GRK5 Deficiency Accelerates β-Amyloid Accumulation in Tg2576 Mice via Impaired Cholinergic Activity*

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Membrane G protein-coupled receptor kinase 5 (GRK5) deficiency is linked to Alzheimer disease, yet its precise roles in the disease pathogenesis remain to be delineated. We have previously demonstrated that GRK5 deficiency selectively impairs desensitization of presynaptic M2 autoreceptors, which causes presynaptic M2 hyperactivity and inhibits acetylcholine release. Here we report that inactivation of one copy of Grk5 gene in transgenic mice overexpressing β-amyloid precursor protein (APP) carrying Swedish mutations (Tg2576 or APPsw) resulted in significantly increased β-amyloid (Aβ) accumulation, including increased Aβ+ plaque burdens and soluble Aβ in brain lysates and interstitial fluid (ISF). In addition, secreted β-APP fragment (sAPPβ) also increased, whereas full-length APP level did not change, suggesting an alteration in favor of β-amyloidogenic APP processing in these animals. Reversely, perfusion of methoctramine, a selective M2 antagonist, fully corrected the difference between the control and GRK5-deficient APPsw mice for ISF Aβ. In contrast, a cholinesterase inhibitor, eserine, although significantly decreasing the ISF Aβ in both control and GRK5-deficient APPsw mice, failed to correct the difference between them. However, combining eserine with methoctramine additively reduced the ISF Aβ further in both animals. Altogether, these findings indicate that GRK5 deficiency accelerates β-amyloidogenic APP processing and Aβ accumulation in APPsw mice via impaired cholinergic activity and that presynaptic M2 hyperactivity is the specific target for eliminating the pathologic impact of GRK5 deficiency. Moreover, a combination of an M2 antagonist and a cholinesterase inhibitor may reach the maximal disease-modifying effect for both amyloid pathology and cholinergic dysfunction.

Alzheimer disease (AD3) is a devastating neurodegenerative disorder clinically characterized by progressive loss of memory and other neurological functions. Given that basal forebrain cholinergic neurons are the fundamental basis for memory function, any of the pathological causes in AD, no matter whether they are the hallmark changes of β-amyloid (Aβ)-enriched senile plaques and neurofibrillary tangles or of the prominent inflammation, have to eventually converge to the extensive basal forebrain cholinergic neuronal loss in AD.

Mounting evidence suggests that the excessive accumulation of Aβ is a paramount pathological event leading to AD, either by direct neuronal toxicity or by indirect exacerbation of inflammatory damage to neurons (1, 2). The amyloid precursor protein (APP) can be proteolytically processed either through the β-amyloidogenic pathway by β- and γ-secretases or via the non-β-amyloidogenic pathway by α-secretase. The regulation of these two mutually exclusive pathways determines the amount of Aβ production and is thus critically important for the pathogenesis of AD. Previous studies have demonstrated that proteolytic APP processing can be regulated by a variety of G protein-coupled receptors, including cholinergic, serotoninergic, and glutamatergic receptors (3–5). In particular, reduced cholinergic activity in AD can promote the β-amyloidogenic pathway of APP processing, producing more Aβ (6–10), thus forming a positive feedback or a vicious cycle to accelerate AD pathogenesis. Therefore, understanding the detailed molecular mechanisms underlying these pathological events is important for design of therapeutic targets that can effectively break this vicious cycle.

It has been recently reported that membrane-associated G protein-coupled receptor kinase-5 (GRK5) deficiency occurs during early AD (11). Although the underlying mechanisms remain elusive, some aging-related factors (i.e. free radicals) and AD-specific alterations (i.e. excess Aβ and homocysteine) are among the known causes (11, 12). GRK5 is one of seven

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3 The abbreviations used are: AD, Alzheimer disease; Aβ, β-amyloid; APP, β-amyloid precursor protein; sAPPβ, secreted β-APP fragment; ACh, acetylcholine; ISF, interstitial fluid; GRK, G protein-coupled receptor kinase; NOI, novel object introduction; ChEI, cholinesterase inhibitor; ChAT, choline acetyltransferase; MT, methoctramine; CREB, cAMP-response element-binding protein; KLC, kinesin light chain.
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GRK family members, whose primary function is to desensitize activated G protein-coupled receptors (13, 14). Although functional redundancy exists among GRK isoforms, individual GRK members can have selective substrates (12, 13). For GRK5, studies using GRK5 knock-out (GRK5KO) mice have demonstrated that one selective substrate is the family of muscarinic acetylcholine (ACh) receptors (15, 16). Moreover, the selectivity of GRK5 for muscarinic ACh receptors also appears to be subtype-specific for the Gq-coupled M2/M4 subtypes, rather than the Gq-coupled M1 receptors (15, 17).

