Brief report

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SORL1 Is Genetically Associated with Late-Onset Alzheimer's Disease in Japanese, Koreans and Caucasians

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Abstract

To discover susceptibility genes of late-onset Alzheimer's disease (LOAD), we conducted a 3-stage genome-wide association study (GWAS) using three populations: Japanese from the Japanese Genetic Consortium for Alzheimer Disease (JGSCAD), Koreans, and Caucasians from the Alzheimer Disease Genetic Consortium (ADGC). In Stage 1, we evaluated data for 5,877,918 genotyped and imputed SNPs in Japanese cases (n = 1,008) and controls (n = 1,016). Genome-wide significance was observed with 12 SNPs in the *APOE* region. Seven SNPs from other distinct regions with p-values $<2\times10^{-5}$ were genotyped in a second Japanese sample (885 cases, 985 controls), and evidence of association was confirmed for one *SORL1* SNP (rs3781834, $P=7.33\times10^{-7}$ in the combined sample). Subsequent analysis combining results for several SORL1 SNPs in the Japanese, Korean (339 cases, 1,129 controls) and Caucasians (11,840 AD cases, 10,931 controls) revealed genome wide significance with rs11218343 ($P=1.77\times10^{-9}$) and rs3781834 ($P=1.04\times10^{-8}$). SNPs in previously established AD loci in Caucasians showed strong evidence of association in Japanese including rs3851179 near *PICALM* ($P=1.71\times10^{-5}$) and rs744373 near *BIN1* ($P=1.39\times10^{-4}$). The associated allele for each of these SNPs was the same as in Caucasians. These data demonstrate for the first time genome-wide significance of LOAD with *SORL1* and confirm the role of other known loci for LOAD in Japanese. Our study highlights the importance of examining associations in multiple ethnic populations.

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Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by cognitive dysfunction and memory loss. Multiple rare mutations in *APP*, *PSEN1*, *PSEN2* and *SORL1* account for most cases of early-onset autosomal dominant AD [1,2]. Risk of late-onset AD (LOAD), the most common type of dementia in the elderly, is associated with complex interactions between genetic and environmental factors. Until recently, *APOE* was the only unequivocally recognized major susceptibility gene for LOAD [1,3]. Several genome-wide association studies (GWAS) each including more than 5,000 Caucasians identified genome-wide significant associations for LOAD with nine other loci including *ABCA7*, *BIN1*, *CD2AP*, *CD33*, *CLU*, *CR1*, *EPHA1*, *MS4A* gene cluster, and *PICALM* [4,5]. To our knowledge, no large GWAS for LOAD has been performed in any Asian population. Because there is a possibility that there exist ethnic-specific LOAD susceptibility variants, we carried out a large-scale GWAS to confirm associations at known loci and identify novel loci for LOAD using a three-stage design including a discovery Japanese cohort and replication cohorts of Japanese, Korean and Caucasian subjects.

Methods

Subjects

Japanese datasets. Clinically defined subjects were recruited by the Japanese Genetic Study Consortium of Alzheimer's Disease (JGSCAD: principal investigator, Y.I.) [6,7]. Probable AD cases were ascertained on the basis of the criteria of the National Institute of Neurological and Communicative Disorders, and Stroke-Alzheimer's Disease and Related Disorders (NINCDS/ADRDA) [8]. The Mini-Mental State Examination [9], Clinical Dementia Rating [10], and/or Function Assessment Staging [11] were primarily used for evaluation of cognitive impairment. Elders living in an unassisted manner in the local community with no signs of dementia were used as controls. DNA was extracted from peripheral blood leukocytes using standard protocols [6]. For the purpose of this study, the Stage 1 genome-wide association study (GWAS) dataset included 2024 subjects (1008 AD cases and 1016

controls) and the Stage 2 dataset included 1870 subjects (885 AD cases and 985 controls).

Korean dataset. A total of 339 subjects with AD were recruited at the Samsung Medical Center in Seoul, Korea. All AD subjects fulfilled NINCDS-ADRDA criteria for probable AD [8]. These subjects underwent a clinical interview and neurological examination that were previously described [12]. The absence of secondary causes of cognitive deficits was assessed by laboratory tests including complete blood count, blood chemistry, vitamin B12/folate, syphilis serology, and thyroid function tests. Conventional brain MRI scans (T1-weighted, T2-weighted, and FLAIR images) confirmed the absence of territorial cerebral infarctions, brain tumors, and other structural lesions. Healthy control subjects (n = 1,129) ages 55 to 85 years were recruited from routine health examination at the same location and showed no evidence of cognitive dysfunction.

Alzheimer Disease Genetics Consortium dataset. Summarized information from tests of genetic association of AD with SNPs located in the candidate gene regions was culled from a recent large GWAS conducted by the Alzheimer Disease Genetics Consortium (ADGC) [5]. Results were computed for SNPs throughout the genome in a sample composed of 11,840 AD cases and 10,931 cognitively normal elders from 15 independent Caucasian data sets. Details of the quality control and statistical analysis procedures and genetic models has been published elsewhere [5].

This study was approved by the Boston University Institutional Review Board, Institutional Review Board of Niigata University, and the Institutional Review Boards of all participating institutions. Written informed consent was obtained from all participants. Next of kin, carer takers or guardians consented on the behalf of participants whose capacity to consent was compromised. All subjects were anonymously genotyped. The basic demographics of the cases and controls before QC in each dataset are presented in Table 1.

Genotyping

GWAS genotyping was performed in the Stage 1 sample using Affymetrix GeneChip 6.0 microarrays containing 909,622 SNPs. Applied Biosystems' (ABI) TaqMan Assays were used to genotype individual SNPs in the Japanese and Korean replication cohorts. *APOE* genotypes in the Japanese and Korean samples were determined by haplotypes derived from rs7412 and rs429358 which were genotyped using TaqMan Assays. Details of *APOE* genotyping in each ADGC dataset were described previously [13].

Quality Control and Population Substructure

In the Stage 1 sample, SNPs with a genotype call rate (GCR) <95%, a minor allele frequency (MAF) <0.05, or significant deviation from the Hardy-Weinberg equilibrium (HWE) in controls ($P<10^{-6}$) were excluded. After excluding 83,673 low quality and 298,304 low frequency SNPs, we removed 196 subjects with a GCR <95% and 41 subjects whose gender as determined by analysis of X-chromosome data using the PLINK program (ver. 1.06) [14] was inconsistent with the reported gender. The same QC procedures were applied to the Japanese and Korean replication datasets. We examined population substructure in the GWAS dataset by analyzing tagging SNPs from the genome-wide panels using the *smartpca* module from EIGENSTRAT software [15] in a manner described previously [5]. Subsequently, we excluded three subjects who were cryptically related to other subjects in the dataset and 49 individuals who were population outliers. The strength of association of the top 10 principal components (PCs) was tested with AD status. The first

Table 1. *Sample size and characteristics of the discovery and replication datasets.*

Population (Stage)	Total	Alzheimer Disease Cases					Cognitively Normal Controls			
		N	Female (%)	Age at onset (mean ± SD)	Age at exam (mean ± SD)	*APOE* ε2/ε3/ε4 Frequency	N	Female (%)	Age at exam (mean ± SD)	*APOE* ε2/ε3/ε4 Frequency
Japanese Discovery (Stage 1)	2,024	1,008	723 (72%)	73.0 (4.28)	NA	0.02/0.65/0.33	1,016	583 (57%)	77.0 (5.89)	0.04/0.87/0.09
Japanese Replication (Stage 2)	1,870	885	574 (65%)	74.3 (6.98)	NA	0.02/0.69/0.29	985	618 (63%)	73.74 (5.84)	0.05/0.86/0.09
Korean (Stage 3)	1,468	339	245 (72%)	NA	73.67 (9.49)	0.03/0.70/0.27	1,129	550 (49%)	71.04 (4.86)	0.06/0.85/0.09
Caucasian (Stage 3)	22,771	11,840	7168 (61%)	76.37 (5.18)	80.59 (4.92)	0.04/0.61/0.36	10,931	6418 (59%)	76.77 (3.55)	0.08/0.78/0.14
TOTAL	**28,133**	**14,072**					**14,061**			

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three PCs were nominally associated with AD status. A total of 574,828 SNPs and 1,735 subjects comprising 891 cases and 844 controls passed the QC and were used for imputation and in further statistical analyses.

Genotype Imputation

Genotypes for all SNPs in Japanese and Caucasians were imputed with the Markov Chain haplotyping (MaCH) software [16] using reference haplotypes in the 1000 Genomes database (version released in February 2012 for Japanese datasets and version released in December 2010 for Caucasian datasets). This procedure also filled in missing data for the genotyped SNPs. Imputation quality was determined as R^2, which estimates the squared correlation between imputed and true genotypes. We applied threshold criteria for quality control assessment of imputed SNPs ($R^2 \geq 0.8$) as recommended for 1000 Genomes imputed data using the IMPUTE2 program [17]. Genotype probabilities for 5,877,918 genotyped and reliably imputed SNPs with a minor allele frequency (MAF) >0.02 were included in the Japanese GWAS.

Statistical analysis

Genotyped and imputed SNPs were tested for association with AD in the Stage 1 dataset using a logistic generalized linear model (GLM) controlling for age-at-onset (cases)/age-at-exam (controls), sex and the first three principal components from analysis of of population substructure. Stage 1 analyses were also performed based on a model adjusting for these covariates and the number of *APOE* ε4 alleles. SNPs in the *APOE* region (between map positions 45,000 kb and 45,800 kb on chromosome 19) were also tested for association in ε3/ε3 and ε3/ε4 subgroups. Genotyped SNPs were coded as 0, 1, or 2 according to the number of minor alleles under the additive genetic model. For imputed SNPs, a quantitative estimate between 0 and 2 for the dose of the minor allele were used to incorporate the uncertainty of the imputation estimates. All analyses were performed using PLINK. SNPs attaining a P value below 5×10^{-5} were considered for replication in Stage 2. Initially, only one SNP per region was tested in the replication sample to minimize the penalty for multiple testing. Additional SNPs from regions meeting the signifcance threshold in the replication sample were also evaluated. SNPs with a P value below 1×10^{-5} in the combined Stages 1 and 2 samples and nominally significant in Stage 2 ($P<0.05$) were advanced to Stage 3.

