NEUROBEHAVIOURAL EFFECTS ASSOCIATED WITH POSTNATAL EXPOSURE TO DECABROMODIPHENYL ETHER IN APOE2, APOE3 AND APOE4 TRANSGENIC MICE

Ingrid Reverté Soler

Dipòsit Legal: T. 162-2012

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Doctoral dissertation

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Departament of Psychology

UNIVERSITAT ROVIRA I VIRGILI

Tarragona
2011

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The Present Dissertation: NEUROBEHAVIOURAL EFFECTS ASSOCIATED WITH POSTNATAL EXPOSURE TO DECABROMODIPHENYL ETHER IN APOE2, APOE3 AND APOE4 TRANSGENIC MICE, presented by Ingrid Reverté Soler, has been supervised by Maria Teresa Colomina Fosch, *Professor at the Departament de Psicologia* of the *Universitat Rovira i Virgili*, in Fulfilment of the Requirements for the degree of Doctor of Philosophy.

5th December, 2011, Tarragona

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This thesis has been done in the Laboratory of Toxicology and Environmental Health of the School of Medicine and the Psychology Department of the University Rovira I Virgili, and has been funded by:

- *Promotion of Biomedical Research Program, Ministry of Health, Instituto de Salud Carlos III (ISCIII); project reference PI07/0823. Cofounded by the European Regional Development Fund (FEDER – "Una manera de hacer Europa").*

- *FI program from the Agency for Administration of University and Research Grants (AGAUR), Commission for Universities and Research of the Department of Innovation, Universities and Enterprise of the Catalan Government; grant reference 2009FI-B 00256. Supported by the European Social Fund (EFS).*

- *BE program from the Catalan government, AGAUR; grant reference 2010 BE-1 00721.*

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Als meus pares,
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Abstract

Gene–environment interaction is the crucial determinant of the structural organisation of the brain that eventually will reflect upon the optimal functionality. During the early stages of life, the central nervous system (CNS) is especially susceptible to environmental stimuli. In particular adverse environmental conditions, such as nutritional deficits or exposure to toxic agents, have detrimental effects over the normal CNS development, contributing to the insurgence of neuropsychiatric disorders (Schmechel et al. 2006; Tau et al. 2010; Miodovnik 2011).

The cumulative impact of technological changes has facilitated an increased pollution. In fact a wide range of chemical substances are present in the environment, among them, Polybrominated diphenyl ethers (PBDEs) are one of concern because of their persistency, bioaccumulation and toxicity on the Endocrine and Nervous systems (Darnerud et al. 2001; Costa et al. 2007; Talsness 2008). Notable levels of PBDEs have been found in human's blood and breast milk, for what is especially worrying its exposure during prenatal and postnatal development (Darnerud et al. 2001; Schuhmacher et al. 2009; Vizcaino et al. 2010). Gender, age or genetic factors may provide distinct protection or vulnerability to illness or adverse conditions. In this sense different polimorphisms of Apolipoprotein E, focus of this dissertation, have been related to different risk for metabolic, cardiovascular or neurodegenerative disease (Arendt 2001; Hatters et al. 2006). Being carrier of ApoE4 allele is the major risk for developing Alzheimer's disease, and also increases the risk of neurocognitive damage after injury or negative conditions (Grootendorst et al. 2005; van Meer et al. 2007; Reitz et al. 2009). We hypothesised that an early exposure to BDE-209, a higher brominated PBDE congener which is supposed to be less toxic, can induce long-term impairments and interact with ApoE genotype, being ApoE4 more vulnerable to the injuries. To test this, mice carrying the Human ApoE isoforms – ApoE2, ApoE3 and ApoE4, were treated with an acute oral dose of 0, 10 or 30 mg/kg of BDE-209 on postnatal day 10, and were assessed for neurobehaviour at three major moments in life; development, young adulthood and old age. Additionally, myelination pattern during development, and thyroid hormone and BDNF levels in adults were also determined. Our findings show that ApoE genotype has strong influence in growth and behaviour during late development, and throughout life in activity, anxiety and learning performance. Mice carrying distinct polimorphisms of ApoE also show different levels of BDNF and its receptor TrkB in the hippocampus and

different thyroid hormone status. Moreover, BDE-209 exposure is able to induce short and long-term effects, but depending on ApoE genotype and age. An acute exposure to BDE-209 on postnatal day 10 produces a delay in growth, motor and sexual development in ApoE2, likely by the interference on myelin compaction which was also altered in these mice. In adulthood, postnatal BDE-209 exposure decreases the levels of free thyroxin in females, enhances the inherent hypoactivity of ApoE4 and worsens good learning scores observed in ApoE3 males. The effects of BDE-209 exposure are also evident at old ages. Exposed ApoE3 aged mice show increased anxiety levels compared with their control counterparts. BDE-209 also impairs learning and memory, especially in ApoE3, at advanced ages. The results of this research point that BDE-209 has potential neurotoxicity and interacts with genetic factors and age. Furthermore, it highlights the importance of longitudinal and multifactorial methodology in toxicological surveys in order to detect long term effects and vulnerable populations to toxic agents.

Key words: gene-environment interaction, toxicity, PBDEs, Apolipoprotein E, development, aging, behaviour, anxiety, learning.

Acknowledgements

Primer de tot vull donar les gràcies en general a totes aquelles persones que generosament m'han ofert consell, recolzament, inspiració i amistat durant el meu pas per la Rovira i Virgili.

La més sincera gratitud cap a la meva directora de tesi i mentora, Dra Maria Teresa Colomina, amb qui ha estat un plaer treballar. Gràcies per compartir el teu coneixement, per introduir-me en el món de la neurociència i la toxicologia, per les oportunitats que m'has donat, per ajudar-me en el meu camí cap al futur, per la teva accessibilitat, l'entusiasme per la ciència i per les teves idees, que son l'eix d'aquesta tesi.

Al Dr. Josep Lluís Domingo per haver-me acollit al seu laboratori i permetre'm desenvolupar el meu treball a la seva unitat, i al Departament de Ciències Mèdiques Bàsiques.

Als membres del Centre de Recerca en Avaluació i Mesura de la Conducta (CRAMC) i a tot el Departament de Psicologia.

Donar les gràcies també a la unitat de bioquímica, en especial a la Dra Mercedes Gómez, i al personal de l'estabulari, Amparo, Esperanza i Juan.

Al Manel per seguir organitzant els seminaris que ens ensenyen molt, ens uneixen i ens ajuden a perdre el pànic escènic.

Agraïr també la col·laboració del personal de l'àrea d'hormones de l'ICS del camp de Tarragona, Victoria Becerra, Concepción Garcia, Socorro Matilla, Concepción Medina, Josep Pinto i en especial el Dr. Andreu Pujol, professor titular de Fisiologia Humana del departament de Ciències Mèdiques Bàsiques.

My gratitude to Cecilia Ratner and especially to Anders Bue Klein from the Neurobiology Research Unit, Righospitalet Cophenaguen University for their collaboration and enthusiasm.

Special thanks to Dr. Jeff Dalley for welcome me as stage student in his unit which was a very enriching experience. Many thanks to Daniele for his useful advices and for shared cappuccinos and jamón.

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Un agraïment molt especial als companys de conducta: a la Diana per saber escoltar, pels teus apreciats “a la marxa” i per estar sempre disposada a ajudar sense demanar res a canvi, al Jose Gregorio por enseñarme todo lo imprescindible en el laboratorio, por tu paciencia y por las horas compartidas de confocal (un beso para Carmen y Abril también), a Luis por echarme siempre una mano con el ordenador, por los ratones que hemos perseguido, por las conversaciones transcendentales en la sala de conducta. Gràcies també a la resta de gent de psicobiologia, Marga i Paloma.

Un agraïment molt particular per les meves nenes de toxico: Bea, Is, Gemma i Lolita, per fer les hores de feina més agradables i també pels bons moments que hem compartit i seguirem compartint fora de la feina.

Una abraçada a les noies de psico: Mireia, Cristina, Núria, Carmen, Carol i Elisa.

Donar les gràcies també a la resta de persones amb qui he coincidit i passat bones estones, Tania, Marta, Meri, Espe, Roser, Núria, Celeste, Elena, Jordi, Laura, Mònica, Nuria, Nacho, Teresa, Merche, Nair i Nikos.

Al Jordi C. per haver compartit amb mi bona part del temps en que la tesi s’ha dut a terme.

Finalment agraïr a tots els que són sempre al meu costat pel seu valuós suport; la meva família, les amigues de tota la vida: Montse, Sònia, Roser i Marina, les meves companyes de carrera: Anabel, Xènia i les dues Cristines, i les meves estimades psikopsikis: Gemma, Eva, Ester, Elena, Juani i Georgina.

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Glossary

5-CSRTT	Five Choice Serial Reaction Time Task
AD	Alzheimer's Disease
ADHD	Attention deficit and Hyperactivity Disorder
Ahr	Aryl Hydrocarbon receptor
ANOVA	Analysis of Variance
ApoE	Apolipoprotein E
APOE	Apolipoprotein E gene
APP	Amyloid Precursor Protein
Aβ	Amyloid Beta
BDE-209	2, 2' ,3, 3' ,4, 4' ,5, 5' ,6, 6' -decabromodiphenyl ether
BDE-47	2,2',4,4'-tetra-bromodiphenyl ether
BDE-99	2,2',4,4',5-penta-bromodiphenyl ether
BDNF	Brain-derived Neurotrophic Factor
BFR	Brominated Flame Retardants
BGS	Brain Growth Spurt
CA1	Cornu Ammonis area 1
CaMKII	Calcium/Calmodulin-dependent protein kinase II
CAR	Constitutive Androstane Receptor
Chat	Choline Acetyltransferase
CLIA	Chemiluminiscent immunoassay
CNS	Central Nervous System
CYP2B	Subfamily of Cytochrome P450
CYP3A	Subfamily of Cytochrome P450
EPM	Elevated Plus Maze
EZM	Elevated Zero Maze
FT4	Free Thyroxine
Gap43	Growth Associated Protein 43
GD	Gestational Day
GFAP	Glial Fibrillary Acidic Protein
HDL	High-Density Lipoprotein
HPA	Hypothalamic Pituitary Axis
ICS	Health Catalan Institute
IDE	Insulin Degrading Enzyme
IQ	Intelligence Quotient
IUPAC	International Union of Pure and Applied Chemistry
LD50	Lethal Dose 50
LDL	Low-Density Lipoprotein

LTP	Long Term Potentiation
MBP	Myelin Basic Protein
MeOPBDE	Polybrominated Methoxy Diphenyl Ether
MRI	Magnetic Ressonance Imaging
MWM	Morris Water Maze
NGF	Nerve Growth Factor
NMDA	N-Methyl-D-aspartic acid
NS	Nervous System
NSE	Neuron-Specific Enolase
NT-3	Neurotrophin 3
OF	Open Field
OH-PBDE	Hydroxylated Polybromodiphenyl Ether
P75NTR	p75 neurotrophic factor
PAH	Polycyclic Aromatic Hydrocarbons
PBDE	Polybrominated Diphenyl Ethers
Pb	Lead
PCB	Polychlorinated Biphenyls
PFA	Paraformaldehide
PFC	Prefrontal Cortex
PKC	Protein Kinase C
PND	Postnatal Day
POP	Persistent Organic Pollutants
PSEN1	Presenilin 1
PSEN2	Presenilin 2
PXR	Pregnane X Receptor
SORL1	Sortilin-related Receptor L
T3	Triiodothyronine
T4	Thyroxine
TrKB	Neurotrophic Tyrosine Kinase Receptor
TSH	Thyroid Stimulating Hormone
UDGPT	Glucoronosyltransferase
USA	United States of America
VLDL	Very Low-density Lipoprotein

Preface

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This dissertation is concerned with the long lasting effects derived from perinatal exposure to environmental toxic chemicals. The repercussion of early exposures to toxic agents on health in adults and in the elderly is difficult to establish from epidemiological studies. That is, the time elapsed between the exposure and the effect, the possibility of multiple other factors contributing to the related effect, and differences in genetic background accounting for individual differences make it complicated, especially when the effect is not salient enough. For these reasons, experimental approaches to assess long lasting effects derived from low or acute exposures are an important tool and an additional source of data for risk assessment in humans.

Several studies on Polibrominated Diphenyl Ether (PBDE) toxicity have been reported since its introduction as flame retardants and its extensive use around the world. The lower brominated forms, penta and octa brominated congeners, have shown neurotoxicity and are banned in Europe and several states of U.S.A. They are also considered endocrine disrupters

as they interfere with the thyroid system, which in turn could be implicated in the developmental effects.

The deca-brominated form (BDE-209) is still in use and differs from the lower brominated forms in its absorption, which is lower, and its excretion, which is higher, showing a short half-life. Its degradation to lower brominated forms in the environment due to thermolability and photosensitivity and its degradation and debromination inside the body after ingestion is an issue of study and debate. In the last years, the literature on the neurotoxic effects of the higher brominated compound has increased and some discrepancies emerged between studies. The diversity of doses, exposure periods, species, strains, time at testing and endpoints assessed are beyond these differences between studies. In general term studies point to some behavioural effects which increase or are more evident with age. Taking these considerations together, an open question is how these perinatal exposures are able to interact with known risk genetic factors for aging, cognitive impairment or other pathologies. To asses this, we planned a longitudinal study in transgenic ApoE mice carrying the human ApoE2, ApoE3 and ApoE4 polymorphisms characterized for presenting different vulnerabilities to metabolic and cardiovascular diseases and neurodegeneration. The study was divided in three experimental phases to assess the effects of postnatal exposure to BDE-209 during development, in 4 months young and in 12 months old transgenic mice. Neurodevelopmental landmarks, activity and anxiety, learning and memory, thyroid hormones, myelination pattern and levels of BDNF neutrophine and its Tirosine Kinase B (TrkB) neurotrophin receptor were studied at different point times.

2. Motivation

Using a top-down approach, 3 major factors motivate the work described in this dissertation:

- The desire to describe longitudinally the degree of severity of behavioural alterations derived from the exposure to industrial chemical agents present in the environment during development.

- Understanding the genetic risk factors in interplay with toxic agent exposure.

- The identification of orderly interaction between age, sex, genetic asset and neurotoxic agent exposure and its mechanism of action.

3. Organization of the thesis

The dissertation is organized in seven chapters as follows;

- In the first chapter there is an overview on the basic issues concerning the Central Nervous System development and plasticity, a review of Polibrominated Diphenil Ethers toxicity and on the role of Apolipoprotein E in normal and pathological brain function, and an outline on the neurobehavioural assessment in rodent animal models.

- In the second chapter are presented the justification of the study, the research hypothesis and the main purpouse and objectives proposed to accomplish them.

- In the third chapter there is a detailed description of the experimental design and used procedures.

- In the fourth chapter are presented and discussed the results obtained in the different experimental phases of the research.

- In the fifth chapter is presented a general discussion on the main findings and are posed the future directions for further investigation.

- In the sixth chapter are pointed out the main conclusions derived from the research.

- In the last chapter is listed the bibliography referred in the present dissertation.

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Chapter I: Introduction

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Approximately 80,000 industrial chemicals are reported by regulatory agencies as present in the environment, and less than 1% of these substances have been examined for its neurotoxicity (Miodovnik 2011). There is evidence that environmental toxic exposure during development may affect health of infants and long-life disabilities. Neurodevelopmental disorders, which include mental retardation, dyslexia, autism and attention-deficit and hyperactivity disorder (ADHD), affect 13-16% of children (Grandjean et al. 2006; Miodovnik 2011; Stein et al. 2011). Chromosome alterations, infections and/or drug exposure during pregnancy, or perinatal anoxia are factors that can cause neurodevelopmental disorders, but the aetiology of 3% of these disorders may be attributed to environmental toxic exposure and another 25% may result from the interaction of environmental exposure with genetic predisposition (Grandjean et al. 2006; Miodovnik 2011). The recognition of genes that provides risk factors is the key to understand the role of the environment in developmental processes and genotypes that give rise to protective or vulnerable phenotypes against disease and toxic effects (Schmechel et al. 2006; Lenroot et al. 2011). Genes and environment interactions across lifespan are time and space dependent,

leading to a great amount of different effects depending on the age at which exposure occurs. Along these lines, there is evidence that some chemicals induce developmental toxicity at low doses that do not affect adult subjects. Frequently, exposure to low doses of those agents do not cause acute effects, but induce permanent changes in Central Nervous System (CNS) which give rise to subtle neurobehavioral effects, called subclinical toxicity. In addition, minimal lesions or functional alterations of the Nervous System (NS) can remain latent until a compensatory capacity of the NS decreases, precipitating the appearance of neurological and/or psychiatric disorders in adulthood or old age. Regarding that, toxic factors can affect normal aging and the onset and progression of neurodegeneration (Schmechel et al. 2006). For example, carbon monoxide, pesticide and heavy metal exposures have been proposed to contribute in developing Parkinson or Alzheimer's Disease (Grandjean et al. 2006; Schmechel et al. 2006). To sum up, toxic exposure during development may affect health of children and/or induce diseases long after exposure in young or older adults. To establish the causal associations between toxic exposure and its effects is challenging because of the high variability due to genetic and environmental interactions and variable time between exposure to noxious agents and their effects. In this sense, experimental studies should allow us to identify the risk factors implicated in the appearance of neurological and neuropsychiatric disorders with multifactorial etiology. The detection of hazardous agents and vulnerable populations to their toxicity is essential because the effects of environmental chemicals entail great personal, social and economic costs and may be preventable (Grandjean et al. 2006).

1. Development and plasticity of the Central Nervous System

The development of the central nervous system depends on multiple interactions between its cells, glial and neurons, and the signals they receive from each other. Among the many factors involved, a pattern of neural activity will be necessary for the establishment of appropriate contacts and to modulate the secretion of different intercellular signalling molecules such as neurotrophins or neurotransmitters.

The timing of development depends on each species. In rats and mice the brain grows rapidly from birth through eleven days and quite slowly thereafter. This period of rapid growth is called Brain Growth Spurt (BGS), and reaches the maximum peak of growth around postnatal day 10. During this period, several processes like axon and dendrites growth, establishment of synaptic contacts and differentiation occurs at a maximum rate. These processes are mediated by multiple signalling mechanisms and the many different molecules such as hormones, neurotrophins or activity dependent factors are implicated. Glial cells also play an important role in the development and function of CNS. In the last years glial cells alterations have been linked to several functional deficits and neurological and psychiatric pathologies.

The proper organization of the brain circuitry is essential for a correct development of the CNS. Myelination, together with neurogenesis, synaptogenesis and synaptic modulation, is a key process involved in establishes an effective connectivity. Myelin has a role in the maintenance of axons it envolves and allows the rapid conduction of the electrical

impulses. The myelination of axons starts before birth but is mainly developed postnatally, and like in all the processes during development, the preservation of the timing at which myelination occurs is critic for an optimal function (Tau et al. 2010). Myelin synthesis in the CNS is carried out by oligodendrocytes. Myelin organization, conditional upon oligodendrocyte maturation, is depending on axon signals, which in turn are mediated by growth factors, thyroid hormones, cell adhesion molecules and neureregulins among others. The formation of myelin sheath requires the coordinated assembly of myelin proteic components, such as myelin basic protein (MBP) and proteolipid protein (Miller 2002). The expression of these proteins reaches the maximum peak around postnatal day 20 in mice (Jordan et al. 1989).

Both, Thyroid Hormones (TH) and Brain-derived neurotrophic factor (BDNF) play an important role in myelination of the CNS (Barres et al. 1994; Cellerino et al. 1997; Xiao et al. 2009). Levels of Neuron-growth factor (NGF), BDNF and Neurotrophin 3 (NT-3) are regulated by thyroid hormones at the level of gene expression during developmental stages. In addition, BDNF and its receptor tyrosine kinase B (TrkB) genes are also expressed in the pituitary (Kononen et al. 1994), so BDNF could regulate the production of pituitary hormones, including thyroid stimulating hormone (TSH). Thyroid hormones are known to regulate the survival of oligodendrocytes and myelination (Barres et al. 1994; Pasquini et al. 1994). Mechanisms of action of thyroid hormones in the brain during development are mainly mediated by the control of gene expression. The genes affected during the postnatal period are not as sensitive to thyroid hormones in adults. The most sensitive period of thyroid hormone in the brain is limited to the first 2-3 postnatal weeks in rodents, which would correspond to the

twentieth week of gestation to 2-3 years after birth in humans. Important developmental events occur during this period, such as myelination, development of the cerebellum, dentate gyrus, glial cell proliferation, differentiation of neurons, synapse formation and axon and dendrite sprouting (Watson et al. 2006). Recent investigations have studied myelin alterations in relation to both behavioural and cognitive deficits (Kodama et al. 2008; Tanaka et al. 2009).

1.2 The role of neurotrophines in the CNS

Neurotrophines and their receptors are a group of molecules involved in multiple signalling processes in the brain; they regulate many aspects of neural development and plasticity. Four neurotrophins are expressed in mammals: NGF, BDNF, neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). BDNF is highly expressed in brain of many mammalian species. During normal development, BDNF regulates dendritic growth of neurons and many growth factors are involved in the proliferation, differentiation, and maturation of the oligodendrocytes which are involved in myelination (Djalali et al. 2005). In the adult brain, BDNF facilitates long term potentiation and synaptic transmission and also induces morphological changes on pyramidal neurons. BDNF is synthesized in neurons as a preproBDNF and then cleaved to form proBDNF, which can be secreted by the cell and is active by itself (Sebastiao et al. 2011). Responses to BDNF are mediated by the interaction with its receptor TrkB. Binding of BDNF rapidly activates TrkB activity, which in turn triggers multiple intracellular signaling pathways. In the other hand, proneurotrophines, the precursors of mature neurotrphines, can activate the neurotrophin receptor p75 (p75NTR) that promotes apoptotic cell death; this pathway is especially relevant after

brain injury or in neurodegenerative processes (Reichardt 2006). Recent studies have demonstrated that proBDNF has also an important role in long term depression, while mature BDNF has an important role in long term potentiation (Sebastiao et al. 2011). There are several factors like exercise, brain activity, hormone levels, cholesterol influencing neurotrophin expression. In this regard, a recent report demonstrate BDNF increases the synthesis of cholesterol in neurons, and in turns pharmacological depletion of cholesterol reduces BDNF-dependent synaptic transmission (Suzuki et al. 2007). In general terms, BDNF is able to modulate multiple processes in the brain and multiple factors are able to regulate BDNF and TrkB expression. Finally, it is important to note the relation found between levels of BDNF and TrkB and some pathologies such as Alzheimer disease, Parkinson disease and depression (Smith 1996; Mattson 2008; Cirulli et al. 2009).

2.2 The role of Thyroid Hormones in the CNS

Thyroid hormones are essential for life both in adults and during development. In adults, modifications of thyroid levels lead to various clinical manifestations. For example, hypothyroidism causes lethargy, hyporeflexia and poor motor coordination, and subclinical hypothyroidism is often associated with memory impairment and to bipolar affective disorders, depression, or loss of cognitive functions, especially in the elderly. Hyperthyroidism causes anxiety, irritability, and hypereflexia. Both, hypothyroidism or hyperthyroidism can lead to mood disorders, dementia, confusion, and personality changes. However, when observed in adults these disorders are usually reversible with proper treatment, indicating that thyroid hormone alterations of adult onset do not leave

permanent structural defects. This is not the same for thyroid alterations produced during development, whose hormones are essential for brain development and to regulate proliferation migration and differentiation of neural cells. In addition, this regulation is time and region dependent, leading to a variety of defects according to the moment at which thyroid alteration is produced (Santisteban et al. 2005). Thyroid hormone deficiencies, even of short duration during development, may lead to irreversible brain damage. For instance, neonatal hypothyroidism results in altered neuronal structure and function, including the reduction in neurite outgrowth, synaptogenesis and dendritic arborisation and altered myelination (Thompson et al. 2000).

The high plasticity of the CNS during developmental period is required for adaptation, but also generates vulnerability to environmental conditions. As seen above, any alteration on multiple factors influencing CNS development may affect its normal course and induce permanent changes. Some environmental pollutants, such as dioxins, pesticides, polychlorinated biphenyls (PCBs) and Polybrominated Diphenyl Ethers (PBDEs), have potential impact on the Endocrine System and the Nervous System (Masuo et al. 2011).

2. Polybrominated diphenyl ethers (PBDEs)

Polybrominated diphenyl ethers (PBDEs) are a set of chemical substances widely used as flame-retardants due to their capacity to delay ignition of fires, their thermal stability and their low cost. PBDEs are present in a large variety of consumer products such as polyurethane foams used in furniture,

mattresses, carpet pads and automobile seats and styrene plastics used in textiles and for electrical appliances, such as computers and televisions. PBDEs are released to the environment by volatilization or waste incineration. They also accumulate in wildlife, rise up the food chain and arrive this way to the human beings. Although they save lives, toxicological studies in the last decade have provided increasing evidence that these chemicals accumulate in the environment and in living organisms and may cause hepatic, endocrine, and neurodevelopmental toxicity (Darnerud et al. 2001; Talsness 2008).

2.1 Chemical structure

PBDEs are a group of brominated compounds within which from 1 to 10 bromine atoms are attached to a diphenyl ether molecule. The general chemical formula of a PBDE is C12H(0–9)Br(1–10)O .

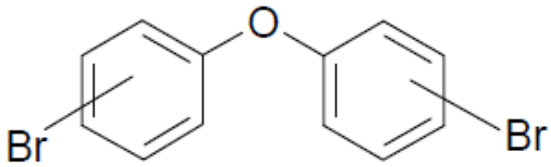

Figure 1. Polybrominated diphenyl ether (PBDE) chemical structure

2.2 PBDE congeners

There are 209 different congeners of PBDEs. These congeners are numbered according to the International Union of Pure and Applied Chemistry (IUPAC) with the same system used for the Polychlorinated

Biphenyls (PCBs) (Darnerud et al. 2001; Darnerud 2003; Fernandez 2008). For convenience, congeners are divided into ten groups from mono- to deca-BDE depending on the number of bromine atoms (Darnerud et al. 2001; Talsness 2008) (Table 1). PBDEs are usually commercialized as mixtures of different congeners named from the predominant form. The most common mixtures are pentabrominated, octabrominated and decabrominated (BDE-209). Penta- and octa- mixtures production was banned in the European Union in 2004, and in several states in the U.S.A. As a consequence, the use of the BDE-209 increased and has become the most widely used PBDE globally (Costa et al. 2007). In the European Union BDE-209 has been banned for use in electrical and electrochemical applications since July 1, 2008 ((EFSA) 2011). Despite these regulations, BDE-209 is still present in the environment and foodstuffs. For this reason it is important to increase our knowledge on possible adverse effects produced by BDE-209.

Table 1. Polybromodiphenyl ether (PBDE) congeners

Form	Bromine atoms	Congeners
Bromodiphenyl ethers	1	BDE-001 – BDE- 003
Dibromodiphenyl ethers	2	BDE-004 – BDE- 015
Tribromodiphenyl ethers	3	BDE-016 – BDE- 039
Tetrabromodiphenyl ethers	4	BDE-040 – BDE- 081
Pentabromodiphenyl ethers	5	BDE-082 – BDE- 127
Hexabromodiphenyl ethers	6	BDE-128 – BDE- 169
Heptabromodiphenyl ethers	7	BDE-170 – BDE- 193
Octabromodiphenyl ethers	8	BDE-194 – BDE- 205
Nonabromodiphenyl ethers	9	BDE-206 – BDE- 208
Decabromodiphenyl ethers	10	BDE-209

2.3 PBDEs in the environment

PBDEs are released into the environment mainly by the incineration of waste and through sewage. Volatilization of PBDEs from consumer products into the surrounding air during their lifecycle is also significant because they are not fixed in the polymer product through chemical binding (Darnerud et al. 2001; Talsness 2008). PBDEs have very low water solubility, tend to accumulate in sediments and are not easily degraded, so they may last in the environment for years, being considered persistent organic pollutants (POPs). Levels of PBDEs have been found in air, sludge, water and sediments in many countries, but also in indoor air and house dust. Levels of PBDEs have also been detected in wildlife including aquatic, terrestrial and avian organisms throughout the planet, supporting the idea that PBDE are spread in the environment. In humans, significant levels of PBDEs have been detected in adipose tissue, serum and breast milk (Darnerud et al. 2001; Costa et al. 2007). Globally, the internal human concentrations vary between countries and continents. Levels in North American are generally 10 to 100 times higher than those in Europe or Asia (Fernandez 2008).

PBDEs accumulate in the biota because they are highly lipophilic and quite resistance to physical, chemical, and biological degradation. Higher brominated compounds, such as BDE-209, are more resistant to degradation but they can suffer debromination by exposure to ultraviolet (UV) light and sunlight. Incineration of PBDEs can develop into the formation of polybrominated dibenzo-p-dioxins and dibenzofurans, compounds that are even more toxic and persistent than PBDEs. Microbial degradation is limited and is also related to the number of bromine atoms of the compound (Darnerud et al. 2001; Costa et al. 2007).

2.4 Human exposure pathways

Diet is considered the main route of PBDE exposure to, but high levels of the contaminant found in human samples cannot be explained solely by dietary intake (Costa et al. 2007; Talsness 2008). The indoor environment has been suggested as another important means of exposure, as significant levels of PBDEs have been detected in house air and dust. Occupational exposure is another matter of concern, affecting mostly electrical and electronic equipment waste management workers and computer technicians (Costa et al. 2007). Dermal uptake is considered a non-significant route of exposure (Fernandez 2008). In babies, oral intake through breast milk is the main pathway of PBDE exposure.

Diet

Diet is considered the main route of PBDE exposure. PBDEs present in the environment biomagnify in the food chain and accumulate in fatty foods due to their lipophilic properties (Darnerud et al. 2001; Costa et al. 2007). Contamination is highest in fish, then in meat and lowest in dairy products (Fernandez 2008). In a study carried out in our laboratory, levels of PBDEs were determined in samples of foodstuffs consumed by the population of Catalonia, Northeast Spain. The highest concentration of total PBDEs was found in fish and shellfish, followed by oils and fats and bakery products. For most food groups, BDE-47 and BDE-99 congeners showed the highest levels. The dietary intake of PBDEs was estimated in 1.1 ng/kg body weight/day (Domingo et al. 2008).

For infants, the main route of exposure to PBDEs is through breast milk intake, which is the largest contributor to lifetime exposure (Talsness 2008). A study determined PBDE concentration in mothers living in the county of Tarragona (Spain) who were breast-feeding. The PBDE concentrations (sum 15 congeners) ranged from 0.57 ng/g fat to 5.9 ng/g fat, with a mean value of 2.5 ng/g fat (Schuhmacher et al. 2009). Another study in California (USA) determined that BDE-209 accumulates in breast milk and is able to transfer from the mother to the infant (Park et al. 2011). There is evidence that humans are exposed to PBDEs even before birth, as similar concentrations have been found in mothers and foetuses (Costa et al. 2007).

Indoor environment

Non-dietary ingestion of dust and soil is also an important contributor of exposure throughout life to brominated flame retardants (BFRs), since diet cannot by itself explain such high levels found in human samples.

The contamination of dust and indoor air occurs because of lower brominated compounds being very volatile and the formation of dust when electronic devices are switched on. This exposure pathway is especially important for young children because they often play on the floor and put objects into their mouth. For toddlers, dust ingestion is estimated to account for more than 80% of the total exposure to PBDE (Costa et al. 2007; Talsness 2008).

Toxicokinetics

The understanding on the kinetics of PBDEs once they entered in the body is essential to determine their potential toxicity and its mechanisms of action. The absorption, distribution, metabolism and excretion of PBDEs are depending on the bromine content of the compound. It has to be noted that those parameters also varies among species, generally metabolism is faster and excretion is higher in rodents compared to humans.

Absorption

Lipophilic compounds with low molecular weight are easily absorbed by passive transport, while those with high molecular weight require transporter proteins. For this reason, the assimilation of lower brominated congeners is favoured, while BDE-209 have been shown poor absorption, estimated to be of only 10% (Darnerud et al. 2001; Morck et al. 2003).

Distribution

Tissue distribution of PBDEs in rodents depends on the congener and the means and period of exposure. In general, the target tissues are fat, muscle, skin, liver, adrenals, kidney and heart. Although concentrations in brain are low, hexaBDE compounds showed a 10 times higher brain concentration than the tetraBDE compounds (Morck et al. 2003; Staskal et al. 2006).

Despite very low water solubility BDE-209 has a limited uptake by adipocytes, unlike lower brominated compounds ((EPA) 2008).

Approximately 9% of BDE-209 has been shown to remain in the body a week after exposure, and the highest concentrations were found in adrenals, kidneys, heart, and liver (Morck et al. 2003). In developing mice BDE-209 distributed all over the body and increased in the brain during the first week after an oral administration. BDE-209 concentrations in the brain were higher in mice exposed in PND3 and PND10 compared to those exposed on PND19, highlighting the importance of the time at exposure, above all during critical periods in development (Viberg et al. 2003). In addition, human and animal data demonstrated that BDE-209 is able to cross the placenta and transfer to foetuses ((EFSA) 2011).

Metabolism

Metabolism of PBDEs occurs in two phases in the intestine. In Phase I, a reactive polar group is introduced, giving rise to a hydroxylated metabolite (OH-PBDE) facilitating the clearance of the compound from the organism. In Phase II, a conjugation reaction in the polar group converts the OH- into a methoxylated metabolite (MeOPBDE). This makes the compound more hydrophilic and increases its excretion. However, PBDE metabolites are quite hydrophobic and remain in the organism, being even more toxic than the original compound (Darnerud et al. 2001; Fernandez 2008).

The highest brominated compound, BDE-209, suffers debromination in the first step of metabolize. After that, the debrominated metabolites undergo hydroxylation to form phenols or catechols. Cathecols are then methylated to form hydroxymethoxylated metabolites, and the reactive intermediates and phenolic compounds are rapidly conjugated via Phase II, giving rise to water-soluble metabolites which would be easily excreted ((EFSA) 2011).

Excretion

Elimination of PBDEs is low, and mainly occurs through fecal excretion. Elimination of BDE-209 is faster compared to low brominated compounds (Darnerud et al. 2001; Morck et al. 2003; Staskal et al. 2006). It is important to note that toxicokinetics of PBDEs are different in developing pups than in adult mice, since the excretion capacity is lower in the pups and this lead to increase the concentrations of PBDEs in target tissues during critical stages of development (Staskal et al. 2006). In humans, there is also evidence of differences between infants and adults in drug metabolism, distribution and excretion (Strolin Benedetti et al. 2005).

2.5 Toxicity of PBDEs

PBDEs are of primary interest to European and American Health Authorities, because of their extensive presence in the environment, their similar structure to substances considered hazardous, such as PCBs, and for the increasing evidence of their toxicity. A great amount of data is available on the toxicity of PBDEs, although little information is known about the human adverse health effects.

Acute toxicity of PBDEs differs between the different congeners and is generally low, with an oral LD50 higher than 5g/kg bw, and they do not induce genotoxicity (Darnerud et al. 2001). After acute exposure rodents show evident signs of toxicity, such as skin and eye irritation, reduced growth and tremors. Different congeners and mixtures show different toxic effects at different dosages. BDE-209 shows the lowest acute toxicity,

probably because of their poor absorption, while pentaDBE show clear toxicity and mortality after exposure to high doses (Darnerud et al. 2001).

After chronic exposure to PBDEs, the target organs are the liver, the kidney and the thyroid gland. These organs are generally enlarged and degenerated, and penta- and octa- BDEs induced an increase of porphyrins in the liver (Darnerud et al. 2001).

Exposure toPBDE during gestation and the neonatal period has been shown to produce physical and neurobehavioural effects and alterations in the endocrine and reproductive systems in the offspring. Some of these effects are similar to those induced by PCBs. Despite their structural similarity with PCB, the toxic effects exerted by PBDEs occur by different mechanisms which are still not fully known (Darnerud et al. 2001; Costa et al. 2007).

Effects on reproductive system

The exposure to penta- and tetraBDEs during gestation causes alterations in the male and female reproductive system.
A reduction in the number of sperm and spermatid in males, and changes in the ovarian maturation and structure in females, were observed in adult rodent offspring administered PBDEs during gestation (Costa et al. 2007; Talsness et al. 2008). Antiandrogenic effects of PBDEs were observed *in vitro* and *in vivo*; delayed puberty and reduced growth of the androgen-dependent tissues have been observed in males (Stoker et al. 2004; Stoker

et al. 2005). Mithocondrial damage in germinal cells has been observed in both males and females (Talsness 2008).

The exposure to the highest brominated compound, BDE-209, has been shown to increase the production of sperm reactive oxygen species (ROS) and to slightly impair sperm motility, but it has not been shown to affect the sperm count, morphology or DNA content (Tseng et al. 2006; Talsness 2008).

PBDEs are able to affect circulating sex steroid levels and to interact with androgen, progesterone and estrogen receptors (Lilienthal et al. 2006; Costa et al. 2007). Androgens and estrogens not only lead to sex differentiation but can also affect brain and behaviour, can promote neuronal viability and can give neuroprotection (Jordan et al. 2008).

Endocrine disruption effects

PBDEs are considered endocrine disruptors since they are able to disturb several points of the system. They have been shown to alter steroid and thyroid hormones, which are crucial for regulation of tissue function, growth and development, behaviour and mood.

The effects of PBDEs on the sexual hormones have been described in the previous section as they mediate the reproductive system impairments. A change in the sexually dimorphic behaviour sweet preference was also observed in rodent males, who exhibited a feminization of this behaviour. Sweet preference behaviour is modulated by the aromatase activity at the pre-optic area of the hypothalamus, whose formation and organization is

affected by testosterone levels during gestation (Lilienthal et al. 2006; Talsness 2008).

PBDEs have also been found to alter the release of vasopressin in the supraoptic nuclei of the hypothalamus. This hormone play a role in NS development and it is implicated in fluid homeostasis, cardiovascular control and learning and memory processes (Coburn et al. 2007).

The thyroid hormone is the principal target of PBDE action in the endocrine system. Decreased concentrations of the total and free thyroxin (T4) hormone have been widely reported after the administration of tetra- and pentaBDE mixtures (Zhou et al. 2002; Branchi et al. 2003; Stoker et al. 2004; Ellis-Hutchings et al. 2006; Darnerud et al. 2007; Kuriyama et al. 2007; Richardson et al. 2008; Driscoll et al. 2009). Several mechanisms have been proposed for the PBDE disruption of thyroid equilibrium. Hydroxylated BDEs show a similar structure to thyroid hormones, so they can compete with the hormone to bind the thyroxin plasma transporter transthyretin or bind directly to thyroid hormone receptors. An increased metabolism of T4, by the induction of UDGPT, has been also suggested to cause the reduction in the hormone levels (Meerts et al. 2000; Richardson et al. 2008; Talsness 2008). Moreover, Talsness and colleagues found persistent histological and morphometric changes in the thyroid gland at 3 months of age after a single low dose administration of BDE-47 at GD6 (Talsness et al. 2008).

Studies on the effects of BDE-209 on thyroid hormone levels are less consistent; some of them found no effect on T4 levels, one study found a

decrease in T4 at high doses, and some studies observed differential gender effects in T3 and T4 levels ((EFSA) 2011).

In humans, data on the effects of PBDE on hormones is scarce and divergent. An American study tested 405 adult males and found increased thyroglobulin antibodies and increased T4 levels associated with PBDEs in blood serum (Turyk et al. 2008). Another study detected decreased TSH levels, but intact T4 related to higher PBDE levels in blood of 270 Californian pregnant women (Chevrier et al. 2010). Nevertheless, a recent study used linear regression to examine TSH levels in 239 neonates and levels of BFRs in their mothers' breast milk in a Norwegian population and they did not find any association between thyroid-stimulating hormone and to PBDE exposure (Eggesbo et al. 2011). More studies are required to determine possible endocrine disruptive effects of PBDEs in the human population at different levels of exposure.

Thyroid hormones have an essential role during brain development and for the normal function of the CNS, as they are implicated in the regulation of neuronal growth and synaptogenesis, and their receptors are distributed throughout the CNS. Dysfunctions of this system during critical developmental periods, cause structural and biochemical changes in the CNS which leads to anomalous or delayed development and can explain the disrupted neurobehaviour observed in animals and humans during thyroid dysfunctions (Ahmed et al. 2008).

