



Glutamate Receptors Modulate Oxidative Stress in Neuronal Cells. A Mini-Review.

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Multiple lines of evidence demonstrate that reactive oxygen species (ROS) are involved in regulation of normal cell metabolism as second messengers. Under extreme conditions, these molecules induce oxidative stress, which may stimulate (or accompany) a number of neurodegenerative processes. In the glutamatergic system, ROS levels are under control of ionotropic and metabotropic glutamate receptors, which modulate ion fluxes through the neuronal membrane. The Na⁺/K⁺-pump is also one of the important participants affecting stationary ROS levels through several distinct mechanisms. This review describes the involvement of the Na⁺/K⁺-pump in intracellular signaling mechanisms via cross-talk between the pump and glutamate receptors in cerebellum granule cells. Selective dysfunction of mGlu II receptors may also lead to abnormal protein phosphorylation (*i.e.*, tau phosphorylation), culminating in neurodegenerative disorders (*i.e.*, Alzheimer disease). Also, unregulated production of intracellular ROS resulting from an imbalance of ionotropic and metabotropic receptors may activate one or more protein kinases. In summary, Glu receptor dysfunction, leading to a deficit in glutamate-mediated signal transduction may represent one of the earliest stages of neurodegenerative disorders. The Na⁺/K⁺-pump is able to prevent over-production of intracellular ROS, thus increasing oxidative stability of neuronal cells.

Keywords: Ionotropic receptors; Metabotropic receptors; Glutamate; Na/K-ATPase; Neurons; Oxidative stress; Reactive oxygen species

Since the end of the 20th century when the glutamatergic nature of interneuronal signal transduction was discovered (for review, see Carpenter, 2002), the main issue still not clearly understood is the biological significance of the multiplicity of glutamate receptors on the neuronal membrane. Two major subtypes of glutamate receptors are currently recognized, associated either with ionic channels (*ionotropic receptors*) or with membrane bound G-proteins involved in signal transduction from the external cell membrane to intracellular metabolic processes (*metabotropic receptors*).

Specific agonists and antagonists for most glutamate receptors are widely used (see Table I), which make possible the inhibitory analysis of individual glutamate receptor functions. Such studies have demonstrated functional diversity of glutamate receptors but their interrelationships in cellular metabolism remain obscure (Boldyrev, 2000; Conn, 2003).

One of the first neurochemical observations illustrating functional heterogeneity of glutamate receptors was made as early as 1996, when intracellular calcium ions in neurons were measured as a function of the concentration of different ligands (Oyama *et al.*, 1996). The comparison of glutamate and kainate effects showed that glutamate starts to activate the neurons at a lower concentration than kainate, while increases in glutamate concentration resulted in a smaller effect compared to that of kainate (FIG. 1). Thus, the former is a milder activator of intracellular calcium release compared to the latter. Kainate is a well-known neurotoxin which induces neuronal death *via* ROS accumulation within the intraneuronal space. The suggestion has been made that, in addition to kainate receptors, glutamate occupies another group of membrane receptors preventing extra release of calcium ions and the

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Table I Classification of glutamate receptors

Glutamate receptors	Functional characteristics of sub-types			
	Isoforms	Main agonist	Obvious antagonist	Metabolic function
Ionotropic	NR1 NR2A NR2B NR2C NR2D NR3A	NMDA	D-AP5 MK-801	Activation of specific ionic channels
	GluR1 GluR2 GluR3 GluR4	AMPA	CNQX	Activation of specific ionic channels
	GluR5 GluR6 GluR7 KA1 KA2	Kainate	DNQX	Activation of specific ionic channels
Metabotropic	Class I (mGlu1, mGlu5)	3,5-DHPG	MCPG	Activation of IP3-dependent Ca-turnover
	Class II (mGlu2, mGlu3)	L-CCG, NAAG	PCCG4	Potentiation of cAMP effects
	Class III (mGlu4, mGlu6, mGlu7, mGlu8)	L-AP4 ACPD	MSOP LY341495	Suppression of cGMP level

excitotoxic action of kainate. Several ionotropic and metabotropic receptors belonging to both subtypes of glutamate receptors are currently recognized (Table I).