SNP association results obtained from individual datasets were combined by meta-analysis using the inverse variance method implemented in the software package METAL (http://www.sph.umich.edu/csg/abecasis/Metal/index.html) [18]. An additive model was assumed and the association results across datasets were combined by summing the regression coefficients weighted by the inverse variance of the coefficients. The meta-analysis P-value of the association was estimated by the summarized test statistic, after applying a genomic control within each individual study. Effect sizes were weighted by their inverse variance and a combined estimate was calculated by summing the weighted estimates and dividing by the summed weights.

Results

The quantile-quantile plot indicated limited genomic inflation ($\lambda = 1.04$in the Stage 1 GWAS results (Fig. S1). A total of 125 SNPs from seven distinct regions showed evidence of association with $P<10^{-4}$ (Table S1, Fig. S2). In addition to *APOE* SNP rs429358 ($P=2.46\times10^{-49}$, OR [95% CI] =5.5 [4.4–6.9]), 12 other SNPs in the *APOE* region were associated with LOAD at the genome-wide significance level of $P<5.0\times10^{-8}$. The two most significant results in this group of SNPs were rs12610605 (*PVRL2*: $P=1.38\times10^{-13}$, OR [95% CI] =1.8 [1.5–2.0]) and rs62117161 (between *CEACAM16* and *BCL3*: $P=3.46\times10^{-12}$, OR [95% CI] =0.47 [0.38–0.58]). Since imputation in the *APOE* region using the 1000 Genomes reference panel is unreliable [6], we genotyped nine SNPs from this region, spanning multiple linkage disequilibrium (LD) blocks (Fig. S3) and that were nominally significant in the *APOE* ε3/ε3 subgroup, in the Japanese discovery and replication samples using TaqMan assays (Table S2). Genome-wide significant results were obtained for five of these SNPs, but only the association with *PPP1R37* SNP rs 17643262 remained nominally significant after adjustment for the number of *APOE* ε4 alleles ($P=3.96\times10^{-4}$) or in analyses stratified by *APOE* genotype (ε3/ε3: $P=0.01$; ε3/ε4: P = 0.0016).

SNPs from six other distinct chromosomal regions met Stage 2 follow-up criteria ($P<5\times10^{-5}$) and the top SNP from each region was genotyped in an independent Japanese sample (Table 2). Two SNPs were nominally significant in the replication sample, however the effect direction for *KIAA0494* SNP rs7519866 differed from the discovery sample. Modest evidence for replication was observed only with *SORL1* SNP rs4598682 ($P\leq0.05$). Subsequently, we selected an additional four *SORL1* SNPs (rs3781834, rs2282647, rs17125523, and rs3737529) for testing in the Japanese replication sample that were among the most significant in the basic or extended models in the discovery sample (Table S1) and not in LD with rs4598682 ($r^2<0.2$, Figure S4). Two of these SNPs (rs3781834 and rs17125523) were chosen also because they were genotyped in the discovery sample and thus would minimize the effects of potential imputation artifacts in meta-analysis of the two Japanese samples. Highly significant results were obtained for *SORL1* SNPs rs4598682 ($P=9.51\times10^{-6}$), rs3781834 ($P=7.33\times10^{-7}$), rs17125523 ($P=5.51\times10^{-6}$), and rs3737529 ($P=4.14\times10^{-6}$) after combining results from the discovery and replication samples (Table S3).

These four *SORL1* SNPs showing significant association in the combined samples from Stages 1 and 2 were considered for further replication in Stage 3. We added rs11218343 to this stage of the analysis because it was the most significant *SORL1* SNP in the large Caucasian dataset ($P=1.0\times10^{-7}$), a result which emerged after pooling the Caucasian discovery GWAS sample and unpublished data in the replication sample from our previously published GWAS [5]. These five SNPs were subsequently evaluated in Stage 3 by meta analysis including the Stage 1 and 2 Japanese, Korean and ADGC Caucasian datasets. SNPs rs11218343 ($P=2.20\times10^{-9}$) and rs3781834 ($P=9.90\times10^{-9}$), attained genome-wide significance in the sample of datasets from all stages (Table 3, Fig. 1). There was modest evidence of replication for rs17125523 (meta $P=3.30\times10^{-6}$) and rs 3737529 (meta $P=5.10\times10^{-6}$). Although the allele frequencies for the top SNPs were very different between the Asian (MAF >0.2) and Caucasian (MAF <0.05) samples (Table 3), there was no evidence of heterogeneity in the magnitude of the odds ratios or effect direction among the population groups ($P>0.15$, Fig. 2). There was no apparent association in the comparably smaller Korean dataset; however, the direction of the effect for each SNP was the same as in the Japanese and Caucasian datsets.

Next, we investigated whether robust genetic associations for LOAD reported previously in Caucasians [4,5] generalize to Japanese. After correcting for 15 tests, SNPs rs3851179 located approximately 90 kb upstream from *PICALM* ($P=1.71\times10^{-5}$) and rs744373 located approximately 30 kb upstream from *BIN1* ($P=1.39\times10^{-4}$) were significantly associated with LOAD risk in the Japanese Stage 1 dataset (Table 4). Nominally significant

Table 2. Top-ranked genome-wide association results in the Japanese discovery (Stage 1) sample ($P<2.5\times10^{-5}$) and their replication in Japanese (Stage 2).

SNP	CH:MB	Nearest Gene	MA	MAF	# SNPs	Discovery (Stage 1)		Replication (Stage 2)		Meta-Analysis (Stages 1+2)	
						OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
rs7519866	1:47.0	*KIAA0494*	G	0.37	52	0.71 (0.61–0.83)	9.70×10^{-6}	1.15 (1.01–1.32)	0.04	0.90 (0.57–1.44)	0.67
rs913360	9:111.7	*PALM2*	G	0.28	20	1.56 (1.43–1.70)	1.83×10^{-7}	1.11 (0.96–1.29)	0.16	1.29 (1.15–1.44)	6.6×10^{-6}
rs1273007	10:9.0	*LOC338591*	T	0.27	39	0.68 (0.62–0.74)	3.08×10^{-6}	0.95 (0.81–1.10)	0.47	0.81 (0.73–0.91)	2.2×10^{-4}
rs10898417	11:85.2	*SYTL2*	G	0.15	2	0.59 (0.53–0.66)	1.17×10^{-6}	1.02 (0.85–1.22)	0.83	0.82 (0.71–0.93)	0.003
rs4598682	11:121.1	*SORL1*	G	0.23	11	0.68 (0.57–0.81)	2.25×10^{-5}	0.83 (0.68–1.00)	0.05	0.75 (0.66–0.85)	9.5×10^{-6}
rs11621843	14:92.2	*RIN3*	G	0.26	19	1.47 (1.35–1.60)	5.19×10^{-6}	1.03 (0.88–1.20)	0.72	1.21 (1.08–1.36)	8.1×10^{-4}

CH:MB, chromosome:position (in megabasepairs, build 19); MA, minor allele; MAF, minor allele frequency; # SNPs, the number of SNPs for which $P\leq1\times10^{-4}$ in the discovery (Stage 1) sample; OR, odds ratio; *P* P-value;
Selected SNPs represent the strongest association within each locus.
doi:10.1371/journal.pone.0058618.t002

associations were also observed for SNPs in *CR1*, *CLU*, and *ABCA7*. Of the eight SNPs tested in the small Korean sample, nominally signficant results ($P<0.05$) were obtained for one SNP in *CLU* and *PICALM*, each with the same pattern of association and comparable effect size as in Japanese.

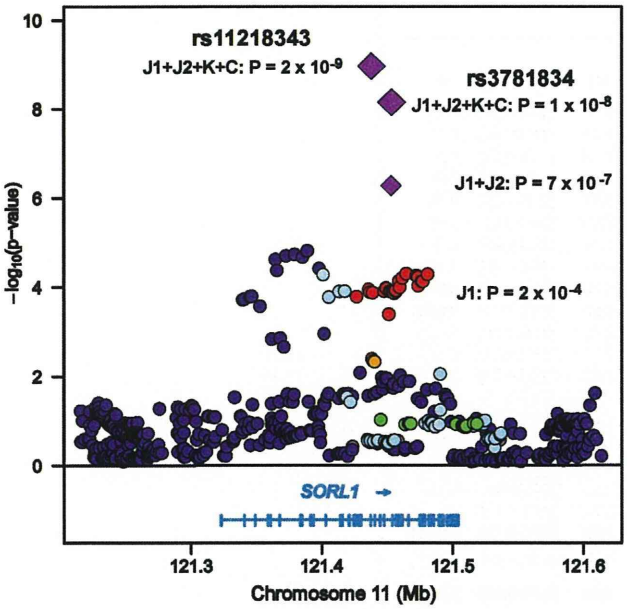

Figure 1. Regional association plot for the *SORL1* region on chromosome 11 in the three-stage design. For each SNP, the chromosomal location is shown on the x-axis and the significance level for association with LOAD is indicated by a $-\log_{10}$P value on the y-axis. P-values are expressed as $-\log_{10}$(P) (y-axis) for every tested SNP ordered by chromosomal location (x-axis). Genomic position was determined using the NCBI database (Build 37.1). Computed estimates of linkage disequilibrium (LD; r^2) between SNPs in this region with the top-ranked SNP (rs3781834) in the Japanese discovery (J1) dataset are shown as red circles for $r^2\geq0.8$, orange circles for $0.5\leq r^2<0.8$, light blue circles for $0.2\leq r^2<0.5$, and dark blue circles for $r^2<0.2$ using hg19/1000 Genomes of Asian populations (ASN; release on November 2010) combined from Han Chinese (CHB) and Japanese (JPT). Meta-analysis *P*-values are shown as purple diamonds for the Japanese datasets (J1+J2) and all datasets (J1+J2+K+C) including Japanese, Korean (K), and Caucasians (C). Two genome-wide significant SNPs in the final stage (rs3781834 and rs11218343) are presented. The gene structure and reading frame are shown below the plot. Exons are denoted with vertical bars. The LD between rs3781834 and rs11218343 is 0.57 in the ASN reference population.
doi:10.1371/journal.pone.0058618.g001

Discussion

Our multi-stage GWAS of LOAD identified for the first-time genome-wide significant association with *SORL1*. Genetic association with *SORL1* was first established in a study focused on genes encoding proteins involved in vacuolar protein sorting [19]. Most, but not all, subsequent studies in Caucsians replicated this finding (summarized in Alzgene database: http://www.alzgene.org/). Confirmatory evidence of association with *SORL1* SNPs has also been reported in comparatively small samples of Chinese and Japanese (reviewed in [20]). These findings are independent of previous candidate gene studies of *SORL1* in Japanese (two subjects in common) and with Caucasians in the Rogaeva et al. study [19] (less than 2% overlap).

