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Insights into cerebrovascular complications and Alzheimer disease through the selective loss of GRK2 regulation

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Abstract

Alzheimer disease (AD) and stroke are two leading causes of age-associated dementia. Increasing evidence points to vascular damage as an early contributor to the development of AD and AD-like pathology. In this review, we discuss the role of G protein-coupled receptor kinase 2 (GRK2) as it relates to individuals affected by AD and how the cardiovasculature plays a role in AD pathogenesis. The possible involvement of GRKs in AD pathogenesis is an interesting notion, which may help bridge the gap in our understanding of the heart–brain connection in relation to neurovisceral damage and vascular complications in AD, since kinases of this family are known to regulate numerous receptor functions both in the brain, myocardium, and elsewhere. The aim of this review is to discuss our findings of overexpression of GRK2 in the context of the early pathogenesis of AD, because increased levels of GRK2 immunoreactivity were found in vulnerable neurons of AD patients as well as in a two-vessel occlusion (2-VO) mammalian model of ischaemia. Also, we consider the consequences for this overexpression as a loss of G-protein coupled receptor (GPCR) regulation, as well as suggest a potential role for GPCRs and GRKs in a unifying theory of AD pathogenesis, particularly in the context of cerebrovascular disease. We synthesize this newer information and attempt to put it into context with GRKs as regulators of diverse physiological cellular functions that could be appropriate targets for future pharmacological intervention.

Keywords: GRK2 • Alzheimer disease • cerebrovascular disease

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 [1]. To date, seven mammalian serine/threonine protein GRKs, which comprise the GRK family, have been described and six members cloned. GRK2 and 3 form the second subfamily, namely, β-adrenergic receptor kinase (βARK) subfamily, members of which are known to phosphorylate and regulate

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agonist-occupied or constitutively active GPCRs [2]. The known homology domains of GRK2, which when recruited to the cell membrane, modulate the simultaneous inhibition of signalling by G-alpha, G-beta and G-gamma subunits. Further, recent studies suggest that GRKs, particularly GRK2, may have more diverse protein/ protein cellular interactions. This notion is based on the identification of a consensus caveolin-binding motif within the pleckstrin homology domain of GRK2 [3].

We speculate that an imbalance in the activity of nitric oxide synthase (NOS) isoforms, endothelin-1 (ET-1) and oxidative stress, as evidenced by several biomarkers of this damage, along with mitochondrial DNA (mtDNA) aberrations and the imbalance of mitochondrial enzymes in vascular wall cells and in neurons, leads to an inadequacy in the antioxidant response capacity to sufficiently abate metabolic and oxidative insults which are two key initial features in the brains of stroke and AD patients [5–7]. We further hypothesize that GRK2 plays a role in these deleterious processes [4]. Additionally, the involvement of chronic brain hypoperfusion (CBH) and physical distortion of the surrounding tissue exacerbate this imbalance and more than likely contribute to the collapse of post-ischaemic/hypoxic vessels. Sustained hypoperfusion and oxidative stress, which are primary features of aged brain tissues during the prodromal stages of AD [5–7], also may further stimulate the expression of various NOS species and subsequent ET-1 in brain cells and probably increase the accumulation of oxidative stress products, thereby contributing to blood-brain barrier (BBB) breakdown and brain parenchymal cell damage (For a more in-depth discussion regarding the interactions of each of these factors, please see our previous work [8].) These findings raise questions regarding the direct relationship between oxidative stress, energy failure (*e.g.* mitochondrial lesions) or metabolic insufficiency, neuronal and vascular damage, BBB breakdown and Aβ deposition during the maturation of AD-like pathology [9, 10].

Normal aging and sporadic, late-onset AD have many features in common, with AD-like symptoms manifesting only when certain quantitative levels of damage attributed to risk factors such as metabolic and oxidative stress, as well as those associated with impaired cerebral perfusion (*e.g.* cardiovascular and cerebrovascular diseases including hypo- & hypertension and stroke) breech the body's ability to adequately cope with further insults [6, 11]. Under conditions associated with advanced aging such as those mentioned above, any imbalance in the activity of NOS isoforms, ET-1 and oxidative stress can lead to a potential and very destructive positive feedback loop in which increased levels of reactive oxygen species (ROS) (1) interfere with NO function and endothelial relaxation by reducing its bioavailability (through ROS scavenging), (2) actually increase the amount of oxidative stress levels through the production of the potent oxidant peroxynitrite, (3) impair endothelial barrier function and promote leukocyte adhesion and (4) induce alterations in normal vascular function thereby further decreasing cerebral blood flow (CBF) [12]. It appears that transient GRK2 activity correlates with compensatory changes to oxidative stress and arterial occlusion, including changes in ET-1 expression [9, 10]. Although we are aware that correlation does not necessarily imply causation, we are equally cognizant of the axiom, which necessitates correlation in order for causation to be proved. With this in mind, we determined the cellular, subcellular and ultrastructural distribution and localization of GRK2 immunoreactivity in cases of human AD as well as in a mammalian model of CBH in order to investigate what roles, if any, GRK2 might play in the early pathogenesis of dementia, which was first seen with cytochemistry at the light level and confirmed by Western blotting for GRK2 [4]. Increasing evidence for the roles of GRKs and angiotensin 1 and 2 (AT_1 and AT_2) in hypertension, stroke, and heart disease and association between these receptors and ligands in heart disease and AD [13] as well as early amyloid-β (Aβ) accumulation *in vitro* [14] and our *in vivo* work with models of hypoperfusion [4] prompts further consideration of AD and AD-like pathology in terms of possible inclusion and classification as disorders of the cerebrovasculature, because they involve common receptor types. Our *in vivo* findings demonstrated the early involvement of this kinase in both cerebrovascular ischaemia and in AD [4]. During ischaemic injury and in the vulnerable neurons of AD patients, we found increased GRK2 immunoreactivity. Therefore, cellular and subcellular investigations into the mechanisms preceding Aβ deposition and progression, as well as the possible accelerating effects of environmental factors such as chronic hypoxia/reperfusion, were crucial to understanding events that precede amyloid deposition and may lead to insights into new pharmacological treatments of AD [4, 15].

