



阿尔茨海默病中 BACE1 相互作用蛋白筛选及功能分析*

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摘要 目的 β 淀粉样蛋白前体蛋白裂解酶 1 (β -site APP cleaving enzyme 1, BACE1) 是阿尔茨海默病 (Alzheimer's disease, AD) 中淀粉样斑块形成的限速酶, 其表达水平与活性在 AD 发生发展中起到关键作用。BACE1 的相互作用蛋白可以通过直接结合、间接结合、参与各种细胞信号转导通路等方式, 直接或间接在 BACE1 的转录、翻译、修饰、胞内运输等各个环节对 BACE1 进行调节, 从而参与 AD 的发生与疾病的进程。本研究拟筛选并验证 BACE1 的相互作用蛋白, 为进一步深入阐明淀粉样斑块形成的机制提供新的依据。**方法** 采用免疫共沉淀 (co-immunoprecipitation, Co-IP) 与质谱联合应用的技术富集并鉴定 AD 模型小鼠海马组织中 BACE1 的相互作用蛋白, 通过生物信息学分析筛选与 BACE1 有潜在相互作用的蛋白质, 采用 Co-IP 实验及免疫荧光共聚焦技术进行初步验证, 并探究 BACE1 相互作用蛋白在 AD 细胞模型中蛋白质表达水平变化。**结果** 本研究在 AD 组中共鉴定到与 BACE1 相互作用的差异表达蛋白 614 个。基因本体 (GO) 富集分析显示, AD 组 BACE1 相互作用蛋白主要参与细胞内信号转导、靶向高尔基体囊泡的转运、浦肯野细胞层发育等生物学过程; 京都基因与基因组百科全书 (KEGG) 分析显示, AD 中 BACE1 相互作用蛋白主要参与了 PI3K-Akt 信号通路、mTOR 信号通路等神经退行性疾病相关通路。通过构建 BACE1 相互作用蛋白网络, 筛选了 12 个可信度较高的相互作用蛋白, 其中 NSF、NUMB、HSP90aa1、SNAP91 为首次发现的 BACE1 相互作用蛋白。经进一步验证, 发现 NSF 与 BACE1 存在相互作用, 并且在 AD 细胞模型中 NSF 表达水平上调。**结论** BACE1 相互作用蛋白参与多种 AD 相关生物途径及信号通路, NSF 为新鉴定到的 BACE1 相互作用蛋白, 与 BACE1 存在相互作用。NSF 在 AD 细胞模型中表达水平上调, 预测 NSF 与 BACE1 蛋白间相互作用参与调节 AD 疾病的进程, 为疾病的机制研究提供新的靶点和方向。

关键词 β 淀粉样蛋白前体蛋白裂解酶 1, 免疫共沉淀, 质谱法, 神经退行性疾病, 阿尔茨海默病

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阿尔茨海默病 (Alzheimer's disease, AD) 是一种以进行性认知功能下降为主要特征的神经退行性疾病, 患者主要表现为记忆力减退、执行力障碍及行为改变等临床特征^[1]。淀粉样斑块形成是 AD 的主要病理特征之一^[2-3]。在正常生理状况下, 淀粉样前体蛋白 (amyloid precursor protein, APP) 主要通过非淀粉样降解途径降解, 而在 AD 患者脑内, β 淀粉样蛋白前体蛋白裂解酶 1 (β -site APP cleaving enzyme 1, BACE1) 蛋白表达水平和酶活性上调, APP 经淀粉样降解途径降解增多。BACE1 是 APP 淀粉样蛋白降解途径的限速酶^[4], BACE1 在 β 位点切割 APP 产生可溶性淀粉样蛋白前体蛋白 β (soluble amyloid precursor protein β , sAPP β) 和 APP 的 C 端片段 C99^[5], C99 进一步被 γ 分

泌酶切割形成 β 淀粉样蛋白 40 (amyloid-beta 1-40, A β ₄₀) 和 β 淀粉样蛋白 42 (amyloid-beta 1-42, A β ₄₂), A β ₄₂ 更具有神经毒性, 其更容易聚集成 A β 寡聚体、A β 原纤维, 最终形成淀粉样斑块。除参与 APP 淀粉样蛋白降解途径, BACE1 通过与维持突触可塑性和突触稳态的底物相互作用, 参与衰老、AD、癫痫以及其他神经系统疾病的病理过程^[6-7]。

研究证实, 相互作用蛋白质间结合水平的改变, 或者异常蛋白质复合物的形成, 将导致 AD 的

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发生。探究在AD疾病发生发展过程中起关键作用的蛋白质功能及其相互作用组对于疾病的治疗以及生物标志物和药物靶点预测具有重要意义^[8-10]。近年来,已有研究发现,BACE1的相互作用蛋白能够调节BACE1酶活性以及转录、翻译、胞内转运等过程,进而影响AD发生与疾病的进程^[11-14]。但现有的证据尚不足以全面且透彻地阐释BACE1参与调控AD的发病机理。目前基于BACE1抑制剂的药物研发处于瓶颈,BACE1抑制剂导致BACE1绝对不足引起的神经功能障碍仍无法解决^[15]。而基于BACE1相互作用蛋白质靶点的药物研发具有巨大的优势,其利用蛋白质间相互作用温和精准地抑制BACE1功能,延缓AD疾病进程^[16]。因此构建BACE1的蛋白质相互作用网络以及探究新的相互作用蛋白对于AD发病机制的探究以及突破AD治疗的瓶颈有重要意义。本研究通过免疫共沉淀-质谱联合应用技术(immunoprecipitation-mass spectrometry, IP-MS)深入探索了BACE1的相互作用蛋白,初步阐明了BACE1相互作用蛋白在AD及其他神经退行性疾病中的功能及作用机制。

1 材料与方法

1.1 细胞

人胚肾细胞HEK293T细胞、人神经母细胞瘤细胞SH-SY5Y细胞购于协和细胞库,小鼠神经母细胞瘤细胞N2a细胞购于武汉普诺赛生物科技有限公司。

1.2 动物

按照国家科技部2006年印发的《关于善待实验动物的指导性意见》规定,对购自北京至善生物科技有限公司的APP^{swe}/PS1^{dE9}小鼠(8月龄,雄性,5只)和C57/BL6野生型(wild type, WT)小鼠(8月龄,雄性,5只)进行脑组织取材,课题伦理获首都医科大学医学伦理委员会审查批准(批件号:临研审[2021]225号)。

1.3 试剂

BACE1-HA、NSF-Flag、APP-Flag、NUMB-Flag、HSP90aa1-Flag、SNAP91-Flag过表达质粒、甘油菌种购自北京合生生物科技有限公司; Anti-BACE1、Anti-Amyloid Precursor Protein、同型对照IgG购于abcam, Flag标签抗体购自Proteintech, 抗体过氧化物酶标记的山羊抗小鼠IgG(H+L)和过氧化物酶标记的山羊抗兔IgG(H+L)购自北京中杉金桥生物技术有限公司; ABflo® 647-

conjugated Goat Anti-Rabbit IgG (H+L)、ABflo® 555-conjugated Goat Anti-Mouse IgG (H+L) 购于武汉爱博泰克生物科技有限公司, 蛋白A/G磁珠购于Merck; 质谱兼容型蛋白酶抑制剂、磷酸酶抑制剂、EDTA、硫磺素S均购于上海碧云天生物技术有限公司。

1.4 实验方法

1.4.1 细胞培养

N2a细胞、SH-SY5Y细胞、HEK-293T细胞培养于含10%胎牛血清及青链霉素双抗的DMEM高糖基础培养基中,于37℃、5% CO₂培养箱环境中培养。

1.4.2 质粒转染

转染前24 h铺板,转染质粒时细胞生长密度达到80%左右,转染前按比例将稀释液、质粒、转染试剂混合后室温孵育10 min再进行转染,转染6 h后,更换新鲜培养基,继续培养,转染后48 h在荧光显微镜下观察转染效率。

