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Overexpression of GRK2 in Alzheimer Disease and in a Chronic Hypoperfusion Rat Model is an Early Marker of Brain Mitochondrial Lesions

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Heterotrimeric guanine nucleotide-binding (G) protein-coupled receptor kinases (GRKs) are cytosolic proteins that are known to contribute to the adaptation of the heptahelical G protein-coupled receptors (GPCRs) and to regulate downstream signals through these receptors. GPCRs mediate the action of messengers that are key modulators of cardiac and vascular cell function, such as growth and differentiation. GRKs are members of a multigene family, which are classified into three subfamilies and are found in cardiac, vascular and cerebral tissues. Increasing evidence strongly supports the hypothesis that vascular damage is an early contributor to the development of Alzheimer disease (AD) and/or other pathology that can mimic human AD. Based on this hypothesis, and since kinases of this family are known to regulate numerous receptor functions both in the brain, myocardium and elsewhere, we explored cellular and subcellular localization by immunoreactivity of G protein-coupled receptor kinase 2 (GRK2), also known as β-adrenergic receptor kinase-1(βARK1), in the early pathogenesis of AD and in ischemia reperfusion injury models of brain hypoperfusion. In the present study, we used the two-vessel carotid artery occlusion model, namely the 2-VO system that results in chronic brain hypoperfusion (CBH) and mimics mild cognitive impairment (MCI) and vascular changes in AD pathology. Our findings demonstrate the early overexpression of GRK2 member kinase in the cerebrovasculature, especially endothelial cells (EC) following CBH, as well as in select cells from human AD tissue. We found a significant increase in GRK2 immunoreactivity in the EC of AD patients and after CBH, which preceded any amyloid deposition. Since GRK2 activity is associated with certain compensatory changes in brain cellular compartments and in ischemic cardiac tissue, our findings suggest that chronic hypoperfusion initiates oxidative stress in these conditions and appears to be the main initiating injury stimulus for disruption of brain and cerebrovascular homeostasis and metabolism.

Keywords: Alzheimer disease; G protein-coupled receptor kinase 2; GRK2; Oxidative stress; Vascular hypoperfusion; Mitochondrial damage; Neurodegeneration

INTRODUCTION

G protein-coupled receptor kinases (GRKs), like GRK2, are cytosolic proteins that are known to contribute to the adaptation of the heptahelical G protein-coupled receptors (GPCRs) and to regulate downstream signals through these receptors. GPCRs mediate the action of messengers that are key modulators of cardiac and vascular cell function (Iacovelli *et al.*, 1999). The various GRK subtypes differ in their localization, regulation and mode of action. To date, seven mammalian serine/threonine protein GRKs have been described and six members cloned, which comprise the GRK family. GRK2 and GRK3 form the second subfamily, namely, β-adrenergic receptor kinase (βARK) subfamily, members of which are known to phosphorylate and regulate agonist-occupied or constitutively active GPCRs (Lodowski *et al.*, 2003). The known homology domains of GRK2, also called the regulator of G protein signaling (RGS) include protein kinase and pleckstrin homology domains, which recruit the enzyme to

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the cell membrane favoring the simultaneous inhibition of signaling by sequestering G-alpha, G-beta and G-gamma subunits. Further, recent studies suggest that GRKs may have more diverse protein/protein cellular interactions. This is based on the identification of a consensus caveolin binding motif within the pleckstrin homology domain of GRK2 (Carman *et al.*, 1999).

These receptors kinases have been found highly expressed in heart, brain and other tissues. In rat and hamster (Arriza *et al.*, 1992) they are known to regulate numerous receptor functions in both the brain and myocardium (Erdtmann-Vourliotis *et al.*, 2001). In the rat brain the mRNA expression pattern of GRKs family of proteins (GRK2, GRK3, GRK4 and GRK6) was found to be widely distributed and have nearly the same expression pattern, although GRK3 was generally more weakly expressed than GRK2 in most tissues. Generally, GPCRs are involved in complex regulatory mechanisms that modulate the receptor's responsiveness and underlie important physiologic phenomena including signal integration and desensitization (Mayor *et al.*, 1998). Desensitization and resensitization of a wide variety of GPCRs are processes involved in numerous brain functions and GPCRs expression is increased in the developing rat brain, which is consistent with an involvement in brain maturation processes (Penela *et al.*, 2000). The apparent recapitulation of ontogeny, as it applies here, is a characteristic increasingly found in response to many pathologic conditions, *e.g.*, in lung angiogenesis in hyperoxia-induced lung injury (Thebaud *et al.*, 2005).

GRK2 has been well-characterized in the heart, where the onset of congestive heart failure (CHF) is associated with characteristic changes in myocardial expression of GRK2 and is known to significantly contribute to myocardial regulation and function in the failing heart (Hata and Koch, 2003). Signaling through cardiac βAdrenergic Receptors (βARs) is significantly impaired in many cardiovascular disorders, including CHF. Further, elevated levels of GRK2 mRNA and GRK2 activity have been reported in human left ventricle explants from heart failure patients (Ungerer *et al.*, 1996). In the heart, βARs control numerous trophic responses to the catecholamine neurotransmitters, norepinephrine and epinephrine. Heart failure onset is characterized by reduced responsiveness to β-adrenoceptor in cardiac tissues (Choi *et al.*, 1997) and by changes in the expression of GRK2 or β-adrenoceptor kinase1 (βARK1) (Harris *et al.*, 2001). When β-adrenoceptor responsiveness was examined in a completely developed reperfused myocardial infarction model, higher levels of tissue catecholamines and GRK2 were observed in the ischemic epicardium (Boucher *et al.*, 2004). It was found that the density of the β-adrenoceptor in the viable ischemic regions can be modified by GRK2 and catecholamines. Conversely, cardiopulmonary intervention was found to decrease GRK expression (Hagen *et al.*, 2003).

G protein-coupled receptor desensitization is emerging as an important feature of several cardiovascular diseases and GRK2 plays a key role in the regulation of a variety of these receptors and, at the promoter level, cardiac muscle expression is altered in pathological situations such as in CHF (Ramos-Ruiz *et al.*, 2000), portal hypertension (Liu *et al.*, 2005) and in other cells and tissues in these conditions, such as lymphocytes (Iaccarino *et al.*, 2005). GRK-dependent receptor desensitization, and regulation of βAR and other GPCRs, is a rapid process, which appears to involve agonist-promoted receptor phosphorylation by GRKs. GRK-mediated receptor phosphorylation promotes the binding of arrestin proteins, such as β-arrestin (Fredericks *et al.*, 1996). β-arrestin binding uncouples G protein-coupled receptors from their respective G proteins by sterically blocking receptor coupling to G proteins. These same regulatory molecules also regulate GPCR endocytosis, which then involves the processes of transient receptor internalization, intracellular trafficking and resensitization (Ferguson, 2001). Further, the processes involving internalization are known to lead to ERK activation as is the case of the β(2)AR and lysophosphatidic acid receptor (Pitcher *et al.*, 1999). Consequently, the β-arrestins play a crucial role in regulating the responsiveness of many GPCRs. GRK2, along with β-arrestin, also play a key role in resensitizing GPCRs by allowing their dephosphorylation and recycling. Data by Mayor, and colleagues indicate that, besides the uncoupling function of β-arrestin, which together with GRK, directly participates in β(2)AR sequestration and may provide the trigger for resensitization (Mayor *et al.*, 1998). GRK2 levels in myocardium and lymphocytes may be associated with β-AR dysfunction and heart failure severity.

Since GRKs, angiotensin 1 and 2 (AT_1 and AT_2) are found to be increasingly important in hypertension, stroke and heart disease, and association between these receptors and ligands have been found in heart disease and AD (Ge and Barnes, 1996), as well as early β-Amyloid accumulation, *in vitro* (Suo *et al.*, 2004), it is important to consider AD and AD-like pathology in terms of possible inclusion and classification as disorders of the cerebrovasculature. In that regard, the goal of the present study was to determine the cellular, subcellular and ultrastructural distribution and localization of GRK2-immunoreactivity in cases of human AD and in a mammalian model of CBH. In keeping with these

findings, the experimental model of CBH in this study was able to delineate early signs of AD-like pathology prior to the appearance of any detectable plaque deposition. To go further, the expression of GRK-2 also would precede any mild cognitive impairment or frank human AD.

MATERIALS AND METHODS

Human AD and Control Brain Tissues

Unless otherwise stated, all chemicals were obtained from Sigma (Sigma Inc., St. Louis, MO) and were of the highest grade available. All AD cases met CERAD criteria (Hirai *et al.*, 2001) for definite AD corresponding to Braak stages V-VI. Control cases were also assigned by CERAD and Braak criteria and, in some cases, showed age-related neurofibrillary pathology identified with mouse monoclonal AT-8 antibody to phosphorylated cytoskeletal tau protein (Hirai *et al.*, 2001). Control cases otherwise showed no significant neuropathological changes. Hippocampal tissues from patients with AD (n=4, ages 60-85 years), along with age matched controls (n=3, ages 60-85 years) with similar *postmortem* intervals (AD, 3-24 h; controls, 6-36 h), were fixed in 4% buffered paraformaldehyde at 4°C overnight. Following fixation, tissue was trimmed and sectioned for future pre-embedding immunocytochemical detection of the localization of GRK2 immunoreactivity using colloidal gold decoration as we have described earlier (Aliev, *et al.*, 2002a,b; de la Torre and Aliev, 2005).