General features of GRKs

GRK function and interaction is complicated and important, compelling an active area of research interest. GRKs are known to regulate numerous receptor functions in both the brain and myocardium [16]. General features of GRK interaction with GPCRs lead to complex regulatory mechanisms that modulate receptor responsiveness and underlie important physiologic phenomena, including signal integration and desensitization [17]. GRKs are members of a multigene family, which are classified into three subfamilies. GRK2 and 3 form the second subfamily [beta-adrenergic receptor kinase (beta ARK) subfamily], which phosphorylate and regulate agonist-occupied or constitutively active GPCRs. BetaARK1 (also known as GRK2) is the most abundant GRK in the heart, and it is increased in several cardiovascular diseases associated with impaired cardiac signalling and function, suggesting that this protein could have pathophysiological relevance in the setting of heart failure.

GRKs critically regulate beta-arrestin signalling *via* receptor phosphorylation and the triggering of desensitization and the beta-arrestins play a crucial role in regulating the responsiveness of multiple GPCRs [17]. The molecular mechanisms of desensitization are quite complex and have been investigated largely with the beta2-adrenergic receptor (beta2AR) used as the main model system. Recent data from Mayor and colleagues indicate that, besides

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the uncoupling function, GRK2 and beta-arrestin also directly participate in beta2AR sequestration, thus providing the trigger for its resensitization. This is followed by binding of uncoupling proteins termed arrestins and transient receptor internalization, which plays a key role in resensitizing GPCR by allowing its dephosphorylation and recycling [17]. A detailed knowledge of the role of GRKs and arrestins in betaAR internalization would make their physiologic role in the modulation of cellular responses to messengers better understood and is much too complex to address in this review. However, recent work has revealed potential phosphorylation-independent regulation of GPCRs by GRK2 and GRK3 [18]. Further, GRKs may themselves be regulated by caveolin [3]. Nevertheless, reduced expression of GRK and beta-arrestins leads to supersensitization of GPCRs and increase the response to neuropeptides, neurotransmitters, chemokines and many other molecules. Thus, overexpression of these GRKs could serve a protective or compensatory response to these stressors and chronic stress conditions during excitotoxicity.

Expression patterns of GRK2

The various GRK subtypes differ in their localization, regulation and mode of action. Many GRKs have been found highly expressed in heart, brain and other tissues. In rat and hamster [19], they are known to regulate numerous receptor functions in both the brain and myocardium [20]. Desensitization and resensitization of a wide variety of GPCRs are processes involved in numerous brain functions and GRK2 expression is increased in the developing rat brain, which is consistent with an involvement in brain maturation processes [21]. The expression in the developing brain and in AD, which is also characterized by ectopic expression of a multitude of cell cycle markers and proteins that are involved in cell division, can be seen as an apparent ontogenic recapitulation as well [22]. These same analogies have been considered in the parallels of AD and cancer [23]. In the rat brain, 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. In our AD and hypoperfusion studies, we observed less positive signals for GRK in control cases, generally. Mostly GRK positive gold label in electron microscopic studies was observed bound to the residues of the different cellular compartments, such as damaged mitochondria, distorted perivascular cells. Some positive signals were observed in perivascular cells associated with damaged vessels as well as in cellular compartments with lesions. Nevertheless, there are pathological cellular structures, such as NFT-like and/or vacuolar degenerative structures (GVD), which colocalized with GRK2 [4]. In some cases, GRK positive signals were bound to degenerated vacuolar structures. Those data were the first known *in vivo* evidence demonstrating GRK2 activation in early cerebrovascular disease, including AD, and thus, GRK2 could serve as a new target for treatment approaches to AD, cerebrovascular dementia or stroke [24].

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 [25]. Signalling through cardiac β adrenergic 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 [26]. 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-adrenoreceptor in cardiac tissues [27] and by changes in the expression of GRK2 or β-adrenoreceptor kinase1 (bARK1) [28]. When β-adrenoreceptor responsiveness was examined in a completely developed reperfused myocardial infarction model, higher levels of tissue catecholamines and GRK2 were observed in the ischaemic epicardium [29]. It was found that the density of the β-adrenoreceptor in the viable ischaemic regions can be modified by GRK2 and catecholamines. Conversely, cardiopulmonary intervention was found to decrease GRK expression [30].

GPCR desensitization is emerging as an important feature of several cardiovascular diseases. 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 [31], portal hypertension [32] and in other cells and tissues in these conditions, such as lymphocytes [33]. 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 [34]. β-arrestin binding uncouples GPCRs from their respective G proteins by sterically blocking receptor coupling to G proteins. These same regulatory proteins also regulate GPCR endocytosis, which then involves the processes of transient receptor internalization, intracellular trafficking and resensitization [35]. 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 [36]. Consequently, the β-arrestins play a crucial role in regulating the responsiveness of many GPCRs. GRK2, along with beta-arrestin, also play a key role in resensitizing GPCRs by allowing its 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, may provide the trigger for resensitization [17]. GRK2 levels in myocardium and lymphocytes may be associated with β-AR dysfunction and heart failure severity.