1.4.3 构建AD细胞模型

通过A β_{42} 诱导SH-SY5Y细胞构建AD细胞模型,使用DMSO溶解A β_{42} 多肽,母液浓度为5 mmol/L,工作液浓度为20 μ mol/L,诱导前24 h将细胞种植于12孔板,细胞生长密度达到70%左右时,实验组加入含20 μ mol/L A β_{42} 的培养基诱导48 h,对照组添加相同体积的DMSO,48 h后提取细胞总蛋白质。

1.4.4 总蛋白质提取

细胞蛋白质提取:加入含蛋白酶抑制剂、磷酸酶抑制剂的1% TritonX-100裂解液冰上裂解30 min后,收取蛋白质至1.5 ml离心管中,4℃、12 000 r/min离心15 min,提取上清用于后续实验。

组织蛋白质提取:每70 mg组织剪碎后加入1 ml含蛋白酶抑制剂、磷酸酶抑制剂的1% TritonX-100裂解液于组织匀浆管中,冰上裂解1 h,匀浆仪中研磨20 s,收取蛋白质至离心管中,离心提取上清用于后续实验。

1.4.5 免疫共沉淀(co-immunoprecipitation, Co-IP)实验

每个体系中加8 μ g抗体、含1 mg蛋白质的蛋白裂解液,4℃旋转孵育过夜。取160 μ l悬浮磁珠至1.5 ml微离心管中。用0.1% PBST溶液清洗磁珠3次,将预先制备好的抗原抗体复合物加入到磁珠中,在混悬仪上4℃孵育过夜捕获免疫复合物。孵育完成后,弃去蛋白质上清,用PBST与盐溶液清

洗磁珠3次,加入1×SDS-PAGE上样缓冲液进行高温变性洗脱,洗脱下的蛋白质用于后续蛋白质印迹(Western blot, WB)实验。

1.4.6 蛋白质印迹(WB)

蛋白质经过SDS-PAGE分离后,转移至硝酸纤维素膜;随后用5%脱脂奶粉室温封闭1 h,加入相应抗体,4℃摇床孵育过夜;加入辣根过氧化物酶标记的二抗(过氧化物酶标记的山羊抗小鼠IgG(H+L)和过氧化物酶标记山羊抗兔IgG(H+L)),室温孵育1 h, TBST洗膜后用显影仪进行显影。

1.4.7 细胞免疫荧光

在24孔板中制备细胞爬片,种植细胞 10^5 /孔,培养24 h待细胞贴壁后,弃掉细胞培养基,PBS洗3次。使用4%预冷PFA进行固定,PBS洗3次。0.5% TritonX-100室温打孔20 min, PBS洗3次,弃掉洗液,滴加10%驴血清封闭30 min。吸水纸吸干封闭液,加入1%驴血清配置的一抗(1:100稀释),4℃孵育过夜。PBST洗3次,加二抗(1:1000稀释),37℃孵育1 h。PBST洗3次,吸干液体,用含DAPI的抗荧光猝灭封片剂封片,共聚焦显微镜下观察。

1.4.8 硫磺素S染色

组织切片脱蜡后,洗涤3次,每次洗涤5 min, DAPI染色10 min。用50%酒精配置浓度为0.3%的硫磺素S溶液,室温孵育10 min。使用80%酒精洗涤10 s,滴染10 s,重复2次。最后使用纯水冲洗1次,冲洗后封片,在荧光显微镜观察并拍照。

1.4.9 高效液相色谱-串联质谱技术检测分析

蛋白质检测:将Co-IP洗脱产物进行SDS-PAGE电泳,电泳结束后进行考马斯亮蓝R-250染色,蛋白质条带清晰可见则进行后续实验。

蛋白酶解:取蛋白质样品,加入DB蛋白溶解液补足体积至100 μ l,加入胰酶和100 mmol/L TEAB缓冲液,混匀后于37℃酶切4 h,加入胰酶和CaCl₂酶切过夜。加入甲酸调节pH小于3,混匀后于室温、12 000g离心5 min,取上清缓慢通过C18除盐柱,之后使用清洗液(0.1%甲酸、3%乙腈)连续清洗3次,再加入适量洗脱液(0.1%甲酸、70%乙腈),收集滤液,冻干。

液质检测:配制流动相A液(100%水、0.1%甲酸)和B液(80%乙腈、0.1%甲酸)。使用10 μ l A液溶解冻干粉末,4℃下14 000g离心20 min,取上清1 μ g样品进样,进行液质检测。使用EASYnLCTM 1200纳升级UHPLC系统,Q

ExactiveTM系列质谱仪,Nanospray FlexTM(ESI)离子源,质谱采用数据依赖型采集模式。

1.5 数据分析

1.5.1 质谱数据分析

数据库检索:质谱数据通过MaxQuant(V1.6.6)软件进行检索,通过Uniprot蛋白数据库搜索结果谱图,主要检索参数如下:项目类型选择LFQ;可变修饰选择Oxidation(M)、Acetyl(Protein N-term)、Deamidation(NQ);固定修饰选择Carbamidomethyl(C);酶切选择Trypsin/P;一级质谱匹配容差在初次检索中设置为20 ppm,主要检索中设置为4.5 ppm;二级质谱匹配容差设置为20 ppm。检索结果以蛋白质和肽段水平分析。

蛋白质鉴定:认为可信度在99%以上的谱肽(peptide spectrum matches, PSMs)为可信PSMs,至少包含一个特有肽段的蛋白质为可信蛋白,只保留可信的谱肽和蛋白质,并做错误发现率(false discovery rate, FDR)验证,去除FDR大于1%的肽段和蛋白质。

1.5.2 生物信息学分析

基因本体(gene ontology, GO)富集分析:利用GO数据库对AD组中BACE1差异结合蛋白的基因功能进行富集分析,使用GO Term Finder,将BACE1差异结合蛋白注释基因与GO Term数据库进行比对,将所有差异注释基因分别从生物学进程(biological process, BP)、分子功能(molecular function, MF)和细胞组分(cellular component, CC)三个方面分配到不同的GO功能分类中,得到基因参与的所有GO,采用超几何分布计算每个GO的显著性水平,从而筛选出注释基因富集的显著性GO Term。

代谢通路分析:将BACE1差异结合蛋白的注释基因基于京都基因与基因组百科全书(Kyoto Encyclopedia of Genes and Genomes, KEGG)数据库进行代谢通路的注释,得到差异注释基因参与的所有Pathway Term,采用超几何分布计算Pathway的显著性水平,从而筛选出注释基因富集的显著性Pathway Term。

1.5.3 候选相互作用蛋白筛选

候选相互作用蛋白筛选条件:a.质谱定量分析数据满足PSMs>99%、特有肽段数 ≥ 1 、FDR<1%、AD组/AD-IgG组表达差异倍数(fold change, FC)>4;b.过滤AD组与WT组共同鉴定到的BACE1相互作用蛋白;c.细胞内空间结构高度有序、相同的

亚细胞定位是蛋白质间相互作用的必要条件, 候选蛋白应与BACE1具有共同亚细胞定位; d. 蛋白质间结合基于蛋白质本身结构特性, 具有蛋白质结合功能的候选蛋白质可信度更高, GO注释文件下载于基因本体论联合网站 (<http://www.geneontology.org/>); e. STRING数据库 (<https://string-db.org>) 通过收录已发表文献通过证据评分构建相互作用蛋白质网络, 构建BACE1相互作用蛋白质网络, 筛选条件设置为置信度评分 high confidence (confidence score>0.7), 与BACE1存在直接或间接关联的蛋白质分子; f. 将相互作用蛋白质的编码基因通过小鼠基因组信息网站 (mouse genomeinformation, MGI, <http://www.informatics.jax.org>) 进行检索, 保留基因异常表达时导致小鼠出现致死、异常的神经精神症状相关表型的基因; g. 通过NCBI数据库 (National Center for Biotechnology Information, NCBI, <https://www.ncbi.nlm.nih.gov/>) 检索蛋白质在各组织的表达情况, 选择高度表达于大脑皮层、海马组织的蛋白质分子。

