (from 2,8 to16,7 mM) is indicated by the arrow.
Insulin release appears after 1-2 minutes, when the glucose metabolism is enhanced. After 4 minutes, release rate has a fast increase, as indicated by the peak, and then it decreases. The second secretion phase is characterized by a slow increase of secretion rate, until it reaches a plateau.

[Straub and Sharp, 2002]

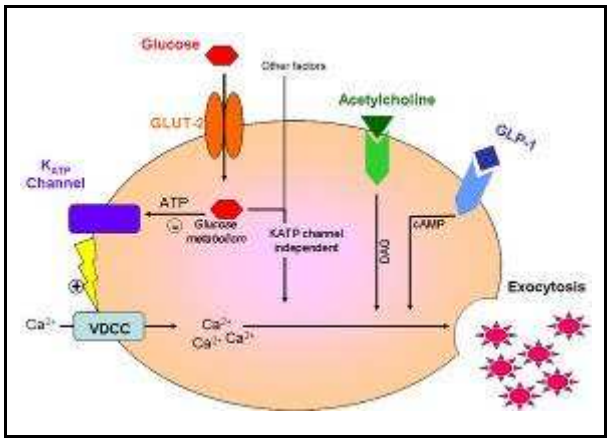

FIG. 3: Simplified representation of the main pathways for insulin release. K_{ATP} Channel dependent and independent pathways are shown. Acetylcholine and GLP1 can enhance insulin release, acting on G-protein receptors.
K_{ATP} Channel: ATP-dependent Potassium Channels.
VDCC: Voltage Gated Calcium Channels.
GLUT-2: Glucose transporter.
DAG: DiAcylGlycerol.

[Straub and Sharp, 2002]

The presence of these two phases is related to two different pathways for vesicle release (Fig. 3): K-ATP channel dependent and K-ATP channel independent, involving different pools of insulin granule and responding to different nutritional stimuli.

The first phase of insulin release, also identified as K-ATP channel dependent pathway, is driven by glucose metabolism, and has been defined Glucose stimulated insulin secretion (GSIS).

Indeed, during GSIS, the glucose metabolism enhances cytoplasmatic ATP/ADP ratio, that in turn reduces the open probability of ATP sensitive K+ channels in the plasma membrane, thus leading to depolarization and subsequent opening of voltage gated Ca^{2+} channels, responsible for exocytosis.

The second phase, K-ATP channel-independent pathway, potentiates the secretory response to the increased $[Ca^{2+}]_I$, and can also be induced by DAG or phorbol esters.

Several metabolic factors downstream glucose entry in the beta cell have been proposed to support the second phase of insulin release.

First, glucose anaplerosis induces an increase in the amount of mitochondrial citrate, which is exported to the cytosol and transformed in malonyl-CoA. The latter induces the inhibition of carnitine palmitoyl transferase 1 (CPT1), thus blocking transport of long-chain acyl-CoA into the mitochondria. Accumulation of long-chain acyl-CoA in the cytosol leads to an increase in intracellular calcium levels [Corkey *et al.*, 1989; Prentki *et al.*, 1992; Chen *et al.*, 1994; Brun *et al.*, 1996].

Second, glucose induces an increase in the production of glutamate in the mitochondria, that is exported to the cytosol. It has been proposed that in the cytosol, glutamate sensitizes the secretory machinery to Ca^{2+}, perhaps by an action on the insulin-containing granules [Maechler and Wollheim, 1999; Rubi *et al.*, 2001; Maechler and Wollheim, 2000]. Another

hypothesis is that glutamate may exert an additive action on mitochondrial malate/aspartate shuttle function [Newsholme *et al.*, 2010].

Another speculation predicts that increased glucose concentrations provoke changes in concentrations and ratios of nucleotides such as ATP, adenosine diphosphate (ADP), GTP, and guanosine diphosphate (GDP) that may participate in regulation of the KATP channel-independent pathway [Henquin, 2000; Sato and Henquin, 1998; Detimary *et al.*, 1996; Detimary *et al.*, 1998].

Other metabolic factors and substances have been shown to play important roles in the regulation of hormone release. We tried to summarize the different regulatory factors in the table below:

Insulin secretagogues	Insulin secretion inhibitors
Glucose	Somatostatin
Mannose	2-Deoxyglucose
Amino acids	Mannoeptulose
Enteric hormones	Galanin
β-ketoacids	Phenitoin
AcetylCholine	Diazoxide
Glucagon	Insulin
cAMP	Tiazolidinic diuretics
β-adrenergic agonist	α-adrenergic agonist

In particular, an emerging role has been attributed to lipids and amino acids, that are thought to be involved in the second phase of insulin release (KATP channel independent).

The role of lipids as insulin secretagogues has been debated, since they seem to have different effects on hormone release, depending on the acute or chronic exposure: the former promotes insulin release, whereas the latter inhibits it [Zhou and Grill, 1994; Gravena *et al.*, 2002]. Lipid effect is also linked to the saturation and the length of the acyl chain [Gravena *et al.*, 2002].

Compared to the other nutrients, plasma amino acids seem to be the more effective on insulin release: in vivo amino acids derived from dietary proteins and those released from intestinal epithelial cells, in combination with glucose, stimulate insulin secretion.

The hypothesis is that the stimulatory action of amino acids on insulin secretion can be attributed to different possibilities [Newsholme *et al.*, 2010; Mc Clenaghan NH *et al.*, 1996]:

1- amino acid metabolism with generation of ATP (glutamine, leucine)

2- direct membrane depolarization (cationic amino acids, arginine)

3- Na+/amino acid co-transport with consequent depolarization (alanine)

4- receptor activation (glutamate, see below)

2.2 Endocrine pancreas and glutamate

2.2.1 Glutamate signalling and hormone release

A particular amino acid that must be analysed for its modulatory action on pancreatic hormone release is glutamate.

In the pancreas, the presence of glutamate as a signalling molecule is well established [Hayashi *et al.,* 2003] and paracrine-like signalling has been proposed as a novel regulatory mechanism for islet hormone secretion [Moriyama and Hayashi, 2003].

Glutamate could function as an intercellular signal mediator in the islets as they possess all the necessary elements of L-glutamate mediated transmission. Several research groups have reported that islets cells and clonal islet cells express functional glutamate receptors [Hayashi *et al.,* 2003; Molnár *et al.,* 1995; Weaver *et al.,* 1996; Tong *et al.,* 2002] and vesicular glutamate transporters [Bai *et al.,* 2003]

iGluRs	mGluRs	VGLUT
Ampa, Kainate, NMDA	Group I, II, III	VGLUT 1 and 2

TABLE 2: Expression of glutamate signalling molecule in pancreas. [Hinoi *et al.,* 2004]

L-glutamate is co-stored and co-secreted with glucagon in alpha cells and the stoichiometric amounts of glutamate and glucagon are 2000:1 (molar ratio), respectively [Bai *et al.*, 2003].

Glutamate in alpha cells might originate not only from intracellular synthesis by means of glutaminase, but it has also been proposed that it can derive by uptake of plasma L-glutamate through a sodium dependent glutamate transporter [Weaver *et al.*, 1998].

For vesicle loading, vesicular glutamate transporters, with a H^+ dependent uptake activity, were found in alpha cells but not in gamma cells, suggesting that only alpha cells are able to store glutamate [Bai *et al.*, 2003].

Several independent lines of evidence indicate expression of both iGluRs and mGluRs in pancreatic islets.

Among ionotropic glutamate receptors, AMPA are functionally distributed in alpha and beta cells [Gonoi *et al.,* 1999; Inagaki *et al.,* 1995; Molnar *et al.,* 1995; Weaver *et al.,* 1996; Morley *et al.,* 2000; Weaver *et al.,* 1998]. On the other hand, the expression of functional kainate receptors is negligible, even though it has been reported that pancreatic β cells express

both AMPA and kainate currents similar to those reported in neuronal cells [Weaver *et al.*, 1996]. Some laboratories have detected the expression of NMDA receptors in the islets, although neither NMDA receptor subunits, nor NMDA evoked ion transport have been observed.

It has been reported that AMPA and kainate stimulate insulin secretion from perfused or isolated islets or clonal cells in the presence of high level of glucose [Gonoi *et al.*, 1999; Inagaki *et al.*, 1995; Bertrand *et al.*, 1992 and 1995]. Considering just the paracrine role of glutamate, this evidence is not completely convincing in normal physiological conditions, because L-glutamate is co-secreted with glucagon when the islets are immersed in low blood glucose levels. In that situation, glutamate signalling should not be directly involved in insulin secretion, that usually occurs in the opposite condition of high glucose levels. In line with this hypothesis, a recent work by Cabrera, did not show any influence of glutamate signalling on the regulation of insulin secretion [Cabrera *et al.*, 2008].

However, the role of glutamate in modulating insulin release can not be completely ruled out, as it could also originate from plasma, where it could reach a concentration of 50-200 μM.

L-glutamate and AMPA have been shown to stimulate glucagon secretion from the perfused pancreas and in mice, where activation of iGluRs is needed for efficient glucagon secretion in response to hypoglycaemia [Brice *et al.*, 2002; Bertrand *et al.*, 1993; Cabrera *et al.*, 2008].

A more widespread expression pattern of mGlu receptors has been reported in the islets and in clonal cells. It has been demonstrated that also mGluRs participate in hormone secretion from pancreatic islets. Agonists of group I and II increase the release of insulin in the presence of glucose at low concentration, whereas a group III agonist inhibits insulin release at high glucose concentrations [Brice *et al.*, 2002]. By contrast, a class III metabotropic receptor antagonist, inhibits glucagon secretion [Uehara *et al.*, 2004; Tong *et al.*, 2002].

Thus, the activation of glutamate receptors does not always correlate with hormone secretion, but these results may be influenced by the metabolic state of the organism wherein the glutamate appears in the interstitial space.

2.2.1.1 ISLET PARACRINE INTERPLAY

A proposed function for glutamate as an extracellular signal mediator is to participate in islet cross-talking, that can be significant in the control of hormone release (Fig. 4).

Indeed, β cells store GABA in Synaptic-like micro-vesicles (SLMVs), which are secretory vesicles distinct from insulin granules, and secrete GABA by Ca^{2+}-dependent exocytosis

[Thomas/Reetz *et al.*, 1993; Anhert-Hilger *et al.*,1996]. On the other hand, in alpha cells glutamate is co-stored with glucagon in stoichiometric amounts.

It has been proven that low glucose conditions result in co-secretion of stoichiometric amounts of glutamate and glucagon from αTC6 and isolated islets, which triggers GABA secretion from β cells [Reetz *et al.*, 1991; Hayashi *et al.*, 2003]. In particular, in the presence of low glucose, stimulation of AMPA receptors facilitates GABA secretion from beta cells with little effect on insulin secretion. [Hayashi *et al.*, 2003]. The GABA mediated signalling from beta cells in turn inhibits glucagon and glutamate secretion from alpha cells, by activation of GABA A receptors on alpha cells [Rorsman *et al.*, 1989]. Also stimulation of mGlu8 receptors on alpha cells inhibits glucagon secretion, suggesting a role of glutamate in autocrine signalling.

It is likely that L-glutamate signalling also triggers somatostatin secretion, which in turn inhibits glucagon secretion, through somatostatin sst2 receptors [Muroyama *et al.*, 2004; Moriyama and Hayashi, 2003].

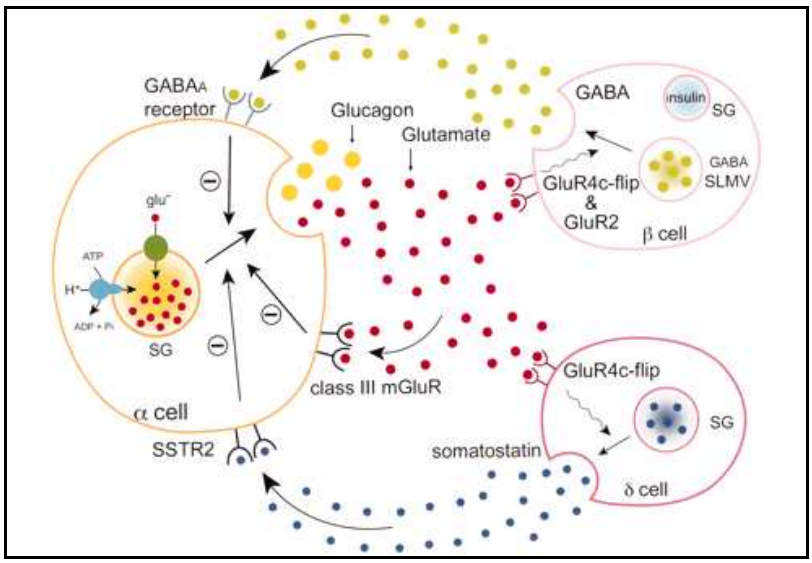

FIG. 4: Hypotetical role of neurotransmitters in regulation of pancreatic hormone secretion.

[Moriyama and Hayashi, 2003]

2.2.1.2 GLUTAMATE TRANSPORTERS

The cellular response to glutamate could be controlled, directly and indirectly, by regulating transport of amino acid across the plasma membrane. In particular, flux through glutamate transporters may act as a modulator of hormone secretion by increasing intracellular amino acid content or by controlling extracellular amino acid concentration and therefore receptor activation.

There are few evidences of glutamate transporter expression in endocrine pancreas and they lack some information regarding their physiological roles.
Sodium dependent glutamate uptake has been detected in the mantle region of islets [Weaver *et al.*,1998], most probably alpha cells, that are able to sequester glutamate from the interstitial space of the islets. The expression of genes encoding sodium dependent glutamate transporters has been detected in isolated islets [Manfras *et al.*, 1994]. Moreover, it has been proposed that a high affinity glutamate transporter expressed in pancreatic islets of Langerhans, modulates insulin secretion stimulated by glucose [Weaver *et al.*, 1998].

2.2.2 Glutamate in islet pathological conditions

Islets of Langerhans show a sophisticated glutamate paracrine system, where all the elements required for glutamatergic transmission, including ionotropic receptors are expressed.
Glutamate derives both from plasma amino acid pool and from alpha cell granules. In addition, it has been demonstrated that during diabetes the islet or plasmatic glutamate level may further increase.

2.2.2.1 DIABETES

Although there are several subtypes of diabetes mellitus, each with a different pathogenesis, it is now recognised that diabetes is characterised by a defect in insulin secretion caused by a deficit and/or functional impairment in insulin producing β cells and by the consequent development of hyperglycaemia. The time of onset, the speed of progression and the nature of precipitating factors (external or genetic) that cause the defect in insulin secretion vary in the different subtypes. It seems that beta cell death may be what connects type 1 and type 2 diabetes [deKoning *et al.*, 2008].

Type I diabetes

Currently type 1 diabetes affects 0,5% of the population in developed countries and it is increasing in incidence. It is not known what triggers it and there is little understanding of the genetic and the environmental factors regulating its progression. Thus, it is not known how to prevent or reverse type 1 diabetes.
Type 1 diabetes is an autoimmune disease resulting from specific destruction of the insulin producing beta cells of the islets of Langerhans in the pancreas [Tisch and McDevitt, 1996]. It has two distinct phases: insulitis, when leucocytes invade the islet, and diabetes, when most of

the beta cells have been killed, and there is no longer sufficient insulin production to regulate blood glucose levels, resulting in hyperglycaemia.
Destruction of beta cells is a crucial event at disease outset, initiating autoimmunity. Indeed, type 1 diabetes is primarily a T-lymphocyte mediated disease [Tisch *et al.*, 1996; Bach and Mathis, 1997].

Type II diabetes

Type 2 diabetes is by far the most common subtype, and its occurrence is increasing rapidly because of an imbalance between food intake and energy expenditure, which leads to obesity and insulin resistance [Wild *et al.*, 2004].
The pathogenesis of the type II diabetes is non-autoimmune in nature and remains controversial, although there is a general agreement that both insulin resistance and a beta cell defect contribute to the disease. Depending on genetic or environmental factors, a fraction of type 2 diabetics may exhibit abnormal beta cell death, that may degenerate to such a degree that insulin therapy becomes necessary in advanced stage of the disease. As a consequence of reduced beta cell mass, type 2 diabetes is characterized by the absence of a first phase insulin response and abnormalities in insulin pulsatility [Leahy *et al.*, 1988].
Many observations indicate a negative effect of high glucose concentration, termed glucotoxicity, on glucose sensing, insulin gene expression, insulin secretion and beta cell survival [Robertson 2004; Leahy *et al.*, 1992].
A support for a progressive decline in beta cell mass during the course of the disease is provided by in vitro experiments, where elevated glucose concentrations, analogous to those seen in type 2 diabetics, can induce apoptosis of cultured human islets [Federici *et al.*, 2001]. Better evidence for beta cell death in late stages of type 2 diabetes comes from animal models: when *Psammomys obesus* (gerbil) is kept in captivity and is fed with high energy diet, an increase in apoptosis occurs and it develops diabetes [Bar-On *et al.*, 1999; Donath *et al.*, 1999]. A modest reduction in the beta cell mass of type 2 diabetes individuals at autopsy has been reported [Clark *et al.*, 1998].
In Caucasians, compared to obese normoglycaemic subjects, relative beta-cell mass is reduced by 40% in obese subjects with impaired fasting glucose, and by 63% in obese subjects with type 2 diabetes [Sakuraba *et al.*, 2002; Yoon *et al.*, 2003]. Moreover, an increased number of apoptotic beta cells was observed in the islets of diabetic subjects compared with those of normoglycaemic subjects [Butler *et al.*, 2003]. One possible mechanism of beta cell death

could be a decrease in the ratio of anti-apoptotic to pro-apoptotic members of the *bcl* 2 family [Federici *et al.*, 2001].

2.2.2.2 GLUTAMATE AND DIABETES

Many studies have underlined alterations in glutamate homeostasis that correlated with both type one and type two diabetes, even though it is not described any clear relationship between the two phenomena.

Variations in plasma glutamate concentration have been reported in T1DM and T2DM, although deriving from different origin.

In children who later progress to type 1 diabetes, alterations in the metabolic profile characterize the early pathogenesis of the disease, suggesting a role in its progression. The appearance of insulin and glutamic acid decarboxilase autoantibodies (IAA and GADA) in the serum is preceded by abnormally high glutamic acid (13 fold increase). Glutamic acid increase is dependent on the time when autoantibodies appeared: 0-9 months before the seroconversion, there is a peak of serum glutamic acid (32-fold), that slightly decreased in the following months (to 5.2 fold), while after the seroconversion, the metabolic profile is partially normalised. Such evidences could suggest that the initial autoimmune response is physiological and aimed at restoring the metabolic homeostasis [Oresic *et al.*, 2008].

During the pathological development and the drug intervention of type 2 diabetes mellitus (T2DM), is involved altered expression of serum low molecular weight metabolites, including carbohydrates, long chain fatty acids and amino acids. In particular, among amino acids, serum glutamate is significantly increased in diabetic subjects. Interestingly, three treatments used for the therapy of T2DM were tested in patients and were able to down-regulate the high level of glutamate [Bao *et al.*, 2009].

In addition, changes in glutamate concentration may occur also in the islet microenvironment and might contribute to disease progression as well as participate in the late stages of the disease.

Indeed, during lymphocyte islet infiltration in type 1 diabetes, glutamate regulates the initiation of immune response [Pacheco *et al.*, 2006]. Human dendritic cells (DC) release significant amounts of glutamate, that acts on mGluR1 expressed on T cells after antigen presentation, and it mediates enhanced T cell proliferation and secretion of Th1 and inflammatory cytokines. Normal human T cells express also high levels of AMPA ion channel receptors, that can trigger integrin mediated T cell adhesion to laminin and fibronectin [Ganor *et al.*, 2003].

Moreover, both T1DM and T2DM are characterised by an increase in beta cell death, thereby leading to a change in alpha/beta cell ratio [Clark *et al.*, 1988; Unger and Orci, 2010]. In particular, in type two diabetes, in the corpus (body, tail and anterior part of the head of the pancreas) the mean α/β-cell ratio increased from 0.27 in control subjects to 0.57 in Type 2 diabetic patients [Clark *et al.*, 1988]. Thus, during the response to physiological stimuli, the increased number of alpha cells is able to secrete more glutamate in the extracellular space.

In addition, in the normal physiology of the islets, glucagon (and therefore glutamate) secretion is under control of a negative paracrine feedback from GABA A receptors present on alpha cells. In an imbalanced situation of low percentage of GABA secreting beta cells, as happens during diabetes, this negative feedback could be impaired, leading to a massive glutamate release.