The relationship of the above-mentioned receptors to intracellular ROS levels has been studied recently. Over-excitation of ionotropic glutamate receptors usually induces over-production of ROS within neuronal cells. At the same time, the effect of metabotropic receptors depends very much on their nature, with activation of neuronal metabolism by 1-aminocyclopentane-trans-1,3-dicarboxylic acid (ACPD, an mGlu III agonist) stimulating *N*-methyl-D-aspartate (NMDA) - dependent production of ROS, whereas (*RS*)-3,5-dihydroxyphenylglycine (DHPG, an mGlu I agonist) suppresses ROS production by NMDA stimulated neurons (Boldyrev *et al.*, 2003). Thus, mGlu III receptors enhance excitotoxic effects of ionotropic NMDA receptors whereas mGlu I receptors protect the cell against excitotoxic effects (Conn, 2003).

One of the targets of ROS accumulated in neuronal

cells is the Na⁺/K⁺-activated ATPase (Huang *et al.*, 1992; Boldyrev and Kurella, 1996; Boldyrev *et al.*, 2003) which generates ionic asymmetry across the plasma cell membrane, a feature which is especially important for excitable cells. Under normal conditions, the neuronal Na⁺/K⁺-pump consumes from 15% to 40% of cell energy to support ionic gradients. Over-activation of glutamate receptors and increases in the intracellular ROS levels result in reversible suppression of the Na⁺/K⁺-pump and partial dissipation of the Na⁺/K⁺-gradient (Boldyrev, 2002). Therefore, oxidative stress diminishes the stability of neurons facilitating cellular death. Similarly, mGlu III receptors increase the inhibitory effect of NMDA on the Na⁺/K⁺-pump, and mGlu I receptors protect the pump against such inhibition (Bulygina *et al.*, 2002; Boldyrev *et al.*, 2003).

Recently, a feedback relationship between the Na⁺/K⁺-pump and ROS production was described. Analysis of the functional role of the Na⁺/K⁺-ATPase in excitable tissues (primary cultures of myocardial cells

or brain neurons) showed that the active state of the pump is sufficient to prevent intracellular ROS accumulation (Bulygina *et al.*, 2002; Xie and Cai, 2003; Boldyrev *et al.*, 2004).

In brain neurons, three isoforms of the Na⁺-pump are present which differ in sensitivity to cardiac glycosides. Thus, α(2+3) possesses a higher affinity to ouabain (K₀₅ ~ 5x10⁻⁶M) whereas α1 is characterized

by a lower affinity for the inhibitor (K₀₅ ~ 10⁻⁴M) (Blanco and Mercer, 1998). We have found that exposure of rat brain neurons to either low (10⁻⁷ M) or high (10⁻³ M) ouabain concentrations resulted in increases of both stationary ROS levels and ionized calcium (FIG. 2). This calcium can be derived from the endoplasmic reticulum (Verkhatsky and Toescu, 2003) or from mitochondria (Zhu *et al.*, 2004), or both. We have used an inhibitory analysis in order to elucidate the consequences of the reactions in which the Na⁺/K⁺-ATPase is involved and which include this enzyme in cell-signaling pathways (Table II).

FIG. 3 demonstrates how different inhibitors affect the ouabain-induced increase in intracellular Ca²⁺ levels both at low (FIG. 3a) and high (FIG. 3b) concentra-

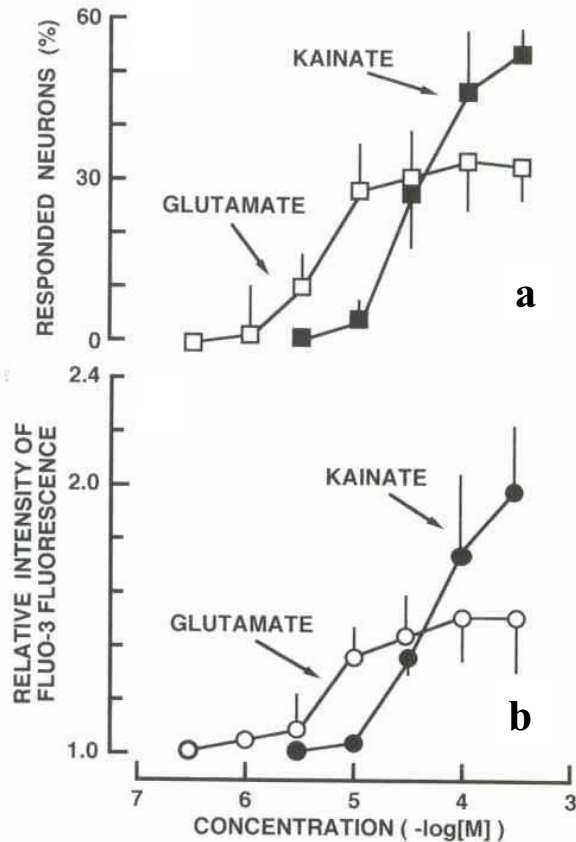


FIGURE 1 Dose-response for glutamate- and kainate-induced changes in the number of responding neurons as a percentage of the whole population (a), and intensity of Ca²⁺-dependent Fluo-3 fluorescence (b) measured in neurons exposed to glutamate or kainate (arbitrary units) (Republished with permission from Elsevier Publishing; in Oyama *et al.*, Brain Res. 728: 121-124, 1996).