Chronic Brain Hypoperfusion (CBH) Model

Male Harlan Sprague-Dawley rats (Harlan SD, Indianapolis, IN) 12 months old were used for the 6 month study. Rats were separated at random into two groups and assigned to receive CBH using a 2-vessel occlusion (2-VO, n=22) or sham non-occlusion (S-VO, n=21). Rats in the 2-VO group had the common carotid arteries doubly-ligated for 6 months and rats in the S-VO group had carotid surgery but no occlusion. Previously, we showed that mean arterial blood pressure (MABP), tympanic membrane temperature, hematocrit and arterial blood gases remain stable in the S-VO control and 2-VO animals for as long as 6 months after surgery (de la Torre and Fortin, 1991; de la Torre *et al.*, 1991; 1995). Our previous studies and those of others have reported that two-vessel occlusion will result in a 22%-30% reduction of hippocampal blood flow that will stabilize after several weeks without further reduction (Tsuchiya *et al.*, 1992; de la Torre *et al.*, 1995; Pappas *et al.*, 1996; de la Torre and Aliev, 2005). All procedures conformed to the National Institutes of Health (NIH) guide for the care and use of laboratory animals and were approved by the CASE Ethics Committee for the humane treatment of animals.

Surgery

Surgery was performed under ketamine hydrochloride (100 mg/kg, IM) and methohexital sodium (50 mg/kg, IP) anesthesia as we have described (de la Torre and Aliev, 2005). Atropine sulfate (0.1 mg IM) was administered to prevent respiratory distress. A ventral midline incision was made, and both common carotid arteries were gently separated from the carotid sheath and vagus nerve (de la Torre *et al.*, 1992a). Each common carotid artery was doubly ligated with 5-0 silk suture just below the internal-external carotid bifurcation (2-VO). Sham vessel occlusion (S-VO) non-occluded control rats underwent similar surgery but no vessel ligation. During surgery, body temperature was maintained at 37°C and monitored with a rectal probe, which was attached to a heating bulb. After surgery, rats were placed on a heating pad to maintain body temperature until full recovery. We have reported that the surgical procedure and recovery period do not modify serum electrolytes, pH, lactate, ATP, creatine phosphate or N-acetyl aspartate levels in the brain (de la Torre *et al.*, 1992b; 1995; de la Torre and Aliev, 2005). By the end of experiments, while under terminal anesthesia, the brain was immediately removed and exposed to the buffered solution of 4% paraformaldehyde overnight at 4°C.

Pre-embedding EM Immunocytochemistry

The rat brain was dissected from connective tissue and sliced with a vibratome to produce 100-200 micron thick sections for future pre-embedding electron microscopy (EM) immunogold decoration using the GRK2 polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) as previously described (Aliev *et al.*, 2002a,b). Briefly, sliced human AD and aged-matched control hippocampal tissue were processed for pre-embedding immunogold decoration by GRK2. Rats subjected to 6 months of 2-VO were processed for routine transmission electron microscopy (TEM) and for preembedding immunogold TEM techniques for GRK2 as we have described previously (Aliev *et al.*, 2002a,b). Human and animal hippocampal vibratome sections were treated with hydrogen peroxide (3% in methanol) for 30 min to block endogenous peroxidase activity, and washed twice with Tris-buffered saline (TBS) 50 mmol/l Tris-HCl (pH 7.6), 150 mmol/l NaCl for 10 min at room temperature (RT), incubated in 10% normal goat serum (NGS) for 2 h at RT, and then incubated with a polyclonal antibody against GRK2

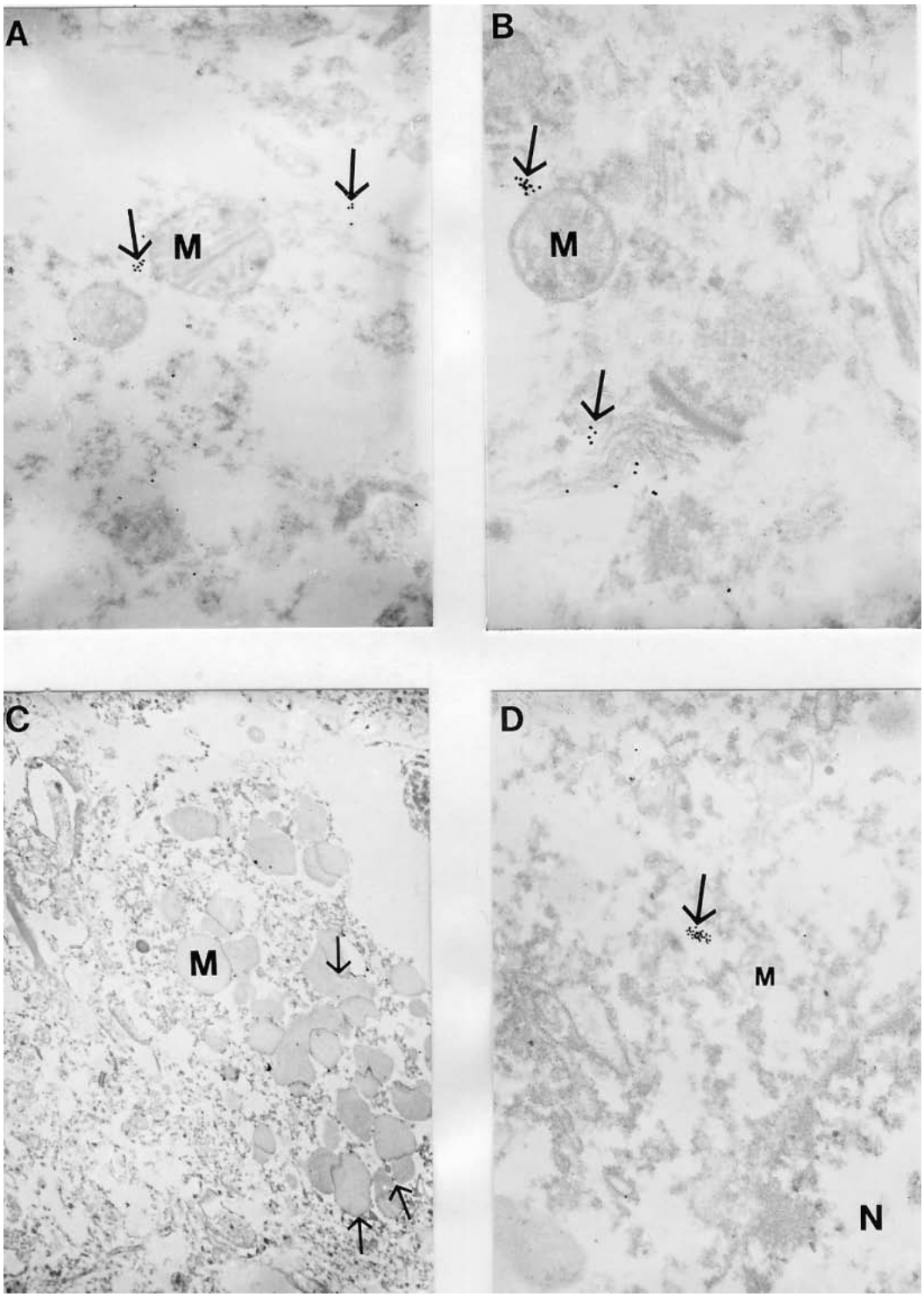

FIGURE 1 Ultrastructural features and localization of GRK2 immunoreactivity (17 nm gold particles) in hippocampus of control (**A, B**) and AD brain (**C, D**) tissues. **A and B** - Control brain hippocampus. The neuronal cell body shows the presence of GRK2 (arrows) attached to the external membrane of partially damaged mitochondria. GRK2 immunoreactivity was seen in the matrix of damaged mitochondria and Golgi cistern, X 30,000 and X 40,000, respectively, A and B. **C** - AD-brain tissue. The neuronal cell body is characterized by the presence of clusters of mitochondria derived lysosomes (M). Diffuse distribution of GRK2 was seen throughout the neuronal cell body (arrows), X 6,000. **D** - AD brain tissue. Glial cell body shows islands of GRK2-immunoreactivity in the matrix of mitochondria derived lysosomes (single arrow), X 20,000. Abbreviations: M-Mitochondria, N-Cell Nucleus.

according to the manufacturer's instruction (1:250) and adapted by us for electron microscopic immunogold labeling procedure, diluted in 1% NGS overnight at 4°C (1:70). After rinses in 10% NGS, gold-conjugated secondary antibody to rabbit IgG (ø=17 nm) was applied for 4 to 24 h and thoroughly rinsed in PBS. Then all samples were postfixed in buffered 2.5% glutaraldehyde for 1 h and again rinsed with PBS. Finally, sections were exposed to 1% osmium tetraoxide for 1 h at RT, rinsed, dehydrated through alcohol and acetone, and flat embedded in Spurr's embedding media. Randomly selected ultrathin sections were stained and viewed in a JEOL 100CX TEM at 80 kV for immunocytochemical detection of GRK2 immunoreactivity.