Furthermore, in both types of diabetes, has been described a paradoxical effect of hyperglycaemia on glucagon release. In normal conditions, immediately after the ingestion of a carbohydrate-containing meal, a spike of insulin release occurs and is believed to be the cause of glucose-induced glucagon suppression (fig.5).

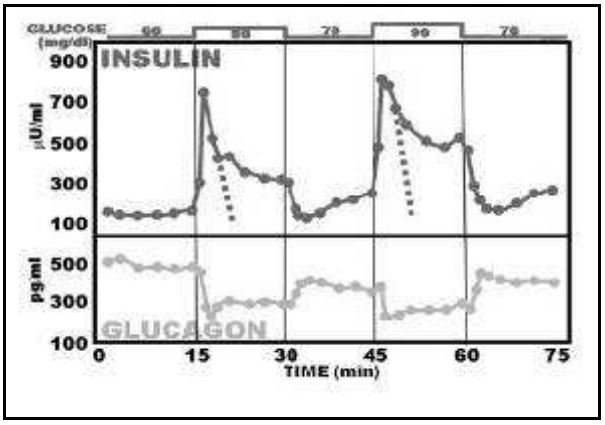

FIG. 5: Demonstration of the reciprocal responses of insulin and glucagon to 20 mg/dL changes in the concentration of glucose perfusing the isolated pancreas of rats. Dotted lines mark the full contour of the first phase.

In T1DM, glucose increases without a parallel insulin increase and hyperglycaemia will paradoxically stimulate glucagon and therefore glutamate secretion [Braaten *et al.*, 1974; Le marchand, *et al.* 2010].

In T2DM the initial spike is lost, thus glucagon remains unsuppressed and elevated relative to the ambient glucose concentration, exaggerating the postprandial hyperglycaemia [Unger and Orci, 2010].

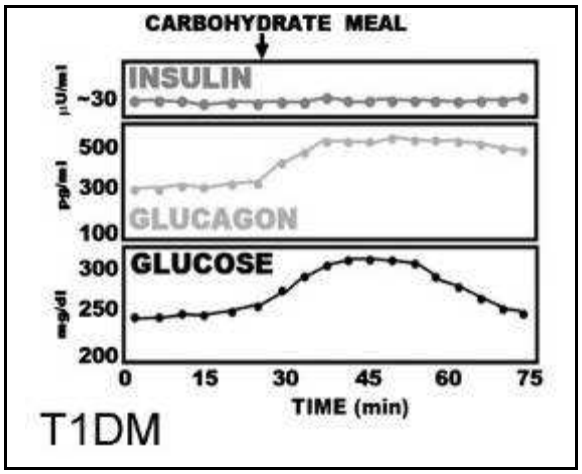

FIG. 6A: In T1DM, due to the absence of juxtaposed insulin-secreting cells, basal glucagon levels are increased and they increase further in a paradoxical response to hyperglycaemia from a carbohydrate-containing meal.

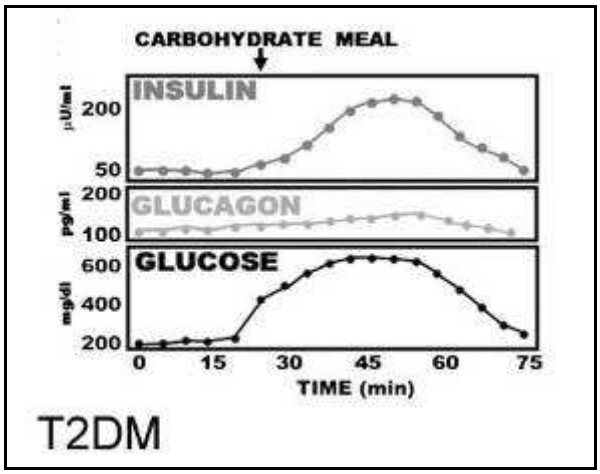

FIG. 6B: In T2DM, α-cells are not suppressed by the hyperglycaemia, because of the loss of the first-phase spike of insulin release immediately after the ingestion of a carbohydrate-containing meal.

In the CNS, activation of glutamate ionotropic receptors has been identified as the mechanism underlying glutamate toxicity [Choi *et al.*, 1987] and it has been reported that glutamate can also be toxic outside the CNS [Said *et al.*, 1996].

The effect of glutamate on islet cell function and survival is presently unknown, thus we thought that it could be interesting to understand whether and how islets can become resistant to the toxicity of blood glutamate.

There are two ways to explain the glutamate resistance: in pancreatic cells, glutamate ionotropic receptors may not have a drastic effect on survival, or an efficient system that removes glutamate from the extracellular space exists.

The presence of glutamate receptors in the islets has been characterised and their subunit assembly and conductances seem to be similar to the one in the CNS.

We previously found that clonal β cells selectively express GLT1 transporter (Fig. 7A). We confirmed the expression of GLT1 in human pancreases, by means of double immunofluorescence analysis (Fig. 7B) [Tesi di Laurea Magistrale-Di Cairano, 2007].

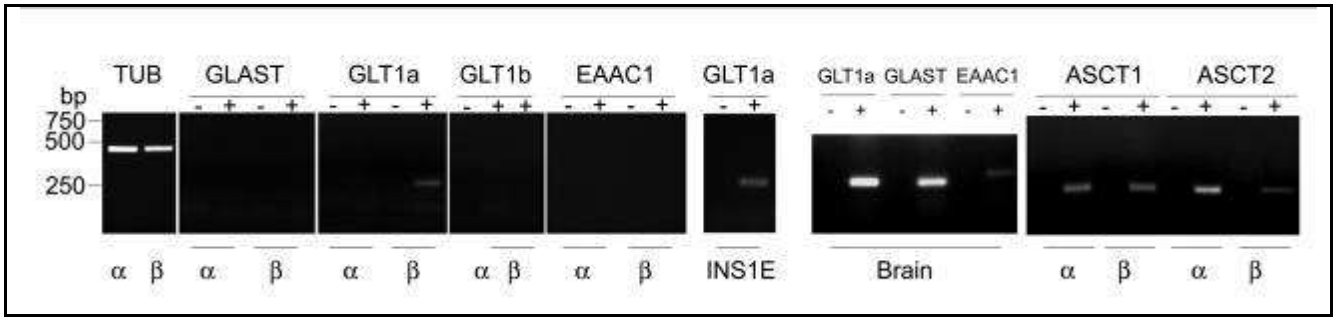

FIG. 7A: Expression of high-affinity glutamate transporters in αTC1 and βTC3 cells. RT-PCR analysis of SLC1A glutamate transporter subtypes (+). Negative controls(-): PCR reactions performed in the absence of cDNA. Positives controls: Tubulin amplification; primers consistency was checked using Brain cDNA as a template; INS1E cDNA to confirm GLT1 expression in β cells. Markers on the left indicate bp.

In this work, we investigate its physiological role in the islets.

Moreover, since alterations in glutamate homeostasis have been observed in patients affected by diabetes and glutamate transporters have been proposed as key regulators of the extracellular glutamate concentration, we investigated whether GLT1 may be involved in diabetes progression.

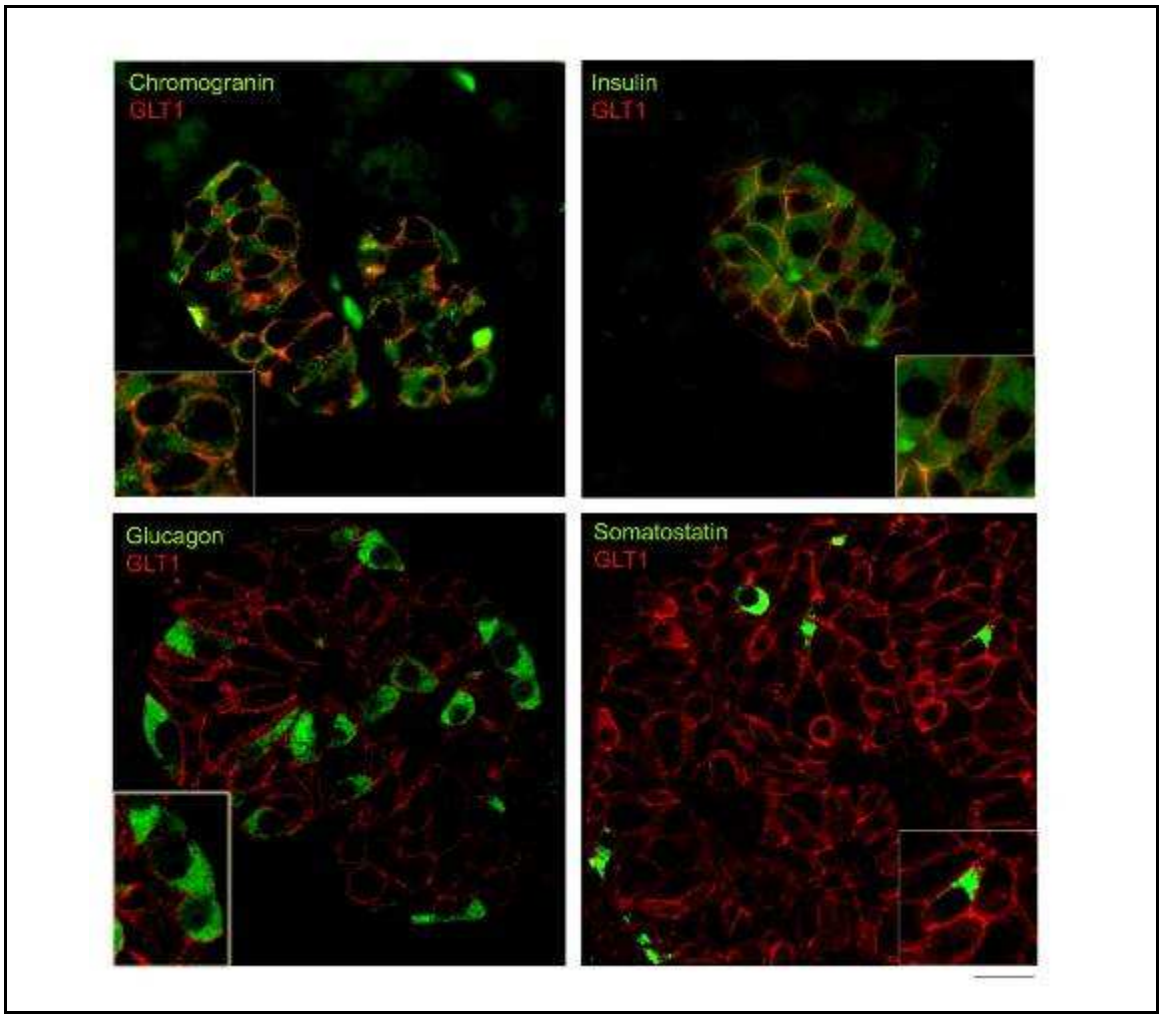

FIG. 7B: GLT1 localizes to the plasma membrane of the β-cells. Immunofluorescence staining of human pancreas sections with anti-GLT1 (red) and hormones (green) as markers of different endocrine cell types. Scale bar = 10 µm. In the inset, a particular of the islet is shown at higher magnification (2x). The yellow/orange staining indicates co-localization between the transporters and the hormones.

3 RESULTS

3.1 βTC3 cells are vulnerable to glutamate-induced cytotoxicity

We first determined whether β- and α-cell lines were vulnerable to extracellular glutamate. βTC3 and αTC1 were cultured in freshly prepared medium in the presence of different glutamate concentrations (ranging from 0.05 to 5 mM) and cell viability was assayed by the MTT test. Exposure to glutamate induced a dose- and time-dependent decrease of βTC3 viability (Fig. 1A, B). As previously shown in MIN6 cells [Morley, 2000], we did not observe glutamate-induced cytotoxicity after a 20 min incubation at 5 mM, as usually occurs in neurons [Perego *et al.*, 2000]. Notably, αTC1 cells were resistant to glutamate-induced toxicity showing a small decrease in viability only after 5-day exposure to 5 mM glutamate (Fig.1A).

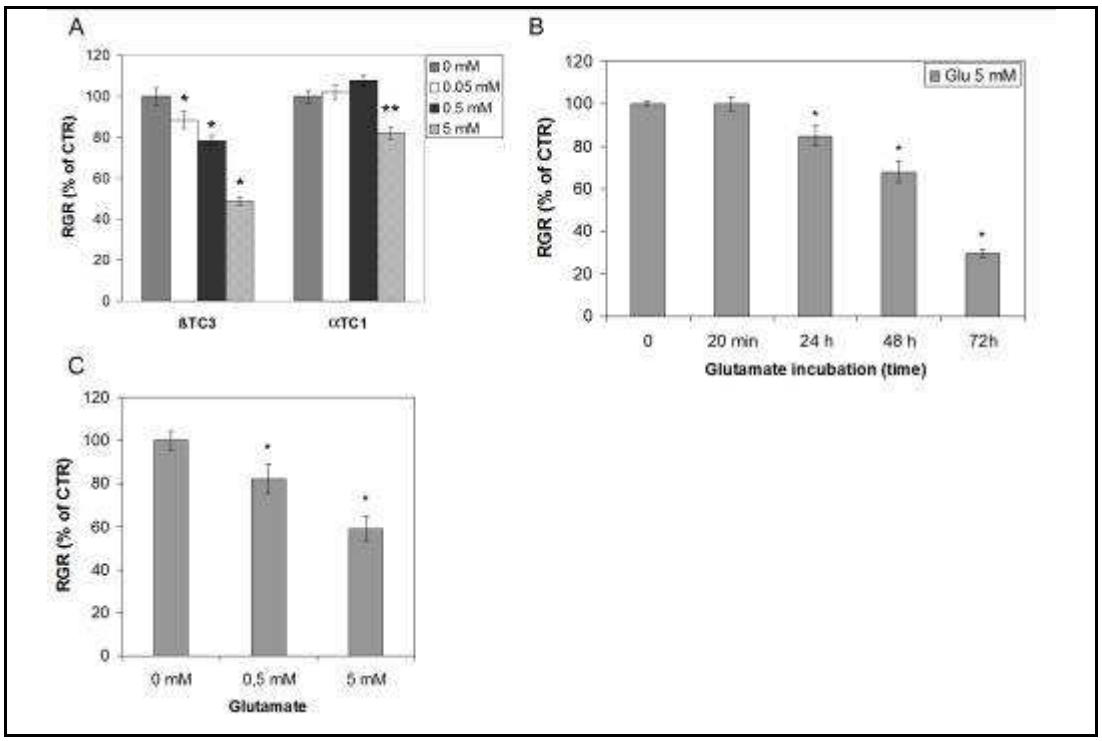

FIG. 1: Chronic incubation with glutamate impairs β-cell viability (A). αTC1 and βTC3 cell viability measured by MTT assay after cell incubation with the indicated glutamate concentrations for 5 days. The Relative Growth Rate (RGR) is presented as percentage of 0 mM glutamate and data are mean ± s.e.m., n=7 with 8 replicates. (* $p<0.0001$ vs Ctr; ** $p<0.01$ vs Ctr). (B). βTC3 viability measured by MTT assay after incubation with 5 mM glutamate for the indicated times. Data are presented as percentage of each relative Ctr (no glutamate) and are mean ± s.e.m. n=4 with 8 replicates. (* $p<0.001$ vs Ctr). (C). βTC3 viability measured by MTT assay after incubation with DMEM media supplemented with the indicated glutamate concentrations. Data are presented as percentage of 0 mM glutamate and data are mean ± s.e.m., n=3 with 8 replicates (* $p<0.05$ or ** $p<0.01$ vs Ctr).

As βTC3 and αTC1 usually grow in different media, RPMI and DMEM respectively, the latter containing no glutamate, we assessed glutamate toxicity in βTC3 in the presence of DMEM media. Also in this media, βTC3 showed a significant decrease in viability, that is similar to that obtained in RPMI media (Fig. 1C).

Glutamate-induced cytotoxicity caused βTC3 apoptosis. In fact, 5-day incubation in the presence of 0.5 mM glutamate, a physiological relevant concentration, significantly increased the number of TUNEL positive βTC3 (Fig. 2).

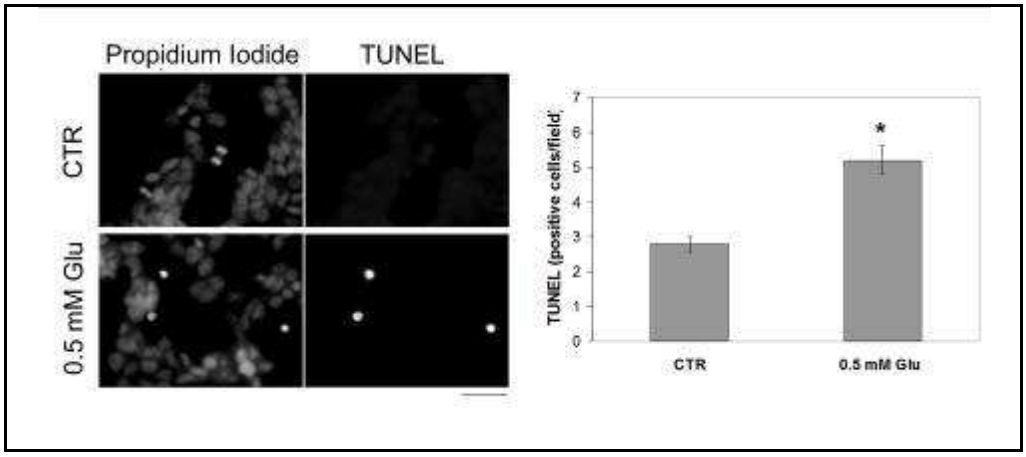

FIG. 2: β-cell death is mediated by apoptosis. Determination of βTC3 cells apoptosis by TUNEL assay after incubation with 0.5 mM glutamate for 5 days. Cell nuclei were labelled with Propidium iodide. On the left panel is shown fluorescence image, scale bar: 50 μm. On the right panel is shown quantification of TUNEL-positive β-cells. Data are the mean ± s.e.m. of 3 independent experiments performed in duplicate (* $p<0.05$)

These data indicate that β-cells, similarly to oligodendrocytes and neurons, are vulnerable to glutamate-induced cytotoxicity while α-cell are resistant [Choi *et al.*, 1987].

3.2 Excitotoxicity is responsible for glutamate-induced βTC3 apoptosis

To determine whether glutamate-induced βTC3 toxicity was mediated by excitotoxicity, we exposed βTC3 to high glutamate concentrations in the presence of specific ionotropic receptors' antagonists (Fig. 3A). We could therefore demonstrate that glutamate toxicity was partially prevented by the co-administration of 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), a non-selective α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptor antagonist (46.3+2.7% increase in RGR relative to 5 mM glutamate; $p<0.005$) but not by D-2-Amino-5-phosphonovaleric acid (APV), a selective N-methyl-D-

aspartate (NMDA) receptor antagonist. In the absence of glutamate supplementation, βTC3 viability was not affected by the presence of both glutamate receptor antagonists. These data indicate that glutamate-induced βTC3 cytotoxicity is in part mediated by chronic activation of AMPA and/or kainate ionotropic glutamate receptors and can be therefore referred as "excitotoxicity", as occurs in the CNS [Choi *et al.*, 1987].

