The effect of cannabinoids on *APP*, *BACE1*, and *ApoE* mRNA expression

Navjit Brar

Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, ON Canada. Faculty supervisor: Dr. Bettina Kalisch.
For correspondence, please email: nbrar@uoguelph.ca.

Abstract

Alzheimer's disease (AD) is an age-related neurodegenerative condition associated with cognitive decline. The pathological hallmark of this disease is the deposition of β -amyloid protein plaques (A β) in the brain, which evoke neuronal cell death and impair inter-neuronal communication. Past studies have suggested that cannabinoids reduce the levels of A β in the brain; however, little is known about the mechanisms involved in this process. In this study, the SH-SY5Y cell line was first examined for expression of amyloid precursor protein (*APP*), beta-site APP cleaving enzyme 1 (*BACE1*), and apolipoprotein E (*ApoE*), genes involved in A β production and clearance. All three genes were expressed and detected in the cell line. We then observed the effects of the endocannabinoid anandamide, a CB1 receptor agonist, on the mRNA expression of *APP*, *BACE1*, and *ApoE* in SH-SY5Y cells. After 48h exposure to anandamide, mRNA levels of *APP* and *BACE1* significantly decreased, which could contribute to reduced A β levels. The mechanism of action by which anandamide reduces mRNA levels of *APP* and *BACE1* should be further investigated. *ApoE* mRNA levels were not found to be significantly changed, suggesting that anandamide does not affect mRNA expression of this gene. The effects of cannabinoids on *ApoE* levels should be further studied as the effects may occur at a level different from mRNA expression and may even occur via a pathway unrelated to CB1 receptor activation.

Keywords: Alzheimer's disease; β -amyloid; anandamide; amyloid precursor protein; beta-site APP cleaving enzyme 1; apolipoprotein E

Introduction

Alzheimer's disease (AD) is known to be a major cause of dementia (Tarawneh and Holtzman 2012). It is characterized by the accumulation of amyloid plaques, comprised of β -amyloid (A β) protein, which play a central role in the pathophysiology of AD (Harvey et al. 2012). A β comes from a larger protein called amyloid precursor protein (APP) found in the cell membrane of nerve cells. APP can be cleaved by two pathways: the non-amyloidogenic pathway, in which A β is not produced, and the amyloidogenic pathway, in which the end product is A β . In patients with AD, there is increased cleavage of APP via the amyloidogenic pathway involving beta-secretases such as beta-site APP cleaving enzyme 1 (*BACE1*). Cleavage of APP by *BACE1* produces a sAPP β fragment and a C-terminal fragment (Cole and Vassar 2008), which is then cleaved by gamma secretase to produce A β (Cole and Vassar 2008). In AD, it is thought that higher concentrations of A β are due to an impaired clearance of A β from the brain, a process normally facilitated by apolipoprotein E (*ApoE*) (Cramer et al. 2012).

As incidence rates of AD are projected to triple in the next fifty years, the development of therapeutics to slow or cure the disease have become crucial to improve the quality of life of patients as well as reduce the health care expenses associated with AD (Hebert et al. 2013). Recently, there has been increasing interest in the use of cannabinoids as a potential treatment for AD as a result of their neuroprotective and anti-inflammatory effects.

Cannabinoids are a class of chemical compounds that activate cannabinoid receptors in the body of which there are two main subtypes – CB1 and CB2. High concentrations of CB1 receptors exist in brain structures such as the hippocampus and basal ganglia. These receptors, which are important in memory, are also impaired in individuals with AD (Mechoulam and Parker 2013). CB2 receptors are found mainly in peripheral tissue and in microglial cells of the brain, but not in neurons (Mechoulam and Parker 2013). Hence, the CB1 receptor is the focus of this study.