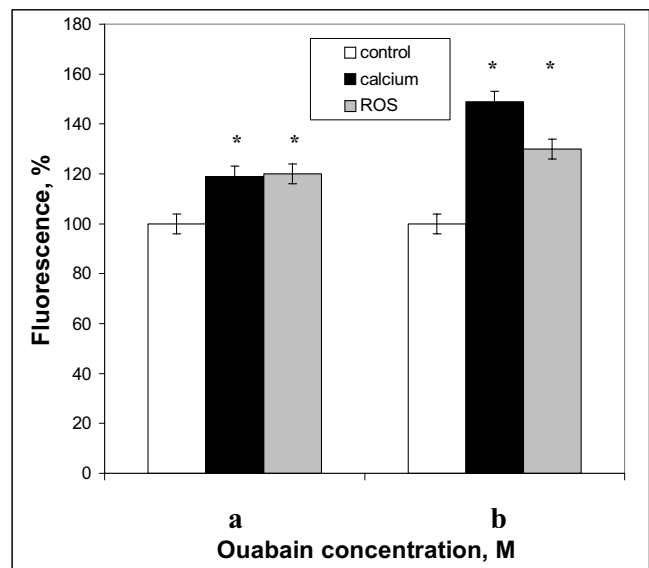


FIGURE 2 Effect of low (10⁻⁷ M in graph a) and high (10⁻³ M in graph b) concentrations of ouabain on intracellular Ca²⁺ and ROS levels in rat cerebellum granule cells. Gray - control, black - Fluo-3 fluorescence (corresponding to calcium levels), white - DCF fluorescence (corresponding to intracellular ROS). Measurements were made using flow cytometry after 30 min incubation. "*" indicates a significant difference (p < 0.05) vs control.

Table II The inhibitors used in the study

Inhibitor	Action mechanism	Active concentration
Ouabain	Inhibitor of (α2+α3) subunits of Na/K-ATPase	K _i = 2.10 ⁻⁷ M
Ouabain	Inhibitor of (α1+α2+α3) subunits of Na/K-ATPase	K _i = 2.10 ⁻⁴ M
MK-801	Inhibitor of NMDA-dependent ionic (Na+Ca) channels	10 μM
Amiloride	Inhibitor of Na/Ca-exchange	20 μM
LY294002	Inhibitor of IP ₃ -Ca Kinase	40 μM
Chelerythrine	Inhibitor of Pkc Family	10 μM
PD98059	Inhibitor of MAP Kinase	30 μM

tions. One can see that only the MAP kinase inhibitor 2'-amino-3'-methoxyflavone (PD98059) does not affect the intracellular Ca^{2+} signal, whereas dizocilpine (MK-801), amiloride or LY294002 prevent the rise in ouabain-stimulated Ca^{2+} release. This actually means that in both cases (inhibition of only highly sensitive ATPase isoforms or of all isoforms), the increase in Ca^{2+} concentration is a Ca^{2+} -induced Ca^{2+} -release from the endoplasmic reticulum, which is controlled by the IP_3 - Ca^{2+} kinase.

The only difference between FIGs. 3a and 3b is that chelerithrine partially inhibits the Ca^{2+} -signal depending on Na^+/K^+ -ATPase, which is highly sensitive to ouabain and does not significantly affect it when all ATPase isoforms are switched off. In other words, Ca^{2+} -

release, which is dependent on $\alpha(2+3)$ subunits of the Na^+/K^+ -pump is only partially dependent on the protein kinase C family (PkC) and this partial dependence may be related to the stimulating PkC effect on NMDA receptors (Boldyrev, 2000). When the $\alpha 1$ subunit of Na^+/K^+ -ATPase is additionally inhibited by ouabain, the larger portion of calcium ions is released, and partially insensitive to chelerithrine. This means that the $\alpha 1$ isoform controls a different pool of intracellular Ca^{2+} ; presumably one which is in the mitochondrial matrix.