RESULTS

Ultrastructural localization of GRK2 in the hippocampus of brain sections was determined using preembedding immunogold techniques. Neurons from the hippocampus control brains showed the presence of GRK2-positive gold particles, which were very often attached to the external membrane of partially damaged but normal mitochondria (FIG. 1A-B). Sometimes the presence of GRK2-positive gold particles was seen in the matrix of damaged mitochondria and Golgi cistern (FIG. 1B). In contrast to this observation, AD brain tissue neuronal cell bodies were characterized by the presence of clusters of mitochondria-derived lysosomes (FIG. 1C). Diffuse distribution of GRK2-positive gold particles was localized in and throughout the neuronal cell body (FIG. 1C). We have found that GRK2 overexpression was not unique to neurons. Very often, clusters of GRK2-containing immunopositive gold particles were seen in the matrix of mitochondria-derived lysosomes in glial cells as well (FIG. 1D).

Another cellular compartment of hippocampal tissue also showed the overexpression of GRK2 in the cytoplasm at early reversible stages of damage, but not when damage became irreversible (FIG. 2A-2C). Perivascular pericytes, but not the non-reversibly damaged vascular endothelium, also contained GRK2 immunopositive gold particles in their cytoplasmic matrix (FIG. 2A). Because age factors affect tissue structures, we found the presence of GRK2-containing positive gold particles in the cytoplasmic matrix of brain tissue affected by age-associated damage (FIG. 2B-2C). The perivascular pericytes show accumulation of GRK2-positive gold particles in their matrix, where most gold particles were associated with the neurofilament or neurofibrillary tangle-like structures (FIG. 2B-2C). However, GRK2 was not localized on the membrane and/or in the matrix of

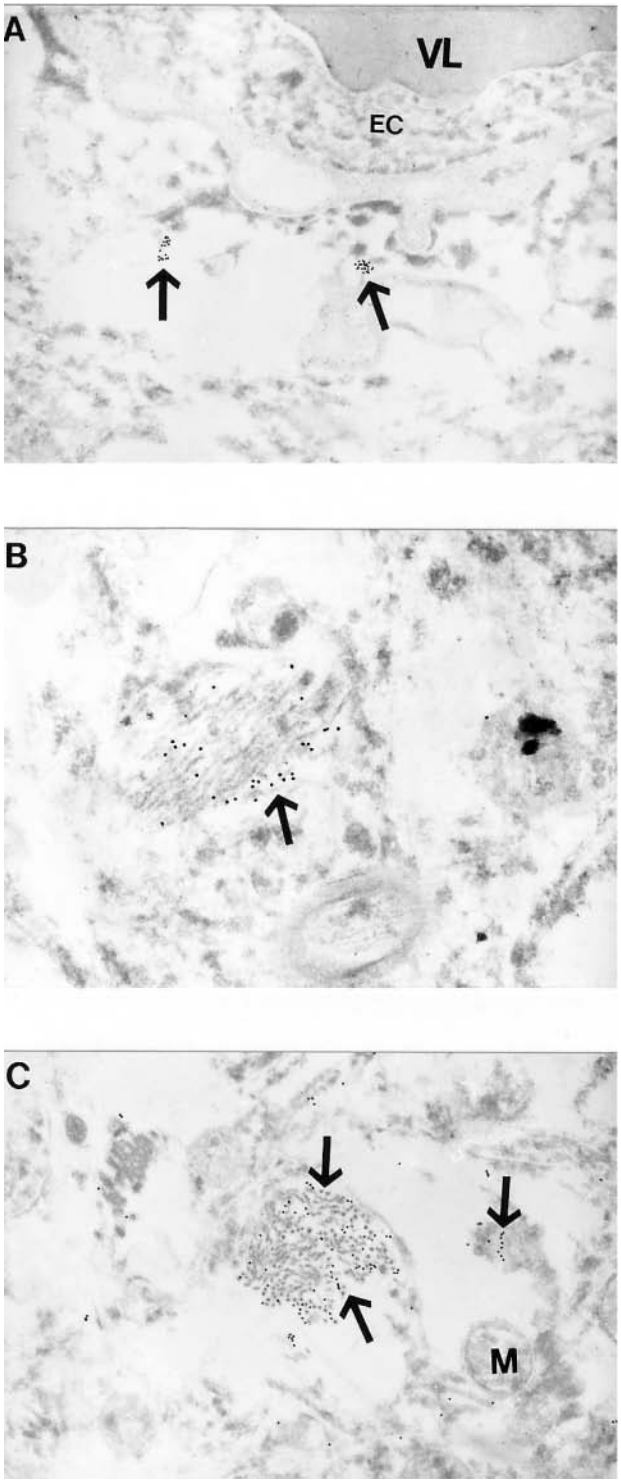

FIGURE 2 Comparative characteristics of the ultrastructural localization of GRK2 containing gold particles in human AD (**A**) and control brain (**B, C**) tissues. **A**- The presence of GRK2 immunoreactivity was seen in the matrix of perivascular pericytes (indicated by single thick arrows) but not in the cytoplasmic matrix of severely damaged vascular endothelium (EC), X 40,000. **B and C** - The neurons close to perivascular spaces show accumulation of GRK2 in their matrix, where most gold particles were associated with the neurofibrillary tangle like structures (arrows). Intact mitochondria (M) did not show any GRK2-immunoreactivity, X 40,000, respectively, B and C.