Table 1. Primers used for reverse transcriptase and real-time polymerase chain reactions

Primer Name	Primer Sequence
APP-Forward	5'-TCCGAGGGGTAGAGTTTGTG-3'
APP-Reverse	5'-TCCACCTCAGCCACTTCTTC-3'
BACE1-Forward	5'-TGGAGGGCTTCTACGTTGTC-3'
BACE1-Reverse	5'-CAGAGTGGCAGCATGAAGAG-3'
ApoE-Forward	5'-CGTCCTTCCCCAGGAGCCGA-3'
Appose-Reverse	5'-GCGACCCAGTGCCAGTTCCTCC-3'
G6PD-Forward	5'-CAACACCTTGACCTTCTCATCAC-3'
G6PD-Reverse	5'-TGGAACCGGGACAACATC-3'
B2M-Forward	5'-TGACCGTGATCTTTCTGGGTG-3'
B2M-Reverse	5'-ATCTGAGGTGGGTGGAACCTG-3'

Cannabinoids, particularly CB1 receptor agonists, have previously been shown to prevent experimental A β -evoked brain pathology in rats *in vivo* (Ramirez et al. 2005) and reduce A β levels in the brains of APP transgenic mice (Martín-Moreno et al. 2012). However, there is currently very little information about the mechanism of action in this process. Cannabinoids have been known to affect the expression of various genes in the brain (Bosier et al. 2007). This study examined whether cannabinoids have a direct cellular effect on the mRNA expression of *APP*, *BACE1*, and *ApoE* in SH-SY5Y cells, which are known to carry the CB1 receptor. This would shed light on the mechanism by which cannabinoids reduce A β levels in the brain and exert their neuroprotective effects.

The first objective of this study was to test whether the SH-SY5Y cells, a human neuroblastoma cell line, expressed the genes of interest: *APP*, *BACE1*, and *ApoE*, as well as to test for a good housekeeping gene in this cell line. The second objective was to treat the cells with anandamide, a CB1 receptor agonist, and see whether there were changes in the mRNA expression of *APP*, *BACE1*, and *ApoE*. By examining the direct effects of anandamide on these genes, it may be possible to identify a mechanism through which cannabinoids decrease A β levels in the brain: by decreasing production of A β , increasing clearance of A β or both. It was hypothesized that anandamide lowers A β levels through decreased expression of *APP* and *BACE1* and increased expression of *ApoE*. The present study focused on mRNA expression. Studies examining the effects of anandamide on APP, BACE1 and ApoE protein levels as well as A β production will be needed to test this hypothesis further.

Materials and Methods

Materials

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin, Trizol, trypsin, trypan blue, DNA purification kits, reverse transcription reagents, and PCR reagents were purchased from Invitrogen (Carlsbad, CA). Anandamide was supplied by Dr. Linda Parker at the University of Guelph. PerfeCTa

SYBR Green Fastmix with ROX (Quanta BioSciences) was purchased from the Genomics facility at the University of Guelph.

SH-SY5Y cell culture

SH-SY5Y human neuroblastoma cell lines were maintained in 75 cm² culture flasks with DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 μ g/mL of streptomycin. Cells were plated or divided twice weekly using trypsin to detach adherent cells. For plating, cell counts were performed using a haemocytometer and trypan blue was used to stain non-viable cells, which were excluded from the count. Cell counts typically fell between 155-200 cells for every four fields on the haemocytometer. All experiments and cell maintenance was performed in a laminar flow hood under sterile conditions. Cells were grown in an incubator with 5% CO₂ at 37°C. Cells were plated onto Corning CellBind coated dishes at a density of 2.0 million cells per 100 mm dish for RNA analysis, 48 hours prior to experiments.

Treatment of SH-SY5Y cells

Anandamide was stored at -20°C in 100% ethanol medium. Six sets of cell plates were treated with 0.1 μ M, 1 μ M, or 10 μ M anandamide. Plates treated with 10 μ L of 100% ethanol medium were used as a control.

RNA isolation and reverse transcription

Total RNA was isolated from each SH-SY5Y cell plate using Trizol 48h after treatment. RNA was purified with RNase-free chloroform and precipitated using RNase-free isopropanol. The RNA levels were subsequently quantified using a spectrophotometer. Extracted RNA (5 μ g) was reverse transcribed with Superscript II for 75 min at 43°C, using oligo-dt as the primer. The cDNA generated was then used for reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR (qPCR).

