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► **To cite this version:**

Fanfan Sun, Jie Zhao, Huajie Zhang, Qihui Shi, Yan Liu, et al.. Proteomics Evidence of the Role of TDMQ20 in the Cholinergic System and Synaptic Transmission in a Mouse Model of Alzheimer's Disease. ACS Chemical Neuroscience, 2022, 13 (21), pp.3093-3107. 10.1021/acchemneuro.2c00455 . hal-03826219

HAL Id: hal-03826219

<https://hal.science/hal-03826219>

Submitted on 6 Nov 2023

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1 **Proteomics evidence of the role of TDMQ20 in the cholinergic**
2 **system and synaptic transmission in a mouse model of Alzheimer's**
3 **disease**

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24

25

26 **Running Title**

27 Proteomics pattern of TDMQ20, an anti-AD drug-candidate, on 5xFAD mice

1 **Abstract**

2 The interaction between copper ions and amyloid peptide A β has been reported to
3 be involved in Alzheimer's disease (AD) pathology. Based on copper coordination
4 biochemistry, we designed specific copper chelators (TDMQs) in order to regulate
5 copper homeostasis in AD brain, and to inhibit the deleterious oxidative stress
6 catalyzed by copper-A β complexes. We previously reported that TDMQ20, a
7 highly selective copper chelator selected as drug-candidate, was able to extract
8 copper from Cu-A β ₁₋₁₆ complex and to restore cognitive and behavioral deficits in
9 AD mouse models. For a better understanding of the mechanism of action of
10 TDMQ20, we decided to investigate the change of profile of proteins expressed in
11 5xFAD mice after an oral treatment of TDMQ20 (dose = 10 mg/kg, once every
12 two days during 3 months, in total 45 times). Clioquinol (CQ), a non-specific
13 chelator, has been used as comparator.

14 Here, we report the proteomic alterations in the cortex of 5xFAD mice using
15 iTRAQ (isobaric tags for relative and absolute quantification) proteomics
16 technology. The results indicated that 178 differentially expressed proteins (DEPs)
17 have been identified in AD mouse group with respect to wild type (WT) animals
18 (AD/WT). After treatment by TDMQ20, 35 DEPs were common in AD/WT and
19 TDMQ20/AD groups in an opposite up- or down-regulated manner, respectively.
20 In addition, among the 35 DEPs mentioned above, 10 common target proteins have
21 been identified in AD/WT, TDMQ20/AD and CQ/AD groups, among which three
22 target proteins were successfully validated by Western blot analysis. In particular,
23 the expression level of ChAT and CHRM4 is significantly increased upon
24 TDMQ20 treatment with respect to 5xFAD mice, while CQ did not significantly
25 change the expression of these proteins. Our study suggests the involvement of this
26 copper chelator TDMQ20 on the cholinergic system, a feature which may explain
27 the improved cognitive and behavioral performance in AD mice upon oral
28 treatment by TDMQ20.

29

1

2 **Keywords**

3 Alzheimer's disease, copper chelator, TDMQ20, iTRAQ proteomics, cholinergic
4 neurotransmission, oxidative stress

5

6 **Highlights**

- 7 • TDMQ20 reverses proteomic profiles through multiple pathways in 5x FAD
8 AD mouse model.
- 9 • TDMQ20 exerts a significant inhibition effect on PI3K-Akt pathway in the
10 5x FAD AD mouse model.
- 11 • TDMQ20 modulates the expression level of ChAT and CHRM4 in cholinergic
12 system of 5x FAD AD mouse model.
- 13 • TDMQ20 dramatically reduces A β plaque load in the cortex of 5x FAD AD
14 mouse model.
- 15 • TDMQ20 can be considered a promising candidate in treating AD via the
16 regulation of cholinergic and synaptic functions.

17

18

19

20 **In brief**

21 Sun *et al.* describe the effect of a new copper chelator TDMQ20 on the profile of
22 protein expression in AD mice. TDMQ20 inhibits the PI3K-Akt pathway in AD
23 mouse cortex at the proteomic level. It has great potential for AD treatment by
24 modulating ChAT and CHRM4 along the cholinergic system and decreasing
25 cortical A β plaques.

26

1 INTRODUCTION

2 Among the 55 million of persons currently living with dementia worldwide,
3 60-70% of them are due to Alzheimer's disease (AD), the most common type of
4 dementia (1). At the moment, AD is the largest unmet medical need in neurology.
5 In AD brains, the main pathology is featured by protein deposits of aggregated
6 amyloid- β (A β) and hyperphosphorylated form of tau protein (p-tau), appearing as
7 amyloid plaques and neurofibrillary tangles, respectively, distributed in several
8 brain regions like cortex, hippocampus, olfactory bulb, amygdala and basal
9 forebrain (2,3). At the moment, there is still a lack of efficient drugs able to stop
10 the development AD in the early stages and/or prevent the cognitive decline and
11 behavioral changes induced by the disease. Since the approval of memantine in
12 2003, and despite huge research efforts made by universities or pharmaceuticals
13 companies, the level of failures in clinical trials of drug-candidates is close to 100%
14 (4). GV-971, a sodium oligomannate, and aducanumab, an antibody targeting β -
15 amyloids, have been approved by China National Medical Products Administration
16 (NMPA) in 2019 and 2021, respectively (5,6). However, this biopharmaceutical
17 has been rejected by the European Agency in December 2021 (7).

18 Therefore, it is still urgent to explore other potential targets to enlarge the drug
19 spectrum with different mechanisms, due to the complexity of the etiology of AD.
20 Consequently, several research groups, including our own, decided to develop
21 different metal chelators as drug candidates for the treatment of AD (8-11). The
22 driving force of this research domain is the rupture of the homeostasis of redox
23 active metal ions such as copper and iron in AD brains and their accumulation in
24 amyloid plaques (12,13). In the presence of endogenous reductants, copper-
25 amyloid complexes are able to catalyze the reduction of dioxygen and to produce
26 reduced, reactive oxygen species (ROS) responsible for neuron death (14). In
27 addition, β -amyloid oligomers induce mitochondrial damage in a copper catalyzed
28 nitric oxide mediated process (15).

1 Based on our previous studies on copper chelators (10) and our understanding
2 of the failure of non-specific copper chelators (16), we designed a new series of
3 tetradentate ligands based on a 8-aminoquinoline specific for copper chelation
4 named TDMQ (17-20). One of these chelators, TDMQ20, has been selected as
5 drug-candidate and evaluated on three different mouse AD-models, two models
6 based on non-transgenic mice, and one being the classical transgenic 5xFAD mice.
7 TDMQ20 was able to reduce memory impairments in all three AD-models and was
8 also able to reduce the brain level of malondialdehyde, a marker of oxidative stress
9 (21).