Signalling through cardiac betaARs is significantly impaired in many cardiovascular disorders, including congestive heart failure. Recent studies in several different mouse models have demonstrated that betaARK1 plays a key role not only in the regulation of myocardial signalling, but also in cardiac function and development.

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Moreover, studies have shown that targeting the activity of GRKs, especially betaARK1, appears to be a novel therapeutic strategy for the treatment of the failing heart and thus we could extrapolate the same to the AD brain. The development of small molecule inhibitors of betaARK1 and GRK activity may advance therapeutic options for heart disease [37], which may be useful for AD as well, perhaps under conditions where excitotoxicity is not the predominant, precipitating or predisposing factor.

GRK, ET-1 and insulin signalling

Because we have hypothesized the existence of an imbalance between the nitric oxide synthases (NOS species) and ET-1, we now suggest a putative role of GRK2 in chronic ET-1-induced insulin resistance in the brain and vascular wall cells, as it has been found for other cells and tissues, which also may contribute to consequences for Alzheimer and stroke patients by similar mechanisms. In that regard, GRKs, which are classical serine/threonine kinases that desensitize agonist-occupied GPCRs, have been found to regulate other receptors such as the insulin receptor (IR), which is a tyrosine kinase receptor. GRK2 was found to negatively regulate glycogen synthesis in mouse liver FL83B cells [38]. This group demonstrated that the IR also couples to G-proteins, specifically GRK2, and utilizes downstream signalling components to negatively regulate IR signalling in those cells. In other tissues and cells, GRK2 can function as a negative regulator of insulin action by interfering with G protein-q/11 alpha-subunit (Galphaq/11) signalling [39], causing decreased glucose transporter 4 (GLUT4) translocation [40]. This same group reported that chronic ET-1 treatment leads to heterologous desensitization of insulin signalling with decreased tyrosine phosphorylation of insulin receptor substrate (IRS)-1 and Galphaq/11, and decreased insulin-stimulated glucose transport in 3T3-L1 adipocytes. Taken together, the importance of GRK2 in AD, vascular dementia and other metabolic diseases, such as diabetes, should not be underestimated.

Recent data suggest possible alternate roles for GRK2 other than as a kinase. In that regard, when the role of phosphorylation of the endothelin B receptor (ETBR) in agonist-induced desensitization was investigated, using a mutant lacking C-terminal 40 amino acids (delta 40 ETBR). In cells expressing the wild-type or delta 40 ETBR, ET-1 caused rapid desensitization of calcium responses [41]. These investigators found the wild-type ETBR was phosphorylated by ET-1, and the phosphorylation was markedly enhanced when coexpressed with GRK2. However, delta 40 ETBR was not phosphorylated regardless of coexpression with GRK2. Phosphatidylinositol 3 formation was ET-1-induced in these cells and was decreased by coexpression with GRK2 or kinase-dead GRK2 by a similar mechanism, by which the authors suggest the presence of phosphorylation-independent desensitization mechanism in delta 40 ETBR as a possible alternate role for GRK2, other than those that are kinase-related in the strict sense.

Of importance in these GRK2 studies in AD are the vascular endothelium, neurons and glia, which all are able to synthesize, store and release ROS, NO and 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. ET-1 is produced by multiple cells and is differentially coupled to G-proteins [42] in response to hypertrophic stimuli *in vitro* and in the development of heart failure *in vivo* [43, 44]. Nevertheless, the endothelin A and B receptors (ET_A-R and ET_B-R) undergo desensitization, most likely also through GRK2 [45]. For example, ET-1 can elicit several responses; it activates EC NOS *via* G-protein beta/gamma subunits signalling through protein kinase B/Akt [46] as well as prolonged physiologic responses, including mitogen-activated protein kinase (MAPK) activation [47] and c-Jun NH2-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) in cultured animal cells and *in vivo* [48]. MAP kinases have been long-associated with AD and ERK activation may be another important early event, perhaps downstream from GRK2 activation [49]. Interestingly, these pathways also have been implicated in cell cycle dysregulation in human AD cases [50, 51]. Recently, we have demonstrated that because successful dysregulation of the cell cycle is also the hallmark of a neoplastic changes, 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 [52]. Further, it has been shown that phosphorylation of GRK2 by MAPK also triggers turnover and GRK2 degradation through the proteasome pathway. GRK2 is targeted for proteolysis by β-arrestin function [53]. Therefore, GRK2 may play a very important role in AD pathogenesis mechanisms through oxidative stress and mitochondrial dysfunction.

GRK studies in AD and CBH

Studies of the details and consequences of GRK's mechanisms have focused heavily on the original beta-adrenoreceptor kinase (beta-ARK) family (GRK2 and GRK3) and, in particular, on phosphorylation-dependent recruitment of adaptor proteins such as the beta-arrestins. Several lines of evidence implicate GRK and beta-arrestin expression in AD and after cerebral hypoxia/ischaemia (HI) [16] and the differential GRK 2 expression in compensated hypertrophy and heart failure after myocardial infarction in the rat. Moreover, GRKs regulate metabotropic glutamate receptor 5 function and expression [54], which has also been implicated in AD pathogenesis and GRKs may offer a mechanism for desensitization of this receptor isoform.