Within hippocampal memory circuits, where M1, M2, and M4 receptors are enriched, the inhibitory M2/M4 receptors are primarily presynaptic autoreceptors, whereas the excitatory M1 subtype is the main postsynaptic muscarinic receptor mediating cholinergic activity (18, 19). As we have recently demonstrated, selective impairment of presynaptic M2 receptor desensitization in young GRK5KO mice causes prolonged or persistent M2 signaling, which leads to reduced ACh release and cholinergic hypofunction without any evident structural degeneration (17). In aged GRK5KO animals, however, there are significant axonal defects and synaptic degenerative changes in cholinergic projection areas, such as hippocampus, piriform cortex, amygdaloid, and anterior olfactory nuclei. Moreover, these moderate pathological alterations are associated with a significant short term working memory deficit (20). Therefore, it seems that GRK5 deficiency specifically affects the function and vulnerability of the cholinergic system.

It is now known that GRK5 deficiency can result from excess Aβ accumulation (11). Once GRK5 deficiency occurs, it selectively impairs desensitization of presynaptic M2 receptors and inhibits ACh release (17). On the other hand, many studies have shown that reduced postsynaptic cholinergic activity affects APP processing in favor of the amyloidogenic pathway (6–10). Therefore, we hypothesized that GRK5 deficiency may be one of the underlying mechanisms that mediates the vicious cycle between Aβ and cholinergic hypofunction. In this study, we used Tg2576 (APPsw) mice deficient in GRK5 (with one copy of the Grk5 gene inactivated) as our model to test this hypothesis by determining whether GRK5 deficiency promotes Aβ accumulation via altered APP processing. We further determined the most effective strategy to combat the impact of GRK5 deficiency and to break the vicious cycle.

EXPERIMENTAL PROCEDURES

Animals—GRK5KO mice were generated by targeted deletion of exons 7 and 8 of the Grk5 gene, encoding critical sub-elements I through III of the protein kinase catalytic domain, as detailed previously (15). GRK5KO mice (C57/BL6 background) were bred with APPsw mice (also with a C57/BL6 background) to produce double heterozygote mutant mice (with the genotype of Swedish mutant human APPsw+/−/− and murine GRK5+/−, abbreviated as Aβ+/−/G−/−), heterozygote GRK5KO control (GRK5Het, A−/−/G+/+), and wild type (WT, A−/−/G+/+) mice for this study. A tail DNA preparation was used for genotyping as described previously (21). We have previously found that the pathological changes caused by GRK5 deficiency are gender-dependent, with females showing worsened changes (22). Others have shown that the APPsw mice also have gender (female)-preferable changes (23, 24). Therefore, only female mice were used for these experiments. All procedures for using these animals were approved by the Kansas City Veterans Affairs Medical Center Institutional Animal Care and Use Committee.

Immunofluorescent Staining—Brain tissue preparation and immunofluorescent staining procedures were performed according to our previous procedures (20, 21). In brief, 18-month-old female mice were anesthetized and perfused with cold phosphate-buffered saline (PBS), their brains were removed, and left hemispheres were post-fixed with 4% paraformaldehyde and processed for histopathology. To minimize experimental variations, multibrain embedding and sectioning techniques were used as we previously described (20, 21). The multibrain embedding allows the subsequent sectioning and staining procedures for all samples in a single block to be performed in a unified condition, which reduces variations that often occur when each brain is manipulated separately. Frozen sections were taken at a thickness of 20 μm in the coronal plane through the hemisphere. All immunofluorescent staining was performed on the floating multibrain sections as described previously (20, 21), except for an additional step of pretreatment with 70% formic acid for 5 min prior to the initial washing and blocking steps. For Aβ staining, several different antibodies (Abs) were used, including two mouse monoclonal mAbs to human Aβ C-terminal (4G8, Invitrogen, 1:500; and Alpha Diagnostics International, San Antonio, TX; 1:600) and one goat polyclonal Ab (pAb) to human Aβ C-terminal (Santa Cruz Biotechnology, Santa Cruz, CA, 1:500). All three Abs are claimed to recognize both human and murine Aβ.