1.5.4 统计分析

统计分析由SPSS20.0进行t检验。所有结果均表示为 $mean\pm SD$ 。 $P<0.05$ 认为具有统计学意义。

2 结 果

2.1 BACE1在AD模型小鼠海马组织中的蛋白质表达水平升高

本文选取8月龄APP/PS1小鼠模拟AD患者脑区病理改变, 通过硫磺素S染色检测APP/PS1小鼠海马组织中淀粉样斑块形成情况 (图1a), APP/PS1小鼠海马组织、皮层组织中淀粉样斑块数量显著增多, 提示8月龄APP/PS1小鼠已经出现了淀粉样斑块沉积的病理改变。接着, 检测8月龄APP/PS1小鼠海马组织中BACE1蛋白表达水平变化,

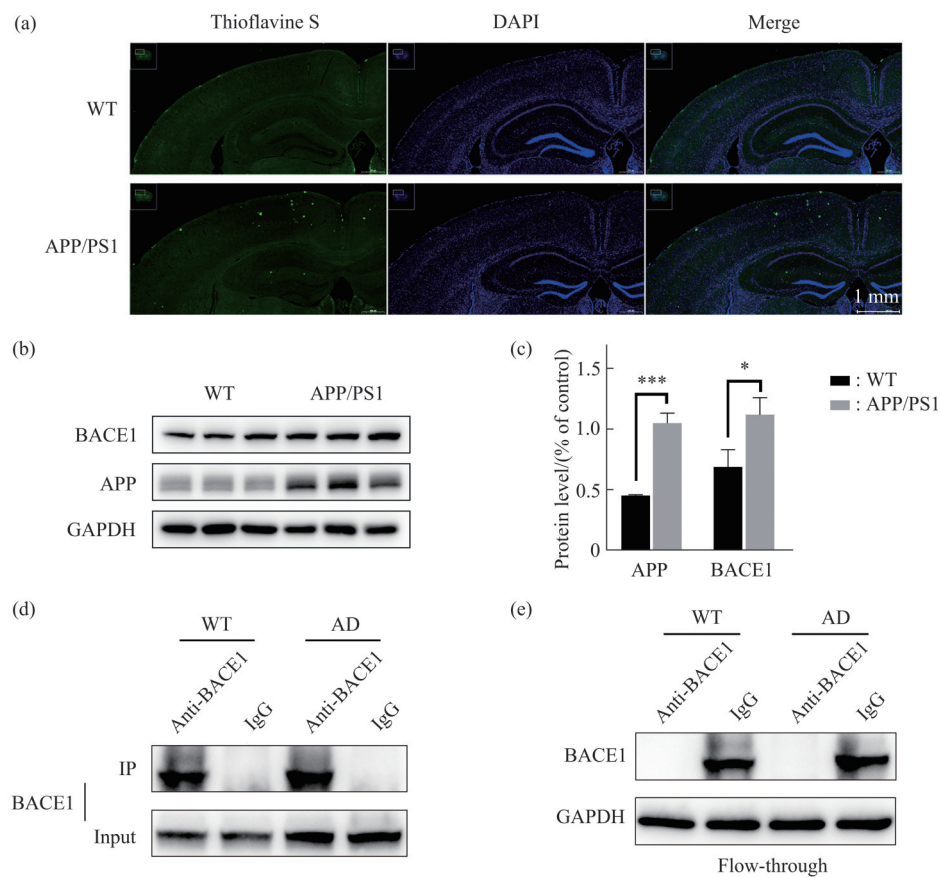


Fig. 1 Validation of Alzheimer's disease pathology models and antibodies, protein expression level of BACE1

(a) Distribution of β -amyloid plaques in the cortex and hippocampus of WT and APP/PS1 mice. Senile plaque formation in 8-month-old WT and APP/PS1 mouse brain tissue examined by thioflavin S staining. (b-c) The expression level of BACE1 is up-regulated in AD. The expression of BACE1 in the hippocampus of APP/PS1 mice was higher than that of WT mice by Western blot assay. Data were shown as $mean\pm SD$. $n=3$. $*P<0.05$, $***P<0.001$ vs. WT group. (d) Immunoprecipitation: BACE1 was captured from hippocampal tissue protein lysate of WT, APP/PS1 mice using BACE1 antibody. (e) Western blot: the residual amount of BACE1 in the filtrate of each group of IP experiment was detected to verify the efficacy of the antibody.

结果显示, 相对于WT小鼠, APP/PS1小鼠海马组织中BACE1蛋白表达水平上调(图1b~c)。

进一步提取小鼠海马组织总蛋白质, 验证BACE1抗体效能。以BACE1抗体进行Co-IP实验, 并选用同型IgG作为对照。将Co-IP洗脱产物以及流穿液进行Western Blot检测, 结果表明, 实验组Co-IP洗脱产物中检测到BACE1的特异性条带, 而对照组中未检测到BACE1的蛋白质条带(图1d), 流穿液检测中实验组BACE1蛋白条带缺失, 证明BACE1被IP抗体捕获至Co-IP洗脱产物中, 流穿液中没有残余, Co-IP实验成功(图1e), 抗体满足Co-IP实验需求。

2.2 免疫沉淀-质谱联合应用技术鉴定BACE1相互作用蛋白

2.2.1 通过IP-MS技术表征BACE1及其相互作用蛋白质组

为探究能够特异在AD疾病中发挥功能的

BACE1相互作用蛋白, 实验设置4个分组: AD组(实验组)、AD-IgG组(实验组阴性对照组)、WT组(对照组)、WT-IgG组(阴性对照组), 每组实验样本为5只8月龄WT、APP/PS1小鼠海马组织混合样本以减少个体差异。为避免数据依赖性采集方式下高丰度的诱饵蛋白影响低丰度相互作用蛋白的采集, 切取诱饵蛋白所在的70 ku分子质量区段胶条单独上机检测, 剩余胶条另行上机检测, 检测及鉴定流程如图2a所示, 检测结果如表1所示, 描述了各组中鉴定到的BACE1特异性肽段数目(unique peptides)、蛋白质序列中氨基酸覆盖度(coverage/%)、蛋白质的丰度标准化值(abundance(normalized))、肽段匹配到二级谱图的数目(PSMs)。WT组、AD组均鉴定到BACE1, WT-IgG、AD-IgG两组阴性对照组未鉴定到BACE1(表1)与诱饵蛋白自拉自抗实验结果一致, 提示Co-IP实验诱饵蛋白被成功富集。