Neurodevelopmental effects

PBDE exposure to during development is the major concern. There are some factors that make this population more vulnerable to toxic insults. Children are exposed to higher levels of contaminants since they drink more, eat more and breathe more in relation to their body weight compared to adults. In addition, many organ systems in children are still immature. Infants have reduced capacity to metabolize, detoxify, and excrete many toxicants. Of special interest are the possible effects of toxicants on the CNS, because the brain is the most immature organ at birth and especially vulnerable to damage.

Some effects were observed on growth and development in mice after PBDE exposure. Gee et al. (2008) found differences in neuromotor development on specific days and in specific tests, after a single oral dose of 10 or 30 mg/kg of BDE-47 on PND10. Branchi et al. (2002) found a delay in the ability to climb in mice that had been exposed to 30mg/kg of BDE-99 from GD6 to PND21. Mice exposed to 20mg/kg of BDE-209 from PND2 to PND15 showed a delay in the acquisition of palpebral reflex (Branchi et al. 2002; Rice et al. 2007; Gee et al. 2008).

The neurobehavioural effects of perinatal exposure to PBDE have been studied widely in adult offspring. Alteration in locomotor activity has been found after administration of tetra-, penta- and decaBDE compounds during the perinatal period in both rats and mice. Impaired habituation to new spaces, characterized by prior low activity followed by hyperactivity, have been reported in neonatal exposed rodents at 1,2,4,6 and 8 months of age (Eriksson et al. 2002; Viberg et al. 2002; Viberg et al. 2003; Viberg et

al. 2003; Viberg et al. 2004; Branchi et al. 2005; Kuriyama et al. 2007; Rice et al. 2007; Viberg et al. 2007; Gee et al. 2008; Johansson et al. 2008; Viberg 2009). Some effects in learning and attention have also been reported after neonatal PBDE exposure. In a 5-choice serial reaction time task (5CSRTT), rats administered pentaBDE chronically since PND1 showed deficits in sustained attention and inhibitory control, while those exposed from PND6 to PND12 showed impairment in learning the task but no effect on sustained attention or inhibitory control (Dufault et al. 2005; Driscoll et al. 2009). Mice exposed to hexaBDE on PND10 showed deficits in spatial learning in the MWM task at six months of age (Viberg et al. 2003), but a single oral dose of BDE-99 in adult rats did not show any effect on the passive avoidance and the MWM tasks (Belles et al. 2010). Mice exposed to decaBDE from PND2 to PND15 showed less efficient performances than controls in an operant fixed interval task, and slower learning and more perseverance errors in a light-dark discrimination task at 16 months of age. The same mice showed only a minimal effect on the light-dark discrimination at young age, indicating that the effects of PBDE exposure appeared much later (Rice et al. 2009).

Some studies on human children also indicate a relationship between PBDE levels and neurobehavioural and cognitive impairment. Herbstman et al. (2010) estimated the median concentrations of three PBDE congeners (BDE-47, 99, and 100) in cord blood and found an association between high levels of PBDEs and lower mental and psychomotor skills at 12, 48 and 72 months of age. In the same direction, Gascon et al. (2011) determined the levels of PBDE-47 in cord blood and at 4 years old, and found a positive association between BDE-47 exposure and a significant increase in the risk of attention deficit symptoms and poor social

competence and non-significant lower cognitive and motor scores (Herbstman et al. 2010; Gascon et al. 2011).

Most alterations in spontaneous behaviour have been noted after PBDE exposure during the brain growth spurt (BGS). The BGS is a fundamental period during which neurite growth occurs, along with the establishment of neural connections, axonal myelinization, changes in the cholinergic system and the acquisition of motor and sensory skills. In humans, the BGS extends from the third term of pregnancy to the first two years of age. In rodents, this period covers the first 3-4 weeks after birth and reaches its peak around the postnatal PND10 (Eriksson et al. 2002; Viberg et al. 2007). In fact, PND10 has been identified as a critical point of time in which PBDE exposure produces neurobehavioural alterations in rats and mice. However, some studies indicate that this critical point might be different for the BDE-209 compound, which would induce its maximum effects when administered on PND3, probably because it exerts its mechanisms by the creation of debrominated forms that take some days to occur (Talsness 2008).

2.6 Mechanisms of action

Among the potential mechanisms for developmental toxicity induced by PBDEs are the direct genotoxicity, gene regulation, epigenetics, cell signalling, abnormal neuronal development, changes in neurotransmitter release and metabolism, neuroinflammation, oxidative damage and neuroendocrine dysregulation (Miodovnik 2011).

Several in vitro and in vivo studies investigated the role of PBDEs on the activation of receptors leading the cytochrome P450 enzyme gene expression. Initial research suggested that PBDEs may activate the Aryl-hydrocarbon receptor (AhR), a receptor that induces xenobiotic metabolizing enzymes, but which have been shown to elicit the production of toxic metabolites in polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzo-p-dioxins and PCBs. More recent studies suggest that PBDEs can bind the AhR but do not activate its signaling pathway or induce AhR-gene expression. Like PCBs, PBDEs activate the pregnane X receptor (PXR) and constitutive androstane receptor (CAR), which lead to an increase in CYP3A and CYP2B expression in rat liver (Talsness 2008). The activation of PXR and CAR receptors may trigger the reproductive and hormone disruptor effects observed in PBDEs ((EFSA) 2011).

Oxidative damage was reported after exposure to pentaBDE congener. BDE-99 was shown to decrease antioxidant enzyme levels and increase free radicals in the hippocampus and cerebellum after perinatal (Cheng et al. 2009) and adult exposure (Belles et al. 2010).

PBDEs have been shown to disturb intracellular signaling events, including calcium homeostasis and subsequent events such as protein kinase C (PKC) in the frontal cortex, cerebellum, and hippocampus, in a similar manner to PCB. These signalling pathways have been implicated in learning and memory and are critical for the normal function and development of the nervous system (Kodavanti et al. 2002; Kodavanti et al. 2005).

Alterations in proteins involved in cytoskeleton and in neuronal survival, growth and synaptogenesis such as BDNF, Gap-43 and CaMKII have also

been demonstrated after PBDE exposure. These proteins are required for cell migration, proliferation and differentiation during the BGS (Talsness 2008). Dingemans et al. (2007) observed that BDE-47 exposure reduced LTP in the CA1 area of the hippocampus and reduced postsynaptic proteins implicated in synaptic plasticity, such as some glutamate receptor subunits and CaMKII (Dingemans et al. 2007).

Altered cholinergic system was proposed as an inductor of motor and learning deficits that result from PBDE exposure, since altered responses to nicotine (nicotinic agonist) and to scopolamine (muscarinic antagonist), and decreased cholinergic receptors have been reported (Viberg et al. 2002; Viberg et al. 2003; Dufault et al. 2005; Viberg et al. 2007; Johansson et al. 2008; Talsness 2008). This disturbance in the cholinergic system might occur after PBDE thyroid hormone disruption, since hypotiroidism has been shown to reduce the expression of the enzyme responsible for acetylcholine production (Chat) (Talsness 2008).

Extensive research has shown that PBDEs affect neurodevelopment at molecular, cellular and functional level and those alterations remain for a long time or even worsen with age.

3. Apolipoprotein E (ApoE)

Human Apolipoprotein E (ApoE) belongs to the family of soluble apolipoproteins and is implicated in cholesterol and phospholipid transport, and it is involved in neuroprotection in the brain. ApoE has three different alleles in the population, one of them, ApoE4, has been identified as a

major genetic risk for developing Alzheimer's disease in the elderly and as more susceptible to cognitive impairments.

3.1 Apolipoprotein E structure and polimorphisms

ApoE is a protein consisting of 299 amino acids, codified in a gene (APOE) located in the 19-chromosome in humans. ApoE contains two independent folded helical lipid-domains, which are able to switch from a lipid free state to a lipid bound state. The C-domain has α-helical structure and is the principal lipid-binding region of the protein. The N-domain is a four-helix bundle and is the receptor-binding region. ApoE is polymorphic with three different structural conformations of the protein that provide different functional properties.

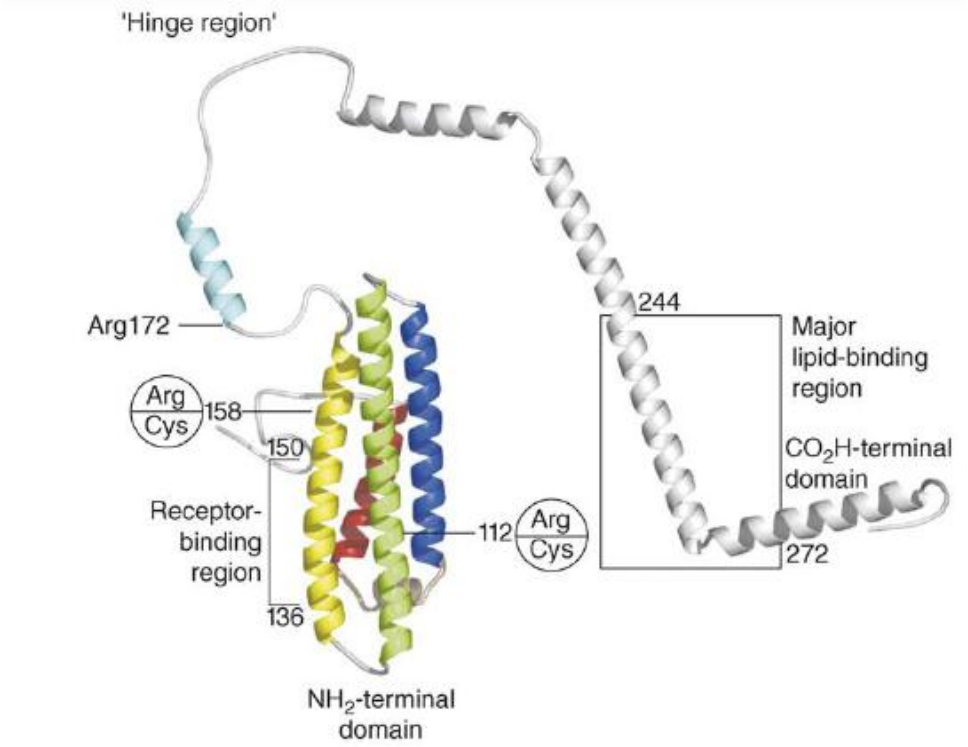

Figure 2. Apolipoprotein E (ApoE) structure

* Source of the image (Hatters et al. 2006)

The three allelic isoforms carry different amino acids at positions 112 and 158; ApoE3, the most common isoform, contains a cysteine and an arginine, while ApoE2, the least common, contains two cysteines, and ApoE4 contains two arginines at these positions. ApoE3 and ApoE4 have a similar low-density lipoprotein (LDL) receptor affinity, while the structural conformation of ApoE2 results in a weak LDL receptor affinity. Therefore ApoE2 is associated to type III hyperlipoproteinemia, a lipid disorder characterized by increased plasma levels of cholesterol and triglycerides and premature cardiovascular disease. The major association of ApoE with disease is attributed to the ApoE4 isoform, which is associated with the greatest risk of developing Alzheimer's disease and other neurological conditions along with higher cardiovascular risk. Two structural properties of ApoE4 might be responsible for its dysfunction. The first is that ApoE4 has less conformational stability than ApoE2 and ApoE3. The second is a domain interaction between the C and N-domain generated by the arginine location at 112 position, which leads to ApoE4 binding preferentially to LDL and very low density lipoproteins (VLDL), while ApoE2 and ApoE3 bind preferentially to high density lipoproteins (HDL) (Hatters et al. 2006).

3.2 Apolipoprotein E function

ApoE is mainly synthesized in the liver and it takes up lipoproteins containing cholesterol and phospholipids and distributes them to where they are used or stored. ApoE transports lipids both in plasma and in the CNS (Arendt 2001; Hatters et al. 2006). In the brain, ApoE is generated in astrocytes and is involved in neuroprotection; it has a role in neuronal growth and maintaining the synaptic integrity after injuries or ageing (Arendt 2001; Lanterna et al. 2009). The ApoE2 allele provides the most

neuroprotection, ApoE3 has an intermediate neuroprotective effect and ApoE4 is the least neuroprotective allele.

The brain is the most cholesterol-rich organ in the body. Cholesterol is an important precursor of hormones and signalling molecules and plays an important role in cells plasma membrane (Jenner et al. 2010; Gibson 2011). Important amounts of cholesterol are required during brain development and in all processes requiring the addition of new membranes such as the growth of neurites or the reparation or regeneration processes (Gibson 2011). Cholesterol cannot cross the blood brain barrier and it is synthesized in neurons and glial cells, that is, cholesterol synthesis and metabolism in brain is dependent of its own regulatory mechanisms and independent of the periphery. Once cholesterol is synthesized, several mechanisms including ApoE transport and interaction with LDL receptors contribute to its distribution and localization properly in the membranes (Jenner et al. 2010). Cholesterol plays a crucial role in the organization of lipid rafts microdomains in the membranes which are specialized in signal transduction, and recent reviews have highlighted a role for lipid rafts in neurotrophin signaling. Moreover, lipid raft signaling and clustering may be important for the assembly of components that form neuronal synapses in the central nervous system (Paratcha et al. 2002). The association of cholesterol and neurotrophines has been reported, but seems to be complex. A recent report demonstrate BDNF increase the synthesis of cholesterol in neurons (Suzuki et al. 2007) and cholesterol deprivation in vitro increases the activity of TrkB (Martin et al. 2008). While other authors refer increased BDNF levels after high cholesterol diet in wild and ApoE Knock out mice and increase levels of TrkB in ApoE Knock out mice (Wang et al. 2011).

3.3 Apolipoprotein E and Alzheimer's disease

Alzheimer's disease (AD) is a neurodegerative pathology characterized by a progressive deterioration of cognitive capacity. A familiar form of the disease is characterized by early onset and mutations in the amyloid precursor protein (APP), presenilin 1 (PSEN1) and presenilin 2 (PSEN2) genes. The late-onset form of Alzheimer's disease is the most common neurodegenerative disease in the elderly in which genetic and environmental factors are involved in its aetiology. Genes that increase the risk of developing late-onset AD are APOE and sortilin-related receptor (SORL1). At the anatomical-pathological level, AD exhibits an abnormal accumulation of tau protein at intracellular level, called neurofibrillary tangles, and aggregates of insoluble β-amyloid protein at the extracellular level, named beta amyloid plaques (Findeis 2007; Reitz et al. 2009; Querfurth et al. 2010). The ApoE4 allele has been identified as a major genetic risk factor for developing Alzheimer's disease, accounting for more than 50% of the risk (Higgins et al. 1997; Raber et al. 2004; Grootendorst et al. 2005). Multiple mechanisms have been proposed to explain the relationship between ApoE and AD. There is evidence that ApoE4 increases the rate and extent of amyloid plaques and can contribute to the development of tau pathologies (Raber et al. 2004; Findeis 2007). ApoE4 seems to have little ability for Aβ clearance since it reduces the expression of Aβ degrading enzyme IDE by binding its receptor and activating NMDA receptor pathway (Du et al. 2009). It has also been suggested that ApoE4 might have insufficient levels of functional ApoE required to maintain neuronal health, since lower levels of ApoE were detected in plasma, brain and cerebrospinal fluid in mice (Sullivan et al. 2009). Colton et al. (2005) found that the ApoE4 gene promotes a pro-inflammatory macrophage

phenotype in neonatal microglia, and furthermore, ApoE4 has been associated with mitochondrial disfunctions, alterations in myelination and increased levels of isoprostanes as oxidative stress markers (Colton et al. 2005; Huang 2006). The deficiency of ApoE4 in synaptic function, energetic metabolism, neuronal repair and protection against reactive oxygen species might explain its association with neurodegenerative processes. Moreover, some data indicate that altered functioning might be related with the lower capacity of androgens to bind the androgenic receptor in ApoE4 carriers (Raber 2008), which suggests that ApoE4 carriers can be especially vulnerable when they are exposed to endocrine disruptors.

3.4 Apolipoprotein E, cognitive performance and neurobehaviour

The effects of the ApoE4 allele are beyond the increased risk of developing a neurodegenerative disease, inasmuch as ApoE4 also modulates the cognitive function. The presence of at least one ε4 allele was associated with faster cognitive decline in AD patients (Reitz et al. 2009). In a sample of mild AD patients, ApoE4 carriers had significantly greater impairment on measures of memory retention and showed greater medial temporal lobe atrophy, whereas non-carriers were more impaired on tests of working memory, executive control and lexical access, and had greater frontoparietal atrophy (Wolk et al. 2010). ApoE also exerts its effect in cognitively normal subjects or preclinical stages of the disease (Reitz et al. 2009). Compared to ApoE3, ApoE4 also increases the risk of developing cognitive impairments after several environmental conditions such as neurotrauma, ischemia, cardiopulmonary bypass surgery, human

immunodeficiency virus infection, and it is also associated with cognitive impairments that occur with normal aging (van Meer et al. 2007). There is evidence that old, non-demented ApoE4 carriers perform worse than non-carriers in object recognition tasks, episodic memory tasks and spatial navigation tests (Berteau-Pavy et al. 2007; De Blasi et al. 2009; Kukolja et al. 2010) and have an impaired attention and working memory (Greenwood et al. 2005). Memory decline associated with the ApoE4 genotype has also been reported in middle-aged populations (Bour et al. 2008). Nowadays, there is an increasing interest in know what the role of ApoE4 in cognitive function is in young people. Human studies have reported changes in activity in prefrontal and middle-temporal areas, and in the hippocampus in young healthy ApoE4 carriers while memory tasks are being performed, although they had equal or even better results in cognitive and memory tasks than non-carriers (Mondadori et al. 2007; Dennis et al. 2009). It was been reported that young human ApoE4 carriers with normal cognitive ratings showed functional abnormalities assessed by MRI in the posterior cingular, parietal, temporal, and prefrontal cortices similar to those found in middle-aged ApoE4 carriers and patients with probable Alzheimer's disease, although they did not find any differences in cognitive and memory measures (Reiman et al. 2004). Similarly, young Apoe4 carriers showed equivalent scores to non-carriers in memory tasks, information-processing speed, attention, and executive skills (Dennis et al. 2009). Contrary to these findings, ApoE4 children aged 7-10 did not show any target preference in the spatial test *Memory island*, which indicates no retention of the task in this group (Acevedo et al. 2010). Moreover, ApoE4 carriers aged 16-30 years old performed worse than ApoE3 carriers in a maze test consisting of learning a route (Alexander et al. 2007). The data suggests that contrary to general cognitive ability, spatial learning and

memory might be particularly sensitive for detecting the negative effects of apoE4 at younger ages (Siegel et al. 2010).

Studies with rodents have reported ApoE isoform-dependent effects on cognitive function. Studies with mice expressing ApoE under the control of the neuron–specific enolase (NSE) and under control of the glial fibrillary acidic protein (GFAP) found sex and age dependent effects. Raber and co-workers found subtle differences in *Morris Water Maze* acquisition in female NSE-ApoE4 at 3 months of age, and these subtle differences became evidently impaired at 6 months of age in NSE-ApoE4 females. 18-month-old NSE-ApoE4 females showed impairments both in acquisition and retention in the water maze. These indicate that ApoE4-related cognitive impairments in the *Morris Water Maze* progressively worsen with age (Raber et al. 2000). Similar results as found in 6-months NSE-ApoE4 females were found in 6-month-old GFAP-apoE4 female mice, proving that the detrimental effects of apoE4 on spatial memory retention in the water maze is independent of the cellular source of ApoE (van Meer et al. 2007). However, they did not find any differences among genotypes in 6-month-old NSE-ApoE males (Raber et al. 1998). Impaired performance in the radial arm maze, but not in the *Morris Water Maze* was found in 11-14 month-old GFAP-ApoE4 male mice (Hartman et al. 2001). On the contrary, (Pfankuch et al. 2005) found subtle impairments in the *Morris Water Maze* retention test in sham-castrated 6-month-old NSE-ApoE4 males.

A human ApoE targeted-replacement mouse model, which allows the physiological expression of human ApoE isoforms, has also been used to assess spatial learning and memory in the *Morris Water Maze*. Mathis and

co-workers found impaired *Morris Water Maze* retention in both ApoE4 males and females at 4-5 months of age, and lower improving acquisition and impaired retention in ApoE4 females of 15 months of age (Grootendorst et al. 2005; Bour et al. 2008). Nevertheless, a recent study did not report any deficits, but better performances in acquisition and retention of the *Morris Water Maze* task in ApoE4 mice (Siegel et al. 2010). These substantial differences among rodent studies could be due to methodological differences in tasks used for the cognitive assessment.

ApoE polimorphisms have also been proposed to modulate anxiety state. Increased measures of anxiety are frequently associated with AD (Siegel et al. 2010) and ApoE4 carriers have been shown higher levels of plasma corticosterone and higher anxiety scores. Anxiety and other non-cognitive behavioural changes are a major cause of institutionalisation of AD patients, a major concern for their caregivers and negative predictor for survival (Robertson et al. 2005; Raber 2007). Increased anxiety levels also might contribute to lower performances on cognitive tests (Siegel et al. 2010).

3.5 Transgenic ApoE models in rodents

The role of ApoE has been studied in different mouse models. First of all, the knockout ApoE (0/0) mice were used to evaluate the functions of this protein. These initial studies suggested an implication of ApoE in learning and memory processes. To evaluate the vulnerability phenotype of ApoE4, new mouse models were generated which expressed ApoE under the control of neuron-specific-enolase (NSE) or the glial-fibrillary-acidic-protein (GFAP) promoter. These mouse models provided information about

the detrimental effects of ApoE4 on learning and anxiety, but they showed differences in expression and distribution of the protein (Grootendorst et al. 2005; Bour et al. 2008). Finally, the ApoE Human Targeted Replacement Mouse model was created to emulate the human condition, since it allows expression of the human ApoE protein in the same pattern and level as non-demented humans and keep the regulatory sequences required for modulate ApoE expression intact. This provides a perfect model for studying the neurobiology of the human ApoE isoforms (Sullivan et al. 2004).

4. Neurobehavioural assessment

Neurobehavioural research has been a very important tool in neuroscience and in the assessment of toxicological effects and genetic factors (Weiss 1994). Rodents are widely used for neurobehavioural assessment in neuropsychiatric and psychopharmacological research since they have similar gene functions and share a number of neuroanatomical, neurochemical and behavioural commonalities with humans. For these reasons multiple tests have been developed to evaluate functional, emotional and cognitive abilities in rodent models (Sartori et al. 2011).

4.1 Anxiety and locomotor activity

Anxiety and fear are considered basic emotions, important for drive behaviour in humans and animals. Despite fear and anxiety being closely related, they are considered different emotions that implicate different mechanisms and responses. In neuroscience anxiety is defined as the response to an undetermined potentially hazardous situation, while fear is

defined as the response to an explicit hazard. Fear and anxiety are adaptive responses essential for survival. However, anxiety becomes pathological when the response is disproportionate in respect to the stressful agent or is inappropriate in a particular context. Both intrinsic and extrinsic factors can affect the anxiety response (Sartori et al. 2011).

Neurobiology of anxiety

The anatomical brain system implicated in the neurobiology of anxiety is the limbic system. The amygdala receives projections from olfactive and visceral afferent pathways and from the thalamus, which provide sensory information about the stressful stimulus. The role of the amygdala is to create conditioning to the sensory stimuli and to generate an emotional response. For this, the amygdala sends projections to the prefrontal cortex (PCF), the hipoccampus, the striatum, the cranial nerve nuclei and the hypothalamus. The PFC enables emotional regulation by the integration of information on the emotional history of a stimulus (amygdala), the context and time (hippocampus), the internal state (brainstem monoamines), and cognitive-mnemonic information (orbital and lateral PFC), and is then able to interpret, modify and predict the behavioural response. The hippocampus has a role in the generation of contextual fear conditioning. The striatum induces changes in the locomotor activity that allows the cessation of the exploration and the escape behaviours. The cranial nerves nuclei and the hypothalamus are responsible for fear facial expressions and the activation of the sympathetic and parasympathetic responses, including the activation of the Hypothalamus-Pituitary-Adrenal axis (HPA) axis. Many neurotransmitter systems are implicated in the modulation of anxiety;

corticosteroids, neuropeptide Y and the noradrenergic system are implicated in the stress response mediated by HPA system. Serotonin, dopamine, GABA and glutamate are other neurotransmitter systems implicated in the emotional response of anxiety (Kenneth L. Davis et al. 2002; Sotres-Bayon et al. 2006).

Assessment of anxiety in rodents

Tests of anxiety-related behaviour developed for rodents can be classified as conditioned or unconditioned. Conditioned paradigms require training to learn the association of the aversive stimulus with a neutral or rewarding stimulus. Examples of conditioned paradigms are Vogel-conflict, Fear-potentiated Startle and Fear Conditioning. Unconditioned paradigms are based on ethological behaviour, and use the natural spontaneous reactions of the animal to an innate aversive stimulus. Examples of unconditioned paradigms include the light/dark test, social interaction, ultrasonic vocalization, the holeboard test, the Open Field test and the Elevated Plus or Zero mazes.

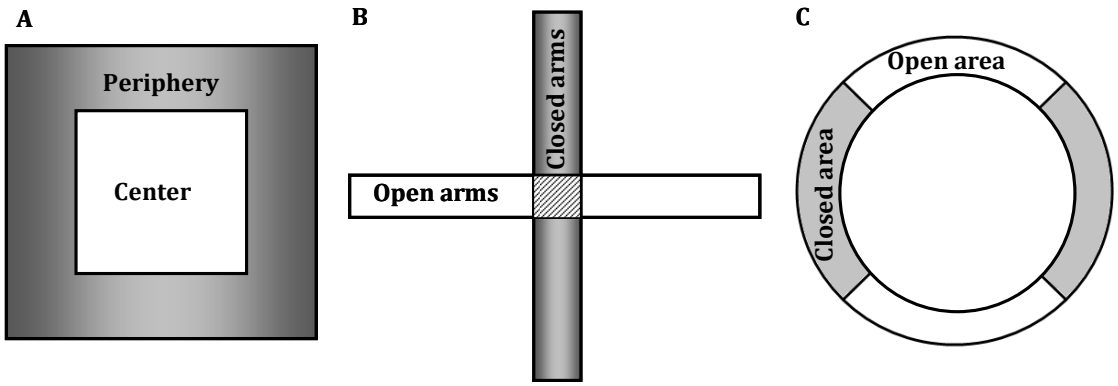

Figure 3. Unconditioned anxiety paradigms.

A) Open Field, B) Elevated Plus Maze, C) Elevated Zero Maze.

In these paradigms, rodent behaviours such as avoidance, escape or freezing behaviour are used as markers of a state of enhanced anxiety, but often referred to as 'anxiety-related' or 'anxiety-like' behaviour rather than as anxiety *per se*. The Open Field test and the Elevated Plus or Zero Maze tests are based on the conflict between exploring a new space and the fear that open, high or brightly lit spaces generate in the animal. In these tests, the animal is allowed to freely explore the space for a determined time, and then the time spent and the activity carried out in the anxious-like spaces (centre or opened arms) are taken as measures of anxiety. Nevertheless the mentioned anxiety tests are based on exploration and locomotor activity, so the activity levels can act as confounders that prevent taking a pure anxiety measure. Discriminate the emotional and non-emotional component of animal behaviour is essential in these tests (Vorhees et al. 2006; Ramos 2008).

4.2 Learning and memory

Neural processes for learning and memory are essential for the individual's adaptation to the environment and may be modulated by different endogenous systems. Learning is defined as the acquisition of a stable change on behaviour that requires interaction with the environment and practice. Memory is the process by which this information is encoded, stored and retrieved (Sharma et al. 2010).

Different types of memory can be distinguished based on time or content. Depending on the time this memory is stored you can divide memory in sensory, working, short-term and long-term. Based on content memory can be classified as non-declarative (implicit) and declarative (explicit). Non-

declarative memory includes skills and habits, conditional learning, non-associative learning (habituation, sensitisation) and priming. Declarative memory comprises semantic (facts) and episodic memory (temporal and spatial context). Working and episodic memory are more likely to be impaired with age or neurodegenerative processes. (Graef et al. 2011). Several mazes have been described to assess spatial memory in rodents. Among them, the Morris Water Maze (MWM) is the most used paradigm (Sharma et al. 2010).

Neurobiology of learning and memory

Non-declarative learning, called emotional or associative learning, involves mainly the amygdala, but also the striatum and nucleus accumbens (Lombroso 2004). Declarative learning involves the hippocampus and other structures of the medial temporal lobe, although definitely storage seems to be located in the cortex (Morgado 2005). Neocortical associative areas send sensorial information to the parahippocampal and perirhinal cortices that in turn send aferences to the enthorinal cortex. The enthorinal cortex gives aferences to the hippocampal formation, which includes the dentate gyrus, CA1-CA4, and the subiculum. The subiculum gives information back to the enthorinal cortex, which sends it to the parahippocampal region and then to the associative areas again (Rudy 2009).

Spatial navigation is a complex process that besides the hippocampus, involves several brain areas such as striatum, basal forebrain, cerebellum and neocortical areas. Many electrophysiological and lesion studies support the proposition that the hippocampus is a critical structure for this kind of

learning (D'Hooge et al. 2001). O'Keefe and Nadel suggested that the hippocampus creates a neural representation of the physical space, called cognitive map, observing that some hippocampal neurons (place cells) were firing when the rat was in a specific location (Eichenbaum et al. 1999). Parietal cortex, whose cells provide information about head position and movement, seem to provide additional information to the place cells (Ruiz-Medina 2007). It was shown that basal forebrain lesions impair the performance in the MWM, which is likely because it provides innervation to the hippocampus and neocortex. The striatum is implicated in the response flexibility, motor control and procedural consolidation in the MWM. Cerebellum participates in the procedural aspects involved in the MWM task. The prefrontal cortex also has a role in the MWM task to the extent that it allows the planning of movements required to solve the task, despite not having a proper role in memory (D'Hooge et al. 2001).

Several neurotransmitter systems are related to the spatial learning and memory. The modulation of the cholinergic system has been shown to affect the performance in the MWM. The suppression of cholinergic activity by lesions or blocking muscarinic receptors impairs the acquisition of the task, but not the recall, but the administration of cholinergic agonists, by contrast, enhances learning in subjects with lesions and age-related deficits. The glutamatergic system is also related to the spatial learning, since the administration of receptor antagonists affects the MWM performance. However, research on glutamate implication is controversial. On one hand, NMDA may contribute to some aspects of the task, but on the other hand, it may not be essential for spatial learning. The amine system does not play a crucial role in spatial navigation, but noradrenaline,

dopamine and serotonin have been shown to affect different aspects of MWM performance in specific cases (Ruiz-Medina 2007).

Assessment of memory and learning in rodents

The Morris Water Maze (MWM) test was described in 1984 by Morris as a test to assess spatial learning and memory in rats. It is a circular pool in which there is a platform hidden 1 cm underwater. The water provides a spatial environment that avoids visible, audible and olfactory cues that may guide the animal to the target, and the animal must successfully navigate itself to it (Morris 2003). The MWM test has become one of the most frequently used tools in behavioural neuroscience, being used in neuropharmacological, neuropathological and physiological studies including aging (Patil et al. 2009). In addition, MWM has been used for the validation of rodent models for several neurocognitive disorders (D'Hooge et al. 2001).

Many diverse protocols and tasks have been developed in the MWM in order to assess different aspects involved in the task or different kinds of learning. Cued or visible platform tasks have been developed to evaluate the motivation to escape or any visual disabilities that might affect the MWM performance. Reversal tasks change the position of the platform once the animal has learned the previous location, in order to assess cognitive flexibility to new learning. Working memory is also assessed in the MWM, by changing the position of the platform in each session. Latent learning tasks place the animal in the platform before the training trial, instead of after. In the standard reference memory task the animal must

learn to navigate to the hidden platform using distal cues (Vorhees et al. 2006). Rodents can use different strategies to locate the escape platform; a praxic strategy, in which the animal learns the sequence of movements required to reach the platform, a taxic strategy, in which the animal uses proximal cues to reach the platform, or a spatial strategy, in which the animal reaches the target using information about the spatial location of the platform according to the spatial configuration of distal cues (D'Hooge et al. 2001; Sharma et al. 2010). To prevent the animal using internal or proximal cues, each trial starts from one of four different positions. However non-controlled proximal cues, for example irregularities in the wall of the pool, can supply additional information to the animal finding the platform. With the aim of avoiding those uncontrolled proximal cues, in our laboratory we added an inner rotating wall to the device, which rotates during each trial. This modification to the Water Maze has proven to be sensitive to detecting differences between Tg 2576 and wild type mice at early stages (Ribes et al. 2008).

Despite the reliability of the test having been widely tested, characteristics of the animals, the apparatus features, the protocol used to train the animals and the quantification of data might all influence the performance in the task. Stable variables, such as gender or strain of the animals can lead to differences in performance in the MWM, and so should be taken into account. Environmental factors affecting the animal such as stress, undernourishment, sickness or age, can all impair performance in the MWM (D'Hooge et al. 2001). Furthermore, differences in the apparatus or training procedure, like the number of trials per day, the location of extra-maze cues, or the control of the intra-maze cues can either increase o decrease the sensitivity and the specificity for this test (Ribes et al. 2008).

4.3 Anxiety and memory systems interaction

There is evidence coming from neurogenetics, neurochemistry, and behavioural pharmacology of interaction between memory and anxiety processes and that this interplay is an essential feature of CNS functioning. Memory consolidation and anxiety both require arousal (Kalueff 2007). The relationship between learning and stress has been shown to be non-linear, whereas an inverted U function, in which memory increases with stress to an optimal point and then decreases, reflects this relationship in greater measure (Kalueff 2007; Salehi et al. 2010). A spatial learning test was carried out under three water temperature conditions, 16^{o}C, 19^{o}C and 25^{o}C. Corticosterone levels increased as the temperature decreased but the best performance was shown for the group training at 19^{o}C. This experiment highlights that certain level of stress is beneficial for learning (Salehi et al. 2010).

The interaction between memory and anxiety has to be taken in account since performance of a mouse in a learning test can be affected by emotional states. Thigmotaxis behaviour in the MWM is considered an effect of high levels of anxiety that leads to poor performance. An inaccurate analysis can lead to confusion between increased anxiety states and poor spatial learning capacity. Water is stressful for rodents, and land tests, such as the T-maze and the Barnes maze, have been shown to be more appropriate for evaluating spatial learning in some mouse strains (Branchi et al. 2004; Sharma et al. 2010).

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Ingrid Reverté Soler
DL:T. 162-2012

Chapter II: Hypothesis and Objectives

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The presence in the environment of chemicals with probable toxic effect increases year on year. The possible effect of these substances on human health is unknown in many cases because of the difficulty and the cost of testing all the new chemicals appearing in the market. Detectable levels of Polybrominated diphenyl ethers (PBDEs) are found in wildlife and human samples, such as breast milk. European legislation forbade the use of lower brominated forms of PBDEs, which implied an increase in the use of higher brominated forms, such as the Decabromodiphenyl ether (BDE-209). Data reveals that BDE-209 levels in the environment have risen in recent decades. Babies are exposed to PBDEs during pregnancy, after birth through breast milk intake and by dust inhalation when toddlers. Children are vulnerable to toxicity due to their physical characteristics - lower body weight, less excretion capacity and so forth – and to the nature of development. Particularly, the CNS is especially susceptible to toxic damage during development (Henck 2002) showing both short term effects in youth but also long lasting effects. In recent years there has been an

increasing interest in identifying developmental exposure that modifies adult vulnerability to neurodegeneration or other disorders. There is little data available on the developmental and long lasting effects of perinatal BDE-209 exposure and less is known about the interaction of toxic exposure with other genetic risk factors. Regarding this, human polymorphisms for ApoE have shown to confer different vulnerability to neurodegeneration; being a carrier of ApoE4 allele is a risk factor to developing Alzheimer's disease and provides more vulnerability to CNS damage. The aim of this study is to provide information about the possible short, middle and long term effects of an early BDE-209 exposure in a rodent ApoE model carrying the three human isoforms (ApoE2, ApoE3 and ApoE4), which are known to confer different vulnerability to develop neurodegenerative disease with age.

1. Hypothesis

The research hypothesis was as follows:

The exposure to BDE-209 during the development stage produces mild alterations in the CNS. There is evidence of alterations in learning and memory or anxiety related behaviours in adulthood or in ageing when the compensatory capacity of the CNS decreases. The effects produced by early BDE-209 exposure interact with the ApoE genotype, the ApoE4 group being the most vulnerable to the injuries.

2. Objectives

2.1 General objective

The main purpose of this research was to assess short, middle and long term neurobehavioural effects of early exposure to BDE-209 in ApoE2, ApoE3 and ApoE4 transgenic mice with distinct vulnerability to the development of neurodegenerative disease, and to evaluate possible effects of the interaction between the genotype, the treatment and gender. Three experimental phases were designed in order to address the objective.

2.2 Specific objectives

Experimental Phase I: Development

The general objective of the first experimental phase was to characterize postnatal maturation of mice carrying one of the three ApoE polymorphisms and to study the possible interactions of early exposure to BDE-209, the ApoE genotype in the neuromotor and the physical development of transgenic ApoE mice.

The specific objectives were as follows:

- To evaluate genotype different parameters of physical, functional and neuromotor maturation during the postnatal period (PND1-PND9) in each ApoE,

- To evaluate the effects of exposure to BDE-209 and its interaction with the ApoE genotype during the postnatal period on physical, functional and neuromotor maturation (PND11-PND40), and
- To evaluate possible alterations in the myeliniation pattern derived from exposure to BDE-209 and its interaction with the ApoE genotype during the postnatal period (PND20).

Experimental Phase II: 4 months old

The general objective of the second experimental phase was to evaluate neurobehavioural effects of the oral administration of 0, 10 or 30 mg/kg of BDE-209 on PND10 in young adult ApoE2, ApoE3 and ApoE4 transgenic mice.

The specific objectives were as follows:

- To assess anxiety and locomotor activity in an EZM.
- To assess anxiety and locomotor activity in an OF test.
- To assess spatial learning and memory in a MWM reference task.
- To determine levels of the thyroid hormones triiodothyronine (T3), thyroxine (T4) and free thyroxine (FT4) in blood serum.
- To determine levels of the growth factor BDNF and its receptor TrkB in frontal cortex and hippocampus.

Experimental Phase II: 12 months old

The general objective of the third experimental phase was to evaluate the neurobehavioural effects of the oral administration of 0, 10 or 30 mg/kg of BDE-209 on PND10 in, 12 months old females ApoE2, ApoE3 and ApoE4 transgenic mice.

The specific objectives were as follows:

- To assess anxiety and locomotor activity in an EZM.
- To anssess anxiety and locomotor activity in an OF test.
- To assess spatial learning and memory in an MWM reference task.

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Chapter III: Material and Methods

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1. Animals

To conduct this study the APOE Targeted Replacement Mouse Model was used. APOE Targeted Replacement mice express the human ApoE protein at the same pattern and level as non-demented humans and keep intact the regulatory sequences required for modulate ApoE expression, which provide a perfect model to study the neurobiology of the human ApoE isoforms (Sullivan et al. 2004).

Adult male and female APOE Targeted Replacement Mice homozygous for the three human alleles (ApoE2, ApoE3, and ApoE4) were obtained from Taconic (Taconic Europe, Lille Skensved, Denmark). After a quarantine period of 7 days, female mice were mated with males of the same genotype. When a pregnant female was detected it was individually housed and allowed to deliver and wean its offspring. The day of delivery was designated as postnatal day 0 (PND0), and thirty days after (PND30) the pups were separated from the mother and housed in plastic cages of 2-4 animals of the same sex. The animal room was maintained at a temperature of 22±2 °C, a relative humidity of 50±10%, and a 12-h light/dark automatic light cycle (light: 0800–2000 h). All animals were allowed free access to food (Panlab rodent chow, Barcelona) and tap water. The use of animals and the experimental protocol were approved by the Animal Care and Use Committee of the “Rovira i Virgili” University (Tarragona, Spain).