The main experimental goal of our previous study was to investigate and better clarify the relationship between GRK2, vascular lesions and the development of pathology in a CBH model and AD, at the cellular and subcellular level [4]. In that regard, we examined a connection between vascular damage and predisposing

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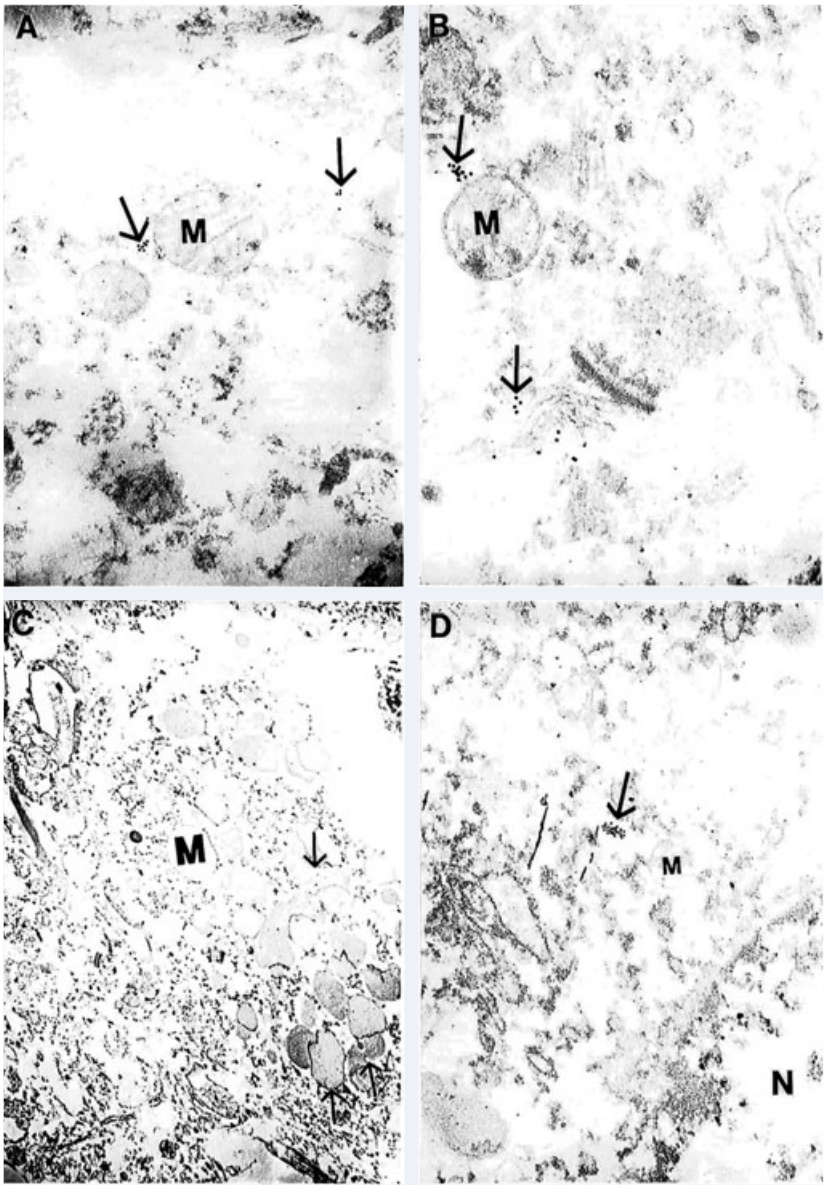

Fig. 1 Subcellular localization of GRK2 immunoreactivity detected by using pre-embedding immunogold decoration in hippocampus of aged matched control (**A, B**) and AD brain (**C, D**). (**A** and **B**) The neuronal cell body from the age-matched control brain hippocampal tissue shows the presence of GRK2 containing gold particles (arrows) attached to the external membrane of partially damaged mitochondria. GRK2 immunopositive gold particles localized in the matrix of damaged mitochondria and Golgi cistern. ×30,000 and ×40,000, respectively, A and B. (**C**) Hippocampal tissue from the AD brain shows that the neuronal cell body is characterized by the presence of large number of mitochondria-derived lysosomes (M), and disperse distribution of GRK2 positive gold particles (arrows). ×6000. (**D**) Glial cell body from the AD brain tissue shows clusters of GRK2-immunoreactivity in the matrix of mitochondria-derived lysosomes (single arrow), ×20,000. Abbreviations: M, mitochondria; N, cell nucleus.

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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 [55–57]. This model is relevant to examining the physiopathology of AD and stroke and enables exploration of the relationship between vascular events and AD.