10 We should keep in mind that the rupture of the homeostasis of copper ions
11 generates an excess of this metal ion in some proteins and a reduction of its
12 availability for other ones. For example, copper is a regulator of muscarinic
13 acetylcholine receptor in the brain of rats and its deficiency has a dramatic effect
14 on both receptor number and binding of antagonists to this receptor (22). This fact
15 might create a link between copper homeostasis and three of the few approved
16 drugs (donepezil, galantamine and rivastigmine) acting as acetylcholinesterase
17 (AChE) inhibitors (23). Cholinergic network in the central nervous system is
18 essential for learning capacity and memory, with most of cell bodies sitting in basal
19 forebrain region and innervations projecting to areas like cortex and hippocampus
20 (24). Cholinergic neurons are lost in AD patients, with dramatically reduced levels
21 of acetylcholine (ACh), a key neurotransmitter for many neurophysiological
22 processes (25). Choline acetyltransferase (ChAT), a key enzyme for the
23 biosynthesis of ACh, was significantly reduced in temporal lobes of patient brains
24 (26) and in dementia induced by hypoxia (27). For all these different reasons, it is
25 essential to understand the origin of the degradation of cholinergic neurons, and in
26 general the neuron death that has been related to the accumulation of copper ions
27 within amyloid proteins when these copper-amyloid complexes are activated by
28 endogenous reductants (9,20,28).

1 So, the different possible modes of action of the TDMQ20 copper regulator
2 may involve reduction of the oxidative stress created by Cu-amyloids, but also a
3 copper-dependent regulation of several proteins involved in the redox metabolism,
4 resulting in normalization of the activity of cholinergic neurons.

5 Here we report proteomics data obtained with transgenic 5xFAD mice that
6 received by oral route 10 mg/kg of TDMQ20 once every two days during 3 months.
7 The reported results suggest that this drug-candidate has a positive effect on the
8 cholinergic system. Consequently, TDMQ20 should be considered as having a bi-
9 modal mechanism of action: the first one as inhibitor of ROS production due to its
10 capacity to extract copper from copper amyloid and the second as provider of
11 copper to copper-deprived cholinergic neurons. This regulation of copper
12 homeostasis, *i.e.* the capacity for a ligand to extract copper from amyloid and to
13 release it for a copper carrier, has been suggested by "in chimico" experiments (29).

14

15 **RESULTS**

16 Relative quantification of proteins in the brain of 5xFAD mice was achieved using
17 iTRAQ based mass spectrometry proteomics analysis. Using the statistical assay
18 of *t*-test, DEPs between two mouse groups were screened with fold change higher
19 than 1.2 or lower than 0.8 as the threshold, and *p* value < 0.05 as significance
20 criterion. A total of 3243 DEPs were identified and classified in four categories:
21 proteins identified in (i) 5xFAD mice compared to wild type mice (AD/WT), (ii)
22 5xFAD mice treated with TDMQ20 compared to untreated 5xFAD mice
23 (TDMQ20/AD), (iii) 5xFAD mice treated with clioquinol compared to untreated
24 5xFAD (CQ/AD) and (iv) common DEPs among the above three categories.

25

26 ***DEPs between 5xFAD mice compared to WT***

27 Among total identified proteins in AD/WT comparison, 178 DEPs were identified,
28 101 being up-regulated (57%) and 77 down-regulated (43%) (Figure 1A and 1B)
29 indicating that there is no significant protein loss in AD mouse brain tissue (raw
30 data: Supplementary 1.xlsx, sheet AD vs WT). Gene Ontology analysis (GO) of

1 biological process (BP), cell component (CC) and molecular function (MF) of
2 these proteins is shown in Figure 1C-E. As shown in Figure 1D, fifteen enriched
3 BP found in GO analysis linked DEPs of AD/WT category mainly included
4 localization, cellular component organization or biogenesis, transport,
5 establishment of localization, single-organism localization, single-organism
6 transport and regulation of biological quality. While establishment of localization
7 in cell, cell-cell signaling, single-organism intracellular transport, chemical
8 synaptic transmission, anterograde trans-synaptic signaling, trans-synaptic
9 signaling, synaptic signaling, and modulation of synaptic transmission were
10 affected to a lesser extent. Regarding to structural stability, fifteen enriched CC
11 processes in GO analysis displayed in Figure 1E mainly linked to DEPs related to
12 intracellular part, cytoplasm, cytoplasmic part and, to a lesser extent, to vesicle,
13 cytosol, extracellular region part, membrane-bounded vesicle, extracellular
14 exosome, extracellular vesicle, extracellular organelle, extracellular membrane-
15 bounded organelle, cell projection, cell body and neuronal cell body. MF DEPs
16 included protein binding, enzyme binding, identical protein binding, binding,
17 kinase binding, anion binding and purine ribonucleoside binding, indicating that
18 these DEPs related to molecular processes were disrupted under AD pathological
19 conditions.

20 AD related pathways associated with these differentially expressed proteins
21 were identified using KEGG (Kyoto Encyclopedia of Genes and Genomes)
22 bioinformatics tool (Figure 2A). The majority of DEPs were involved in
23 organismal systems (Figure 2A, red bars), human diseases (violet), environmental
24 information processing (green) and, in a minor way, in metabolism (blue) and
25 genetic information processing (orange). Among all the enriched pathways, the
26 synaptic vesicle cycle pathway had the lowest p value ($p=2.69e-04$), with 5
27 enriched proteins: Ap2a1, Atp6v1b2, Syt1, Cacna1a and Slc18a2 (Supplementary
28 Figure 1). Besides, important pathways also included choline metabolism in cancer,
29 endocrine and other factor-related calcium, dopaminergic synapse, cysteine and
30 methionine metabolism, serotonergic synapse, thyroid hormone synthesis, ErbB

1 signaling pathway, hepatitis B, Wnt signaling, protein processing in endoplasmic
2 reticulum, axon guidance, pancreatic secretion, insulin resistance (Figure 2A).
3 Protein-protein interaction (PPI) network analysis (Figure 2B) confirms that these
4 AD affected proteins are associated with synaptic vesicle cycle and also with
5 metabolism, protein processing, choline metabolism, neurotransmitters and
6 signaling, these pathways are closely related with each other, and expressional
7 disturbance are expected to influence many functions.