2. Chemical compound

The compound used for the study is DE-USC 209, a decabromodiphenyl ether (BDE-209) (technical) purchased in LGC Promochem (Barcelona, Spain) (IUPAC nomenclature: 2,2',3,3',4,4',5,5',6,6'-decabromodiphenyl ether). The compound was dissolved in corn oil and the concentrations were adjusted to give 10mg/kg or 30 mg/kg in 1µl/g of body weight.

3. Treatment

At postnatal day 10 (PND10) the litters, were treated at doses of 0, 10 or 30 mg/kg of BDE-209 dissolved in corn oil. The treatment was administered by a micro-pipette of 2-20µl as a single oral dose. The range of doses was chosen based on previously published data; the dose corresponds to an equimolar dose of that used in the lower brominated compounds (Eriksson et al. 2001).

4. Experimental schedules

4.1 Experimental Phase I: Development

This study was conducted to determine the development timeline of the three ApoE genotypes and to detect possible effects of BDE-209 exposure in the development of the mice. Pregnant females were individually housed and checked once a day. The delivery day was designated as postnatal day

0 (PND0) and the number of living and dead pups was recorded. From PND1 to PND36, a battery of tests was administered in order to evaluate the maternal care, the growth and several physical, functional and neuromotor parameters. On PND20, two males in each group from different litters were sacrificed and the brains were collected to determine the myelin pattern by Immunohistochemistry.

Behavioural assessment, animal disposal, sample collection and histological determinations are detailed in following sections.

4.2 Experimental Phase II: 4 months old

This study was conducted to evaluate neurobehavioural effects of early postnatal exposure to BDE-209 in young adult ApoE2, ApoE3 and ApoE4 mice.

At 4 months of age, ten animals for each sex, treatment and genotype were weighed and tested in an Elevated Zero Maze (EZM) and an Open-field (OF) in two consecutive days. Three days after, spatial learning and memory in a Morris Water Maze (MWM) were evaluated for two consecutive weeks.

A group of undisturbed animals were used for biochemical determinations. At the age of 5-6 months, five animals for each sex, treatment and genotype were killed by decapitation, and blood and brain samples collected for thyroid hormone and BDNF determinations.

Behavioural assessment, animal disposal, sample collection and biochemical determinations are detailed in following sections.

4.3 Experimental Phase III: 12 months old

This study was conducted to evaluate neurobehavioural effects of early postnatal exposure to BDE-209 in old ApoE2, ApoE3 and ApoE4 female mice.

At 12 months of age, ten females for each treatment and genotype were weighed and tested in an Elevated Zero Maze (EZM) and an Open-field (OF) in two consecutive days. Three days after, spatial learning and memory in a Morris Water Maze (MWM) were evaluated for two consecutive weeks. At the end of the study animals were 13 months old. The behavioural procedure and the data analysis are explained above.

5. Behavioural assessment

5.1 Physical and functional assessment during development

A battery of different tests was administered during the postnatal period in order to determine the development timeline for each group. These tests included maternal care evaluation, body weight monitoring and physical, functional and neuromotor assessment. The use of functional observation batteries to assess development has been extensively used in toxicology (Moser 2000; Fuentes et al. 2007).

Pregnant females were individually housed and checked once a day. The delivery day was designated as postnatal day 0 (PND0) and the number of living and dead pups was recorded. The body weight of the pups was

recorded throughout the developmental period, on PND1, PND4, PND8, PND10, PND12, PND21 and PND30.

A maternal care test was conducted on PND1 and PND3. The pups were separated from the dam for one minute and then returned to the homecage and the latency to collect the first pup and the time required by the dam to collect all of them were recorded. The quality of nest was also scored on PND1 and PND3 as follows *(0= no nest 1 =some nest, but not distinct 2= nest obvious: all pup are not in the nest area, 3= well defined nest; all pups in the nest area.*

From PND3 to PND8 the incisor eruption and the pinna detachment were recorded for each pup. From PND5, surface-righting ability was timed in each pup until all of them got it in less than 2 seconds.

On PND10 pups were orally treated with 0, 10, or 30 mg/kg of BDE-209 dissolved in corn oil, but no tests were carried out.

Eye opening was scored from PND12 to PND20 as follows (0= *both eyes closed 1= more closed than open 2 = more open than closed 3 = both eyes open).*

From PND12, neuromotor development was also assessed; the pups were placed near the top on a vertical screen and pulled backward by a gentle pull on its tail. The resistance offered by the pup during the pull was recorded. The marks for tail pull reflex were given as follows: *0, the pup offers no resistance; 1, some resistance; 2, good resistance during part of the pull; and 3, good resistance during most of the pull*. In order to test

geotaxis, cling and climb ability, the pups were placed near the bottom on a vertical screen and allowed to climb. The time that pups take to turn was recorded. The marks for cling skill were given as follows: *0, the pup falls off immediately; 1, falls within 15 s; 2, holds on 15 s but slips back, and 3, spends 15 s at higher or same position*. The marks given for climb skill were: *0, the pup falls off immediately; 1, climbs a body length using only forepaws; 2, does not reach the half of the screen, and 3, climbs to the top half of the screen*.

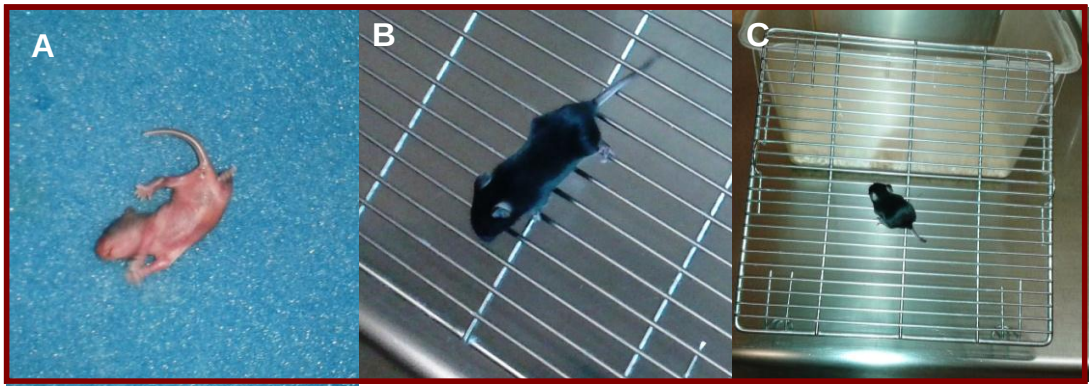

Figure 4. Neuromotor tests

A) Surface righting. B) Geotaxis. C) Cling and climb.

From PND21, the testicle descent in males and the vaginal opening in females were checked. The marks for the testicle descent were given as follows: *0, no testicle presence; 1, testis detected pressing the abdomen; 2, extracorporeal location of testicles starts becoming evident; 3, testicles are detected at first sight.* The vaginal opening in females was scored *as 0, no opening; 1, vaginal opening.*

At PND30 the pups were separated of the dam, and males and females were housed separately.

Table 2: Developmental assessment

Test	Assessment day (PND)	Score
Body weight	1, 4, 8, 10, 12, 21, 30	Weight (g)
Maternal care	1,3	Latency to collect the first pup Time (s) required to collect all pups
Nest quality	1,3	*0= no nest; 1 =some nest, but not clear;* *2= nest obvious: all pup are not in the nest area;* *3= well defined nest: all pups in the nest area.*
Pinna detachment	3-8	Presence/Absence
Incisor eruption	3-8	Presence/Absence
Surface righting	5-12	Time (s)
Eye opening	12-20	0= *both eyes closed; 1= more closed than open;* *2 = more open than closed; 3 = both eyes open*
Tail pull reflex	12-20	*0= no resistance; 1= some resistance;* *2=good resistance during part of the pull;* *3=good resistance during most of the pull*
Geotaxis	12-20	Time (s)
Cling ability	12-20	*0= the pup falls off immediately;* *1=falls within 15 s; 2=holds on 15 s but slips back;* *3=spends 15 s at higher or same position*
Climb ability	12-20	*0= the pup falls off immediately; 1=climbs a body length using only forepaws; 2= does not reach the half of the screen; 3= climbs to the top half of the screen*
Testicle descent	21-36	*0=no testicle presence; 1= testis detected pressing the abdomen; 2=extracorporeal location of testicles starts becoming evident; 3= testicles are detected at first sight.*
Vaginal opening	21-36	Presence/Absence

5.2 Elevated Zero Maze test

Anxiety responses and exploration activity were measured in an Elevated Zero Maze (EZM) for mice, which is a modification of the Elevated Plus Maze (EPM) model of anxiety for rodents. The circular shape of EZM provides two advantages over the EPM. Firstly, it avoids the ambiguity in interpretation of the time spent in the central zone of the EPM, and secondly, it allows uninterrupted exploration (Shepherd et al. 1994). The apparatus is a white annular metacrylate platform (internal diameter 350 mm. external diameter 460 mm.) elevated 50 cm off the floor. The total distance of the pathway is 500 mm, separated into two opposite enclosed quadrants and two open. A white methacrylate wall of 160 mm in height surrounds the enclosed quadrants and the open arms have a 0.5 cm edge to avoid falls.

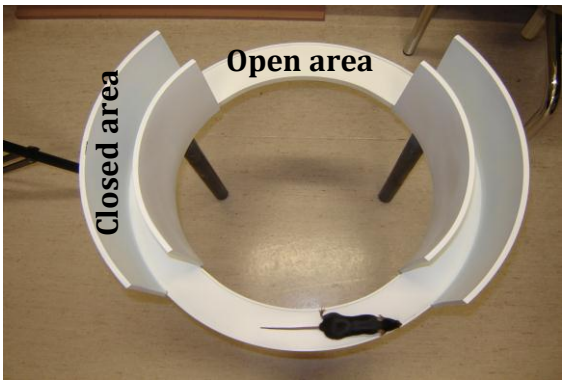

Figure 5. Elevated Zero Maze test (EZM)

Mice were placed in the open area at the beginning of the test and allowed to move freely around the maze for 5 min. Experimenters were placed 50 cm from the maze, controlling the time and recording the animal behaviour. Parameters recorded were latency to the first enter into the closed area, the number of crossings from closed to open areas, the time spent in the open

area, the number of head-dips from closed to open area, the number of rearings, the number of freezings, the number of groomings and the number of defecations.

5.3 Open Field test

General motor activity and behavioural responses to a new environment were measured in an Open Field apparatus, which is a 60 × 60 cm wooden square surrounded by a 50 cm-high wall. Two areas are virtually differentiated in the Open Field: the periphery (the area 10 cm from the wall) and the central area (the rest of the open-field). Mice were placed in the centre of the arena at the beginning of the test being allowed to move freely around the open-field and to explore the environment for 30 min. The path and movements of the animals were recorded by a video camera (Sony CCD-IRIS model) that was placed above the square. The video tracking program Etho-Vision© (Noldus Information Technologies, Wageningen, The Netherlands) was used to measure the total distance travelled, the time spent in each area, and the number of rearings as a measure of vertical activity.

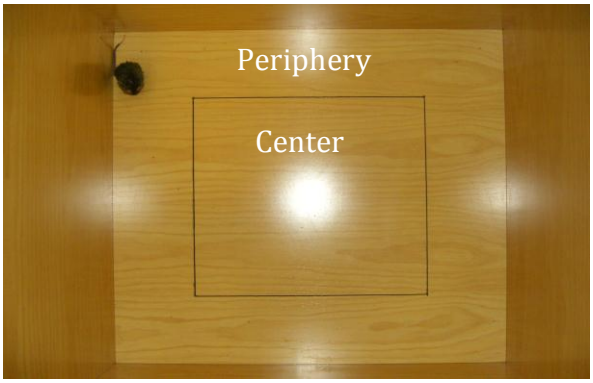

Figure 6. Open Field test (OF)

5.4 Morris water maze reference memory task

The Water Maze is in a circular tank (diameter: 1m, height: 60 cm), virtually divided in four quadrants. An escape platform of 10 cm diameter was located 1cm below the water surface in the target quadrant. To acquire the task (locate the hidden platform) the animals performed 2 trials per day, with a 60 min inter-trial interval, for ten days. During each trial, mice were allowed 90 s to find the hidden platform and had to remain on it for 30 s. If the animal failed to find the platform at this time, the experimenter then put it onto it. The starting position was changed for each trial, from four different positions; none of them were placed in the target quadrant. To avoid proximal cues and prevent egocentrical learning, an internal mobile wall was added to the maze, and the wall was rotated between trials. The retention of the task was assessed by four probe trials during acquisition, which consisted of a 60 s free swim without the escape platform. The probe trials were performed on acquisition days 3, 5, 8 and 10 before the training session. Seventy-two hours after the last training session, long-term retention of the task was again tested using the same procedure. Animal performance was video recorded, with the camera (Sony CCD-IRIS model) placed above the maze, and data was analysed by the video tracking program Etho-Vision© (Noldus Information Technologies, Wageningen, The Netherlands). The latency to find the escape platform, distance travelled, and swim velocity during the training sessions were measured. During the probe trials, total time spent in the target quadrant and time spent in other quadrants were analysed as a measure of unbiased recall for spatial memory.

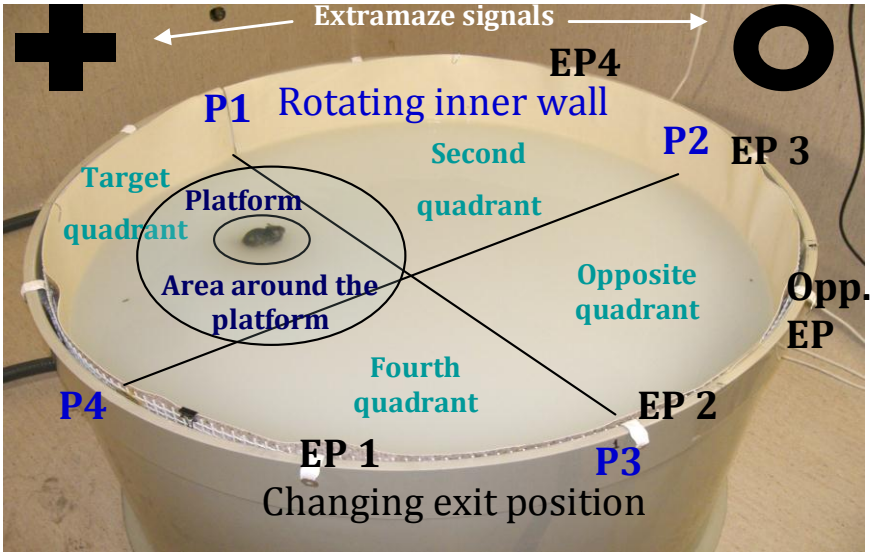

Figure 7. Morris Water Maze test (MWM)

MWM apparatus modified to control intra maze cues.

6. Disposal of the animals and sample collection

For the myelin determination, two males in each group from different litters were sacrificed on PND20. The pups were anaesthetised with ketamine/xylazine and perfused through the heart with 4% paraformaldheyde (PFA). Brains were removed and fixed overnight in 4% PFA at 4ºC. Subsequently they were placed into 30% sucrose in 0.1 M PO_4 for 48h at 4ºC and then snap frozen in methibuthane and kept at -20ºC.

For the thyroid hormone and BDNF determinations, mice were sacrificed by decapitation. Blood was collected in 5 ml tubes and brains were immediately removed, frozen in liquid nitrogen and stored at −80ºC. Blood was centrifuged at 4000 rpm for 30-40 min and the temperature was maintained at 4ºC. The serum was extracted and stored at -20ºC.

7. Myelin Basic Protein (MBP) Immunohistochemistry

The MBP Immunohistochemistry was performed according to the methods described previously (Franco-Pons et al. 2006; Fuentes et al. 2007). For myelin basic protein (MBP) immunostaining, free-floating 40µm coronal sections of brain were rinsed in PBS and H2O2 to inactivate endogenous peroxidase activity. Sections were blocked in 10% goat serum (GS)–PBS 0.5% Triton X-100 buffer (Sigma-Aldrich, Barcelona, Spain) for 2-3 h. Antibody against MBP (Chemicon, Barcelona, Spain) was diluted at 1/1000 in blocking buffer and incubated for 48 hours at 4ºC. Tissue sections were then washed in PBS 0.5% Triton X-100 buffer and incubated with 1:200 biotinylated goat anti-rabbit antibody for 1 h at 37ºC. After additional washes, the secondary antibody was detected using the avidin–biotin complex reaction (ABC Elite Kit, Vector Laboratories, Barcelona, Spain) and developed with peroxidase substrate kit DAB, SK-4100 (Vector Laboratories, Barcelona, Spain). The sections were dehydrated with 70%, 96%, 100% ethanol and xylene, and mounted with DPX.

8. Biochemical determinations

8.1 Thyroid hormones determination in blood

Thyroid hormones in serum were determined in ICS Camp de Tarragona Laboratory (Hospital Joan XXIII- Departament de Ciencies Mediques Basiques, URV). Triiodothyronine (T3), thyroxin (T4) and free thyroxin (FT4) levels were determined in blood serum by a chemiluminiscent

immunoassay (CLIA) (commercial methods and reactives Advia Centaur. Siemens). In this competitive immunoassay, the hormone competes with the one that is linked covalently to paramagnetic particles of the solid phase, for a limited amount of mouse monoclonal antibody against the hormone. The antibody is marked with a luminescent reactive. After the automatic dispensations and the incubation time, the final equilibrium of the reaction Ag-Ac is achieved. The system detects the quantity of lighting units, which is inversely related with the amount of hormone of the sample (Alonso et al. 2010).

8.2 BDNF determination

BDNF determination was carried on in the Neurobiology Research Unit, Rigshospitalet, Copenhagen University Hospital (Denmark). The growth factor BDNF and its receptor TrkB were determined in the hippocampus of ApoE2, ApoE3 and ApoE4 male mice treated with 0 or 30 mg/kg BDE-209. After decapitation, the brain was quickly removed and frozen in liquid nitrogen. Hippocampus and frontal cortex were dissected out, homogenized and sonicated in ice-cold protein extraction buffer (Pierce, Rockford, IL) containing protein inhibitor cocktail and sodium orthovanadate (Sigma). The protein concentration was measured with modified Lowry method (DC Protein Assay, BioRad Laboratories, Herlev, Denmark). Samples were protein-matched and dissolved in 2 x SDS (sodium dodecyl sulphate) sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol), heated for 5 min, 100 °C and run on AnyKd SDS-polyacrylamide gel (BioRad) (60 minutes, 150 V, RT). The proteins were transferred onto a PVDF membrane (350 mA, 60 min, using cooled wet transfer, BioRad) and the membrane was blocked for

unspecific binding with 5% non-fat dry milk (1 h, RT) and then incubated overnight at 4°C with either primary antibody (GAPDH, 1:50.000, MAB374, Millipore, MA; BDNF, 1:1000, sc-546, Santa Cruz, CA; TrkB, 1:1000, #610101, BD Biosciences, NJ) Washed 3×10 min in TBS, blocked with 5% BSA in TBS for 1 h at room temperature (RT), incubated with horseradish peroxidase-linked secondary antibody (GαM or GαR, DAKO Cytomation, Glostrup, Denmark) diluted at 1:2000 in blocking solution for 1 h at RT, and finally developed with ECL detection kit (Amersham Biosciences, RPN2106PL). Band intensity was quantified using the Quantity One software (BioRad). For each sample, the optical density of each band was normalized by dividing with the optical density of the corresponding band of GAPDH.

9. Statistical analyses

Data were analyzed by the SPSS Statistics 17.0 software. A three-way analysis of the variance (ANOVA) was performed using the genotype, the treatment and sex as main factors. Repeated measures multivariate analysis of variance with day or period of time as the within-subject factor was also used when appropriate. Post-hoc Tukey tests were used to analyze differences between groups. Analyses of variance homogeneity using a Levenne test were performed and non-parametrical Kruskal–Wallis test and Mann–Whitney U test were used when appropriate. Significance was set at $p<0.05$.

Chapter IV: Results

UNIVERSITAT ROVIRA I VIRGILI
NEUROBEHAVIOURAL EFFECTS ASSOCIATED WITH POSTNATAL EXPOSURE TO DECABROMODIPHENYL ETHER IN APOE2, APOE3 AND APOE4 TRANSGENIC MICE
Ingrid Reverté Soler
DL:T. 162-2012

The evaluation of the effects of the early postnatal BDE-209 exposure was carried out in ApoE targeted replacement mice – ApoE2, ApoE3 and ApoE4 – which have distinct vulnerability to develop neurodegenerative impairment with age. The research was divided in three experimental phases; Experimental Phase I: Development, Experimental Phase II: 4 months of age and Experimental Phase III: 12 months of age. Different animals were used in each experimental phase.

In the first phase ApoE2, ApoE3 and ApoE4 pups were evaluated during the development period, before of the BDE-209 exposure from PND1 to PND9 and after BDE-209 administration from PND11 to PND36. On PND20 two males of each group from different litters were killed and their brains were removed to determine the myelin pattern.

In the second experimental phase ApoE2, ApoE3 and ApoE4 mice exposed on PND10 to a single oral dose of 0, 10 or 30 mg/kg BDE-209 were evaluated at 4 months old. Anxiety and activity were assessed in an EZM and an OF tests. Spatial learning and memory were assessed in a MWM memory reference task. At 5-6 months old, undisturbed animals were disposed by decapitation and blood and brains collected to determine thyroid hormones and BDNF respectively.

In the third experimental phase ApoE2, ApoE3 and ApoE4 female mice exposed on PND10 to a single oral dose of 0, 10 or 30 mg/kg BDE-209 were evaluated at 12 months old. Anxiety and locomotor activity were assessed in an EZM and an OF tests. Spatial learning and memory were assessed in a MWM memory reference task.

In the current chapter you will find the results and discussion of each Experimental Phase.

Experimental Phase I: Development

UNIVERSITAT ROVIRA I VIRGILI
NEUROBEHAVIOURAL EFFECTS ASSOCIATED WITH POSTNATAL EXPOSURE TO DECABROMODIPHENYL ETHER IN APOE2, APOE3 AND APOE4 TRANSGENIC MICE
Ingrid Reverté Soler
DL:T. 162-2012

1. Experimental Phase I: Development

The first Experimental Phase comprised neurodevelopmental assessment of the ApoE transgenic mice from birth to early adulthood. We administered a battery of tests to assess litter characteristics, maternal care, and physical, functional and neuromotor development of the pups, before and after the BDE-209 treatment on PND10. In the analyses, the litter was taken as the study unit, and both sexes were evaluated separately. The results of *Experimental Phase I: Development* are explained in the following pages, organized as follows: Litter features, Maternal care, Physical development, Neuromotor development, Post-weaning development: Sexual features, and Myelination study.

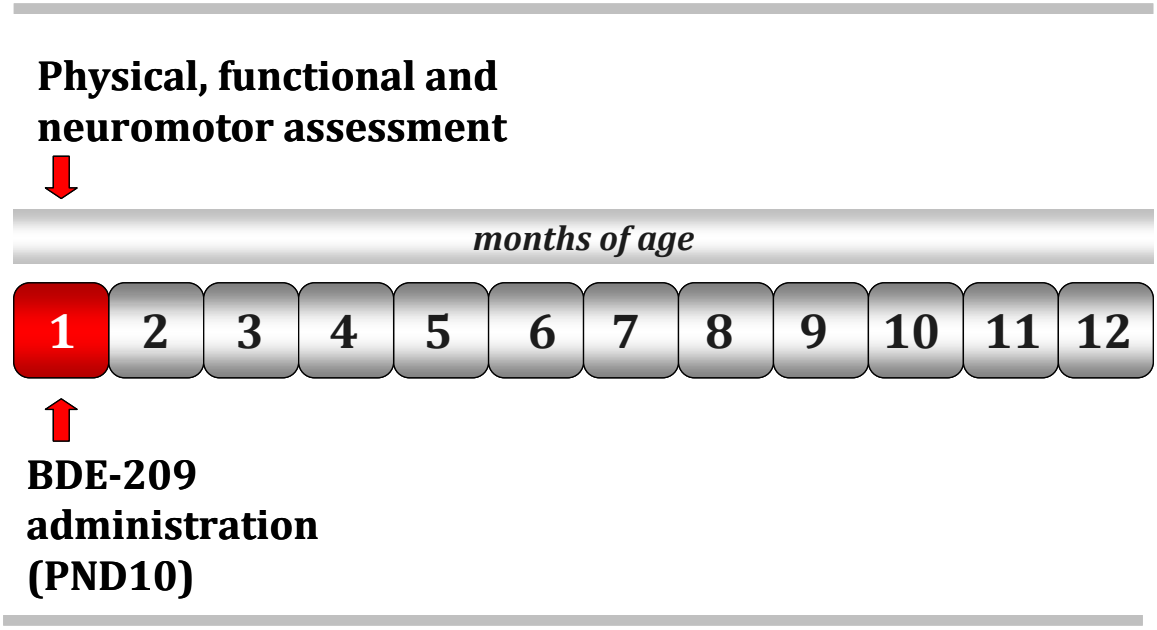

Figure I. Scheme of Experimental Phase I: Development

1.1 Results of the Experimental Phase I: Development

Litter features

The litter size, the ratio of male pups and pup mortality were analyzed by a one-way ANOVA with the ApoE genotype as factor. As the litter size did not show variance homogeneity, a non-parametrical Kruskal-Wallis test was used for this variable. An effect of the genotype was significant in the litter size [X^2=12.128, p=0.002], which was significantly smaller in ApoE4 compared to the other genotypes. No effect of the genotype was found in male ratio or pup mortality (Figure I.1). Litters found with all pups dead shortly after birth were: one litter in ApoE3 (10 pups) dead on PND3, one litter in ApoE4 (8 pups) dead on PND1 and another litter in ApoE4 (2 pups) dead on PND2. None of them were included in the statistical analyses and complete data of pup mortality is detailed in Table 2.

In brief, the litter size at birth depends on the genotype. In the ApoE4 genotype the size of the offspring is reduced but maintains the sex ratio and mortality is similar in all the genotypes.

Table 3: Neonatal mortality

	Pups (litter)	**Total number of litters**
ApoE2	7 (6)	11
ApoE3	17 (9)	20
ApoE4	11 (4)	10

Table 3. Distribution of dead pups among genotypes, number of litters affected and total number of litters studied.

FIGURE I.1

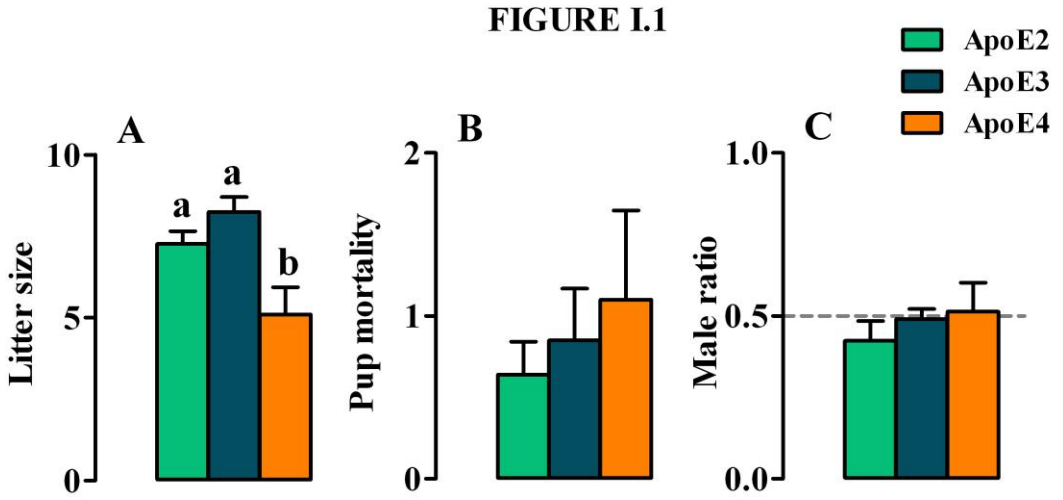

Figure I.1. Litter features

Number of alive (A), dead (B) and male pups/total pups (C) on PND0. Data are expressed as mean and S.E.M. Groups showing different letters a,b differ from each other at $p < 0.05$. The dashed line shows the theoretical expected male ratio.

Maternal care

To analyze the quality of maternal care the dam provides to the litter, a one-way ANOVA using the genotype as factor, was performed on the nest score, the latency (s) to collect the first pup and the time spent (s) on collecting and grouping all the pups in the nest area (corrected for the number of pups in each litter), on postnatal days 1 and 3. The effect of the genotype was not statistically significant in any of the measures and the nest did not score less than the maximum in any of the litters studied. A one-way ANOVA for repeated measures was also carried out on maternal care variables on PND1 and PND3. A decrease in the time to collect the pups from PND1 to PND3 was observed [$F(1,40)=8.099, p=0.007$] and an effect of the genotype [$F(2,40)=6.710, p=0.003$] was found. A paired t-test between maternal performance on day 1 and 3 showed that ApoE2 and

ApoE3 mothers significantly decreased the time to collect the pups in PND3, while the mothers ApoE4 did not (Figure I.2). The maternal care quality was quite good in mothers of all the genotypes, but ApoE2 and ApoE3 mothers improved their efficiency during the first days, while ApoE4 did not.

FIGURE I.2

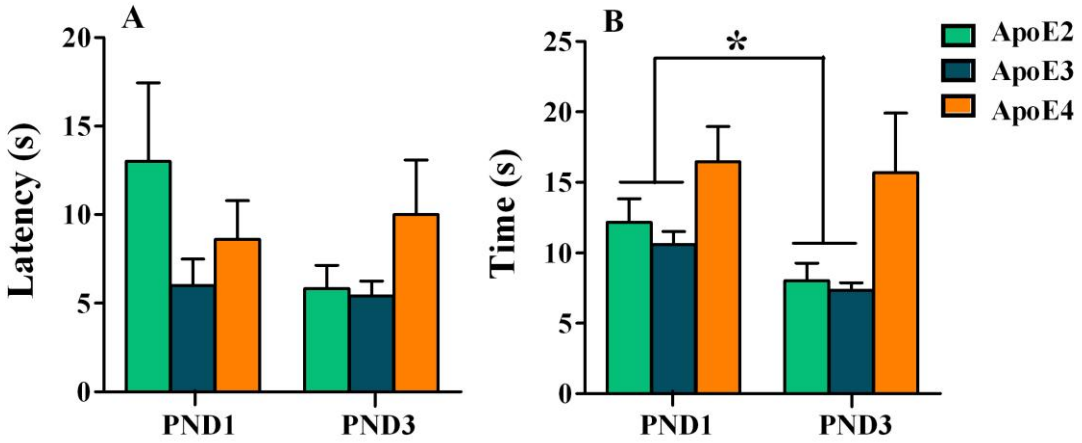

Figure I.2. Maternal care

Latency to collect the first pup (A) and time spent to collect and joint all the pups in the nest area (corrected for the number of pups of the litter) (B). Data expressed as mean and S.E.M. Variables showing an asterisk are different from each other at $p < 0.05$.

Physical development

Early growth (PND1-PND10)

To analyze the growth of the animals, the litter median of body weight (g) was analyzed by a two-way ANOVA (genotype x sex) for repeated measures on postnatal days 1, 4, 8 and 10. The body weight increased significantly by the day [$F(3,40)=641.359$, $p<0.001$] and genotype and sex did not show any significant effect or interaction (Figure I.3). The day at

pinna detachment and incisor eruption were analyzed by a two-way ANOVA (genotype x sex). No effects of genotype, sex or interactions were statistically significant. All the animals presented detached pinna and incisor eruption around PND4.

FIGURE I.3

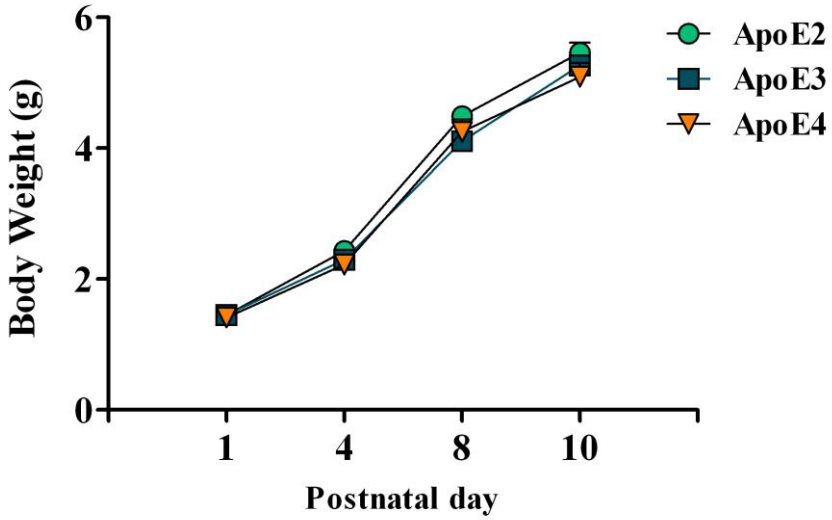

Figure I.3. Early growth

Body weight at PND 1, 4, 8 and 10. Data are expressed as mean and S.E.M.

Post-treatment growth (PND12-PND30)

To detect possible effects of the treatment on the growth, a three-way ANOVA (genotype x treatment x sex) for repeated measures was performed on the litter median body weight recorded on PND 12, 21 and 30. As expected body weight increased significantly during the period [$F(2,40)=746.258$, $p<0.001$]. An effect of the genotype [$F(2,40)=4.336$, $p=0.017$] was significant, indicating differences between ApoE4 and the other genotypes (Figure I.4).

FIGURE I.4

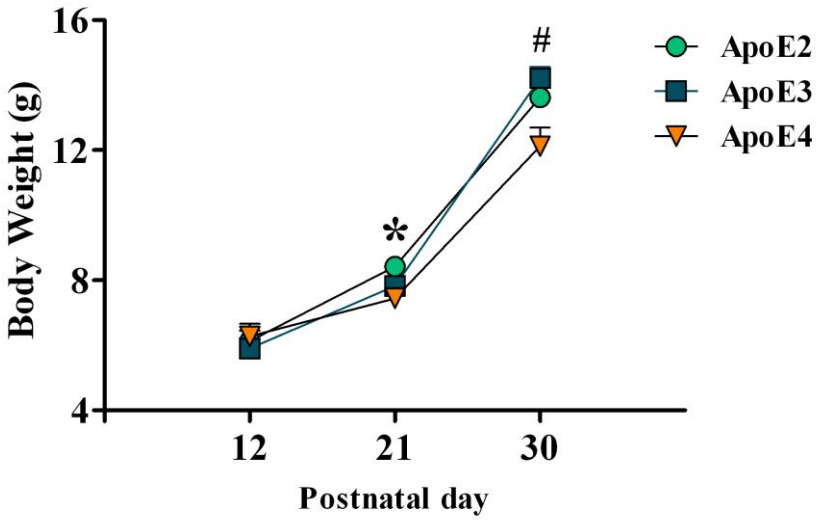

Figure I.4. Post-treatment growth by genotype

Body weight at PND 12, 21 and 30. Data expressed as mean and S.E.M. ApoE4 genotype differs from ApoE2 (*) and from ApoE3 (#) at $p<0.05$.

Additionally, an overall effect of the treatment [$F(2,40)=3.547$, $p=0.035$] is shown in Figure I.5. Interaction between the PND and the genotype [$F(4,40)=7.049$, $p=0.002$], the PND and the treatment [$F(4,40)=4.131$, $p=0.021$] and the PND, the genotype and the treatment [$F(6,40)=2.566$, $p=0.047$] were also significant, indicating different growth patterns depending on the genotype and the treatment. Therefore the analysis of post-treatment body weight change was carried out in each genotype separately by a two-way ANOVA (treatment x sex) for repeated measures. An effect of the treatment [$F(2,10)=11.236$, $p=0.01$] (Figure I.6) and an interaction between the PND and the treatment [$F(3,10)=4.007$, $p=0.04$] were observed in ApoE2 body weight. No effects of treatment or sex were observed in ApoE3 and ApoE4 mice.

FIGURE I.5

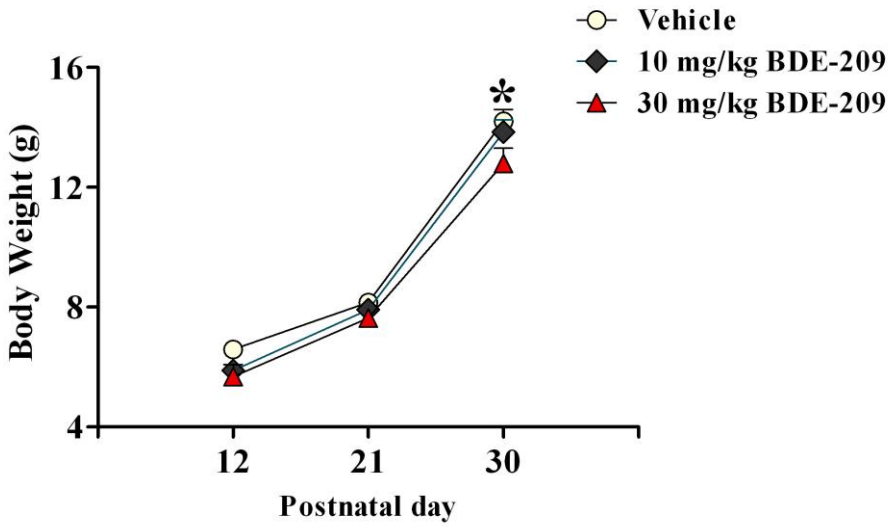

Figure I.5. Post-treatment growth by treatment

Body weight (g) registered at PND 12, 21 and 30. Data are expressed as mean and S.E.M. The asterisk indicates a trend of difference between the control and high dose exposed group ($p<0.065$).

Eye opening was scored from 0 to 3 from PND12 to PND2 and was analyzed by a three-way ANOVA (genotype x treatment x sex) for repeated measures. An effect of the day was found in the eye-opening score [$F(9,40)=2128.657$, $p<0.001$], and an interaction between the genotype and the treatment was statistically significant [$F(4,40)=2.998$, $p=0.025$]. Hence the analysis of eye opening was performed separately in each genotype by a two-way ANOVA (treatment x sex). An effect of the treatment was significant in ApoE2 group, showing a delay in eye opening in high dose exposed mice (Figure I.7).

To summarize, early growth does not differ between genotypes, but ApoE4 showed a smaller increase in body weight compared to the other genotypes from PND21. The 30 mg/kg BDE-209 treatment significantly reduced body weight and delayed eye opening in ApoE2 genotype.