Figure 1. Representative expression of *APP*, *BACE1*, *ApoE*, *G6PD*, and *B2M* in SH-SY5Y cells. (A) *APP* expression in water blank (wb), control sample (cntl), sample treated with 10 μ M anandamide (10 μ M), and sample treated with 1 μ M anandamide (1 μ M). (B) *BACE1* expression in wb, cntl, 10 μ M, and 1 μ M anandamide. (C) *ApoE* expression in wb, cntl, 10 μ M, and 1 μ M anandamide. (D) *G6PD* expression in wb, cntl, 10 μ M, and 1 μ M anandamide. (E) *B2M* expression in wb, cntl, 10 μ M, and 1 μ M anandamide.

Figure 2. (A) *APP* sequencing results. (B) Comparison of *APP* sequence (query) with *Homo sapiens APP* transcript variant 7 sequence (subject) published under National Centre for Biotechnology Information. Nucleotides 18-148 from the *APP* sequencing results were compared against the published sequence.

Figure 3. (A) *BACE1* sequencing results. (B) Comparison of *BACE1* sequence (query) with *BACE1* transcript variant f sequence (subject) published under National Centre for Biotechnology Information. Nucleotides 17-198 from the *BACE1* sequencing results were compared against published sequence.

Figure 4. RT-PCR analysis using housekeeping gene *G6PD*. (A) RT-PCR visualized using gel electrophoresis for independent sample sets 1-3 (reverse transcribed twice) treated with 100% ethanol for control, 0.1µM anandamide, 1 µM anandamide, or 10 µM anandamide. (B). RT-PCR visualized using agarose gel electrophoresis for independent samples sets 4-6 treated with 100% ethanol for control, 0.1 µM anandamide, 1 µM anandamide, or 10 µM anandamide.

The effect of cannabinoids on APP, BACE1, and ApoE mRNA expression (Brar)

Figure 5. Analysis of *APP*, *BACE1*, and *ApoE* mRNA expression (A) Real-time PCR analysis of total *APP* mRNA relative to *G6PD* levels following treatment with 0.1, 1, or 10 μM anandamide. *APP* mRNA expression was significantly decreased with 10 μM anandamide treatment compared to control (* $p < 0.05$). (B) Real-time PCR analysis of total *BACE1* mRNA relative to *G6PD* levels following treatment with 0.1, 1, or 10 μM anandamide. mRNA expression of *BACE1* was significantly decreased with 10 μM anandamide treatment when compared to control (** $p < 0.01$). (C) Real-time PCR analysis of total *ApoE* mRNA relative to *G6PD* levels following treatment with 0.1, 1, or 10 μM anandamide. Changes in mRNA expression of *ApoE* were not statistically significant ($p > 0.05$). Statistical significance was determined by performing a one-way ANOVA followed by the Dunnett Multiple Comparisons Test. Data are presented as the mean $\pm$ standard error of mean ($n = 6$).

RT-PCR and gel electrophoresis

The RT-PCR reactions were performed to determine whether the SH-SY5Y cell line expressed *APP*, *BACE1* and *ApoE* gene products at detectable levels, as well as to test whether either glucose-6-phosphate dehydrogenase (*G6PD*) or beta-2-microglobulin (*B2M*) would make a better housekeeping gene. For each sample, 5 μL of cDNA was mixed with a master mix containing 15 mM MgCl₂, 10 mM dNTPs, 5 μM of forward and reverse primers, 10x PCR buffer, Platinum Taq and water to create a final volume of 50 μL. For all genes, RT-PCR reactions were performed in a MyCycler (Bio-Rad Laboratories) repeating 35 cycles of 94°C for 30s, 56°C for 30s, and 72°C for 1 min. The primers used for each gene are listed **Table 1**.

The PCR products of each gene were electrophoresed on a 1.5% agarose gel against a 100 bp DNA ladder. The products of *APP* and *BACE1* were purified using a DNA purification kit and sent for sequencing to verify that the gene products

amplified from these primers were, in fact, those of *APP* and *BACE1*. Gene products of *ApoE* and *G6PD* primers had already been sequenced and verified to be those of their corresponding genes. The sequences were compared with published sequences from the National Centre for Biotechnology Information database.

qPCR

The qPCR reactions were carried out to quantify changes in mRNA expression of *APP*, *BACE1*, and *ApoE*. The qPCR reactions were performed with Steponeplus (Applied Biosystems) and Vii7 Software (Applied Biosystems) using 96-well plates. The cDNA (5 μL) of each sample was mixed with SYBRGreen Fastmix, primers, and water for a final volume of 15 μL. The cycling conditions were 1 cycle of 30s at 95°C followed by 40 cycles of 3s at 95°C and 30s at 60°. The primers used to amplify *APP*, *BACE1*, *ApoE*, and *G6PD* were the same as those used for RT-PCR (**Table 1**).