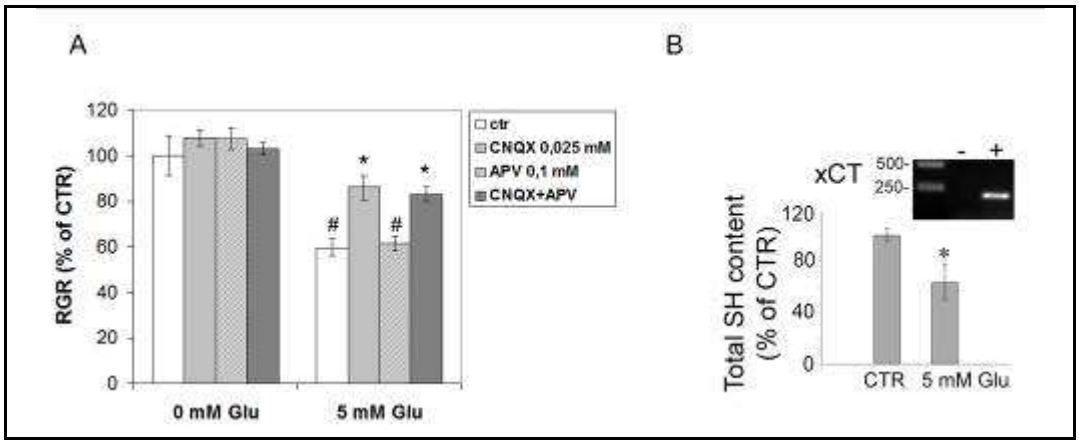

FIG. 3: Mechanisms of β-cell death: ionotropic receptors' activation and oxidative stress. (A). Viability of βTC3 cells after incubation for 5 days with 0.025 mM CNQX or 0.1 mM APV, with or without 5 mM glutamate measured by MTT assay. Data are presented as RGR and are mean ± s.e.m. n=3 with 8 replicates. (#$p<0.05$ vs 0 mM glutamate Ctr; *$p<0.05$ vs 5 mM glutamate Ctr). (B). Evaluation of total SH content in βTC3 cells after incubation with 5 mM glutamate. Data are expressed as % of CTR and are the mean ± s.e.m. of 4 independent experiments performed in duplicate (*$p<0.05$). In the inset is shown RT-PCR analysis of xCT subunit in βTC3 cells.

As CNQX was not able to completely restore βTC3 viability during glutamate exposure, we investigated if other mechanisms were involved in glutamate toxicity. It has been demonstrated that increased extracellular glutamate may also lead to cell death by oxidative stress (oxidative glutamate toxicity) which is mediated by the glutamate/cysteine exchanger (x^{-c}) [Tan *et al.*, 2001]. Thus, we first demonstrated that xCT subunit of the x^{-c} exchanger is expressed in βTC3 cells by RT-PCR analysis (Fig. 3B, inset). Given xCT expression in βTC3, after a 5 day cell treatment with 5 mM glutamate, we measured total SH content as an indicator of oxidative stress. We found that chronic incubation with glutamate caused a significant reduction in the total SH content (Fig. 3B), thus suggesting that glutamate may induce β-cell death also via oxidative stress.

3.3 Excitotoxicity can be prevented by treatment with Exendin-4

To confirm the excitotoxic mechanism of β-cell death, we have exposed βTC3 cells to high glutamate and Exendin-4 (Ex-4). Ex-4 is a synthetic long lasting analogue of the enteric hormone glucagon-like peptide 1 (GLP-1), recently approved by the Federal Drug Administration for the treatment of diabetes. It is known to preserve β-cell mass by reducing apoptosis [Farilla *et al.*, 2003; Wajchenberg *et al.*, 2007] and to protect cultured rat hippocampal neurons against glutamate induced apoptosis [Perry *et al.*, 2002]. We demonstrated that Ex-4 alone did not alter the viability of βTC3 cells at the concentrations tested, but significantly reduced glutamate-induced excitotoxicity (Fig. 4). This mechanism can be considered specific, as treatment with glutamate and DPP-IV (dipeptidyl-peptidase IV), the enzyme responsible for GLP-1 inactivation, did not show any improvement in βTC3 viability.

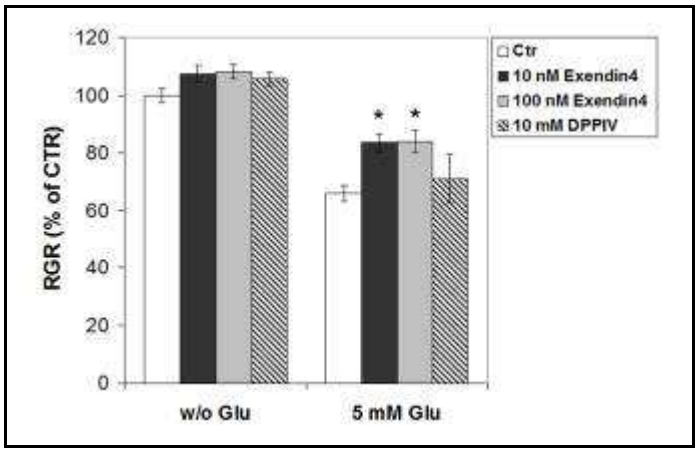

FIG. 4: Pharmacological prevention of excitotoxicity restores β-cell survival. Determination of β-cell viability by MTT assay after incubation for 5 days in 10 or 100 nM Exendin-4 and 10 mM DPPIV with or without 5 mM glutamate. Data are presented as RGR and are mean ± s.e.m.; n=3 with 8 replicates. (*$p < 0.005$, vs Ctr in 5 mM Glutamate).

3.4 GLT1 protects βTC3 from excitotoxicity

The selective expression of GLT1 transporters in β cells has been established in previous studies [Tesi di Laurea Magistrale-Di Cairano, 2007]. We then investigated its physiological relevance in islets. Therefore, we tested whether a function of GLT1 is to protect against excitotoxicity, as occurs in the CNS [Choi *et al.*, 1987]. To this aim, we thought to assess beta cell viability during pharmacological inhibition of the transporter.

First, we tested the inhibition ability of dihydrokainate (DHK), a selective GLT1 inhibitor [Arriza *et al.*, 1994], in βTC3 cells, where acute exposure to DHK induced a dose-dependent inhibition of Na^+-dependent [^{3}H]-D-aspartate uptake, with an IC_{50}= 0.05 mM DHK, in line with previous reported Ki [Kawahara *et al.*, 2002] (Fig. 5A). We avoided higher concentrations (1 mM), known to affect ionotropic receptor activation [Wang *et al.*, 1998], to isolate the specific effect on GLT1 transporter.

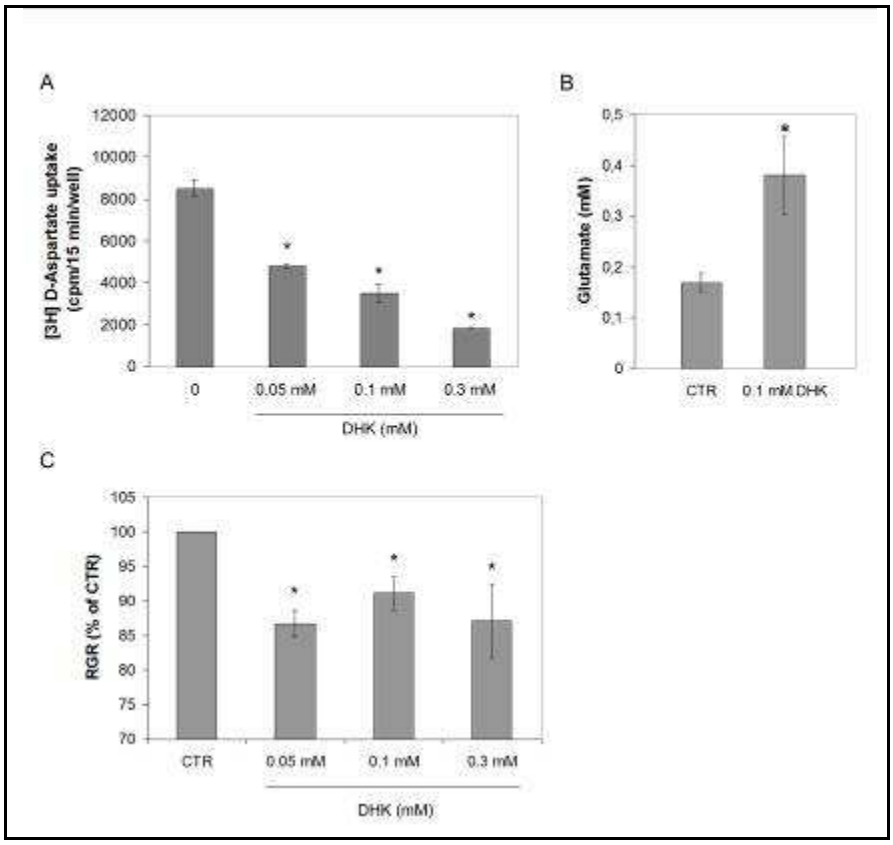

FIG. 5: The GLT1 transporter controls the extracellular concentration of glutamate and β-cell viability. (A). The Na-dependent [^{3}H]-D-Aspartate uptake in βTC3 cells was measured in the presence of increasing DHK concentrations. Data are expressed as cpm/well/15 min and are mean ± s.e.m.; n=3 performed in triplicate. (*P<0.01: vs CTR). (B). Determination of extracellular glutamate concentration by enzymatic assay after βTC3 cells incubation with or without 0.1 mM DHK for 5 days. Data are expressed in mM and are mean ± s.e.m.; n=4, performed in triplicate (*p<0.05). (C). Determination of βTC3 cells viability by MTT assay after incubation with DHK for 5 days. Data are expressed as RGR and are mean ± s.e.m. n=3 with 8 replicates. (*p<0.05, vs CTR).

In presence of GLT1 inhibition (0.1 mM DHK in cell medium for 5 days), we measured in the medium of βTC3 cells a significant increase of glutamate level, that reached concentration of the same order of magnitude of that toxic for beta cells (Fig. 5B). In agreement with this observation, DHK induced a decrease in βTC3 viability that was

already maximal at 0.05 mM DHK (Fig. 5C), indicating a role of GLT1 in prevention of glutamate toxicity. Cytotoxicity was due to apoptosis, as revealed by a TUNEL assay performed on βTC3 incubated with 0.1 mM DHK for 5 days (Fig. 6). An increase in the number of apoptotic nuclei was observed after DHK treatment.

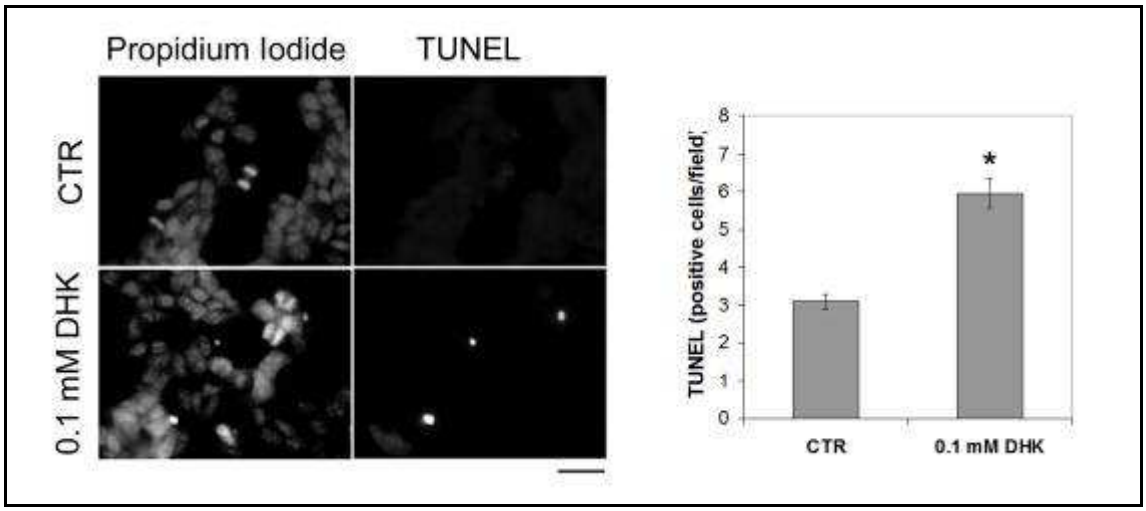

FIG. 6: GLT1 inhibition by DHK induces β-cells apoptosis. Assessment of β-cells apoptosis by TUNEL assay after 5 days incubation with 0.1 mM DHK. Cell nuclei were labelled with Propidium Iodide. Scale bar: 50 μm. In the right panel, quantification of TUNEL-positive β-cells is shown. Data are mean ± s.e.m. of 3 independent experiments performed in duplicate. (*P<0.05, vs CTR).

Similar results were obtained by reducing the expression of the GLT1 gene in βTC3 by means of shRNA (Fig. 8). We first tested whether shRNAs (small hairpin RNAs) were effective in reducing the total expression of GLT1, by means of western blotting and uptake experiments (Fig. 7A, B). From that analysis, we concluded that SH1 and SH3 were the more promising shRNA to be used in the following experiments of GLT1 down-regulation.

A 35% down-regulation of GLT1 activity achieved by SH1 was sufficient to increase βTC3 cell apoptosis after a 24-hour incubation in 0.5 mM glutamate, by two to four folds (Fig. 8). More interestingly, the shRNA constructs increased βTC3 apoptosis also in the absence of glutamate supplementation, suggesting that impaired GLT1 activity is "per se" sufficient to induce βTC3 cell death (Fig. 8), as previously demonstrated by DHK treatment.

Altogether, these data demonstrate a pivotal role of GLT1 in β-cell physiology and in protecting β-cells from excitotoxicity.

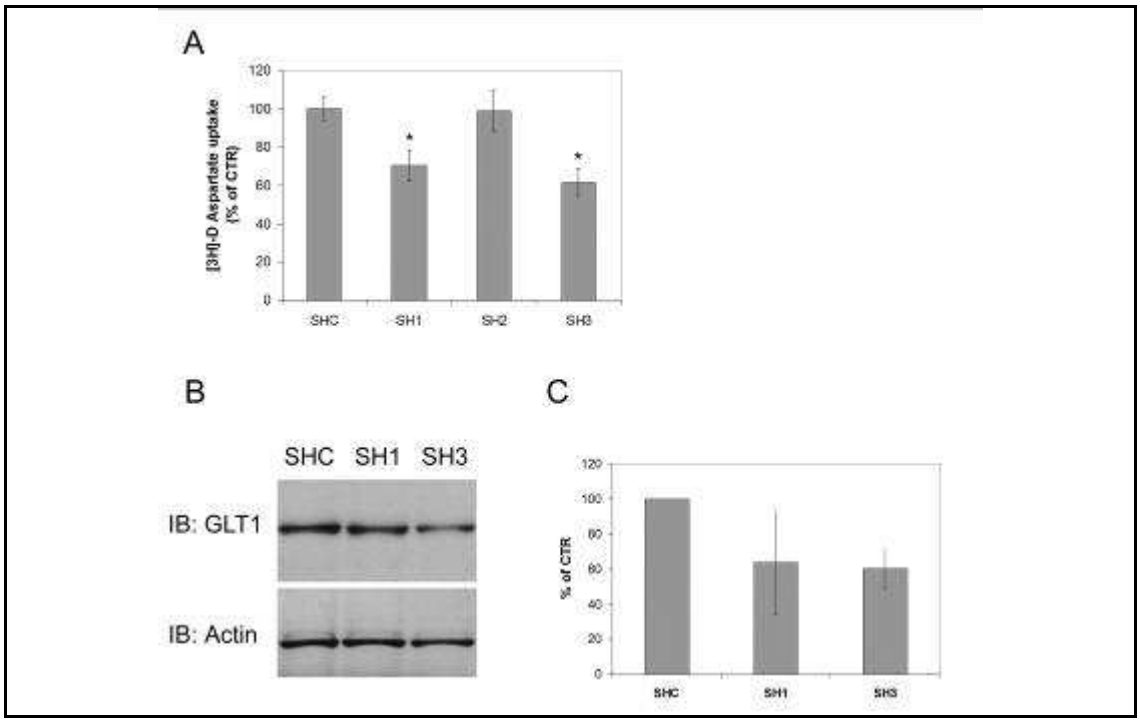

FIG. 7: Validation of shRNAs against GLT1 transporter. βTC3 cells were transfected with different GLT1 shRNAs (SH1, SH2 and SH3) or a control shRNA (SHC). (A). Determination of GLT1 surface expression by means of Na-dependent [^{3}H]-D-Aspartate uptake. Data are expressed as percentage of SHC and are mean ± s.e.m. of 3 independent experiments performed in triplicate (*p<0.01, vs SHC). (B). Immunoblotting of 30 µg of whole βTC3 lysate with anti-GLT1 and anti-actin antibodies (internal standard). A representative blot is shown. (C). Quantification of GLT1 expression by densitometry. Data were normalized for actin content and expressed as percentage of SHC and are mean ± s.e.m. of 3 independent experiments.

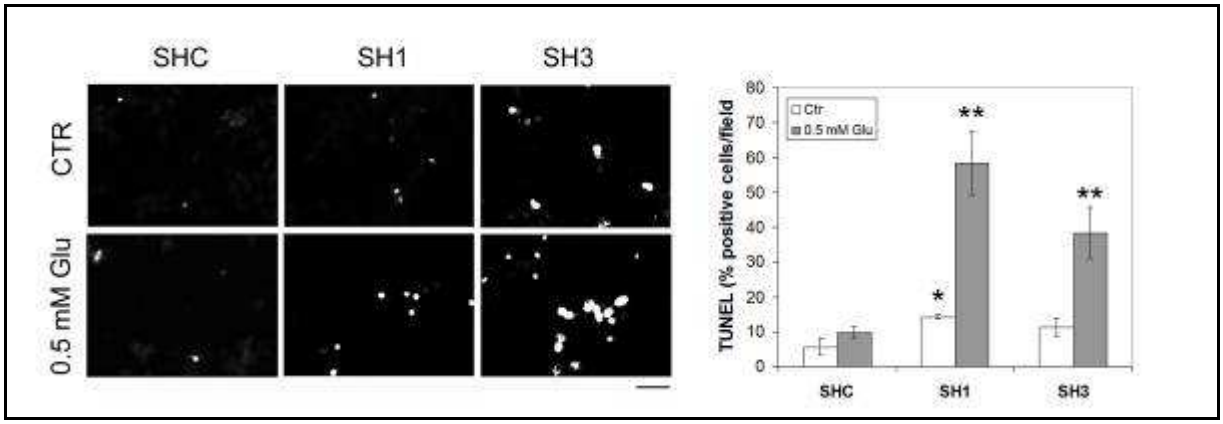

FIG. 8: GLT1 knock down by shRNA causes β-cells death by apoptosis. One day after shRNAs transfection, βTC3 cells were incubated for 2 days with or without 0.5 mM glutamate, and apoptosis assessed by TUNEL. Cell nuclei were labelled with Propidium iodide. Scale bar: 50 µm. In the left panel, quantification of TUNEL-positive β-cell is reported. Data are expressed as percentage of total cells/field and are mean ± s.e.m. of 3 independent experiments performed in duplicate. (*p<0.05 vs SHC Ctr; **p<0.005 vs SHC 0.5 mM glutamate).

3.5 β-cells death can be pharmacologically prevented by treatment with Ceftriaxone

To further support the involvement of GLT1 in the control of β-cell survival, we tried the opposite approach compared to DHK/shRNA, that aimed at up-regulating GLT1 expression or function.

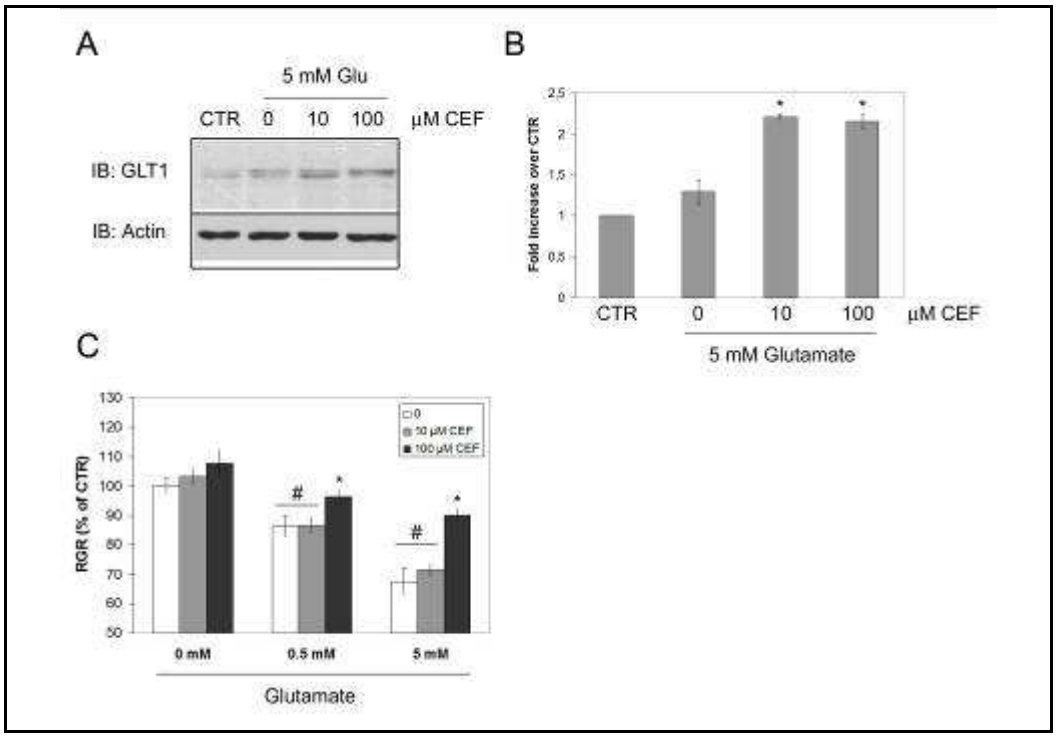

FIG. 9: GLT1 upregulation by Ceftriaxone restores β-cell survival. (A). After incubation of βTC3 cells with the indicated CEF and glutamate concentrations, 100 µg of each cell lysate were immunoblotted with anti-GLT1 and actin antibodies. A representative blot is shown. (B). Quantification of GLT1 expression by densitometry. Data were normalized by actin content and are expressed as mean ± s.e.m. of 3 independent experiments. (*p<0.01, vs CTR). (C). Determination of β-cell viability by MTT assay after incubation for 5 days with the indicated Ceftriaxone (CEF) and glutamate concentrations. Data are expressed as RGR and are mean ± s.e.m. n=3 with 8 replicates. (*p<0.05, 100 µM CEF vs relative Ctr; # p<0.05, vs 0 mM glutamate 0 CEF).