Our study is the first to show ultrastructural localization and overexpression of GRK2 during the early stages of damage in aged human and AD cases (see Figs. 1 and 2), and also in a our 2-VO model of CBH (see Figs. 3–5). 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 (see Figs. 1 and 2). The immunopositive reactivity was observed in damaged vessel wall cells and their subcellular compartments (Figs. 1 and 2). We have found that neurons that contain neurofibrillary tangles (NFT)

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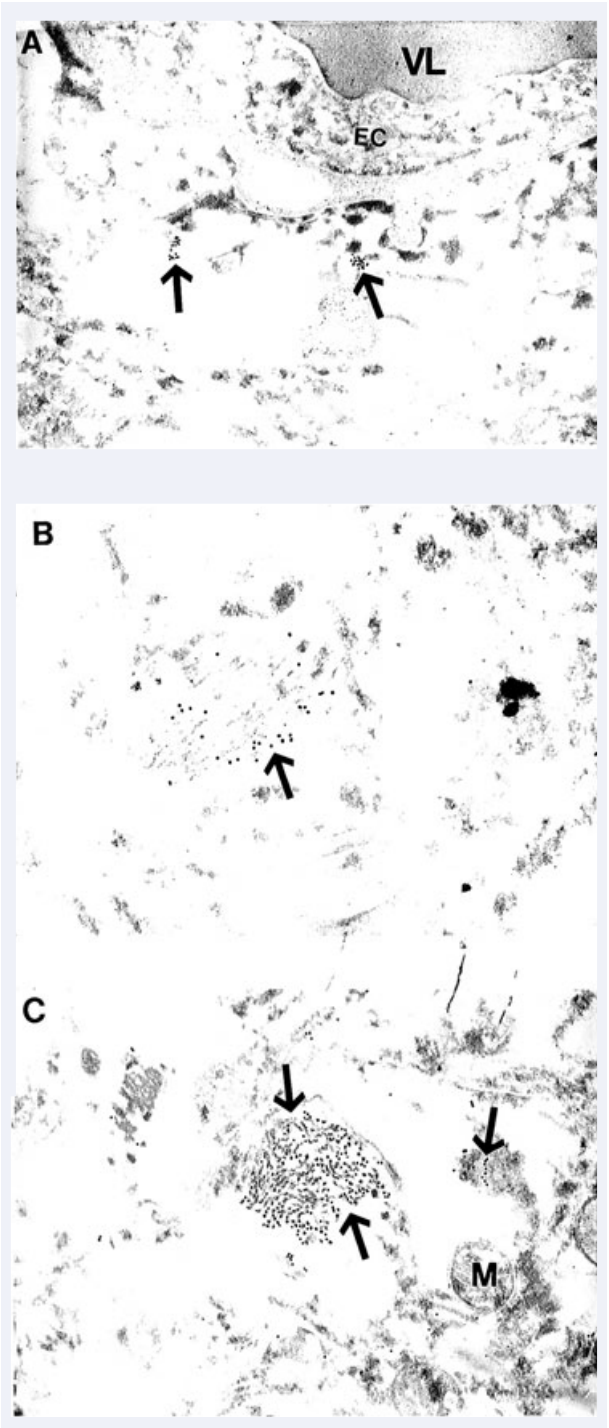

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show abundant GRK2 immunopositive reactivity (Fig. 2). The intensity of the reaction varied from cell to cell and within cellular compartments as well (Figs. 1 and 2). However, cellular lipofuscin was not associated with any GRK2 immunoreactivity. 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. Late stages of damage reveal scarce GRK2-immunoreactivity in areas that were previously abundant, which suggest that overexpression of GRK2 is reduced. GRK2 reduction was confirmed by Western blotting [4]. Thus, this protein can serve as an earlier marker of the brain damage that typifies cerebral-vascular and/or mild cognitive impairment, 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 [4, 67].

A parallel study reported abnormal GRKs *in vitro* for 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 [14]. This led to reduced membrane GRK2/5, which subsequently led to retarded GPCR desensitization, prolonged GPCR signalling, and cellular supersensitivity to GPCR agonists [14]. 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 *versus* 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. Nevertheless, 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 have been explored in various rat models [55, 58]. In this regard, we have demonstrated that abnormal mitochondria (mitochondria with electron dense matrix and mitochondrial-derived lysosomes) and lipofuscin appear to be features of damaged hippocampal neurons in aged Tg (+) mice

Fig. 2 The ultrastructural localization of GRK2 immunopositive gold particles in postmortem human AD (**A**) and age-matched control brain (**B, C**) tissues. (**A**) The GRK2 immunopositive containing gold particles in the matrix of perivascular pericytes (indicated by single thick arrows) but not in the cytoplasmic matrix of severely damaged vascular endothelium (EC), ×40,000. (**B** and **C**) The neurons close to perivascular regions show the presence of GRK2containing gold particles in their matrix, where most gold particles were associated with the neurofibrillary tangle (NFT)-like structures (arrows). However, the intact mitochondria (M) were free from GRK2-immunopositive gold particles, ×40,000, respectively, B and C.