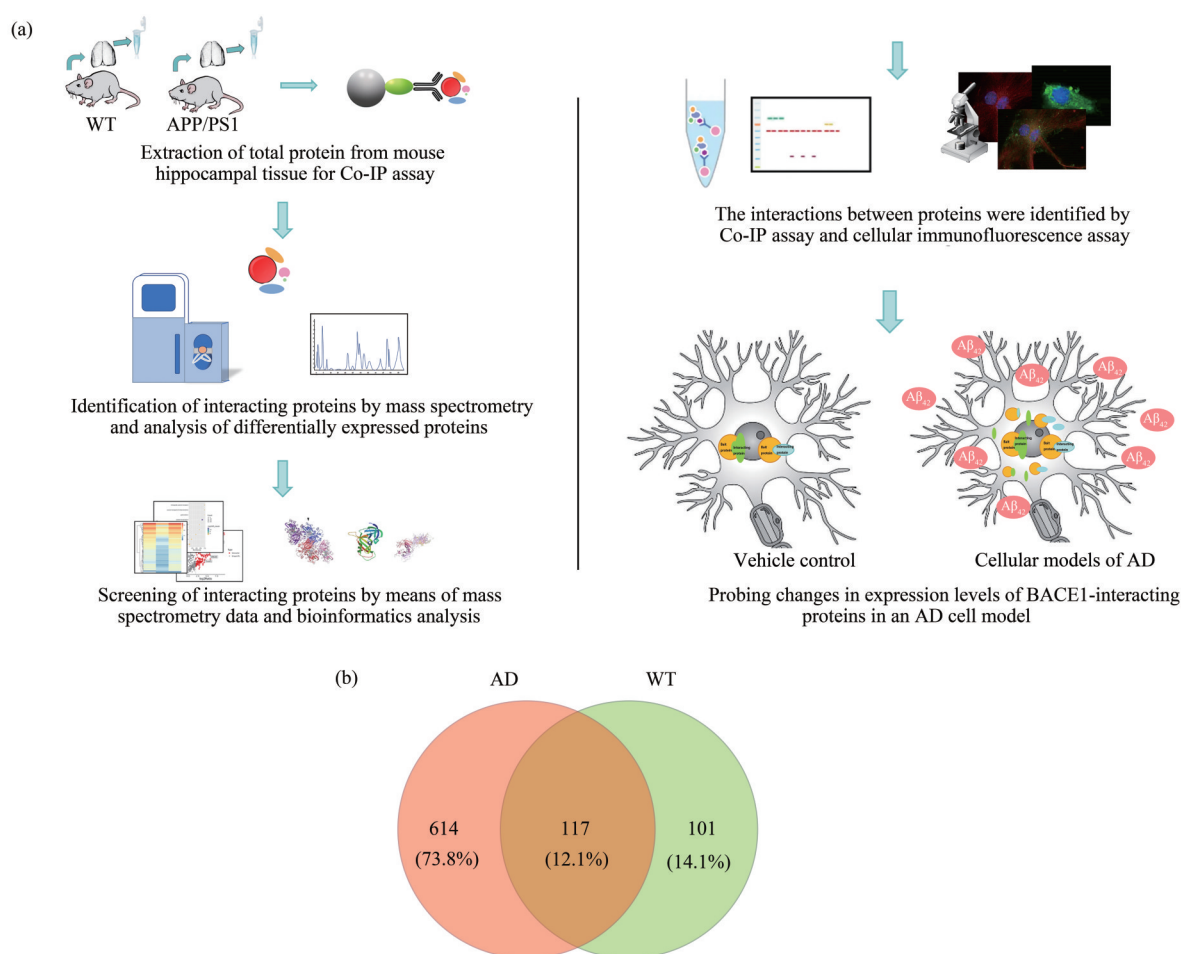


Fig. 2 Protein statistics determined by mass spectrometry

(a) Schematic of the immunoprecipitation-mass spectrometry approach to identify BACE1-interacting proteins. (b) Protein statistics identified by mass spectrometry: interacting proteins identified after removal of common contaminating proteins, where red represents proteins enriched in the AD group sample; green represents proteins enriched in the WT sample; the middle segment is the protein identified by both.

Table 1 Mass spectrometry identification of bait protein				
Group	Unique peptides	Coverage/%	Abundance (normalized)	PSMs
WT	19	36	1316128856.56	48
WT-IgG	—	—	—	—
AD	20	36	1666896281.25	52
AD-IgG	—	—	—	—

对质谱鉴定数据进行过滤以去除假阳性非特异性结合蛋白, 通过实验检测与数据分析, AD组共鉴定到731个蛋白质与BACE1相互作用, WT组鉴定到218个蛋白质与BACE1相互作用, 其中仅于AD组鉴定到的BACE1相互作用蛋白614个(73.8%), 仅于WT组鉴定到的BACE1相互作用蛋白101个(14.1%), 两组均鉴定到的BACE1相互作用蛋白117个(12.1%) (图2b)。

2.2.2 BACE1相互作用蛋白参与神经退行性疾病相关通路

进一步对AD组鉴定到的614个与BACE1存在相互作用的差异表达蛋白进行GO注释和分类, 分析其显著富集的GO条目, 以揭示AD中BACE1差异结合蛋白的基因功能, 包括基因的分子功能、所

处的细胞组分以及参与的生物过程。通过气泡图展示显著富集结果, 横轴数值与圆圈颜色表示富集显著性, 纵轴为功能节点或通路, 圆圈大小表示功能分类或通路中蛋白质个数。GO富集分析显示: AD中BACE1差异结合蛋白的功能在分子功能层面主要集中于离子通道调节、蛋白激酶活性、转录因子结合、被动跨膜转运蛋白活性等; 在所处的细胞位置层面主要定位于高尔基体、内质网、内体、溶酶体、囊泡等具膜细胞器; 在参与的生物过程层面主要集中于免疫应答调节细胞表面受体信号通路、靶向高尔基体囊泡的转运、昼夜节律的调节、浦肯野细胞层发育等生物学过程 (图3a~c)。

接着对AD组与BACE1存在相互作用的差异表达蛋白, 根据KEGG数据库进行信号通路的富集

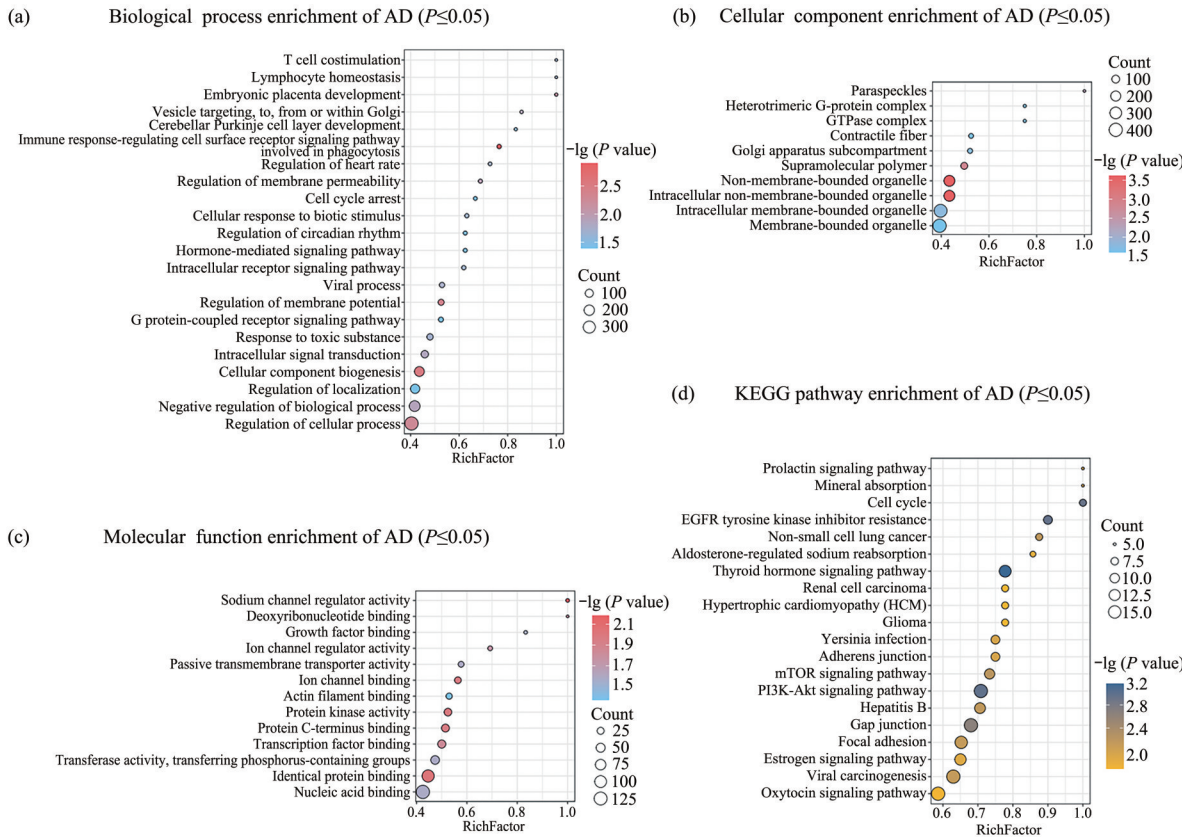


Fig. 3 Functional enrichment analysis of AD group-specific differentially expressed proteins assessed by GO and KEGG pathway

(a) Biological process, (b) cellular component, (c) molecular function, (d) KEGG pathway.