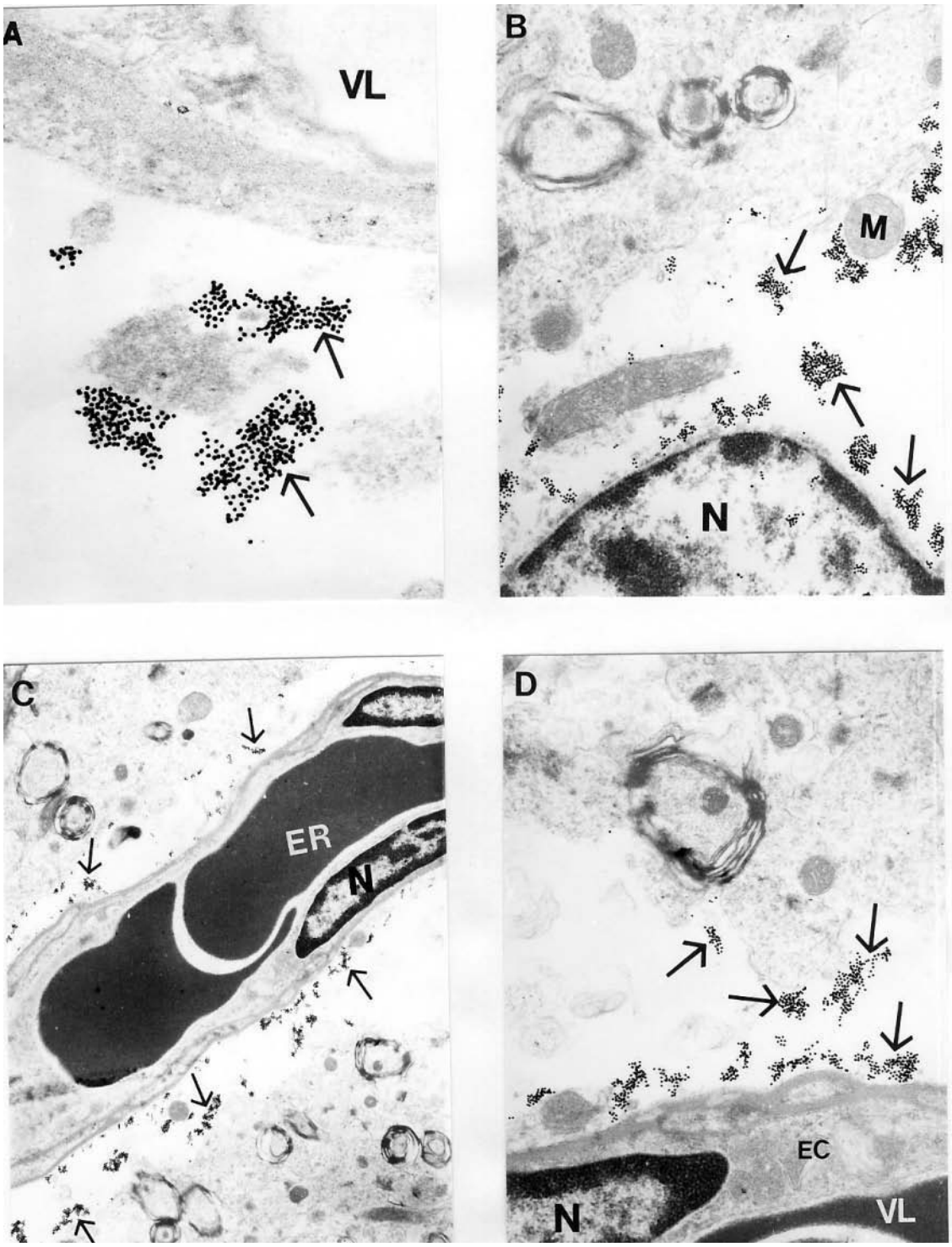

FIGURE 3 Ultrastructural localization of GRK2 immunoreactivity in control (same-operated: **A, B**) and 2-VO (**C, D**) rat brain hippocampal tissue. **A** - Islands of GRK2 was seen in the cytoplasmic matrix of perivascular pericytes (arrows) but not in the vascular EC, X 20,000. **B** - GRK2 immunoreactivity is associated with the edematous portion of the perivascular pericytes cytoplasmic matrix (arrows). Non-damaged mitochondria (M) are free from any GRK2-containing positive gold particles. However, the external membrane of giant mitochondria shows some GRK2 immunopositive gold particles, X 30,000. **C** - Diffuse distribution of GRK2 was seen in the hippocampal tissues from CBH rat brains. GRK2-immunoreactivity was seen throughout the matrix of damaged perivascular pericytes (arrows), X 8,000. **D** - Perivascular regions of this vessel (figure C) under higher magnification. Islands of GRK2-immunoreactivity can be seen associated with the cytoplasmic matrix, where the damage occurs (arrows). Nucleus (N) and non-damaged mitochondria did not show GRK2-immunoreactivity, X 30,000.

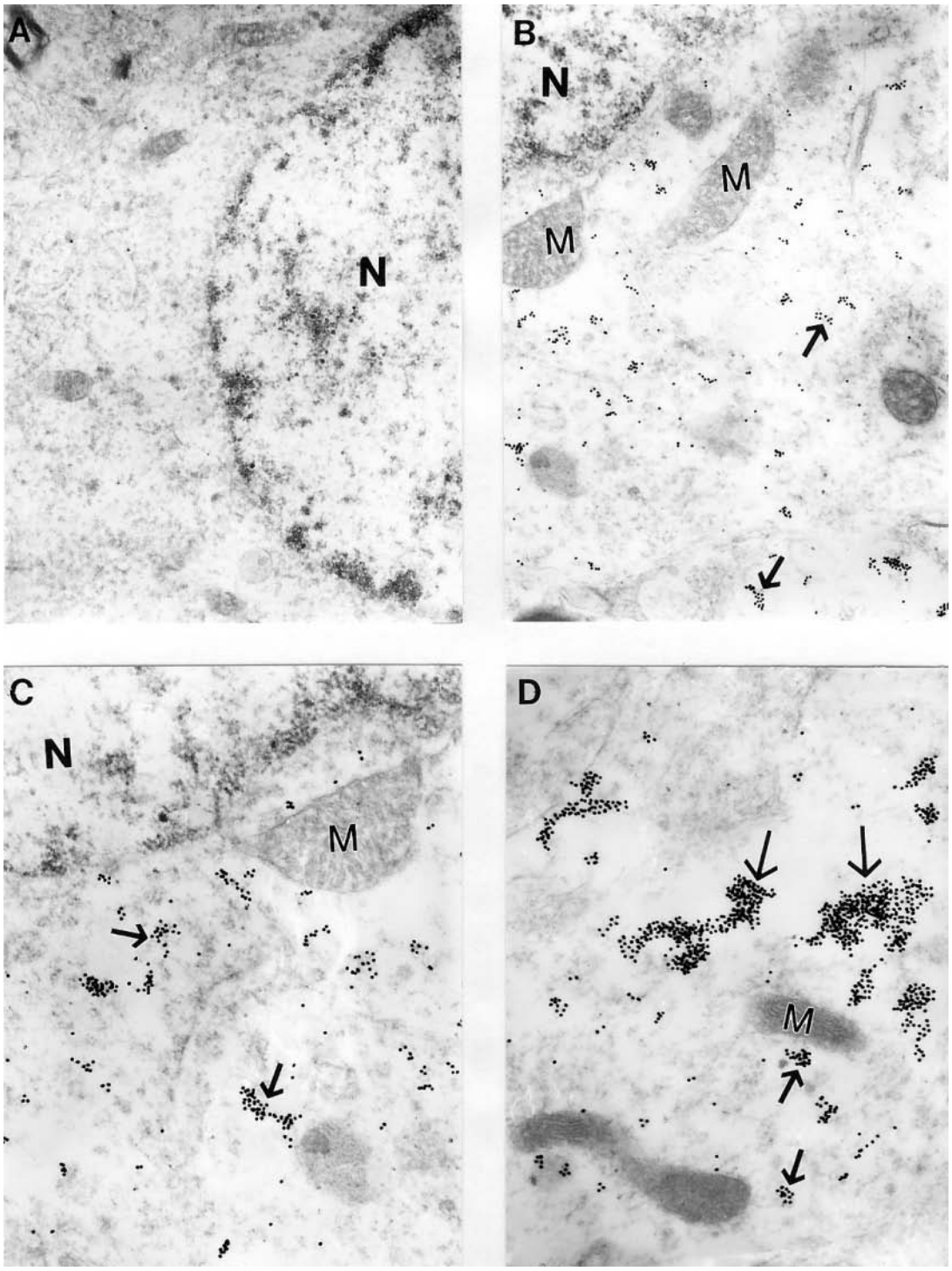

FIGURE 4 Comparative characteristics of the distribution of GRK2-immunoreactivity in the hippocampus of the rat subjected to 2 vessel occlusion. **A** - Non-damaged neurons show absence any of GRK2 positive immunoreactivity in their cytoplasmic matrix, X 15,000. **B** - Hypoperfusion-exposed neurons show GRK2 overexpression (arrows) throughout the cell body. Normal mitochondria (M) were free from any GRK2 positive containing gold particles, X 30,000. **C** - Another neuronal cell body shows islands of GRK2 positive immunodecoration in the external membrane and in the matrix of damaged mitochondria and mitochondria-derived lysosomes (arrows). The cell nucleus is free from any GRK2 containing gold particles, X 40,000. **D** -Neurons at the early stages of severe damage shows the presence of islands of GRK2 containing immunopositive gold particles that associated with mitochondria-derived lysosomal structures (arrows). Non-damaged mitochondria (intact and giant) are free from GRK2 positive immunodecoration, X 40,000.