The two genome-wide significant *SORL1* SNPs, rs11218343 and rs3781834 are located at chromosome positions 121,435,587 base pairs and 121,445,940 base pairs, respectively, and thus between the two previously reported strongly associated 3-marker haplotypes that extend upstream from rs641120 (121,380,965 base pairs) and downstream from rs1699102 (121,456,962 base pairs) [19]. A recent meta-analysis including more than 30,000 Caucasian and Asian subjects demonstrated that multiple *SORL1* SNPs in distinct regions are associated with AD [20], a finding substantiated in an association study of *SORL1* SNPs with brain MRI traits in LOAD families [21]. Further analysis of our large Caucasian sample suggests that the association peak at rs3781834 is independent of at least one of the two distinct haplotypes previously associated with AD in an independent sample of non-Hispanic Caucasians, Caribbean Hispanics and Israeli-Arabs (Fig. S5) [19], Since all of the SNPs at the association peaks reported in this study and previously are intronic, functional studies are required to determine the identity of pathogenic variants at these locations.

Remarkably, the less frequent alleles at rs11218343 and rs3781834 are protective in both Japanese and Caucasian datasets with very similar odds ratios (range 0.74 to 0.83) despite the fact that these alleles are much rarer in Caucasians (4% and 2%, respectively) than in Japanese (34% and 23%, respectively). The rarity of these SNPs in Caucasians, as well as allelic heterogeneity, may explain why *SORL1* did not previously emerged as a genome-

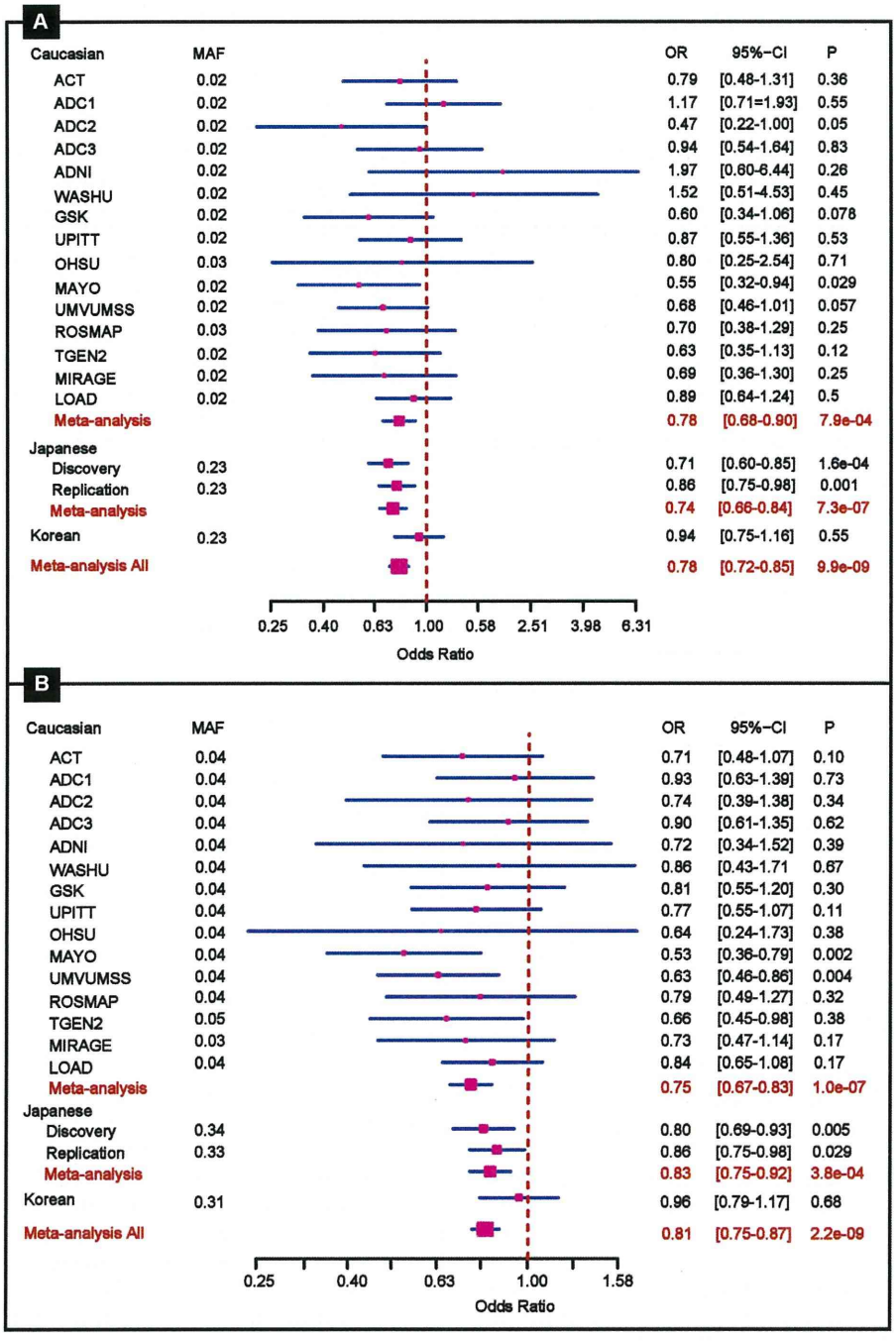

Figure 2. Forest plots of the two most strongly associated SNPs, rs3781834 (A) and rs11218343 (B), in the *SORL1* region showing the strength and pattern of significance in the Japanese discovery and each replication dataset in the model of adjusting for population structure, age, and sex.
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wide significant AD locus in much larger GWAS [4,5]. Given the discovery sample size, effect size (odds ratio [OR] = 0.74) and MAF (0.23) of the top *SORL1* SNP (rs3781834) in the Japanese sample, and a significance level of 2×10^{-5} (i.e., threshold for including a SNP in the Stage 2 replication phase), calculation of power *post hoc* using the PAWE-3D program [22] confirmed that the discovery sample had sufficient power (83.7%). By comparison, the Caucasian sample of 22,771 subjects had only 52.8% power to detect association with this SNP at the observed significance level of 7.9×10^{-4} and OR (0.78) and a much lower MAF (0.02) than in Japanese.

The most significant result in the GWAS in Japanese was obtained for *PALM2* SNP rs913360 ($P=1.8\times10^{-7}$), but this SNP was not significant in the Japanese replication sample ($P=0.16$) and the result for the combined Japanese datasets was less significant than in the discovery sample ($P=6.6\times10^{-6}$). There was no evidence in the large Caucasian dataset supporting association for rs913360 ($P=0.38$) or other *PALM2* SNPs.

Table 3. Meta-analysis of top-ranked association results with *SORL1* in Japanese, Korean, and Caucasian datasets.

SNP	MA	Japanese (Stage 1+2)			Korean (Stage 3)			Caucasian (Stage 3)			Meta-Analysis (Stages 1–3)	
		MAF	OR (95% CI)	P	MAF	OR (95% CI)	P	MAF	OR (95% CI)	P	OR (95% CI)	P
rs4598682	G	0.23	0.75 (0.66–0.85)	9.5×10^{-6}		not available		0.02	1.04 (0.85–1.28)	0.68	0.82 (0.72–0.93)	3.6×10^{-3}
rs11218343	C	0.34	0.83 (0.75–0.92)	3.8×10^{-4}	0.31	0.96 (0.79–1.17)	0.68	0.04	0.75 (0.67–0.83)	1.0×10^{-7}	0.81 (0.75–0.87)	2.2×10^{-9}
rs3781834	G	0.23	0.74 (0.66–0.84)	7.3×10^{-7}	0.23	0.94 (0.75–1.16)	0.55	0.02	0.78 (0.68–0.9)	7.9×10^{-4}	0.78 (0.72–0.85)	9.9×10^{-9}
rs17125523	G	0.25	0.77 (0.68–0.86)	5.5×10^{-6}	0.23	0.96 (0.78–1.19)	0.72	0.02	0.85 (0.74–0.99)	0.034	0.82 (0.76–0.89)	3.3×10^{-6}
rs3737529	T	0.25	0.77 (0.68–0.86)	4.1×10^{-6}	0.26	1.04 (0.84–1.29)	0.70	0.02	0.83 (0.71–0.97)	0.016	0.82 (0.76–0.89)	5.1×10^{-6}

CH:MB, chromosome:position (in megabase pairs, build 19); MA, minor allele; MAF, minor allele frequenc; OR, odds ratio; *P* P-value.
doi:10.1371/journal.pone.0058618.t003

We obtained evidence in Japanese and Korean populations for association of AD with the same SNPs in the *PICALM* and *BIN1* regions that were identified as genome-wide significant in multiple large GWAS in Caucasians [4,5]. There are no previously reported association studies of these loci in Japanese. Several small association studies of *PICALM* in comparatively smaller Chinese samples have yielded conflicting results [23–25]. We also found nominally significant associations in the Japanese sample for previously associated SNPs in *CR1*, *CLU*, and *ABCA7*. Lack of association with *EPHA1*, *CD2AP*, *MS4A6A*, and *CD33* may be due to insufficient power, different linkage disequilibrium structure of these regions than in Caucasisans, locus heterogeneity or intragenic heterogeneity.