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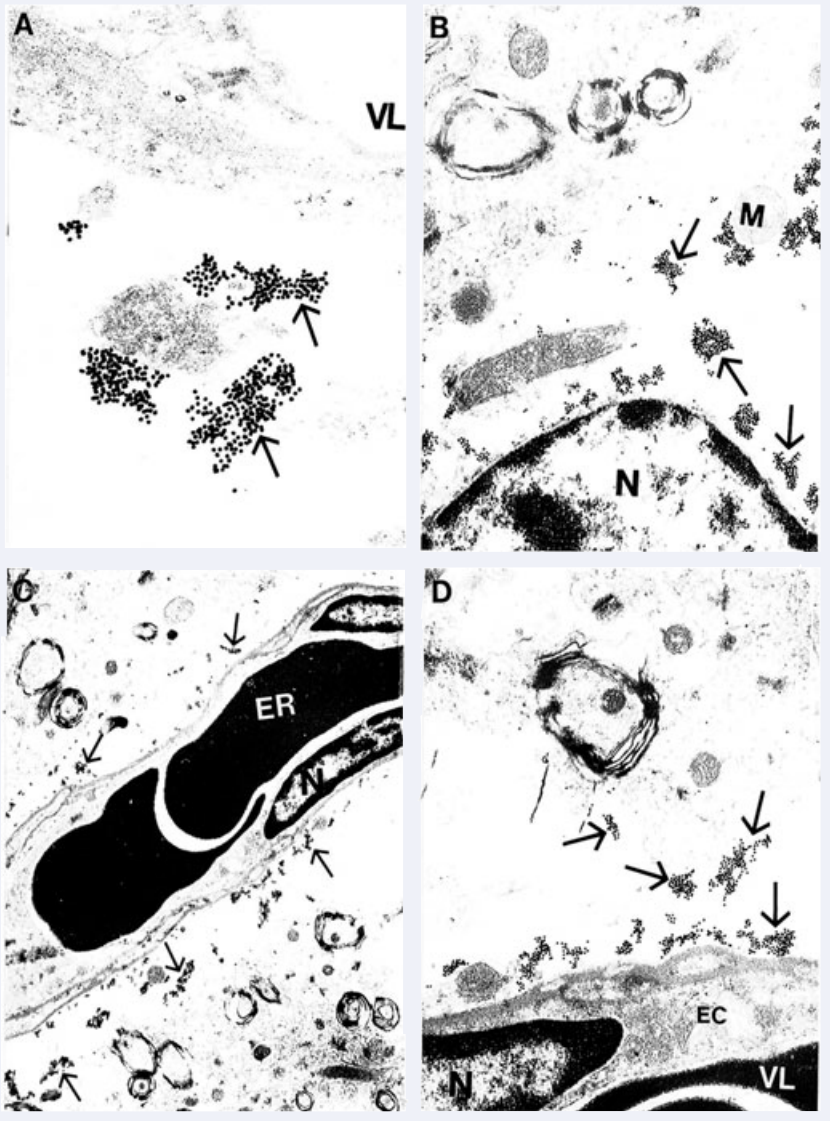

Fig. 3 Ultrastructural characteristics of GRK2 immunopositive gold particles from the rat brain in control (same-operated: **A, B**) and 2 vessel occlusion (2-VO; respectively **C, D**) experiments. (**A**) Clusters of GRK2 immunopositive gold particles in the cytoplasmic matrix of perivascular pericytes (arrows) but not in the vascular EC, ×20,000. (**B**) The presence of GRK2 immunopositive gold particles associated with the oedematous portion of the perivascular pericytes cytoplasmic matrix (arrows). Intact, but not giant mitochondria (M) are free from any GRK2-immunopositive positive gold particles, ×30,000. (**C**) The GRK2 containing positive gold particles was seen in the hippocampal tissues from rat exposed to 2-VO. The presence of GRK2-immunopositive gold particles was seen throughout the matrix of damaged perivascular pericytes (arrows), ×8,000. (**D**) Perivascular regions of this area from the figure C under higher magnification display the presence of islands of GRK2-containing gold particles, which are associated with the damaged regions of the cytoplasmic matrix (arrows). Nucleus (N) and intact mitochondria are from the GRK2-immunoreactivity, ×30,000.

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and human AD, suggesting a direct relationship between vascular abnormalities, BBB breakdown, neuronal loss and amyloid deposition [8–10, 59, 60].

Our *in vivo* data discussed in light of another study involving amyloid [14], our model shows a similar effect, but attributes the overexpression of GRK2 to oxidative stress and events prior to Aβ deposition. While the effect observed in the Sou study involved 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, failing to control for glia or other immunologic cells that may be involved. However, because 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. Therefore, the appearance Aβ is unlikely to be the primary predisposing factor for GRK2 overexpression, as Aβ deposition occurs much later in the disease process. Any cytotoxicity may emanate from mechanisms other than amyloid directly as earlier events seem to be more crucial in the disease pathogenesis. Perhaps early cytotoxicity resulting from non-amyloid-mediated mechanisms may be more crucial in the etiopathology and suggest Aβ is less likely to be the primary predisposing factor for GRK2 overexpression [59, 61].