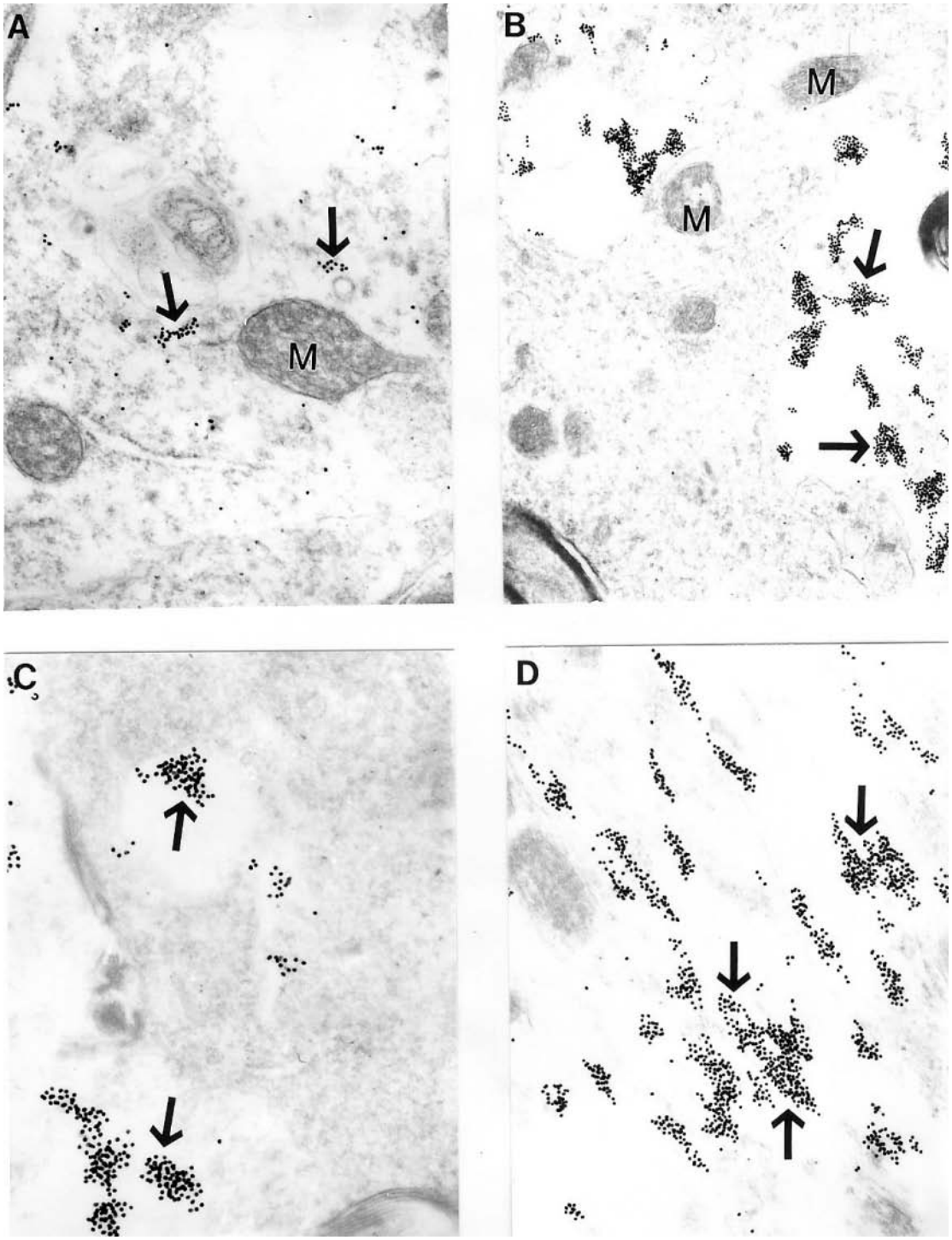

FIGURE 5 Ultrastructural distribution of GRK2-immunoreactivity in rat brain hippocampal tissues exposed to two vessel occlusion. **A** - Neurons show GRK2 positive immunodecoration (arrows) in the external membrane and or in the matrix of damaged but not intact mitochondria (M), X 40,000. **B** - Another neuron containing granular vacuolar degenerative-like structures also shows island of GRK2 containing positive immunogold labeling (single arrow), X 30,000. **C** - The glial cell body shows overexpression of GRK2-immunoreactivity in the matrix of granular vacuolar degenerative structures (single arrow), X 50,000. **D** - Neurons contain GRK2 immunopositive decoration (single arrows) that is associated with neurofilament, X 40,000.

intact mitochondria (FIG. 2C).

In the CBH model, we have found that, generally, ultrastructural localization of GRK2 is characterized by heterogeneous morphology (FIG. 3-5). In the sham operated groups, only occasionally, did we see GRK2 immunoreactivity in the cytoplasmic matrix of perivascular pericytes (FIG. 3A). In some cases, GRK2 was seen in the matrix of partially damaged mitochondria (FIG. 3B). However, in most cases, control brain tissues did not show the overexpression of GRK2-immunoreactivity in any cellular compartment. In sharp contrast to this observation, animals exposed to CBH showed overexpression of immunopositive GRK2-containing gold particles in nearly all cellular compartments of the hippocampus (see FIGs. 3C-3D, 4 and 5). When noted, the overexpression of GRK2-immunoreactivity coexists with damaged cellular components such as mitochondria (FIG. 3C-3D). In these cases, the endothelial cells (EC) occupy only a small portion of the vessel wall. Their nucleus is sharply contracted and chromatin condensation was evident in the abluminal part of the nuclear membrane (FIG. 3C-3D). Moreover, we did not find the overexpression of GRK2 immunoreactivity when the neuron did not show any particular damage (FIG. 4A). CBH exposed neurons had a heterogeneous distribution of GRK2-containing gold particles in their cell body (FIG. 4B-4D). The normal mitochondria did not show any GRK2 immunoreactivity (FIG. 4B-4D). The GRK2-immunoreactivity was associated with the cellular compartment at the beginning, but not at later stages in the damage process (FIG. 4B-4D). Very often, large islands of GRK2 positive gold particles were seen in the cytoplasmic matrix of neuronal and glial cells (FIGs. 4D, 5C). Notably, neurons that contain neurofilaments or neurofibrillary-like structures show abundant GRK2-immunoreactivity (FIG. 5D). Moreover, damaged glial cells also were strongly GRK2 positive gold particles (FIG. 5C). Islands of immunopositive GRK2 containing gold particles appeared to be a permanent feature of these cells (FIG. 5B-5D). Granular vacuolar degenerative structures (GVD) also were strongly positive for GRK2 (FIG. 5B-5C). However, non-damaged cellular compartments, such as mitochondria and/or granular and non-granular endoplasmic reticulum did not show GRK2-immunoreactivity (FIGs. 3-5). After omission of the primary antibody, we did not find GRK2 positive containing gold particles in the any compartment of control, AD or rat brain (data not shown).

DISCUSSION

The main experimental goal of this study was to investigate and better clarify the relationship between GRK2, vascular lesions and the development of pathology in a CBH model and in AD, at the cellular and subcellular level. In that regard, we examined a connection between vascular damage and predisposing factors for AD, where we explored the changes in brain distribution of GRK2 in microvessel wall cells and neurons using a CBH model and in AD cases. Our previous studies, and those of others, have reported that CBH will result in a 22%-30% reduction of hippocampal blood flow that will stabilize after several weeks without further reduction (Tsuchiya *et al.*, 1992; de la Torre *et al.*, 1995; Pappas *et al.*, 1996). This serves as a model of AD, stroke and AD-related pathology. The purpose of the study was to further explore the relationship between vascular events and AD. In that regard, several lines of evidence implicate GRKs and beta-arrestin expression in vascular dementia and after cerebral hypoxia/ischemia (HI) and the differential expression of GRK2 in compensated hypertrophy and heart failure after myocardial infarction in the rat (Theilade *et al.*, 2003). This is the basis for the connections we draw from in making our conclusions.

In this study we have, for the first time, demonstrated that the ultrastructural localization and overexpression of GRK2 occurs during the early stages of damage in aged human and AD cases, and in a two-vessel occlusion model of CBH. This overexpression is an early event, occurring at prodromal stages, before and up to a point when the damage is reversible. Usually, GRK2-immunoreactivity was found to be associated with damaged cellular compartments, especially mitochondria and/or mitochondria-derived lysosomes or granular/vacuolar degenerative structures. The positive reaction was observed in damaged vessel wall cells and their subcellular compartments. We have found that neurons that contain neurofibrillary tangles (NFT) show abundant GRK2 positive immunoreactivity associated with the membranous structures of the NFT. The intensity of the reaction varied from cell to cell and within cellular compartments as well. However, cellular lipofuscin was free from GRK2-immunoreactivity. Nevertheless, there are pathological hallmarks of AD present in harvested neurons, *e.g.*, neuronal inclusions, or those neurons containing structures such as NFTs, granular vacuolar degeneration (GVD), as well as in microvascular wall cells, which show a highly-intense immunopositive reaction. At the late stages of damage, the same areas with abundant GRK2-immunorectivity become absent, which indicates that overexpression of GRK2 can be lost. Thus, this protein can serve as an earlier marker of the brain damage that typifies cerebral-vascular and/or mild cognitive impairment (MCI),

human AD and damage in an animal model that mimics AD. In addition, the overexpression of GRK2-immunoreactivity complements our earlier observation that oxidative stress-induced damage is observed in mitochondria and or other cellular compartments before any amyloid deposition occurs.

A very recent paper by Suo and colleagues report abnormal GRKs at the prodromal and early stages of AD, which is associated with early amyloid beta (Aβ) accumulation *in vitro* and showed that subthreshold Aβ pretreatment disrupts binding of GRKs to activated GPCRs. This led to reduced membrane GRK2/5, which subsequently led to retarded GPCR desensitization, prolonged GPCR signaling, and cellular supersensitivity to GPCR agonists (Suo *et al.*, 2004). The same group went on to report in a transgenic mouse model of AD, where the double-mutant form of APP695 is overexpressed under the regulation of a prion promoter, the overexpression of GRK2, and to a lesser extent GRK5, occurred in the cytosolic *vs* membrane fractions from hippocampal and cortical brain homogenates with increasing age and plaque deposition. While the *in vitro* observation is quite likely to occur within microglia, the increase in the overexpression of GRK2 and GRK5 in the cytosol of neurons was not differentiated in this study. Here, we report the subcellular localization of GRK2 in neurons and the earlier involvement of vascular lesions, *in vivo*, as a key event in this process and, thus, in the development of human AD and AD-like pathology. Data to support this notion has been explored in various animal models (de la Torre *et al.*, 1992a; Pappas *et al.*, 1996). In this regard, we have demonstrated that the abnormal mitochondria (mitochondria with electron dense matrix, mitochondrial-derived lysosomes) and lipofuscin appears to be features of damaged hippocampal neurons in aged Tg (+) mice and human AD, and suggest a direct relationship between vascular abnormalities, blood-brain barrier (BBB) breakdown, neuronal loss and amyloid deposition (Aliev *et al.*, 2002a,b; 2003; 2004; Zhu *et al.*, 2004).