In addition, our analyses showed numerous highly significant results for imputed SNPs in the *APOE* region (including *CEACAM/BCL3*, *PVRL2*, *TOMM40*, and *LOC284352)* even after adjustment for the dose of the ε4 allele. However, recognizing that the reliability of imputation is poor for SNPs in this region [13], we genotyped 10 of the significant SNPs in the Japanese discovery and replication datasets. Only one of these results, a *PPP1R37* SNP, was nominally significant after adjustment for dose of ε4. Association of AD with this SNP, which is located approximately 225 kb from *APOE*, has not been observed previously. *PVRL2* and *APOE* are located in a genomic region sandwiched between two recombination hotspots [26], where strong association signals for LOAD have been reproducibly detected in Caucasians [1,5], but dissipate almost completely for all non-*APOE* loci after conditioning on *APOE*, suggesting that no other loci in this region influence LOAD susceptibility [13]. This conclusion is consistent with the observation of moderate linkage disequilibrium between the SNPs determining *APOE* genotype, rs7412 and rs429358 (Fig. S5), SNPs showing genomewide significant evidence for association with LOAD without adjustment for *APOE* genotype, and our prior LOAD association studies with SNPs in this region among Caucasians [13].

SorL1, also known as SorLA and LR11, and APP proteins are co-localized in the endosomal and Golgi compartments [27]. SorL1 through its co-dependent interaction with vps26 regulates the intracellular transport and processing of APP, resulting in reduction of amyloid beta (Aß) peptide production [20,27,28]. *SORL1* knock-out mice carrying both pathogenic mutations in the *PSEN1* (exon 9 deletion) and *APP* (Swedish, K595M/N596L)

Table 4. Association of LOAD in Asians with SNPs showing genome-wide significance in Caucasians.

Gene	CH	BP	SNP	MA	Japanese			Korean		
					MAF	P	OR (95% CI)	MAF	P	OR (95% CI)
CR1	1	207,692,049	rs6656401	A	0.04	***9.02E-03***	1.38 (1.08–1.76)	0.04	3.75E-01	1.24 (0.77–1.99)
CR1	1	207,784,968	rs3818361	A	0.39	2.54E-01	0.94 (0.85–1.04)	0.31	4.08E-01	0.92 (0.76–1.12)
BIN1	2	127,894,615	rs744373	G	0.33	***1.39E-04***	1.25 (1.11–1.4)	0.36	8.05E-01	0.98 (0.81–1.18)
CD2AP	6	47,453,378	rs9349407	G	0.14	3.83E-01	0.94 (0.82–1.08)	NT	–	–
EPHA1	7	143,109,139	rs11767557	C	0.17	6.47E-01	1.03 (0.9–1.17)	NT	–	–
CLU	8	27,456,253	rs2279590	T	0.25	***7.01E-03***	0.85 (0.76–0.96)	0.2	9.70E-02	0.82 (0.65–1.04)
CLU	8	27,464,519	rs11136000	T	0.28	***1.09E-02***	0.87 (0.78–0.97)	0.23	***3.61E-02***	0.79 (0.63–0.98)
CLU	8	27,468,862	rs9331888	G	0.41	6.97E-02	1.1 (0.99–1.22)	0.47	1.92E-01	0.89 (0.74–1.06)
MS4A6A	11	59,939,307	rs610932	T	0.3	7.99E-01	0.99 (0.89–1.1)	NT	–	–
MS4A6A	11	59,971,795	rs670139	T	0.4	8.23E-01	0.99 (0.89–1.09)	NT	–	–
MS4A6A	11	60,034,429	rs4938933	C	0.27	3.23E-01	1.06 (0.95–1.18)	NT	–	–
PICALM	11	85,868,640	rs3851179	T	0.39	***1.71E-05***	0.8 (0.73–0.89)	0.34	***1.99E-02***	0.79 (0.66–0.96)
ABCA7	19	1,046,520	rs3764650	G	0.42	***3.66E-02***	1.13 (1.01–1.27)	NT	–	–
EXOC3L2	19	45,708,888	rs597668	C	0.43	***8.23E-03***	0.88 (0.79–0.97)	0.37	7.31E-01	0.97 (0.8–1.17)
CD33	19	51,727,962	rs3865444	A	0.2	4.92E-01	1.04 (0.92–1.18)	NT	–	–

NT not tested; P<0.05 was italized.
doi:10.1371/journal.pone.0058618.t004

exhibited increased production and accumulation of Aß [29]. *SORL1* variants might influence the CSF Aß42 level in AD patients [30]. Recently, Pottier et al. sequenced the exomes of 29 index cases with autosomal dominant early-onset AD who lacked mutations in *APP, PSEN1* and *PSEN2* [2]. Seven of these subjects had private *SORL1* mutations (2 nonsense and 2 missense) that were predicted to have a pathogenic effect. By comparison, the two genome-wide significant SNPs in this study are both intronic. It is expected that future large resequencing studies of *SORL1* will identify the functional variants, thus providing important clues about the mechanisms governing normal and abnormal action of SorL1 on processes leading to LOAD. The emergence of *SORL1* as a genome-wide significant locus for AD confirms existing genetic and functional evidence and elevates the importance of intracellular trafficking involving retromer and the Golgi-to-endosome as a key pathway leading to AD [31,32].

Supporting Information

Figure S1 Quantile-quantile (Q-Q) plot of observed (y-axis) vs. expected (x-axis) *P*-values from tests of association genome-wide (5,877,918 SNPs) adjusted for population structure, age and sex for LOAD in the Japanese discovery sample. Genomic inflation was low ($\lambda = 1.047$).
(TIF)

Figure S2 Manhattan plot of observed $-\log_{10}$ *P*-values for genome-wide SNP association tests for LOAD (y-axis) according to chromosomal location (x-axis) in the Japanese discovery sample adjusted for population structure, age, and sex. All genome-wide significant SNPs (above the horizontal line corresponding to $P = 5 \times 10^{-8}$ on the y-axis) are located in the *APOE* region on chromosome 19.
(TIF)

Figure S3 Linkage disequilibrium (r^2) among SNPs in the *APOE* region genotyped using TaqMan calculated in the Japanese discovery (A) and replication (B) datasets. *APOE* genotype is derived from haplotypes of coding SNPs rs429358 and rs7412.
(TIF)

Figure S4 Linkage disequilibrium (r^2) among SNPs in the *SORL1* region genotyped in the Japanese discovery (A) and replication (B) datasets.
(TIF)

Figure S5 Comparison of *SORL1* association findings in the current study with association signals previously identified by Rogaeva et al. [20]. (**A**) Regional association plot of the *SORL1* region. P-values are expressed as $-\log_{10}$(P) (y-axis) for every tested SNP ordered by chromosomal location (x-axis) and represented as blue rectangles for the Japanese discovery set (J1), light blue diamonds for the ADGC Caucasian set (C), pink circles for meta-analysis of Japanese discovery and Caucasian sets (J1+C), and red circles for meta-analysis of Japanese discovery, Japanese replication (J2), Korean (K), and Caucasian sets (J1+J2+K+C). The numbers below the line showing the orientation of *SORL1* are the designations for associated SNPs in the Rogaeva et al. study: 8 = rs668387, 9 = rs689021, 10 = rs641120, 11 = rs4935775, 19 = rs2070045, 22 = rs1699102, 23 = rs3824968, 24 = rs2282649, and 25 = rs1010159. Recombination hotspots are indicated by the continuous blue line behind the symbols for the SNP P-values. (**B**) Linkage disequilibrium (r^2) of the previously associated SNPs in the *SORL1* region [20] in the HapMap 2 reference Japanese population (JPT). The association signal with rs3781834 (contained in Block 2) appears to be independent of one of the distinct AD-associated haplotypes reported by Rogaeva et al. [20] (including SNPs in Block 1), but not necessarily independent of the other AD-associated haplotype reported by Rogaeva et al which includes rs1699102 in Block 2 and the SNPs in Block 3.
(TIF)

Table S1 Top-ranked GWAS results in the Japanese GWAS dataset ($P<1\times10^{-4}$ and imputation quality ≥0.8) with and withut adjustment for the number of *APOE* ε4 alleles.
(DOCX)

Table S2 Association of individually genotyped SNPs in the *APOE* region in models with and without adjustment for the number of *APOE* ε4 alleles.
(DOCX)

Table S3 Association results for *SORL1* SNPs genotyped in the Japanese replication sample.
(DOCX)

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Unintended Effects of Cardiovascular Drugs on the Pathogenesis of Alzheimer's Disease

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Abstract

Alzheimer's disease (AD) is rapidly becoming one of the leading causes of disability and mortality in the elderly. As life-expectancy increases, an increasing number of people will rely on modern medicines to treat age-associated disorders. Among these medications, some might benefit, while others might exacerbate, the pathogenesis of AD. We screened 1,600 FDA approved drugs for β-amyloid (Aβ)-modifying activity and identified drugs that can potentially influence amyloid precursor protein processing. In this study, we focused on cardiovascular drugs and demonstrated that some hypertensive medication can differentially modulate Aβ, both *in vitro* and *in vivo*. Our study suggests that some commonly prescribed drugs might exert unintended effects and modulate AD and provides the basis for continuing investigation of the role of individual drugs on a case-by-case basis. This line of investigation will lead to the identification of common medications that are potentially beneficial or detrimental to AD as a reference for physicians to consider when prescribing the most appropriate drugs for their patients, particularly for treating chronic disorders among the growing geriatric population.

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Introduction

Alzheimer's disease (AD) is one of the most persistent and devastating disorders of old age, often leading to severe memory loss and functional impairment [1]. Its prevalence increases dramatically with aging. It is estimated that up to ~5 million people in the US currently have AD and it is projected that up to 14 million people will be affected by AD by the middle of this century.