Ceftriaxone (CEF) is a β-lactam antibiotic which has been shown to increase brain GLT1/EAAT2 expression and activity and to induce neuroprotection in models of ischemic injury and motor neuron degeneration by protecting from excitotoxicity [Rothstein *et al.*, 2005; Lipski *et al.*, 2007]. This compound may exert its protective function acting on GLT1 promoter and increasing its expression. Actually, western blot analysis proved that a 5-day incubation with CEF induced a 100% increase in GLT1 expression also in βTC3 cells, reaching saturation already at 10 µM (Fig. 9A,B). A representative blot is shown in figure 9A

whereas band quantification by densitometry was performed on three independent experiments using Actin as an internal standard (Fig. 9B). In those conditions, CEF determined a dose-dependent protection from glutamate-induced toxicity, as shown by MTT experiments carried after a 5-day treatment with glutamate in presence of CEF at different concentrations (Fig. 9C).

Our data show that Ceftriaxone exerts a significant anti-excitotoxic effect on βTC3 cells, probably by increasing GLT1 expression.

3.6 Human islets express GLT1

Given the possible implications in β-cell physiology and pathology, we next validated our findings in humans.

We confirmed the expression of GLT1 in native human tissues by immuno-histochemistry performed on human and monkey pancreatic sections. As shown in Fig. 10A, in both human and monkey (Cercopiths) pancreases anti-GLT1 reactivity was limited to the islets while absent in the exocrine tissue, indicating that GLT1 is a selective target in endocrine tissue. In both species, but even more clearly in the human, GLT1 staining was almost exclusively localized to the cell membrane.

We tested specificity of these staining by incubating human pancreatic sections with a preimmune serum or in the presence of a blocking peptide. In these conditions, islets did not show GLT1 stain, confirming the specificity of the previous results (Fig. 10B).

To rule out cross reactions with other members of the high affinity glutamate transporter family, we stained sections with an anti-EAAC1/EAAT3 or anti-GLAST1/EAAT2 antibodies (Fig. 10C). Also in this case, results were in agreement with a selective staining, indicating also that GLT1 is the only high affinity glutamate transporter expressed in the islet of Langerhans.

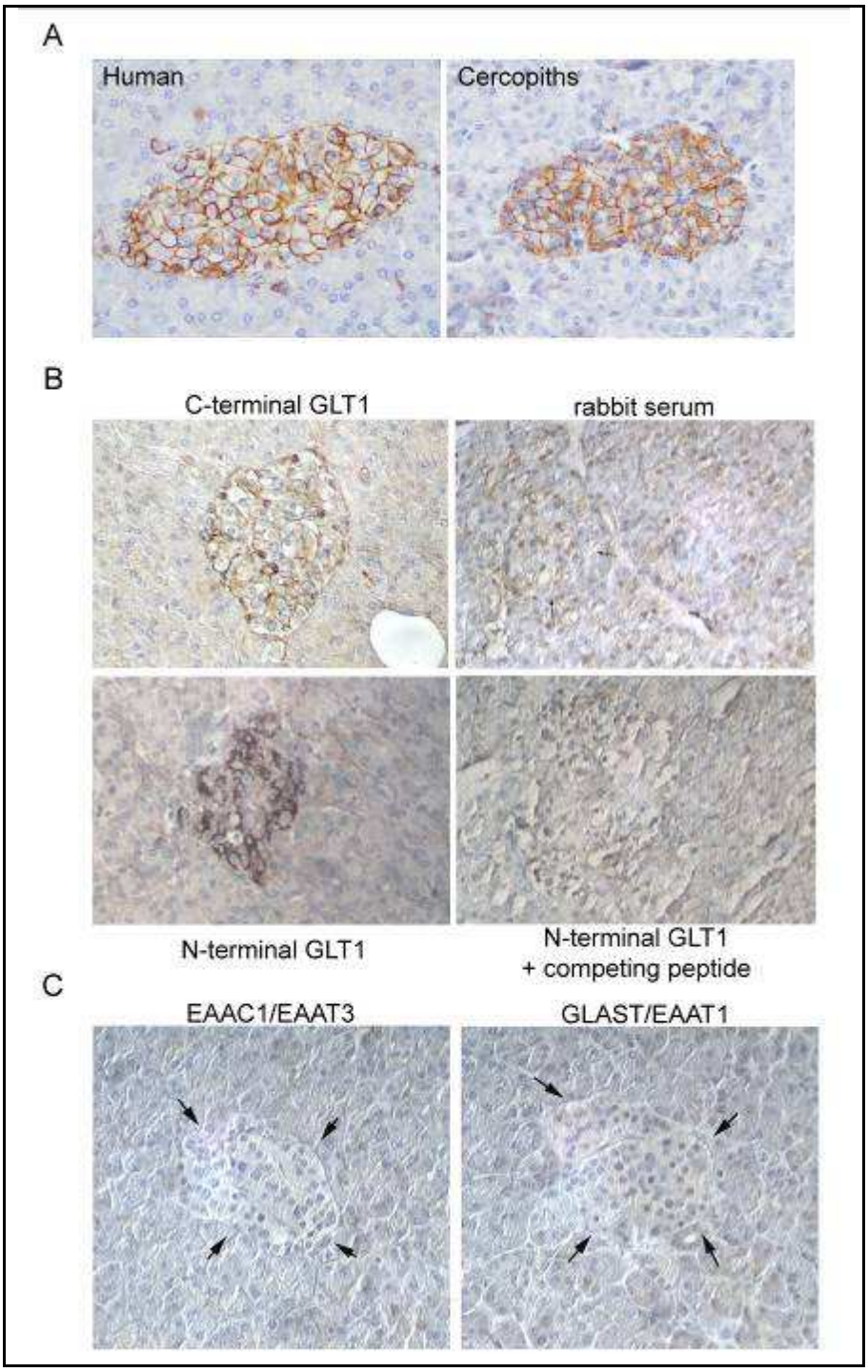

FIG. 10: GLT1 is the only high affinity glutamate transporter expressed in the islet of Langerhans (A). Immunohistochemistry staining of human or cercopiths pancreas sections with a selective rabbit anti-GLT1 antibody. Slides were counterstained with Mayer's haemalum, to stain cytoplasm and nuclei. 40x image magnifications are shown. (B). Human pancreas sections were stained with anti-GLT1 antibodies directed against different epitopes (C-terminal epitope and N-terminal epitope). Staining with the N-terminal anti-GLT1 antibody was prevented by incubation with 10 fold excess of N-terminal blocking peptides. No labelling was detected using a rabbit serum. 40x image magnifications are shown. (C). No reactivity was detected after staining of human pancreas sections with anti-GLAST or anti-EAAC1 antibodies.

3.7 Human islets are vulnerable to excitotoxicity that is exacerbated by GLT1 inhibition

Having established the presence of GLT1 in human tissues, we next tried to confirm our data on glutamate toxicity and GLT1 cytoprotection in human isolated islets, an *in vitro* model system which has been proven useful to explore the mechanisms involved in β-cell death induced by high glucose (glucotoxicity) [Hinoi *et al.*, 2004].

Isolated islets were isolated from human cadaveric donors using the method by Ricordi at San Raffaele Scientific Institute and Ospedale Niguarda Cà Granda. Islets purity was checked and only islets with purity above 80% were used in our experiments.

The first step was to confirm GLT1 expression in the model. By RT-PCR we found that GLT1 mRNA is expressed by purified human islets of Langerhans (Fig. 11A) and immunoprecipitation experiments confirmed protein presence (Fig. 11B). The bands corresponding to oligomer and monomer (120 and 60 kDa, respectively) were clearly identified in the immunoprecipitate and matched perfectly with those of the brain lysate. On the other hand, in the islet lysate, only the monomer was detectable, probably due to the different enrichment in GLT1 between the two samples. A non specific band was also detected in islets samples, but it did not affect our observations, as it was well segregated from the GLT1 bands.

Uptake experiments revealed that islets expressed glutamate transport systems, both sodium dependent and independent, but the former prevailed; we found that GLT1 is functional in the islets and it is the main regulator of glutamate clearance in the islet as shown by the fact that the selective inhibitor DHK almost completely inhibited the sodium dependent glutamate uptake activity (Fig. 11C).

The cellular-specific localisation in the islets was assessed by immunofluorescence experiments performed on isolated islets plated onto glass coverslips and using hormone staining as markers of the different cell subtypes. Staining confirmed the membrane expression of the transporter and showed that GLT1 co-localised with Insulin, as underlined by yellow-orange colour in the merge image (Fig. 11D).

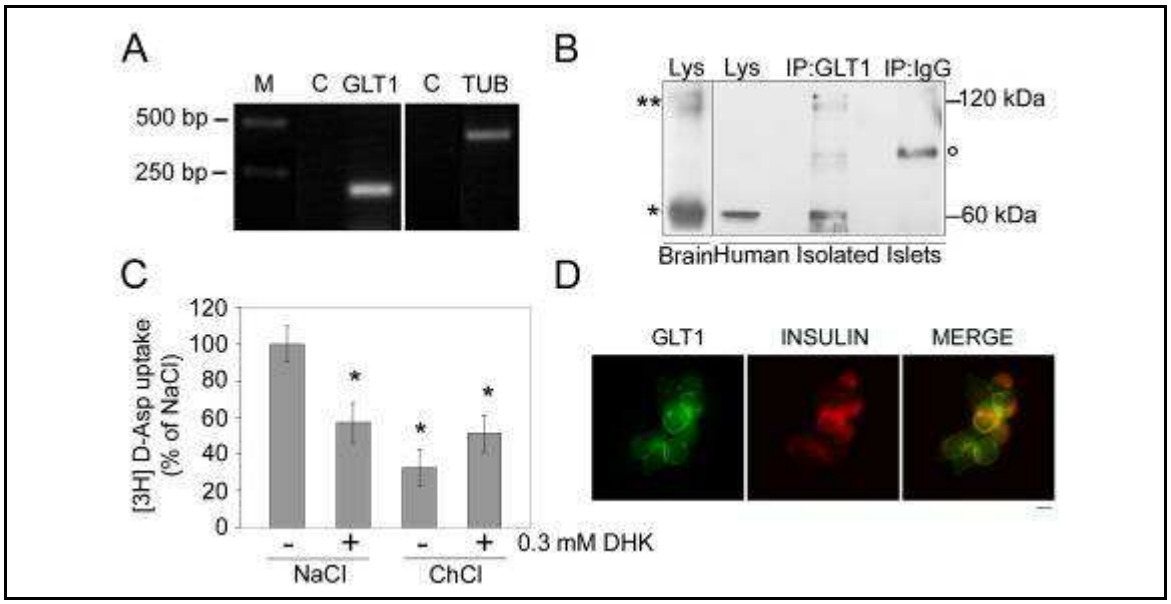

FIG. 11: GLT1 is expressed in human isolated islets (A). Determination of GLT1A expression in human isolated islets by means of RT-PCR. PCR product were analysed on a gel. Negative control "C": PCR amplification performed in the absence of cDNA. Positive control is represented by Tubulin amplification (TUB). Left: DNA Marker (M). (B). Immunoprecipitation of human islets whole lysate extract with rabbit serum (IgG) or GLT1 antibody. 50-100 µg of brain or islet extract (Lys) was loaded in the same gel. Markers on the right indicate kDa; **oligomer; *monomer; ° non-specific band. (C). [^{3}H]-D-Aspartate uptake measurements in 40 human islets. 0.3 mM DHK was added to the uptake solution. Data are expressed as a percentage of the Na-dependent uptake (NaCl) and are mean ± s.e.m. of at least 3 independent experiments performed in triplicate. (*P<0.001, vs NaCl). (D). Immunolocalization of GLT1 (green) and insulin (red) on dispersed human islets seeded on glass coverslips. Scale bar: 10 µm.

Taken together these results indicate that GLT1 is mainly expressed in the beta cells of the isolated human islets of Langerhans, it is functional and it represents the main glutamate uptake system present in the islets.

We next explored the vulnerability of human islets to glutamate toxicity.

As isolated islets are more susceptible to insults and show degenerative features when cultured in vitro, we choose to reduce the time of exposure to glutamate, to avoid the extreme results deriving from excessive islet stress.

After 3-day exposure to glutamate, we assessed human islets viability by MTT test, that was decreased in a dose dependent manner only in the presence of high glucose, and it was statistically significant at 5 mM glutamate (Fig. 12A). These results may indicate an additive effect of glutamate on glucose toxicity. The glutamate-induced islet toxicity, observed in the presence of high glucose, increased further in the presence of 0.1 mM DHK, suggesting that also in the human isolated islets GLT1 is important to prevent glutamate toxicity (Fig. 12B).

By TUNEL assay performed in dispersed islet cells, we observed that the cell death was due to apoptosis and was restricted to the β-cells as shown by double staining for insulin (Fig. 12C).

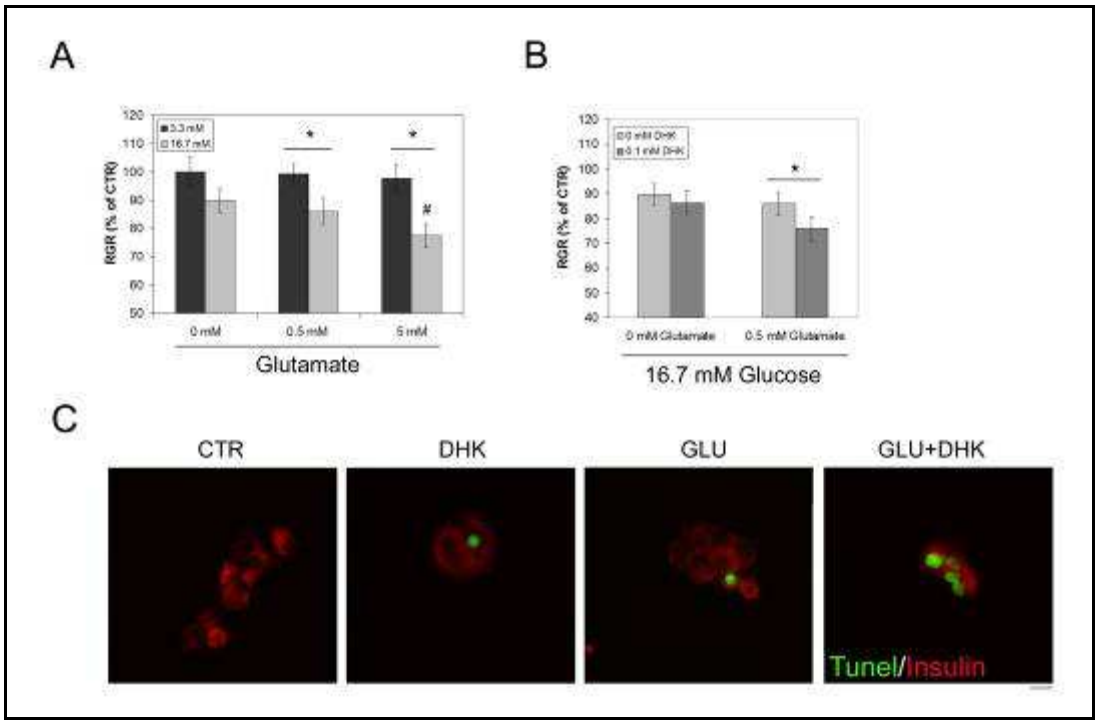

FIG. 12: GLT1 inhibition induces β-cell death by apoptosis in human isolated islets. (A). Determination of β-cell viability by MTT assay after incubation of 20 human islets for 3 days with the indicated glutamate concentrations in the presence of 3.3 mM or 16.7 mM glucose. Data are expressed as RGR and are mean ± s.e.m. n=4 with 8 replicates (*p<0.01 16.7 vs 3.3 mM glucose; #5 mM vs 0 mM glutamate in 16.7 mM glucose). (B). Determination of β-cell viability by MTT assay after incubation for 3 days with the indicated glutamate and DHK concentrations in the presence of 16.7 mM glucose. Data are expressed as RGR and are mean ± s.e.m.; n=4 with 8 replicates (*p<0.01 0.1 mM vs 0 mM DHK). (C). Determination of β-cell apoptosis (green) by TUNEL assay after 3 days incubation with 0.5 mM glutamate (GLU), 0.1 mM DHK (DHK) or both (GLU+DHK) in the presence of 3.3 mM glucose. β-cells were stained with insulin (red). Scale bar = 10 μm.

3.8 Type 2 diabetes patients show impaired GLT1 expression

Type 2 Diabetes Mellitus (T2DM) is characterised by insulin resistance and impaired glucose homeostasis, but in the advanced stage of the disease it is also present an increased beta cell death [Federici *et al.*, 2001]. Glucose represents the exemplary toxic stimuli for beta cells in T2DM, but we have proved that also glutamate can at least in part contribute to beta cell death. Thus, we reasoned that GLT1, as the main system for glutamate clearance in the islets, could be involved in type 2 diabetes.

To verify this hypothesis, we carried out immunohistochemistry experiments on pancreases from normal and type 2 diabetic subjects, aiming at determining whether patients have different GLT1 expression (Fig. 13).

Pancreases from normal individuals confirmed GLT1 expression in the islet and the subcellular localisation was mainly at the cell membrane, as indicated by the brown colour

concentrated at cell-cell boundaries (Fig. 13). In contrast, type 2 diabetic pancreases revealed an altered expression of GLT1. In particular, it is evident that GLT1 is not exclusively expressed at the plasma membrane but it is also present in intracellular compartments, suggesting that the transport activity can be impaired in these patients, as only plasma membrane GLT1 can be functional for glutamate clearance.

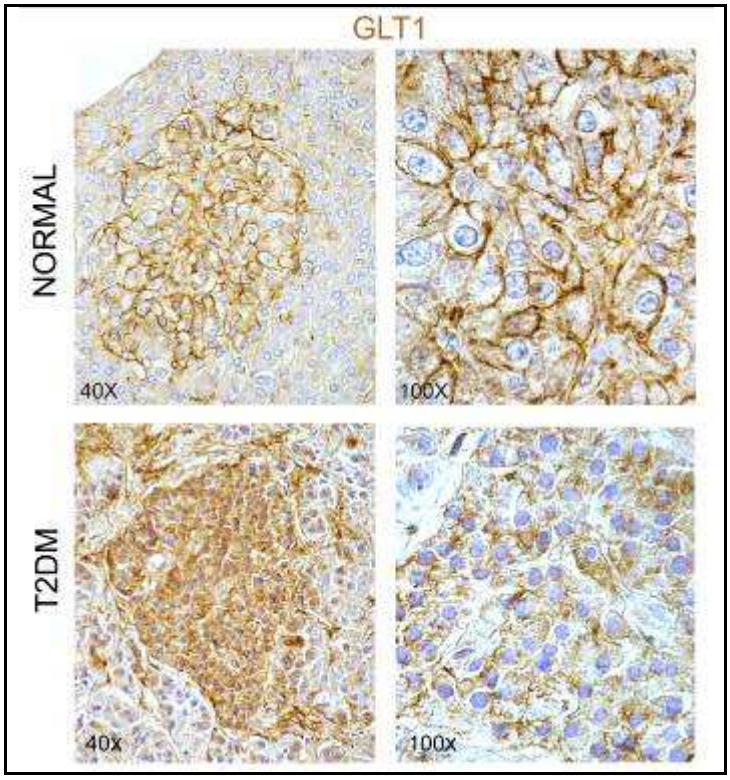

FIG. 13: GLT1 membrane expression is impaired in T2DM pancreases. Immunohistochemistry staining of human pancreas sections from normal subjects and T2DM patients with a selective rabbit anti

4 DISCUSSION

The main finding of our research is that chronic exposure to high glutamate concentrations exerts a cytotoxic effect on the pancreatic β-cells. This effect is physiologically prevented by the activity of the glutamate transporter GLT1.