FIGURE I.6

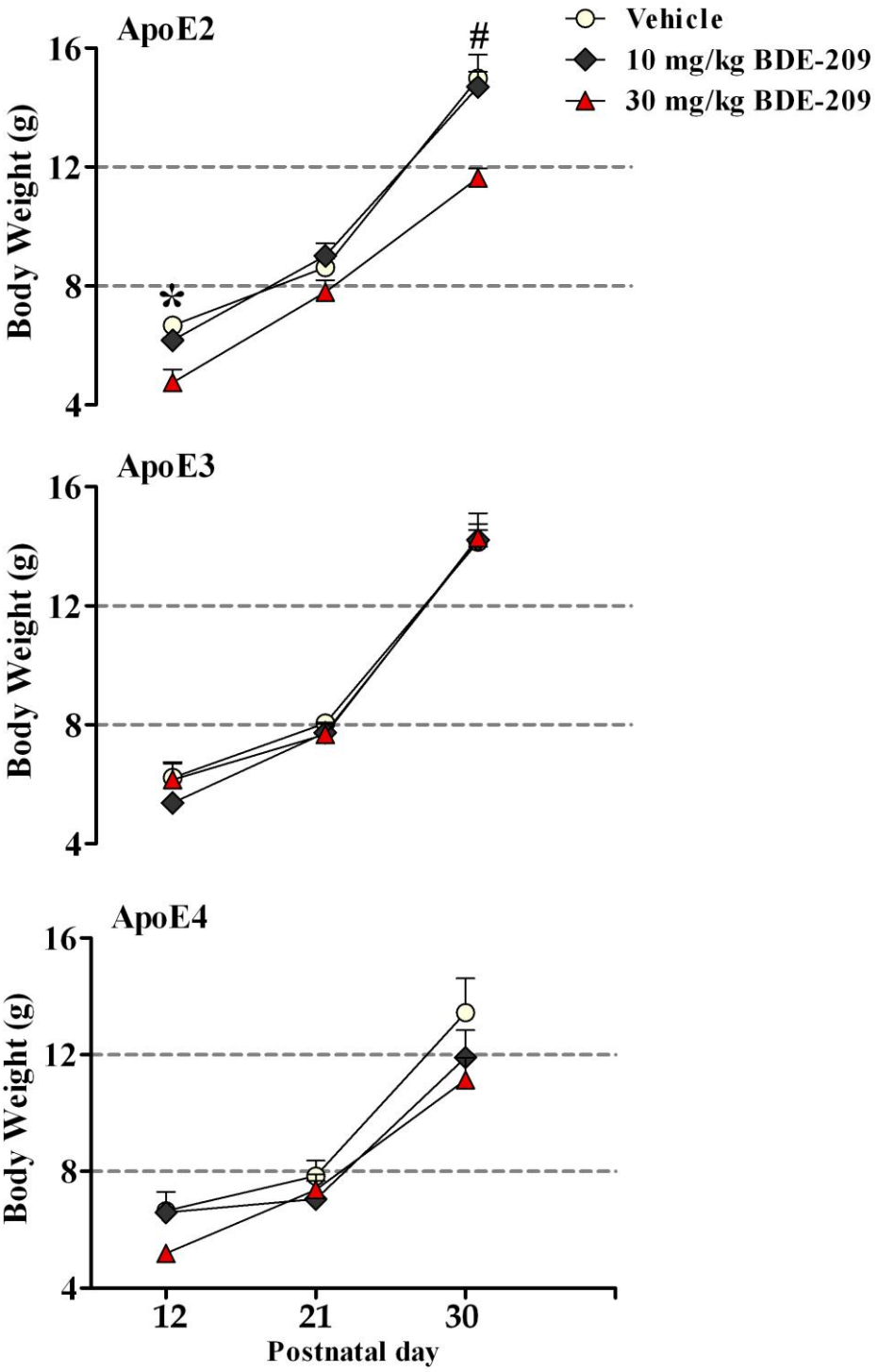

Figure I.6. Post-treatment growth by genotype and treatment

Body weight (g) registered at PND 12, 21 and 30. Data are expressed as mean and S.E.M. High dose exposed mice differ from the control (*) and from both the control and the low dose exposed (#), at $p<0.05$.

FIGURE I.7

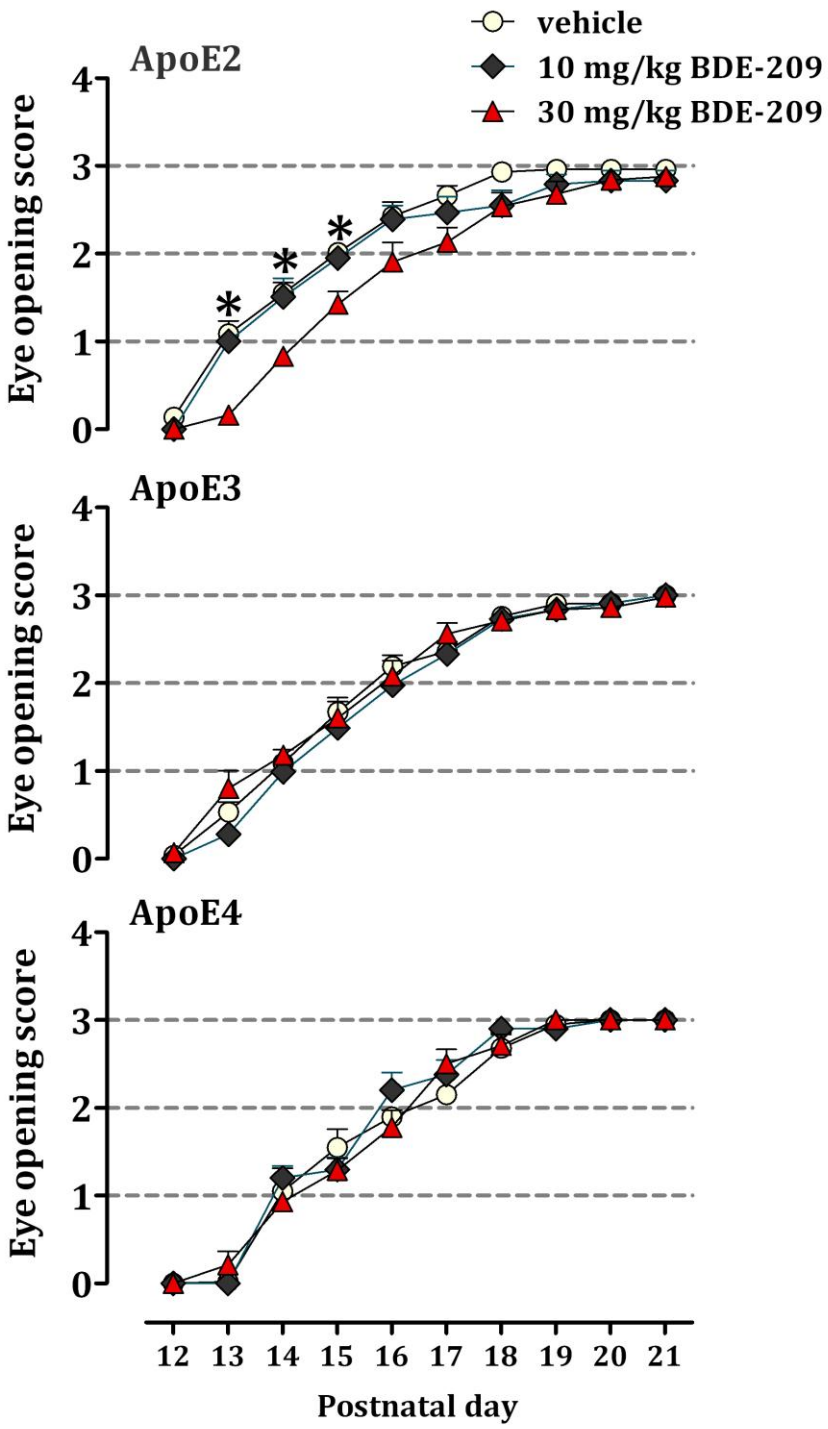

Figure I.7. Physical development by genotype and treatment

Eye opening score at PND 12,21 and 30. Data are expressed as mean and S.E.M. The asterisk indicates the high dose exposed mice differ from the control and the low dose exposed group at $p<0.05$.

Neuromotor development

Several tests were carried out to assess the effects of the genotype, the treatment and the sex on the neuromotor development. For all variables studied, the litter has been used as the unit of analysis.

Motor reflexes

Surface righting was analyzed by a two-way ANOVA (genotype x sex) for repeated measures. The day at testing (PND5 -13) was taken as the within-subject factor, while the dependent variable was the time (s) required for the pups to turn from the supine position to all fours. Surface righting in PND11, 12 and 13 was also analyzed by a three-way ANOVA (genotype x treatment x sex) to detect any possible effect of the BDE-209 administered at PND10. As expected, all the animals improved their ability over the days [$F(7,40)=61.052$, $p<0.001$] to acquire the ability to turn in less than two seconds on about PND11. No effects of genotype, sex or treatment were statistically significant.

Geotaxis was analyzed by a three-way ANOVA (genotype x treatment x sex) for repeated measures. The day of testing (PND12 -16) was taken as the within-subject factor, while the dependent variable was the time (s) required for the pup to turn up when placed in front of a cliff. A significant effect of the day was found in geotaxis [$F(4,40)=14.647$, $p<0.001$]. An overall effect of the treatment [$F(2,40)=3.323$, $p=0.043$] was observed, but the post-hoc analyses did not show any differences between groups. Moreover, an overall effect of sex [$F(1,40)=7.270$, $p=0.009$] and an interaction between sex and genotype [$F(3,40)=4.602$, $p=0.014$] were

observed. The analysis of geotaxis conducted split by sexes showed an effect of genotype only in males [F(2,40)=4.678, p=0.017 (Figure I.8).

FIGURE I.8

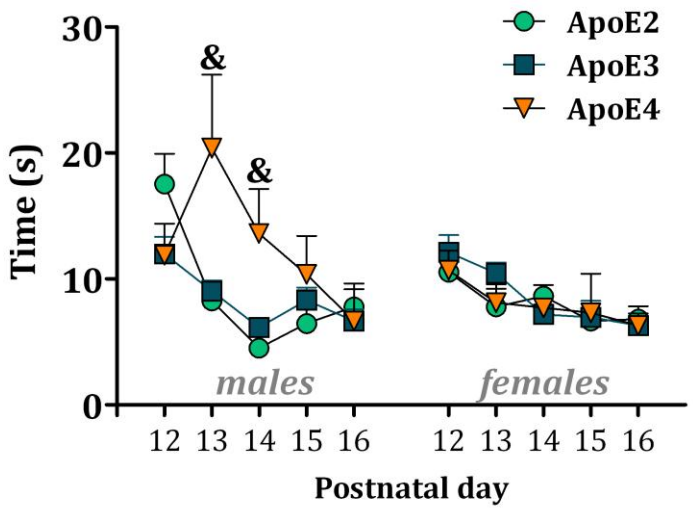

Figure I.8. Negative geotaxis by sex and genotype

Mean of time the pups took to turn up when placed in front of a cliff. Data are expressed as mean and S.E.M. The litter is the unit of analyisis. The symbol & indicates differences between ApoE4 and the other genotypes at $p<0.05$.

Motor skills

The resistance offered by the pup when pulled by the tail (tail pull test), the ability of the pup to hold onto a grid inclined at 45 degrees (cling test), and the ability of the pup to climb the mentioned grid (climb test), were tested from PND12 to PND21. Motor skills tests were analyzed by a three-way ANOVA (genotype x treatment x sex) for repeated measures taken as the within-subject factor on the day of testing, and the mean score of litter pups as a dependent variable in each test.

Results in the tail pull test showed that the animals improved as time went on [F(9,40)=237.369, p<0.001]. An effect of the genotype [F(2,40)=4.334, p=0.017] and an interaction between the genotype and the treatment [F(4,40)=3.067, p=0.023] were found to influence the test score. This interaction was studied by analyzing each genotype separately by a two-way ANOVA (treatment x sex), which showed ApoE2 was the only group affected by the treatment [F(2,40)=9.554, p=0.002] (Figures I.9 and I.10).

FIGURE I.9

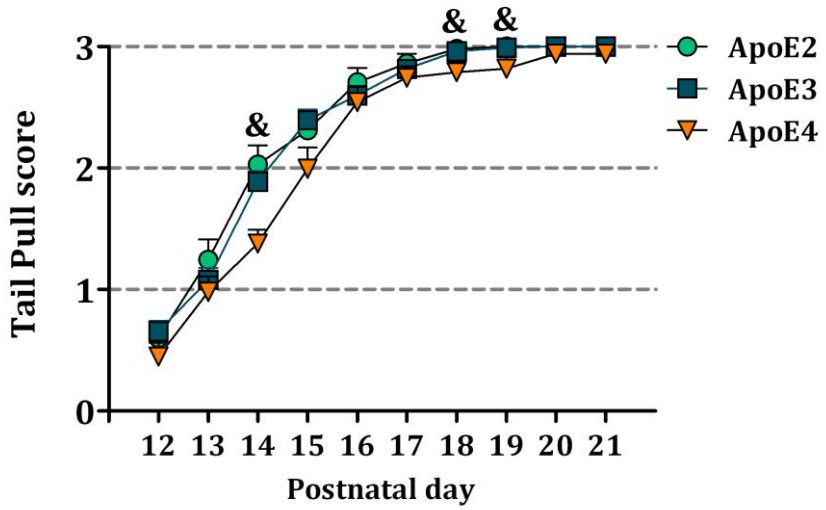

Figure I.9. Tail pull test by genotype

Mean of tail pull score of the litter. Data are expressed as mean and S.E.M. The symbol & indicate differences between ApoE4 and the other genotypes at p<0.05.

Results of the cling test showed that all the animals improved over time [F(6,40)=80.429, p<0.001]. An overall effect of the genotype [F(2,40)=5.466, p=0.006] and an overall effect of the sex [F(2,40)=5.829, p=0.019] were observed (Figure I.11). Moreover, trends towards treatment effects [F(2,40)=3.117, p=0.051] and interactions between the genotype and treatment [F(4,40)=2.273, p=0.071] justified a more detailed analysis.

FIGURE I.10

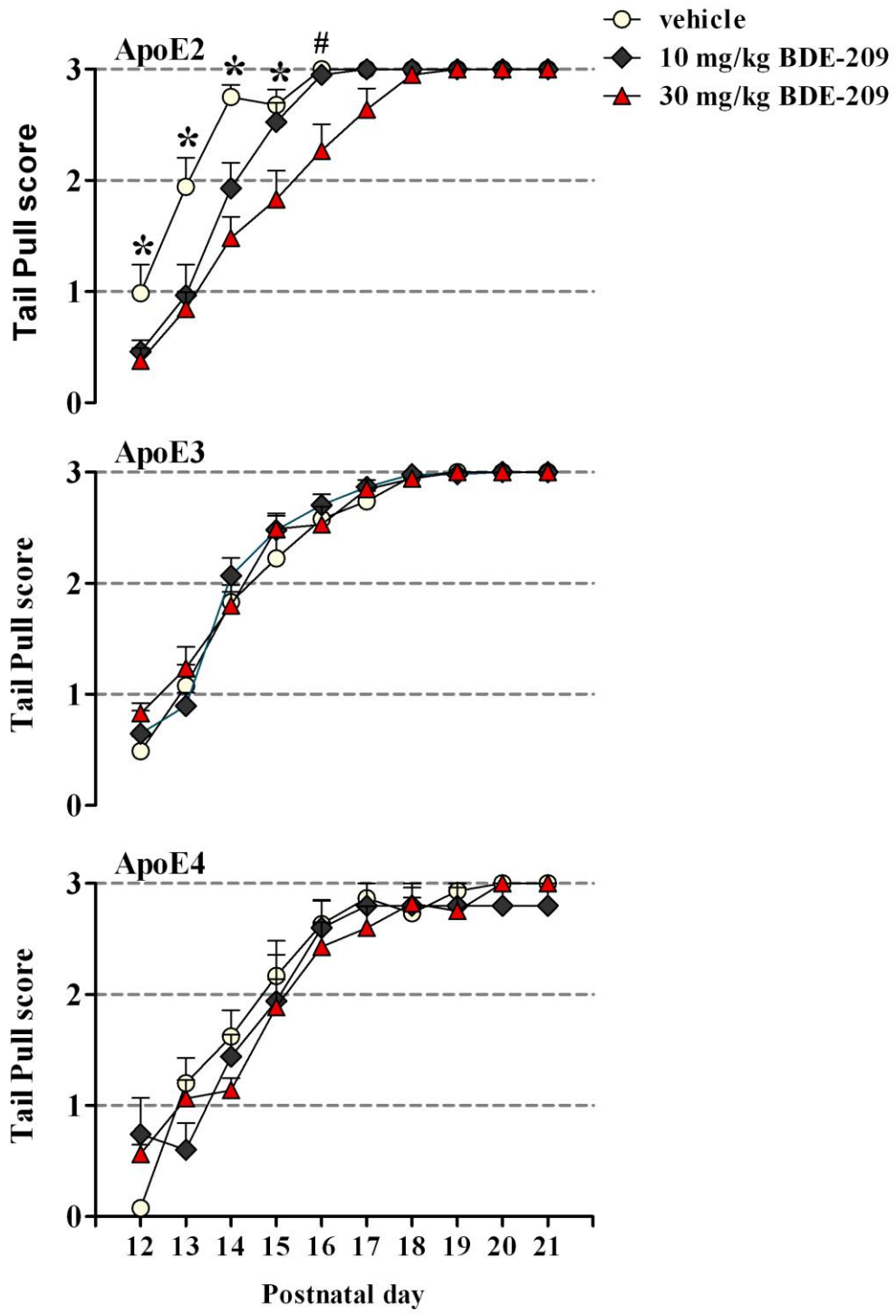

Figure I.10. Tail pull test by genotype and treatment

Mean of tail pull score of the litter. Data are expressed as mean and S.E.M. High dose exposed animals differ from control group (*) and both control and low dose exposed groups (#) at $p<0.05$.

FIGURE I.11

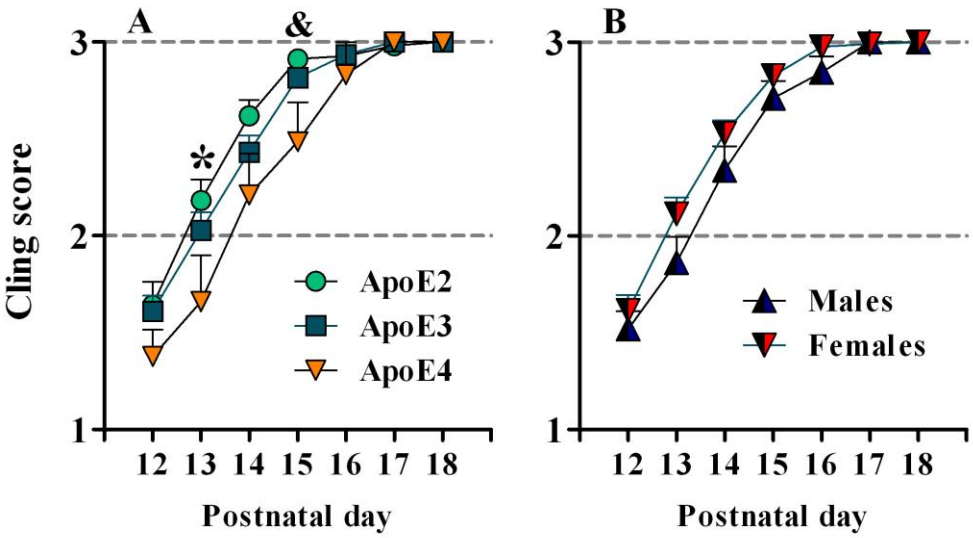

Figure I.11. Cling test

(A) Mean cling score in each genotype. (B) Mean of cling score in each sex. Data are expressed as mean and S.E.M. ApoE4 differ from ApoE2 (*) and from the other two genotypes (&) at $p<0.05$.

Cling test performance was analyzed separately in each group (genotype and sex) to determine differential effects of the treatment. Results showed the BDE-209 treatment effects are evident in ApoE2 males [$F(2,11)=5.050$, $p=0.038$] and there is also a trend towards a treatment effect in ApoE4 females [$F(4,9)=4.321$, $p=0.069$] (Figure I.12). The high variability observed in both males and females cling score in ApoE4 genotype is noteworthy.

FIGURE I.12

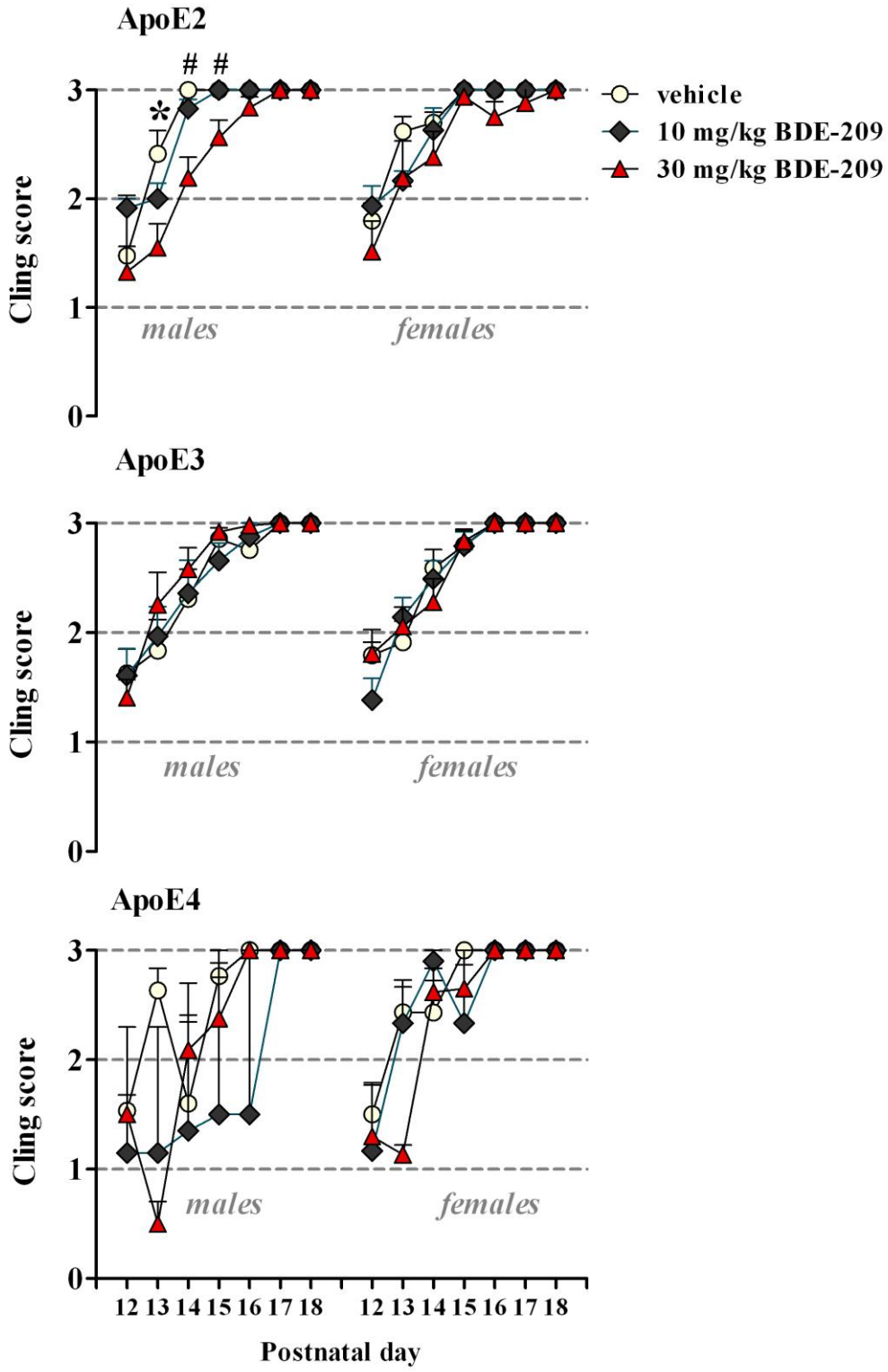

Figure I.12. Cling test by genotype, treatment and sex

Data are expressed as mean and S.E.M. The asterisk indicates the high dose exposed ApoE2 mice differ from their control, and # indicates the high dose exposed ApoE2 mice differ from control and low dose exposed ApoE2 mice, at $p<0.05$.

Climb ability improved over time [$F(9,40)=153.865$, $p<0.001$] in all the groups. An overall effect of the treatment [$F(2,40)=4.896$, $p=0.011$] and an interaction between the treatment and the genotype [$F(4,40)=5.100$, $p=0.001$] were observed, indicating different maturation patterns depending on genotype and treatment. The analysis of the climb test carried out in each genotype separately by a two-way ANOVA (treatment x sex) for repeated measures, revealed a significant effect of treatment in ApoE2 mice (Figures I.13 and I.14).

Taking all neuromotor data together, ApoE4 mice showed delayed negative geotaxis, tail pull and cling abilities. The BDE-209 treatment at high doses clearly produced a delay in neuromotor development in ApoE2 mice.

FIGURE I.13

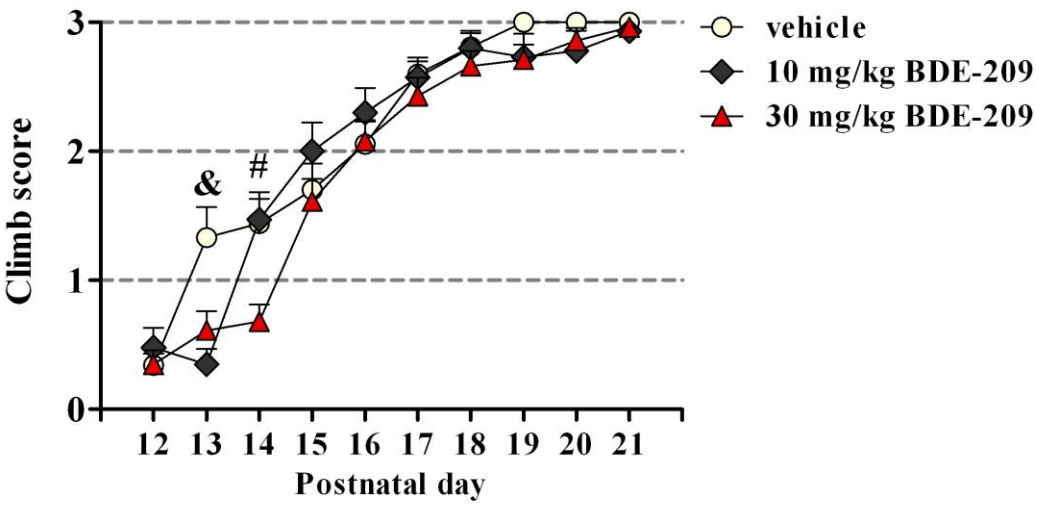

Figure I.13. Climb test by treatment

Data are expressed as mean and S.E.M. The symbol (&) indicates control mice differ than treated, and (#) indicates differences between high dose exposed mice respect the other two groups at $p<0.05$.

FIGURE I.14

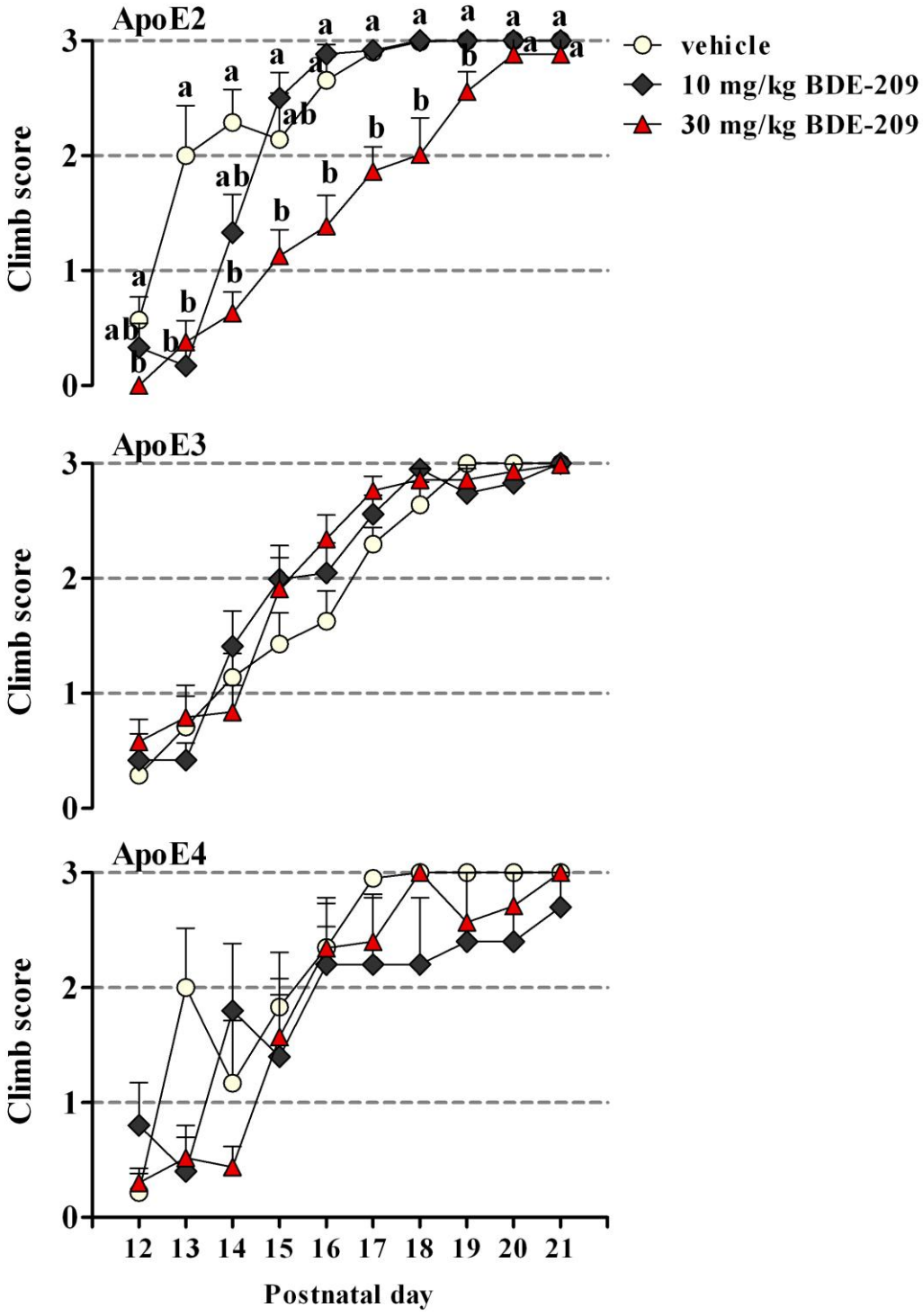

Figure I.14. Climb test by genotype and treatment

Data are expressed as mean and S.E.M. Groups showing different letters a, b, differ among them at $p<0.05$.

Post-weaning development: sexual maturation

Testicle descent was scored in males from PND21 to PND 42 and analysed by a two-way ANOVA (genotype x treatment) for repeated measures, using the day of testing as the within-subject factor, and the score of testicle descent as dependent variable. An effect of genotype was observed [$F(2,40)=3.085$, $p=0.044$]. An effect of BDE-209 exposure appeared when the low and the high dose exposed groups were taken together [$F(2,40)=4.752$, $p=0.037$]. However, analyzing each genotype separately no significant effects between groups were observed (Figure I.15).

FIGURE I.15

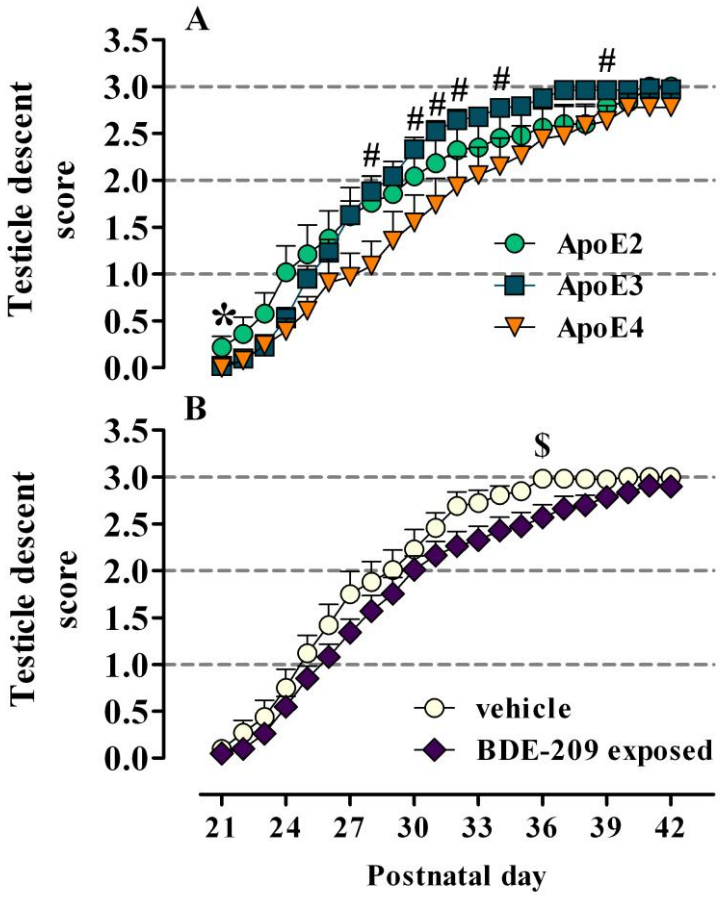

Figure I.15. Sexual maturation

Testicle descent by genotype (A) and by treatment (B). Data are expressed as mean and S.E.M. Symbols indicate differences at $p<0.05$ between; ApoE2 and the other genotypes (*), ApoE4 and ApoE3(#), control and BDE-209 exposed mice ($).

Vaginal opening day was checked in females from PND21 and analyed by a two way ANOVA (genotype x treatment) on the mean day at vaginal opening of the litter. Results did not show any significant effect of the genotype or the treatment. As in males, the effect of BDE-209 exposure (low dose and high dose exposed groups together) was examined and an effect of BDE-209 exposure in vaginal opening day [$F(1,40)=5.448$, $p=0.026$] and a trend towards an interaction between genotype and BDE-209 exposure [$F(2,40)=3.271$, $p=0.051$] were observed. Analyzing this effect in each genotype separately, BDE-209 exposure showed a non significant trend in ApoE2 females [$F(1,40)=4.008$, $p=0.08$] (Figure I.16).

In short, genotypes showed differences in the rhythm of sexual maturation in males; ApoE2 matures faster than ApoE4. BDE-209 exposure also delayed maturation in males, but did not affect differently the genotypes tested. Female sexual maturation was similar in all the genotypes, but the BDE-209 exposure affected the vaginal opening depending on the genotype, ApoE2 females are likely to be the most affected.

FIGURE I.16

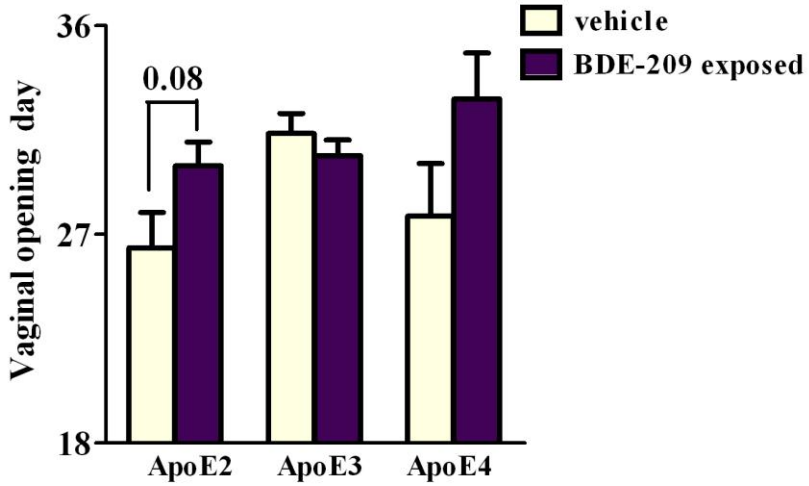

Figure I.16. Sexual maturation

Vaginal opening day in control and BDE-209 exposed, ApoE2, ApoE3 and ApoE4 female groups. Data are expressed as mean and S.E.M.

Myelination study

During maturation and the myelination process, MBP is present in the cytoplasm of the soma and prolongations of the oligodendrocytes. As the myelination process advances, MBP localize in the membrane of the oligodendrocyte prolongations and myelin sheaths become compacted. Results for immunohistochemical MBP, performed at PND20 in males, were very similar between genotypes and the treatment effects were only evident in ApoE2 group.

In Figures I.17 to I.20 the coronal sections show two medial sections including the hippocampal formation and striatal fibres, and an anterior section showing corpus callossum, fornix and anterior comissure.

The MBP signal was present in all the sections stained, but a more intense and a more spread signal is compatible with a reduced compaction of the myelin sheaths in BDE-209 treated ApoE2 subjects compared to controls. Reduced compaction is visible in all sections, especially in the anterior one. This possible delay in myelination in ApoE2 is more evident in the anterior section probably because of the rostrocaudal pattern myelin maturation.

Figure I.17

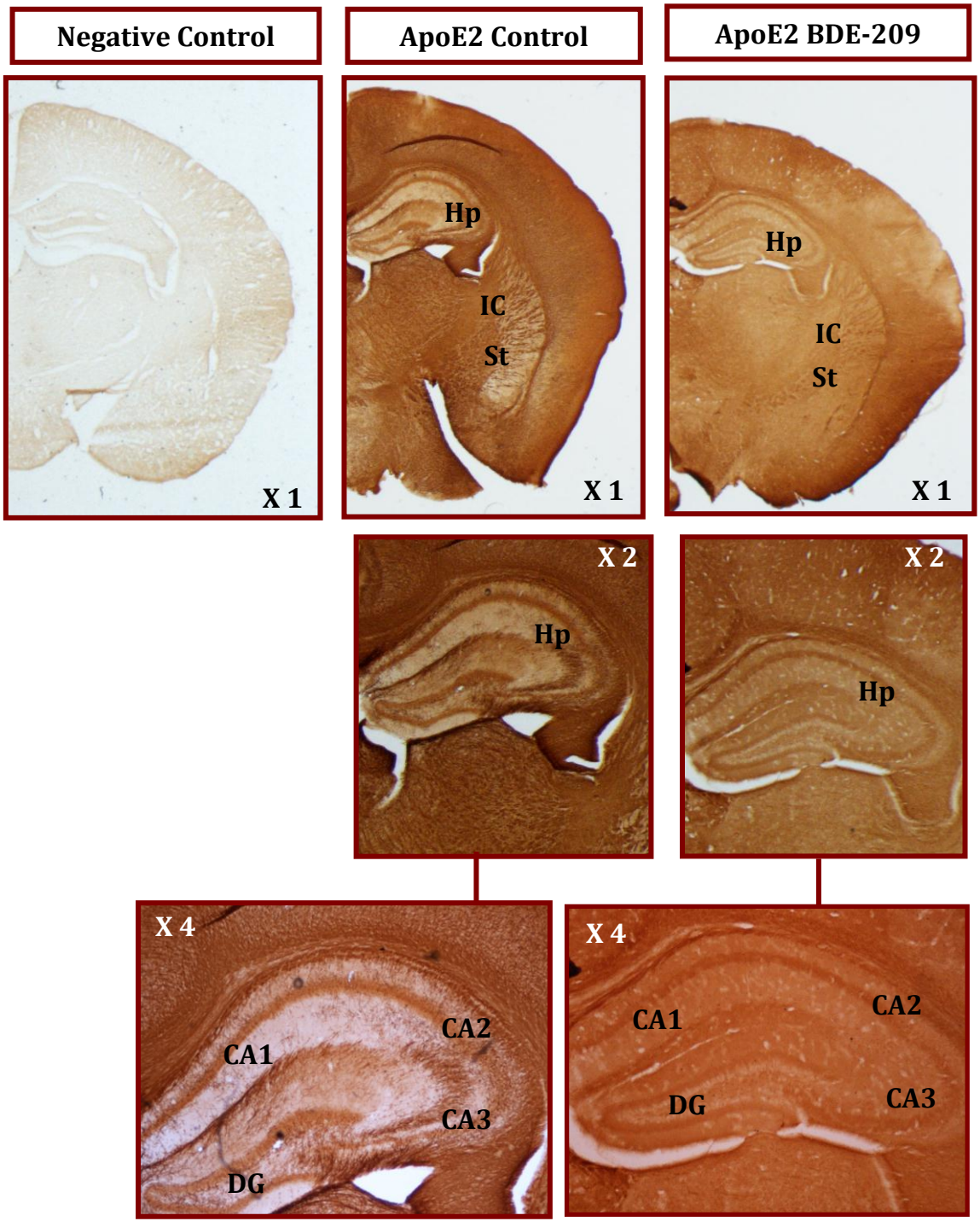

Figure I.17. MBP immunostainning

Coronal brain slices from ApoE2 male pups (PND20) administered 0 or 30 mg/kg BDE-209 on PND10. Images show medial sections in which is observable the hippocampus formation (Hp), the striatum body (St) and the internal capsule (IC), consisting in descending cortical fibres. The enlargement allows distinguishing different parts of the hippocampus; CA1, CA2 and CA3 fields and dentate gyrus (DC).