Quantification of Aβ+ Plaque Load—The systematic quantifications of the Aβ+ plaque number and area in these sections were performed in hippocampus, parietal, and temporal cortices, respectively, essentially as described previously (20, 25). Briefly, every sixth section throughout the entire left hemisphere was taken for systematically counting the number of Aβ+ plaques in the regions of interest, in a manner similar to stereological cell counting. However, due to the relatively low number of plaques, all the plaques in the outlined regions, instead of those within a designated optical dissector counting frame, were counted. This appears to be the most accurate stereological quantification method (26). In practice, we have found that counting the plaques in the entire set of the series sections was often unnecessary because according to our analysis, the data randomly chosen from any six sections from all the series revealed the same statistical significance as revealed by using the data from the entire series. Moreover, the statistical power when using six sections was already greater than 99%. Computer-assisted image capture and analysis were performed in a blinded fashion as described previously (21), using a Leica DMI 6000 B microscope (Leica DFC 340 FX, Heerbrugg, Germany) with the aid of Image-Pro Plus software (Media Cybernetics, Bethesda, MD). The Aβ+ plaque area burden was converted to percentage for final analysis.
Western Blotting—At euthanasia, the cold PBS-perfused right hemispheres were further dissected into anterior cortex, posterior cortex, striatum, hippocampus, brainstem, and cerebellum. Protein extract preparation, total protein content determination, Western blotting, and semiquantitative analyses were performed as described previously (20, 21). Primary Abs used were mouse monoclonal anti-Aβ antibody 6E10 (Covance, Emeryville, CA; 1:1000), mouse anti-human secreted APPβ (sAPPβ, clone 6A1, IBL-America; 1:20), goat anti-human APP (Serotec, Raleigh, NC; 1:1000), and clone 6A1, IBL-America; 1:20), goat anti-human APP (Serotec, Raleigh, NC; 1:1000).

Microdialysis and Interstitial Fluid (ISF) Aβ Measurement—3–4-month-old female APPsw control and the double mice were anesthetized using pentobarbital sodium injection (50 mg/kg) and immobilized in a stereotaxic frame (Kopf Instruments). MBR-10 guide cannula (Bioanalytical Systems, West Lafayette, IN) was stereotactically inserted into the right hippocampus and secured on the skull with screw and dental cement. The coordinates for the guide cannula tip were as follows: bregma, 3.2 mm, 2.5 mm lateral, −0.6 mm relative to dura mater, according to a stereotaxic atlas (27). After surgery, mice were placed in Plexiglas chambers (24 cm wide, 25 cm deep, and 26 cm tall) and allowed to recover for 7 days before the microdialysis experiments.

On the day of the microdialysis experiments, microdialysis probes (BR 4-10 probes; membrane length, 4 mm; cutoff, 35 kDa; Bioanalytical Systems) were connected to a Harvard 22 syringe pump (Harvard Apparatus, Holliston, MA) using fluorinated ethylene propylene tubing (inner diameter, 0.12 mm; CMA Microdialysis Inc.) and flushed with 0.15% bovine serum albumin (BSA)-artificial cerebrospinal fluid perfusion buffer (in mM: 122 NaCl, 3 KCl, 1.3 CaCl2, 1.2 MgSO4, 0.4 KH2PO4, 25 NaHCO3, pH 7.35). After the guide cannula stylets were removed, the flushed probes were manually inserted through the guide cannula into the target regions. The perfusion rate was 1.5 μl/min, and dialysate samples were collected hourly using a refrigerated fraction collector for subsequent measurements of Aβ1–42 by sandwich ELISA. After a 3-h equilibration, three samples of dialysate were obtained to establish baseline values of Aβ1–42. After baseline assessment, novel objects were introduced into the experimental chamber. Novel object introduction (NOI) has been previously shown to stimulate ACh release (28), which was used here to differentiate the responses between the APPsw control and the double mice. For treatments, the perfusion fluid was switched to artificial cerebrospinal fluid containing methoctramine tetrahydrochloride or eserine (both drugs were obtained from Sigma) through the UniSwitch® liquid switch syringe selector (IBL-America). Microdialysis samples were analyzed for Aβ1–42 using the human Aβ1–42 ELISAs kit, and tests were performed according to the manufacturer’s instructions (Immuno-Biological Laboratories). After the experiments, mice were euthanized by pentobarbital overdose and were perfusion-fixed with 4.0% paraformaldehyde. Coronal sections were then cut on a cryostat and stained with cresyl violet to verify probe locations.