There are several reasons that prompted us in the study of glutamate and its clearance system in endocrine pancreas.

First, glutamate-mediated signalling as been recognised as a modulator of hormone secretion in the islets, but there were few and incomplete evidences regarding the expression and physiological function of high affinity glutamate transporters. In the CNS, these transporters control the concentration of glutamate in the extracellular space and therefore they limit activation of receptors and prevent the overstimulation of ionotropic receptors, that triggers excitotoxicity. We reasoned that it could be of particular interest studying the effect of glutamate on cell viability in endocrine cells and to understand if glutamate uptake may represent an efficient clearance system in the islets.

Glutamate is present in the islets and in the blood circulation, and it is toxic to neuronal cells, that share common features with endocrine cells [Alpert *et al.*, 1988]. The precise concentration of extracellular glutamate in the islet microenvironment is presently unknown but it is likely remarkably high. Blood glutamate concentration is relatively high (50-200 μM) as compared to the CNS [Federici *et al.*, 2001] and can increase up to ~500 μM under glutamate-enriched diet [Stegink *et al.*, 1987; Graham *et al.*, 2000]. Moreover, islets are highly vascularised structures [Bonner-Weir *et al.*, 1982] and glutamate may reach very high concentrations in islet's capillaries, especially after a meal. In addition, glutamate is physiologically co-secreted with glucagon [Hayashi *et al.*, 2003], thus it is released any time the glucagon is secreted, i.e. during hypoglycaemia. Extracellular glutamate concentrations may increase further in the islets of humans with T2DM which are characterized by inverted β-cell/α-cell ratio and glucagon hypersecretion [Clark *et al.*, 1988; Dunning *et al.*, 2007; Guardado-Mendoza *et al.*, 2009] but also in T1DM islets since dendritic (DC) and T cells release glutamate to activate and improve chemotactic migration [Pacheco *et al.*, 2006; Ganor *et al.*, 2003]. DC-T cell interaction at the early stages of lymphocytic islet infiltration might trigger a vicious cycle that increases islet inflammation and extracellular glutamate levels. Of note, increased glutamate levels have been found in sera of subjects with both T1DM [Oresic *et al.*, 2008] and T2DM [Bao *et al.*, 2009] thus suggesting that glutamate may represent a common factor underlying the two types of diabetes.

We reasoned that if β-cells are exposed to high extracellular glutamate levels they might be vulnerable to excitotoxicity, unless they also express an efficient glutamate clearance system.

Actually, we demonstrate that clonal β-cells, whereas not α-cells, are susceptible to glutamate-induced toxicity, that mediates apoptosis. The different sensitivity of the two cell types may be ascribable to different receptors and signalling pathways in alpha and beta cells, as the two cell types express different subtypes of ionotropic and metabotropic glutamate receptors [Hayashi *et al.*, 2003; Molnár *et al.*, 1995; Weaver *et al.*, 1996; Tong *et al.*, 2002].

It is not surprising that glutamate mediated toxicity does not occur at short incubation time, as happens in the CNS, because pancreatic cells are more frequently exposed to high levels of glutamate and may have developed a sort of glutamate resistance, necessary for their survival.

In beta cells, glutamate toxicity is mediated by the activation of the ionotropic AMPA/kainate glutamate receptors, as inhibition of these receptors significantly improved β-cell survival. On the contrary, we did not measured any change in beta cell survival during NMDA receptors inhibition. In line with this finding, we did not detect NMDA expression in our cell model (data not shown).

Nonetheless, glutamate receptor inhibitors did not completely prevent β-cell death, suggesting that, other mechanisms might be involved in glutamate-induced toxicity, as the oxidative glutamate toxicity. Originally described in astrocytes, excess of extracellular glutamate reverts the activity of the glutamate/cystine-antiporter system x^{-c}, thus depleting the cells of cysteine, a building block of the antioxidant glutathione [Chen *et al.*, 2000]. A similar mechanism can be relevant for β-cells which express the x^{-c} exchanger (Fig.3B, inset) and are particularly vulnerable to oxidative stress [Numazawa *et al.*, 2008]. In agreement with this possibility, we measured reduced free SH content after incubation with glutamate (Fig.3B). Oxidative stress can at least in part explain the need of chronic glutamate exposure to observe changes in beta cell viability, in contrast to the acute glutamate action in neurons.

Glutamate-induced toxicity also affects human islets (Fig. 12), but it occurs only in the presence of high glucose and is quantitatively small as islets viability decreases of only 10%. However, these data most likely underestimate the real impact of excitotoxicity in human β-cells because isolated islets preparations are an heterogeneous cell population including endocrine non-β and contaminant non-endocrine cells which are resistant to glutamate toxicity. Indeed, the TUNEL assay performed on dispersed human islet cells stained for insulin showed a considerably higher number of apoptotic β-cells, because we were able to distinguish among the cell types present in the islets (Fig. 12C).

Alike in neurons, also in the β-cells, glutamate-induced toxicity is physiologically prevented by GLT1 activity. By using molecular, biochemical, pharmacological and physiological approaches we found that GLT1 is selectively expressed by the β-cells, while it is virtually absent in all the other islet cell types. Immunohistochemistry and confocal microscopy of human pancreatic sections showed that GLT1 is prevalently located to the plasma membrane [Tesi di Laurea Magistrale-Di Cairano, 2007 and present data]. None of the other known high affinity glutamate transporter subtypes (EAAT1/GLAST, EAAT3/EAAC1) were detected in the islets. Our observation that GLT1 is selectively expressed in the β-cell contrasts with previous data showing a Na^+-dependent glutamate/aspartate transport activity confined to the non-β islet's cell mantle [Weaver *et al.*, 1998]. This discrepancy may be explained by considering that in the latter study the localization of the transporter's substrate (glutamate), and not the transporter itself was assessed. In agreement with the lack of GLT1 expression, we did not detect measurable Na^+-dependent glutamate uptake in αTC1 cells, neither we revealed the presence of any other high affinity glutamate transporter by RT-PCR [Tesi di Laurea Magistrale-Di Cairano, 2007]. Interestingly, our findings and recent results indicate that α-cells express a functional glutamine uptake system (ASCT2 and SAT2) and a phosphate-dependent glutaminase activity suggesting that glutamate in these cells may prevalently derive by glutamine transamination [Montero *et al.*, 2007].

In this study, GLT1 was identified as the main regulator of the extracellular glutamate clearance in the islet, and it is demonstrated that its normal function is critical for β-cell survival. Indeed, GLT1 down-regulation by pharmacological blockade or by shRNA interference is sufficient to determine β-cell death also in normal physiological growing conditions. The most important function of GLT1 in the CNS is to regulate the extracellular glutamate concentration and to prevent excessive glutamate receptor activation and excitotoxicity [Rothstein *et al.*, 1996; Tanaka *et al.*, 1997]. Accordingly, data show that pharmacological blockade of GLT1 by the selective inhibitor DHK, induces an increase in extracellular glutamate concentration to a degree which is toxic for the β-cells. *Vice versa* GLT1 upregulation by CEF, a β-lattamic known for its ability to increase GLT1 expression in neurons and to provide neuroprotection against excitotoxicity, increased GLT1 expression also in beta cells and therefore reduced glutamate-induced β-cell death (Fig. 9).

Thus, CEF, and other compounds capable to increase GLT1 expression or/and activity may represent novel therapeutic strategies to achieve β-cell cytoprotection. Interestingly, β-cell toxicity could be also prevented by Ex-4, a GLP1 analogue employed for the treatment of

T2DM and known for its glucose-dependent insulinogenic action and its antiapoptotic effect on the β-cell [Wajchenberg *et al.*, 2007]. Thus, the anti-excitotoxic action mediated by Ex-4 provides a novel mechanism through which it may exert its known β-cell cytoprotective effect.

Because of GLT1 selective localization in insulin positive cells and its involvement in β-cell survival, we reasoned that it could be involved in diabetes. In particular we hypothesised that its expression may be altered in patients with diabetes, a disease characterised by beta cell death and impaired glutamate homeostasis. The time of beta cell death appearance is different in the two types of diabetes: it is generally recognised that beta cell death occurs before the onset of impaired glucose homeostasis in type 1 diabetes, whereas in type 2, it characterizes later stage of the disease.

Our data show that GLT1 subcellular localisation is altered in pancreases of type 2 diabetes patients. In particular, the plasma membrane localisation of GLT1 is lost and the transporter seems to accumulate in intracellular compartments, where it can not accomplish to its uptake function.

Although these evidence need further support, they suggest that the transporter may play a role in T2DM progression.

The hypothesis to be confirmed is that glutamate homeostasis can be altered in islets of diabetic patients. Glutamate clearance may be low, due to an increased beta cell death or the impaired GLT1 localisation, whereas the high alpha/beta cell ratio could lead to an increase in glutamate release from alpha cells, thereby exacerbating islet conditions.

In conclusion, our findings demonstrate that β-cells are sensitive to glutamate-induced cytotoxicity and the high affinity glutamate transporter GLT1 is fundamental to physiologically prevent β-cell death. These results may be of particular interest, as GLT1 plasma membrane localisation seems altered in type 2 diabetes patients.

Any novel indication in the islet physiology could be potentially interesting for application in diabetes, one of the more widespread pathology and cause of morbidity in the population of occidental countries. In particular, could be remarkable to find elements controlling hormone release and beta cell survival, as the common notes between the two types of diabetes are represented by beta cell death and impaired glucose homeostasis.

5 MATERIAL AND METHODS

5.1 Cell lines

Cell lines represent a useful model to isolate and study the peculiar characteristic of each cell type present in the islet of Langerhans. Moreover, they can be grown in vitro for a considerable number of passages, maintaining their characteristic and their similarity to the native cells. However they can not be the only model for this work as they differ from the physiological conditions, where exist interaction and cross-talk between all the cell types in the islet.

Mouse βTC3 and αTC1 cells were kindly provided by Prof. Douglas Hanahan (Department of Biochemistry and Biophysics, University of California, San Francisco, CA). αTC1 and βTC3 cells derive from pancreas of transgenic mice generated with a fusion gene between SV40 large T antigen and glucagon and insulin promoters, respectively [Powers 1990; Efrat 1988].

ßTC3 were grown in RPMI 1640 (from the name of the institute where the media was developed: Roswell Park Memorial Institute) 11 mM glucose supplemented with 10% heat inactivated foetal bovine serum, 2 mM L-glutamine, and 100 IU/ml streptomycin/penicillin.

αTC1 were cultured in DMEM (Dulbecco's Modified Eagle's Medium) 25 mM glucose supplemented with 10% heat inactivated foetal bovine serum, 2 mM glutamine, and 100 IU/ml streptomycin/penicillin.

Cultures were performed under standard humidified conditions of 5% CO_2 at 37° C.

All media were supplied by Sigma-Aldrich.

5.2 Cell viability assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

MTT is a water soluble tetrazolium salt, with a yellow colour. When added in the cell media, active mitochondrial dehydrogenases of living cells convert the MTT in an insoluble purple formazan by cleavage of the tetrazolium ring; in contrast, death cells do not cause this change. Thus, this method is useful to measure cytotoxicity, as previously demonstrated [Carmichael *et al.*, 1987].

The MTT assay was performed on ßTC$_3$ cells seeded at a density of 8 x 10^3 cells/well onto 96-well culture plates. Cells were allowed to attach and grow for 24 h in standard medium, then medium was replaced with fresh medium containing glutamate, DHK, Ceftriaxone, Exendin 4, DPPIV and glutamate receptor inhibitors APV and CNQX at the indicated concentrations. If not differently stated, after five days incubation, cell viability was assessed

using the MTT method, according to the manufacturer's protocols. Briefly, MTT solution was added to the media in each well, to a final concentration of 0,5 mg/ml; after a 4 hour incubation, the media was removed and the formazan precipitates were solubilised in 100% DMSO. The coloured formazan product was determined spectrophotometrically at 540 nm, with a reference wavelength of 655 nm, yielding a function of the formazan concentration. DMSO absorbance in empty wells was taken as a blank and subtracted from each well.
Data were analysed as percentage of viability relative to control treatment (%RGR, Relative Growth Rate). Results are shown for a at least three independent experiments, performed with eight replicates.
All reagents are from Sigma Aldrich.

5.3 Quantification of apoptosis in β-cells and isolated human islets

The apoptosis of human isolated islets and βTC3 cells was estimated using the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labelling assay (TUNEL, Promega). This method, measures nuclear DNA fragmentation, an important biochemical hallmark of apoptosis. Fragmented DNA is enzymatically labelled at 3'-OH end with FITC (Fluorescein IsoTioCyanate) conjugated nucleotides.
Cells and islets were plated on glass coverslips and, following each indicated treatment, they were fixed in 100% methanol, washed in PBS (150 mM NaCl, 10 mM PO_4 buffer pH 7.4), permeabilized in PBS-0,2% Triton X-100 and incubated with Equilibration Buffer (200 mM Potassium Cacodylate pH 6.6, 25 mM TrisHCl pH 6.6, 0.2 mM DTT, 0.25 mg/ml BSA, 2.5 mM Cobalt chloride). Then the coverslips were incubated for 1 hour at 37°C with terminal deoxynucleotidyltransferase (rTdT) in the presence of FITC labelled dUTPs. After abundant washes, cell were incubated with Propidium Iodide (1 μg/ml) or with insulin antibodies (see immunofluorescence protocol for details), as a positive control and as a marker for beta cells, respectively. Tunel-positive cells were counted by two independent observers using a 40X objective from at least 40 randomly selected fields per coverslips. The data were plotted as number of TUNEL positive cells/field.

5.4 Quantification of total SH content

To evaluate the redox conditions in the βTC3 cells after treatment with glutamate, we performed Ellmann test, in which quantification of SH groups is related to protein oxidative stress.

Cells were incubated for 5 days in 5 mM Glutamate and then lysed for 45 minutes in lysis solution (150 mM NaCl, 30 mM Tris-HCl, 1 mM $MgCl_2$, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, and 1 µg/ml aprotinin and leupeptin). Cleared lysate was then incubated in reaction buffer (0.1 M sodium phosphate, pH 8; 1 mM EDTA) and DTNB (5'5'-dithiobis (2-nitrobenzoic acid); stock solution 4 mg/ml) at room temperature. This reagent is able to react with SH groups, resulting in colour variation from colourless to yellow, depending on SH concentration. Absorbance at 412 nm was measured after 30 minutes. Lysis buffer absorbance was taken as a blank and subtracted from each sample. Standard curve: 0-0.5 mM reduced GSH. Data deriving from interpolation are presented as a ratio between total SH and total protein in the sample.

5.5 RNA isolation and RT-PCR analysis

To assess transporter expression in our models, we performed RNA isolation and RT-PCR analysis.

Total RNA represents about 1% of the total weight of mammal cells and is composed of coding RNA, i.e. tRNA, rRNA and mRNA, and non-coding RNA, which represents the major amount. We are interested in mRNA, because it can be taken as an indicator of gene expression.

TOTAL RNA EXTRACTION

Beta TC3 were grown in a 6 cm plate until confluence or 1500 isolated human islets of Langerhans, from post-mortem sampling were harvested.

Total RNA was extracted with RNA fast isolation system following manufacturer's protocol (Molecular Systems-San Diego, CA). This reagent is composed of guanidine salts and urea as denaturating agents, phenol to selectively isolate total RNA from chromosomal DNA.

The system consists mainly of distinct phases: cell/islets homogenisation, followed by RNA extraction, using chloroform and centrifugation and finally RNA precipitation by adding isopropanol. After over night RNA precipitation, pellet was washed twice with 75% Ethanol and resuspended in 15 µl of UltraPure™ DNase/RNase-Free Distilled Water (GIBCO, Invitrogen) to obtain adequate concentration for subsequent reactions.

Quality of total RNA wash checked by electrophoresis and its concentration was measured using spectrophotometric absorbance (260-280 nm) and a molar extinction coefficient of 40 µg/ml. The RNA purity was confirmed by the relative absorbance at 260 versus 280 nm.

DNASE DIGESTION

This step was useful to remove any DNA contamination from our RNA extract.

We treated 2 µg of total RNA with DNAse (Promega) and its suitable buffer in presence of RNAse OUT (Invitrogen) for 30 minutes at 37°C. The reaction was stopped adding Stop Solution (Promega) and followed by 10 minutes incubation at 65°C to inactivate the enzyme.

RT-PCR

For cDNA synthesis, 2 µg of digested RNA was reverse-transcribed using random primers (to a final concentration 12.5 ng/µl; Promega) and 200 U of M-MLU reverse transcriptase (Invitrogen) in presence of RNAse OUT, DTT 0,1 M (Invitrogen) and dNTPs (Promega).

The first reaction step was performed with RNA, oligos and UltraPure™ DNase/RNase-Free Distilled Water (GIBCO, Invitrogen) at 65°C for 5 minutes; then the other reagents were added and the reaction was incubated for 50 minutes at 37°C. Finally the reaction was led to 95°C for 5 minutes to inactivate the reverse transcriptase.

PCR

PCR amplification of the reverse-transcribed RNA was carried out using specific primers in the 3' end of cDNA, to obtain about 200 bp PCR product, crossing the STOP codon. The reason why we choose to have a short fragment is to have more possibility to check the expression of the gene products. Indeed, bad quality RNA can be really short, thus impairing the annealing of the forward primers even if the gene has been transcribed, therefore generating false negatives. Choosing primers near each other increases the probability to have a PCR product. The primers were selected with a melting temperature near 60 °C and keeping G/C near 40% of the whole primer.

In mouse cell lines we used the following primer:

Mouse		Primer	PCR product
xCT	Forw Rev	CCCAGATATGCATCGTCCTT CGTCTGAACCACTTGGGTTT	207

The primers used in human islet of Langerhans are listed below:

Human		Primer	PCR product
GLT1-A	Forw Rev	CTTTTGGGGCTGGGATAGTC TTGGCTGCCAGAGTTACCTT	211
Tubulin	Forw Rev	CCTCACCATTGCCATTATCC GCTTCCACTTTCACCTCAGC	451

Reaction was performed with PCR Master Mix (Promega) adding cDNA, oligos and water to a final volume of 25 µl. Cycling conditions were 2 minutes at 95°C; 30 seconds at 95°C, 30 seconds at 60°C, 1 minute at 72°C, for 40 cycles; and a final elongation at 72°C for 10 minutes. To confirm absence of genomic contamination in the RNA samples, reverse transcriptase-negative controls were introduced in each experiment (no M-MLU reverse transcriptase).
Amplified DNA fragments were analyzed by electrophoresis in a 1.5% agarose gel and compared to a 250 bp ladder (Invitrogen).

5.6 Cell lysis and Western blotting analysis

ßTC$_3$ cells were seeded onto 6-cm tissue culture plates and allowed to attach and grow until confluence. Cells or 1500 isolated human islets were harvested and lysed in 100 µl lysis buffer (150 mM NaCl, 30 mM Tris-HCl, 1 mM $MgCl_2$, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, and 1 µg/ml aprotinin and leupeptin). After 1 h at 4° C, lysates were centrifuged at 13000 rpm for 10 min, the extracted proteins were solubilised with denaurating β-mix (5% SDS, 20% Glycerol, 0.3M β-mercaptoethanol, blue bromophenol) and separated by SDS-PAGE (Tris-Gly/ SDS buffer: 25 mM Tris-Base, 192 mM Glycine, 0.1% SDS). Finally, proteins were transferred to a nitrocellulose membrane (Transfer buffer: 25 mM Tris-Base, 192 mM Glycine, 20% Methanol). After membrane incubation with blocking buffer (2% non-fat milk, 0.1% Tween 20, 20 mM tris HCl pH 7, 150 mM NaCl), the blots were probed with rabbit anti-GLT1 (Alpha Diagnostic), an affinity purified rabbit anti-GLT1 (kindly provided by Dr. Grazia Pietrini [Perego *et al.*, 2000]) or anti-actin (Sigma) antibodies as a primary reagents in the blocking solution. This incubation was followed by anti-rabbit HRP-conjugated IgG (80 ng/ml; Amersham, GE Healthcare), and visualised by ECL (Perkin-Elmer Life Science, Boston, MA).
Band quantification was performed using NIH J-image software and results were normalised for actin content and shown in bar graphs.