Our *in vivo* data should be discussed in light of the Suo study (Suo *et al.*, 2004), where we show a similar effect with our model, but attribute the overexpression of GRK2 to oxidative stress and earlier events other than Aβ deposition. While the effect observed in the Suo study was with subthreshold levels of the protein, which may reflect an early event as well, the use of total homogenates from the transgenic model of Aβ overexpression does not indicate which cells are affected, but argues that glia or other immunologic cells may explain that data (Suo *et al.*, 2004). However, since Aβ deposition is a later hallmark lesion in AD, we suspect that the appearance of Aβ along with the loss of GRK2-immunoreactivity may be linked somehow, but the role of Aβ on GRK2 translocation may be cell-specific and has not been characterized. In this regard, the appearance Aβ is unlikely to be the primary predisposing factor for GRK2 overexpression, as Aβ deposition occurs much later in the disease process, and any cytotoxicity may emanate from mechanisms other than amyloid directly, as earlier events seem to be more crucial in the disease process (Aliev *et al.*, 2004; de la Torre and Aliev, 2005).

Our study demonstrates an increase in GRK2 localization to the cytosol, but in particular to subcellular components and only those components with evident damage or pathology. When GRKs are recruited to the cell membrane simultaneous inhibition of signaling follows. Therefore, the sequestration of GRKs to subcellular locations may indicate a compensatory adaptation to AD. However, other studies suggest that GRKs may have more diverse protein/protein cellular interactions, and the β-arrestins together with GRKs play a crucial role in regulating the responsiveness of many GPCRs. Further, GRK2 levels in myocardium and lymphocytes may be associated with β-AR dysfunction as well, which is one area that should be addressed in AD. One explanation for the subsequent loss of GRK2 may lie in the ability of Aβ to act as a bioflocculant (Robinson and Bishop, 2002) and in this study Aβ may have a role in the sequestration of GRK2, thereby limiting downstream phosphorylation events, as well, or lead to translocation of GRK2 to the cytosol. Regardless, the reduced availability of GRK2 and β-arrestins to regulate GPRC signaling most likely would lead to a state of GPCR supersensitization, thereby increasing response to neuropeptides, neurotransmitters, chemokines, and many other molecules, all of which could have deleterious consequences. Conversely, it may be plausible that increased GRK2 expression, and particularly localization, would impart a compensatory or survival response to excitotoxicity, a claim made for Aβ as well. GRK2, and the action of specific phosphatases, have been implicated in other neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) (Hu *et al.*, 2003). In this regard, there are numerous parallels that can be made between neurodegenerative and cerebrovascular disorders with heart disease and systemic vascular disorders, such as AD (Aliev *et al.*, 2003; 2004; Paris *et al.*, 2003).

Of importance in these GRK2 studies in AD are the vascular endothelium, neurons and glia, which all are able to synthesize, store and release reactive oxygen species (ROS), nitric oxide (NO), and endothelin-1 (ET-1), a vasoactive peptide, in response to certain stimuli. Their contribution to the pathophysiology

of stroke or stroke-like conditions and AD cannot be understated. For example, ET-1 can elicit several responses; it activates EC nitric-oxide synthase (NOS) via G-protein betagamma subunits signaling through protein kinase B/Akt (Liu *et al.*, 2003) as well as prolonged physiologic responses, including mitogen-activated protein kinase (MAPK) activation (Sarnago *et al.*, 1999) and c-Jun NH_2-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) in cultured animal cells and *in vivo* (Kwon *et al.*, 2004). MAP kinases have been long associated with AD, and ERK activation may be another important early event, perhaps downstream from GRK2 activation. Interestingly, these pathways also have been implicated in cell cycle dysregulation in human AD cases (Roder *et al.*, 1993; Zhu *et al.*, 2002; Obrenovich *et al.*, 2005). Recently we demonstrated that since successful dysregulation of the cell cycle is also the hallmark of a neoplastic change, early cell-cycle pathophysiology in AD may recruit oncogenic signal transduction mechanisms and, hence, could be viewed as pseudo-neoplastic transformation, which is eventually aborted (Raina *et al.*, 2001). Further, it has been shown that phosphorylation of GRK2 by MAPK also triggers turnover and GRK2 degradation through the proteasome pathway, in which GRK2 is targeted for proteolysis by ß-arrestin function, Src-mediated phosphorylation and by Raf (Elorza *et al.*, 2003). ET-1 is produced by multiple cells and is differentially coupled to G proteins (Arai *et al.*, 2003) in response to hypertrophic responses *in vitro* and in the development of heart failure *in vivo* (Sakai *et al.*, 1996; Feldman, 2002). Nevertheless, the endothelin A and B receptors (ET_A-R and ET_B-R) undergo desensitization, most likely also through GRK2 (Freedman *et al.*, 1997).

AD cases are dominated by abnormal mitochondria when compared to controls, where deleted mtDNA is a main feature (Aliev *et al.*, 2004; Zhu *et al.*, 2004) and mitochondrial-derived lysosomes in regions closely associated with lipofuscin, which suggests that proliferation, deletion and duplication of mtDNA occurs in mitochondria in human AD and transgenic mouse models of neurodegeneration (Aliev *et al.*, 2002a,b; 2003; 2004; Zhu *et al.*, 2004). *In situ* hybridization with a chimeric mouse and human mitochondrial cDNA probes for the 5kb common deletion indicated that the deletion is increased at least 3 fold in AD cases as compared to controls and in yeast artificial chromosome (YAC) AβPP mouse hippocampus (Hirai *et al.*, 2001; Aliev *et al.*, 2002a,b; 2003; 2004), which is strongly and positively correlated (r=0.934) with the marker of DNA oxidation, 8-OH deoxyguanosine. While markers of oxidative stress were not explored in this study, common features of the mitochondrial abnormalities are seen in the brain during chronic hypoperfusion and AD progression. These findings indicate that the mitochondrial DNA overproliferation and/or deletion are key initiating factors for disruption of the BBB and the development of pathology, and GRK2 immunoreactivity overexpression would be coincident with these processes.

Earlier in a 2-VO model (de la Torre and Aliev, 2005), we reported that ultrastructural examination of hippocampal CA1 capillaries in rats revealed a smaller size EC containing damaged mitochondria, characterized by transformation of lysosomal structures within the EC and in the perivascular area (de la Torre and Aliev, 2005). Along with mitochondrial abnormalities these changes appeared to be associated with amyloid deposition found surrounding the capillary vessel wall. EM immunostaining showed almost an absence of endothelial-specific NOS (eNOS)-containing positive gold particles in the matrix of the vascular endothelium, which is in contrast to increased labeling in the cytoplasmic matrix of perivascular cells, together with electron-dense mitochondria, indicative of a hypoxic insult (de la Torre and Aliev, 2005). Immunoreactive eNOS-containing positive gold particles were found markedly expressed in hippocampal neurons and in glial cells, when compared to non-occluded controls (de la Torre and Aliev, 2005). Of interest is the comparison between the eNOS overexpression pattern to that of GRK2 (Liu *et al.*, 2005), which has the same pattern as our previous observation with eNOS.

The EM findings in rat hippocampus after CBH also support the general hypothesis that chronic oxidative stress caused the EC structural changes, and the mitochondrial and immunoreactive eNOS changes, since such changes were observed only in 2-VO rats. In addition, previously we demonstrated that oxidative damage is the earlier event in AD (Nunomura *et al.*, 2001). The present findings support our working hypothesis that oxidative stress-induced vascular changes, such as an abnormality in vascular NO, is an important molecule in spatial memory functions, at least in this CBH model. Further, this damage coexists with overexpression of GRK2-immunoreactivity. Therefore, chronic oxidative stress-mediated inhibition of eNOS may coexist with the early overexpression of GRK2-immunoreactivity and would appear to support a compensatory role or reaction in brain tissue to potentially mitigate chronic injury stimuli, such as oxygen depletion and nutrient deficiency or imbalance in metabolic homeostasis found in 2-VO conditions (de la Torre and Aliev, 2005). This data supports the present observation that it is chronic injury stimuli that not only initiates damage and compensatory changes

but accelerates brain damage in tissues, which can contribute to some types of mental retardation and cognition deficits involving the consequence of Aβ accumulation in the brain. The connection to a cerebrovascular component to AD is further borne out in other rat studies, where differentially expressed cardiac GRK2 expression and activity has been found. Here, GRK2 expression has been reported to be inhibited in animals with cardiac hypertrophy without heart failure, whereas animals with heart failure had elevated GRK2 (Theilade *et al.*, 2003). This expression pattern indicates differential regulation in hypertrophic non-failing and hypertrophic failing hearts. Nevertheless, it is now a commonly held belief that GRKs may likely become effective therapeutic targets for heart disease (Iaccarino and Koch, 2003) and should be considered for AD or related neuropathology as well.