Data analysis and statistics

Data from qPCR were analysed using StepOne Software (Applied Biosystems) and Viia7 Software (Applied Biosystems). *APP*, *BACE1*, and *ApoE* levels were quantified relative to *G6PD* levels in the same sample. Data are representative of six experiments and are presented as the mean $\pm$ standard error of the mean (SEM). All data were tested for normality, and statistical analysis was carried out on GraphPad InStat (GraphPad Software Inc.) using a one-way analysis of variance (ANOVA). ANOVA was followed by the Dunnett Multiple Comparisons Test to determine significant differences between treatment groups. Mean values were considered different if $p < 0.05$.

Results

Expression of target genes in SH-SY5Y cell line

To qualitatively test for expression of *APP*, *BACE1*, and *ApoE* as well as the housekeeping genes *G6PD* and *B2M* in the SH-SY5Y cell line, a few of the RNA samples collected were subjected to RT-PCR. Products from the RT-PCR reactions were visualized by agarose gel electrophoresis. One control sample, one sample treated with 10 μ M anandamide, and one sample treated with 1 μ M anandamide, were used for each gene. As seen in **Figure 1**, amplification of DNA products was detected for each primer pair, indicating that all of the genes were expressed in the cell line. From the intensity of the bands, it is also evident that *G6PD* (**Figure 1D**) was amplified at higher concentrations compared to *B2M* (**Figure 1E**). Thus, the *G6PD* gene was used as a housekeeping gene in further experiments. Although this qualitative analysis revealed there was some contamination of the water blank of *G6PD* (**Figure 1D-wb**), subsequent PCR and qPCR analysis did not result in the detection of any PCR products in the water blank controls.

Gene sequencing of APP and BACE1

The gene products of *APP* and *BACE1* were purified and sent for sequencing to verify that the gene products amplified from these primers were, in fact, those of *APP* and *BACE1* (**Figures 2A and 3A**). Gene products of *ApoE* and *G6PD* primers had been sequenced previously and verified to be those of their respective primers. The sequences were compared with published sequences from the National Centre for Biotechnology Information database. As seen in **Figures 2B and 3B**, sequences of gene products of *APP* as well as *BACE1* matched published sequences of these genes with 99% identity.

Effect of anandamide on APP, BACE1 and ApoE mRNA levels

Six sets of SH-SY5Y cell plates were treated with 0.1 μ M, 1 μ M, or 10 μ M anandamide (or 100% ethanol for the control samples), and RNA was isolated 48h after treatment. The samples were subjected to RT-PCR to qualitatively test

for sufficient quantity and integrity of cDNA of each sample prior to running qPCR with the samples. The housekeeping gene, *G6PD*, was used for relative quantification. As seen in **Figures 4A and 4B**, bands were visible for all samples, indicating that there was a sufficient quantity of gene product in each sample; in addition, it also verified that the integrity of the gene products of all the samples had been conserved.

Next, qPCR was carried out on all the samples to measure changes in mRNA expression of *APP*, *BACE1*, and *ApoE*. The *APP* mRNA levels were significantly decreased ($*p < 0.05$) with 10 μ M anandamide treatment in comparison to control (**Figure 5A**). The *BACE1* mRNA levels also showed a very significant decrease ($**p < 0.01$) with 10 μ M anandamide treatment (**Figure 5B**). The *ApoE* mRNA levels appeared to increase with anandamide treatment; however, this increase was not statistically significant (**Figure 5C**).