8

9 ***DEPs between TDMQ20-treated 5xFAD mice compared to untreated animals***

10 Compared to untreated 5xFAD AD group, 183 proteins were altered after
11 TDMQ20 treatment, 72 proteins being up-regulated (39%) and 111 proteins being
12 down-regulated (61%) (Figure 3A and 3B) (raw data: Supplementary 1.xlsx, sheet
13 TDMQ20 vs AD). Gene Ontology analysis of BP, CC and MF of these proteins is
14 shown in Figure 3C-E.

15 As shown in Figure 3D, BP linked to DEPs in the TDMQ20/AD category
16 mainly included cellular component organization or biogenesis, cellular
17 component organization, and nervous system development. Then follows in order
18 of diminishing impact: protein complex subunit organization, nitrogen compound
19 transport, generation of neurons, neuron development, neuron projection
20 development, response to organonitrogen compound, chemical synaptic
21 transmission, anterograde trans-synaptic signaling, trans-synaptic signaling,
22 synaptic signaling and modulation of synaptic transmission.

23 Cellular components linked to DEPs of TDMQ20/AD comparison included
24 cytoplasm and cytoplasmic part and, to a lesser extent, vesicle, cytosol,
25 extracellular region part, extracellular exosome, extracellular vesicle, extracellular
26 organelle, extracellular membrane-bounded organelle, cell projection, neuron part,
27 somadendrites, cell body and neuronal cell body (Figure 3E). Molecular function
28 included protein binding, binding and, to a much lesser extent, nucleoside-,
29 ribonucleoside- purine ribonucleoside-binding, identical protein binding (Figure 3
30 C).

1 KEGG pathway analysis (Figure 4A) showed that the majorities of DEPs were
2 involved in the human diseases (brown bars), then in cellular processes (Figure 4A,
3 red bars) and environmental information processing (green bars), organismal
4 systems (violet bars), genetic information processing (orange bars) and metabolism
5 (blue bars). Among all the enriched pathways, the PI3K-Akt signaling pathway had
6 the highest significantly (lowest p value, $p = 3.88e-04$), with 11 enriched proteins:
7 Ywhae, Hsp90ab1, Ywhaz, Ywhag, Hras, Cdc37, Rac1, Gnb4, Tsc1, Tnc and
8 Ppp2r5b (Supplementary Figure 2).

9 PPI network of TDMQ20/AD is depicted in Figure 4B. Putting together with
10 the above analysis, these DEPs were found to be associated with the following
11 pathways which were closely related with each other: PI3K-Akt signaling pathway
12 and synaptic vesicle cycle, as well as protein processing in endoplasmic reticulum,
13 regulation of actin cytoskeleton, focal adhesion, hippo signaling pathway, and
14 inositol phosphate metabolism.

15

16 ***DEPs between CQ-treated 5xFAD mice compared to untreated animals***

17 When compared to the untreated AD group, 226 proteins were altered after CQ
18 treatment of AD mice (Figure 5). Among them, 73 proteins (32%) were up-
19 regulated and 153 proteins (68%) were down-regulated (Figure 5A and B) (raw
20 data: Supplementary 1.xlsx, sheet CQ vs AD). As shown in Figure 5C-D,
21 biological process of GO analysis linked to DEPs of CQ/AD groups mainly
22 included establishment of localization, transport, single-organism localization,
23 single-organism transport, cellular localization and regulation of localization.
24 Protein localization, regulation transport, establishment of protein localization,
25 protein complex subunit organization, amide transport, peptide transport, protein
26 complex assembly protein complex biogenesis and nucleobase containing small
27 molecule metabolic process were also altered to a lesser extent (Figure 5D).
28 Cellular components significantly linked to DEPs of CQ/AD groups included
29 cytoplasm, cytoplasmic part, vesicle, extracellular region, extracellular region part,
30 membrane-bounded vesicle, extracellular exosome, extracellular vesicle,

1 extracellular organelle, extracellular membrane-bounded organelle, neuron part,
2 cell projection, neuron projection and cell body (Figure 5E). Molecular function
3 mainly included protein binding and binding, and also enzyme binding, small
4 molecule binding, purine ribonucleoside binding, purine nucleoside binding and
5 several other binding functions. KEGG pathway analysis (Figure 6A) showed that
6 the majorities of DEPs were involved in metabolism, genetic information
7 processing, environmental information processing, cellular processes, organismal
8 systems, human diseases. Among all the enriched pathways, the endocrine and
9 other factor-regulated calcium reabsorption pathway had the lowest *p* value, and
10 accounted for 8 proteins as Ap2b1, Plcb1, Atp2b1, Atp1b1, Atp1b2, Calb1, Prkca
11 and Adcy6. PPI network is shown in Figure 6B. Putting together with the above
12 analysis, these proteins were found to be associated with fatty acid degradation,
13 cysteine and methionine metabolism, carbon metabolism, thyroid hormone
14 synthesis, phospholipase D signaling pathway, endocrine and other factor-
15 regulated calcium reabsorption, synaptic vesicle cycle and GABAergic synapse,
16 and cholinergic synapse.

17

18 ***Common DEPs in AD/WT, TDMQ20/AD and CQ/AD categories***

19 We further analyzed the commonly altered DEPs in the AD/WT, TDMQ20/AD
20 and CQ/AD series. Among the 35 common DEPs (Supplementary Figure 4)
21 identified in AD/WT and TDMQ20/AD categories, and 30 common DEPs
22 (Supplementary Figure 5) identified in AD/WT and CQ/AD categories, 10 proteins
23 were found common in all three AD/WT, TDMQ20/AD and CQ/AD categories,
24 with DEPs in AD/WT that were reversed in TDMQ20/AD and CQ/AD (Figure 7,
25 panels A and B). Five proteins which were down-regulated in AD/WT, namely
26 Chrm4, Chat, Rin1, Lgalsl, and Mrps9 displayed a consistent up-regulated pattern
27 both in TDMQ20/AD and CQ/AD categories. Conversely, proteins Plp1, Vps29,
28 Rac1, Ptpnz1 and Cmpk1, which were overexpressed in AD/WT were down-
29 regulated in both TDMQ20/AD and CQ/AD (Figure 7B). According to PPI
30 network analysis, these 10 common DEPs are mainly involved in pathways related

1 to cognition, behavior, learning and memory, anterograde trans-synaptic signaling
2 as well as neuron projection development (Figure 7C). Most significantly regulated
3 proteins with gene names *Chat*, *Rin1*, *Chrm4*, *Ptprz1*, *Rac1* and *Plp*, were
4 associated with these pathways.