分析,对差异表达蛋白富集显著的信号通路进行统计分析,以揭示AD疾病进程中BACE1相互作用差异结合蛋白参与调控的信号通路(图3d)。结果显示,BACE1相互作用蛋白参与了多种活化的信号通路,包括PI3K-Akt信号通路、mTOR信号通路、细胞周期调控、细胞缝隙连接的形成等,这些信号通路在调节细胞增殖、细胞凋亡以及炎症反应中发挥了重要的作用,其中PI3K-Akt信号通路、mTOR信号通路是AD、帕金森病以及其他神经退行性疾病治疗的关键靶点,对于抑制蛋白质异常聚集具有重要作用。

2.2.3 筛选BACE1相互作用蛋白

细胞高度有序的空间结构是维持胞内正常生理功能的基础,共同的亚细胞定位是蛋白质相互作用的必要条件,BACE1主要定位于内体、细胞膜以及囊泡中,根据GO注释保留与BACE1具有相同亚细胞定位的蛋白质条目,排除位于细胞核、核糖体等结构的蛋白质条目。AD组鉴定到的614个蛋白质分子中,40个定位于内体,226个定位于细胞膜,21个定位于囊泡(表S1)。具有蛋白质结合功

能是蛋白质间相互作用的基础,对与BACE1存在共同亚细胞定位的蛋白质分子进一步筛选,其中具有蛋白质结合功能的蛋白质177个(表S1)。将以上177条蛋白质信息导入STRING数据库,构建BACE1相互作用蛋白质网络(protein-protein interaction networks, PPI),展示与BACE1存在直接或间接关联的蛋白质分子(置信度评分 high confidence (confidence score>0.7)),排除与BACE1无任何关联的蛋白质分子(图4),每个节点代表由单个蛋白质编码基因位点编码的蛋白质,各节点间连线代表蛋白质与蛋白质之间的关联。共有3个蛋白质分子与BACE1存在直接相互作用,121个蛋白质分子与BACE1存在间接相互作用(表S1)。候选的BACE1相互作用蛋白应在神经系统中存在功能作用,将PPI网络中编码蛋白质的基因通过MGI数据库检索,保留基因异常表达时导致小鼠出现致死、异常的神经精神症状等相关表型的基因;通过NCBI数据库检索蛋白质在各组织的表达情况,选择高表达于大脑皮层、海马组织等中枢神经系统的蛋白质分子。

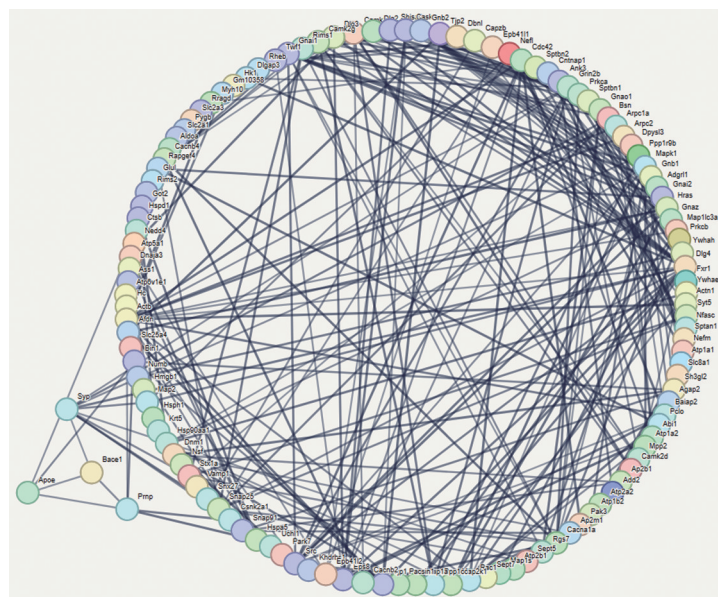


Fig. 4 The PPI network diagram of proteins interacting with BACE1

经筛选认为PRNP、APOE、SYP、NSF、NUMB、SNAP91、HSP90aa1、UCHL1、BIN1、SNX27、Rheb、Ap2m1 12个蛋白质可信度较高,其中NSF、NUMB、SNAP91、HSP90aa1为新发现的BACE1相互作用蛋白,蛋白质信息如表2所示,表2描述了BACE1与以上BACE1相互作用蛋白的

序列数据编号(accession)、蛋白质名称(name)、蛋白质类型(type)、亚细胞定位(cellular component)、分子功能(molecular function)、参与的生物学过程(biological process)、哺乳动物表型(mammalian phenotype)、蛋白质分子主要表达分布(expression)。这些蛋白质主要定位于细胞

膜、内体、突触囊泡等亚细胞结构, 特异地表达于中枢神经系统, 与神经系统疾病发病密切相关, 在调控淀粉样蛋白前体蛋白代谢过程、调节突触可塑性、内体转运、突触中囊泡介导的转运、调节突触后神经递质受体内化等生物途径中发挥重要的作用。

Table 2 Basic informations about the bait protein and its potential interacting proteins

Accession	Name	Type	Cellular component	Molecular function	Biological process	Mammalian phenotype	Expression
P56818	BACE1	Bait protein	Cytoplasmic vesicle membrane, early endosome, plasma membrane, etc.	Enzyme binding, aspartic-type endopeptidase activity, etc.	Protein processing, amyloid-beta binding, amyloid-beta metabolic process, etc.	Abnormal spatial learning, decreased exploration in new environment, etc.	Cerebellum, ovary, etc.
Q3TZI7	PRNP	Interactor of group AD	Cell surface, plasma membrane, etc.	Protein-containing complex binding, protein-folding chaperone binding, amyloid-beta binding, etc.	Learning or memory, response to amyloid-beta, negative regulation of long-term synaptic potentiation, etc.	Abnormal hippocampus morphology, ataxia, etc.	Cortex, frontal lobe, etc.
P08226	APOE4	Interactor of group AD	Endosome Golgi apparatus, neuronal cell body, late endosome, etc.	Amyloid-beta binding, tau protein binding, signaling receptor binding, protein-containing complex binding, etc.	Amyloid precursor protein metabolic process, regulation of amyloid precursor protein catabolic process, etc.	Synaptomorphism, etc.	Liver, brain, etc.
Q62277	SYP	Interactor of group AD	Membrane, presynapse, synaptic vesicle, etc.	Identical protein binding, protein-containing complex binding, SNARE binding, etc.	Synaptic vesicle endocytosis, regulation of neuronal synaptic plasticity, endocytosis, etc.	Increased vertical activity, etc.	Cortex, frontal lobe, etc.
Q3TCH2	UCHL1	Interactor of group AD	Plasma membraneendoplasmic reticulum membrane, etc.	Ubiquitin binding, ubiquitin protein ligase binding, etc.	Ubiquitin-dependent protein catabolic process, axonogenesis, etc.	Tremors, paralysis, ataxia, neurodegeneration, premature death, etc.	CNS, brain, etc.
O08539	BIN1	Interactor of group AD	Endosome, plasma membrane, synapse, synaptic vesicle, etc.	Function identical protein binding, protein-containing complex binding, tau protein binding, etc.	Endosome to lysosome transport, negative regulation of amyloid-beta formation, etc.	Neonatal lethality, complete penetrance, etc.	Cerebellum, cortex, etc.
Q3UHD6	SNX27	Interactor of group AD	Early endosome, endosome, glutamatergic synapse, etc.	Ionotropic glutamate receptor binding, protein binding, etc.	Endosomal transport, endosome to lysosome transport, regulation of postsynaptic membrane neurotransmitter receptor levels, etc.	Postnatal lethality, etc.	CNS, cerebellum, etc.
Q921J2	Rheb	Interactor of group AD	Neuronal cell body, plasma membrane, synapse, etc.	Protein kinase binding, GTP binding, GDP binding, etc.	Positive regulation of TOR signaling, small GTPase mediated signal transduction, etc.	Abnormal brain development, demyelination, premature death, etc.	Bladder, CNS, etc.