Figure I.18

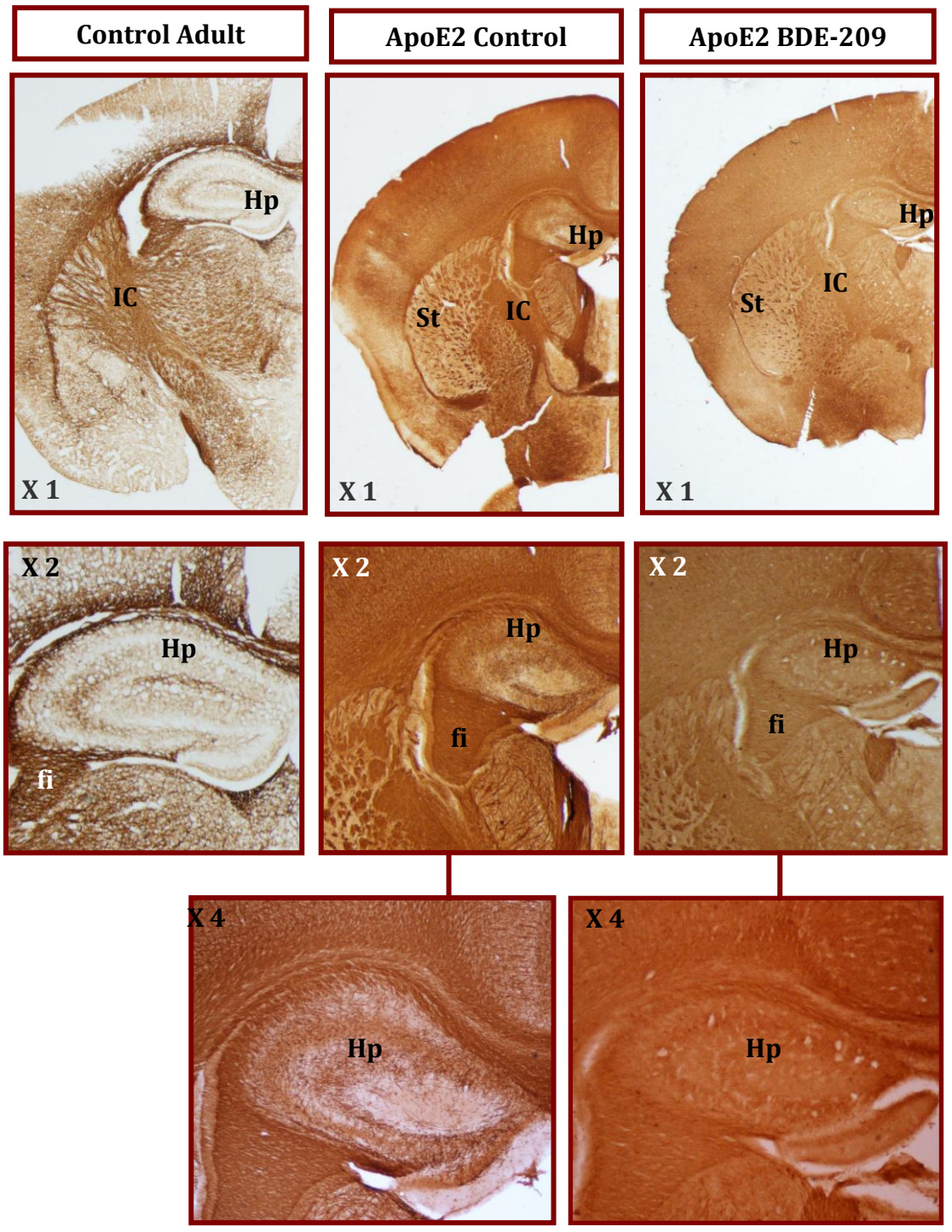

Figure I.18. MBP immunostainning

Coronal brain slices from ApoE2 male pups (PND20) administered 0 or 30 mg/kg BDE-209 on PND10 and a control adult. Images of medial sections show the hippocampus (Hp), the striatum body (St) and cortical fibres of the internal capsule (IC). The enlargement allows seeing in detail the anterior dorsal hippocampus.

Figure I.19

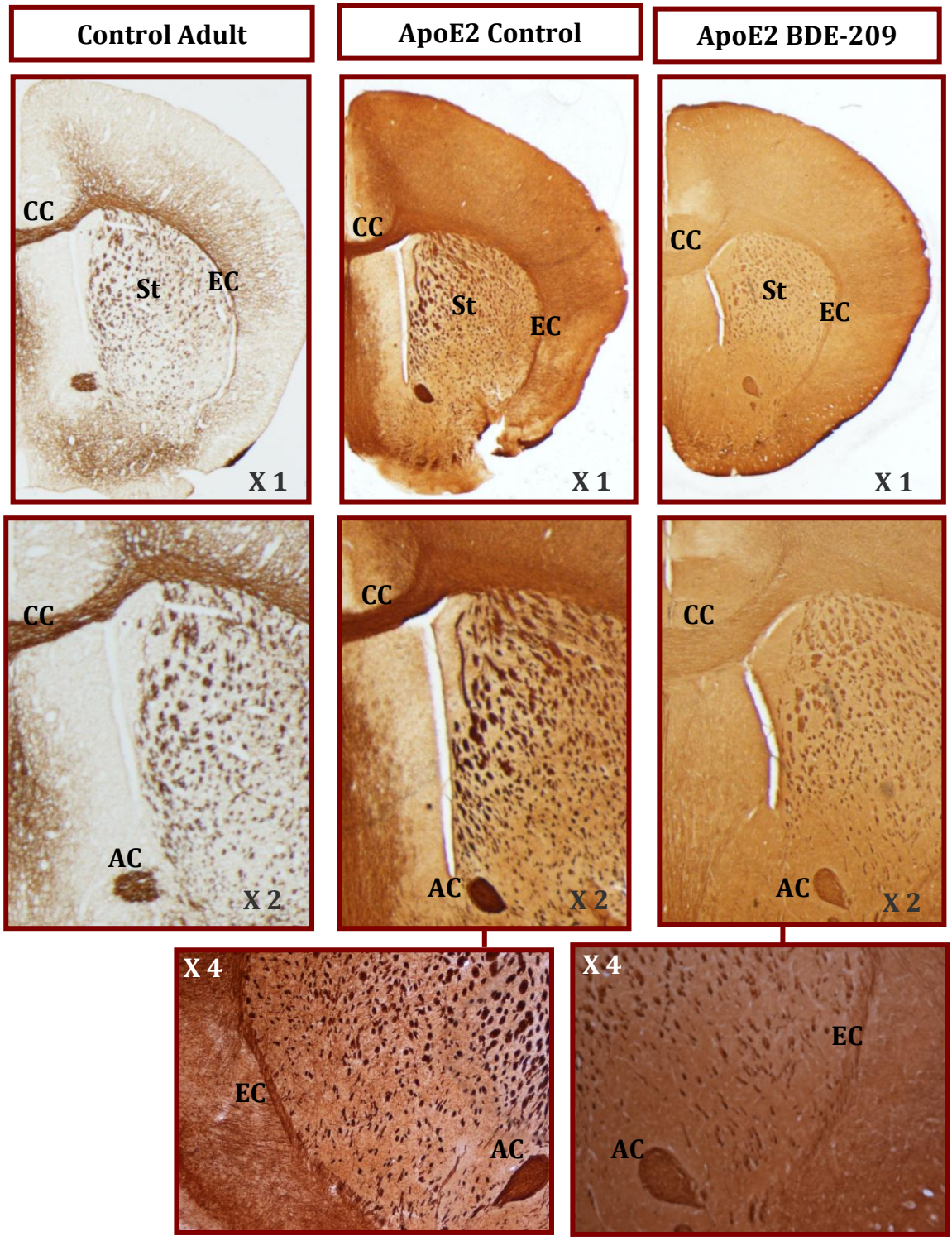

Figure I.19. MBP immunostainning

Coronal brain slices from ApoE2 male pups (PND20) administered 0 or 30 mg/kg BDE-209 on PND10 and a control adult. Anterior sections show fibres of the corpus callossum (CC) connecting the hemispheres, the striatum (St), and ascending cortical fibres of the external capsule (EC). In the enlargement anterior commissure (AC) fibres travelling in the anterio-posterior direction are prominent as “spots” from the cross sectional cut.

Figure I.20

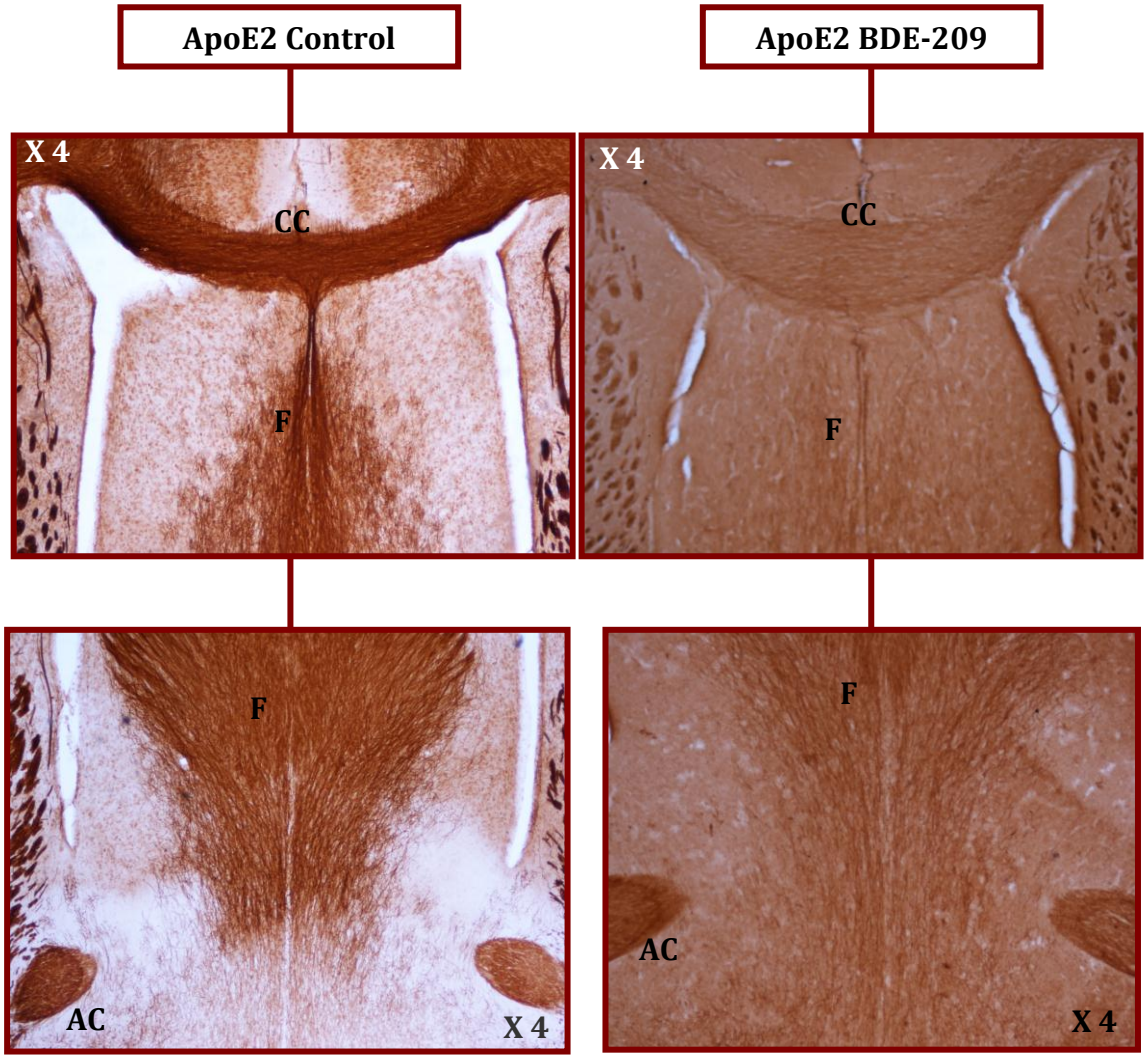

Figure I.20. MBP immunostainning

Coronal brain slices from PND20 male ApoE2 pups administered vehicle or 30 mg/kg DBDE on PND10. Images show enlargements of the anterior sections. In superior images are observable the interhemispheric fibres of the corpus callossum (CC) and the precommissural fornix (F), containing fibres from the hippocampus travelling to the septum and nucleus accumbens. In the inferior images the anterior commissure (AC) fibres are observable as two "spots" and the diffusely spread fibres of the precommissural fornix (F) in the midline.

1.2 Discussion of the Experimental Phase I: Development

The developmental assessment of the ApoE transgenic mice exposed to BDE-209 on PND10 show that both factors, the ApoE genotype and postnatal exposure to BDE-209, influence developmental traits.

Different ApoE genotypes showed differences in the size of the litter, maternal care, and physical and functional development of mice. Differences in development according to ApoE polymorphisms have not been previously studied in animal models, but there is some information available from humans. A study in Oregon found ApoE4 children carriers were ten times more likely to be placed in an Intensive Care Unit after birth and to have other pregnancy complications (Acevedo et al. 2010). In our study ApoE4 dams had fewer pups per litter and do not improve over time in their maternal care skills. However, ApoE4 pups did not show differences compared to the other genotypes in early development. Only a slight delay in body weight increase and a delay in the achievement of motor skills in late development were observed in ApoE4. There is no available data related to neurological development in humans carrying the different polymorphisms for the ApoE, but some studies investigate children's performance in cognitive and behavioural traits related to the ApoE genotype. Alexander and coworkers assessed cognition in a 6- to 65-year-old cohort and did not find association between ApoE genotype and brain function across the entire age range (Alexander et al. 2007). Similarly, a recent study failed to find any relationship between ApoE genotype and IQ, memory function or school attainment in children aged 7-14 years (Taylor et al. 2011). On the other hand, Wright and colleagues observed better scores on the Bayley development scale in 2-year-old

children carrying ApoE4 (Wright et al. 2003) and Bloss and coworkers did not find any differences among genotypes in language or mathematical skills, but the ApoE2 genotype showed lower visuospatial scores (Bloss et al. 2010). Conversely, some studies found a positive association between the ApoE4 allele and lower cognitive execution. Impaired spatial memory retention was observed in children aged 7-11 years carrying the ApoE4 allele (Acevedo et al. 2010), and slower speed processing in children aged 11 was reported by Luciano and colleagues (Luciano et al. 2009). Furthermore, ApoE4 carrier children have also shown reduced cortical thickness in the left enthorrinal cortex, while ApoE2 showed the thickest. ApoE4 cognitive deficits were also observed in children with other risk factors associated. Children carrying ApoE4 plus a family history of AD were also found to show lower cognitive scores (Bloss et al. 2008). Another study observed poorer memory scores correlated with the ApoE4 allele and a specific BDNF genotype in children who had suffered a sexual trauma (Savitz et al. 2007). Likewise, a worse neurological outcome was found in ApoE4 children after a brain injury (Brichtova et al. 2008). Those studies suggest that in children the ApoE4 genotype may be associated with an impaired performance in specific abilities or under certain risk conditions. Our results show a slight delay in ApoE4 development, which does not implicate a severe impairment, since all the genotypes acquired all the development milestones assessed.

Sexual development was also slightly delayed in ApoE4 male mice, but again no information is available, either in humans or animals, on sexual development and ApoE genotypes.

BDE-209 exposure showed to interact with ApoE genotype to exert its effects, since ApoE2, but not the other genotypes, was affected by the treatment. ApoE2 mice exposed to BDE-209 on PND10 showed decreased growth and delayed eye opening. In contrast, most studies do not find differences in pup body weight after perinatal PBDE exposure (Branchi et al. 2002; Zhou et al. 2002; Staskal et al. 2006; Tseng et al. 2006; Rice et al. 2007; Kodavanti et al. 2011). There are two studies that have reported increases in body weight after neonatal PBDE exposure, which were not manifested in developing pups but became evident from one or two months of age (Dufault et al. 2005; Gee et al. 2008). These results would be in agreement with our findings, since ApoE2 exposed animals did not show decreased body weight until post-weaning period. However, there is a discrepancy, since pentaBDE showed body weight increasing while our animals exposed to the BDE-209 compound showed a decrease.

Two studies attempted to relate ApoE genotype with the vulnerability to toxic insults in adult subjects, which found that ApoE4 allele was associated with higher lead and mercury body burdens and more persistent CNS toxic effects (Stewart et al. 2002; Godfrey et al. 2003). This relationship has been considered to be due to the diminished ability of ApoE4 to eliminate heavy metal (Mutter et al. 2004). On the other hand, one study found greater negative effects of Pb in the Development Index of the Bayley Scale in children carrying ApoE2 or ApoE3 compared to ApoE4 children. A strong association was found between ApoE levels and Pb in children, at levels of Pb exposure below the thresholds of concern established by regulatory agencies (Birdsall et al. 2010). Discrepancies between studies in lead toxicity could be related to the age at testing and to the levels of lead exposure, however they point to a relationship between

lead toxicity and the ApoE genotype. Some studies found a higher susceptibility for development disturbances after cardiac surgery in children who were ApoE2 carriers. These infants showed lower mental and psychomotor indexes at one year of age and lower growth and behavioural and social interaction problems at 4 years (Gaynor et al. 2003; Gaynor et al. 2007; Gaynor et al. 2009; Burnham et al. 2010). Data previously reported suggest that ApoE2 might be more vulnerable during development after an environmental challenge. In the present study, ApoE2 mice exposed to BDE-209 showed delays in all neuromotor tests carried out and those detrimental effects were more pronounced in high dose exposed mice. Alterations in motor development have been already reported after PBDE exposure. Subtle affectation in coordination, grip time and inverted screen test from PND12-18 in C57BL/J6 mice exposed to BDE-47 has been reported (Gee et al. 2008), and delayed appearance of climbing ability was found in Swiss CD1 females exposed to 30 mg/kg of BDE-99 (Branchi et al. 2003). Neonatal exposure to BDE-209 did not show any motor retardation in C57BL/J6 mice, but there was an observed delay in palpebral reflex acquisition (Rice et al. 2007).

Sexual development was also delayed in mice exposed to BDE-209 in the current study. In females, but not in males, this delay was also related to ApoE2 genotype. PentaBDE congener exposure has been shown previously to retard the onset of puberty in rodents of both sexes (Stoker et al. 2005; Lilienthal et al. 2006).

The myelination pattern was also altered in ApoE2 mice exposed to 30mg/kg of BDE-209. The myelination process occurs during BGS and any environmental interference during foetal and early life period could affect

myelin synthesis and compaction. Malnutrition during critical periods, such as iron or fatty acid deficiency, has been shown to alter myelination (Di Biase et al. 1997; Georgieff 2007) as well as exposure to toxic agents, for example the copper chelator cuprizone induces a severe brain demyelination (Pott et al. 2009). PBDE effects on myelination process have not been studied, but PBDE is recognized as thyroid hormone disruptor and hypothyroidism leads to delayed myelination (Dufault et al. 2005). The developing myelination process related to ApoE isoforms has not been explored either, but ApoE and cholesterol are known to play a critical role in myelin maintenance, integrity and recovery (Vila-Rodriguez et al.; Bartzokis et al. 2007; Li et al. 2010; Vila-Rodriguez et al. 2011). White matter relative to the ApoE genotype has been studied in healthy and demented adults, finding a correlation between diffused white matter, cognitive decline and presence of the Apoe4 allele (Nierenberg et al. 2005; Heise et al. 2010; Ryan et al. 2011). The altered pattern of myelination found in ApoE2 mice exposed to BDE-209 may be responsible for the delayed acquisition of neuromotor abilities observed. These results deserve more experimentation to explore possible effects or interactions between genotype, treatment and sex using semi-quantitative methods or electronic microscopy techniques.

This research highlights that genetic factors, such as the ApoE genotype, influence development and modulate neurodevelopmental toxic effects induced by PBDEs. Moreover, the full-brominated BDE congener, which is supposed to be less toxic, appeared able to affect development in vulnerable subjects.

Taking these results together, ApoE2 can potentially be used as a risk biomarker for toxic exposures to PBDE during development. In this regard, this genotype has also been shown to be a risk factor to other toxic insults, such as Pb exposure as mentioned above.

Experimental Phase II: 4 months old

UNIVERSITAT ROVIRA I VIRGILI
NEUROBEHAVIOURAL EFFECTS ASSOCIATED WITH POSTNATAL EXPOSURE TO DECABROMODIPHENYL ETHER IN APOE2, APOE3 AND APOE4 TRANSGENIC MICE
Ingrid Reverté Soler
DL:T. 162-2012

2. Experimental Phase II: 4 months-old

Experimental Phase II consisted in the evaluation of young adult ApoE transgenic mice exposed to BDE-209 on PND10. Anxiety and locomotor activity were assessed in an EZM and an OF. Spatial learning and memory were assessed in a MWM spatial reference task. The levels of thyroid hormones and the levels of BNDF in in the hippocampus of control and exposed ApoE mice were studied. The results of the second experimental phase are detailed in subsequent pages organized as follows: body weight, activity and anxiety measures in the Elevated Zero Maze and in the Open Field, learning and memory in the Morris Water Maze reference memory task, Thyroid Hormones and BDNF and TrkB levels.

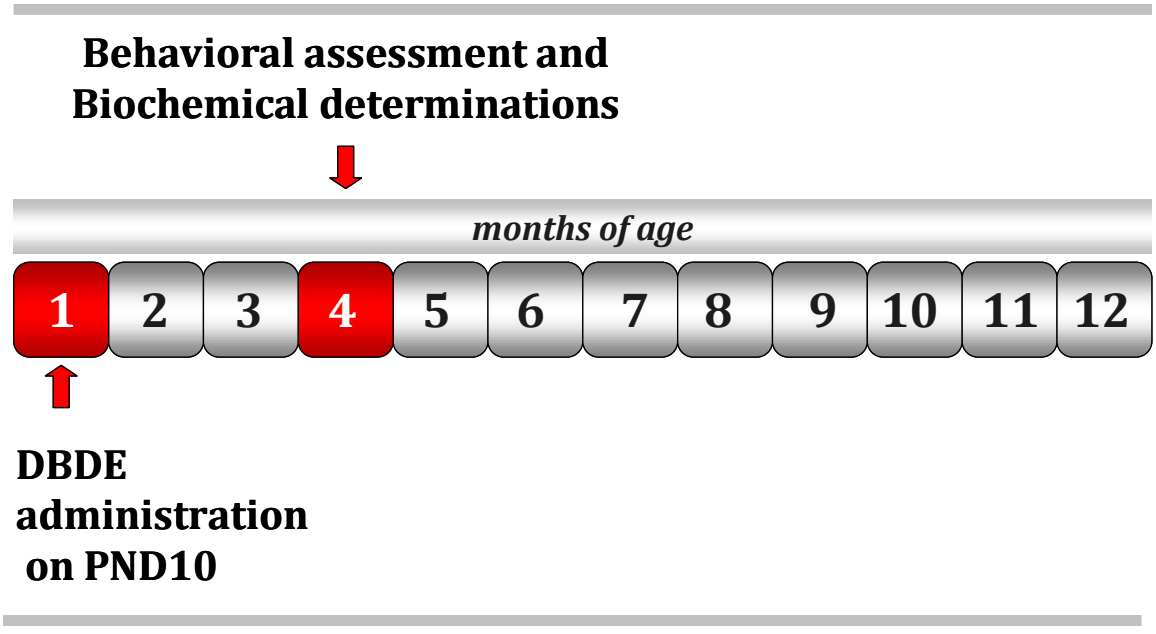

Figure II. Scheme of Experimental Phase II: 4 months of age

2.1 Results of Experimental Phase II: 4 months old

Body weight

Body weight (g) at 4 months of age was analyzed by a three-way ANOVA (genotype x treatment x sex). As was expected, males weighed more than females [$F(1, 191) = 80.892$, $p<0.001$]. An overall effect of the genotype [$F(2, 191) = 9.681$, $p<0.001$], an interaction between genotype and sex [$F(2, 191) = 4.383$, $p=0.009$] and an interaction between genotype and treatment [$F(2, 191) = 4{,}161$ $p=0.017$] were also observed. Genotype differences in body weight were restricted to females [$F(2, 97) = 29.139$, $p<0.001$] (Figure II.1).

FIGURE II.1

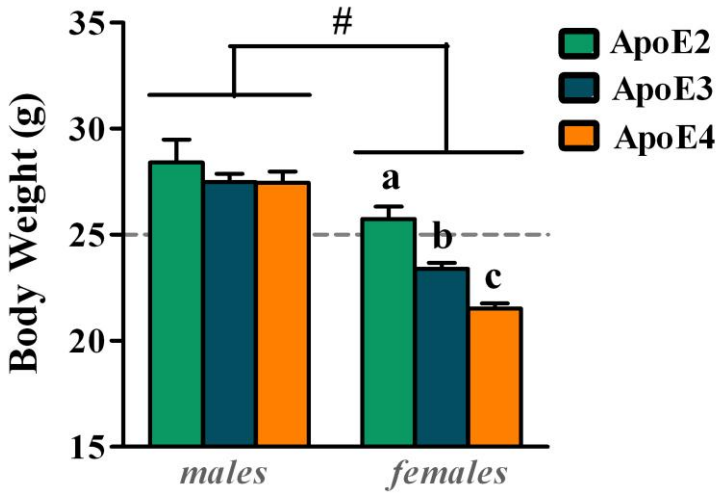

Figure II.1. Body weight at 4 months of age by genotype and sex

Data are expressed as mean and S.E.M. Groups showing different letters a,b,c are different from each other at $p < 0.05$.

BDE-209 postnatal exposure affected body weight differently depending on both genotype and sex. Significant differences were observed in ApoE4 males [$F(2, 33) = 6.381$, $p=0.005$], whose treated with BDE-209 showed a decrease in body weight, and in ApoE3 females [$F(2, 31) = 3.718$, $p=0.037$], of which those exposed to the low dose showed a slight increase in their body weight (Figures II.1 and II.2).

FIGURE II.2

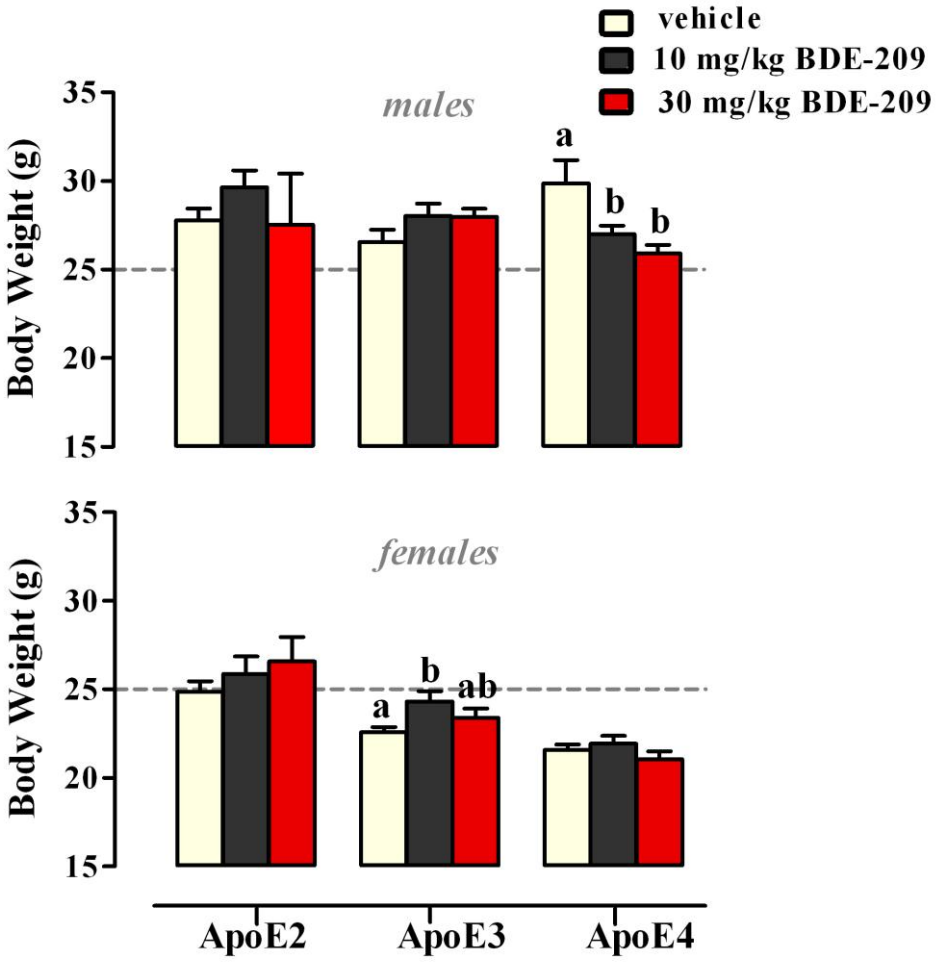

Figure II.2. Body weight at 4 months of age by genotype, sex and treatment

Data are expressed as mean and S.E.M. Groups showing different letters a,b are significantly different from each other at $p < 0.05$.

To summarize, the female body weight is clearly affected by the genotype. The effects of BDE-209 treatment on PND10 remain in young adults depending on the genotype, effects which are evident in ApoE4 males and ApoE3 females.

Elevated Zero Maze

The evaluation of anxiety at four months of age was conducted in an EZM test. Ten animals were excluded from the analyses because they fell off the apparatus (Table 4).

Table 4: Mice excluded of the EZM analysis at 4 months of age

	ApoE2 males	ApoE2 females	ApoE3 males	ApoE3 females	ApoE4 males	ApoE4 females	
0 mg/kg	0	0	2	0	2	0	4
10 mg/kg	0	0	1	1	0	0	2
30 mg/kg	0	1	2	0	0	1	4
	0	1	5	1	2	1	
	1		6		3		

Table 4. Number of the animals removed from the statistical analyses because they fell off the EZM apparatus and its distribution among groups (genotype, treatment and sex). Data is expressed as absolute values.

The analysis of the EZM was conducted by a three-way ANOVA (genotype x treatment x sex) on the latency (s) to enter the closed area, the time (s) spent in the open area, the number of crossings from the closed to the open area, the number of head dips, rearing, grooming, freezing and defecation. A correction was made in the time (s) spent in the open area, by

subtracting the latency to enter the closed area from the total time spent in the open area. This correction was made in order to find a measure of the time the animals spent in the open area, when they crossed out voluntarily.

As none of the variables, with the exception of the grooming, showed variance homogeneity, the non-parametrical Kruskall-Wallis test was used. Results showed an overall effect of the genotype in the latency [X^2 = 15.422, p<0.001], in the corrected time spent in the open area [X^2 = 4.652, p<0.001], in the number of crossings [X^2 = 63.107, p<0.001], in the number of head dips [X^2 = 22.054, p<0.001], in the number of rearings in the closed area [X^2 = 1.930, p=0.012], in the number of freezings [X^2 = 20.429, p<0.001]and in the number of defecations [X^2 = 6.111, p=0.047] (Figure II.3). With respect to grooming behaviour, a significant interaction was observed between treatment and sex [F(2, 164) = 3,515, p=0.032], and there was a trend towards an interaction between sex and genotype [F(2, 164) = 2,854, p=0.061], and between treatment, sex and genotype [F(4, 164) = 2,134, p=0.079].

ApoE4 showed an increased latency to enter the closed area, and thereafter made fewer crossings and spent less time in the open area (corrected time in open area) than the other genotypes. ApoE4 also did less rearing and head dips and displayed more freezing than the other genotypes.

Additionally, we analyzed the effect of the treatment separately in each genotype and sex. An effect of the treatment in the number of crossings was observed in ApoE3 females [X^2 = 6.383, p=0.041] (Figure II.4).

FIGURE II.3

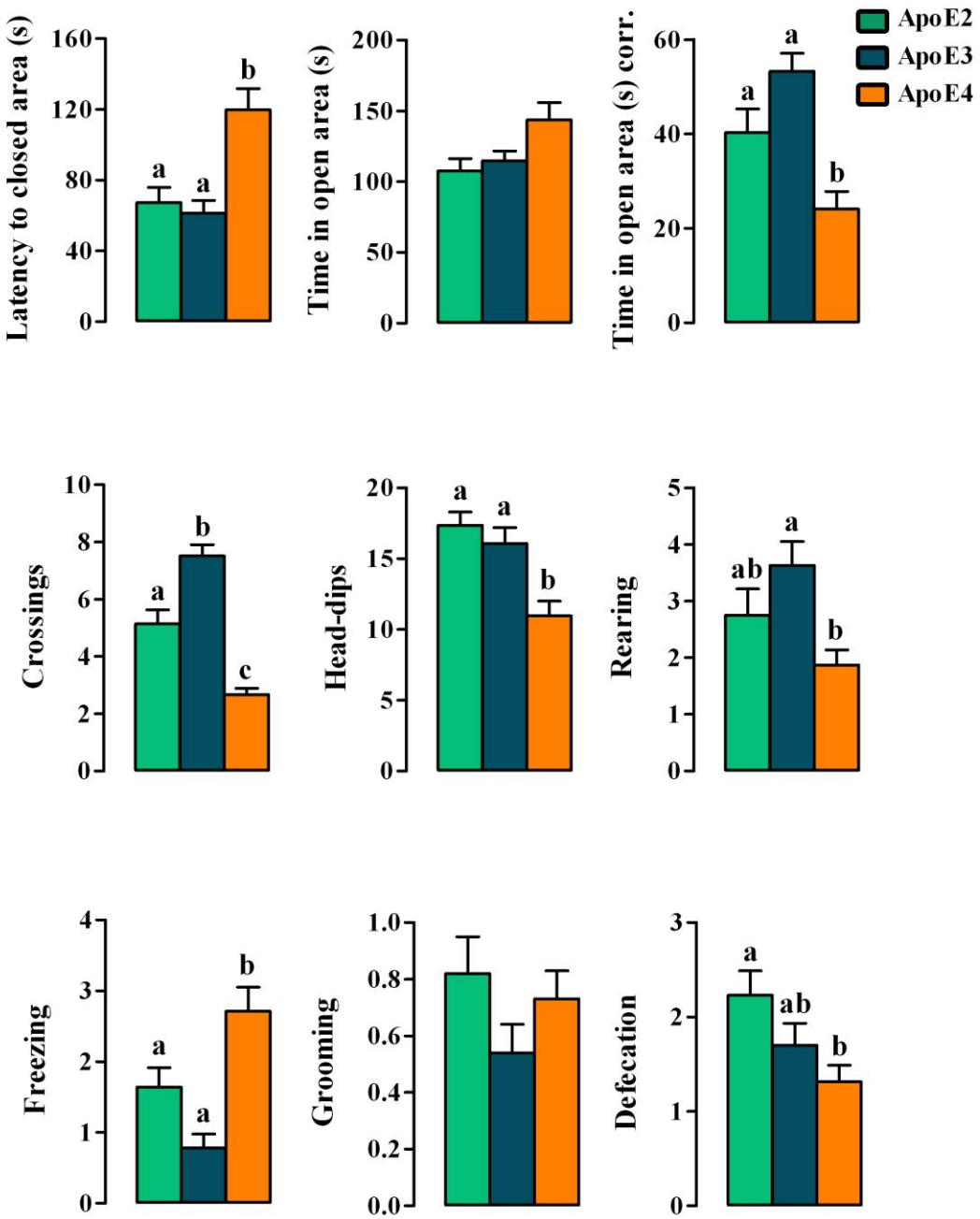

Figure II.3. Behavior in an EZM by genotype

Data are expressed as mean and S.E.M. Groups showing different letters a,b,c are different from each other at $p < 0.05$.

FIGURE II.4

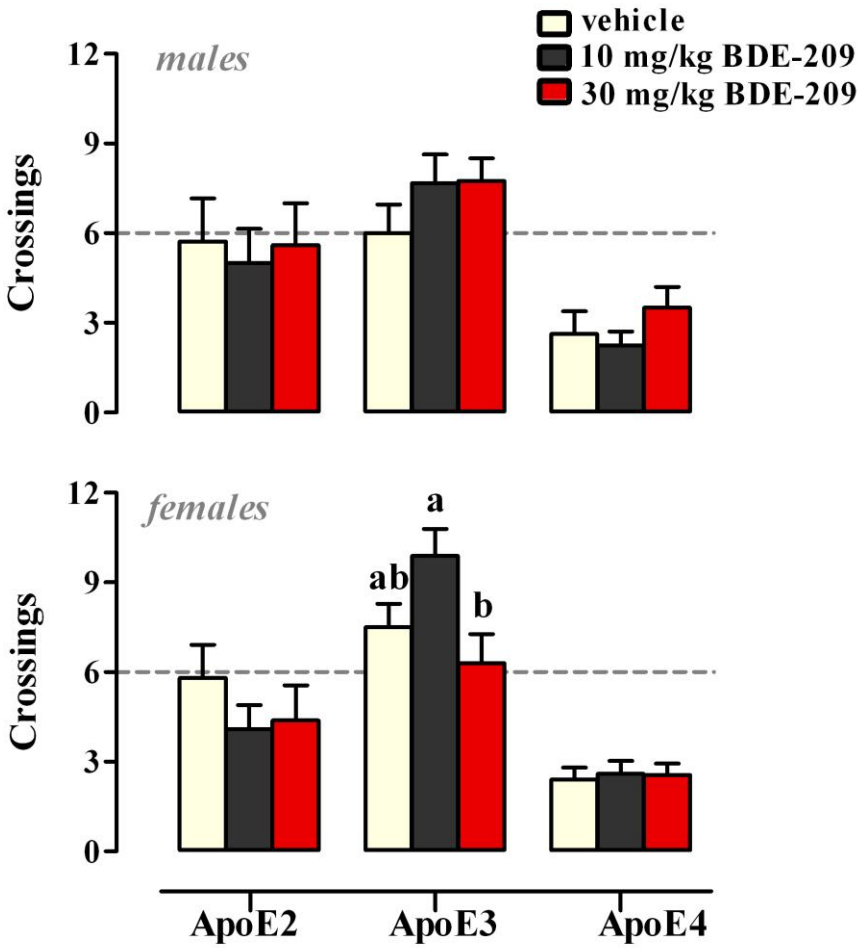

Figure II.4. Behaviour in the EZM by genotype, treatment and sex

Number of times the animals crossed from the closed to the open area. Data are expressed as mean and S.E.M. Groups showing different letters a,b,c are significantly different from each other at $p < 0.05$.

Briefly, the analysis in the EZM showed a strong effect of the genotype in exploratory and anxiety-like behaviour measures of the test, the ApoE4 mice being those more anxious and less active. The treatment showed only a slight effect in ApoE3 females, in which those exposed to the low dose showed an increase in the number of crossings to the open area.

Open Field

General activity in the Open Field

General activity was analyzed by a three-way ANOVA (genotype x treatment x sex) for repeated measures using six 5-min periods as the within-subject factor and the distance travelled as dependent variable. The period of time had a significant effect on the distance travelled [$F(6, 181) = 70.978$, $p<0.001$]. All the animals shortened the distance travelled over time indicating habituation to the new space. An overall effect of the genotype [$F(2, 181) = 29.308$, $p<0.001$] and an interaction between the treatment and sex [$F(2, 181) = 4.522$, $p<0.012$] were also observed (Figure II.5).

FIGURE II.5

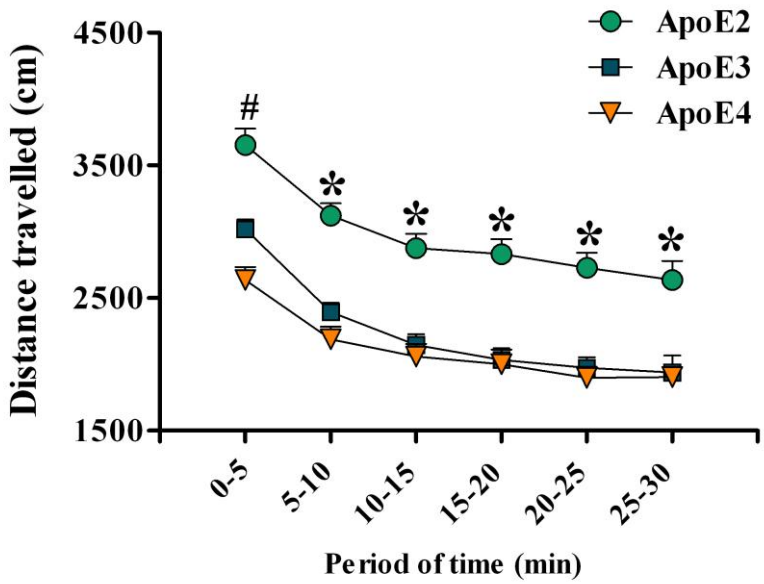

Figure II.5. Distance travelled in the OF by genotype

Data is expressed as mean and S.E.M. The symbol # indicates that all three genotypes differ among them; the asterisks indicate differences between ApoE2 and the other genotypes. $p<0.05$.