Variations of intracellular ROS levels stimulated by low and high ouabain concentrations in the presence of different inhibitors are presented in FIG. 4. It is seen that inhibition of $\alpha(2+3)$ subunits of Na^+/K^+ -ATPase

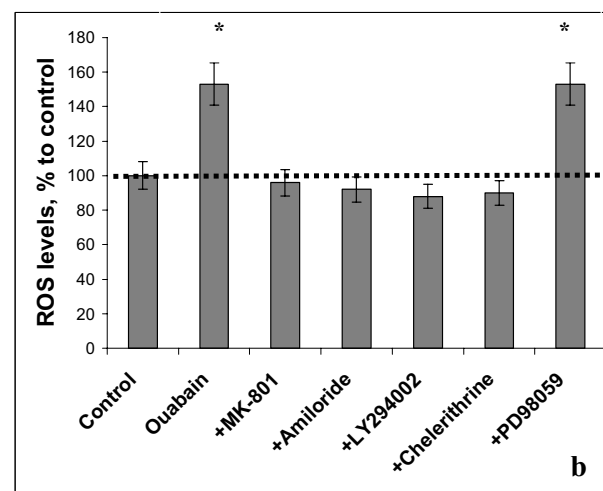
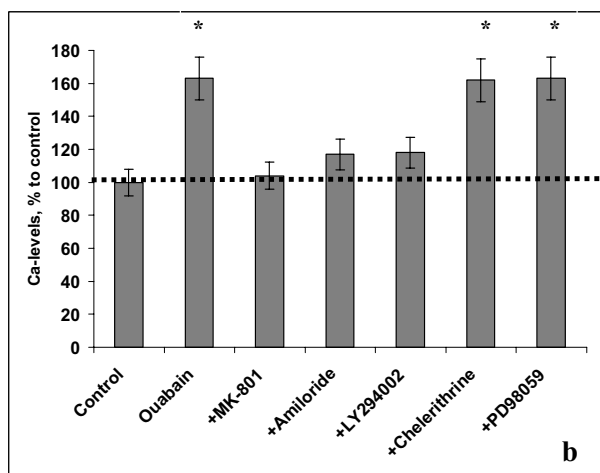
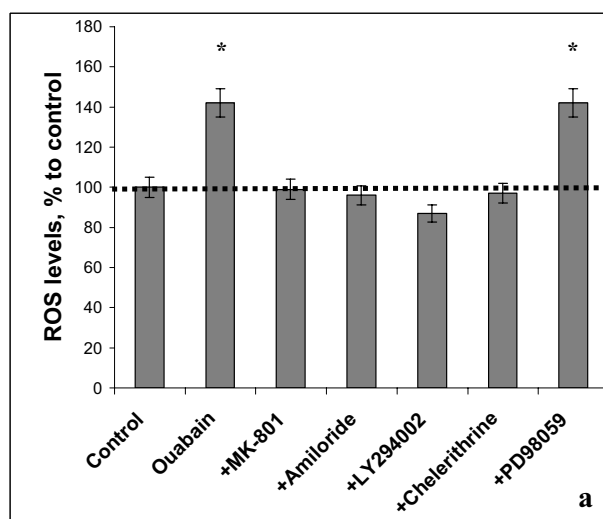
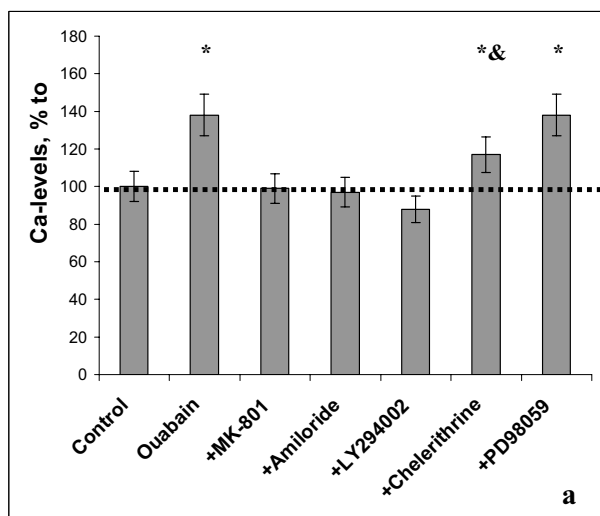


FIGURE 3 Effects of several metabolic inhibitors on Fluo-3 (intracellular Ca^{2+} levels) fluorescence in suspensions of cerebellum granule cells in the presence of low (10^{-7} M in graph a) or high (10^{-3} M in graph b) ouabain concentrations. Incubations were performed for 30 min at 37°C. "*" indicates a significant difference ($p < 0.05$) vs control, and "&" - indicates a significant difference from the ouabain value (2); The horizontal dotted line corresponds to the control value.