5 To further validate the proteomic results, the expression of seven proteins with
6 the gene names *Chrm4*, *Chat*, *Rin1*, *Lgalsl*, *Mrps9*, *Ptprz1* and *Cmpk1*, were
7 evaluated by Western blot analysis in WT, AD, TDMQ20 and CQ mice. Results
8 are reported in Figure 8.

9 Among these seven proteins, three proteins, which are significantly
10 downregulated in AD mice with respect to control animals (*ChAT*, *CHRM4* and
11 *Lgalsl*) were found up-regulated after treatment of AD mice with TDMQ20 with
12 respect to untreated AD mice (Figure 8A, C-E). Specially, the significantly
13 increased level of *ChAT* and *CHRM4* (Figure 8C and 8D, respectively) clearly
14 suggests a role of TDMQ20 in the cholinergic system. This result is consistent with
15 improved memory and behavioral performance of TDMQ20 treated 5xFAD mice
16 that was previously reported (21). Conversely, treatment by CQ failed to efficiently
17 reverse the low level of *ChAT* and *CHRM4* in AD mice. The level of *Lgalsl*,
18 downregulated in AD mice, was efficiently reversed by treatment with TDMQ or
19 CQ (Figure 8E). *Ptprz1* level was somewhat lower in AD mice than in control WT,
20 and its level trends to be reversed by TDMQ20 and CQ. However, these differences
21 indicate a tendency but are not statistically significant (Figure 8G). *RIN1*, *Mrps9*
22 and *Cmpk1* levels were found similar in WT control mice and in 5xFAD mice. So,
23 these proteins cannot be considered as relevant of AD targets in this animal model
24 (Figure 8F, 8H, 8I).

25

26 ***Immunofluorescence staining of A β plaques in the cortex***

27 As one of the important pathological hallmarks in AD brains, we additionally
28 quantified amyloid plaques in the mouse cortex of each group. The amyloid plaque
29 load of each mouse group was quantified as the percentage of area covered by
30 6E10-immunoreactivity (Figure 9) in each image sample, acquired using a

1 florescent microscope. According to the result, the cortex of mice treated with
2 TDMQ20 exhibited a drastically reduced plaque load compared to untreated AD
3 mice (roughly -70% compared to AD mice), suggesting a lower amount of
4 uncontrolled copper ions promoting plaque formation in the brain of treated mice.
5 This result is consistent with a lower concentration of malondialdehyde, a marker
6 of oxidative stress, in the cortex of AD mice treated with TDMQ20 we reported in
7 a previous study (30). The reduction of plaque load upon treatment with CQ was
8 also significant (-57%), which is also consistent with previous report (31).

9

10 **DISCUSSION**

11 TDMQ20 is a highly specific copper chelator able to extract Cu from Cu-A β
12 complex, thus inhibiting Cu-A β induced activation of dioxygen and ROS
13 production, and to release Cu from Cu-TDMQ20 into normal physiological
14 circulation (19). Furthermore, oral treatment of TDMQ20 on 5xFAD mice had a
15 clear beneficial effect the short-term memory and cognitive function of this
16 transgenic model of Alzheimer's disease (21). In order to investigate further the
17 underlying mechanism of action of TDMQ20, and specially the possible
18 interaction with the copper dependent cholinergic system, the current study was
19 designed as an exploration based on proteomic and bioinformatic analysis. By
20 using iTRAQ, proteomic alterations in the cortex of 5xFAD mice with respect to
21 WT animals were investigated, leading to identification of DEPs in AD with
22 respect to healthy animals. The fate of these DEPs proteins in AD mice treated by
23 TDMQ20 (or CQ used as comparator) was then investigated in order to evaluate
24 the relationship between (i) the pattern of protein modifications induced by
25 TDMQ20 treatment, (ii) copper regulation in brain and (iii) ability of this drug to
26 reverse AD impairment.

27

28 ***Proteomics of AD 5xFAD mice compared to healthy WT mice (AD/WT)***

29 Amyloid precursor protein (APP) was highly upregulated with a fold change of 6.8
30 in our 5xFAD mice with respect to WT (raw data: Supplementary 1.xlsx, sheet AD

1 vs WT), result consistent with plaque accumulation in AD condition (Figure 9).
2 More importantly, Rin1 (Ras and Rab interactor1), known to take part in the long-
3 term potentiation (32) and neuronal plasticity (33), is downregulated with a fold
4 change of 0.3 in 5xFAD mice. Several other DEPs identified in AD/WT were
5 involved in synaptic vesicle cycle: proteins Slc18a and Cacna1a were upregulated,
6 and Ap2a1, Syt1 and Atp6v1b2 were down-regulated, indicating alterations of
7 synaptic signaling and plasticity that are likely linked to impairment of learning,
8 memory and cognitive functions. Another important set of DEPs are related to
9 choline metabolism pathway: ChAT, Chrm4, Slc44a1 and Plpp3 were
10 downregulated, and Prkcg was upregulated. Reduced expression of ChAT (fold
11 change = 0.2), already reported (34-36), is consistent with low Ach level and
12 degeneration of cholinergic neurons in AD brain (37). The significant lower level
13 of Chrm4 (fold change = 0.2) in our 5xFAD mice with respect to healthy animals
14 confirms impairment of cholinergic system at this stage of AD.

15

16 ***Proteomics of AD mice treated with TDMQ20 compared to untreated AD mice***
17 ***(TDMQ20/AD)***

18 After treatment of AD mice by TDMQ20, 183 DEPs, with 72 up-regulated and 111
19 down-regulated with respect to untreated animals (39% and 61%, respectively).
20 Among these 183 DEPs, 35 DEPs were found common in AD/WT and
21 TDMQ20/AD comparisons and are analyzed below. Most of these 35 DEPs were
22 involved in PI3K-Akt signaling pathway involved in cellular survival and
23 proliferation. In addition, several DEPs link to PI3K-Akt signaling pathway, with
24 the gene names Hsp90ab1, Ywhaz, Ywhag, and Rac1 also present properties in
25 synaptic plasticity, such as axon extension (38), synapse formation (39), dendritic
26 spine enlargement and morphogenesis (40,41). The downregulation in AD of
27 another protein involved in PI3K-Akt pathway, galectin-related protein Lgalsl (=
28 HSPC159) (42) was also reversed upon treatment by TDMQ20.