A three-way ANOVA (genotype x treatment x sex) was carried out on the total distance travelled and the total number of rearings. Since none of the variables showed variance homogeneity, the non-parametrical Kruskall-Wallis test was used. An overall effect of the genotype was found in the total distance travelled (cm) [$X^2 = 63.272$, $p<0.001$] and in the total number of rearings [$X^2 = 44.607$, $p<0.001$]. The ApoE2 group travelled further than the other genotypes and reared more compared with ApoE4 (Figure II.6). A treatment x sex interaction was also significant in the total distance travelled [$F(2,181)=4.552, p=0.012$] and in the total number of rearings [$F(2,181)=5.158, p=0.007$].

FIGURE II.6

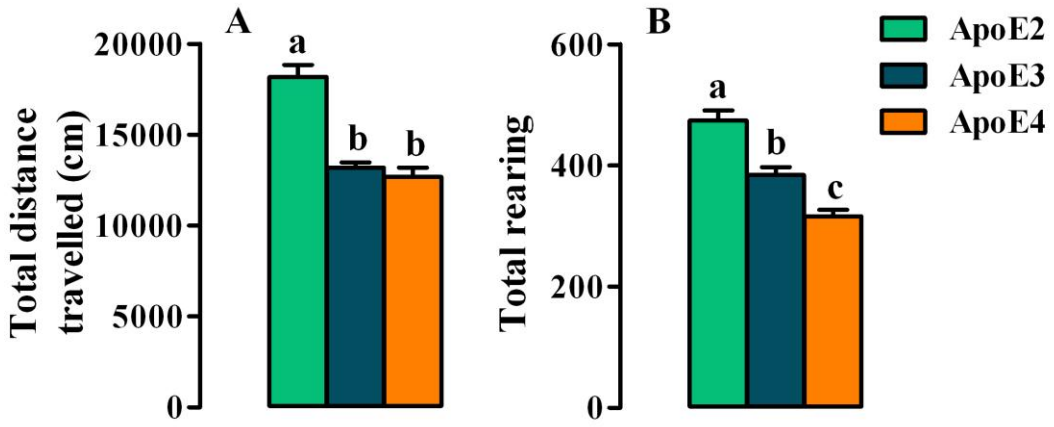

Figure II.5. General activity in the OF by genotype

(A) Total distance travelled. (B) Total rearing performed. Data are expressed as mean and S.E.M. Groups showing different letters a,b,c are different from each other at $p < 0.05$.

A more detailed analysis was carried out to assess the effects of the treatment in each genotype and sex for the distance travelled in each 5-min period. An overall effect of treatment was found in ApoE4 females [$F(2, 29) = 5.377$, $p<0.010$] (Figure II.7).

FIGURE II.7

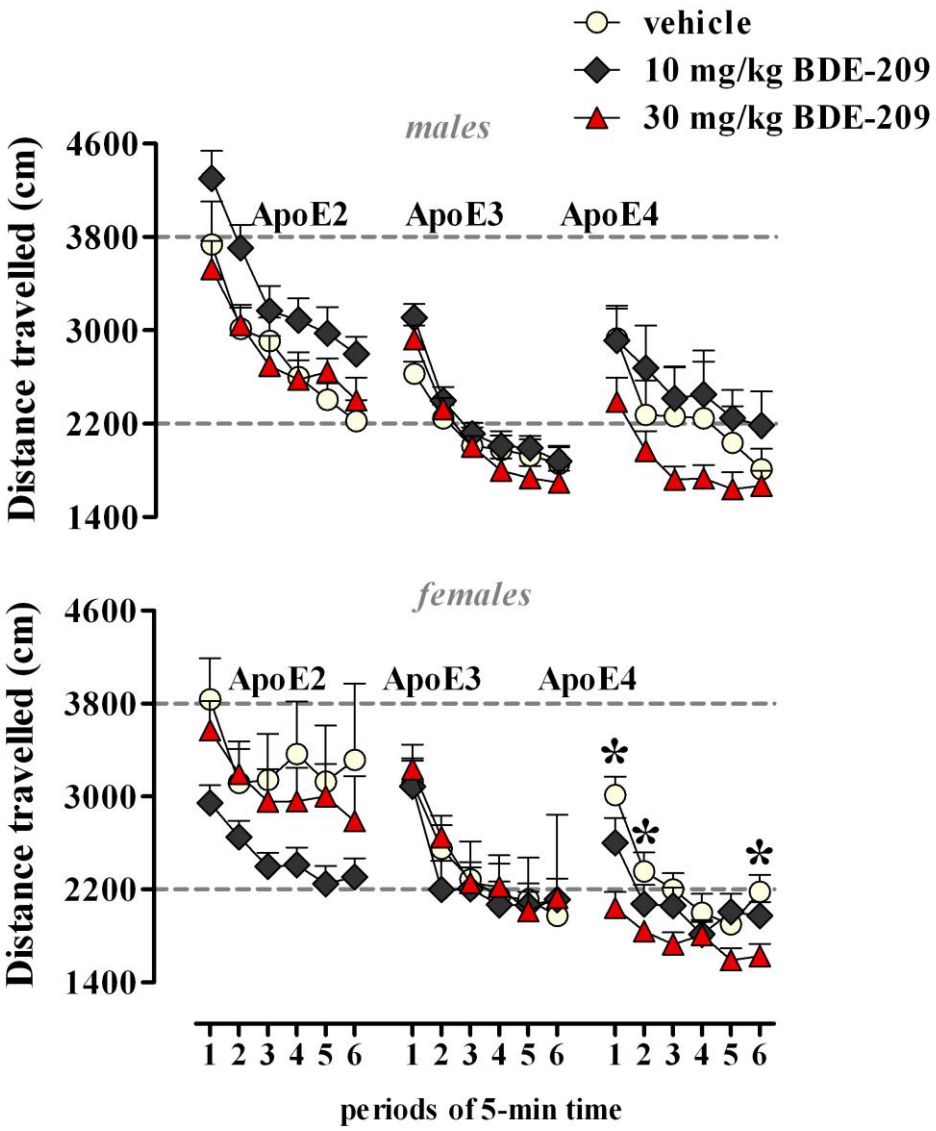

Figure II.7. Distance travelled in the OF by genotype, sex and treatment

Data are expressed as mean and S.E.M. The asterisk indicates differences between ApoE4 control and ApoE4 high dose exposed female mice at $p < 0.05$.

The treatment effects on the total distance travelled and the total rearing were also analyzed in each genotype and sex group. An overall effect of the treatment was found in the total number rearing in ApoE2 males [$F(2, 29) = 3.552$, $p=0.043$], in ApoE3 females [$F(2, 29) = 3.835$, $p=0.034$] and in

ApoE4 females [F(2, 30) = 3.686, p=0.037]. Total distance travelled was affected by the treatment in ApoE4 females [F(2, 30) = 5.377, p=0.010] (Figure II.8 and II.9).

In general terms, the effect of the treatment is a reduced activity in females. This reduction is significant in ApoE4 females for both vertical and horizontal activity in the highest dose.

FIGURE II.8

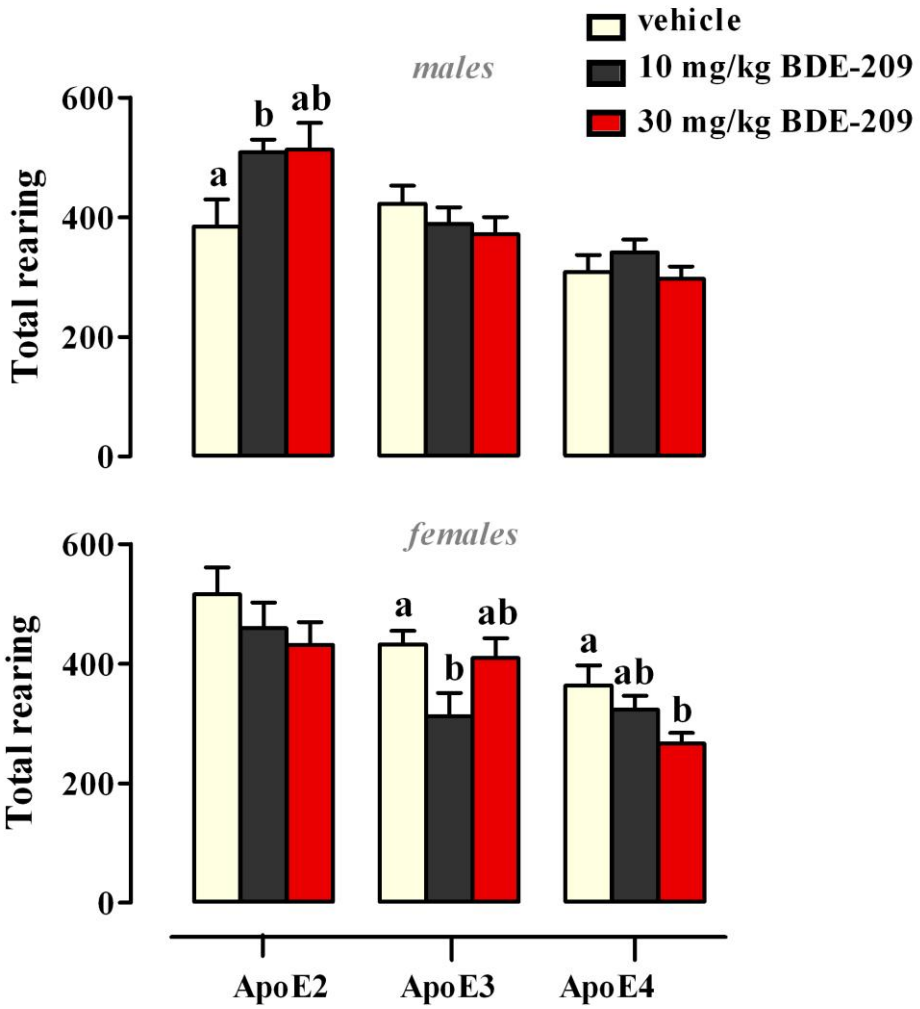

Figure II.8. General activity in the OF by genotype, treatment and sex

Total rearings performed. Data are expressed as mean and S.E.M. Groups showing different letters a,b,c are different from each other at $p < 0.05$.

FIGURE II.9

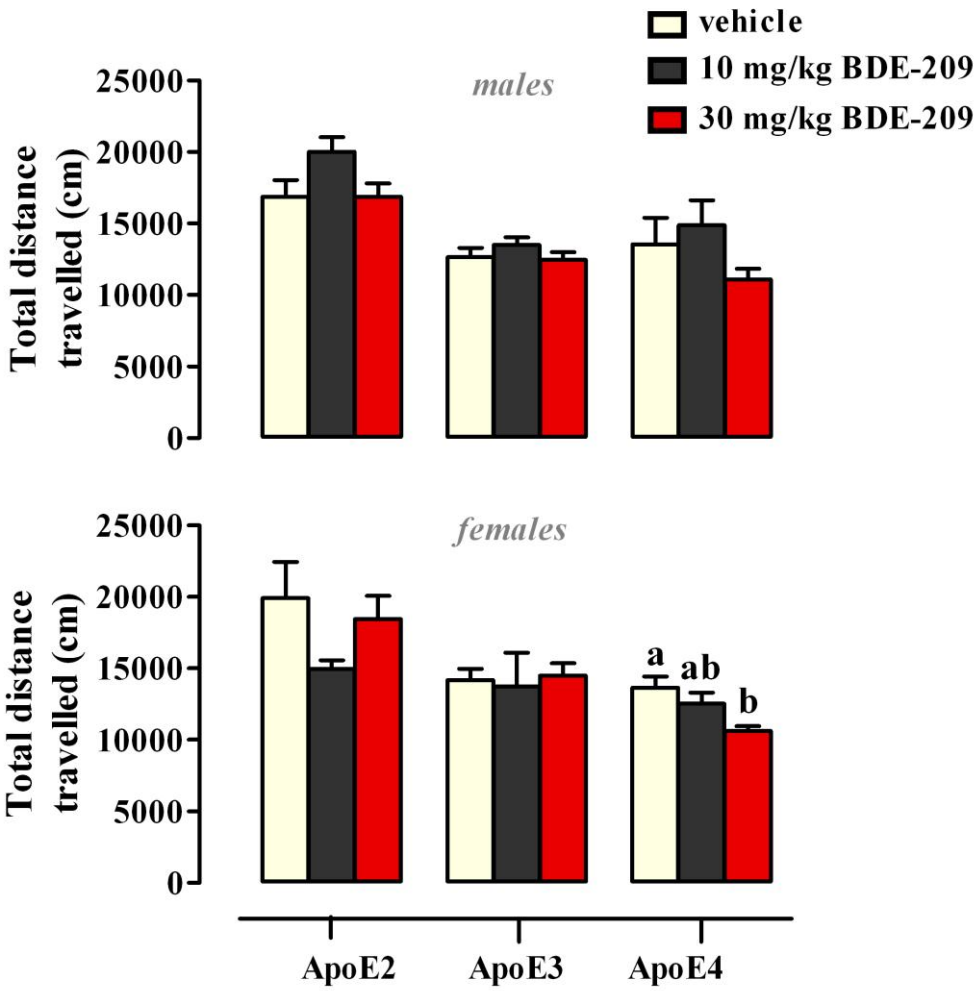

Figure II.9. General activity in the OF by genotype, treatment and sex

Total distance travelled. Data are expressed as mean and S.E.M. Groups showing different letters a,b,c are different from each other at $p < 0.05$.

Activity in the centre of the OF

A three-way ANOVA (genotype x treatment x sex) was performed on the number of entries to the centre and the time (s) spent in the centre. In addition, a ratio of the rearings in the centre (rearing in the centre / total

rearing) and a ratio of the distance travelled in the centre (distance in the centre/total distance) were calculated and analyzed. The ratio of distance travelled in the centre did not show variance homogeneity, so the non-parametrical Kruskall-Wallis test was used for this variable. An overall effect of the genotype was found in the number of entries to the centre [$F(2,181)=17.480$, $p<0.001$], the time spent in the centre [$F(2,181)=4.655$, $p=0.011$] and the ratio of distance travelled in the centre [$X^2 = 15.393$, $p<0.001$]. ApoE2 showed more entries to the centre, but ApoE3 spent more time and travelled more distance in the centre compared with the other genotypes. (Figure II.10). A treatment x sex interaction was found in the number of entries to the centre [$F(3,181)=7.281$, $p=0.001$].

FIGURE II.10

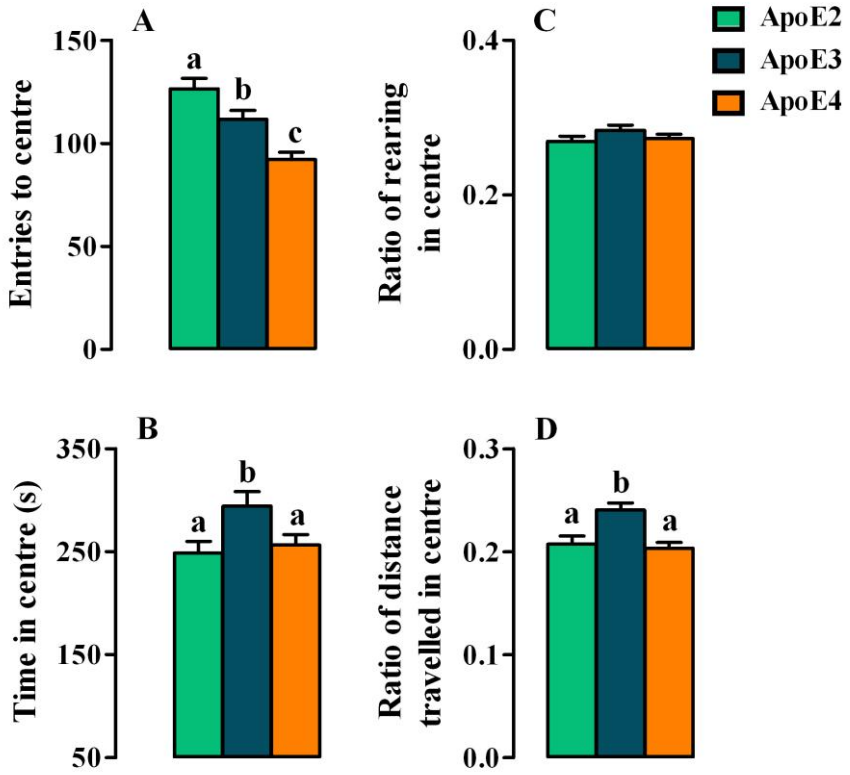

Figure II.10. Activity in the centre of the OF by genotype

Number of entries (A) and time spent (B) in the centre. Ratio of rearing in the centre (C) and ratio of distance travelled in the centre (D). Data are expressed as mean and S.E.M. Groups showing different letters a,b,c are different from each other at $p < 0.05$.

The activity in the centre for each group (genotype and sex) was analyzed by a one-way ANOVA. The BDE-209 treatment affected exclusively the number of entries to the centre of ApoE4 females [F(2, 30) = 3.583, p=0.041] (Figure II.11).

FIGURE II.11

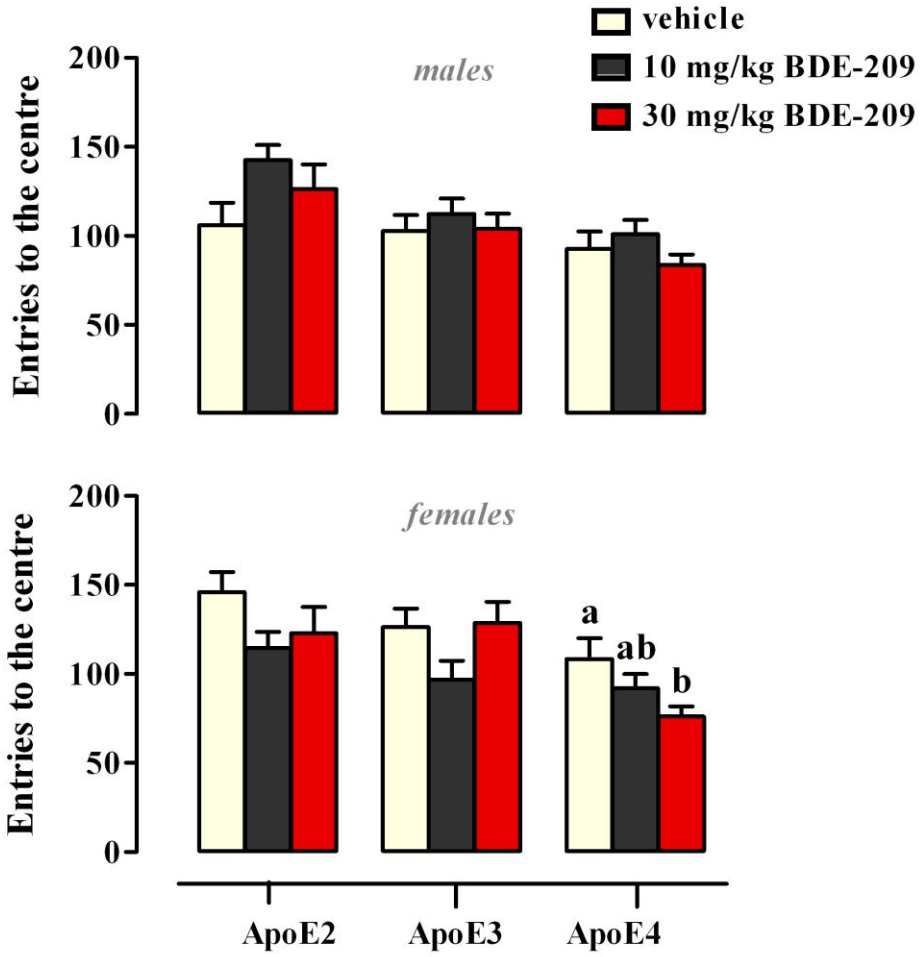

Figure II.11. Activity in the centre of the OF by genotype, treatment and sex

Number of times the animals entered to the centre. Data are expressed as mean and S.E.M. Groups showing different letters a,b are different from each other at $p < 0.05$.

Summarizing, the results in the OF test indicate differences in exploratory behaviour profiles related to the ApoE genotype. ApoE2 mice showed higher general activity, while ApoE3 mice spent more time and explored the centre of the field more compared with mice of the other genotypes. ApoE4 mice showed the lowest activity in the entire field. The treatment increased the activity in ApoE2 males and decreased the activity in ApoE3 females and ApoE4 females, the latter being the group most affected by the treatment.

Morris Water Maze reference memory task

General analyses in the Acquisition of the MWM task

To evaluate learning capacity we analyzed performance in each daily session during the ten days of the acquisition in the MWM task. Data was analyzed by a three-way ANOVA (genotype x treatment x sex) for repeated measures using the session as a within-subject factor, while the dependent variables were the escape latency (s), the distance travelled (cm) to get the platform, and the search velocity (cm/s).

There was an effect of the session on the escape latency [$F(9,191)= 102.921$, $p<0.001$] and on the distance travelled [$F(9,191)= 166.241$, $p<0.001$], which indicates that animals improved their performance over the acquisition days. There was also an effect of the session on the search velocity [$F(9,191)=26.383$, $p<0.001$], indicating changes in velocity through acquisition.

Multivariate analysis showed an interaction between session and genotype in escape latency [F (18,190)= 1.887, p=0.016], in distance travelled [F (18,190)= 2.108, p=0.006] and in search velocity [F (18,190)= 4.694, p<0.001]. An interaction between session and sex [F (9,191)= 2.971, p=0.003] and an interaction between session, treatment and sex [F (18,191)= 1.700, p=0.038] were also observed in the escape latency. An interaction between session, genotype and sex appeared in the distance travelled [F (18,191)= 1.639, p=0.049], and finally an interaction between session, genotype, treatment and sex was found on search velocity [F (36,191)= 1.671, p=0.009].

An overall effect of genotype was observed on the escape latency [F (2,191)= 34.127, p<0.001], on the distance travelled [F (2,191)= 23.227, p<0.001] and on velocity [F (2,191)= 74.803, p<0.001] (Figures II.12, II.13, II.14). An interaction between genotype and treatment [F (4,191)= 3.514, p=0.009] affected the distance travelled. Velocity was also affected by sex [F (1,191) = 4.219, p=0.041].

General analyses showed a clear effect of the genotype in the task acquisition. The three genotypes differed among them in the escape latency and the search velocity. Moreover, ApoE3 mice travelled less distance than the mice of the other two genotypes (Figure II.12 D-F). Although genotype exerts a major effect on this task, the effect of sex and the interactions between genotype and treatment on the performance acquisition should also be taken in account. Because multiple interactive effects emerged, we carried out a more detailed analysis by gender genotype and treatment to better analyze differences between experimental groups.

FIGURE II.12

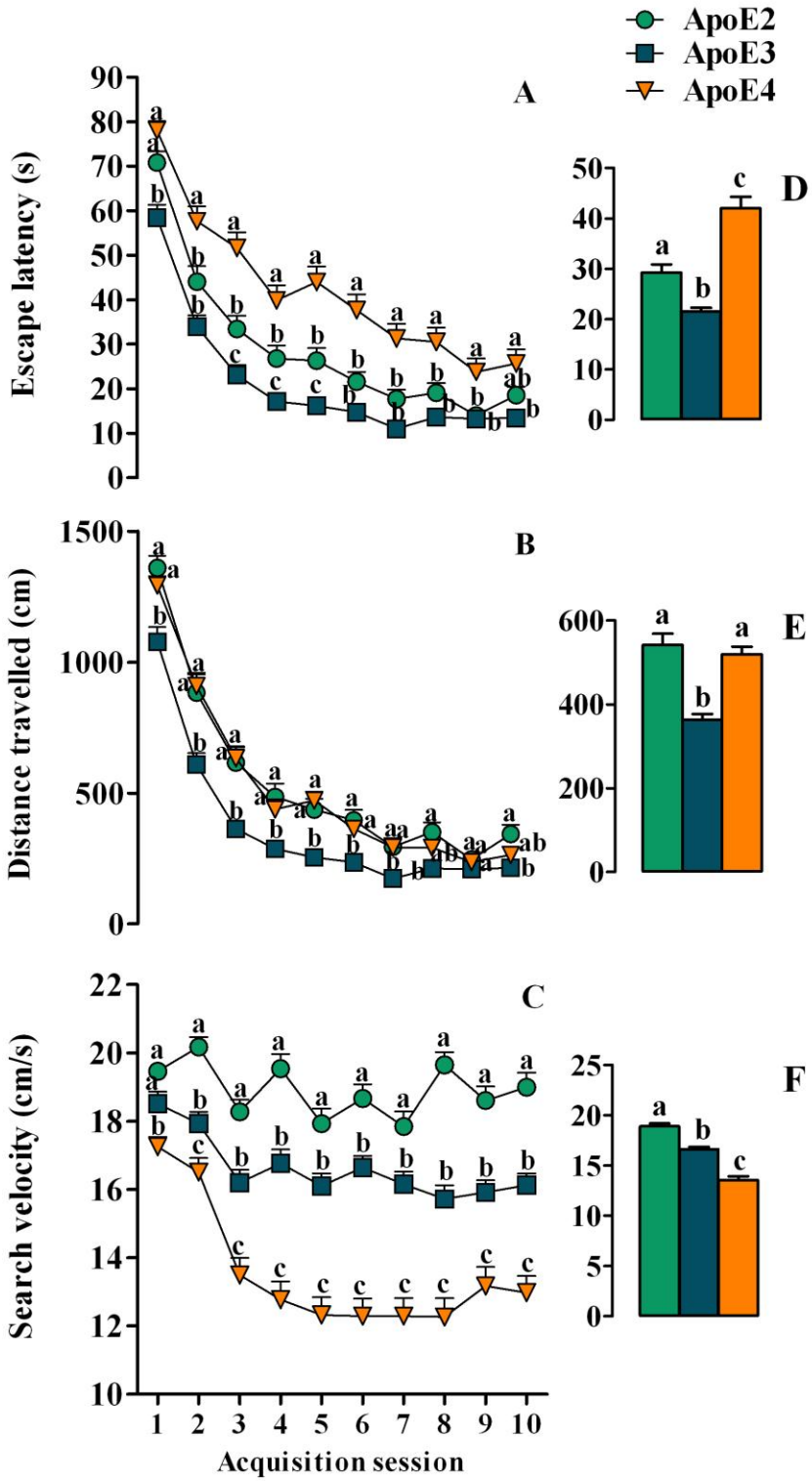

Figure II.12. MWM acquisition by genotype

Escape latency (A), distance travelled (B) and search velocity (C) and their mean of ten sessions respectively (D, E, F). Data are expressed as mean and S.E.M. Groups showing different letters a,b,c are different from each other at $p < 0.05$.

Gender Analyses on the acquisition of the MWM

We analyzed the performance by sex during the ten daily acquisition sessions of the MWM in order to find out whether the genotype or the BDE-209 treatment can affect males or females in a different way. Data, separately by sex, was analyzed by a two-way ANOVA (genotype x treatment) for repeated measures using the session as a within-subject factor and the escape latency (s), the distance travelled (cm), and the search velocity (cm/s) as variables.

As observed in the general analyses, both males and females improved over the sessions ($p>0.001$). The in-between subject analysis showed an effect of the genotype in both males and females for all the variables studied ($p<0.001$). ApoE4 males differed from the other two genotypes in escape latency, while in distance travelled ApoE3 males were who differed from the other genotypes, and the three genotypes were different in their search velocity among males. ApoE4 females differed from the other two genotypes in escape latency, ApoE3 females differed from the other genotypes in distance travelled, and the three genotypes were different in their search velocity among females. BDE-209 exposure affected acquisition in males, which was observed in distance travelled [$F(2,93)= 3.780$, $p=0.027$] An interaction between genotype and treatment [$F(2,93)= 2.711$, $p=0.035$] was also found in males.

In summary, the acquisition performance was affected by the genotype in the same way in both males and females, which reflects the overall genotype effect found in the general analyses above. However, the BDE-209 treatment effect is more evident in males.

Group Analyses on the acquisition of the MWM

Previous analyses showed some interactions within the genotype, the sex and the treatment on the acquisition performance. We analyzed performance during the ten sessions of acquisition by genotype and sex in order to find any differential effects of BDE-209 effects by group. A one-way ANOVA for repeated measures was carried out, using the session as a within-subject factor and the escape latency (s), the distance travelled (cm), and the search velocity (cm/s) as variables.

An effect of the treatment on the escape latency was observed in ApoE3 males [$F(2,28)= 9.165$, $p=0.001$]. An effect of the treatment on the distance travelled was found in both ApoE2 males [$F(2,30)= 4.581$, $p=0.019$] and ApoE3 males [$F(2,28)= 4.241$, $p=0.025$]. This result indicates that the effect of BDE-209 exposure found in ApoE2 and ApoE3 is attributable only in males of both groups.

In summary, only ApoE2 and ApoE3 males showed a general treatment effect on the MWM acquisition performance.

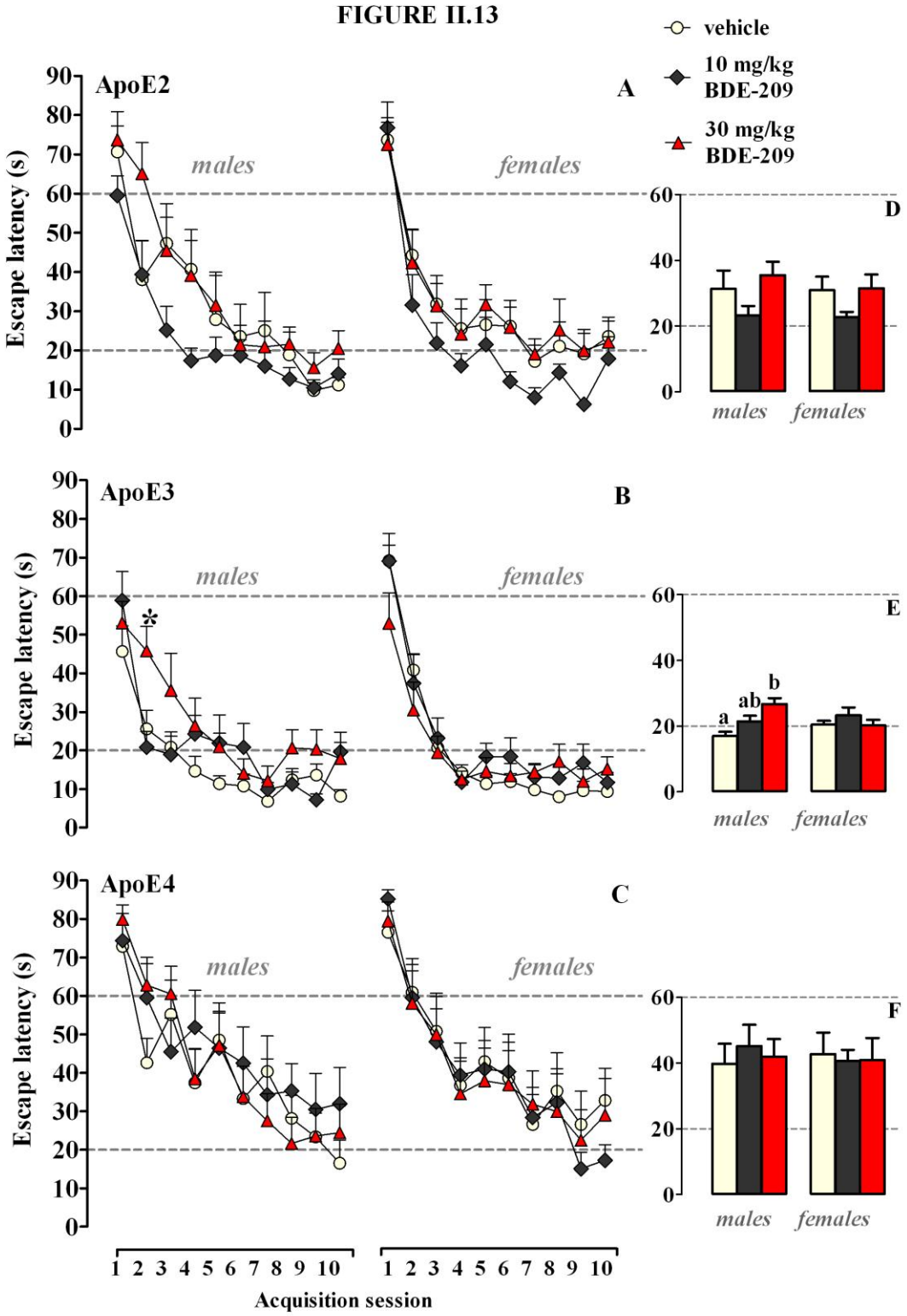

Figure II.13. MWM acquisition by genotype, sex and treatment

Escape latency (A, B, C) and their mean of ten sessions (D, E, F). Data are expressed as mean and S.E.M. The asterisk indicates the high dose group differs from the respective control. Different letters a, b indicate differences between groups. $p < 0.05$.

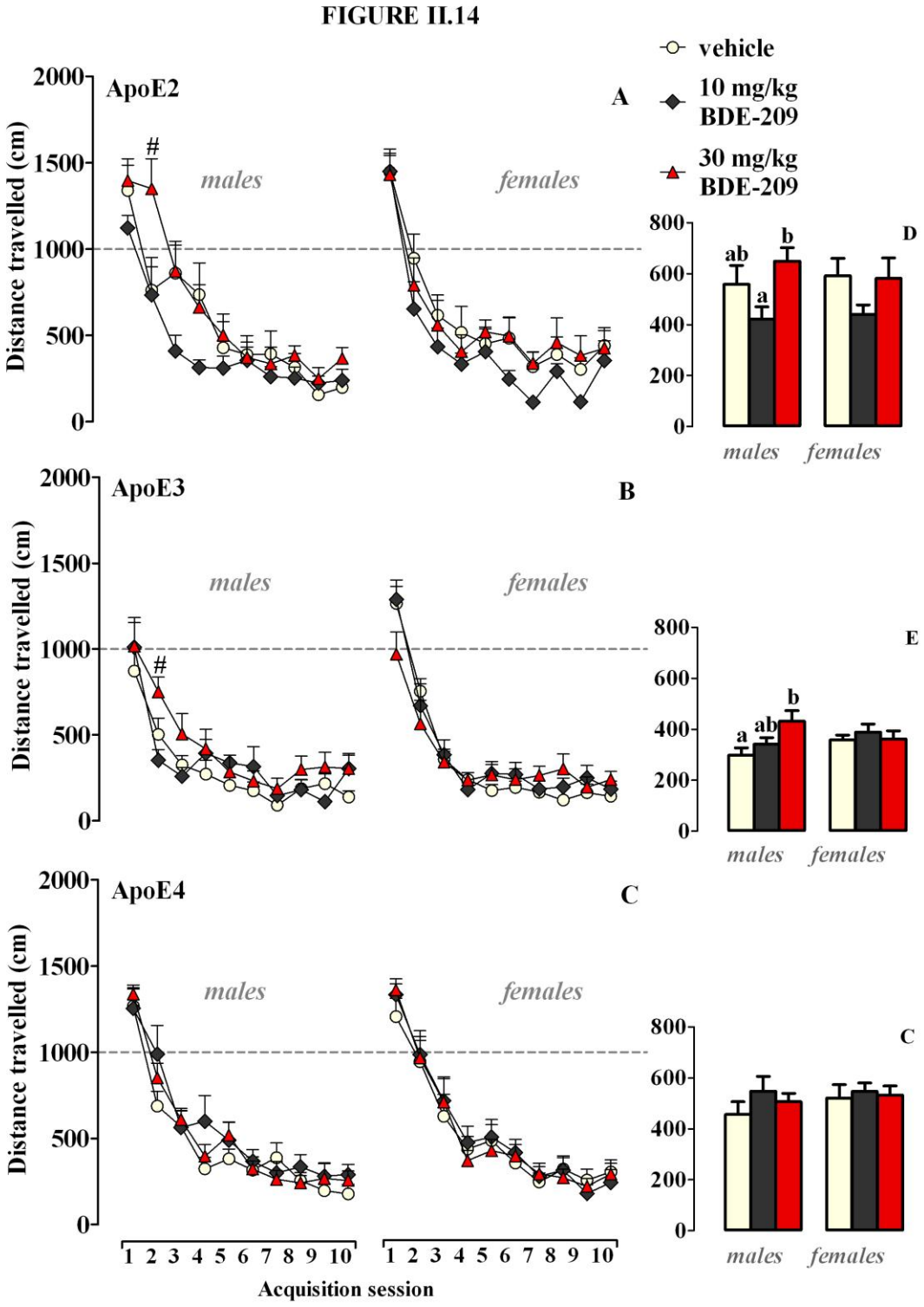

Figure II.14. MWM acquisition by genotype, sex and treatment

Distance travelled (A, B, C) and its mean of ten sessions (D, E, F). Data are expressed as mean and S.E.M. The symbol (#) indicates differences between the high and the low dose groups. Different letters a, b indicate differences between groups. $p < 0.05$.

FIGURE II.15

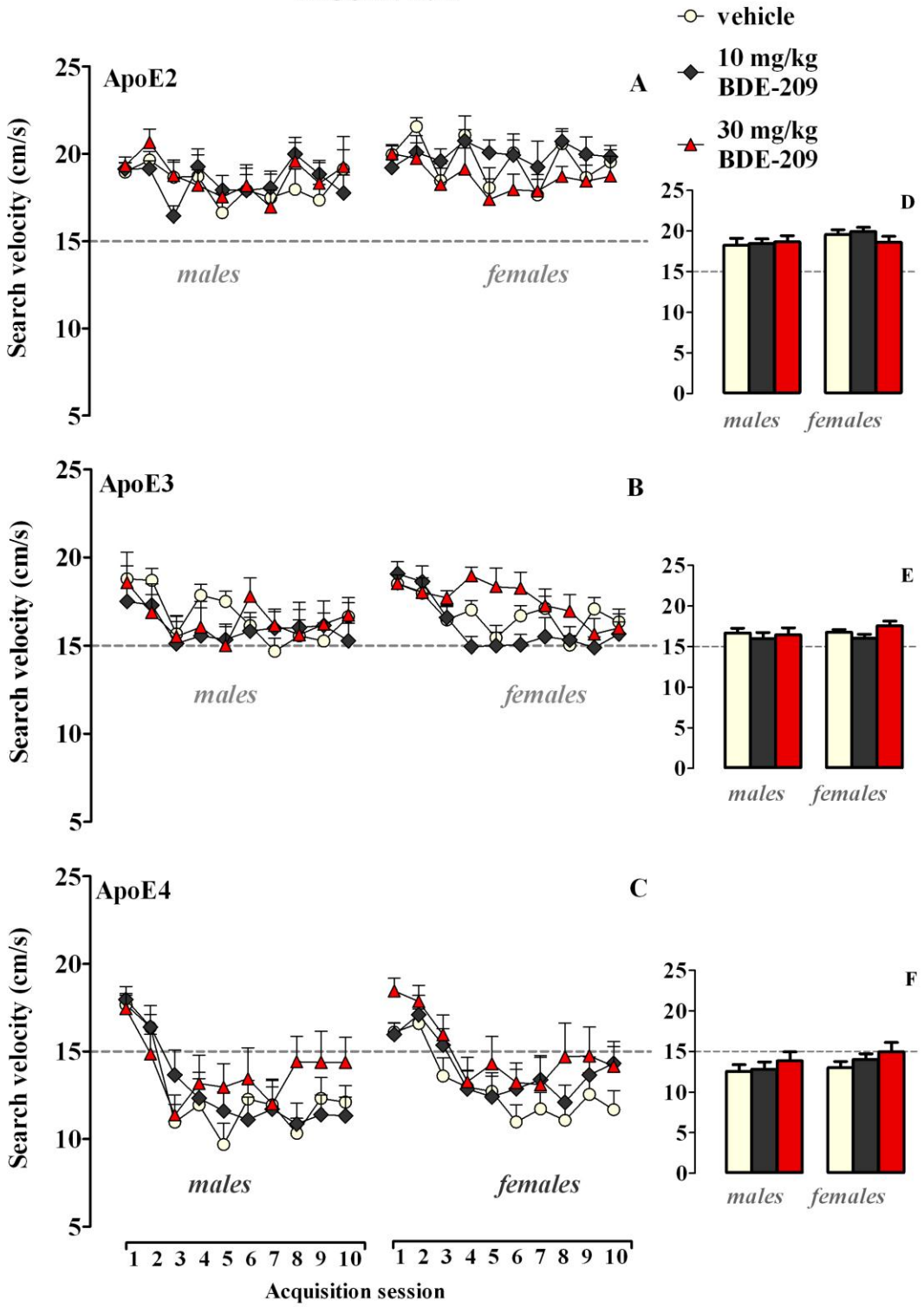

Figure II.15. MWM acquisition by genotype, sex and treatment

Search velocity (A, B, C) and their mean of ten sessions (D, E, F). Data are expressed as mean and S.E.M.