AD is characterized neuropathologically by the accumulation of extracellular neuritic plaques composed of β-amyloid (Aβ) protein, intracellular neurofibrillary tangles of hyperphosphorylated tau protein, and neuron loss [2]. A major hypothesis regarding the pathogenesis of AD is that abnormally elevated Aβ content in the brain of AD patients is critical for the development of AD dementia. This hypothesis, commonly referred to as the "amyloid hypothesis," suggests that increasing accumulation of Aβ peptides promotes assembly of Aβ proteins into neurotoxic, extracellular soluble oligomeric Aβ aggregates that are largely responsible for cognitive deterioration and neuronal loss in AD [3–9]. Continuing recruitment of Aβ peptides to oligomeric Aβ aggregates leads to the formation of larger, insoluble Aβ fibrils that contribute to the formation of AD type neuritic plaques in the brain [10]. The amyloid hypothesis is supported by substantial genetic [11] and preclinical evidence [12]. However, to date, clinical trials based on amyloid hypothesis with the target mechanism of reducing brain amyloid load have produced null results including the most recently completed antibody therapies (bapineuzamab), suggesting that the amyloid hypothesis and the optimal molecular targets or the optimal timing for intervention remains to be elucidated. Nonetheless, Aβ remains to be one of the major disease modifying targets for AD drug discovery.

In addition to AD, the aged population is associated with higher risks for many chronic diseases, such as cardiovascular disorders, diabetes, arthritis, cancer or cognitive impairment. Many elderly individuals require one or more medications to treat and/or manage age-related chronic disease. A survey conducted by Boston University Epidemiology Center showed that over 80% of people 65 years and older take at least one medication and almost 50% take three or more - As life-expectancy increases with advances in medicine the number of elderly continues to increase. Thus, the incidence and prevalence of chronic disease is rising dramatically, together with the number of elderly requiring one or more medications.

We note that many drugs such as some pain medications, antihistamines and anti-psychotic medications might have adverse effect on cognition, especially in elderly. For example, a recently published prospective study involving thirteen thousand participants over 65 years of age showed that the use of anticholinergic medication increases the cumulative risk of cognitive deterioration and mortality [13]. This also raises concerns for other medications that have mild anticholinergic activities, such as cardiovascular drugs digoxin, warfarin, analgesics, codeine and prednisone. On the other hand, some medications might have positive effects on

cognition. For example, recent evidence strongly supports the possibility that the use of some antihypertensive drugs such as β-blockers or Ca^{++} channel receptor antagonists, and certain potassium-sparing antihypertensive diuretics, may decrease the incidence of AD [14–17]. More recently, Hajjar et. al found that patients with or without AD, treated with angiotensin receptor blockers (ARBs), showed much lower amyloid burden compared to patients treated with other anti-hypertensisive medication [18].

Despite evidence associating some drugs with unintended activities on cognitive dysfunction, we do not know the potential role of how specific medications promote or inhibit the generation of Aβ.

Since Aβ is one of the major contributory factors responsible for AD-type dementia, we surveyed 1600 FDA approved drugs that are commonly used by the general population for their ability to modulate Aβ accumulation. Our intention is to identify medications that might be potentially beneficial or harmful for Aβ-mediated cognitive dysfunction. Outcomes from our studies will provide important information for physicians to consider when making decisions on prescribing the most appropriate drugs for their patients, particularly for treating chronic disorders among the growing geriatric population. Moreover, our observations also provide the impetus for future studies to systematically evaluate FDA- approved medications for potential repurposing as novel reagents to prevent or treat AD dementia.

Materials and Methods

Drug Screening Procedure

Embryonic-day (E)16 cortico-hippocampal neuronal cultures were prepared from heterozygous Tg2576 transgenic mice (Tg2576 neurons) [19;20]. Neurons were seeded onto poly-D-lysine–coated 96-well plates at 1.0×10^5 cells per well and cultured in Neurobasal medium supplemented with 2% B27, 0.5 mM L-glutamine and 1% penicillin-streptomycin (Gibco-BRL) in the tissue culture incubator at 37°C with 5% CO_2. The absence of astrocytes (<2%) was confirmed by the virtual absence of glial fibrillary acidic (GFAP) protein immunostaining (data not shown). For primary screening, cultured neurons were treated with 100 μM of drug in duplicates; all drugs were obtained in stock from MicroSource Discovery Systems Inc (Gaylordsville, CT). Conditioned medium was collected for Aβ detection. For secondary screening, primary neurons prepared in 96-well plates were treated with 0.1 μM, 1 μM, 10 μM, 50 μM, and 100 μM of each drug in duplicate for ~16 hours and conditioned medium was tested for Aβ content. Cell viability was assessed using a commercial available LDH assay kit according to the manufacture's instruction (Promega) and by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

Animals and Treatment

Female Tg2576 mice transgenic mice carrying a human amyloid precursor protein (APP) containing the familial Swedish KM670/671NL double mutation [21] were purchased from Taconic. Tg2576 mouse is a well characterized rodent of model with Aβ-mediated neuropathology and cognitive impairment and were previously used in our studies on valsartan and carvedilol [20;22].

Testing drugs were prepared to desired concentrations and delivered to mice by diluting them directly into the drinking water. Typically, a 25~30 g mouse drinks ~4–5 ml per day. The amount of testing drugs to be diluted into the drinking water was based on 1) average daily water consumption and 2) the targeted dose of testing drugs to be delivered to animals. For example, a 25 g mouse would need to take 0.375 mg propranolol per day to achieve 15 mg/kg body weight/day as in the instance of chronic propranolol treatment. Typically a 25 g mouse drinks 4.5 ml liquid per day, therefore, propranolol was diluted in the drinking water to a concentration of 0.083 mg/ml or 83 mg/L. The average liquid consumption was monitored and doses were adjusted accordingly. We acknowledge that there is variability in the amount of liquid each animal consumed. Animals under treatment were provided *ad libitum* access to testing drug-infused water as the sole source of drinking fluid.

For short-term treatment, Tg2576 mice were treated with individual compounds starting at 6 months of age and the treatment lasted one month.

For chronic treatment, Tg2576 mice were treated with 17.2 mg/kg/day nicardipine (equivalent to human 100 mg/day) or 15 mg/kg/day propranolol (equivalent to human 90 mg/day) delivered through their drinking water for 6 months, starting at 8 months of age.

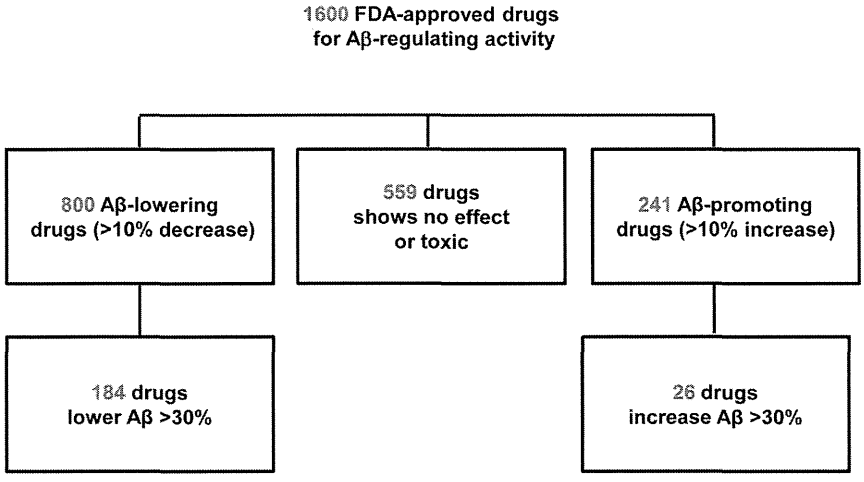

Figure 1. Schematic diagram of primary screening of 1600 FDA approved drugs.
doi:10.1371/journal.pone.0065232.g001

Table 1. 115 commonly prescribed cardiovascular drugs used in screening for potential Aβ-modifying activity.