Continued to Table 2

Accession	Name	Type	Cellular component	Molecular function	Biological process	Mammalian phenotype	Expression
Q3TH69	Ap2m1	Interactor of group AD	Plasma membrane, AP-2 adaptor complex, synapse, etc.	Signal sequence binding, lipid binding, disordered domain specific binding, transmembrane transporter binding, etc.	Synaptic vesicle endocytosis, vesicle budding from membrane, etc.	Lethal, etc.	CNS, brain, etc.
P46460	NSF	Interactor of group AD	Postsynaptic density, plasma membrane, lysosomal membrane, Golgi apparatus, etc.	Protein-containing complex binding, PDZ domain binding, etc.	Vesicle-mediated transport, intracellular protein transport, etc.	Abnormal cerebellum development, embryonic lethality, increased anxiety- related response, etc.	Cerebellum, frontal lobe, etc.
Q9QZS3	NUMB	Interactor of group AD	Early endosome, cytoplasmic vesicle, plasma membrane endosome membrane, etc.	Protein binding, cytoplasm, multicellular organism development, etc.	Nervous system development, regulation of postsynaptic neurotransmitter receptor internalization, etc.	Postnatal lethality, ataxia, etc.	Cerebellum、 cerebral cortex, etc.
E9QLK9	SNAP91	Interactor of group AD	Endosome, plasma membrane, etc.	Protein binding, vesicle-mediated transport, etc.	Vesicle-mediated transport in synapse, synaptic signaling; postsynaptic specialization, etc.	Abnormal CNS synaptic transmission, abnormal response to novel object, etc.	Frontal lobe, CNS, etc.
P07901	HSP90aa1	Interactor of group AD	Plasma membrane, Cellular Component, neuronal cell body, etc.	Protein binding, protein folding, response to endogenous stimulus, etc.	Transmembrane transport, acting on a protein;export from cell, etc.	Prewaning lethality, etc.	CNS, placenta, etc.

2.3 NSF结合BACE1与APP，并在AD细胞模型中蛋白质表达水平上调

进一步对新发现的4种BACE1相互作用候选蛋白，NSF、NUMB、HSP90aa1、SNAP91，与BACE1的相互作用方式进行验证，构建真核过表达载体以及其对照载体，通过将BACE1过表达质粒与候选蛋白过表达质粒共转染HEK293T细胞，提取细胞蛋白质样本进行正向（BACE1作为诱饵蛋白）、反向（候选蛋白作为诱饵蛋白）Co-IP实验以验证蛋白质间的相互作用关系，正向、反向Co-IP实验均为阳性的蛋白质组合认为具有相互作用。结果显示（图5a~c），NUMB、HSP90aa1、SNAP91与BACE1的反向Co-IP实验均为阴性，认为NUMB、HSP90aa1、SNAP91并未与BACE1稳定结合；图5d显示，BACE1与NSF正反向Co-IP实验均为阳性，认为二者存在相互作用关系。为明确NSF在BACE1切割APP过程中的作用关系，本

文进一步探究了APP与NSF的相互作用关系，实验结果显示NSF与APP相互结合（图5e）。

通过激光共聚焦显微镜观察BACE1、APP、NSF三者 在细胞内的定位关系，NSF-HA质粒转染N2a细胞后，对细胞进行间接免疫荧光标记，在激光共聚焦荧光显微镜下观察两种蛋白质荧光共定位情况。结果显示，NSF分别与APP、BACE1存在免疫荧光共定位（图5f），提示NSF与BACE1以及APP均存在于相同的空间结构，具有一定相互作用关系。

通过Aβ₄₂诱导SH-SY5Y细胞构建AD的细胞模型，进一步探究AD病理情况下NSF的表达水平变化。相较于对照组，以浓度为20 μmol/L的Aβ₄₂诱导SH-SY5Y细胞48 h，NSF表达水平上调（图5g，h），提示NSF表达水平与AD病理密切相关，NSF可能参与AD的发生发展。

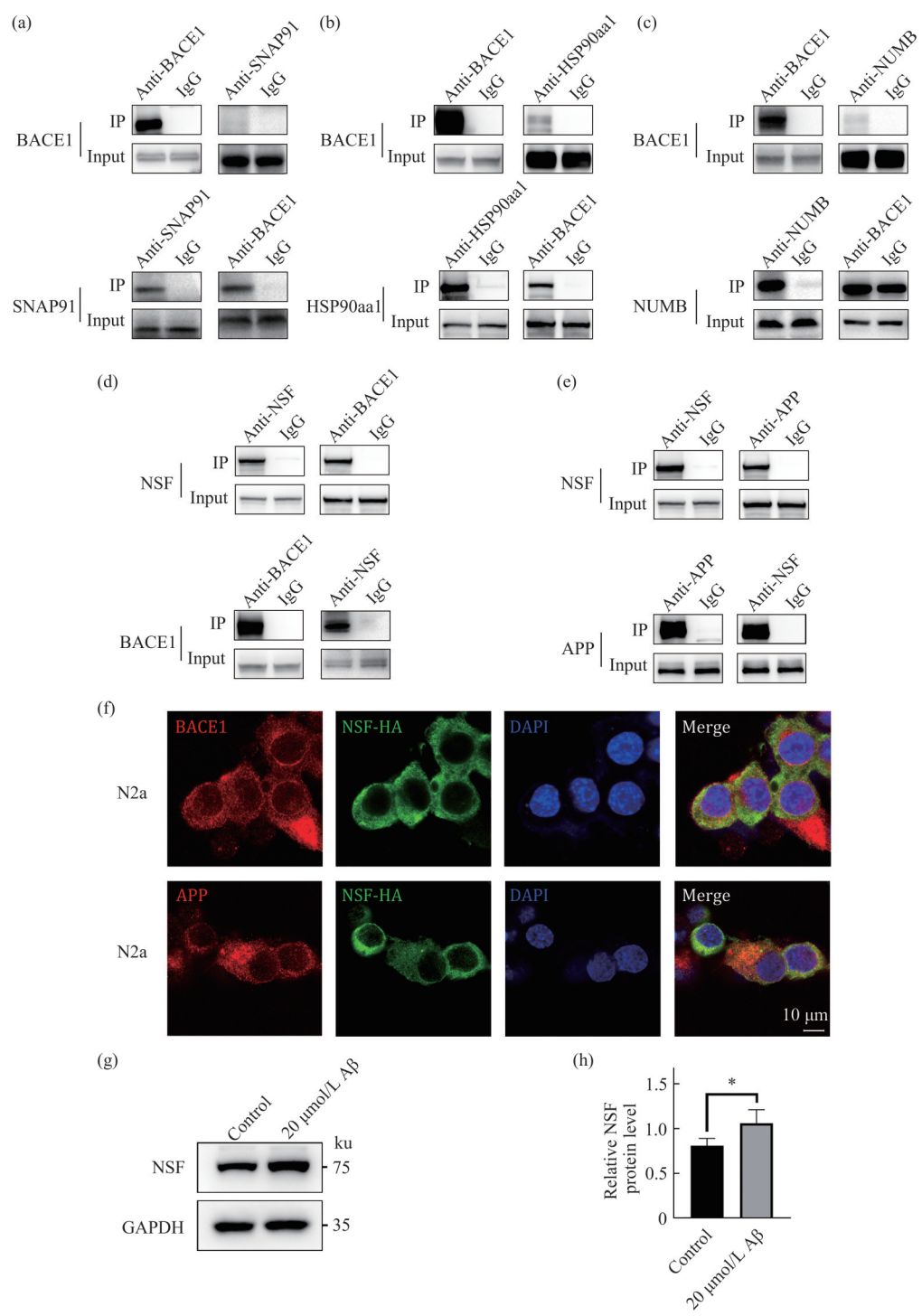


Fig. 5 Co-immunoprecipitation and immunofluorescence for detection of protein interactions