P2 brain extract: Total homogenates of rat brain tissues were prepared as previously described [Perego *et al.*, 2000]. Briefly, rat cortex were suspended and homogenised mechanically in Homogenisation buffer (0.32 M Saccarose, 10 mM Tris-HCl pH 7.5, 5 mM EDTA, 5 mM EGTA and fresh protease inhibitors as above). After multiple centrifuge steps and a ultracentrifugation, the final pellet (called P2) was resuspended in 5 volumes of lysis buffer (RIPA buffer: 150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1% NP-40, 0.5%

Deoxycholate, 0.05% SDS and freshly prepared protease inhibitors) and incubated under constant agitation at 4°C for 1 hour. The cleared lysate was diluted 1:1 with the denaturating β-mix (5% SDS, 20% Glycerol, 0.3M β-mercaptoethanol, blue bromophenol).

5.7 iRNA and Cell transfection

To suppress GLT1 gene expression, the retroviral silencing plasmid (pRS) encoding short hairpin against GLT1(Origene, see Table below) was transfected in βTC3 cells. The short hairpin SH1, SH2 and SH3 are against GLT1, targeting different portions of the transcribed. The SH C (control) contain a non-effective shRNA cassette.

Sequences of the tree short hairpin against GLT1 are listed in the table below:

Name	5'-3' Sequence
SH1	GGATGGAGGACAGATTGTGACTGTAAGCC
SH2	ATCAAGGACTTAGAAGTGGTTGCTAGGCA
SH3	GGTGTATTACATGTCCACGACCATCATTG

The shRNA expression cassette consist of 29 bp target gene specific sequence, a 7 bp loop, another 29 bp reverse complementary sequence and a termination sequence polyT, to terminate the transcription by RNA polymerase (Fig. 1).

Once the plasmid is inserted into the cells, the short hairpin RNA is expressed as a RNA duplex, thus achieving RNA interference and inhibiting GLT1 expression.

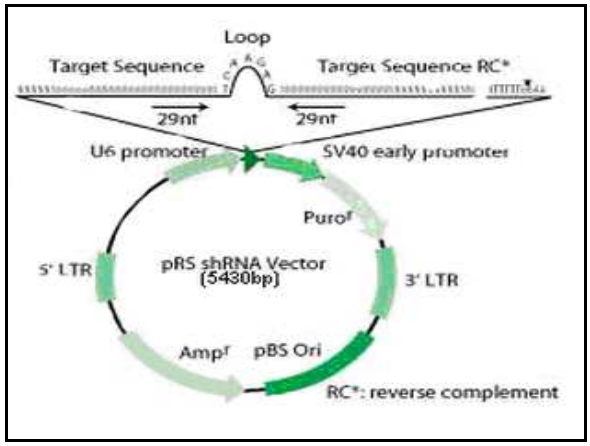

FIG. 1

10^5 or 3 x 10^5 βTC3 cells were plated into 24 well plates and 3.5 diameter Petri's plates, respectively. After 24 hours, cells were transfected with pRS-SH Ctr / 1 / 3 using Lipofast Reagent (Promega).

A mixture of DNA (pRS-SH C /1/3) and Lipofast Reagent was prepared in a serum free media (RPMI supplemented only with 1% glutamine):

Well	DNA	Lipofast Reagent	1%Gln RPMI
24-well plates, each well	1 µg	3 µl	0.2 ml
3.5 cm Petri dishes	5 µg	15 µl	1 ml

Cells were washed twice in the serum free media and then the mixture was added. After 4 hours of incubation, media was substituted with complete RPMI.

24 hours later, cells were assessed by ^{3}[H]-Asp uptake experiments or were lysed to verify GLT1 down-regulation.

After the silencing has been verified, the silencing experiments were performed on βTC3 plated on glass coverslips in 3.5 cm Petri dishes. Transfection was followed by a 2 day treatment in standard complete medium supplemented with 0.5 mM glutamate and analysed by TUNEL (Promega).

5.8 Immunofluorescence

Immunofluorescence technique is a powerful tool for the identification and localisation of transporters in cells and tissues, by means of antibodies conjugated to fluorescent dyes. Antibodies allow to recognise each type of protein in the sample, due to their specificity and selectivity. On the other hand, fluorescent dyes let to display proteins through fluorescence microscopy.

In isolated islets

20 hand-picked islets were seeded onto glass coverslips covered with polylisine (Sigma-Aldrich) to facilitate adhesion and cultured in RPMI medium. After 24 hours islets were fixed in ice-cold 100% methanol and immunostained with anti-GLT1 and anti insulin antibodies, as follows.

Fixed cells were then incubated with primary antibody for 2 hours at room temperature in GDB solution (150 mM NaCl, 10 mM Phosphate Buffer pH 7.4, 0.2% Triton, 0.2% gelatine). Following incubation, cells were washed in PBS and then incubated in GDB with the appropriate fluorochrome-conjugated secondary antibodies (Jackson) for 1 hour at room temperature, in dark. Then, coverslips were mounted on glass slides with Phenilendiammine (1 mg/ml in Glycerol-PBS; Sigma-Aldrich) as antifade reagent and sealed with nail-polish.

5.9 Immunohistochemistry

Immunohistochemistry has been performed in formalin fixed human pancreas paraffin embedded sections.

The ABC immune complex served for the identification of GLT1. After removal of paraffin and rehydratation of tissue, the pancreas sections were first treated with an hydrogen peroxide solution to suppress possible endogenous peroxidase activity and then heated in citrate buffer 10 mM pH 6 using a microwave oven to expose antigens. This was followed by permeabilization with TBS-Triton 0,2% (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 0.2% Triton X-100) and by an incubation with normal serum to quench nonspecific protein binding; finally the sections were incubated with a primary antibody, o/n at 4°C. Unbound antibodies were washed with TBS-Triton 0,2% and then the signal was amplified using secondary antibodies biotin conjugated, incubated at room temperature for 2 hours, followed by an incubation with Peroxidase conjugated-streptavidin (Chemicon). The reaction was performed with freshly activated 40% DAB (Diaminobenzidine, Sigma-Aldrich). The colour development was stopped by washing the slides thoroughly in tap water. To stain cytoplasm and nuclei, the sections were then counterstained with Mayer's haemalum, turned with tap water and dishydratated. Coverslips were mounted with an hydrophobic mounting medium (Dako Corp.).

5.10 Antibodies

The followed primary antibodies were used: rabbit anti-GLT1 (Perego *et al.*, 2000; and Alpha Diagnostic); rabbit anti-EAAC1 (Alpha Diagnostic); rabbit anti-GLAST (Alpha Diagnostic); guinea pig anti-insulin (Roche). Secondary antibodies: biotin-conjugated anti-rabbit IgG; Rhodamine-conjugated anti guinea pig (Jackson Immunoresearch: West Grove, PA); Peroxidase conjugated-streptavidin (Chemicon).

Incubation with GLT1 competing peptide (Alpha Diagnostic): 20 µg of competing peptide+ 2 µg of GLT1 antibody were mixed in TBS and incubated over night at 4°C under constant agitation. The following day, the mixture was used to stain pancreatic section.

Negative control: rabbit pre-immune serum [Perego *et al.*, 2000] and rabbit normal serum (Sigma-Aldrich).

5.11 [^{3}H]D-aspartic acid uptake

Radioactive amino acid uptake is a reliable method to verify the expression and the functionality of transporters expressed at the plasma membrane. Changes in amino acid uptake can either reflect changes in amount of expression and either changes in transporter activity.

150.000 cells/well were plated in a 24-wells plate and grown until confluence. Cells were incubated for 10 minutes in 200 µl of Na^+-dependent (150 mM NaCl 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10mM Hepes pH 7.5) or Na^+-independent (150 mM ChCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10mM Hepes pH 7.5) uptake solution containing 5 µCi/ml of [^{3}H]D-Aspartic acid (specific activity 37 Ci/mmol; Amersham Biosciences). The amino acid uptake was stopped by washing the cells twice in ice-cold sodium-free solution. Cells were dissolved in 150 µl of SDS 1% for liquid scintillation counting. For transport inhibition, DHK was added to the uptake solution at the indicated concentrations. For uptake measurements in human isolated islets, 20 islets were selected and uptake experiments were performed as described.

5.12 Human islet isolation and culture

The islets used in this study were kindly provided by San Raffaele Scientific Institute and Ospedale Niguarda Ca' Granda. Islets were isolated from seven cadaveric multiorgan donors by using the procedure already described [Ricordi *et al.*, 1988] in conformity to the ethical requirements approved by the Niguarda Ca' Granda Ethical Board and HS Raffaele.

When necessary, islets were cultured in RPMI 1640 media, 5.5 mM glucose, supplemented with 10% heat inactivated foetal bovine serum, 2 mM L-glutamine, and 100 IU/ml streptomycin/penicillin. To mimic a high glucose condition, cells were cultured in 16.7 mM glucose, whereas 3.3 mM glucose was used as a low glucose condition, as described [Marchetti *et al.*, 2002].

5.13 Immunoprecipitation

2500 isolated islets were harvested and resuspended in 300 µl of RIPA buffer without SDS (150 mM NaCl, 50 mM Tris/HCl, pH 7.6, 1 mM EDTA, 1% NP-40, 0.5% deoxycholate) supplemented with 100 µg/ml PMSF and a cocktail of protease inhibitors. After 6 minutes of sonication, islets were lysed for 1 h at 4°C under constant agitation. The lysate was then cleared by centrifugation and 100 µg of the total lysates were incubated over night with anti-

GLT1 rabbit antibody (kindly provided by Dr. Grazia Pietrini) or a preimmune rabbit serum as a negative control. The following day, samples were subjected to immunoprecipitation incubating 20 µl of Protein A-Sepharose conjugate (Pierce) for 2 hours at 4°C under constant agitation. Then, Sepharose was washed once with RIPA buffer, twice with Buffer 4 (50 mM Tris-HCl, pH 8) and dried using a Pasteur Pipette. Proteins were solubilised by adding the denaturating β-mix (5% SDS, 20% Glycerol, 0.3M β-mercaptoethanol, blue bromophenol).

5.14 Glutamate Determination

To assess GLT1 effect on extracellular glutamate, cells were treated with DHK and then we measured glutamate concentration in the medium by enzymatic assay (Glutamate-Glutamine Determination Kit; Sigma-Aldrich). The reaction consists in dehydrogenation of L-glutamate to α-ketoglutarate, accompanied by a reduction of NAD^+ to NADH.

The βTC3 cells were plated on 6-cm Petri dishes, grown in standard medium for 24 hours and then treated for 5 days with 0.1 mM DHK (Sigma-Aldrich). Cell media from treated and control plates were collected and used to assess glutamate concentration following the manufacturer's protocols. Essentially, media or standard solutions were incubated 1 hour at room temperature in the presence of Tris-EDTA Hydrazine Buffer, 1.5 mM NAD, 0.5 mM ADP and GLDH 0.1 U/ml. The standard curve was carried out from 0 to 1 mM glutamate. The conversion of NAD+ to NADH is measured spectrophotometrically at 340 nm and is proportional to the amount of glutamate.

5.15 Statistical Analysis

Statistical significance of difference between groups was determined by unpaired Student's *t*-test. Differences were considered significant at $P \leq 0.05$.

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CHAPTER IV: Yeast Two Hybrid Screening

The experimental work presented in this chapter was performed under the supervision of Franco Folli MD PhD, Professor of Medicine and Director of Metabolic and Molecular Research, at the Diabetes Division, Dept. of Medicine, University of Texas Health Science Center at San Antonio (UTHSCSA), located at San Antonio, TX-USA. This work is part of and was supported by NIH grant RO1 DK080148 and UTHSCSA start-up funds, to Franco Folli.
I thank also Lily Dong PhD, Associate Professor of Cellular and Structural Biology, UTHSCSA, for kindly providing the library employed in the screening and helpful advice throughout this work.

The period spent at UTHSCSA, offered me the opportunity to learn a technique useful to study protein-protein interactions, that may be relevant to unravel the proteins interacting with glutamate transporters. Yeast two hybrid screening has first been described in 1989 [Fields and Song, 1989], and now it is a consolidated tool to identify proteins involved in direct interactions in a high-throughput scale.

1 ABSTRACT

IAPP (Islet Amyloid Pancreatic Polipeptide) is the major constituent of pancreatic amyloid deposits, which correlate with β cells apoptosis and α cells replication in Type 2 Diabetes Mellitus (T2DM). Abnormal IAPP processing, folding and secretion could contribute to the formation of islet amyloidosis (IA). In this kind of aggregates, protein-protein interaction could play a key role in the folding and aggregation pattern and therefore in the severity IA.
Aim of the work is to find out which proteins can interact with hIAPP, by means of Yeast Two Hybrid screening from a brain library.

2 INTRODUCTION

2.1 Protein misfolding and diseases

Many human diseases arise from the conversion of specific peptides or proteins, from soluble forms, their native functional conformational state, to highly ordered fibrillar aggregates. In general, these pathological conditions are defined as protein misfolding diseases and may include neurodegenerative diseases, such as Alzheimer and Parkinson, or non-neuropathic diseases both systemic or localised, as Type II Diabetes Mellitus.

The impairment in folding efficiency may result in a loss of function linked to the reduction in the quantity of the protein available to play its normal role. However, the largest group of misfolding diseases is associated with the presence of insoluble fibrillar aggregates.

For most of the pathologies related with any of the amyloid disease, it is known the predominant component of the deposits, that forms the core, but also additional associated species are present [Hirschfield *et al.*, 2003; Alexandrescu *et al.*, 2005].

It is generally recognized that amyloid formation shows a nucleated growth mechanism [Pedersen *et al.*, 2004].

Polypeptide chains can adopt a multitude of conformational states and interconvert between them on a wide range of timescales and not all the conformations are in the form of insoluble aggregates (Fig.1).

The protein sequence influences the relative stabilities of all conformational states and will thereby contribute to the susceptibility of a given polypeptide chain to convert into amyloid fibrils. Also hydrophobicity, charge and secondary structures can strongly influence amyloid formation.

It has been hypothesized that misfolded oligomers may interact with cellular components, such as membranes, small metabolites, proteins or other macromolecules. Such events, may in turn lead to the malfunctioning of crucial aspects of cellular machinery.

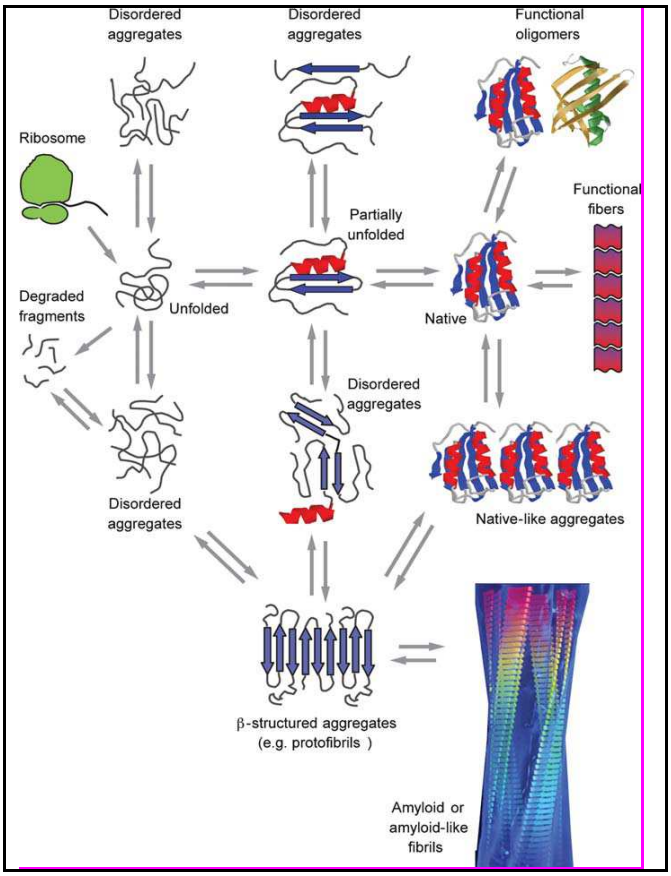


FIG. 1: Schematic representation of some conformational states that can be adopted by polypeptide chains and of the possible interconversion passages. [Chiti and Dobson, 2006]

2.2 Type II diabetes mellitus and amyloid

Type II Diabetes Mellitus (T2DM) is a highly prevalent metabolic disorder, characterized by impaired glucose homeostasis. It is generally recognised that both insulin resistance and reduced mass of insulin producing beta cells contribute to the disease.

In T2DM, β cells apoptosis correlates with the presence of Islet Amyloidosis (IA), a term created to describe pancreatic amyloid deposits in the islets of Langerhans. Amyloid deposits have been observed in both the cytoplasm of beta cells and extracellular space [Guardado-Mendoza *et al.*, 2009; Zucker-Franklin and Franklin, 1969] and they are mainly composed of Islet Amyloid Pancreatic Polypeptide (IAPP or amylin) [Westermark *et al.*, 1987; Cooper *et al.*, 1987].

IAPP is a 37-amino acid polypeptide hormone of the calcitonin family, localised and cosecreted with insulin in secretory granules of pancreatic islet cells [Kahn *et al.*, 1990]. Its physiological function is to reduce food intake and regulate meal size.

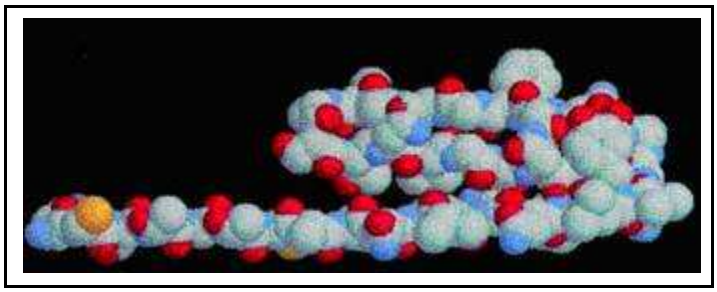


FIG. 2: IAPP in its native conformation.

The primary sequence of IAPP is closely conserved between species, but there are some important differences in amino acids 20–29, the region believed to be important for its propensity to form oligomers [Westermark *et al.*, 1990]. Primates and humans share close homology in IAPP 20–29 region, and synthetic forms of these peptides form amyloid. In contrast, in rats and mice amino acids 20–29 are identical but they are not amyloidogenic, due to three proline residues that render rat and mouse IAPP water soluble [Westermark *et al.*, 1990].

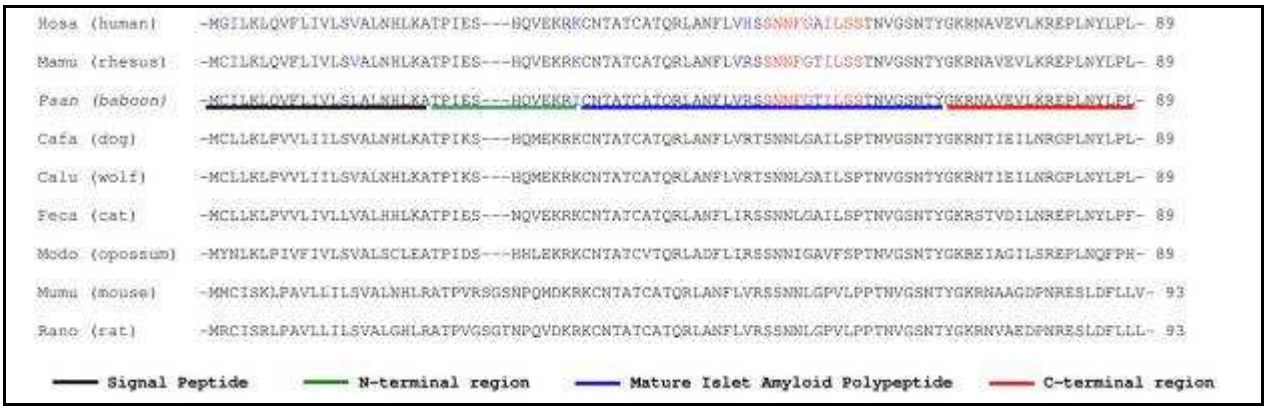


FIG. 3: IAPP protein sequence in different species. The amylodogenic region is reported in red. [Guardado-Mendoza *et al.*, 2009].