Discussion

In the present study, *APP*, *BACE1*, and *ApoE* mRNA were successfully amplified by RT-PCR from the SH-SY5Y cell line. These genes have not been extensively analyzed in this cell line, so determination of their expression was crucial in the steps following in this study. As all genes were expressed in the cell line, the human neuroblastoma cell line can be used as a model to assess the regulation of expression of *APP*, *BACE1*, and *ApoE*. This study also demonstrated that anandamide significantly decreased the mRNA levels of *APP* and *BACE1* but not *ApoE*. This suggests that anandamide-mediated decreases in A β result from decreased synthesis, rather than increased clearance of this peptide. The physiological concentration of anandamide in various parts of the brain ranges from 0.35-0.45 μ M (Muguruza et al. 2013). Studies that previously found decreased A β levels in the brain following cannabinoid treatment administered doses both above and below this range (Ramirez et al. 2005; Harvey et al. 2012). In the present study, the doses of anandamide administered were not only those that had decreased A β levels in previous studies, but were also doses that fell near the physiological range.

Initial RT-PCR reactions indicated that *APP*, *BACE1*, *ApoE*, *B2M* and *G6PD* mRNA were all present in control and anandamide-treated cells. There was some contamination in the water blank of the *G6PD* as seen in **Figure 1D-wb**. This was most likely due to contamination of the primers that were used, or possibly transfer of gene product while loading the wells. The use of newly prepared *G6PD* primers in subsequent experiments did not indicate contamination. Furthermore, as the bands for *G6PD* were of much greater intensity compared to *B2M*, it was decided that *G6PD* should be used as the housekeeping gene in future experiments conducted on SH-SY5Y cells.

The *APP* and *BACE1* gene products were sequenced to verify their identities and to test the efficiencies of the primers used. If the *APP* and *BACE1* sequencing results were not a definite match with published sequences for their corresponding genes, then this would have indicated an issue

with primer specificity. The sequencing of *APP* and *BACE1* resulted in a 99% match with the genes published in the National Centre for Biotechnology Information database (**Figures 2B and 3B**), signifying that the primers were amplifying the correct genes.

In the sequencing results, N represented nucleotides that were undetermined. The nucleotide was marked as undetermined if there was more than one signal detected during the sequencing. However, the graphs of the sequencing results (**Figures 2A and 3A**) could be retrospectively analyzed to determine the nucleotide that was most likely in that position. In the *APP* sequencing graph (**Figure 2A**), the 25th nucleotide was found to be undetermined. At this position in the graph, a red peak, representative of the nucleotide thymine (T), can be observed. Thymine was also the nucleotide found in the same position in the published sequence (**Figure 2B**). From the *BACE1* sequencing graph (**Figure 3A**), the 29th and 189th nucleotide were undetermined. The *BACE1* sequencing graph clearly depicts a green peak at the position of the 29th nucleotide, representing adenine (A), and a red peak at the position of the 189th nucleotide, representing thymine (T). These were also the nucleotides found in the same positions in the *BACE1* published sequence. This indicates that the match between the sequencing results and the published sequences is in actuality greater than 99% due to the identity of the previously undetermined nucleotides during sequencing.

Treatment with 0.1 μ M and 1 μ M anandamide did not show any statistically significant changes in the mRNA expression of *APP* when compared to control. However, as shown in **Figure 5A**, treatment with 10 μ M anandamide corresponded to a significant decrease in *APP* mRNA levels (* $p < 0.05$). This is in accordance with our hypothesis. If *APP* mRNA levels decrease, then perhaps there is less APP protein being produced, and consequently less APP being cleaved by the amyloidogenic pathway. As a result, one would expect decreased A β formation, which is consistent with what has previously been reported to occur during cannabinoid treatment (Bisogno et al. 2008). The effects of cannabinoids on *APP* mRNA expression have not been studied at length, so there is very little information as to how *APP* mRNA levels might decrease due to anandamide administration. However, it has been found that the cytokine interleukin-1 stimulates the promoter region of the *APP* gene (Donnelly et al. 1990). Since anandamide has been shown to significantly decrease pro-inflammatory cytokines, such as interleukin-1 expression (Ortega-Gutiérrez et al. 2005), it is possible that anandamide down-regulates the transcription of the *APP* gene by decreasing interleukin-1. If future studies find that this mechanism of action holds true, it might provide an explanation for the anti-inflammatory and neuroprotective effects of cannabinoids.