29 Regarding nervous system, TDMQ20/AD DEPs also showed a significant
30 connection with the synaptic vesicle cycle. Cplx1 and Cplx3, which regulate

1 synaptic vesicle exocytosis and neurotransmitter secretion (43,44), were
2 significantly up-regulated in TDMQ20 treated AD mice. The above-mentioned
3 downregulation of Rin1, involved in synaptic plasticity in AD, was significantly
4 reversed upon treatment by TDMQ20. Specially, the muscarinic receptor Chrm4
5 was up-regulated with a high fold change value of 6.6, with respect to untreated
6 AD mice. Chrm4 is localized at the pre-synapse of axon terminals and on dendritic
7 membranes at the post-synapse (45), with high density in striatum, cortex and
8 hippocampus (31), and alterations of Charm4 have been reported in AD as early as
9 1982. This confirms the very likely role of TDMQ20 in regulation of cholinergic
10 signaling in the brain, possibly through modulation of synaptic neurotransmission
11 as indicated by biological process from GO analysis.

12

13 ***Proteomics of AD mice treated with CQ compared to untreated AD mice (CQ/AD)***

14 Despite clioquinol (CQ) was reported to exhibit beneficial effects on cognition in
15 a transgenic mouse model of AD (46), it was discarded due to severe neurotoxicity
16 due to its lack of metal selectivity (46). As historical prototype of copper chelator,
17 it was used as comparator in the present study. AD mice treated with CQ exhibited
18 73 (32%) upregulated and 153 (68%) downregulated DEPs with respect to
19 untreated AD mice. DEPs were mostly related to establishment of localization,
20 transport and single-organism localization in biological process and mostly link to
21 cytoplasmic (Cell Components). Unlike TDMQ20 treated mice, KEGG pathway
22 analysis showed a very high percent of DEPs involved in metabolic pathways
23 which might be related to detrimental side-effects of CQ in clinics. Other pathways
24 like endocytosis, adrenergic signaling in cardiomyocytes, pancreatic secretion,
25 carbon metabolism, purine metabolism, are also involved. Among all the analyzed
26 pathway, the endocrine and other factor-regulated calcium reabsorption pathway
27 showed the most significance, with eight proteins including Ap2b1, Plcb1, Atp2b1,
28 Atp1b1, Atp1b2, Calb1, Prkca and Adcy6. In the nervous system, the effect of CQ
29 on several types of synapse pathways. Rin1 displayed significant higher level in
30 CQ treated mice than in untreated AD animals. In cholinergic synapse pathway,

1 similarly to TDMQ20, Chrm4 was highly upregulated with respect to AD. Other
2 proteins related to cholinergic system: Map2k1, Gng3 and Kcnq2 were
3 downregulated.

4

5 ***Differentially expressed proteins in common to AD/WT, TDMQ20/AD and***
6 ***CQ/AD categories***

7 Ten target proteins were found common to these three groups, with similar
8 expression pattern either upregulated or downregulated from one to another.
9 Compared to AD/WT category, five proteins Chrm4, Chat, Rin1, Lgalsl, Mrps9
10 displayed a consistent up-regulated pattern, while five proteins Plp1, Vps29, Rac1,
11 Ptpnz1 and Cmpk1 were downregulated in both TDMQ20/AD and CQ/AD
12 categories. Based on the KEGG pathway analysis, these common DEPs were
13 mainly involved in cognition, learning and memory, behavior, anterograde trans-
14 synaptic signaling and neuron projection development. According to the
15 commercial availability of the antibodies of these common DEPs, seven proteins
16 with the gene names Chrm4, Chat, Rin1, Lgalsl, Mrps9, Ptpnz1 and Cmpk1 were
17 selected and validated by Western blot analysis. Results showed that three proteins,
18 with the gene names Chrm4, Chat and Lgalsl, were found significantly up-
19 regulated after the treatment of TDMQ20, and the decreased level of Lgalsl in AD
20 mice was also reversed by CQ treatment.

21 Among the ten proteins reversed to normal levels by both TDMQ20 and CQ,
22 three of them were successfully validated by Western blot analysis for TDMQ20
23 treatment. Specially, the relative abundance levels of ChAT and CHRM4 (impaired
24 in 5xFAD mice) were significantly increased upon TDMQ20 treatment (with fold
25 changes of 4.3 and 6.6, respectively), while CQ did not induce significant changes
26 of these protein levels (Figure 8), suggesting a specific action of TDMQ20 on these
27 two masterpiece proteins of the cholinergic system.

28

29 Table 3. List of top 10 common DEPs reversed by treatment by TDMQ20 or CQ

30

No.	UniProt ID	Gene names	Protein names	TDMQ:AD Fold change	AD:WT Fold change	CQ:AD Fold change
1	P32211	Chrm4	Muscarinic acetylcholine receptor M4 (Mm4 mAChR)	6.6	0.2	5.6
2	Q03059	Chat	Choline O- acetyltransferase (CHOACTase) (ChAT) (Choline acetylase) (EC 2.3.1.6)	4.3	0.2	4.8
3	Q921Q7	Rin1	Ras and Rab interactor 1 (Ras interaction/interference protein 1)	3.1	0.3	4.0
4	Q8VED9	Lgalsl	Galectin-related protein (Galectin-related protein A) (Lectin galactoside-binding-like protein A)	2.3	0.5	1.7
5	Q9D7N3	Mrps9	28S ribosomal protein S9, mitochondrial (MRP-S9) (S9mt)	1.9	0.6	1.5
6	D3Z645	Vps29	Vacuolar protein sorting-associated protein 29 (Vesicle protein sorting 29)	0.7	1.4	0.6
7	Q3TLP8	Rac1	Ras-related C3 botulinum toxin substrate 1	0.7	1.3	0.5
8	B9EKR1	Ptprz1	Receptor-type tyrosine- protein phosphatase zeta (R-PTP-zeta) (EC 3.1.3.48)	0.8	1.3	0.8
9	A0A0R4J093	Cmpk1	UMP-CMP kinase (EC 2.7.4.14) (Deoxycytidylate kinase) (CK) (dCMP kinase) (Nucleoside- diphosphate kinase) (EC 2.7.4.6) (Uridine monophosphate/cytidine monophosphate kinase)	0.8	1.2	0.8

			(UMP/CMP kinase)			
			(UMP/CMPK)			
			Myelin proteolipid			
10	P60202	Plp1	protein (PLP)	0.5	0.7	0.5
			(Lipophilin)			

1

2

3 ***Plaque load in the mouse cortex***

4 Amyloid plaque, consisting of misfolded and aggregated amyloid β peptides is one
5 of the well-known hallmarks of AD pathological states. In our present study, we
6 calculated the plaque load in mouse cortex as the ratio of area covered by the anti-
7 A β antibody 6E10 to the entire imaged area acquired by a fluorescent microscope.
8 Treatment by TDMQ20 remarkably reduced plaque load in the cortex by about
9 70%, suggesting a beneficial role of TDMQ20 in clearing pathological A β deposits
10 in AD mice. CQ treatment also contributed to an approximately 57% reduction of
11 plaque load in mouse cortex, though not as much as TDMQ20 did. These results
12 further consolidate TDMQ20 as a good candidate for AD treatment.