Statistics—Quantitative data were expressed as the means ± S.E. and analyzed by analysis of variance using SPSS by Tukey’s method where appropriate.

RESULTS

**GRK5 Deficiency Increases Aβ Accumulation**—Aβ accumulation in mice was evaluated using two different strategies. Fibrillar Aβ accumulation was estimated using Aβ1–42 plaque burdens in cortical areas, whereas soluble Aβ was assessed using semiquantitative Western blotting in SDS-containing brain homogenates.

For Aβ1–42 plaque burdens, immunofluorescent staining with antibody against Aβ was performed in brain tissues from 18-month-old mice. We found that the GRK5/APPsw double heterozygote mice had more plaques than the APPsw control heterozygote mice (having normal GRK5) in hippocampal and cortical areas, whereas neither WT nor the GRK5KO/APPsw double heterozygote mice showed any plaques (Fig. 1). It is worth noting that we used three different Abs to Aβ and tests were performed according to the manufacturer’s instructions (Immuno-Biological Laboratories). After the experiments, mice were euthanized by pentobarbital overdose and were perfusion-fixed with 4.0% paraformaldehyde. Coronal sections were then cut on a cryostat and stained with cresyl violet to verify probe locations.

Statistics—Quantitative data were expressed as the means ± S.E. and analyzed by analysis of variance using SPSS by Tukey’s method where appropriate.

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![FIGURE 1. Elevated Aβ plaque burden in the GRKS/APPsw mice. A–D are Aβ1–42 (red) plaque in the aged (18 months) wild type (WT, Aβ1–42/G1–42), GRK5Het (KO, Aβ1–42/G1–42), APPsw (Aβ1–42/G1–42), and the GRK5KO/APPsw double heterozygote (DB, Aβ1–42/G1–42) mice, respectively. Three different Aβ Abs were tested, as detailed under “Experimental Procedures.” The results shown were stained with mAb clone 4G8. Blue = DAPI. Scale bar, 100 μm.](image-url)
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Consistent with the observation of fibrillar Aβ, Western blots revealed that the SDS-soluble Aβ in the cortical homogenates was also significantly increased (p < 0.05) in the double heterozygote mice, as compared with the APPsw-only mice, whereas both the APPsw and the double heterozygote mice showed dramatically higher Aβ levels than those in WT and GRK5Het mice (Fig. 3, A and B).

GRK5 Deficiency Alters APP Processing in Favor of β-Amyloidogenesis—Given that increased Aβ accumulation can result from increased Aβ production, we examined the levels of full-length APP and the sAPPβ fragment to determine whether there was an alteration of APP processing in the double heterozygote mice. Because our antibody for sAPPβ specifically recognizes Swedish mutant human sAPPβ, whereas the WT and GRK5Het control mice do not express human APP at all, this experiment was performed only in the double heterozygote and APPsw-only mice. As demonstrated by Western blots, the full-length APP levels did not change between the double heterozygote and the APPsw-only mice, but sAPPβ level in the double heterozygote mice was significantly (p < 0.05) higher than that in the APPsw control (Fig. 4, A and B). These results suggest that the APP processing in the double heterozygote mice is shifted in favor of β-amyloidogenic pathway.

GRK5 Deficiency Promotes Aβ Production via Impaired Cholinergic Activity—To investigate the mechanism by which GRK5 deficiency promotes Aβ production, we first measured acute dynamic changes of Aβ in ISF from hippocampus using ELISA. Previous studies have shown that the half-life of ISF Aβ in young mice is significantly shorter than that in aged ones (29), indicating that changes of Aβ production can be captured more readily in young animals than in old ones. Therefore, this experiment was performed using 3–4-month-old double heterozygote and APPsw-only mice. In addition, sleep-wake cycle has been reported to affect ISF Aβ levels (25); therefore, all our ISF Aβ levels were measured from microdialysates collected at the same clock time (i.e., morning for baseline and afternoon for treatments). Without any challenge, basal ISF Aβ in young mice was not significantly different between the double heterozygote and the APPsw control mice (Fig. 5). However, when the mice were behaviorally challenged with NO1 using a die, a piece of wood, and a small ball, which has been used previously to stimulate ACh release (28), the ISF Aβ levels in the APPsw mice declined significantly (p < 0.05) within 3 h, whereas that in the double heterozygote mice remained high. These results indicate that cognitive challenge or environmental enrichment can down-regulate Aβ production, whereas GRK5 deficiency in the double heterozygote mice impairs this response.