Changes in mRNA levels of *BACE1* were not statistically significant with 0.1 and 1 μ M of anandamide treatment, but there was a significant decrease corresponding to treatment with 10 μ M anandamide (** $p < 0.01$) (**Figure 5B**). This was

also supportive of our original hypothesis. If lower levels of *BACE1* mRNA correspond to lower levels of BACE1 synthesis, there should be less APP being cleaved through the amyloidogenic pathway, thus resulting in lowered A β levels. Ly et al. (2013) reported that specific inhibition of glycogen synthase kinase 3 (*GSK3*) reduced A β production by decreasing *BACE1* gene transcription and expression. However, no research has yet been published on the effect of cannabinoids on *GSK3* or *BACE1* mRNA expression. Researchers conducting studies on this subject may find that it is through inhibition of *GSK3* signalling that cannabinoids exert their effects on *BACE1* mRNA, which could in turn lower A β levels. Thus, the effect of cannabinoids on *GSK3* signalling merits further study.

This study determined slightly higher *ApoE* mRNA levels with anandamide treatment (**Figure 5C**), which once again supported our hypothesis. However, these results were not statistically significant. Synthetic cannabinoids, such as WIN 55,212-2 and JWH-133 have previously been shown to enhance A β transport across choroid plexus cells *in vitro*. This suggests that administration of cannabinoids can increase A β clearance (Martín-Moreno et al. 2012). Because ApoE plays a key role in the clearance of A β from the brain, the increased clearance of A β seen by Martín-Moreno and colleagues may have been due to the ability of cannabinoids to increase ApoE levels. However, an increase in ApoE may also be a result of cannabinoids exerting their effects at the level of translation or protein stability, as opposed to at the level of transcription. If this were the case, no changes in ApoE would be detected at the mRNA level, as occurred in this study. Changes in ApoE that occur at levels of regulation other than transcription should be further studied.

There is strong supporting evidence that cannabinoids provide neuroprotection through the CB1 receptors (Ramirez et al. 2005). Most of the effects of cannabinoids that take place in the central nervous system rely on CB1 receptor activation, as it is particularly abundant in discrete areas of the brain (Howlett et al. 2007). However, new receptors have been found in the brain, such as the novel GPR55 receptor, through which cannabinoids could potentially exert their effects (Pertwee et al. 2010). Thus, using a cannabinoid other than anandamide that would target receptors selectively may demonstrate significant changes in mRNA and protein levels of ApoE and consequently reduce A β levels.

It is important to note from the trends in data that mean values of *APP*, *BACE1*, and *ApoE* mRNA levels were most different from control when cells were treated with 10 μ M anandamide. This observation may indicate that there is an optimal concentration at which cannabinoids exert their effects. Future studies should include finding an optimal concentration of cannabinoids that is both physiologically relevant and appropriate for use in patients with AD.

Conclusion

As treatment of SH-SY5Y cells with anandamide significantly reduced the mRNA levels of both *APP* and *BACE1*, anandamide has the potential to considerably reduce levels of A β *in vitro* and also in the brain, as hypothesized. However, anandamide does not appear to significantly alter mRNA levels of *ApoE*. The mechanism of action by which anandamide reduces the mRNA levels of *APP* and *BACE1* should be further investigated. Results from these studies would contribute to a better understanding of how cannabinoids reduce A β levels. This may lead to the identification of novel therapeutic targets that would be useful in treating AD.

Acknowledgements

I would like to express my deepest appreciation to all those who provided me with the opportunity to complete this paper. Special thanks to my faculty advisor, Dr. Bettina Kalisch, who offered invaluable assistance, guidance, and encouragement. I would like to acknowledge Dr. Linda Parker at the University of Guelph, who provided the cannabinoids used in this study as well as crucial advice on treatment with cannabinoids. I would also like to thank the Genomics facility and the Martino Lab at the University of Guelph, who allowed the use of equipment necessary for the completion of this research project. This work was supported by a Discovery Grant from NSERC.