13

14 **EXPERIMENTAL PROCEDURES**

15 ***Experimental design and statistical rationale***

16 5xFAD mice (genetic background: C57BL/6 x SJL) were used as AD model. These
17 mice express human APP and PSEN1 transgenes with a total of five AD-linked
18 mutations: the Swedish (K670N/M671L), Florida (I716V), and London (V717I)
19 mutations in APP, and the M146L and L286V mutations in PSEN1. Starting when
20 they were two-month-old, these AD mice were orally treated either with TDMQ20
21 or CQ and compared with untreated mice. The expression of proteins in the cortex
22 of these three AD mouse groups and in the healthy control WT C57BL/6 x SJL
23 mice was compared using iTRAQ labeling and bioinformatics analysis described
24 below. GraphPad Prism 8.0.2 software was used for statistical analysis. Data were
25 expressed as mean \pm SEM unless otherwise indicated, with $P < 0.05$ defining
26 differences as statistically significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).
27 Differences among means were determined using the two-tailed t test.

1

2 ***Materials and animals***

3 TDMQ20 was synthesized according to Reference 18. Clioquinol (5-chloro-7-
4 iodo-8-hydroxyquinoline, abbreviated as CQ) was purchased from Sigma (Sigma-
5 Aldrich Co., St. Louis, USA). All other reagents were supplied by Sigma-Aldrich,
6 AB Sciex or Promega, except otherwise stated.

7 Thirty specific pathogen-free (SPF) 5xFAD mice and 10 non-transgenic
8 C57BL/6 x SJL control mice (male/female ratio = 1/1, weighing 20-25 g, 8 weeks)
9 have been used. Animals were housed by groups in standard laboratory conditions:
10 air-conditioned room (20-25°C), 12 hours light/dark illumination cycle, free access
11 to food and water. Food was provided by Guangdong Medical Experimental
12 Animal Center. All protocols were implemented in accordance with NIH
13 guidelines (NIH publication No. 85-23, revised 1985) for animal care and welfare.
14 All animal care and experiments were approved by the Institutional Animal Care
15 and Use Committee of the Institute for Nutritional Sciences in China and approved
16 by the Regional Ethical Committee for Animal Experimentation at Shenzhen
17 University (Permit Number: AEWC-20140615-002) and performed according to
18 the animal protection law.

19

20 ***Animal grouping and treatment***

21 Two-month-old mice were separated into four groups and drug-treated as indicated
22 in Table 1. Drugs were delivered to stomach of mice once every two days (oral
23 administration by gavage, volume of each gavage = 100 μ L, of a dose = 10 mg/kg)
24 for three months (in total 45 times), and followed with behavioral tests as
25 conducted previously (21).

26

27 Table 1 Mice grouping and drug administration

Group	Drug	Drug dose (mg/kg)
WT (C56BL/6J control)	0.9% NaCl	0
AD (5xFAD mice)	0.9% NaCl	0

TDMQ20 (5xFAD treated with-)	TDMQ20 in 0.9% NaCl	10
CQ (5xFAD treated with-)	Clioquinol in DMSO	10

1

2 ***Sample preparation***

3 Fourteen days after the end of treatment (5.5 months old mice), mice were killed
4 by cervical dislocation. Brains were rapidly excised and placed on an ice-cold plate
5 for dissection of cortices. Six cortex tissue samples were taken from each group of
6 mice for proteomics analysis. Tissue samples were lysed in lysis buffer containing
7 7 M urea, 2 M thiourea, 4 w/v% 3-[(3-cholamidopropyl)dimethylammonio]-1-
8 propanesulfonate hydrate (CHAPS), 40 mM dithiothreitol (DTT), and 40 mM Tris
9 base), sonicated 10 times for 5 s with 10 s pause in an iced water bath, and
10 centrifuged for 10 min at 12 000 rpm, 4 °C. The supernatant was withdrawn and
11 stored at –80 °C until use.

12

13 ***Trypsin digestion and iTRAQ labeling***

14 For proteomics analysis, equal amounts of above protein samples from 6 mice
15 cortex (random selection) in each group were pooled separately to yield two
16 distinct pools in each group. Protein digestion and iTRAQ labeling were performed
17 as previously reported (30,47). In brief, approximately 100 µg of each processed
18 tissue protein sample was reduced with 10 mM DTT (Sigma Aldrich, Missouri,
19 USA) for 1 h at 37 °C, followed by alkylation using 50 mM iodoacetamide (IAA,
20 Sigma Aldrich, Missouri, USA) for 15 min at room temperature in the dark. The
21 samples were then desalted and buffer-changed three times with 100 µL of 0.5 M
22 triethylammonium bicarbonate (TEAB) (B Sciex, Foster City, CA, USA) by
23 Amicon® ultra centrifugal filters (10 kDa cutoff; Millipore, Billerica, MA). The
24 proteins were digested with trypsin (Promega, Madison, WI, USA) at a ratio of
25 1:30 at 37 °C overnight. The digested peptides were then labeled with iTRAQ
26 reagents as follows (Table 2): the WT group was labeled with iTRAQ 117 and 118,
27 while the AD group with iTRAQ 119 and 121, the TDMQ20 treated AD group
28 with iTRAQ 113 and 114, and the CQ treated AD group with iTRAQ 115 and 116.

1 The labeled samples were mixed together and lyophilized. After labeling, the
2 peptides were subjected to reverse-phase chromatography at pH 10, to remove the
3 excess of labeling reagents and salts by HPLC system (Agilent Technologies, Santa
4 Clara, CA, USA). Peptides were eluted and combined into 16 batches. They were
5 then lyophilized and stored at -80 °C.