(a–c) Co-IP assay was used to verify the interaction between BACE1 and SNAP91 (a), HSP90aa1 (b), NUMB (c). (d–e) Co-IP assay was used to verify the interaction between BACE1 and NSF (d), APP and NSF (e). (f) Immunofluorescence: NSF-HA plasmid was transfected into N2a cells, BACE1/APP was labelled with BACE1/APP rabbit antibody and goat anti-rabbit IgG (H+L) (red), NSF was labelled with HA-tag mouse antibody and goat anti-mouse IgG (H+L) (green), and DAPI was used to label the nucleus (blue). Laser confocal microscopy was used to detect protein co-localisation. (g–h) NSF expression levels increase in SH-SY5Y in cell models of AD (* P <0.05, t -test).

3 讨 论

神经退行性疾病是一类具有广泛临床特征和相似病理学特征的疾病^[17],包括癫痫、AD、帕金森病、亨廷顿病、肌萎缩性侧索硬化症、不同类型脊髓小脑共济失调以及Pick病等^[18-21]。神经退行性疾病具有相同或相似神经元进行性变性的特征,导致认知功能、运动功能和情绪控制能力等受损^[22-23]。神经退行性疾病具有共同的细胞和分子机制^[24],如蛋白质聚集和突触功能障碍等^[7, 25-26]。研究表明,导致蛋白质聚集和突触功能障碍的关键蛋白A β 在多种神经退行性疾病的发生发展过程中起重要作用^[27],A β 是APP经BACE1与 γ 分泌酶顺序切割产生,因此,研究BACE1在疾病中的作用机制及其调节因素在神经退行性疾病特别是AD的诊断及治疗中具有重要意义。

蛋白质间的相互作用随生物个体生理状态不同处于动态变化的过程中,Co-IP实验能够极大地探究蛋白质相互作用网络,包括蛋白质间的直接相互作用与间接相互作用。本研究通过IP-MS的方法鉴定并筛选BACE1相互作用蛋白,对鉴定到的蛋白质进行GO富集分析以及KEGG通路富集分析,分析结果提示,AD组特有的与BACE1相互作用的差异表达蛋白可能作为AD疾病的危险因素,当AD疾病发生时,参与细胞内信号转导、生物过程的负调控过程的蛋白质表达水平发生上调或与BACE1相互作用水平上调,胞内出现异常的囊泡转运,异常激活了PI3K-Akt、mTOR等信号通路,参与细胞稳态以及代谢等生理过程的BACE1相互作用蛋白表达水平下调,或与BACE1相互作用水平发生下调^[28-29],引起机体发生AD病理性改变。

在构建的BACE1 PPI网络中,突触囊泡蛋白众多,既往研究证实,BACE1功能活性具有pH依赖性,主要在内体的酸性环境中对APP进行切割^[30]。突触活动通过调节APP胞内转运途径,将APP转运到BACE1阳性的内体中,加速A β 的产生^[31-33]。经研究证实,BACE1与APP在突触中具有相似的分布,突触囊泡同样是BACE1的主要表达部位^[34],并能够调控突触功能以及APP的切割^[35-37]。因此,研究突触囊泡各蛋白质成分、APP、BACE1之间的相互作用为疾病的诊断和治疗提供新的靶点和方向。

在筛选出的可信度较高的候选BACE1相互作用蛋白中,先前的研究已阐明了其中部分蛋白质与

BACE1的相互作用关系,其中朊病毒蛋白(major prion protein, PRNP)又称PRP(c)^[38],能够与BACE1相互作用,差异调节BACE1对野生型和瑞典突变型APP的降解活性^[39]。载脂蛋白E(apolipoprotein E, APOE)羧基端截短的APOE4形式能够调控神经元中BACE1表达水平促进A β 的产生^[40]。泛素羧基端水解酶L1(ubiquitin carboxyl-terminal hydrolase L1, UCHL1)与BACE1相互作用,负性调控BACE1表达水平与BACE1半衰期^[41]。桥接因子1(bridging integrator 1, BIN1)通过调节BACE1从早期内体到溶酶体靶向转运来调节A β 的产生^[42]。分选连接蛋白27(sorting nexin family member 27, SNX27)通过调控BACE1的结合蛋白,A型重复序列的分选相关受体(sorting-related receptor with A-type repeats, SORLA),以及调节 γ 分泌酶对APP的降解方式^[43-46]。脑中富集的Ras同源物蛋白(Ras homologue enriched in brain, Rheb)与BACE1相互作用,促进BACE1通过溶酶体和蛋白酶体途径降解,缩短BACE1蛋白半衰期^[47]。蛋白复合物2(the protein complex-2, AP-2)通过调控BACE1在内体、溶酶体、轴突中的转运,促进神经元中BACE1的运输和降解,从而减缓AD的发展^[48]。以上蛋白质分子通过与BACE1直接或间接相互作用调控BACE1的酶活性、胞内转运、降解等过程,从而影响AD的发生发展。

乙基马来酰亚胺敏感因子(N-ethylmaleimide-sensitive factor, NSF)是本研究经验证后新发现的BACE1结合蛋白,NSF是ATP酶AAA家族的主要成员,主要定位于突触囊泡、胞浆、高尔基体、溶酶体及质膜中。由于其有ATP酶活性,在最初的研究中被认为是驱动膜融合的动力因素,随后的研究表明,NSF通过与多种蛋白质结合发挥蛋白质伴侣作用^[49]。参与高尔基体到质膜、囊泡和细胞内蛋白质的转运,以及突触囊泡循环过程与神经递质释放过程,在细胞内的膜融合过程中起重要作用,是囊泡运输的关键分子^[50]。

激活N-乙基马来酰亚胺敏感因子附着蛋白受体(N-ethylmaleimide-sensitive factor attachment protein receptors, SNARE)启动膜融合过程,是NSF的主要功能^[51]。NSF通过可溶性NSF附着蛋白(soluble N-ethylmaleimide-sensitive factor attachment protein, SNAP)与SNARE结合,调节SNARE复合物构象^[52],调节膜融合过程^[53-54]。

SNARE复合物各蛋白质组分对于APP的胞内转运, 以及在细胞器结构中如何调控APP向含BACE1的膜区域转运过程具有重要意义^[55-56]。NSF能结合其他蛋白质和蛋白质复合物, NSF及其相互作用蛋白质的分子功能提示NSF在胞内成分的转运以及胞内循环过程有广泛的作用。

NSF与AD、癫痫性脑病、脑缺血损伤等神经系统疾病发病密切相关。NSF在细胞内囊泡转运中起关键作用, NSF基因中的新生杂合致病变异可引起婴儿癫痫性脑病, 这与NSF在神经递质释放过程中功能失常导致突触神经传递失调密切相关^[57]。NSF在内体系统的膜融合过程中起着至关重要的作用, NSF失活导致晚期内体与溶酶体融合过程的中断, 影响缺血性脑损伤期间细胞内的生物降解效率^[58]。

基于上述研究, 本文推测NSF作为AD发病的危险因素, 可能参与调节突触前囊泡BACE1与APP的循环过程或BACE1与APP的胞内转运过程进而影响A β 的合成, 同时通过影响囊泡融合与释放调节A β 的胞内转运以及胞外释放过程, 最终影响AD的疾病进程。