FIGURE 4 Effects of several metabolic inhibitors on DCF (intracellular ROS levels) fluorescence in suspensions of cerebellum granule cells in the presence of low (10^{-7} M in graph a) or high (10^{-3} M in graph b) ouabain concentrations. Incubation was performed for 30 min at 37°C. "*" indicates a significant difference vs control, which is shown as a horizontal dotted line.

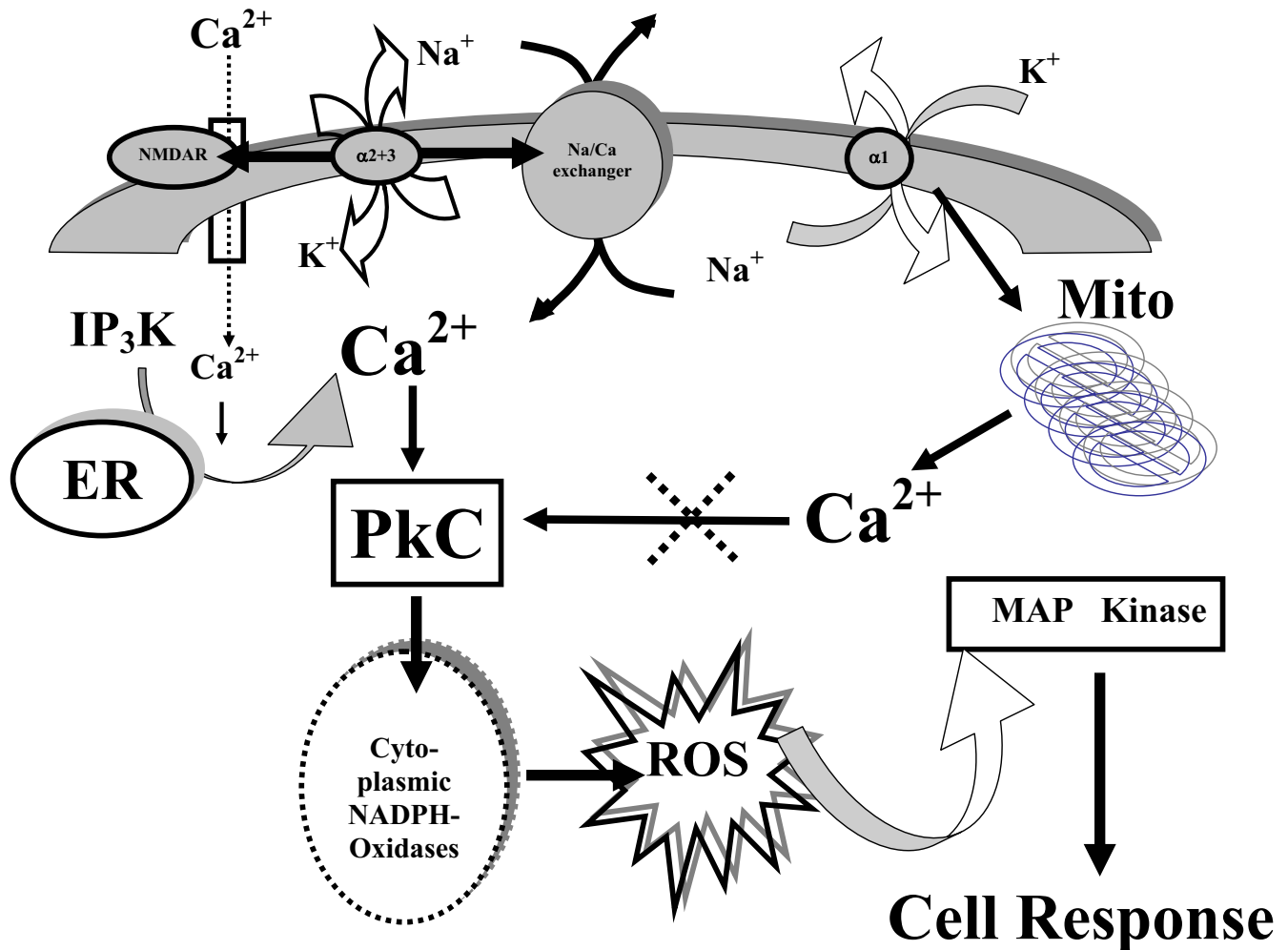


FIGURE 5 Schematic representation of participation of the Na⁺/K⁺-ATPase in the regulation of intracellular calcium and ROS levels (see text for explanation).