Drug Name	Aβ	Drug Name	Aβ	Drug Name	Aβ
ANTIHYPERTENSIVE					
A. β-Adrenergic blocker		**D. Vasodilator**		**G. Angiotensin converting enzyme inhibitor**	
RESERPINE	–	PAPAVERINE HYDROCHLORIDE	–	PERINDOPRIL ERBUMINE	83.8
CARVEDILOL	**38.3**	**HYDRALAZINE**	**66.1**	FOSINOPRIL SODIUM	94.7
PROPRANOLOL HYDROCHLORIDE (−)	**55.0**	DIPYRIDAMOLE	72.2	ENALAPRIL MALEATE	100.7
PROPRANOLOL HYDROCHLORIDE	**65.2**	ISOXSUPRINE HYDROCHLORIDE	78.3	CAPTOPRIL	104.5
NYLIDRIN HDROCHLORIDE	70.5	NICOTINYL TARTRATE	80.0	RAMIPRIL	107.4
LABETALOL HYDROCHLORIDE	84.9	ISOSORBIDE DINITRATE	80.5	BENAZEPRIL HYDROCHLORIDE	116.3
ATENOLOL	90.4	PROTOVERATRINE A	91.7	QUINAPRIL HYDROCHLORIDE	119.3
METOPROLOL TARTRATE	91.3	MINOXIDIL	94.2	*TRANDOLAPRIL*	*136.7*
ALPRENOLOL	93.8	VINCAMINE	99.1	**H. Diuretic**	
NADOLOL	94.2	METHYLDOPA	104.0	BUMETANIDE	–
PINDOLOL	99.0	MOLSIDOMINE	118.3	ETHACRYNIC ACID	–
PRACTOLOL	99.7	DIAZOXIDE	113.5	TRIAMTERENE	–
TIMOLOL MALEATE	101.3	ADENOSINE PHOSPHATE	121.8	**AMILORIDE**	**60.4**
GUANETHIDINE SULFATE	125.5	**E. Ganglionic blocking agent**		CYCLOTHIAZIDE	71.4
ACEBUTOLOL HYDROCHLORIDE	133.6	PEMPIDINE TARTRATE	80.9	ALTHIAZIDE	74.4
B. a-Adrenergic Blocker		HEXAMETHONIUM BROMIDE	84.8	BENDROFUMETHIAZIDE	76.2
PRAZOSIN HYDROCHLORIDE	–	PENTOLINIUM TARTRATE	121.0	SPIRONOLACTONE	77.5
PHENTOLAMINE HYDROCHLORIDE	83.4	MECAMYLAMINE HYDROCHLORIDE	124.4	TRICHLORMETHIAZIDE	89.3
PHENOXYBENZAMINE HYDROCHLORIDE	87.5	**F. Ca channel blocker**		HYDROCHLOROTHIAZIDE	89.5
TOLAZOLINE HYDROCHLORIDE	90.8	AMLODIPINE BESYLATE	–	METOLAZONE	91.4
URAPIDIL	96.4	BEPRIDIL HYDROCHLORIDE	–	CLOPAMIDE	92.0
TAMSULOSIN HYDROCHLORIDE	106.5	FENDILINE HYDROCHLORIDE	–	HYDROFLUMETHIAZIDE	92.4
GUANABENZ ACETATE	131.2	FLUNARIZINE HYDROCHLORIDE	–	UREA	92.8
C. Angiotensin receptor blocker		TETRANDRINE	–	CANRENOIC ACID, POTASSIUM SALT	100.6
CANDESARTAN CILEXTIL	–	**NICARDIPINE**	**10.7**	THEOBROMINE	101.3
VALSARTAN	**51.6**	NITRENDIPINE	33.0	BENZTHIAZIDE	105.7
LOSARTAN	**70.0**	VERAPAMIL HYDROCHLORIDE	70.9	INDAPAMIDE	107.0
OLMESARTAN CILEXTIL	83.9	NIMODIPINE	79.1	CHLOROTHIAZIDE	107.3
IRBESARTAN	89.7	NIFEDIPINE	83.4	TORSEMIDE	107.4
TELMISARTAN	91.1	DILTIAZEM HYDROCHLORIDE	107.8	CHLORTHALIDONE	108.5
		BERBAMINE HYDROCHLORIDE	119.1	*FUROSEMIDE*	*138.5*
ANTIARRHYTHMIC					
PROPAFENONE HYDROCHLORIDE	–	DISOPYRAMIDE PHOSPHATE	83.5	AJMALINE	114.0
AMIODARONE HYDROCHLORIDE	–	MEXILETINE HYDROCHLORIDE	88.8	PROCAINAMIDE HYDROCHLORIDE	118.1
QUINIDINE GLUCONATE	63.3	HYDROQUINIDINE	96.5		
ANTITHROMBOTIC and FIBRINOLYTIC					
A. Coagulant		**B. Antifibrinolytic**		**C. Antihyperlipidemic**	
SULOCTIDIL	–	TRANEXAMIC ACID	84.9	FENOFIBRATE	–
DICUMAROL	51.3	AMINOCAPROIC ACID	124.4	SIMVASTATIN	–
SCOPOLETIN	71.9			ATORVASTATIN CALCIUM	66.0
WARFARIN	89.4			ROSUVASTATIN	83.6
ANISINDIONE	90.1			PROBUCOL	86.8
PENTOXIFYLLINE	97.1			CLOFIBRATE	90.9
PHENINDIONE	104.0			NIACIN	101.1
				BEZAFIBRATE	107.2
CONGESTIVE HEART FAILURE					
LANATOSIDE C	–	DOPAMINE HYDROCHLORIDE	85.0	PERUVOSIDE	115.3
DIGITOXIN	68.3	DOBUTAMINE HYDROCHLORIDE	90.1		

Table 1. Cont.

The 115 cardiovascular agents were obtained as part of the Spectrum Collection from MicroSource Discovery Systems Inc. and listed in 4 pharmacological categories (source: Physician's Desk Reference and Martindale Complete Drug Reference). Potential Aβ-modifying activity was assessed using primary cortico-hippocampal neuron cultures derived from embryonic Tg2576 AD mice and the levels of Aβ in the conditioned medium were measured and expressed as percentage of vehicle-treated control (Aβ column). In the high-throughput screening studies, 13 drugs lowered the Aβ content greater than 30% and two drugs (in bold and Italic) increased Aβ content greater than 30% compared to the vehicle controls. The 13 Aβ-lowering drugs were proceeded to a follow-up dose dependent test and the 8 drugs in bold were found to exert significant dose dependent Aβ -lowering activity in the absence of cellular toxicity. "–" indicates that the drug is toxic for primary neurons at 100 μM.

doi:10.1371/journal.pone.0065232.t001

All mice were housed with food *ad libitum* and maintained on a 12:12-h light/dark cycle with lights on at 07:00 h in a temperature-controlled (20±2°C) room prior to experimental manipulation. Mice were group housed with 3–5 mice per cage and average food and water intake was measured weekly. All procedures and protocols were approved by the Mount Sinai School of Medicine's Institutional Animal Care and Use Committee (IACUC) through the Center for Comparative Medicine and Surgery.

Blood Pressure Measurements

Blood pressure and heart rate were measured using a non-invasive commercial blood pressure analysis system designed specifically for small rodents (Hatteras Instruments, NC) as previously described [20;23]. Mice were temporarily immobilized in a restraining chamber with the tail inserted through the tail cuff, laid down into the tail slot and secured with a piece of tape. Every mouse underwent 5 preliminary cycles for acclamation and the following 10 measurements of systolic, diastolic, and mean arterial pressure (MAP) and heart rate was recorded.

Behavioral Assessment of Cognitive Functions by the Morris Water Maze (MWM) Test

Spatial learning memory was assessed by the Morris water maze behavioral test, as previously described [20;24]. Briefly, mice were tested in a circular pool filled with water mixed with non-toxic white paint (Dick Blick Art Materials, IL). The water temperature was kept between 70 and 74 °F. Mice were first tested in a visible trial for 3 consecutive days where the escape platform was clearly marked with a white sail. Following the visible trial, the white sail was removed and replaced by local visual cues. Mice were trained to mount the submerged escape platform in a restricted region of the pool using the visual cues. Mice were given 4 trials per day with 60 seconds per trial. Each day, the mice would start at different quadrant and if the testing mouse failed to reach the platform in 60 seconds, it would be gently led to the platform and let stay on the platform for 15 seconds before returning to the home cage. Spatial memory was assessed by recording the latency time for the animal to escape from the water onto a submerged escape platform as a function of the number of learning trials during the learning phase. Twenty-four hours after the last learning session, mice were subjected to a 45 second probe trial wherein the escape platform is removed. The water maze activity was monitored with the San Diego Instrument Poly-Track video tracking system (San Diego, CA). The cued-platform learning curve was used as control for the non-spatial factors on MWM performance, e.g., sensory-motor performance, motivation, anxiety etc., which can be influenced by the potential effect of the testing drug. The mentstrual cycle was not controlled for the behavior testing.

Assessment of AD-type Amyloid Neuropathology

Total Aβ1-40 or Aβ1-42 in the brain and in plasma were quantified by sandwich ELISA, as previously described [25]. Specifically, frozen pulverized tissue was homogenized in 5.0 M guanidine buffer, diluted (1:10) in phosphate-buffered saline containing 0.05% (v/v) Tween-20 and 1 mM Pefabloc protease inhibitors (Roche Biochemicals, Indianapolis, IN) and centrifuged for 20 min at 4°C. Supernatant was subjected to $A\beta_{1-40}$ or $A\beta_{1-42}$ quantification by sandwich ELISA (BioSource, Camarillo, CA).

Statistical Analysis

Differences between means were analyzed using two-tailed Student t-test. For behavior testing, data were analyzed using two-way repeated measures ANOVA followed by Newman-Keuls post-hoc analysis. In all analyses, the null hypothesis was rejected at the 0.05 level. All values are expressed as mean and standard error of the mean (SEM). All statistical analyses were performed using the prism Stat program (GraphPad Software, Inc.).

Results

Identification of Cardiovascular Drugs with AD-modifying Activity

Our high throughput screening study assessed 1600 FDA approved drugs for their ability to modulate Aβ activity. We found 559 drugs of the 1600 had no effect on APP processing or were toxic to neurons at the testing concentration, while 800 drugs could reduce Aβ content over 10% in primary neurons derived from Tg2576 mice, among which, 184 drugs were able to reduce Aβ content greater than 30% compared to vehicle. We also found 241 drugs could potentially promote Aβ accumulation including 26 drugs that could increase the level of Aβ greater than 30% compared to vehicle treatment (Figure 1).

Cardiovascular medications are one of the most commonly prescribed drugs, especially in the elderly. Among the 1600 drugs we tested, 115 are cardiovascular drugs representing all pharmacological classes of antihypertensive, antithrombotic and fibrinolytic, antianginal, antiarrhythmic and congestive heart failure medications (Table 1). Primary screening of these drugs identified 13 cardiovascular drugs that could reduce ≥30% Aβ accumulation in the conditioning medium from Tg2576 neurons following 16 hours treatment at 100 μM concentration without toxicity as tested by MTT assay and LDH release assay (Table 1). We continued our secondary screening for dose response on these candidates and found that among the 13 candidates, carvedilol, propranolol, valsartan, losartan, hydralazine, nicardipine and amiloride demonstrated a concentration-dependent reduction of $A\beta_{1-40}$ and $A\beta_{1-42}$ in primary embryonic cortico-hippocampal neuron cultures. All 7 drugs are antihypertensive agents of different pharmacological subclasses. We also found that 2 drugs,

Table 2. Short-term in vivo treatment dosage conversion in Tg2576 mice.