4 结 论

在AD中, BACE1表达水平上调, 促进APP通过淀粉样蛋白降解途径降解, 引起淀粉样蛋白在脑内的沉积。本文探究了AD模型小鼠海马组织中BACE1的相互作用蛋白质组, 生物信息学分析结果显示, BACE1的相互作用蛋白参与了细胞内信号转导、生物过程的负调控过程、靶向高尔基体囊泡的转运、昼夜节律的调节、浦肯野细胞层发育等生物学过程, 参与了PI3K-Akt、mTOR等神经退行性疾病相关的信号通路。通过构建BACE1的PPI网络, 探究与BACE1存在密切相互作用关系的蛋白质, 发现了新的可能与BACE1存在相互作用的蛋白质NSF、HSP90aa1、SNAP91、NUMB。进一步验证表明, NSF与BACE1、APP存在相互作用, 其在A β_{42} 诱导的细胞AD病理模型中表达水平上调, 预测NSF可能对APP、BACE1胞内转运过程存在调节作用, 后续将进一步探究与验证NSF在AD疾病中发挥的作用及其作用机制。

附件 见本文网络版 (<http://www.pibb.ac.cn> 或 <http://www.cnki.net>):

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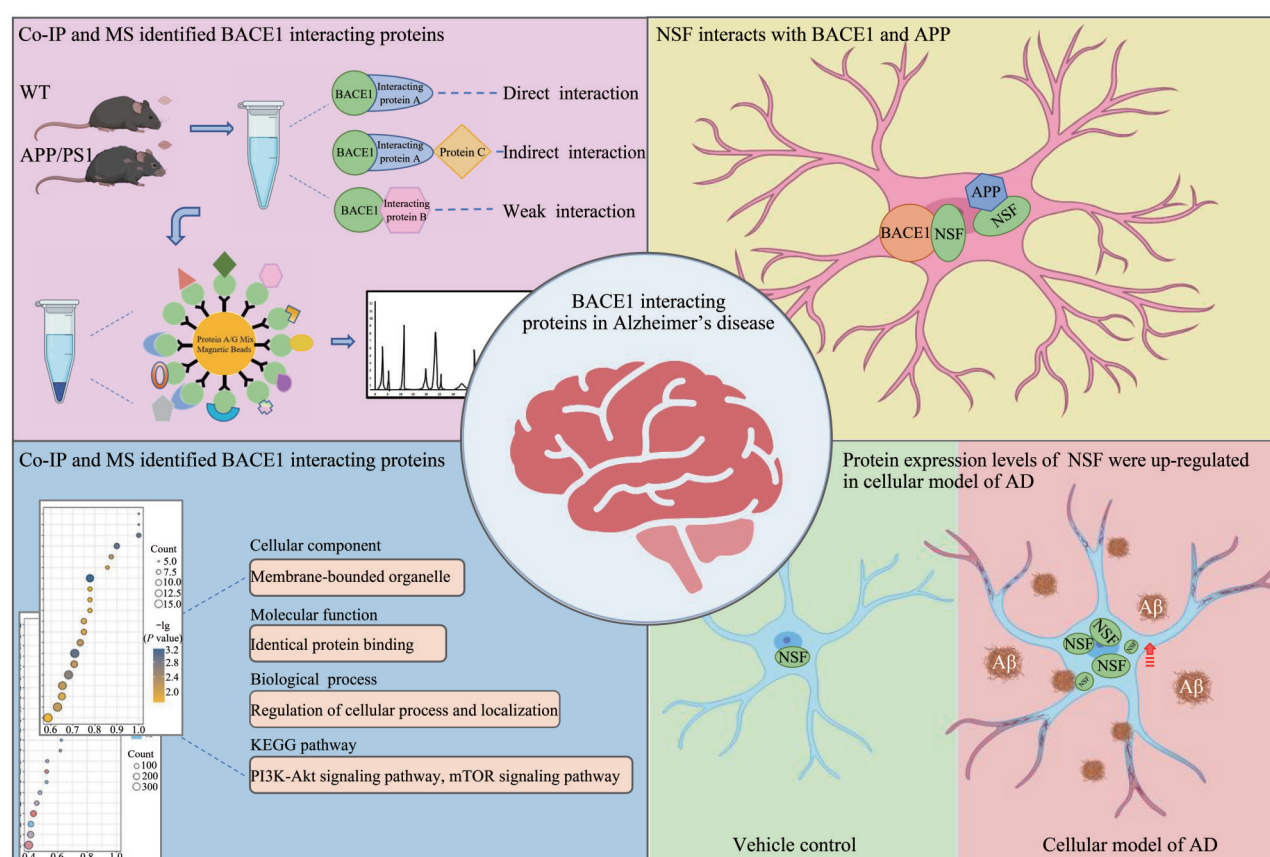
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Screening and Functional Analysis of BACE1 Interacting Proteins in Alzheimer's Disease*

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Graphical abstract



Abstract Objective β -Site APP cleaving enzyme 1 (BACE1) is a rate-limiting enzyme involved in the formation of amyloid plaques in Alzheimer's disease (AD), and its expression and activity play a crucial role in the development of AD. The interacting protein of BACE1 can directly or indirectly regulate BACE1 in the transcription, translation, modification, intracellular transport and other links of BACE1 by directly binding, indirectly binding, and participating in various cell signal transduction pathways, so as to participate in the

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occurrence of AD and the process of disease. This study aimed to screen and validate the interacting proteins of BACE1, providing new insights into the mechanisms of amyloid plaque formation. **Methods** Co-immunoprecipitation (Co-IP) and mass spectrometry (MS) were used to enrich and identify BACE1 interacting proteins in the hippocampus of wild type (WT) mice and AD model mice. For candidate BACE1 interacting proteins, GO enrichment analysis and KEGG pathway enrichment analysis were used to explore the subcellular localization, molecular function, participating biological processes and participating signaling pathways of BACE1 interacting proteins. The protein-protein interaction (PPI) network of BACE1 was further constructed to explore the potential proteins interacting with BACE1. By searching the mouse genome information (MGI) website and NCBI database, the more reliable proteins among the potential BACE1 interacting proteins were screened. Co-IP assay and immunofluorescence confocal technology were used to preliminarily verify the interaction between the proteins, and the changes in protein expression levels of the interacting proteins in AD cell models were explored. **Results** A total of 614 differentially expressed proteins interacting with BACE1 were identified in AD group. GO enrichment analysis showed that the BACE1 interacting proteins in the AD group were mainly located in membrane organelles such as Golgi apparatus, endoplasmic reticulum, endosome, lysosome and vesicles, which had molecular functions such as ion channel regulation, protein kinase activity, transcription factor binding and passive transmembrane transporter activity. It is mainly involved in the biological processes of immune response regulation cell surface receptor signaling pathway, targeting Golgi vesicles transport, circadian rhythm regulation, Purkinje cell layer development, *etc.* KEGG analysis showed that BACE1 interacting proteins in AD were mainly involved in the PI3K-Akt signaling pathway, mTOR signaling pathway and other neurodegenerative disease-related pathways. The PPI network of BACE1 showed that a total of 12 proteins were identified as high confidence binding proteins, including PRNP, APOE, SYP, NSF, NUMB, SNAP91, HSP90aa1, UCHL1, BIN1, SNX27, Rheb, Ap2m1, of which, NSF, NUMB, SNAP91, HSP90aa1 were newly identified candidate proteins. After further verification, we found that NSF not only interacts with BACE1, but also interacts with amyloid precursor protein (APP), the substrate of BACE1, and the expression level of NSF is up-regulated in the AD cell model constructed by A β_{42} induction. **Conclusion** BACE1 binding proteins participate in multiple AD-associated biological processes and signal pathways. NSF is a newly identified BACE1 binding protein that interacts with BACE1, and the protein expression level of NSF is up-regulated in the AD cell model. It is predicted that the interaction between NSF and BACE1 is involved in regulating the course of AD, providing a new target and direction for the study of the mechanism of AD.

Key words BACE1, co-immunoprecipitation, mass spectrometry, neurodegenerative disease, Alzheimer's disease

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