It is tempting to contemplate how GRK2, and its cognate regulatory proteins, may together, offer new insight into a unifying hypothesis of AD, where these proteins may play a pivotal role, one that links the many phenomenological observations into a conceptual framework and add to a growing body of evidence favoring the reclassification of AD as a, largely, cerebrovascular disorder. For example, one clue may lie in the finding that GRK2 is a microtubule-associated protein, and tubulin was identified as a novel GRK2 substrate (Pitcher *et al.*, 1998; 1999). These results suggest that tubulin is most likely phosphorylated *in situ* by GRK2 and that the phosphorylation may affect the interaction of microtubules with microtubule-associated proteins (MAPs) (Yoshida *et al.*, 2003). Phosphor-ylation by GRKs may have downstream consequences for neuronal cell death and perhaps contribute to the hyperphosphorylated state of tau protein, as seen in AD or in earlier events as well, perhaps one that would predispose to neuronal toxicity via NFT formation. However, recent work has revealed potential phosphorylation-independent regulation of GPCRs by GRK2 and GRK3 (Willets *et al.*, 2003), and GRK2 was not found to phosphorylate MAPs under conditions where MAPs were already well-phosphorylated by endogenous kinases, which copurified with tubulin (Haga *et al.*, 1998). Nevertheless, the role of this kinase in early phosphorylation of tau cannot be discounted. Therefore, GRK2-mediated desensitization, may involve many diverse mechanisms. However, the role of GRKs, may be a pivotal one in AD pathology, as GRK-mediated desensitization, in the absence of phosphorylation and arrestin binding, has been reported for metabotropic glutamate receptor 1 (mGluR1), the gamma-aminobutyric acid B receptors (Sallese *et al.*, 2000), and regulation of metabotropic glutamate receptor 5 function and expression (Lea *et al.*, 2003), both of which have been implicated in AD pathogenesis. Therefore, GRKs may hold hope as therapeutic targets for AD and related pathologies. Taken together, this line of evidence strongly supports our findings of a role for GRK2 as an earlier marker in AD pathogenesis and may couple the contribution of oxidative stress, NO, eNOS and ET-1 to the pathobiology of AD.

Our findings also suggest a role for GRK2 as a GPCR signal transducer, which may mediate the effects of GPCR activation on cytoskeletal structure and function in AD. Our study is the first to demonstrate the cellular and subcellular localization and offer *in vivo* evidence for GRK2 activation as an early sign of cerebrovascular aging complications in age-associated diseases involving cerebrovascular abnormalities, neurodegeneration and cognitive impairment before any amyloid deposition can be seen. GRKs as physiological regulators could become an appropriate target for future pharmacological intervention. Moreover, determining the mechanisms of the damage, or potential protective nature of GRK2 receptor antagonist, may provide crucial information in the development of new and more effective therapies for stroke and AD patients. Expanding future research in this direction may enable GRKs to serve as a new target for the treatment approach to AD, stroke, mild cognitive impairment or related cerebrovascular disorders.

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References

Aliev G, D Seyidova, ML Neal, J Shi, BT Lamb, SL Siedlak, HV Vinters, E Head, G Perry, JC LaManna, RP Friedland and CV Cotman (2002a) Atherosclerotic lesions and mitochondria DNA deletions in brain microvessels as a central target for the development of human AD and AD-like pathology in aged transgenic mice. *Ann. NY Acad. Sci.* **977**, 45-64.

Aliev G, MA Smith, D Seyidova, ML Neal, BT Lamb, A Nunomura, EK Gasimov, HV Vinters, G Perry, JC LaManna and RP Friedland (2002b) The role of oxidative stress in the pathophysiology of cerebrovascular lesions in Alzheimer's disease. *Brain*

Pathol. **12**, 21-35.

Aliev G, MA Smith, ME Obrenovich, JC de la Torre and G Perry (2003) Role of vascular hypoperfusion-induced oxidative stress and mitochondria failure in the pathogenesis of Azheimer disease. *Neurotoxicity Res.* **5**, 491-504.

Aliev G, MA Smith, JC de la Torre and G Perry (2004) Mitochondria as a primary target for vascular hypoperfusion and oxidative stress in Alzheimer's disease. *Mitochondrion* **4**, 649-663.

Arai K, Y Maruyama, M Nishida, S Tanabe, S Takagahara, T Kozasa, Y Mori, T Nagao and H Kurose (2003) Differential requirement of G alpha12, G alpha13, G alphaq and G beta gamma for endothelin-1-induced c-Jun NH_2-terminal kinase and extracellular signal-regulated kinase activation. *Mol. Pharmacol.* **63**, 478-488.

Arriza JL, TM Dawson, RB Simerly, LJ Martin, MG Caron, SH Snyder and RJ Lefkowitz (1992) The G-protein-coupled receptor kinases beta ARK1 and beta ARK2 are widely distributed at synapses in rat brain. *J. Neurosci.* **12**, 4045-4055.

Boucher M, S Nim, C de Montigny and G Rousseau (2004) Alterations of beta-adrenoceptor responsiveness in postischemic myocardium after 72 h of reperfusion. *Eur. J. Pharmacol.* **495**, 185-191.

Carman CV, MP Lisanti and JL Benovic (1999) Regulation of G protein-coupled receptor kinases by caveolin. *J. Biol. Chem.* **274**, 8858-8864.

Choi DJ, WJ Koch, JJ Hunter and HA Rockman (1997) Mechanism of beta-adrenergic receptor desensitization in cardiac hypertrophy is increased beta-adrenergic receptor kinase. *J. Biol. Chem.* **272**, 17223-17229.

de la Torre JC and G Aliev (2005) Inhibition of vascular nitric oxide after rat chronic brain hypoperfusion: spatial memory and immunocytochemical changes. *J. Cereb. Blood Flow Metab.* **25**, 663-672.

de la Torre JC and T Fortin (1991) Partial or global rat brain ischemia. The SCOT model. *Brain Res. Bull.* **26**, 365-372.

de la Torre JC, J Saunders, T Fortin, K Butler and M Richard (1991) Return of ATP/PCr and EEG after 75 minutes of global brain ischemia. *Brain Res.* **542**, 71-76.

de la Torre JC, T Fortin, GA Park, KS Butler, P Kozlowski, BA Pappas, H de Socarraz, JK Saunders and MT Richard (1992a) Chronic cerebrovascular insufficiency induces dementia-like deficits in aged rats. *Brain Res.* **582**, 186-195.

de la Torre JC, T Fortin, GA Park, JK Saunders, P Kozlowski, K Butler, H de Socarraz, BA Pappas and M Richard (1992b) Aged but not young rats develop metabolic, memory deficits after chronic brain ischaemia. *Neurol. Res.* **14**, 177-180.

de la Torre JC, K Butler, P Kozlowski, T Fortin and JK Saunders (1995) Correlates between nuclear magnetic resonance spectroscopy, diffusion weighted imaging, and CA1 morphometry following chronic brain ischemia. *J. Neurosci. Res.* **41**, 238-245.

Elorza A, P Penela, S Sarnago and F Mayor Jr (2003) MAPK-dependent degradation of G protein-coupled receptor kinase 2. *J. Biol. Chem.* **278**, 29164-29173.

Erdtmann-Vourliotis M, P Mayer, S Ammon, U Riechertand and V Hollt (2001) Distribution of G-protein-coupled receptor kinase (GRK) isoforms 2, 3, 5 and 6 mRNA in the rat brain. *Mol. Brain Res.* **95**, 129-137.

Feldman RD (2002) Deactivation of vasodilator responses by GRK2 overexpression: a mechanism or the mechanism for hypertension? *Mol. Pharmacol.* **61**, 707-709.

Ferguson SS (2001) Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol. Rev.* **53**, 1-24.

Fredericks ZL, JA Pitcher and RJ Lefkowitz (1996) Identification of the G protein-coupled receptor kinase phosphorylation sites in the human beta2-adrenergic receptor. *J. Biol. Chem.* **271**, 13796-13803.

Freedman NJ, AS Ament, M Oppermann, RH Stoffel, ST Exum and RJ Lefkowitz (1997) Phosphorylation and desensitization of human endothelin A and B receptors. Evidence for G protein-coupled receptor kinase specificity. *J. Biol. Chem.* **272**, 17734-17743.

Ge J and NM Barnes (1996) Alterations in angiotensin AT_1 and AT_2 receptor subtype levels in brain regions from patients with neurodegenerative disorders. *Eur. J. Pharmacol.* **22**, 299-306.

Haga K, H Ogawa, T Haga and H Murofushi (1998) GTP-binding-protein-coupled receptor kinase 2 (GRK2) binds and phosphorylates tubulin. *Eur. J. Biochem.* **255**, 363-368.

Hagen SA, AL Kondyra, HP Grocott, H El-Moalem, D Bainbridge, JP Mathew, MF Newman, JG Reves, DA Schwinn and MM Kwatra (2003) Cardiopulmonary bypass decreases G protein-coupled receptor kinase activity and expression in human peripheral blood mononuclear cells. *Anesthesiology* **98**, 343-348.

Harris CA, TT Chuang and CA Scorer (2001) Expression of GRK2 is increased in the left ventricles of cardiomyopathic hamsters. *Basic Res. Cardiol.* **96**, 364-368.

Hata JA and WJ Koch (2003) Phosphorylation of G protein-coupled receptors: GPCR kinases in heart disease. *Mol. Interv.* **3**, 264-272.