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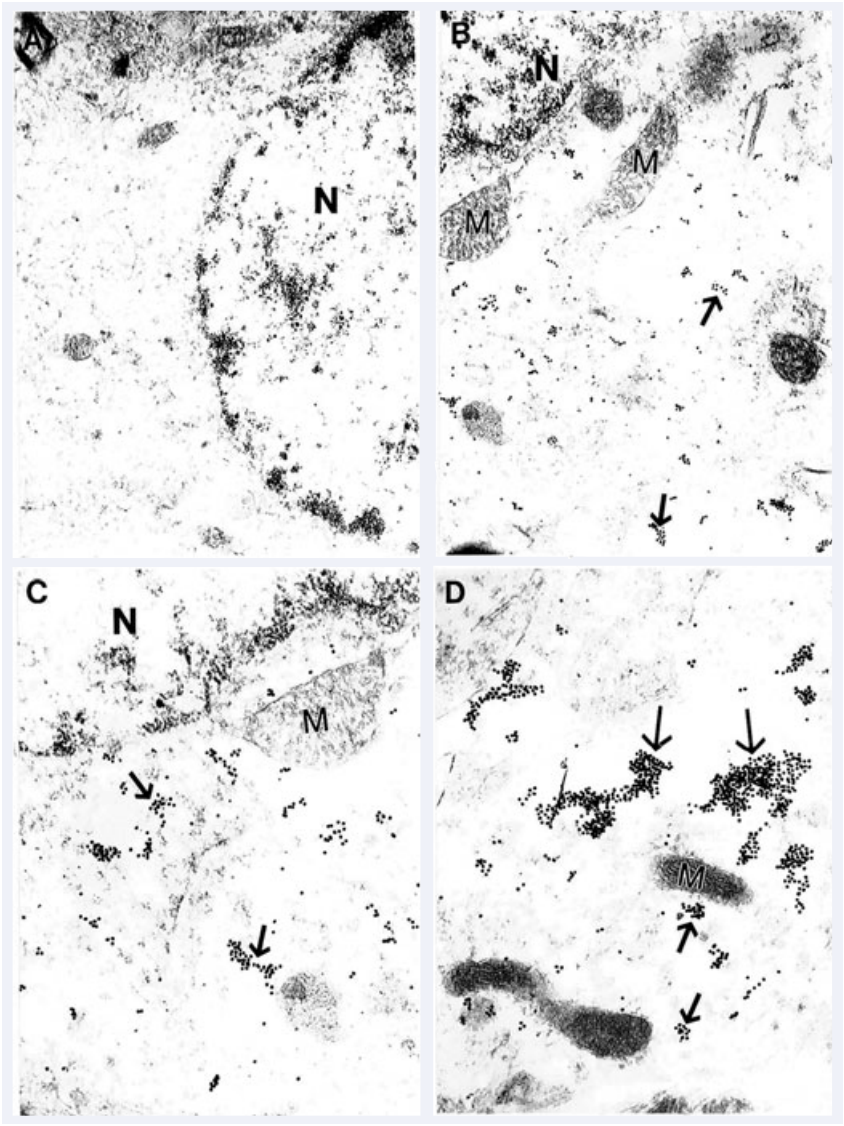

Fig. 4 The subcellular features of the GRK2-immunoreactivity in the hippocampus of the rat subjected to 2-vessel occlusion. (**A**) Intact neurons show absence of any GRK2 immunopositive gold particles in their cytoplasmic matrix, ×15,000. (**B**) Neuron with the effect of chronic cellular hypoperfusion demonstrate the presence of a GRK2 overexpression (arrows) throughout the cell body, however the intact mitochondria (M) were free from any GRK2 immunopositive gold particles, ×30,000. (**C**) *'Hypoperfusion'* affected neuronal cell body shows the presence of islands of GRK2 positive immunodecoration in the external membrane and in the matrix of damaged mitochondria and mitochondria-derived lysosomal structures (arrows), ×40,000. (**D**) Neurons with severe damage shows the presence of islands of GRK2 containing immunopositive gold particles that associated with the completely damaged (mitochondria-derived lysosomal structures) (arrows), but not with non-damaged mitochondria (intact and giant), ×40,000.

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Our studies demonstrate an increase in GRK2 localization to the cytosol, but in particular to subcellular components and only those components with damage and/or pathology evident (see Figs. 1–5). Recruitment of GRK to the cell membrane is followed by inhibition of signalling. Therefore, the sequestration of GRKs to subcellular locations may indicate a compensatory adaptation to AD. However, other studies suggest that GRKs have more diverse protein/protein cellular interactions, and that 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 [62] and a possible role in the sequestration of GRK2, thereby limiting downstream phosphorylation events as well, or leading to translocation of GRK2 to the cytosol. Regardless, the reduced availability of GRK2 and beta-arrestins to regulate GPRC signalling 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 would impart a compensatory or survival response to excitotoxicity, a claim made for Aβ [63]. Finally, neurodegeneration can have numerous overlapping features, and GRK2 along with the action of specific phosphatases

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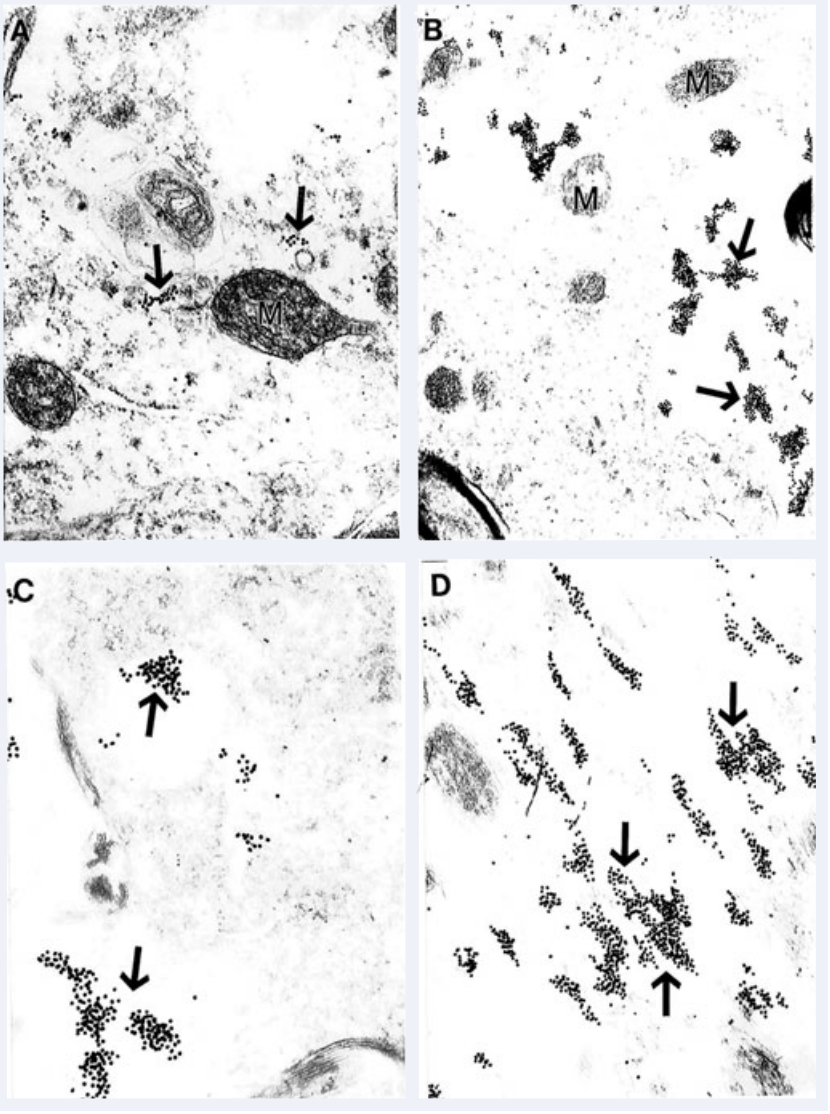