Longitudinal studies in *Macaca nigra* and *Macaca mulatta* showed that progression to T2DM correlated with the IA severity and that IA may play a role in beta cell death [de Koning *et al.*, 1993, Howard *et al.*, 1986; Weir and Bonner-Weir 2004; Federici *et al.*, 2001]. In baboons, a well known non-human primate model for T2DM that shows insulin resistance [Chavez *et al.*, 2008 and 2009], severe IA (>50%) was not only associated with increased beta cell apoptosis and decreased relative beta cell volume, but also with alpha cell replication and increased relative alpha cell volume [Guardado-Mendoza *et al.*, 2009].

The toxic form of amyloidogenic proteins appears not to be the extracellular amyloid fibrils detected by light microscopy, but rather smaller nonfibrillar oligomers [Janson *et al.*, 1999; Konarkowska *et al.*, 2006]. It has been proposed that IA can cause beta cell death by occupying extracellular space, thereby impairing nutrients and oxygen uptake. In addition, small IAPP oligomers can form non-selective ion permeable membrane pores, leading to increased intracellular calcium concentrations, endoplasmic reticulum stress and apoptosis [Clark at al., 1988; Huang *et al.*, 2007; Lorenzo *et al.*, 1994; Mirzabekov et al, 1996; Ritzel *et al.*, 2007; Westermark *et al.*, 1978].

2.2.1 Role of interactions in IAPP misfolding

Abnormal IAPP processing, folding and secretion could contribute to the formation of islet amyloidosis (IA). In this kind of aggregates, protein-protein interactions could play a key role in the folding and aggregation pattern and therefore in the severity of IA.

Specific interactions between soluble forms of IAPP and insulin were demonstrated in vitro by immunoprecipitation and surface plasmon resonance (SPR) experiments [Jaikaran *et al.*, 2004]. The structural basis of this interaction indicated that insulin binds to IAPP (residues 1-18) via salt bridges

and hydrophobic interactions (Fig. 4) [Wei *et al.*, 2009].

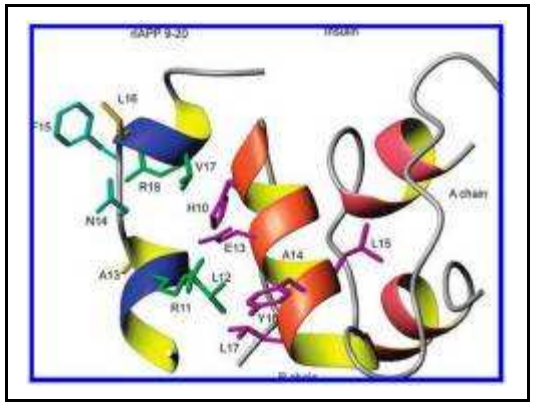


FIG. 4: Structural representation of the insulin-IAPP complex. [Wei *et al.*, 2009].

It has also been proposed that Annexin A5, a Ca^{2+}-dependent membrane-binding protein, is codeposited with misfolded proteins. It likely interacts with a subset of h-IAPP molecules that are in a pathogenic conformation and/or on the pathway toward fibril formation and interferes with this process [Bedrood *et al.*, 2009]

Moreover, other cellular components are involved in IAPP interactions. Zinc, which is found at millimolar concentrations in the secretory granule, significantly inhibits IAPP fibrillogenesis, increasing the lag-time for fiber formation and decreasing the rate of addition of IAPP to existing fibers. However, zinc at higher concentrations has the opposite effect on IAPP fibrillogenesis [Brender *et al.*, 2010].

Mature IAPP has been shown to bind both heparin and heparan sulfate equally, and such interaction promotes aggregation [Abedini *et al.*, 2006; Castillo *et al.*, 1998; Watson *et al.*, 1997].

Characterization of the role of interaction within protein aggregates and between aggregates and the various components of living organisms could provide novel insights into folding physiology and the rationale for alternative therapeutic strategies.

Aim of this project was to find out which proteins can interact with hIAPP, by means of Yeast Two Hybrid screening from a brain library.

3 EXPERIMENTAL PROCEDURES AND PRELIMINARY RESULTS

3.1 Yeast two hybrid

Yeast two hybrid screening, represents a powerful tool to identify protein-protein interactions in a high-throughput scale. The protein of interest, whose interacting partners are still unknown, can be used as a bait to get one (or more) prey from a library of proteins, as described below.

In this kind of screening, the two eucariotic transacting transcriptional regulators DNA binding domain (DNA-BD) and Activation Domain (AD) are physically separated in two different vectors: pLexA and pB42AD, respectively. When the two vectors are co-expressed in the same yeast cells, the two regulators can interact and activate transcription only if they are in close proximity. Generating fusions of these domains to genes encoding proteins that potentially interact, it's possible to detect an interaction between the two proteins with two reporter systems: nutritional selection and X-Gal assay.

Reporter gene	Phenotype
Leu	Ability to grow in Leucine lacking media
Lac Z	Development of blue colour during X-Gal assay

There is also an additional system to control the experiments: the pB42AD library of proteins is under the control of an inducible promoter, that can be activated only in the presence of Galactose in the media. Once the promoter is activated, the library proteins are transcribed and translated and they may interact with the bait (Fig. 5). If the interaction occurs, the transcription of the reporter genes can be verified from the yeast phenotype, as stated above.

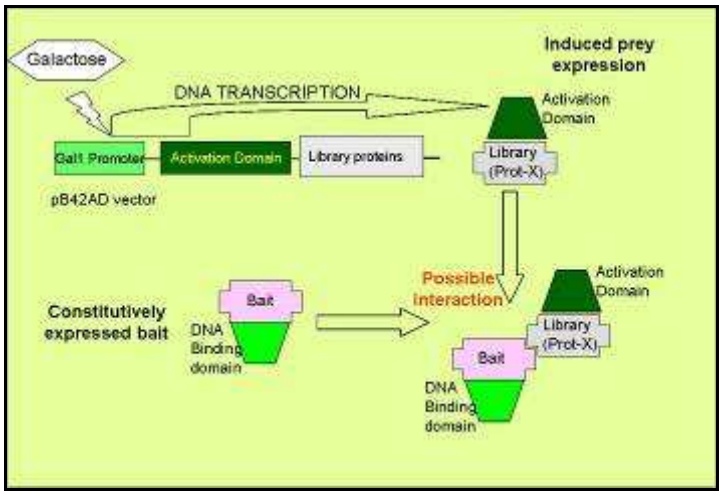
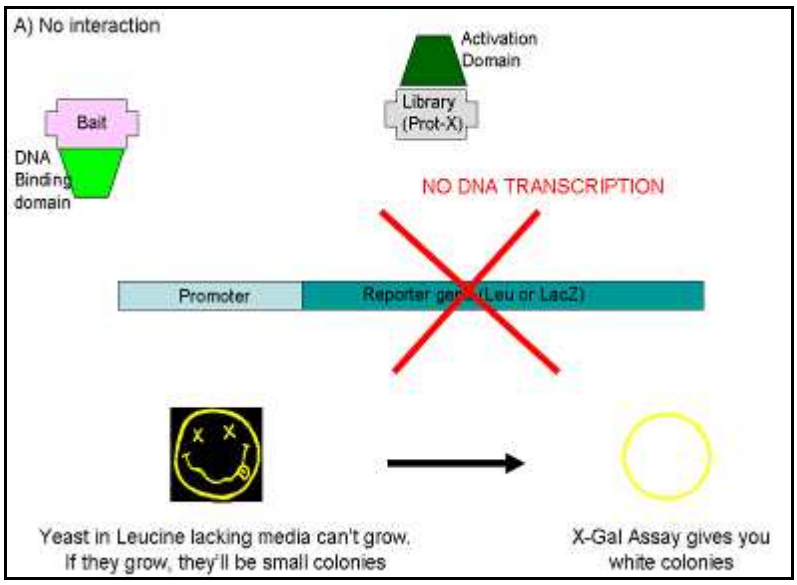


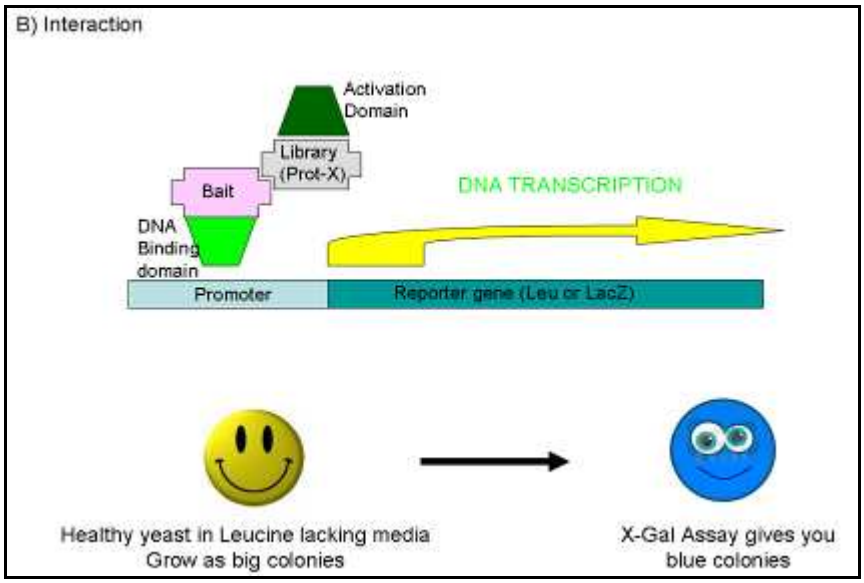
FIG. 5: Schematic representation of pB42AD inducible expression

Accordingly, we expected two main effects during the screening after the induction with Galactose media:

1) If there was not interaction between our bait and the protein X (pray) cloned in the library, we would find out none or few, small and pale colonies, that then would result negative at the X-Gal assay.



2) If there was an interaction between the bait and the prey, we would find out many big healthy colonies, with a yellowish colour, that would give blue colour during X-Gal assay.



However, this technique presents some drawbacks, mainly linked to the high number of false positives that may arise during the screening. Some of the false positives depend on technical aspects, on protein sequence or are casual. In addition, it has to be taken into account that all the potential interactions that are found *in vitro* have to be checked also *in vivo*. Indeed, many proteins may not be available for interaction *in vivo*, due to expression in different cell types or diverse localisation in cell compartments and therefore those interaction are not of physiological relevance.

3.1.1 Bait construction

We inserted hIAPP as a bait in the pLexA vector (Clontech), and a brain library containing a pool of possible interacting proteins (prays) was inserted in the pB42AD vector (Clontech, kindly provided by Prof. Lily Dong, UTHSCSA) [Mao *et al.*, 2006; Dong *et al.*, 2000].

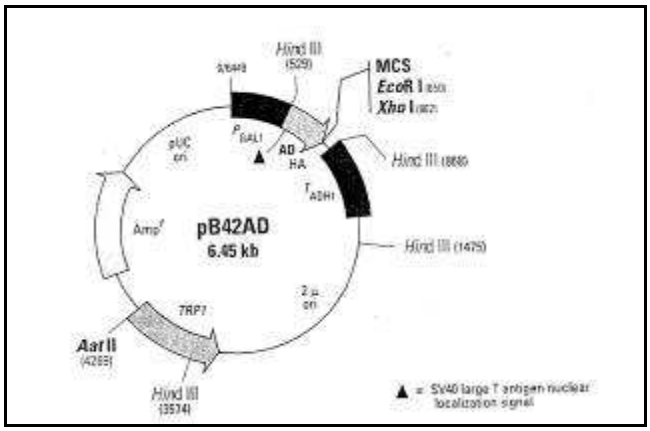


FIG. 6A: The library vector pB42AD. In yeast cells it confers Tryptophan Auxotrophy.

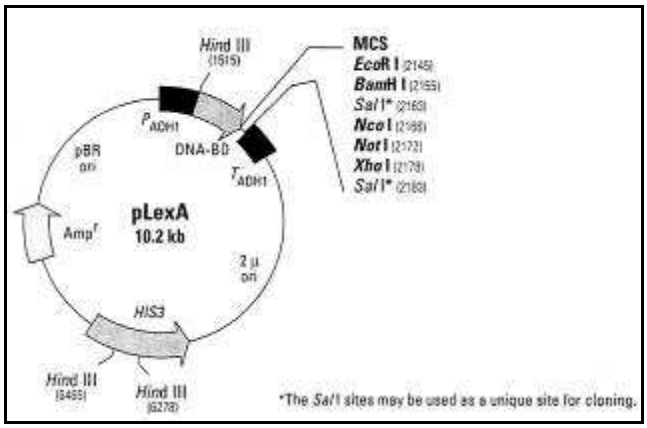


FIG. 6B: The bait vector pLexA. In yeast cells it confers Histidine Auxotrophy.

We were interested in the mature form of hIAPP, from 1 to 37th amino acid, but it was too short to be cloned in a vector easily. Thus, we decided to use a multi-step procedure to obtain the minimum length for the plasmid construction (I thank Dr. Carla Perego for the useful advices in molecular biology).

- hIAPP was previously cloned in Prof. Folli laboratory and inserted in pTOPO vector (Invitrogen).
- Starting from pTOPO-hIAPP as a template, a STOP codon was introduced after the 37th amino acid of mature IAPP (362nd bp) using PCR-site directed mutagenesis with the following primers:

Primer	Sequence 5'-3'
Forward	GGATCCAATACATATTGAAAGAGGAATGCAGTAGAGG
Reverse	CCTCTACTGCATTCCTCTTTCAATATGTATTGGATCC

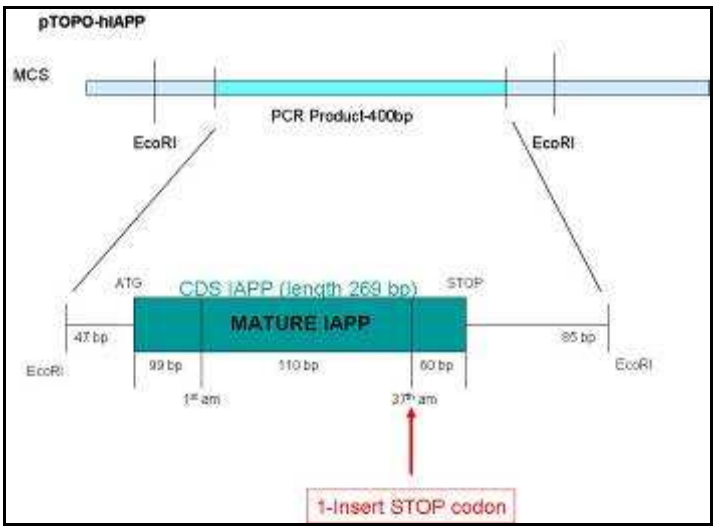
The product was called pTOPO-hIAPP STOP and checked by sequencing. It was an intermediate.

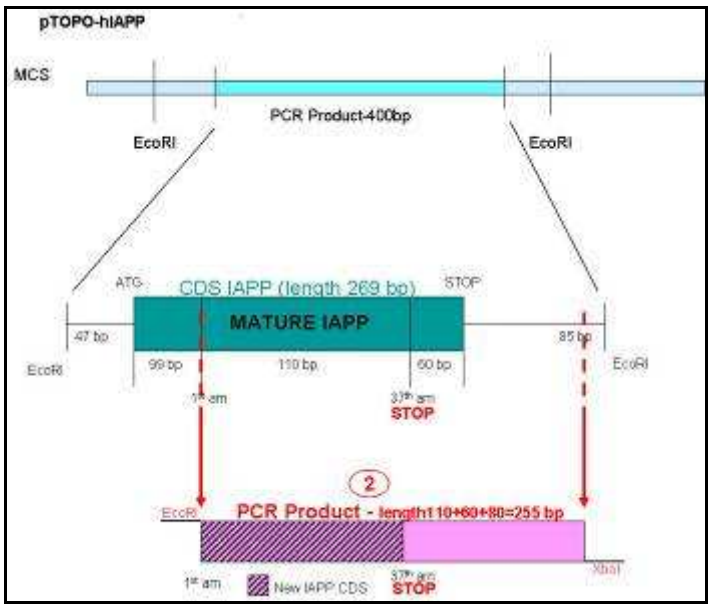
- Using pTOPO-hIAPP STOP as a template, by means of PCR, a small portion of the vector was amplified: from the 1st amino acid of mature IAPP (252nd bp) until 507th bp, generating a product of 255 bp and inserting *EcoRI* (5' end) and *XhoI* (3' end) restriction sites.

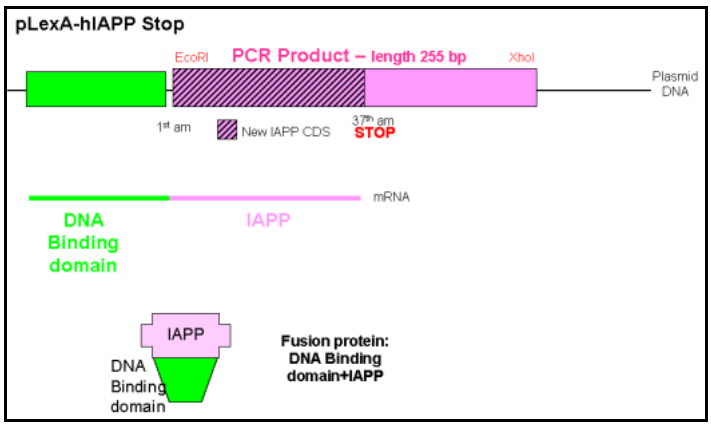
The following primers were used:

Primer	Sequence 5'-3'
Forward	GGAATTCAAATGCAACACTGCCACAGT
Reverse	CCGCTCGAGCACACTGGAGATCAAAAG

- The PCR product, as well as pLexA vector, were digested with *EcoRI* and *XhoI*, and the two DNA were then ligated by T4 ligase (Promega) with a 1 to 3 ratio, respectively.







Note: all the base pair numbers refer to NM_000415.2 in nucleotide NCBI database

The final product was: pLexA-hIAPP STOP, in which mature hIAPP was inserted in *EcoRI-XhoI* restriction sites of pLexA vector. The resulting clones were sequenced and the frame for the chimeric protein DNA-Binding Domain/IAPP was checked.

3.1.2 Bait test

Before the screening, the first step was to verify if the bait was not toxic for the yeast and if the bait alone could activate transcription and therefore turn on reporter genes. To this purpose, a small scale yeast transformation was carried out. In the case of a positive result, the bait would not be suitable for the screening.
We used a yeast strain (EGY48) already transformed for p8OPLAC7 (from Prof. Dong laboratory), a plasmid that provided the ability to respond to the X-Gal assay in case of protein-protein interactions.

EGY48 (p8OPLAC7) yeast cells were transformed in a small scale with:

- pLexA-hIAPP stop
- empty pLexA + empty pB42AD
- pLexA-hIAPP stop + empty pB42AD

And as positive controls:

- pLexA-APPL2
- pLexA-APPL2 + empty pB42AD

The co-transformants were selected in SD-His-Trp-Ura plates: this minimum media is lacking the three indicated amino acids, thus only colonies carrying the LacZ operon and the two plasmids can grow.
Expected results:

Transformation	Growth in SD-His-Trp-Ura plates
pLexA-hIAPP stop	-
Empty pLexA + Empty pB42AD	+
pLexA-hIAPP stop + Empty pB42AD	+
pLexA-APPL2	-
pLexA-APPL2 + empty pB42AD	+

In these conditions, the assay gave the supposed results and yeast grew also in the presence of the pLexA-hIAPP stop, suggesting that the bait is not toxic for the yeast cells.
Then, after the incubation in the induction media (SD Gal-Raf -His-Leu-Trp-Ura), that induced expression of the pB42AD vector, 10 colonies of each transformation were assessed with X-Gal Assay.

Transformation	Growth in SD-Gal-Raf -His-Leu-Trp-Ura	X-GAL
pLexA-hIAPP stop	-	-
Empty pLexA + Empty pB42AD	-	-
pLexA-hIAPP stop + Empty pB42AD	-	-
pLexA-APPL2	+	+
pLexA-APPL2 + empty pB42AD	+	+

The yeast gave positive results at X-Gal Assay using the positive control APPL2, but it gave negative results using the bait, indicating that the bait itself can't activate the reporter gene, therefore is suitable for yeast two hybrid screening.