We have previously demonstrated that GRK5 deficiency selectively impairs desensitization of presynaptic M2 receptors, leading to inhibition of ACh release (17). Our results here have demonstrated that GRK5 deficiency promotes β-amyloidogenic APP processing and Aβ production. Because reduced ACh is also known to promote the β-amyloidogenic APP processing and Aβ production (6–10), we sought to determine whether the increased Aβ production resulting from GRK5 deficiency is mediated by the reduced ACh re-
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Despite various views on detailed causes and processes of AD, two major hypotheses have driven pharmaceutical research of AD in recent decades: the amyloid hypothesis of mice corrected, but levels were further decreased in both mice (Fig. 6C). Moreover, the combined effects were approximately the sum of each treatment, indicating that ChEI and M2 antagonist can work together additively to reach the maximal effects on reducing Aβ production. Taken together, these results not only demonstrate that GRK5 deficiency promotes Aβ production via impaired cholinergic activity but also suggest that modifications of cholinergic hypoactivity via presynaptic and postsynaptic sites may have distinct roles so that combined management appears to be the most effective way to reduce Aβ production.

**DISCUSSION**

Despite various views on detailed causes and processes of AD, two major hypotheses have driven pharmaceutical research of AD in recent decades: the amyloid hypothesis

**FIGURE 5. Dynamic changes of ISF Aβ in the young adult GRK5KO/APPsw mice.** ISF Aβ in the microdialysis of hippocampus from the APPsw and the double heterozygote mice (DB) were collected in free-moving animals and measured using the human Aβ, ELISA kit, as detailed under “Experimental Procedures.” (n = 7). The basal ISF Aβ levels did not differ between the APPsw and the double heterozygote mice. After NOI, however, the ISF Aβ levels in the APPsw mice decreased over time and became significantly lower at 3 h after NOI. By contrast, the ISF Aβ levels in the double heterozygote mice barely decreased at all, indicating that the double heterozygote mice lost their ability to down-regulate the ISF Aβ in response to NOI stimulation, *, p < 0.05, as compared with either the basal level in the APPsw mice or the stimulated Aβ level at 3 h after NOI in the double mice.

**FIGURE 6. Effects of MT and eserine on ISF Aβ in the young adult GRK5KO/APPsw mice.** The treatment drugs were administered into the hippocampus via reverse microdialysis under NOI conditions, and the effects of eserine (A), MT (B), and their combination (C) on ISF Aβ in the young adult GRK5KO/APPsw mice were assessed as detailed under “Experimental Procedures.” A, treatment with eserine (1 μM) decreased the ISF Aβ significantly (*, p < 0.01) as compared with their own non-treated vehicle; n = 6) in both the APPsw and the double heterozygote mice but failed to correct the difference between the APPsw and the double (DB) mice. B, treatment with MT (1 μM) brought the ISF Aβ in the double heterozygote mice down to the level equivalent to that in the APPsw mice but did not significantly affect the ISF Aβ in the APPsw mice. Therefore, the MT treatment efficiently corrected the difference between the APPsw and the double heterozygote mice. n = 6, **, p < 0.001 as compared with vehicle; #, p < 0.001, for interactions between the MT treatment and the GRK5 deficiency analyzed using two-way analysis of variance. C, the combined effects of eserine and MT were larger than each treatment alone, but not significantly larger than their sum, indicating that these two treatments can additively work together to maximally decrease the ISF Aβ levels. n = 4, **, p < 0.001 as compared with vehicle.
and the cholinergic hypothesis (30). The cholinergic hypothesis states that central cholinergic neuronal dysfunction is largely responsible for the cognitive decline in AD (31). The amyloid hypothesis proposes that Aβ is the central pathogenic molecule in AD (1). Although the details of these two hypotheses may evolve over time with increasing insights into disease pathogenesis, the principal concepts of these distinct hypotheses appear to stand solidly (1, 30, 32–37).