General analyses on retention during the MWM task acquisition

We analysed the retention ability in the probe trials carried out during the acquisition before the daily sessions on days 3, 5, 8 and 10, in order to find out whether progressive training affects the retention capacity and to find any effect of the genotype, treatment or sex. A three-way ANOVA (genotype x treatment x sex) for repeated measures was carried out, using the session as a within-subject factor and the time spent (s) in the target quadrant (Tgq) and the distance travelled (cm) in the Tgq as variables. As expected, there was a statistically significant effect of the session on the time spent in the Tgq [$F(3,190)= 36{,}934$, $p<0.01$] and the distance travelled in the Tgq [$F(3,190)= 32.841$, $p<0.001$]. An overall effect of genotype was found on the time spent in the Tgq [$F(2,191)= 4{,}619$, $p=0.011$] and on the distance travelled in the Tgq [$F(2,191)= 39.266$, $p<0.001$] (Figure II.16). No effects of the treatment or sex were observed. All the animals improved retention during the acquisition. ApoE3 showed better performance in the eighth session. ApoE4 travelled less distance in the Tgq compared to the other two genotypes in all probe trials (Figure II.16).

Genotype and group analyses on the retention of the MWM

As the genotype effect was significant in both acquisition and retention of the MWM task, we analysed separately the retention ability in each genotype using a two-way ANOVA (treatment x sex) for repeated measures.As observed in the general analysis, there was an effect of the probe trial in the time spent and the distance travelled in the Tgq ($p<0.001$) for all genotypes. No effects of treatment or sex were found in any of the

genotypes in the time spent in the Tgq, but an effect of treatment was observed in distance travelled in the Tgq in ApoE3 mice [F (2,60)= 3.160, p=0.05]. When analysing this effect in each group (genotype and sex), it was observed only in ApoE3 males [F (2,28)= 3.479, p=0.046], whom exposed to the high dose of BDE-209 differed from their controls. Main differences were found in session 5 (Figures II.17 and II.18).

FIGURE II.16

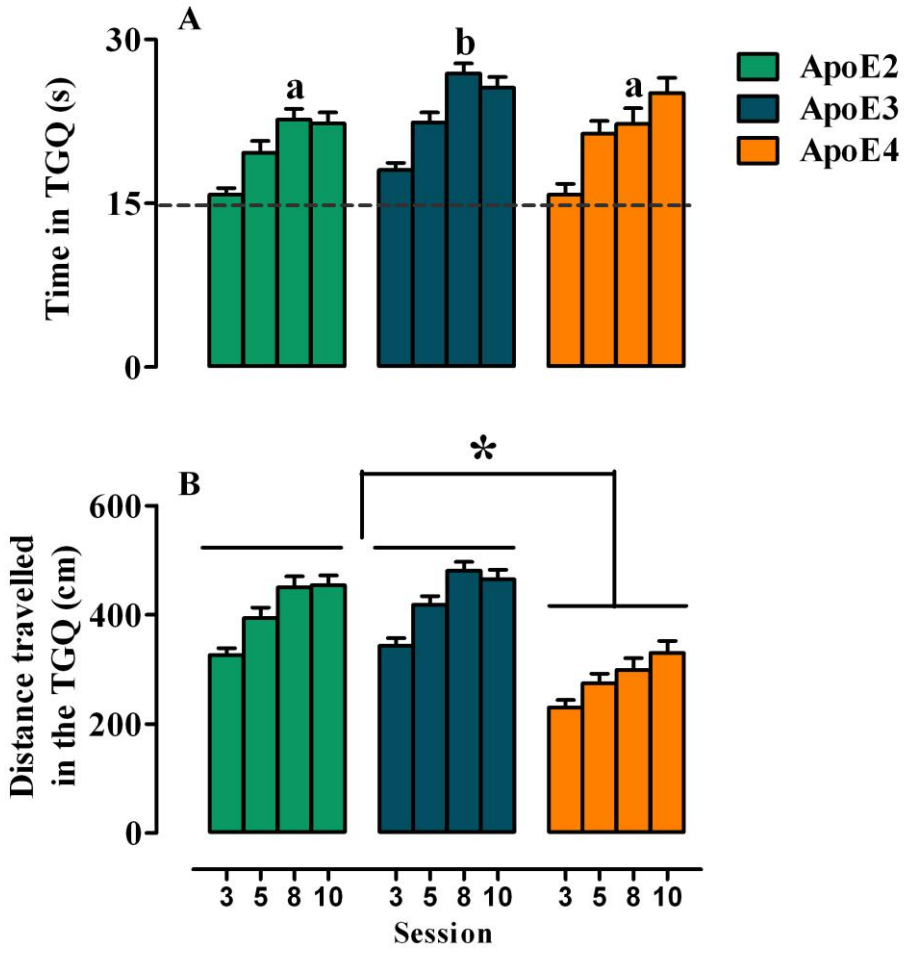

Figure II.16. MWM retention by genotype

Time spent (A) and distance traveled (B) in the Tgq in each probe trial. Data are expressed as mean and S.E.M. Groups showing different letters a,b, or an asterisk are different from each other at $p < 0.05$. Dashed line indicates execution by chance.

FIGURE II.17

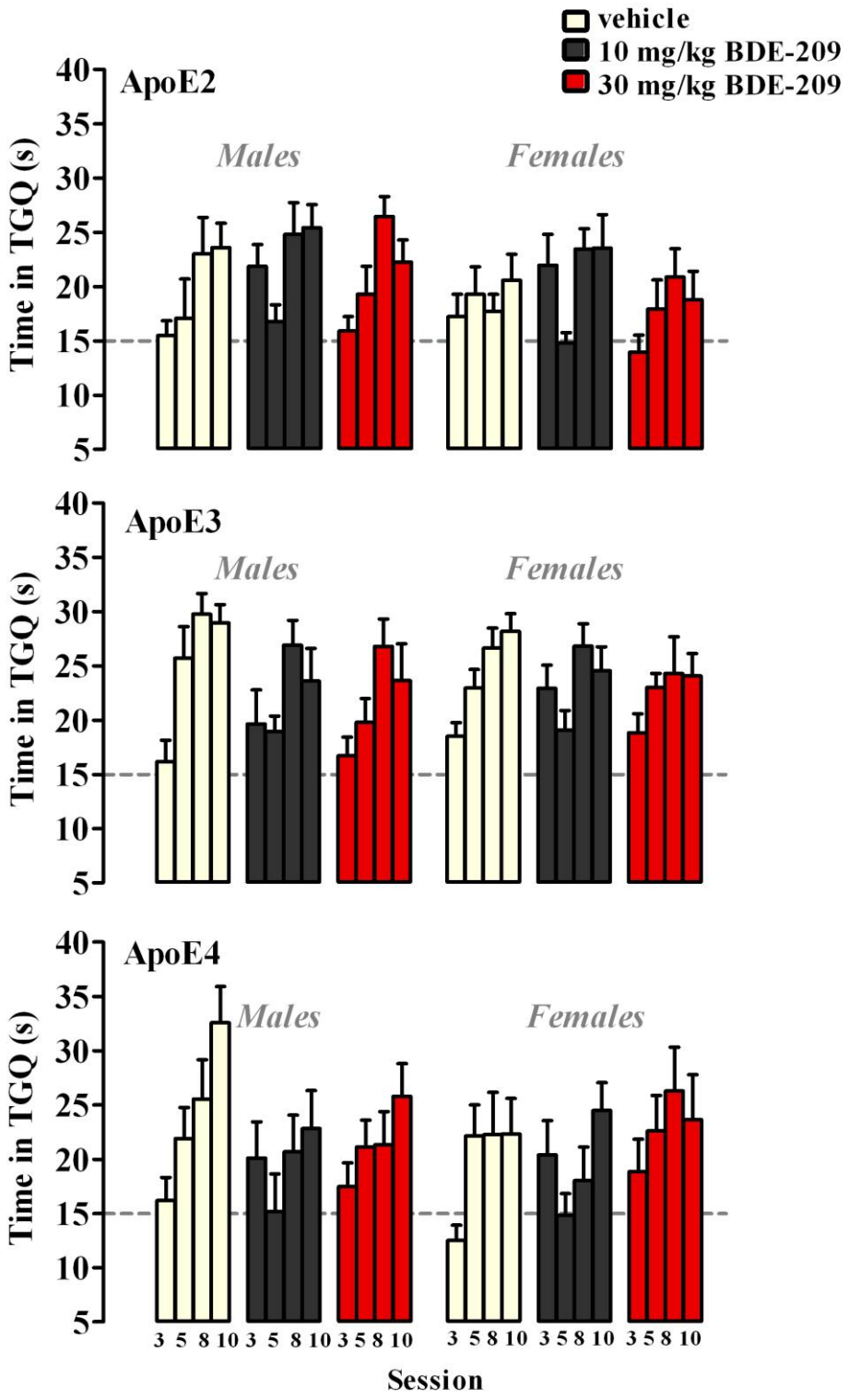

Figure II.17. MWM retention by genotype, sex and treatment

Time spent in the Tgq in each probe trial in sessions 3, 5, 8 and 10. Data are expressed as mean and S.E.M. The dashed line indicates execution by chance.

FIGURE II.18

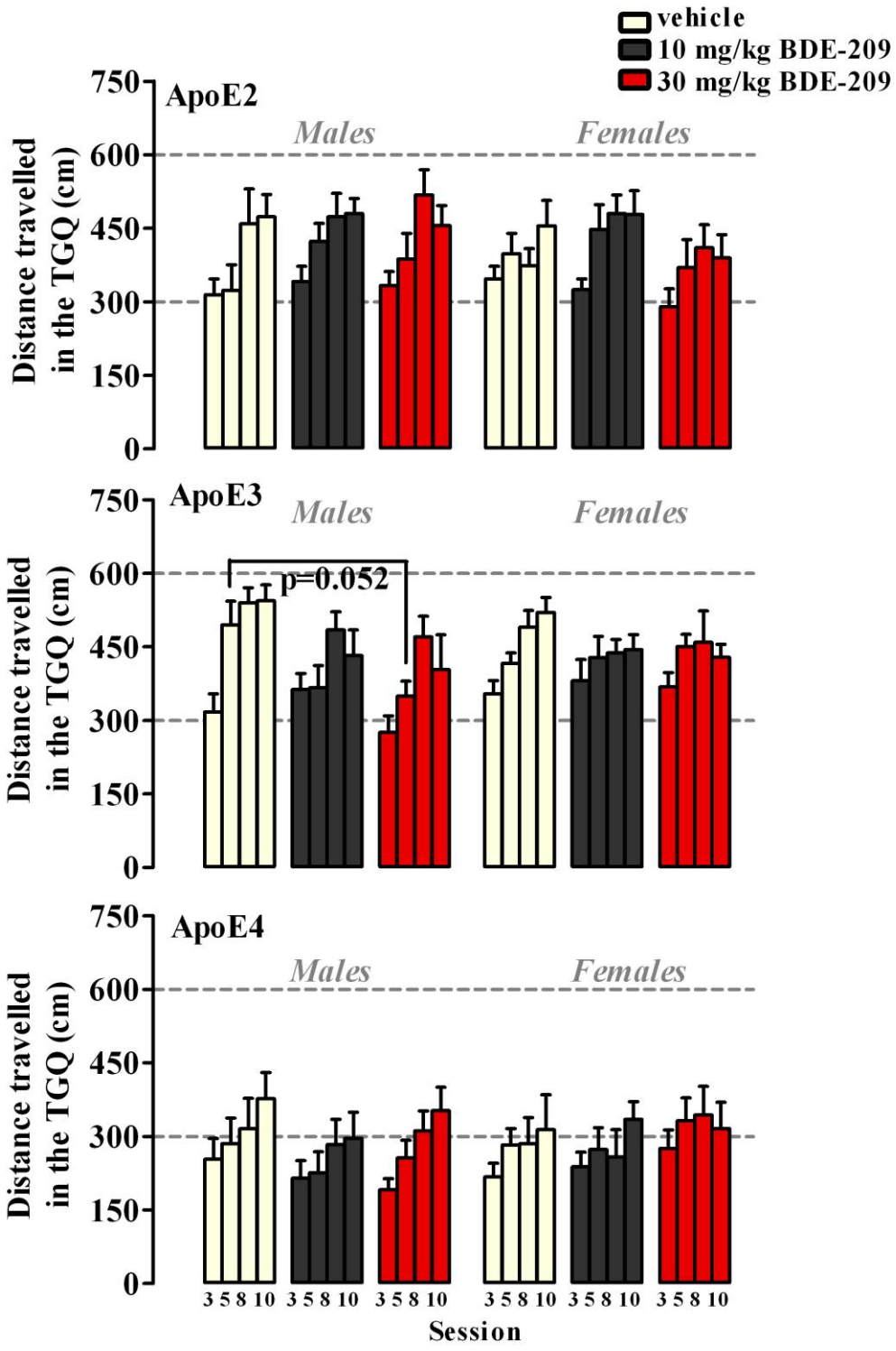

Figure II.18. MWM retention by genotype, sex and treatment

Distance travelled in the Tgq in each probe trial in sessions 3, 5, 8 and 10. Data are expressed as mean and S.E.M.

In summary, performance of retention probes during acquisition sessions improve over time, as expected. Males obtain better scores than females in the ApoE2 and ApoE4 groups, although sex differences are only statistically significant for ApoE4 mice not exposed to BDE-209 in the last probe trial ($p = 0.040$). Treatment effects were limited to one of the genotypes tested, the ApoE3.

Long-term retention of the MWM

Long-term memory assessed in a probe trial carried out 72h after the last training session was analysed by a three-way ANOVA (genotype x treatment x sex) on the time spent and the distance travelled in the Tgq. An overall effect of genotype was found in the distance travelled in the Tgq [$F(2,190)= 15.149$, $p<0.001$]. A trend towards an interaction between genotype, treatment and sex was also observed in the distance travelled in the Tgq [$F(4,190)= 2.404$, $p=0.052$]. Nevertheless all the animals performed above the chance level.

FIGURE II.19

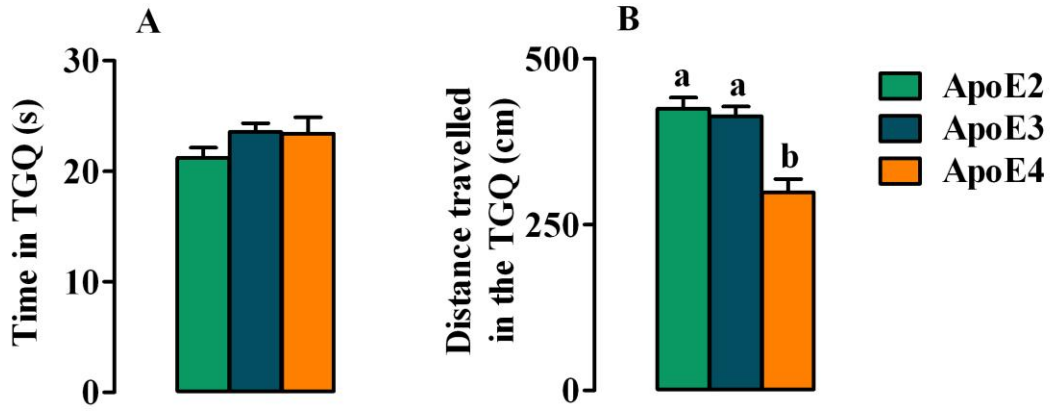

Figure II.19. Long-term retention in the MWM by genotype

Time spent (A) and distance travelled (B) in the Tgq 72h after the last session. Groups showing different letters a,b are different from each other at $p < 0.05$.

The analysis of the long-term retention by group (genotype and sex), showed a treatment effect that appeared only in ApoE3 females in the time spent in Tgq [F (2,31)= 3.807, p=0.034] and in the distance travelled in the Tgq [F (2,31)= 5.205, p=0.012]; but post-hoc analyses failed to show any differences among groups.

FIGURE II.20

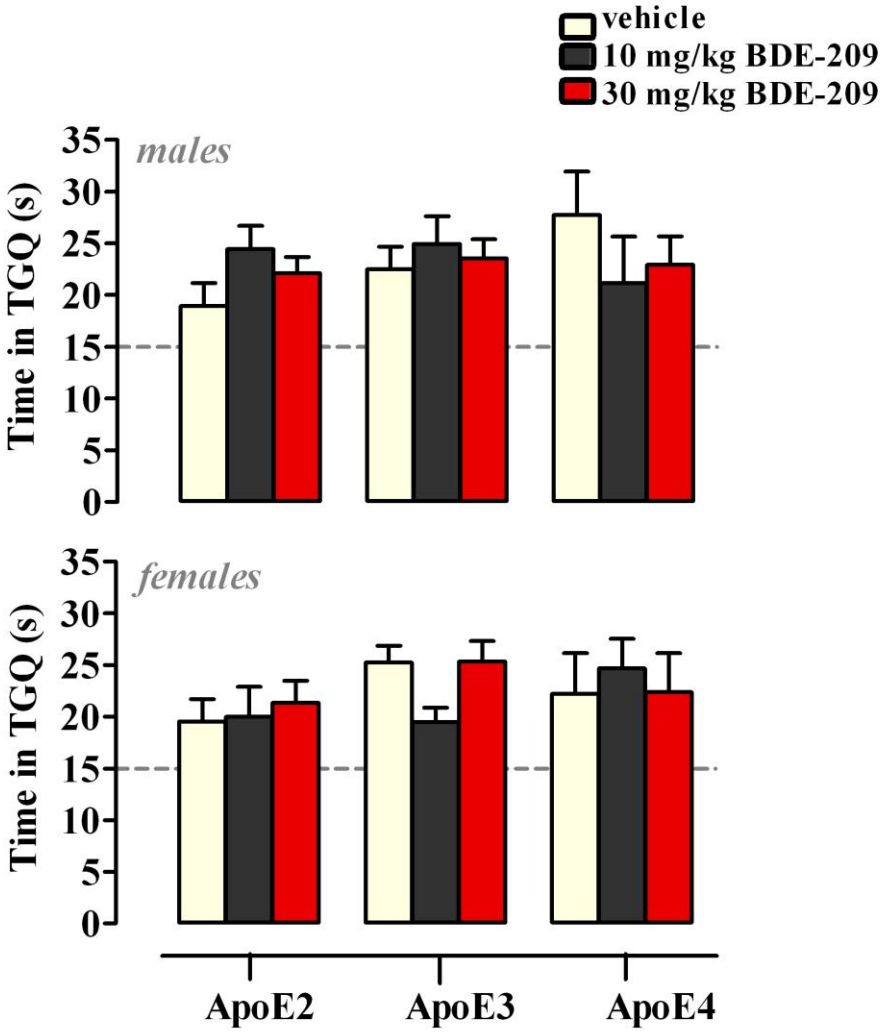

Figure II.20. Long-term retention in the MWM by genotype, treatment and sex

Time spent in the target quadrant in the 72h probe trial. Data are expressed as mean and S.E.M.

Briefly, the ApoE4 genotype travelled less distance in the Tgq during the long-term retention probe trial, although the time spent searching for the platform in the Tgq is similar to the other genotypes. In general terms, treatment effects on long-term retention are isolated and showed high variability between genotypes.

Thyroid hormones

The level of thyroid hormones, T3 (nmol/L), T4 (nmol/L) and free FT4 (pmol/L) was analysed by a three-way ANOVA (genotype x treatment x sex) on the quantity of T3 (nmol/L), on the quantity of T4 (nmol/L) and on the quantity of FT4 (pmol/L). Reference values for the forms analysed are: FT4 (10-20); T4 (58-140); T3 (0.9-2.8).

An overall effect of genotype was found in T3 [(F (2,51) = 4.090, p=0.026)]. ApoE2 showed inferior levels of T3 compared with ApoE3 and ApoE4. No effects of the treatment or interactions were observed (Figure II.21).

The effect of BDE-209 exposure on the hormones levels was analysed separately in each sex by a one-way ANOVA. No effect of the treatment was found, but using the BDE-209 exposure (control animals vs. animals exposed to low and high dose) as a factor a significant effect of the exposure was shown in female FT4 levels [F(1,24)=6.237, p=0.02]. Females exposed to BDE-209 showed a decrease in FT4 hormone levels (Figure II.22). The same analyses were carried out by group (genotype and sex), but no significant differences among treatment conditions were found in any group.

FIGURE II.21

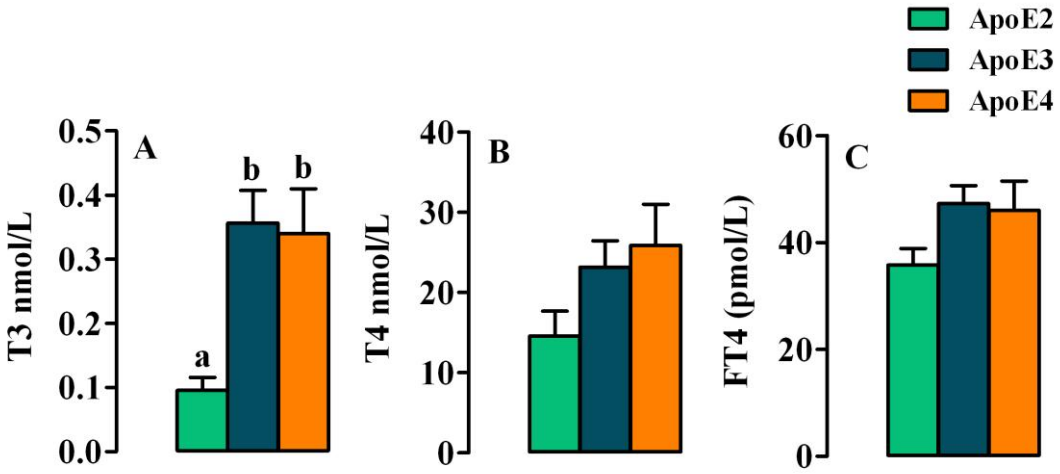

Figure II.21. Levels of thyroid hormones by genotype

Data are expressed as mean and S.E.M. Groups showing different letters a,b,c are different from each other at $p < 0.05$.

FIGURE II.22

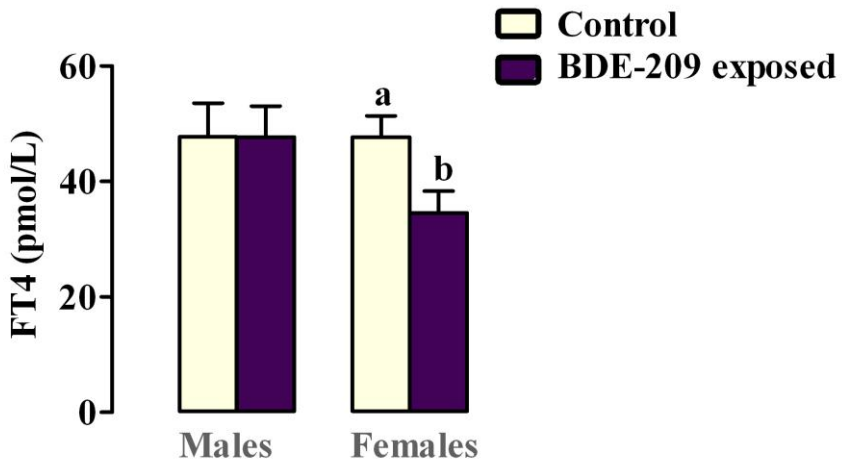

Figure II.22. Levels of free T4; Effects of sex and treatment

Data are expressed as mean and S.E.M. Groups showing different letters a,b are different from each other at $p < 0.05$.

BDNF and TrkB receptor levels

A two-way ANOVA (genotype x treatment) was performed on the levels of BDNF and TrkB in the hippocampus. Results showed an overall effect of the genotype on both the BDNF levels [$F(2,37)=3.845$, $p=0.032$] and the TrkB receptor levels [$F(2,37)=23.613$, $p<0.001$] (Figure II.24). No effects of BDE-209 treatment were observed.

FIGURE II.23

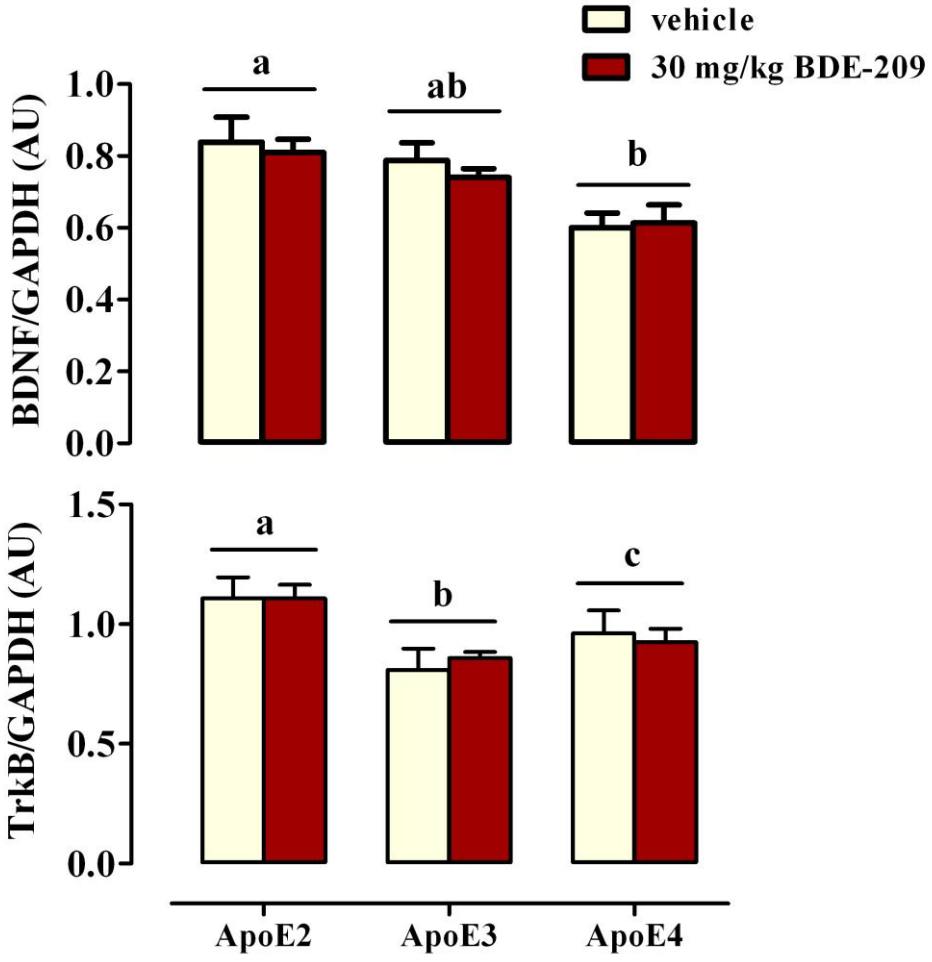

Figure II.23. BDNF and TrkB levels in the hippocampus

Data are expressed as mean and S.E.M. for control and exposed ApoE2, ApoE3 and ApoE4 male mice. Groups showing different letters a,b,c are different from each other at $p<0.05$.

2.2 Discussion of Experimental Phase II: 4 months old

ApoE genotype showed a strong influence on behaviour and interacted with sex and with neonatal exposure to BDE-209 effects in young adults.

In this study, differences in body weight between ApoE genotypes at 4 months of age were only observed in female mice. ApoE4 females showed lower body weight compared to both ApoE2 and ApoE3 females. Accordingly, the French group of Mathis and co-workers found lower body weight in ApoE4 mice compared to ApoE3 at 4 months of age, especially in females (Grootendorst et al. 2005). Human studies also support the role of ApoE in the regulation of the metabolism and associate the ApoE4 allele with lower body weight (Long et al. 2003; Arbones-Mainar et al. 2008; Pendse et al. 2009; Ferreira et al. 2011). Exploratory behaviour, locomotor activity and anxiety were also affected by the ApoE genotype in mice. ApoE4 mice showed less exploration activity in the EZM and in the OF compared to ApoE2 and ApoE3 mice. In agreement with this result, Grottendorst and collaborators found less activity in ApoE4 mice compared to ApoE3 at 4-5 months of age, and Siegel and coworkers found that at 6-8 months of age ApoE4 female mice moved less in the Open Field than ApoE2 and ApoE3 (Grootendorst et al. 2005; Siegel et al. 2010). On the contrary, ApoE4-GFAP mice did not differ in activity levels from ApoE3-GFAP mice (Hartman et al. 2001). Surprisingly, our results revealed that ApoE2 travelled longer distances in Open Field compared to ApoE3 and ApoE4; this increased activity was observed especially in ApoE2 females, which maintained high levels of activity during the whole testing period. ApoE2 hyperactivity has not been reported before and differs from a recent study where ApoE2 females aged 6-8 months moved like ApoE3 females

(Siegel et al. 2010). As we have not found higher activity in ApoE2 in the EZM, which is a more anxiogenic test, we speculated that differences in the protocol might be the reason for these discrepancies. That is to say that the anxiety component might mask differences in the levels of activity. These effects should be further investigated. Interestingly, in our study differences found in activity are not likely to be beyond differences in body weight, which are probably more related to metabolic rate characteristics in these genotypes.

Anxiety-like behaviour was also affected by the genotype. In the EZM, ApoE4 mice made fewer crossings into the open area, spent less time there, and showed more freezing compared to ApoE2 and ApoE3. In the OF, ApoE4 also made fewer entries into the centre compared to the other genotypes and spent less time in the centre compared to ApoE3. Broadly, ApoE3 exhibited the less anxious phenotype among the three genotypes, while ApoE4 showed the most anxious one. Differential effects on measures of anxiety related to ApoE isoforms have been reported previously, likewise indicating high anxiety scores in ApoE4 mice (Robertson et al. 2005; Raber 2007; Siegel et al. 2010).

The three ApoE genotypes also showed differences in spatial learning in the MWM spatial reference task. ApoE4 took longer to reach the hidden platform throughout the training, while ApoE3 achieved very good latencies from the fourth session onwards. ApoE3 also reached the platform in the shortest distance and had better retention ability compared to the other genotypes. Other studies that have investigated spatial learning related to ApoE isoforms did not find any differences in acquisition at young ages, but impaired retention in ApoE4, especially in females (Raber

et al. 2000; Grootendorst et al. 2005; van Meer et al. 2007). On the other hand, Siegel et al (2010) obtained a better performance in the MWM acquisition in young ApoE4 females, and no genotype differences in retention (Siegel et al. 2010). These differences between studies might be due to differences in the spatial task protocol. . In this study, we added a rotational inner wall to the apparatus in order to avoid intra-maze cues, which has been shown to be more sensitive to detect spatial learning impairments in a transgenic animal model for Alzheimer's disease (Ribes et al. 2008). This modification prevents the animals from using a strategy based in proximal cues, and might account for the differences in spatial learning performance observed among the genotypes. Fewer trials per day and a long training period in our experiment, might explain why all the groups achieve good learning results at the end of the training and comparable retention performance among genotypes.

Thyroid hormone levels in mice were also influenced by the ApoE genotype. In our study, ApoE2 mice showed lower levels of thyroid hormones in blood serum compared with ApoE3 and ApoE4, although a significant difference was observed only for T3 levels. This suggests there might be a reduction in hormone synthesis in the ApoE2 group. There is evidence in the literature of an interaction between thyroid hormones and apolipoproteins, although studies relating ApoE polimorphisms with thyroid hormone levels are rare. Lipoproteins, especially HDL, are plasma carriers for a small number of thyroid hormones, which bind by interactions with the specific apolipoproteins including ApoE (Benvenga 1997; Benvenga et al. 1999). Furthermore, the expression of the ApoE gene can be controlled by thyroid hormones (Vandenbrouck et al. 1994). Among children with hypothyroidism, no differences have been reported linked to

ApoE genotypes (Ciomartan et al. 1999). Instead, a recent study found low levels of FT4 in postmenopausal women carrying ApoE2 or ApoE4 alleles compared with the E3/3 genotype. On the other hand, ApoE2 carriers showed higher levels of anti-TG antibodies (Lambrinoudaki et al. 2009). Taken together, the data suggest that the ApoE genotype is related to differences in thyroid hormone levels, which modulate brain organization and function and consequently may influence behaviour. More studies are needed to assess thyroid hormones among ApoE genotypes in humans.

Finally, we found differences in BDNF levels in the hippocampus, among ApoE2 mice compared to ApoE4 mice, while levels of ApoE3 were similar to the other two genotypes. We also observed differences between genotypes in levels of the BDNF receptor TrkB. ApoE2 showed the highest receptor levels, while ApoE3 showed the lowest. Nichol and co-workers reported similar levels of BDNF in the hippocampus of ApoE3 and ApoE4 mice, but they did not report data on ApoE2 mice. They also observed lower TrkB receptor levels in sedentary ApoE4 mice compared to ApoE3, but these levels increased with exercise in ApoE4 mice (Nichol et al. 2009). There are some differences related to the age and housing of the mice between that study and our study. In Nichol's study mice were 10-12 months and housed alone, while in our study, mice were 6 months old and housed in a group, and we did not introduce any exercise conditions. BDNF is involved in synaptic plasticity, neuronal differentiation and survival of neurons (Klein et al. 2011), so any challenge inducing activity in the CNS might modify these levels.

It should be highlighted that in our study, neonatal BDE-209 exposure has been shown to affect body weight, behaviour and learning in young adults, depending on the genotype and sex.

ApoE4 males exposed to low and high doses of BDE-209 showed a significant decrease in body weight compared to ApoE4 control males. Instead, ApoE3 exposed females showed a slight increase in body weight. Few studies report changes in body weight after perinatal PBDE exposure in young adult rodents (Eriksson et al. 2002; Viberg et al. 2002; Viberg et al. 2003; Viberg et al. 2003; Viberg et al. 2004; Viberg et al. 2007; Johansson et al. 2008), or after post-weaning administering or chronic exposure of PBDE (Stoker et al. 2004; Darnerud et al. 2007; Richardson et al. 2008; Driscoll et al. 2009). Only two investigations report a higher body weight in animals postnatally exposed to low-brominated compounds, which appeared after the developing period at 1 or 2 months of age (Dufault et al. 2005; Gee et al. 2008).

Postnatal exposure to BDE-209 showed to affect activity levels at 4 months, depending on the genotype and sex. Rearing increased in treated ApoE2 male mice, while it decreased in ApoE3 and ApoE4 females. ApoE4 females were more affected by the treatment since they showed diminished rearing, decreased distance travelled and fewer crossings to the centre. Effects of PBDEs in locomotor activity have been widely reported in rodents, usually characterized by hyperactivity and lack of habituation (Eriksson et al. 2002; Viberg et al. 2002; Viberg et al. 2003; Viberg et al. 2004; Branchi et al. 2005; Kuriyama et al. 2007; Gee et al. 2008). The BDE-209 compound has also been shown to disrupt locomotor activity in adulthood, after an early postnatal exposure. Regarding this, the Swedish group of Eriksson and coworkers observed initial hypoactivity followed by

hyperactivity due to the lack of habituation in rats and mice (2,4 and 6 months old) exposed to a single oral dose of BDE-209 on PND3 (Viberg et al. 2003; Viberg et al. 2007; Johansson et al. 2008). Rice and collaborators observed an effect of hyperactivity in 2-months-old C57BL/6J male mice exposed to BDE-209 from PND2 to PND15. In our experiment BDE-209 affected the pattern of habituation, inducing hyperactivity in ApoE2 males, while ApoE4 exposed females showed hypoactivity.

In our study, spatial learning and memory at 4 months was affected by BDE-209 treatment only in ApoE3 males. ApoE3 males exposed to high doses showed higher latencies and distance travelled to reach the hidden platform during acquisition and showed worse retention in the second probe trial compared to their control counterparts. Learning and memory have been also studied after neonatal exposure to PBDEs. Impaired learning in a visual discrimination task was observed in adult rats after postnatal exposure to penta DE-71. Exposed rats, compared to controls, committed more errors and omissions and required more trials to reach the criterion in the five choice serial reaction task (Dufault et al. 2005). Referring to spatial learning, tetra BDE-47 exposure impaired both acquisition and retention in a MWM (He et al. 2009), while animals exposed to penta, hexa and octa congeners had higher latencies during acquisition and impaired re-learning processes (Eriksson et al. 2001; Viberg et al. 2003; Viberg et al. 2006; Cheng et al. 2009). The effect of the BDE-209 exposure in the MWM performance has not previously been assessed, but impaired performance in a light-dark discrimination task was observed in old mice (16 months) although not in the young, after postnatal exposure to BDE-209 (Rice et al. 2009). In the current study, we have seen that the ApoE3 mice, which were the best learner, showed poorer

acquisition performance and retention in the MWM, four months after acute exposure to BDE-209 at PND10.

Collecting all behavioural data, we can determine that BDE-209 affects specific functional domains depending on the genotype. The weak point of ApoE4, which was their lower activity levels, was disrupted by BDE-209, causing more hypoactivity in females. Instead, BDE-209 affected the strong point in ApoE3 male mice, impairing their good learning and memory. For the latter, we suspect that effects in other genotypes are masked by the strong genotype influence.

Levels of thyroid hormone were also affected by BDE-209 exposure in female mice. Females exposed postnatally to BDE-209 showed significant lower levels of FT4 compared to control females at four months. The effects of PBDE as endocrine disruptors are well known, and their effects on thyroid hormones are widely reported. Total serum and free T4 have been found to decreases in humans and rodents after exposure to lower bromine compounds during both the gestational period and after birth (Zhou et al. 2002; Branchi et al. 2005; Darnerud et al. 2007; Kuriyama et al. 2007; Rice et al. 2007; Richardson et al. 2008). The effects of BDE-209 on thyroid hormones have recently been studied. Lower T4 and T3 was found in dams (Chi et al. 2011) and in female rat offspring (Kim et al. 2009) exposed to very high doses of BDE-209 during gestation. As far as we know, this is the first study to report changes in thyroid hormone levels after acute postnatal exposure to BDE-209 at relevant doses for human exposure. Because the only hormone affected is FT4, the reduction of FT4 levels in exposed females might be related to an increase in its degradation by UDGPT, which was reported by Zhou et al (2002).

On the whole, results of the second experimental phase indicate that early postnatal exposure to BDE-209 produces long-term derangements in different aspects of the behavioural spectrum and hormonal alterations, depending on distinct genetic vulnerabilities and sex.

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NEUROBEHAVIOURAL EFFECTS ASSOCIATED WITH POSTNATAL EXPOSURE TO DECABROMODIPHENYL ETHER IN APOE2, APOE3 AND APOE4 TRANSGENIC MICE
Ingrid Reverté Soler
DL:T. 162-2012

Experimental Phase III: 12 months old

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3. Experimental Phase III: 12 months-old

Experimental Phase III consisted in the evaluation of ApoE transgenic female mice at 12 months of age that had been exposed to BDE-209 on PND10. Anxiety and locomotor activity were assessed in an EZM and an OF. Spatial learning and memory were assessed in a MWM spatial reference task. The results of the third experimental phase are explained in the following pages, organized as follows: body weight, anxiety and activity measures in the Elevated Zero Maze and in the Open Field, and learning and memory in the Morris Water Maze reference memory task.