References

- Bisogno T, Di Marzo V. 2008. The role of the endocannabinoid system in Alzheimer's disease: facts and hypotheses. *Current Pharmaceutical Design*. 14(23): 2299-3305.
- Bosier B, Tilleux S, Najimi M, Lambert DM, Hermans E. 2007. Agonist selective modulation of tyrosine hydroxylase expression by cannabinoid ligands in a murine neuroblastoma cell line. *Journal of Neurochemistry*. 102(6): 1996-2007.
- Hebert LE, Weuve J, Scherr PA, Evans DA. 2013. Alzheimer disease in the United States (2010-2050) estimated using the 2010 census. *Neurology*. 80(19): 1778-1783.
- Cole SL, Vassar R. 2008. The role of amyloid precursor protein processing by BACE1, the β -secretase, in Alzheimer disease pathophysiology. *Journal of Biological Chemistry*. 283(44): 29621-29625.
- Cramer PE, Cirrito JR, Wesson DW, Daniel Lee CY, Karlo JC, Zinn AE, Casali BT, Restivo JL, Goebel WD, James MJ, Brunden KR, Wilson DA, Landreth GE. 2012. ApoE-directed therapeutics rapidly clear β -amyloid and reverse deficits in AD mouse models. *Science*. 335(6075): 1503-1506.
- Donnelly RJ, Friedhoff AJ, Beer B, Blume AJ, Vitek MP. 1990. Interleukin-1 stimulates the beta-amyloid precursor protein promoter. *Cellular and Molecular Neurobiology*. 10(4): 485-495.
- Eubanks LM, Rogers CJ, Beuscher AE, Koob GF, Olson AJ, Dickerson TJ, Janda KD. 2006. A molecular link between the active component of Marijuana and Alzheimer's disease pathology. *Molecular Pharmaceutics*. 3(6): 773-777.
- Harvey BS, Ohlsson KS, Maag JLV, Musgrave IF, Smid SD. 2012. Contrasting protective effects of cannabinoids against oxidative stress and amyloid- β evoked neurotoxicity in vitro. *Neurotoxicology*. 33(2): 138-146.
- Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, Mechoulam R, Pertwee RG. 2002. International Union of Pharmacology: XXVII. Classification of cannabinoid receptors. *Pharmacological Reviews*. 54(2): 161-202.
- Ly PTT, Wu Y, Zou H, Wang R, Zhou W, Kinoshita A, Zhang M, Yang Y, Cai F, Woodgett J, Song W. 2013. Inhibition of GSK3 β -mediated BACE1 expression reduces Alzheimer-associated phenotypes. *The Journal of Clinical Investigation*. 123 (1): 224-235.
- Martín-Moreno AM, Brera B, Spuch C, Carro E, García-García L, Delgado M, Pozo MA, Innamorato NG, Cuadrado A, de Ceballos ML. 2012. Prolonged oral cannabinoid administration prevents neuroinflammation, lowers β -amyloid levels and improves cognitive performance in Tg App 2576 mice. *Journal of Neuroinflammation*. 9(8).
- Mechoulam R, Parker LA. 2013. The endocannabinoid system and the brain. *Annual Review of Psychology*. 64: 21-47.
- Muguruza C, Lehtonen M, Altonen N, Morentin B, Meana JJ, Callado LF. 2013. Quantification of endocannabinoids in postmortem brain of schizophrenic subjects. *Schizophrenia Research*. 148(1-3): 145-150.
- Ortega-Gutiérrez S, Molina-Holgado E, Guaza C. 2005. Effect of anandamide uptake inhibition in the production of nitric oxide and in the release of cytokines in astrocyte cultures. *Glia*. 52(2): 163-168.
- Pertwee RG, Howlett AC, Abood ME, Alexander SPH, Di Marzo V, Elphick MR, Greasley PJ, Hansen HS, Kunos G, Mackie K, Mechoulam R, Ross RA. 2010. International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: Beyond CB1 and CB2. *Pharmacological Reviews*. 62(4): 588-631.

- Ramirez BG, Blazquez C, Gomez del Pulgar T, Guzman M, de Caballos ML. 2005. Prevention of Alzheimer's disease pathology by cannabinoids: neuroprotection mediated by blockade of microglial activation. *Journal of Neuroscience*. 25(8): 1904–1913.
- Tarawneh R, Holtzman DM. 2012. The clinical problem of symptomatic Alzheimer Disease and mild cognitive impairment. *Cold Spring Harbor Perspectives in Medicine*. 2(5): a006148.