Previously, we have shown that Aβ is one of the main causes for functional GRK5 deficiency in AD (11). In this study, we demonstrated that inactivation of one copy of the GRK5 gene in APPsw mice significantly increased Aβ accumulation, as measured by SDS-soluble Aβ and by Aβ” plaque burden in brain. Therefore, GRK5 deficiency, resulting from excess Aβ accumulation, is expected to further accelerate Aβ production, forming a positive feedback that exacerbates Aβ accumulation, the key molecule in the amyloid hypothesis. Thus GRK5 appears to be a key link between amyloid and cholinergic dysfunctions in AD.

Mechanistically, we have previously demonstrated that GRK5 deficiency causes a selective cholinergic dysfunction due to presynaptic M2 hyperactivity that inhibits ACh release, leading to postsynaptic cholinergic hypoactivity (17). In this study, we extended our mechanistic explorations by measuring changes in sAPPβ and testing the effects of cholinergic modulators (the cholinesterase inhibitor eserine and the M2 autoreceptor antagonist methoctramine) on acute ISF Aβ dynamics. The increased sAPPβ and unchanged full-length APP in the double heterozygote mice indicate that the GRK5 deficiency promotes β-amyloidogenic APP processing and therefore Aβ production. In fact, many other studies have previously shown that cholinergic dysfunction with postsynaptic cholinergic hypoactivity affects APP processing in favor of the β-amyloidogenic pathway, promoting Aβ production (6–10, 38). Therefore, it now seems clear that GRK5 deficiency exacerbates the Aβ self-promoting vicious cycle through a specific cholinergic dysfunction.

Consistent with increased β-amyloidogenic APP processing, measurements of acute dynamic change of ISF Aβ showed that the GRK5 deficiency impaired the ability of the double heterozygote mice to down-regulate Aβ production as a response to cognitive environmental enrichment by novel object introduction. Moreover, this lost function in the double heterozygote mice can be restored by antagonizing presynaptic M2 receptors (with MT) but not by simply preserving the ACh at synaptic clefts using a cholinesterase inhibitor (eserine). Therefore, from a mechanistic point of view, these data confirm that the Aβ self-promoting vicious cycle caused by GRK5 deficiency was indeed mediated through the cholinergic dysfunction initiated at the presynaptic site. Beyond the mechanistic significance, moreover, these experiments also compared the therapeutic efficiencies in eliminating the pathogenic impact of GRK5 deficiency by targeting either presynaptic receptors or the postsynaptic site or both. When targeted at presynaptic autoreceptors using MT, the impact of the GRK5 deficiency on ISF Aβ in the double heterozygote mice was efficiently corrected. However, when targeted at potentially hypoactive postsynaptic cholinergic receptors using eserine, elevating ACh failed to correct the impact of the GRK5 deficiency. On the other hand, although eserine treatment did not specifically eliminate the impact of GRK5 deficiency, it did reduce Aβ production non-selectively in both the double heterozygote and the APPsw mice. Furthermore, combined treatment with the M2 antagonist and ChEI led to complementary effects and reached a maximal disease-modifying response by inhibiting Aβ production through the muscarinic pathway.

Therefore, the findings from this study, together with previous results, directly connect Aβ → GRK5 deficiency → cholinergic dysfunction → Aβ into a self-promoting loop or vicious cycle. In this vicious cycle, Aβ and cholinergic dysfunction each can serve as the causes and consequences, whereas GRK5 deficiency is the pivotal mediator. Considering the dominating importance of both amyloid and cholinergic hypotheses in AD, the importance of GRK5 deficiency should no longer be overlooked.