Hirai K, G Aliev, A Nunomura, H Fujioka, RL Russell, CS Atwood, AB Johnson, Y Kress, HV Vinters, M Tabaton, S Shimohama, AD Cash, SL Siedlak, PL Harris, PK Jones, RB Petersen, G Perry and MA Smith (2001) Mitochondrial abnormalities in Alzheimer's disease. *J. Neurosci.* **21**, 3017-3023.

Hu JH, H Zhang, R Wagey, C Krieger and SL Pelech (2003) Protein kinase and protein phosphatase expression in amyotrophic lateral sclerosis spinal cord. *J. Neurochem.* **85**, 432-442.

Iaccarino G and WJ Koch (2003) Transgenic mice targeting the heart unveil G protein-coupled receptor kinases as therapeutic targets. *Assay Drug Dev. Technol.* **1**, 347-355.

Iaccarino G, E Barbato, E Cipolletta, V De Amicis, KB Margulies, D Leosco, B Trimarco and WJ Koch (2005) Elevated myocardial and lymphocyte GRK2 expression and activity in human heart failure. *Eur. Heart J.* **26**, 1752-1758.

Iacovelli L, R Franchetti, D Grisolia and A De Blasi (1999) Selective regulation of G protein-coupled receptor-mediated signaling by G protein-coupled receptor kinase 2 in FRTL-5 cells: analysis of thyrotropin, alpha(1B)-adrenergic, and A(1) adenosine receptor-mediated responses. *Mol. Pharmacol.* **56**, 316-324.

Kwon S, LH Fang, B Kim, TS Ha, SJ Lee and HY Ahn (2004) p38 Mitogen-activated protein kinase regulates vasoconstriction in spontaneously hypertensive rats. *J. Pharmacol. Sci.* **95**, 267-272.

Lea P, MT Faden and IA Faden (2003) Modulation of metabotropic glutamate receptors as potential treatment for acute and chronic neurodegenerative disorders. *Drug News Perspect.* **16**, 513-522.

Liu S, RT Premont, CD Kontos, S Zhu and DC Rockey (2003) Endothelin-1 activates endothelial cell nitric-oxide synthase via heterotrimeric G-protein betagamma subunit signaling to protein jinase B/Akt. *J. Biol. Chem.* **278**, 49929-49935.

Liu S, RT Premont, CD Kontos, S Zhu and DC Rockey (2005) A crucial role for GRK2 in regulation of endothelial cell nitric oxide synthase function in portal hypertension. *Nat. Med.* **11**, 952-958.

Lodowski DT, JA Pitcher, WD Capel, RJ Lefkowitz and JJ Tesmer (2003) Keeping G proteins at bay: a complex between G pro-

tein-coupled receptor kinase 2 and G betagamma. *Science* **300**, 1256-1262.

Mayor F Jr, P Penela and A Ruiz-Gomez (1998) Role of G protein-coupled receptor kinase 2 and arrestins in beta-adrenergic receptor internalization. *Trends Cardiovasc. Med.* **8**, 234-240.

Nunomura A, G Perry, G Aliev, K Hirai, A Takeda, EK Balraj, PK Jones, H Ghanbari, T Wataya, S Shimohama, S Chiba, CS Atwood, RB Petersen and MA Smith (2001) Oxidative damage is the earliest event in Alzheimer disease. *J. Neuropathol. Exp. Neurol.* **60**, 759-767.

Obrenovich ME, AK Raina, O Ogawa, CS Atwood, L Morelli and MA Smith (2005) *Cell-cycle Mechanisms in Neuronal Death: a New Beginning or a Final exit?* (Nicoletti, Ferdinando, Agata Copani, Eds.) (Landes Bioscience) 8, 1-15.

Pappas BA, JC de la Torre, CM Davidson, MT Keyes and T Fortin (1996) Chronic reduction of cerebral blood flow in the adult rat: late-emerging CA1 cell loss and memory dysfunction. *Brain Res.* **708**, 50-58.

Paris D, J Humphrey, A Quadros, N Patel, R Crescentini, F Crawford and M Mullan (2003) Vasoactive effects of Abeta in isolated human cerebrovessels and in a transgenic mouse model of Alzheimer's disease: role of inflammation. *Neurol. Res.* **25**, 642-651.

Penela P, M Alvarez-Dolado, A Munoz and F Mayor Jr (2000) Expression patterns of the regulatory proteins G protein-coupled receptor kinase 2 and beta-arrestin 1 during rat postnatal brain development: effect of hypothyroidism. *Eur. J. Biochem.* **267**, 4390-4396.

Pitcher JA, RA Hall, Y Daaka, J Zhang, SS Ferguson, S Hester, S Miller, MG Caron, RJ Lefkowitz and LS Barak (1998) The G protein-coupled receptor kinase 2 is a microtubule-associated protein kinase that phosphorylates tubulin. *J. Biol. Chem.* **273**, 12316-12324.

Pitcher JA, JJ Tesmer, JL Freeman, WD Capel, WC Stone and RJ Lefkowitz (1999) Feedback inhibition of G protein-coupled receptor kinase 2 (GRK2) activity by extracellular signal-regulated kinases. *J. Biol. Chem.* **274**, 34531-34534.

Raina AK, A Hochman, X Zhu, CA Rottkamp, A Nunomura, SL Siedlak, H Boux, RJ Castellani, G Perry and MA Smith (2001) Abortive apoptosis in Alzheimer's disease. *Acta Neuropathol. (Berl.)* **101**, 305-310.

Ramos-Ruiz R, P Penela, RB Penn and F Mayor Jr (2000) Analysis of the human G protein-coupled receptor kinase 2 (GRK2) gene promoter: regulation by signal transduction systems in aortic smooth muscle cells. *Circulation* **101**, 2083-2089.

Robinson SR and GM Bishop (2002) Abeta as a bioflocculant: implications for the amyloid hypothesis of Alzheimer's disease. *Neurobiol. Aging* **23**, 1051-1072.

Roder HM, PA Eden and VM Ingram (1993) Brain protein kinase PK40erk converts TAU into a PHF-like form as found in Alzheimer's disease. *Biochem. Biophys. Res. Commun.* **193**, 639-647.

Sakai S, T Miyauchi, M Kobayashi, I Yamaguchi, K Goto and Y Sugishita (1996) Inhibition of myocardial endothelin pathway improves long-term survival in heart failure. *Nature* **384**, 353-355.

Sallese M, L Salvatore, E D'Urbano, G Sala, M Storto, T Launey, F Nicoletti, T Knopfel and A De Blasi (2000) The G-protein-coupled receptor kinase GRK4 mediates homologous desensitization of metabotropic glutamate receptor 1. *FASEB J.* **14**, 2569-2580.

Sarnago S, A Elorza and F Mayor Jr (1999) Agonist-dependent phosphorylation of the G protein-coupled receptor kinase 2 (GRK2) by Src tyrosine kinase. *J. Biol. Chem.* **274**, 34411-34416.

Suo Z, M Wu, BA Citron, GT Wong and BW Festoff (2004) Abnormality of G-protein-coupled receptor kinases at prodromal and early stages of Alzheimer's disease: an association with early beta-amyloid accumulation. *J. Neurosci.* **24**, 3444-3452.

Thebaud B, F Ladha, ED Michelakis, M Sawicka, G Thurston, F Eaton, K Hashimoto, G Harry, A Haromy, G Korbutt and SL Archer (2005) Vascular endothelial growth factor gene therapy increases survival, promotes lung angiogenesis, and prevents alveolar damage in hyperoxia-induced lung injury: evidence that angiogenesis participates in alveolarization. *Circulation* **112**, 2477-2486.

Theilade J, C Strom, T Christiansen, S Haunso and SP Sheikh (2003) Differential G protein receptor kinase 2 expression in compensated hypertrophy and heart failure after myocardial infarction in the rat. *Basic Res. Cardiol.* **98**, 97-103.

Tsuchiya M, K Sako, S Yura and Y Yonemasu (1992) Cerebral blood flow and histopathological changes following permanent bilateral carotid artery ligation in Wistar rats. *Exp. Brain Res.* **89**, 87-92.

Ungerer M, K Kessebohm, K Kronsbein, MJ Lohse and G Richardt (1996) Activation of beta-adrenergic receptor kinase during myocardial ischemia. *Circ. Res.* **79**, 455-460.

Willets JM, RA Challiss and SR Nahorski (2003) Non-visual GRKs: are we seeing the whole picture? *Trends Pharmacol. Sci.* **24**, 626-633.

Yoshida N, K Haga and T Haga (2003) Identification of sites of phosphorylation by G-protein-coupled receptor kinase 2 in beta-tubulin. *Eur. J. Biochem.* **270**, 1154-1163.

Zhu X, HG Lee, AK Raina, G Perry and MA Smith (2002) The role of mitogen-activated protein kinase pathways in Alzheimer's disease. *Neurosignals* **11**, 270-281.

Zhu X, MA Smith, G Perry and G Aliev (2004) Mitochondrial failures in Alzheimer's disease. *Am. J. Alzheimer's Dis. Demen.* **19**, 345-352.