increase intracellular ROS levels, which can be diminished by MK-801, amiloride, LY294002 or chelirithrine. The MAP-kinase inhibitor, PD98059 again does not affect the signal. It is clear that production of ROS is mainly induced by intracellular Ca²⁺ release (*cf.* FIGs. 3a and 4a). The only difference is that chelirithrine inhibits Ca²⁺ release to a lower extent than ROS production. This again confirms that not all of the calcium ions are responsible for free radical generation, suggesting that the mitochondrial calcium pool has no relation to ROS production in cerebellum granule cells.

It is important to stress that an increase in ouabain concentration resulting in inhibition of the resting (*i.e.*, low sensitivity to ouabain) portion of the Na⁺/K⁺-ATPase does not modify the dependence, as seen by the close similarity of data in FIGs. 4a and 4b. These results demonstrate that the α1 isoform is not involved

in regulation of intracellular ROS as we suggested recently (Boldyrev *et al.*, 2004).

Taken together, the data suggest the following chain of the events: i) inhibition of α(2+3) sub-population of Na⁺/K⁺-pump induces an increase in intracellular Ca²⁺ first because of Ca²⁺ entry through the NMDA-activated ionic channels and secondly because of activation of IP₃-Ca²⁺ kinase and Ca²⁺-induced release of Ca²⁺ from endoplasmic reticulum; ii) increased Ca²⁺ levels activate PkC, which leads in turn to an increase in cytoplasmic NADPH oxidase (Jones and Hancock, 2000) and in intracellular ROS levels, which activate MAP kinase. Thus, the proposed hypothetical scheme explains the participation of α(2+3) subunits of the Na⁺/K⁺-pump in signal transduction mechanisms. At the same time, the population of α1 subunits regulates the mitochondrial pool of Ca²⁺, which does not take

part in the intracellular ROS generation under normal conditions. These relationships are schematically presented in FIG. 5.

It is difficult to explain why Ca^{2+} released after inhibition of the $\alpha 1$ subunit do not result in ROS generation. It may mean that Ca^{2+} derived from the mitochondrial pool is not accessible to the signal transduction reactions of the cell. One explanation of this may be that $\alpha 1$ subunits are located in the neuronal cell in cellular compartments not involving in these reactions. Another possibility may be related to different localizations of Na^+/K^+ -pump isoforms. According to several studies (Sweadner, 1992; Juhaszova and Blaustein, 1997), $\alpha(2+3)$ subunits are preferentially located in neurons whereas $\alpha 1$ subunits are found in glial cells where signal transduction mechanisms should not be present. In either event, the results obtained confirm the functional difference between low and highly sensitivity of isoforms of the Na^+/K^+ -pump to ouabain, which was previously reported (Blanco and Mercer, 1998).

The scheme presented in FIG. 5 is in good agreement with literature data. First of all, this continues the ideas of Askari *et al.* (for review, see Xie and Askari, 2002) concerning the participation of the Na^+/K^+ -pump in signal transduction pathways. Moreover, a functional relationship was demonstrated recently between NMDA receptors and the Na^+ -pump (Boldyrev *et al.*, 2003; 2004). Different metabotropic receptors were found to structurally interact with ionic channels on the neuronal membrane (Fagni *et al.*, 2004). Evidently, these interactions may be used for signal transduction from the external face of the membrane to Ras-MAP kinase cascade *via* IP_3 - Ca^{2+} kinase (Kometiani *et al.*, 1998; Wang *et al.*, 2004).

Based on the above information, we speculate that selective dysfunction of mGlu II receptors can be a primary cause for neurodegenerative disease leading to abnormal tau protein phosphorylation and Alzheimer disease (Lee *et al.*, 2004). In addition, unregulated production of intracellular ROS taking place during an imbalance between ionotropic and metabotropic receptors may activate a novel type of protein kinase (Stress Activated Protein kinase-SAP kinase) (Zhu *et al.*, 2004). This may open the way for a neurodegenerative cascade starting initially with a deficit of glutamatergic transmission. Therefore, we conclude that a deficit in glutamate-mediated signal transduction may be one of the earliest key signs of several neurodegenerative diseases.

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Footnote

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In memory of Professor Vincenzo Lombardi (1930-2003) stimulating multidiscipline research studies in modern neurochemistry.

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