It is worth noting that cholinergic dysfunction is a relatively broad term describing the deficiency of neurotransmitter ACh at cholinergic terminals. Cholinergic dysfunction in AD, according to the cholinergic hypothesis, is characterized by cholinergic hypofunction or a reduction of ACh, which may result from known changes in AD brains, such as reduced choline acetyltransferase (ChAT) and choline uptake, cholinergic neuronal and axonal abnormalities, and degeneration of cholinergic neurons (31, 35). These pathological characteristics of AD observed from postmortem brain tissues of AD patients are evident changes at very late stages of the disease. Some late stage changes may not necessarily reveal or reflect more causative alterations that occur early in the disease process. For example, activity of cholinergic markers, such as ChAT and acetylcholinesterase, does not decrease until very late stages of the disease (39). Because neither ChAT nor acetylcholinesterase is rate-limiting, changes in these markers do not necessarily reflect cholinergic function (40). In fact, ChAT can be inhibited up to 90% with no measurable effects on ACh synthesis or release (32), whereas pharmacological and neurophysiological deficits in cholinergic response can exist without significant changes in ChAT activity during normal aging (40). In line with the latter situation, we have previously shown that cholinergic hypofunction (reduced ACh release) can exist in the absence of cholinergic structural degeneration in young GRK5KO mice (17), and this only turns into a cholinergic dysfunction with structural degenerative changes in aged GRK5KO mice (20). We have thus questioned whether early non-structural cholinergic dysfunction that precedes the structural cholinergic dysfunction/degeneration could also be causative for the latter (17). Although this study did not specifically address this question, the demonstration of a GRK5 deficiency-mediated vicious cycle between Aβ and cholinergic dysfunction certainly adds a strong rationale to address this important question in the future. In addition, we have proposed a hypothetic model that links GRK5 de-
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**FIGURE 7. Hypothetical model that links GRK5 deficiency to the amyloid and cholinergic hypotheses.** This hypothetical model schematically illustrates the links of GRK5 deficiency to the amyloid and cholinergic hypotheses, with key references cited and abbreviations spelled out in this legend. The key pathologic molecule of the amyloid hypothesis, Aβ (1), is the main cause of GRK5 deficiency (11); the latter selectively impairs M2 receptor desensitization and leads to presynaptic M2 hyperactivity (17), which initiates cascading pathological events that ultimately result in selective cholinergic dysfunction and degeneration (12, 20–22), the key event of the cholinergic hypothesis (31). In brief, the presynaptic M2 hyperactivity has both anterograde and retrograde impact; anterogradely, it persistently inhibits ACh release, which causes postsynaptic cholinergic hypofunction, and retrogradely, it persistently suppresses cAMP-dependent signaling pathways, which, although inhibiting ACh release (41), may also promote cholinergic axonopathy and apoptosis. The cAMP-dependent signaling pathways have important roles in neuronal survival via 1) cAMP/PKA/CREB/Bcl-2 anti-apoptotic signaling (42–46), and 2) PKA also phosphorylates GSK3, including GSK3β, which increases Tau and KLC phosphorylation and contributes to axonopathy (51–55). Moreover, the inhibited anti-apoptotic pathway may also lead to increased vulnerability for the cholinergic neurons. On the anterograde direction, the postsynaptic cholinergic hypofunction, mainly mediated through M1 receptor (postsynaptic M1 hypofunction), leads to deficiencies in a number of important functions in cholinceptive neurons, including: 1) memory deficiency (33); 2) decreased cAMP/PKA/CREB/Bcl-2 anti-apoptotic signaling strength (42–46, 56); 3) decreased cAMP/PKA signaling, which leads to decreased pGSK3β and increased GSK3β activity, as well as subsequently increased Tau and KLC phosphorylation (51–55, 57, 58); and 4) decreased activities of PKC and α-secretase (ADAM17/TACE) and increased β-secretase (BACE1) activity, which shifts APP processing in favor of β-amyloidogenesis (6, 57, 59). This last consequence of the cholinergic dysfunction leads to more Aβ production (as we demonstrated in this study), which pushes the GRK5 deficiency further and closes the loop of the vicious cycle between Aβ, GRK5 deficiency, and cholinergic dysfunction. Of course, more Aβ production during aging means more Aβ deposits, inflammation, and non-selective toxic effects. All these toxic degenerative insults, along with increased free radicals during aging, will come back to attack the already vulnerable cholinergic system, eventually leading to the cholinergic selective neurodegeneration in AD. Abbreviations used are: acetyl-CoA, acetyl-coenzyme A; ADAM17, a disintegrin and metalloproteinase domain 17; BACE1, β-site of APP-cleaving enzyme 1; Aβ, fibrillar Aβ; pCREB, phosphorylated cAMP response element-binding protein; pGSK3β, phosphorylated glycogen synthase kinase 3β; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; P-KLC, phosphorylated KLC; P-Tau, phosphorylated Tau; TACE, tumor necrosis factor-α-converting enzyme; VACHT, vesicular ACh transporter.

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