6

7 Table 2. iTRAQ labeling of the samples

Group	TDMQ20	CQ	WT	AD
iTRAQ label	113/114	115/116	117/118	119/121

8

9 *Nano LC-MS/MS (Mass Spectrometry) analysis and iTRAQ quantification*

10 Nano LC-MS/MS analysis was conducted as previously described (30,47). Briefly,
11 the 16 pooled batches were reconstituted in 30 μ L of 0.1% formic acid and 2%
12 acetonitrile, among which 8 μ L of each batch was added to a Triple TOF 6600
13 system fitted with a NanosprayIII source (AB Sciex). Data were obtained with a
14 2.4 kV ion spray voltage, 30 psi curtain gas, 5 psi nebulizer gas, and an interface
15 heater temperature of 150 °C. TOF-MS was conducted with a scan scope of 350-
16 1500, and for MS/MS was 400-1250. Smart information-dependent acquisition
17 was activated with automatic collision energy and automatic MS/MS accumulation.
18 The protein was identified and quantified by ProteinPilot v5.0 (AB Sciex) with the
19 Paragon algorithm against the Uniprot “complete proteome” mouse proteins
20 database. Based on a 95% confidence level, at least one unique peptide per protein
21 group was required for identifying proteins, and two quantified peptides were
22 required for quantifying proteins. The cutoff values of 1.2-fold for up-regulated
23 and 0.8-fold for down-regulated proteins, p value <0.05, were considered as
24 significant results. This significant change was further determined by conversion
25 of log₂ ratios, followed by data normalization and performance of a statistical t test
26 by using OMICSBEAN online tools (<http://www.omicsbean.cn>)(48).

27

28 *Bioinformatics analysis*

1 Bioinformatics analysis was performed by using OMICSBEAN online tools and
2 STRING (Search Tool for the Retrieval of Interacting Genes/Proteins, version 9.1,
3 <http://string-db.org/>) database. Differentially expressed proteins (DEPs) between
4 the different mouse groups were analyzed using Gene Ontology annotation (GO),
5 Kyoto Encyclopedia of Genes and Genomes pathways (KEGG) and protein-
6 protein interaction (PPI) networks.

7

8 ***Western Blot Analysis***

9 The validation of targeted proteins was performed by Western blot analysis.
10 Proteins were extracted from the cortex tissue after homogenizing and their
11 concentrations were measured by PierceTM BCA Protein Assay Kit (ThermoFisher,
12 MA, USA). After optimizing the protein concentration, samples were boiled for 10
13 min at 100 °C in water bath together with a 4x loading buffer (BOSTER, California,
14 USA). Proteins were separated on denaturing gel electrophoresis (10% SDS-PAGE
15 (Epizyme, Shanghai, China) for 50 minutes. Separated proteins were further
16 transferred into a nitrocellulose membrane (0.45 μm, Millipore, Darmstadt,
17 Germany). Then, membranes were gently shaken in a blocking solution (Beyotime,
18 Shanghai, China) for about two hours. Primary antibodies (anti-CHRM4, anti-
19 CMPK1, anti-LGALS1, anti-MRPS9, rabbit polyclonal, ABClonal, Wuhan, China;
20 anti-PTPRZ, mouse monoclonal, BD Biosciences, NJ, USA; anti-ChAT, rabbit
21 polyclonal, Cell signaling Technology, Massachusetts, USA; anti-RIN1, rabbit
22 polyclonal, Invitrogen, MA, USA) were added and incubated with membranes
23 overnight, at 4 °C . Corresponding secondary antibodies (anti-rabbit/mouse
24 horseradish peroxidase conjugated, Abmart, NJ, USA) were added after washing 3
25 × 10 min with 4% TBST buffer (tris-buffered saline and Tween 20, pH 7.50) and
26 further incubated for 2 hours at room temperature. After washing 3 × 10 min with
27 TBST buffer, blots were visualized by incubating with ECL reagent (Advansta,
28 CA, USA) and exposed with the Tanon-5200 chemiluminescence imaging system
29 (Tanon, Shanghai, China).

30

1 ***Immunofluorescence staining and image acquisition***

2 Mouse brains were removed after cervical dislocation and were prepared as
3 paraffin-embedded slices cut with ~4 μm thickness. Six slices from each group
4 were selected and deparaffinized by immersing in xylene (2×5 min), and then
5 rehydrated by immersing in the following solutions for 3 min each: 100% ethanol,
6 95% ethanol, 85% ethanol, 75% ethanol, 50% ethanol, 30% ethanol and then
7 deionized water. Slices were then washed in PBS for 2×5 min. After drawing a
8 circle around each slice with a hydrophobic barrier pen, permeabilization buffer
9 containing 0.8% Triton X-100 in PBS was added to each slice for 30 min. Slices
10 were further incubated in blocking solution containing 10% normal goat serum
11 (Solarbio, Beijing, China) at RT for 2 h, and then with primary antibodies (anti-
12 6E10, mouse polyclonal, Biolegend, San Diego, USA; anti-MAP2, chicken
13 polyclonal, Invitrogen, MA, USA) overnight at 4 °C. Slices were washed 3×10
14 min with PBS and then incubated with the secondary antibodies (goat anti-chicken
15 Alexa Fluor 488, goat anti-mouse Alexa Fluor 568, Life Technologies GmbH) for
16 2 h at RT in the dark. After 3×10 min washing in PBS, slices were further stained
17 with DAPI (10 $\mu\text{g}/\text{mL}$, Beyotime, Shanghai, China) for 15 min in the dark. Then
18 slices were washed for 3×10 min with PBS before mounting on glass coverslips
19 with Dako fluorescence conserving medium (Dako, Hamburg, Germany). The
20 dilution of antibodies used here were 1:500 in blocking solution. Images were
21 acquired by using Olympus BX53 fluorescence microscope under a $20 \times$ objective,
22 with resolution of 1920×1900 pixels.

23

24 **CONCLUSIONS**

25 Our current study has been designed as an investigation of protein-based molecular
26 mechanism underlying the improved cognitive and behavioral performance in a
27 transgenic 5xFAD mouse model of Alzheimer's disease upon treatment with the
28 copper chelator TDMQ20. Abnormally expressed proteins in the cortex of AD
29 mice are closely involved in several key functional brain pathways, such as choline

1 metabolism, trans-synaptic signaling, chemical synaptic transmission, and synaptic
2 vesicle cycle.

3 Among DEPs common to AD/WT, TDMQ20/AD categories, ChAT and
4 CHRM4 were further validated by Western blot analysis, suggesting a recovery of
5 the cholinergic system functioning induced by TDMQ20, a feature which may
6 account for improved cognitive, behavioral performances and spatial memory of
7 AD mice upon treatment by TDMQ20. Especially, after treatment of AD mice by
8 TDMQ20, ChAT level was similar to that observed in healthy WT mice. Down-
9 regulation of Lgals1 observed in AD mice was also fully reversed upon TDMQ20
10 treatment. These results suggest that, beside "traditional" targeting of AChE, ChAT
11 and Chrm4 involved in cholinergic system also deserve consideration as potential
12 targets for AD chemotherapy. Noteworthy, cortex of mice treated with TDMQ20
13 do not present the panel of metabolic DEPs that might to be responsible for CQ
14 toxicity.