3.1.3 Screening

Once the bait was validated, we proceeded with the screening, using a big scale transformation.

Day 1: Transformed in a library scale EGY48 (p8OPLAC7) yeast cells with:

- pLexA-hIAPP stop
- pB42AD-library (prepared in Prof. Dong laboratory)

Plated all the co-transformants on 60 150 mm-plates using SD-His-Trp-Ura as a selection media, to select the co-transformants carrying p8OPLAC7, pLexA-hIAPP stop and pB42AD-library. Let them grow for 4 days at 30°C.

Day 5: Harvested the library co-transformants and combined them in a unique tube to make a glycerol stock (stored in aliquotes at -80°C). Titered the glycerol stock in 100 mm-plates, with SD -His-Trp-Ura or SD Gal-Raf-His-Leu-Trp-Ura (Induction media, containing Galactose), to have a ratio of the growing colonies. Let them grow until they were easy to count.

Day 9: Screening library co-transformants for two-hybrid interactions. 2×10^6 library transformants were plated in each of the 60 150 mm-induction plates (SD Gal-Raf-His-Leu-Trp-Ura). We let them grow at 30°C and every huge, healthy colony that showed up in the following 10 days was harvested and plated on a 100 mm replica plate with the same media. In this way, the nutritional selection was done: only the colony in which the two proteins interacted could grow in a Leu lacking media as big colonies. After 24 hours, the replica colonies were tested also with Colony Lift Filter Assay, to check the activation of the other reporter system (X-Gal conversion to a blue product by β-galactosidase, the second reporter gene).

In total, 92 colonies were positive for X-Gal assay, with a peak between those harvested from day 6 to day 7.

In the following days, the positive yeast colonies from the master plates of the replica were inoculated in SD -His-Trp-Ura liquid media and grown o/n at 30°C under constant agitation. The next day, the positive clones were stored at -80°C as glycerol-MgSO4 stocks and the DNA was extracted using the standard protocol. The DNA was stored at -20°C in 20µl aliquots.

Retested all the positive yeast clones: all the positive clones were plated in a unique 150 mm induction plate (SD Gal-Raf-His-Leu-Trp-Ura) and, after 48 hour growth, tested with colony lift filter assay. The X-Gal incubation was performed for 8 hours at 30°C and then the filter was dried under the chemical hood. A filter image was acquired with the scanner and the optical density of each spot was measured using Scion Image Software. Each optical density was corrected for the background and then normalized to the positive control intensity. Not all the positive colonies were confirmed.

3.1.4 Clone identification

3.1.4.1 KC8 COLITRANSFORMATION

KC8 Coli are a particular bacteria strain that can be transformed with just one molecule of DNA for each cell. This is particularly useful to select DNA extracted from yeast, that is a mixture of the two plasmidic DNA used in the transformation, i.e. pLexA and pB42AD. Indeed, at this stage we were interested in identifying the protein encoded by pB42AD vector, the one deriving from the library and that showed a potential interaction with the bait.
Moreover, this strain is unable to grow in Trp lacking media, unless the plasmid pB42AD is present, as it confers Trp auxotrophy.
Thus, exploiting both this characteristics, we were able to select only the bacteria clones carrying pB42AD vector with the relative insert to be identified.
In addition, the step in bacteria allowed us to amplify the yeast DNA, that was extracted with a low yield.
After o/n growth in plates with M9 Minimum media lacking Trp, 10 KC8 coli colonies were harvested and grown o/n in the corresponding liquid media. The following day, clones were lysed and the plasmidic DNA extracted.

3.1.4.2 DNA DIGESTION

To verify that the plasmid pB42AD contained an insert, that could encode for a protein of the library, we digested extracted DNA with restriction enzymes, as well as the empty vector and a positive control. To excide the library inserted fragment, we used *EcoRI* and *XhoI* restriction enzymes (New England Biolabs). After 3 hour digestion, the products were loaded on a 1% agarose gel and the presence of two bands was checked. As expected, two bands were found for the digested products: one for the linear plasmid, around 6 kb, and another band for the insert, the putative interacting protein, of various length. In all cases, the 10 clones reveled the same pattern in the gel, thus only two KC8 colonies from each yeast DNA transformation were selected for the sequencing.

3.1.4.3 DNA SEQUENCING

KC8 clones were grown o/n at 37°C under constant agitation. The following day, plasmidic DNA was extracted using the MiniPrep Kit (Promega) to obtain a high purity suitable for the subsequent reaction of sequencing (DNA Core Facility, UTHSCSA). The reaction time was based on insert length determined by the previous gel electrophoresis.

The primer used for the sequencing was: 5'- CCAGCCTCTTGCTGAGTGGAGATG -3', that is 40 bp before the fusion between Activation Domain and the protein.

3.1.4.4 SEQUENCE ANALYSIS

Chromatograms obtained from the DNA core facility were checked with Sequence Scanner v1.0 (Applied Biosystems) and when some areas were indefinite, the sequencing reaction was repeated.

First, the reading frame of the sequences were determined comparing the known sequence of the Activation Domain with the insert sequences. Then, the sequences were translated with the correct reading frame using a resource available on-line at the following website: http://nbc11.biologie.uni-kl.de/framed/left/menu/auto/right/JDT/. Only the sequences that gave more than 6 amino acids were analysed in the subsequent steps.

Both the nucleotide sequences and the relative translated protein were matched with the databases by the nucleotide/protein BLAST tool in NCBI website (http://ncbi.nlm.nih.gov). Also the ExPasy on-line resources (http://expasy.org/) were used to check translated proteins, but only in few cases they gave different results compared to BLAST results.

At this step, the insert sequences were identified, but especially for the short sequences, some of the identified proteins were from bacteria or other inferior organisms and were rejected.

The next step will be to confirm the potential interaction between the identified protein and the bait, by transforming yeast cells in a small scale with this two constructs. This step is necessary to isolate the effect of that particular interacting protein from the whole library, because during the library screening more than one pB42AD plasmid could enter the yeast and by chance we could have isolated the one that is not responsible for the interaction.

Then, if the interaction was confirmed, further studies would be needed to demonstrate that it occurs also *in vivo*, by means of affinity chromatography or immunoprecipitation from native tissues.

4 ADDITIONAL METHODS

4.1 Yeast Transformation

EGY48 yeast cell with p8OP-LacZ reporter plasmid were grown overnight to the stationary phase in YPD medium at 30 °C under constant agitation. The following day, the yeast culture was transferred to fresh YPD medium and grown to produce an $O.D._{600\ nm}$ close to 0.5/0.6. Cells were then centrifuged and resuspended in 1X TE/LiAc solution to render them competent. Next, a mixture of DNA and 1X PEG/LiAc was added to the cells and we let them grow for 30 minutes at 30°C under constant agitation. Recovered cells were heat shocked for 15 minutes at 42°C in the presence of DMSO and then plated in the appropriate plates.

All yeast media were supplied by Clontech and prepared following the manufacturer protocols.
10X TE solution: 0.1 M Tris HCl pH 7.5, 10 mM EDTA
10X LiAc solution: 1M LiAc
PEG/LiAc solution: 40% PEG, 1X TE, 1X LiAc

4.2 Colony Lift Filter Assay

24 hours after plating the yeast in 100 mm induction plates (Gal/Raf-His-Leu-Trp-Ura), colonies were lifted on a filter presoaked with Z Buffer/X-Gal solution (Z-Buffer, 38 mM β-mercaptoethanol, 0.3 mg/ml X-Gal). Yeast cells were then lysed by thermal shock, immersing the filters in liquid nitrogen two times and then incubated for 8 hrs maximum at 30 °C.

Z-Buffer: 16.1 g/l $Na_2HPO_4x7\ H_2O$, 5.5 g/L x H_2O, 0.75 g/l KCl, 0.246 $MgSO_4x7H_2O$- pH 7

4.3 Yeast DNA Extraction

Yeast clones positive for X-Gal assay were grown overnight in SD-His-Trp-Ura at 30°C under constant agitation. Cells were then sedimented and resuspended in the freshly prepared suspension buffer: 10 mM Tris-HCl pH 8, 1 mM EDTA, 4.5 U/µl Lyticase. After an incubation at 37°C for 30 minutes, lysis buffer was added (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8, 1 mM EDTA). Following mechanical disruption of yeast cells with glass beads, the DNA was extracted with Phenol-Chloroform-Isoamylalchool and

precipitated with Sodium Acetate and Ethanol. The final pellet was resuspended in 20 µl of water.

4.4 Yeast Glycerol stock

30%Glycerol/$MgSO_4$ solution

4.5 KC8/JM109 competent cells transformation

To insert plasmidic DNA into bacterial competent cells we used electroporation. Competent KC8 or JM109 cells were incubated with 2 µl of DNA in a cuvette on ice and then subjected to electroporation (Biorad apparatus) using the program "EC2" from the manufacturer. Only products with a time constant near 5 were plated. Pre-warmed SOC media was immediately added to the cells, that were grown for one hour at 37°C under constant agitation. Then, bacteria were plated on agarose plates.

SOC MEDIA: 2% Tryptone, 0.5 % Yeast extract, 0.05% NaCl, 0.5 % $MgSO_4$, 0.04% Glucose
LB plates (JM109): Luria agar (Sigma Aldrich) prepared following the manufacturer protocol
M9 Media (KC8): 1X M9 salts (Sigma Aldrich), 1.8 % Agar, DO-Trp (Clontech), 1 mM $MgSO_4$, 0.4 % glucose, 0.1 mM $CaCl_2$, 0.05 mg/ml Ampicillin, 0.03 mM thiamin

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APPENDIX

1 ADDITIONAL RESEARCH ACTIVITY PERFORMED

1.1 Expression and function of EAAC1 in Schwann cells

Schwann cells represent the myelinating cells of the Peripheral Nervous System (PNS) and recently glutamate has been involved in the proliferation and differentiation of these cells [Fink *et al.*, 1999; Kinkelin *et al.*, 2000]. Thus, the control of the extracellular glutamate level by glutamate transporters may be crucial for Schwann cell physiology.

Aim of this study was to identify the glutamate transporters expressed in Schwann cells and to verify if they were functional.

By means of RT-PCR, we observed that the high affinity glutamate transporter EAAC1 was expressed by Schwann cells. EAAC1 was present and functional at the cell surface and modulated by exposure to 10 nM allopregnanolone (ALLO), a progesterone's metabolite, known to influence PNS myelinogenesis and myelin protein expression [Melcangi *et al.*, 2005; Schumacher *et al.*, 2007]. We found that transport up-regulation did not involve protein neo-synthesis and was prevented by actin depolimerization, suggesting a change in the transporters' trafficking. To verify this hypothesis, Schwann cells were transfected with the GFP-tagged EAAC1 transporter and its surface density was measured by Total Internal Reflection Microscopy (TIRFM). ALLO induced the actin cytoskeleton reorganization and caused the EAAC1 redistribution from intracellular compartments to the plasma membrane, at the tips of filopodia structures.

Taken together, our data demonstrate that EAAC1 is present in Schwann cells as a functional glutamate uptake system. Its surface activity can be dynamically up-regulated by ALLO through a mechanism which involves increased delivery of EAAC1 to the cell surface. Novel findings on glutamate uptake modulation might be relevant in neurophaties, where glutamate excitotoxicity is involved [Watkins, 2000], and in regenerative processes, since glutamate has a role in Schwann cell proliferation.

1.2 References

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2 PUBLICATIONS

2010

D' Amico A, Soragna A, **Di Cairano E**, Panzeri N, Anzai N, Vellea Sacchi F, Perego C. (2010) The Surface Density of the Glutamate Transporter EAAC1 is Controlled by Interactions with PDZK1 and AP2 Adaptor Complexes. *Traffic*. Aug 17. doi: 10.1111/j.1600-0854.2010.01110.x. [Epub ahead of print]

Di Cairano ES, Davalli A, Perego L, Sala S, Sacchi VF, La Rosa S, Placidi C, Capella C, Conti P, Centonze V, Casiraghi F, Bertuzzi F, Franco Folli F, Carla Perego C. Glutamate-toxicity is a novel mechanism of pancreatic β-cell death and is prevented by the glutamate transporter GLT1/EAAT2. *J Biol Chem*. Under revision.

Perego C, Ballabio M, **Di Cairano ES**, Magnaghi V. Allopregnanolone regulates Schwann cells EAAC1-mediated glutamate uptake and triggers actin changes. *J Neurochem*. Under revision.

3 PATENT

2009

PCT Patent: PCT/EP2009/008256- Inventors: Perego C, Di Cairano ES, Davalli A, Folli F

4 CONFERENCE PARTICIPATIONS AND CONTRIBUTIONS

4.1 Oral communications

2010

Di Cairano ES, Davalli A, Sala S, Sacchi VF, La Rosa S, Folli F, Perego C.
Glutamate toxicity is a novel cause of beta-cells death and is prevented by the activity of the excitatory amino acid transporter 2 (EAAT2) in the pancreas.
SIF National Meeting 2010. Varese, 15-17 September

2009

Di Cairano Eliana S, Fantin Giorgio, D'Amico Anna, Soragna Andrea, Sacchi V.Franca and Perego Carla
The adaptor protein PDZK1/NHERF3 interacts with the glutamate transporter EAAC1 and regulates its surface expression.
Physiology 2009. Dublin, 7-10 July

4.2 Posters

(underlined when presenting author)

2010

F.B. Franco, F. Casiraghi, M. Palomo, G. Abrahamian, G. Halff, M. Abdul-Ghani, P. Frost, **E. Di Cairano**, R. Bastarrachea, A. Comuzzie, D. Tripathy, A. Gastaldelli, A. Davalli, R.A. DeFronzo, A.O. Chavez.
Early alterations of insulin and glucagon secretion during a two-step hyperglycemic clamp with arginine stimulation in obese insulin-resistant baboons
46th EASD Meeting 2010. Stockholm, 20-24 September

S. Kamath, A. Chavez, F. Casiraghi, A. Davalli, G. Halff, G. Abrahamian, G. Hubbard, E. Dick, **E. Di Cairano**, R. Bastarrachea, A. Comuzzie, D. Tripathy, A. Gastaldelli, R.A. DeFronzo, F. Folli. Intrahepatic fat and impaired Insulin-Akt-GSK-3 beta signalling are key determinants of non-alcoholic fatty liver disease in non-human primates
46th EASD Meeting 2010. Stockholm, 20-24 September

Perego C, Ballabio M, **Di Cairano ES**, Magnaghi V.
High affinity glutamate transporters are expressed in Schwann cells and regulated by allopregnanolone.
SIF, National meeting 2010. Varese, 15-17 September

2009

Perego C, Ballabio M, **Di Cairano ES**, Magnaghi V.
High affinity glutamate transporters GLT1, GLAST and EAAC1 are present in Schwann cells. IUSP-XXXVI International Congress of Physiological Sciences 2009. Kyoto, 27 July - 1 August

Perego C, Ballabio M, **Di Cairano ES**, Magnaghi V
High affinity glutamate transporters GLT1, GLAST and EAAC1 are present in schwann cells
IV Meeting on the Molecular Mechanisms of Neurodegeneration 2009, Milano May 8-10th

E.S. Di Cairano, G. Fantin, A. D'Amico, A. Soragna, V.F. Sacchi, C. Perego
PDZK1 is a new EAAC1 binding partner and regulates its surface expression.
SIF Riunione Nazionale Dottorandi in Fisiologia 2009. Pisa, June 22-25

Perego C, Ballabio M, **Di Cairano ES**, Magnaghi V.
High affinity glutamate transporters are expressed in Schwann cells and regulated by allopregnanolone
XII National Congress of the Italian Society of Neuroscience 2009. Milan, October 2-5

2008

Di Cairano Eliana S, D'Amico Anna, Soragna Andrea, Panzeri Nicola, Sacchi V. Franca e Perego Carla.
Modulazione dell'espressione dei trasportatori del glutammato: ruolo delle proteine PDZ
SIF Riunione Nazionale dei Dottorandi di Fisiologia 2008, Bertinoro June 18-21st

D'Amico A, Soragna A Panzeri N, **Di Cairano ES**, Sacchi VF and Perego C
A PDZ protein target sequence and a clathrin-dependent endocytosis signal concur in regulating the surface expression of the glutamate transporter EAAC1 in epithelial cells
Gordon Conference 2008. Lucca, 20-25 July

Andrea Soragna, Anna D'Amico, Naohiko Anzai, Nicola Panzeri, **Eliana Sara Di Cairano**, Vellea Franca Sacchi, and Carla Perego
The surface expression of the glutamate transporter EAAC1 in epithelial cells is controlled by balanced interactions between PDZ proteins and a clathrin adaptors
SFB35 "Transmembrane Transporters in Health and Disease" 2008. Wien, 26-27 September

Perego Carla, **Di Cairano Eliana S**, Fantin Giorgio, D'Amico Anna, Soragna Andrea, Sacchi V.Franca
The adaptor protein PDZK1/NHERF3 interacts with the glutamate transporter EAAC1 and controls its surface activity in epithelial cells
FIRB Metodologie E Tecnologie Innovative Per La Farmaceutica" 2008. Verona, 23-24 April

4.3 Published abstracts

2009

Di Cairano ES, Fantin G, D'amico A, Soragna A, Panzeri N, Sacchi Vf, And Perego
A PDZ protein target sequence and a clathrin-dependent endocytosis signal concur in regulating the surface expression of the glutamate transporter EAAC1 in epithelial cells
60° Congresso Nazionale della Società Italiana di Fisiologia (S.I.F.) 2009. 19-21 settembre 2009, Siena. Acta Physiologica. 197, supp 672, settembre 2009, p58 pag 59

Perego C, Ballabio M, **Di Cairano ES**, Magnaghi V.
High affinity glutamate transporters are expressed in Schwann cells and regulated by allopregnanolone
60° Congresso Nazionale della Società Italiana di Fisiologia (S.I.F.) 2009. 19-21 settembre 2009, Siena -Acta Physiologica. 197, supp 672, settembre 2009, p127 pag 93

2007

D'Amico A, Soragna A, Panzeri N, **Di Cairano ES**, Anzai N, Sacchi VF and Perego C.
PDZ-domain interactions control the surface stability and degradation of the EAAC1 glutamate transporter
FEBS Journal. 2007 274, supp.1, B4-44- 351

Soragna A, D'Amico A, Panzeri N, **Di Cairano E.S**., Sacchi V.F. and Perego C.
A PDZ interaction domain regulates the membrane stability and the degradation of the EAAC1 glutamate transporter in MDCK cells.
Acta Physiologica. 2007 191, supp 657, OC37 pag 33

5 VISITING SCIENTISTS AND SCIENTIFIC INSTITUTIONS

January 2010-May 2010

Research Scholar.
Identification of direct protein-protein interactions by means of yeast two hybrid screening.
Department of Medicine-Division Diabetes at UTHSCSA (University of Texas, Health Science Center at San Antonio, TX)-USA

6 FELLOWSHIPS

2009

European Bursary Scheme: Young Physiologist Bursary to attend Physiology 2009 meeting in Dublin

7 PROFESSIONAL COURSES

2010, Molecular Imaging in Drug Discovery and Preclinical Development-A training course in molecular imaging (Università degli Studi di Milano)

2009, Applicazioni della Fluorescenza nell'imaging, nella microscopia e nella citofluorimetria (Università degli Studi di Milano)

2008, Approfondimenti e confronti sull'uso del microscopio confocale (GIM)

2007, Corso per la gestione e la valorizzazione dei risultati della ricerca accademica e industriale: trasferimento di innovazione dai laboratori di ricerca all'impresa (Università degli Studi di Milano)

8 PARTICIPATING IN RESEARCH PROJECTS

FIRST 2007
Project title: Modulazione dell'interazione tra il trasportatore del glutammato EAAC1 e proteine PDZ in epiteli e neuroni.
Area 05: Scienze biologiche

9 TEACHING AND TUTORING:

2007-2010

Taught two sections of Physiology laboratory for Students of Pharmaceutical Biotechnology at the Institute of General Physiology and Biological Chemistry, Università degli Studi di Milano.

Provided scientific tutoring for graduating students in Pharmaceutical Biotechnology (I Level) and Drug Biotechnology (II Level) at DISMAB, Dept. of Molecular Sciences Applied to Biosystems, Università degli Studi di Milano.

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…a proposito: esiste un premio fedeltà???

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