	Tg2576 treatment (mg/kg/day)	Equivalent human dose Prescribed clinical dose (mg/day)	Recommended dose for hypertension treatment in human (mg/day)
propranolol	41.3	240	120~240
carvedilol	8.6	50	25~50
nicardipine	17	100	50~100
losartan	20.6	120	60~120
amiloride	3.4	20	5~20
hydralazine	51.7	300	100~300
furosemide	14.8	80	20~80
trandolopril	0.74	2	1~2

The dosage recommended for hypertension treatment in humans is listed as prescribed clinical dose. The equivalent dosage in animals is calculated using FDA criteria for converting drug equivalent dosages across species, based on body surface area [39]. The mice equivalent dosage was used in the short-term treatment.
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namely trandolapril and furosemide, could promote ≥30% Aβ accumulation compared to vehicle control.

Short-term *in vivo* Efficacy Study using Tg2576 Mouse Model of AD

Based on the *in vitro* dose-dependent results, we tested 8 drugs (6 with Aβ-lowering activity and 2 with Aβ-promoting activity, Table 2) to explore whether these drugs can exert similar Aβ modification activity in an experimental model of AD.

We performed a short-term feasibility study using the Tg2576 mice model of AD. We used the doses equivalent to the recommended dose for treating cardiovascular disease in humans (Table 2). All dosage ranges were obtained from online Physicians' Desk Reference (http://www.pdr.net) and the conversions of drug equivalent dosages across species were derived using FDA criteria based on body surface area [26]. The detailed dosage for the treatment is listed in Table 2.

Table 3. Effect of short-term in vivo on body weight and liquid consumption in Tg2576 mice.

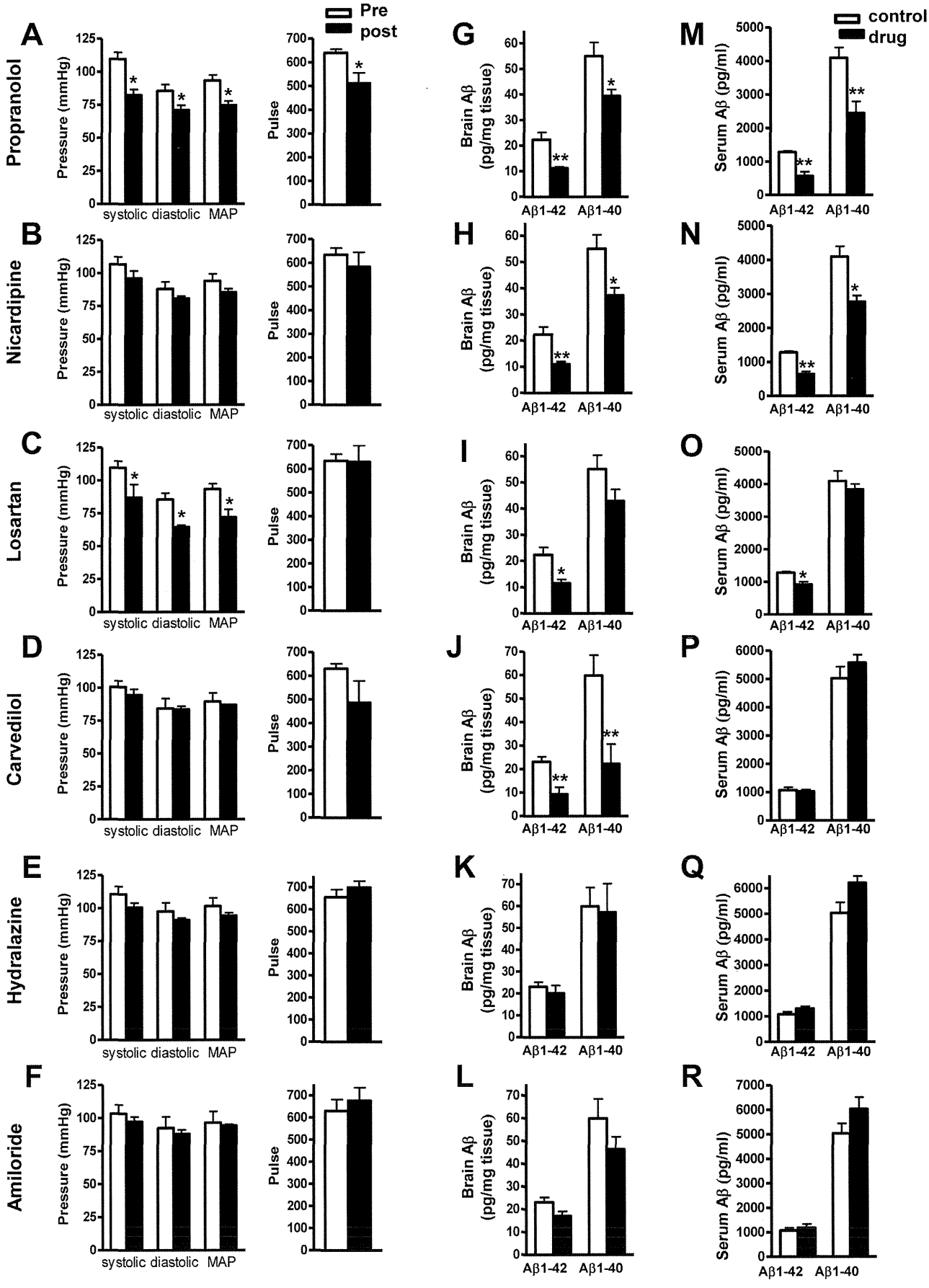
Drug	Body Weight (g)		Liquid Consumption (ml/day)
	Pretreatment	Posttreatment	
Propranolol	23.2±1.0	19.9±2.6	4.8±0.3
Nicardipine	21.9±1.9	19.7±1.6	4.8±0.2
Losartan	24.7±2.6	23.7±2.0	4.6±0.5
Carvedilol	27.4±1.8	27.3±1.5	4.3±0.5
Hydralazine	27.6±1.0	24.3±1.0*	4.5±0.5
Amiloride	30.3±8.5	27.4±9.8	4.8±0.3
Trandolapril	32.9±13.6	32.9±12.7	4.5±0.8
Furosemide	34.5±3.8	32.2±6.8	4.9±0.6

Animals were treated with the anti-hypertensive drugs for four weeks and body weight and liquid consumption were monitored weekly. Data presented here are the end point body weight and the average liquid consumption throughout the study.
*P<0.05, 2-tailed student t-test, n = 3–5 for each treatment group.
doi:10.1371/journal.pone.0065232.t003

We found that short-term drug treatment for one month in Tg2576 mice, delivered in the drinking water at clinical dosage, did not significantly influence animal body weight, except for hydralazine treatment (Table 3), which showed a significant body weight drop following 1 month treatment. However, propranolol and losartan treatment resulted in a significantly influenced blood pressure, ~20% drop in systolic, diastolic and mean arterial pressure (MAP) measurements was observed, while other drugs showed no effect on the blood pressure measurements in the normotensive mice (Figure 2, A-F). The lack of hypotensive effect of the other drugs can be due to: 1) some of the drugs, such as nicardipine, significantly reduce the blood pressure in hypertensive subjects, but have minimal effect on blood pressure in normotensive subjects. 2) the drug dose conversion between species we used in the study is based on the body surface area. It is possible that the absorption and metabolism of certain drug might be different in mice compared to humans.

Consistent with the in vitro data, treatment of Tg2576 mice with propranolol, nicardipine or carvedilol resulted in a ~40% reduction in total guanidine-extractable Aβ1-42 peptide and Aβ1-40 peptide in the brain (Figure 2G, 2H, and 2J), while treatment with hydralazine and amiloride had no effect on brain amyloid peptides levels. Losartan treated mice showed a significant reduction of Aβ1-42 in the brain while no change in Aβ1-40 level. We also found that propranolol, nicardipine and losartan treatment resulted in significant reductions of plasma level of Aβ (Fig 2M, 2N and 2O).

To our surprise, the drugs that increased Aβ *in vitro*, at the dosage equivalent to human prescription dosage (14.8 mg/kg/day for furosemide and 0.74 mg/kg/day for trandolapril), did not increase total Aβ1-40 or Aβ1-42 in the brain following one-month treatment. On the contrary, both furosemide and trandolapril treatment significantly reduced the levels of total brain amyloid content in the Tg2576 mice following one-months short-term treatment (Figure 3B and 3E) without significantly changing of blood pressure. The reduction of brain Aβ content in both treatments was associated with significant increases of plasma levels of Aβ (Figure 3C and 3F). In parallel studies, we confirmed that the drug treatments did not alter the expression of APP in the brains of the mice using western blot analysis (data not shown).


A
Propranolol
Pressure (mmHg)
systolic diastolic MAP
Pulse
Pre
post
B
Nicardipine
C
Losartan
D
Carvedilol
E
Hydralazine
F
Amiloride
G
Brain Aβ (pg/mg tissue)
Aβ1-42 Aβ1-40
H
I
J
K
L
M
Serum Aβ (pg/ml)
control
drug
N
O
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Q
R

Figure 2. Effect of Aβ-lowering drug treatment on blood pressure and amyloid neuropathology in Tg2576 mice. (A–F) Measurements of systolic, diastolic blood pressure, and mean arterial blood pressure (MAP) and heart beat (pulse) in response to ~4 weeks of drug treatments. (G-L) Assessment of Aβ1-42 and Aβ1-40 peptide concentrations in the brain of drug treated mice *vs.* the control mice. (M-R) Assessment of Aβ1-42 and Aβ1-40 in peripheral blood of drug treated mice *vs.* the control mice. Blood pressure determination for each animal was calculated as the mean of 10 individual measurements. Values represents group mean values (±SEM); n = 3–5 mice per group. **P<0.01, *P<0.05, 2-tailed student t-test.
doi:10.1371/journal.pone.0065232.g002

Chronic Administration of Nicardipine and Propranolol on Cognitive Function and Brain Neuropathology

The validation of the effect of any medication on AD pathology is cognitive function.