15 Moreover, the load of amyloid plaques in mouse cortex was drastically
16 reduced after treatment by TDMQ20, suggesting a lower amount of uncontrolled
17 copper ions promoting plaque formation in the brain of treated mice. This result is
18 consistent with the decreased level of malondialdehyde observed in the three
19 different AD mouse models upon TDMQ20 treatment in our previous study. The
20 simultaneous reversal of the deprivation of proteins involved in the copper
21 dependent cholinergic system, suggests the capacity of TDMQ20 to act as regulator
22 of copper homeostasis *in vivo*. The correlation of such a mechanism of action of
23 TDMQ20 and drastic improvement of the cognitive performances of treated
24 animals validates copper homeostasis as a priority target and TDMQ20 as a drug
25 candidate.

26

27 **Author Contributions**

28 B.M., Q.L., and A.R. designed the research; Y.L. and colleagues performed the
29 chelator synthesis; F.S., J.Z., and H.Z. performed biological assays, animal

1 experiments, and analyzed the data; F.S., Q.L., A.R., and B.M., wrote the
2 manuscript.

3

4 **Conflicts of Interest**

5 None.

6

7 **Supporting Information**

8 Figure S1-S3, heat-map of DEPs with expression intensities in AD/WT,
9 TDMQ20/AD, and CQ/AD categories, respectively; Figure S4, common DEPs in
10 AD/WT and TDMQ20/AD categories; Figure S5, common DEPs in AD/WT and
11 CQ/AD categories (PDF)

12

13 **ACKNOWLEDGEMENT**

14 This study was financially supported by grants from the Guangdong Provincial
15 Key S&T Program (2018B030336001), the Shenzhen Science and Technology
16 Innovation Commission (JCYJ20200109110001818), the Shenzhen-Hong Kong
17 Institute of brain Science-Shenzhen Fundamental Research (2021SHIBS0003) and
18 by the CNRS, France. The Instrumental Analysis Center of Shenzhen University
19 (Xili Campus) is acknowledged for access to the experimental instruments.

20

21 **ABBREVIATIONS**

22 The abbreviations used are: A β , amyloid- β peptide; Ach, acetylcholine; AChE,
23 acetylcholine esterase; AD, Alzheimer's disease; APP, amyloid precursor protein;
24 ChAT, choline *O*-acetyltransferase; Chrm4, muscarinic acetylcholine receptor M4;
25 Cmpk1, UMP-CMP kinase; CQ, clioquinol; DEPs, differentially expressed
26 proteins; GO, gene ontology; iTRAQ, isobaric tag for relative and absolute
27 quantitation; KEGG, Kyoto encyclopedia of genes and genomes; Lgls1, galectin
28 related protein; Mrps9, 28S ribosomal protein S9; PPI, protein-protein interactions;
29 Ptpnz1, Receptor-type tyrosine protein phosphatase zeta; Rin1, Ras and Rab

1 interactor 1; SFP, specific pathogen free; TDMQ, tetradentate monoquinoline
2 chelator.

3

4 **FIGURE LEGENDS**

5 **Figure 1. Proteomic and bioinformatic analysis of DEPs in AD/WT category.**

6 (A) Volcano-diagram of DEPs. (B) Number of up-and down-regulated DEPs. (C)
7 GO (Gene Ontology) analysis of DEPs including BP (biological process), CC (cell
8 component), MF (molecular function). (D) Biology process. (E) Cell component.

9

10 **Figure 2. Bioinformatic analysis of DEPs in AD/WT category.** (A) KEGG
11 pathway analysis. (B) PPI network of DEPs.

12

13 **Figure 3. Proteomic and bioinformatic analysis of DEPs in TDMQ20/AD**
14 **category.** (A) Volcano-diagram of DEPs. (B) Number of up-and down-regulated

15 DEPs. (C) GO (Gene Ontology) analysis of DEPs including BP (biological
16 process), CC (cell component), MF (molecular function). (D) Biology process. (E)
17 Cell component.

18

19 **Figure 4. Bioinformatic analysis of DEPs in TDMQ20/AD category.** (A) KEGG
20 pathway analysis. (B) PPI network of DEPs.

21

22 **Figure 5. Proteomic and bioinformatic analysis of DEPs in CQ/AD category.**

23 (A) Volcano-diagram of DEPs. (B) Number of up-and down-regulated DEPs. (C)
24 GO (Gene Ontology) analysis of DEPs including BP (biological process), CC (cell
25 component), MF (molecular function). (D) Biology process. (E) Cell component.

26

27 **Figure 6. Bioinformatic analysis of DEPs in CQ/AD category.** (A) KEGG
28 pathway analysis. (B) PPI network of DEPs.

29

30 **Figure 7. Common DEPs in AD/WT, TDMQ20/AD and CQ/AD categories.** (A)

1 Venn diagram of common DEPs among three categories. (B) Heat-map of common
2 DEPs among three categories with expression intensities. (C) PPI analysis of
3 common DEPs among three categories.

4

5 **Figure 8. Western blot validation of proteins.** (A), (C)-(G), Choline O-
6 acetyltransferase (ChAT), Muscarinic acetylcholine receptor M4 (Chrm4),
7 Galectin-related protein (Lgalsl), Ras and Rab interactor 1 (Rin1), 28S ribosomal
8 protein S9 (Mrps9). (B), (H)-(I), UMP-CMP kinase (Cmpk1), Receptor-type
9 tyrosine-protein phosphatase zeta (Ptrpz1). Quantification was conducted by
10 ImageJ software as ratio of optic density between each band and their
11 housekeeping proteins (α -Tubulin or β -Actin), data analysis and graph were done
12 with Prism GraphPad 8.0.2.2, n=3.

13

14 **Figure 9. Plaque load in mouse cortex of each group.** (A) Representative images
15 of immunohistochemical stainings of amyloid- β plaque (stained with 6E10, red),
16 nuclei (stained with DAPI, blue), microtubule associated protein 2 (MAP2, green)
17 in the cortex. (B) Plaque load in mouse cortex. Analysis was conducted by Image
18 J, calculated as the proportion of area covered by 6E10 staining. Scale bar, 50 μ m.

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