Fig. 5 The GRK2-immunoreactivity in rat brain hippocampal tissues exposed to 2-vessel occlusion and determined by using pre-embedding immunogold cytochemistry techniques. (**A**) Subcellular determination of GRK2 in the neuronal cell body shows the presence of GRK2 immunopositive gold particles (arrows), which associates with the external membrane and the matrix of damaged but not intact mitochondria (M), ×40,000. (**B**) Neurons containing granular vacuolar degenerative structures shows island of GRK2 immunopositive gold particles (single arrow), ×30,000. (**C**) The glial cell body shows overexpression of GRK2-immunoreactivity in the matrix of granular vacuolar degenerative structures (single arrow), ×50,000. (**D**) Neurofilament from the damaged neurons shows the presence of GRK2 immunopositive gold particles (single arrows), ×40,000.

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has been implicated in other neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) [64]. Thus, there are numerous parallels that can be drawn between the neurodegenerative and cerebrovascular disorders with heart disease and systemic vascular disorders, which drives home the important connection the role GRK can have in all disorders, particularly AD [65].

In comparison to controls, ultrastructure in AD and animal models are predominated by abnormal mitochondria. Studies examining deleted mtDNA and mitochondrial-derived lysosomes in regions closely associated with lipofuscin suggest that proliferation, deletion and duplication of mtDNA occur in mitochondria in human AD and transgenic mouse models of neurodegeneration [8–10, 59, 60]. *In situ* hybridization with a chimeric mouse and human mitochondrial cDNA probes for the 5 kb common deletion indicate that the deletion is increased at least three-fold in AD cases as compared to controls and in yeast artificial chromosome (YAC) APP mouse hippocampus [8–10, 66], which is strongly positively correlated ($r = 0.934$) with the marker of DNA oxidation, 8-OH deoxyguanosine. These findings indicate that the mtDNA 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, we reported that ultrastructural examination of hippocampal CA1 capillaries in rats revealed a smaller

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size EC containing damaged mitochondria, characterized by transformation of lysosomal structures within the EC and in the perivascular area [61]. Along with mitochondrial abnormalities these changes appeared to be associated with amyloid deposition found surrounding the capillary vessel wall. Electron microscopic immunoassaying showed sparse endothelial-specific NOS (eNOS)-containing positive gold particles in the matrix of the vascular endothelium, in contrast to substantial labeling in the cytoplasmic matrix of perivascular cells and electron-dense mitochondria, indicative of a hypoxic insult [61]. Immunoreactive eNOS-containing positive gold particles were found markedly expressed in hippocampal neurons and in glial cells, when compared to non-occluded controls [61]. Of interest is the comparison between the eNOS overexpression pattern to that of GRK2 [32], which has the same pattern as our previous observation of 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, because such changes were observed only in 2-VO rats. In addition, previously we have demonstrated that oxidative damage is the earlier event in AD [67]. These 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 [61]. These data support 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 and in some types of mental retardation, such as Down syndrome [68]. 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 [16]. 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 [37] and should be considered for AD or related neuropathology as well.

Conclusions

Our growing understanding of GRK2 and its cognate regulatory proteins provides support for a unifying hypothesis of AD where these proteins play a pivotal role by linking the many pheno-menological observations into a conceptual framework that contributes to a growing body of evidence favouring the reclassification of AD as, primarily, a cerebrovascular disorder. For example, one clue also may lie in the finding that GRK2 is a microtubule-associated protein, and tubulin was identified as a novel GRK2 substrate [69]. 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) [70]. Phosphorylation 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 [18] and GRK2 was not found to phosphorylate MAPs under conditions where MAPs were already well-phosphorylated by endogenous kinases, which copurified with tubulin [71]. 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 [72] and in regulation of metabotropic glutamate receptor 5 function and expression [73]. Both of these receptors have been implicated in AD pathogenesis as well [74, 75]. 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 [4]. Our study is the first to demonstrate the cellular and subcellular localization and offers *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. Further, research in this direction may enable GRKs to serve as a new target for treatment approaches to AD, stroke, mild cognitive impairment or related cerebrovascular disorders.

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