Based on the results from the short-term study (Figure 2), we chose nicardipine and propranolol, both of which significantly changed the levels of total Aβ1-40 and Aβ1-42 in the brain following one-month treatment for chronic studies, to evaluate their effect on cognitive function. Since short-term treatment with propranolol resulted in a significant hypotensive effect in the normatensive mice and significantly reduced heart rate (Figure 2A), we adjusted the dose to 15 mg/kg/day for chronic studies. We used the same dose for nicardipine as short-term treatment since it did not have any adverse effect.

Following 6 months treatment, we tested spatial memory function in the Tg2576 mice using the Morris water maze test. First, we used the visible trial to confirm that propranolol treatment did not affect any non-spatial factors e.g., sensory-motor performance, motivation, anxiety etc. which might affect their water maze performance. Both groups were able to identify the target platform and both groups had similar swimming speed (Figure 4A and 4B). In the hidden platform training session, we found that 6 months of chronic propranolol treatment did not affect AD-type cognitive deterioration reflected by equally impaired spatial memory function between the treated and non-treated control Tg2576 mice. Neither group showed significant learning during the 7 day hidden platform testing (Figure 4C). This lack of learning was also evident during the probe trial, as neither group spent more than 25% chance time in the target quadrant (Figure 4D). Since propranolol is a non-selective beta adrenergic receptor blocker that has been used for memory relief [27–29], in parallel

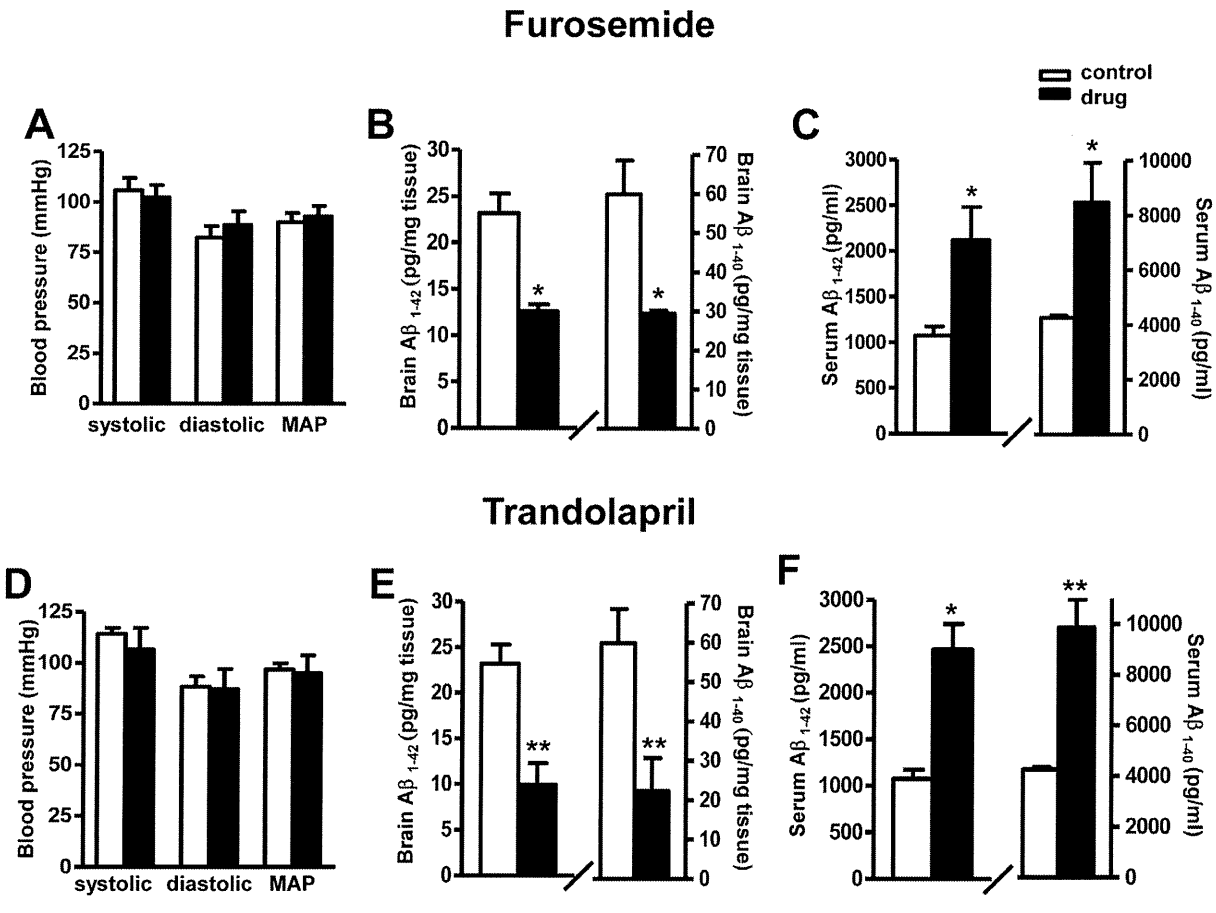
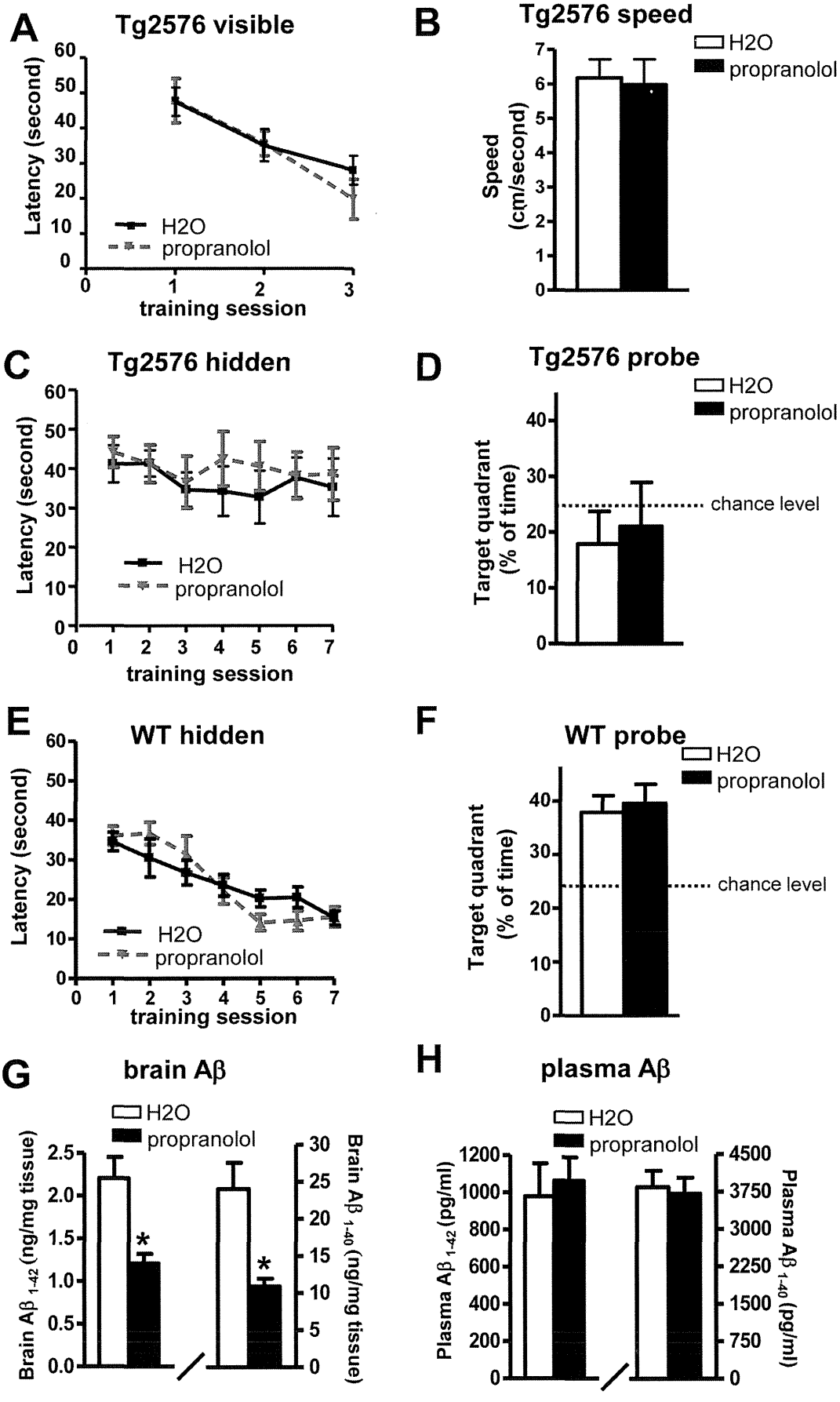


Figure 3. Effect of Aβ -promoting drugs treatment on blood pressure and amyloid neuropathology in Tg2576 mice. (A and D) Measurements of systolic, diastolic blood pressure, and mean arterial blood pressure (MAP) in response to ~4 weeks of drug treatments. (B and E) Assessment of Aβ1-42 and Aβ1-40 peptide concentrations in the brain of drug treated mice *vs.* the control mice. (C and F) Assessment of Aβ1-42 and Aβ1-40 in peripheral blood of drug treated mice *vs.* the control mice. Values represents group mean values (±SEM); n = 3–5 mice per group. **P<0.01, *P<0.05, 2-tailed student t-test.
doi:10.1371/journal.pone.0065232.g003


A
Tg2576 visible
Latency (second)
training session
H2O
propranolol
B
Tg2576 speed
Speed (cm/second)
H2O
propranolol
C
Tg2576 hidden
Latency (second)
training session
H2O
propranolol
D
Tg2576 probe
Target quadrant (% of time)
chance level
H2O
propranolol
E
WT hidden
Latency (second)
training session
H2O
propranolol
F
WT probe
Target quadrant (% of time)
chance level
H2O
propranolol
G
brain Aβ
Brain Aβ 1-42 (ng/mg tissue)
Brain Aβ 1-40 (ng/mg tissue)
H2O
propranolol
H
plasma Aβ
Plasma Aβ 1-42 (pg/ml)
Plasma Aβ 1-40 (pg/ml)
H2O
propranolol