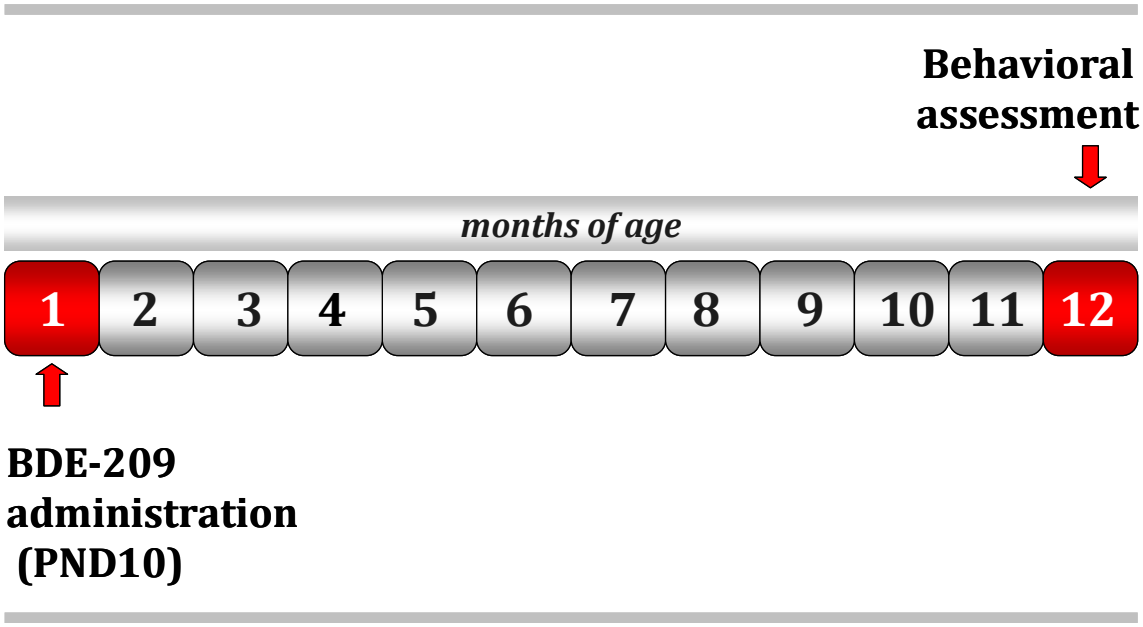

Figure III. Scheme of Experimental Phase III: 12 months old

3.1 Results of the Experimental Phase III: 12 months old

Body weight

Body weight of ApoE female mice at 12 months of age was analysed by a two-way ANOVA (genotype x treatment) and an overall effect of the genotype was observed [F(2,89)=4.957, p=0.009] (Figure III.1).
Analysing the effect of the treatment in each genotype separately, an effect of BDE-209 exposure emerged in the ApoE4 group when control group was compared to the low and the high doses taken together [F(1,36)=4.504, p=0.041] (Figure III.2).

FIGURE III.1

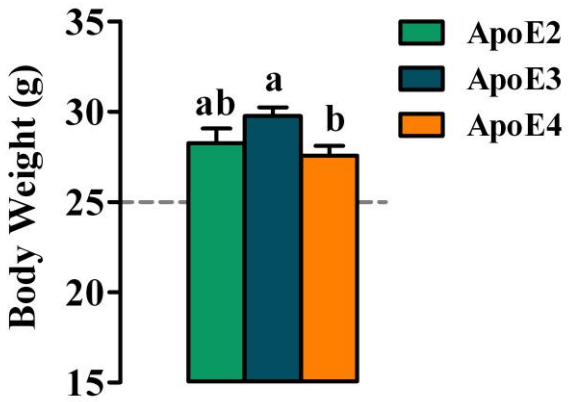

Figure III.1. Body weight at 12 months of age by genotype

Data is expressed as mean and S.E.M. Groups showing different letters a,b are significantly different from each other at $p < 0.05$.

FIGURE III.2

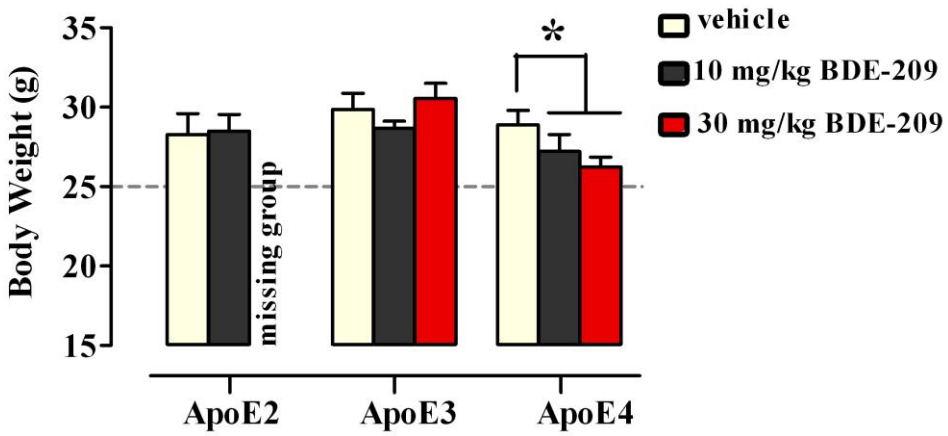

Figure III.2. Body weight at 12 months of age by genotype and treatment

Data is expressed as mean and S.E.M. Groups showing an asterisk differ at $p < 0.05$.

Elevated Zero Maze

The evaluation of anxiety at twelve months of age was conducted in an EZM test. Nine animals were excluded from the analyses because they fell off the apparatus (Table 5).

Table 5: Mice excluded of the EZM analysis at 12 months of age

	ApoE2 females	ApoE3 females	ApoE4 females	
0 mg/kg	1	2	4	7
10 mg/kg	0	0	0	0
30 mg/kg	-	0	1	1
	1	2	5	

Table 5. Number of animals excluded from the statistical analyses because they fell off the EZM, and its distribution among groups. Data are expressed as absolute values.

The analysis of the behaviour in the EZM was carried out by a two-way ANOVA (genotype x treatment) on the latency to enter the closed area, the time spent in the open area, the number of crossings from the closed to the open area, and the number of head dips, rearing, grooming, freezing and defecation. A correction was made to the time spent in the open area; we subtracted the latency to enter the closed area from the total time spent in the open area. This correction was made in order to find a measure of the time the animals spent in the open area when they crossed voluntarily. The number of crossings from the closed to the open area and the corrected time in the open area did not show variance homogeneity, so the non-parametrical Kruskall-Wallis test was used to analyse these variables.

An overall effect of the genotype was found in the corrected time in the open area [X^2=19.945, p<0.001], in the number of crossings [X^2=25.484, p<0.001], in the number of head-dips [F(2,80)=4.744, p=0.012], in rearing [F(2,80)=4.463, p=0.015] and in freezing [F(2,80)=3.884, p=0.025]. ApoE4 made fewer crossings and spent less time in the open area and also carried out less rearing and more freezing than the other genotypes. ApoE2 did more head-dips compared to the other genotypes (Figure III.3). No effects of the BDE-209 treatment were observed.

A one-way ANOVA was performed in each genotype separately, but no differential effects of the BDE-209 treatment were observed in any of the genotypes (Figure III.4).

In summary, the results of the EZM showed increased levels of anxiety and decreased exploration in ApoE4 females at 12 months of age. BDE-209 treatment does not affect behaviour in the EZM at 12 months of age.

FIGURE III.3

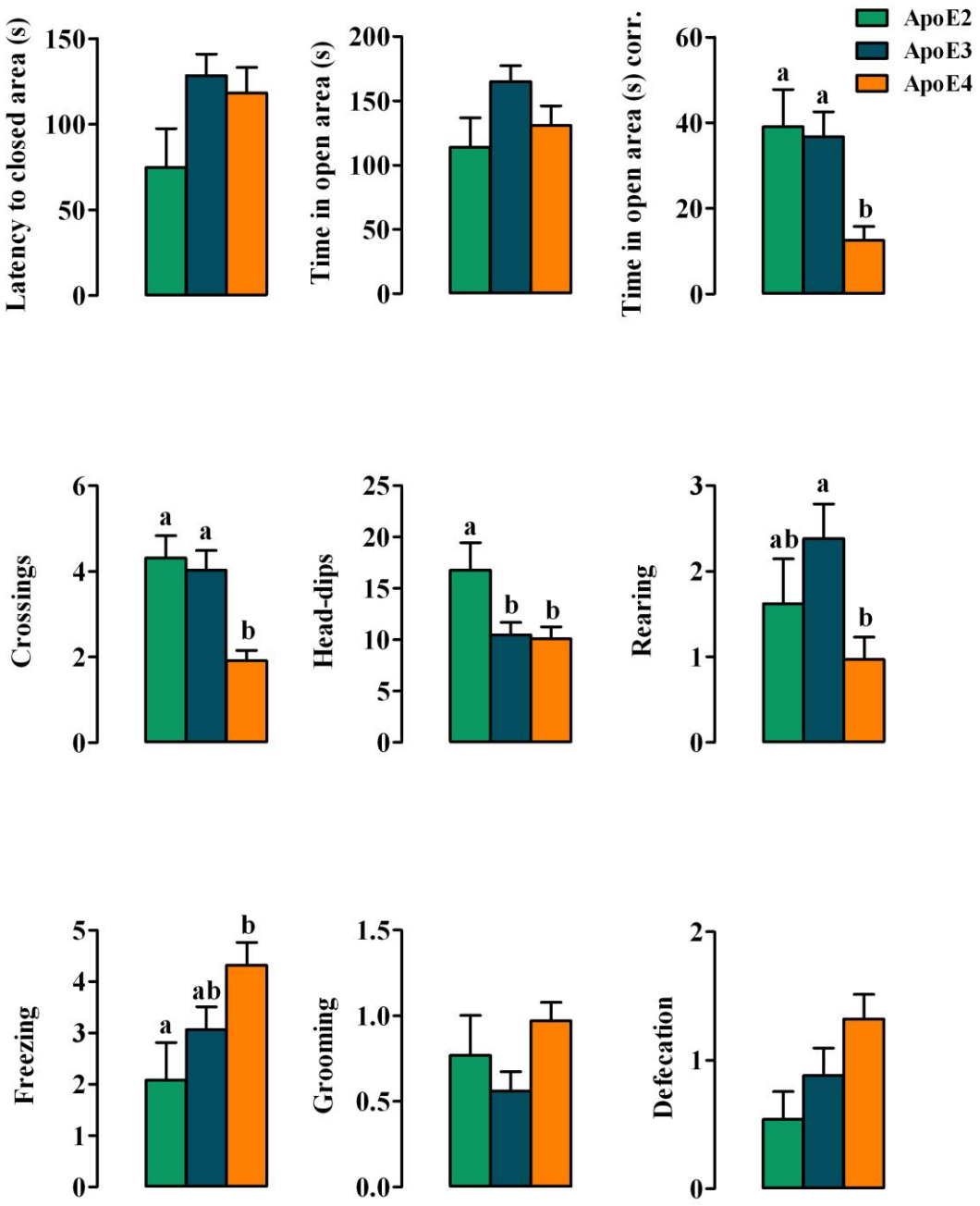

Figure III.3. Behaviour in an EZM at 12 months of age by genotype

Data are expressed as mean and S.E.M. Groups showing different letters a,b,c are different from each other at $p < 0.05$.

FIGURE III.4

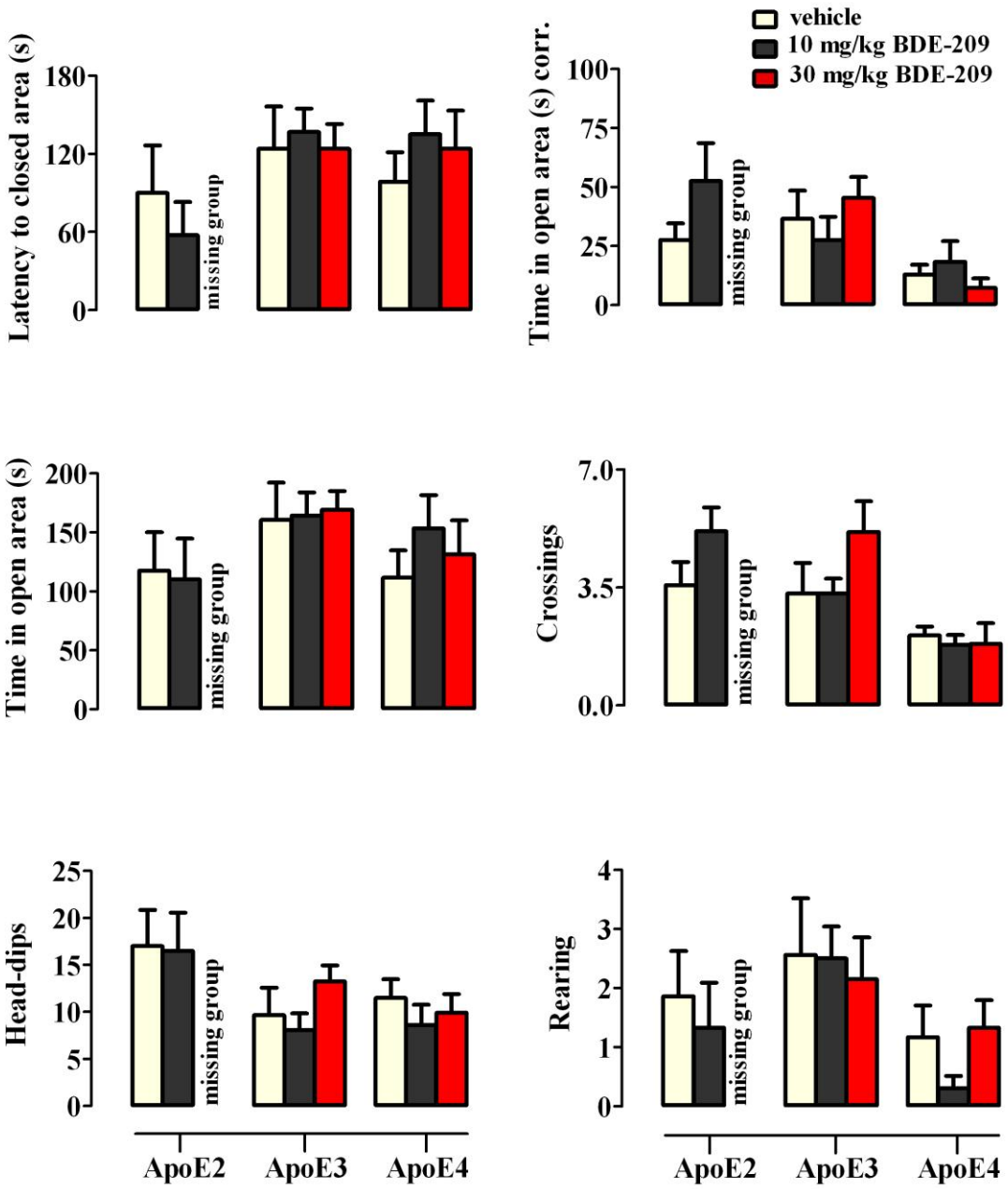

Figure III.4. Behaviour in the EZM at 12 months old by genotype and treatment

Data is expressed as mean and S.E.M.

Open Field

The evaluation of anxiety and locomotor activity at 12 months old was also carried out in an OF test. General activity was analysed by two-way ANOVA (genotype x treatment) for repeated measures using six 5-min periods as a within-subject factor and the distance travelled (cm) as a dependent variable. There was a significant effect of the period of time on the distance travelled [$F(5, 89) = 25.902$, $p<0.001$], indicating that the animals habituate to the new space. An overall effect of genotype [$F(2,89)= 10.395$, $p<0.001$] was found in the distance travelled throughout the test period (Figure III.6). No effects of the treatment or interactions were observed.

FIGURE III.5

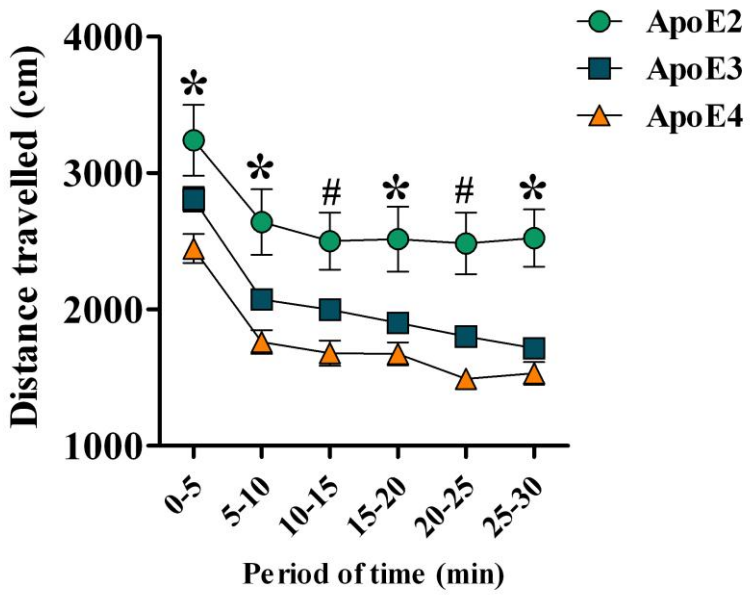

Figure III.5. Distance travelled in the OF by genotype at 12 months

Data is expressed as mean and S.E.M. The asterisk indicates ApoE2 differ from the other genotypes, and the symbol # that all genotypes differ among them, at $p < 0.05$.

A two-way ANOVA (genotype x treatment) was performed on the total distance travelled and the total rearing performed. These variables did not show variance homogeneity, so the non-parametrical Kruskall-Wallis test was used. An overall effect of the genotype was found in the total number of rearings [$F(2,89)=12.495$, $p<0.001$] and the total distance travelled (cm) [$F(2,89)=10.395$, $p<0.001$], showing lower levels of activity in ApoE4 females (Figure III.6). No effects of the BDE-209 treatment were observed in general activity measures.

FIGURE III.6

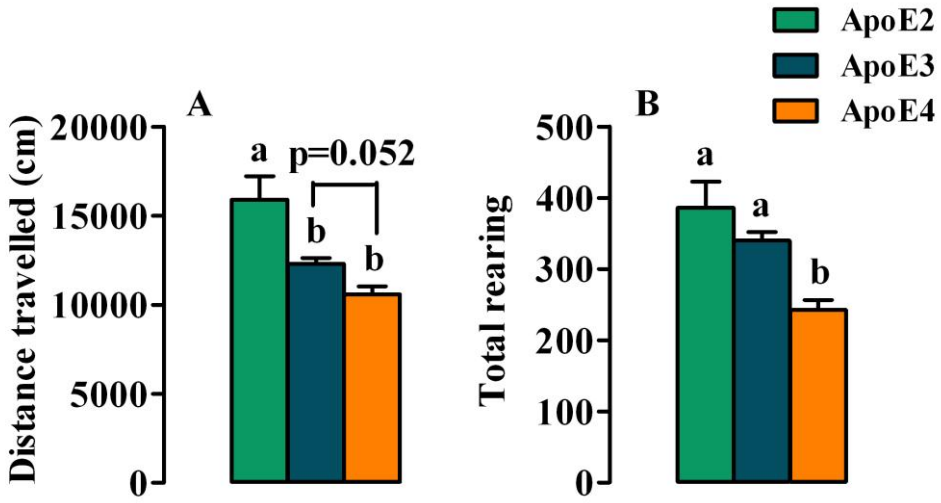

Figure III.6. General activity in the OF by genotype at 12 months of age

Total distance traveled (A) and total rearing (B). Data are expressed as mean and S.E.M. Groups showing different letters a,b are different from each other at $p < 0.05$.

Since all the genotypes showed different activity levels in the OF, a more detailed analysis was performed in each genotype. A one-way ANOVA for repeated measures was carried out, using six 5-min periods as a within-subject factor and the distance travelled as a dependent variable. Results

showed an effect of time on distance travelled in all the genotypes; ApoE2 [$F(5, 16) = 5.830$, $p=0.011$], ApoE3 [$F(5, 36) = 44.303$, $p<0.001$] and ApoE4 [$F(5, 36) = 25.358$, $p<0.001$] (Figure III.7), indicating that there were no genotype differences in habituation, as we have seen above. No effect of the treatment was found in any of the genotypes.

FIGURE III.7

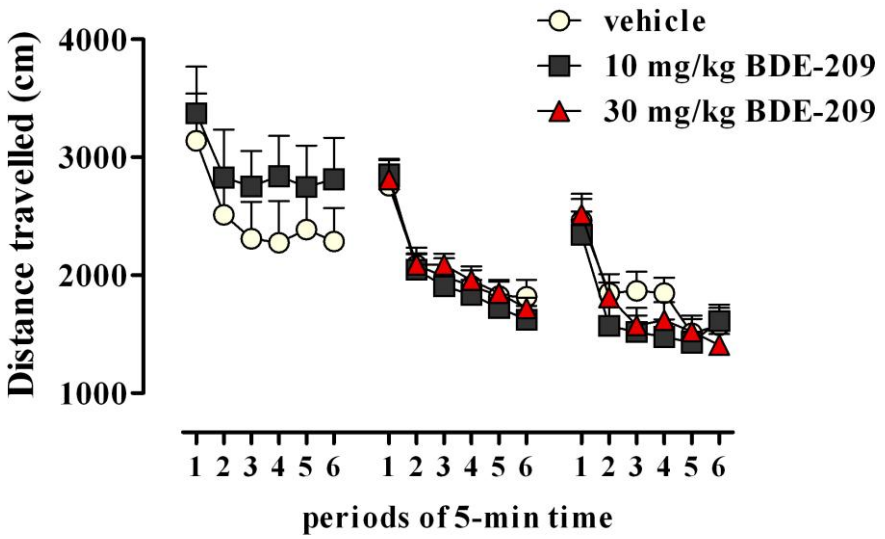

Figure III.7. Distance travelled in the OF by genotype and treatment

Data is expressed as mean and S.E.M.

To evaluate anxiety behaviour in the OF, a two-way ANOVA (genotype x treatment) was performed on the number of entries to the centre, the time spent in the centre, the ratio of rearing in the centre (rearing in the centre / total rearing) and the ratio of distance travelled in the centre (distance in the centre/total distance). The non-parametrical Kruskall-Wallis test was used for the distance travelled in the centre. An overall effect of the genotype was significant in the entries to the centre [$F(2,89)=10.072$, $p<0.001$], the

time spent in the centre [F(2,89)=5.240, p=0.007], the ratio of rearing in the centre [F(2,89)=4.359, p=0.016] and the ratio of distance travelled in the centre [F(2,89)=10.242, p<0.001] (Figure III.8). No effects of the BDE-209 treatment were observed.

FIGURE III.8

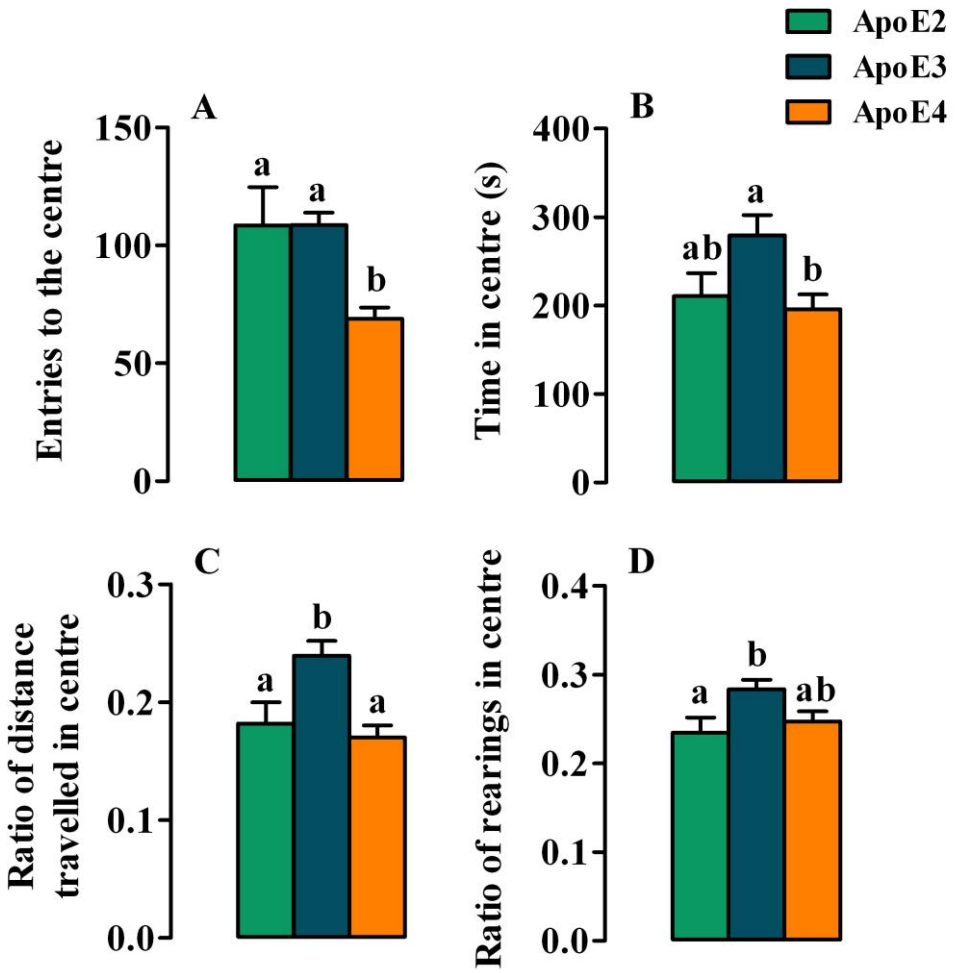

Figure III.8. Activity in the centre of the OF by genotype

Number of entries (A) and time spent into the centre (B) Ratio of distance travelled (C) and ratio of rearing (D) in the centre. Data are expressed as mean and S.E.M. Groups showing different letters a,b are different from each other at $p < 0.05$.

An analysis of the activity in the centre was also carried out in each genotype separately. Taking together the low and the high dose groups, an effect of the BDE-209 exposure was observed in ApoE3 on the ratio of the distance travelled in the centre [F(2,36)=7.023, p=0.012] and on the ratio of rearing in the centre [F(2,36)=4.929, p=0.033] (Figure III.9).

FIGURE III.9

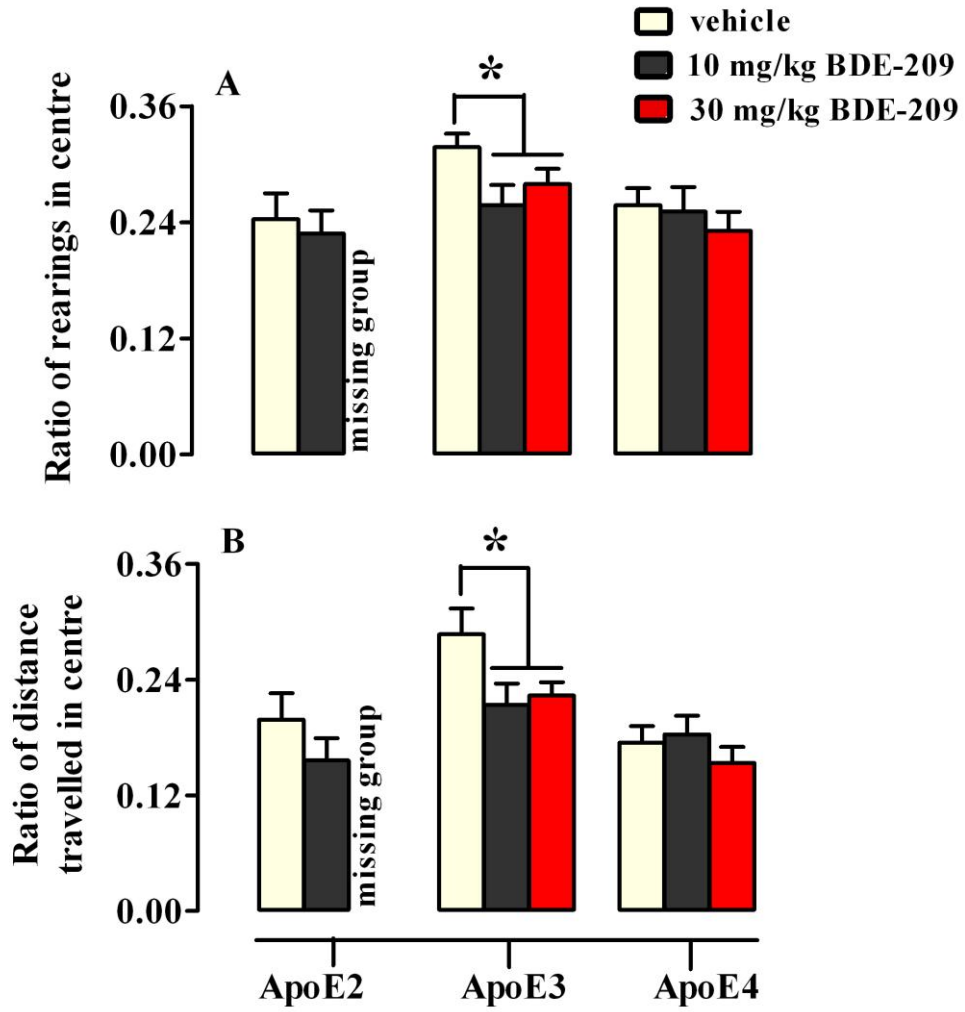

Figure III.9. Activity in the center of OF by genotype and treatment

Data are expressed as mean and S.E.M.

In summary, OF results show that ApoE4 displays lower activity levels. The ApoE3 mice showed less anxiety-like behaviour, but BDE-209 exposure might increase their anxiety levels, equating them with the other genotypes.

Morris Water Maze reference memory task

Spatial learning in the MWM at 12 months of age was analysed by a two-way ANOVA (genotype x treatment) for repeated measures on the latency (s) to get to the hidden platform, the distance travelled (cm), and the search velocity (cm/s) through the ten sessions of acquisition.

There was an effect of the session on the latency [$F(9,89)= 80.393$, $p<0.001$], on the distance travelled [$F(9,89)= 72.726$, $p<0.001$] and on the search velocity [$F(9,89)= 9.292$, $p<0.001$], indicating that the mice improved over the sessions during the acquisition period.

An overall effect of the genotype was found in the latency [$F(2,89)= 7.704$, $p<0.001$], the distance travelled [$F(2,89)= 6.184$, $p=0.004$] and the search velocity [$F(2,89)= 11.480$, $p<0.001$]. ApoE4 group differed from the ApoE3 in the total latency and from the other two genotypes in the distance travelled to get to the platform during the acquisition, while ApoE2 differed from the other two genotypes in the search velocity (Figure III.10). A significant effect of the treatment was also found in the distance travelled (cm) to the platform [$F(2,89)= 5.253$, $p=0.008$], in that the females exposed to a high dose differed from the control (Figure III.11).

FIGURE III.10

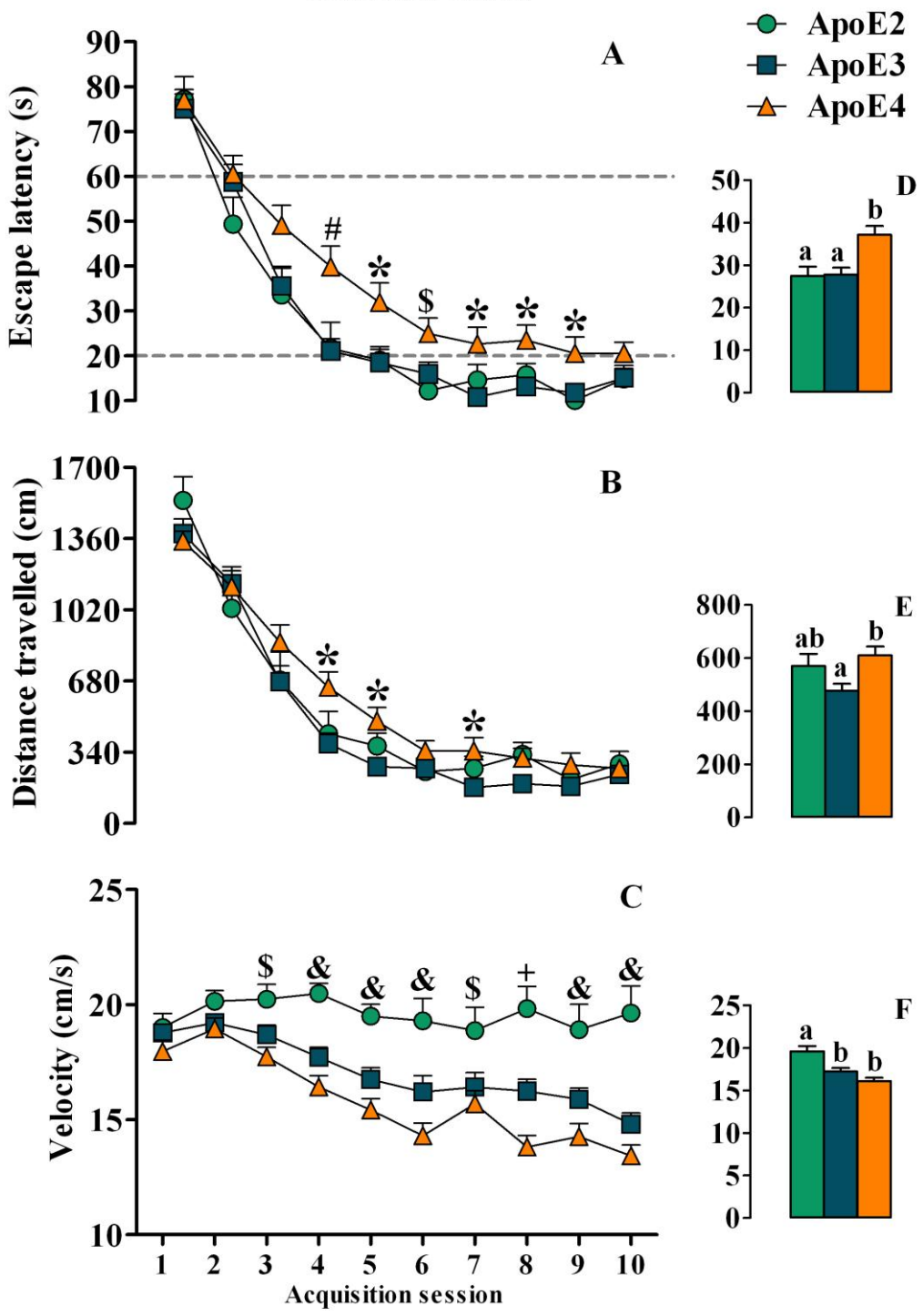

Figure III.10. MWM acquisition by genotype at 12 months of age

Escape latency, distance travelled and search velocity (A, B, C) and their mean of ten sessions (D, E, F). Data are expressed as mean and S.E.M. The symbols express differences at $p < 0.05$ between: ApoE4 and the other genotypes (#), ApoE4 and ApoE3 (*), ApoE4 and ApoE2 ($), ApoE2 and the other grenotypes (&), all the genotypes differ among them (+).

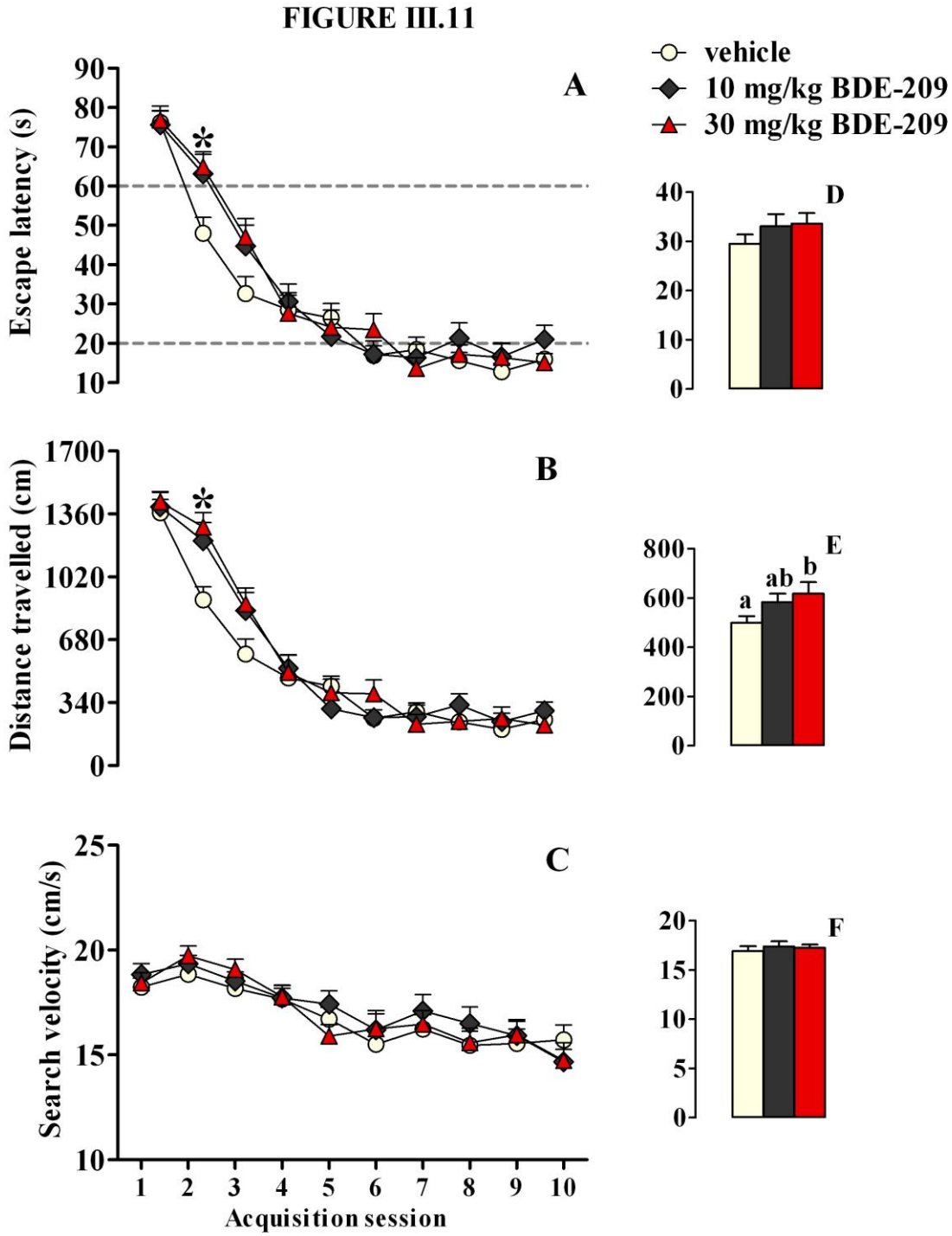

Figure III.11. MWM acquisition by genotype at 12 months of age

Escape latency, distance travelled and search velocity (A, B, C) and their mean of ten sessions (D, E, F). Data are expressed as mean and S.E.M. The asterisk indicates differences ($p < 0.05$) between the control and the exposed mice at both the low and high dose.

We also analysed separately the effect of BDE-209 exposure on the acquisition performance in each genotype. A significant effect of the session was observed in the escape latency for each genotype ($p<0.01$), likewise observed in the general analysis. In the ApoE4 group an interaction between the session, distance and treatment was also observed [$F(9,36)= 1.916$, $p=0.048$].

In ApoE2 females, an overall effect of the treatment was found in escape latency [$F(2,15)= 7.917$, $p=0.015$], as well as a trend towards a treatment effect in distance travelled [$F(2,15)= 4.739$, $p=0.057$]. Those effects indicate differences in the acquisition of the task between the control ApoE2 females and the exposed to a low dose. A trend towards a treatment effect in distance travelled was also found in ApoE3 female mice [$F(2,36)= 3.099$, $p=0.066$], pointing to differences between control and high dose exposed mice (Figure III.12).

In summary, results of the MWM acquisition showed that at 12 months ApoE4 females performed worse in a spatial task compared to the other genotypes. The treatment increases the escape latency in all the genotypes, especially in ApoE2 and ApoE3 females during the first training sessions.

FIGURE III.12

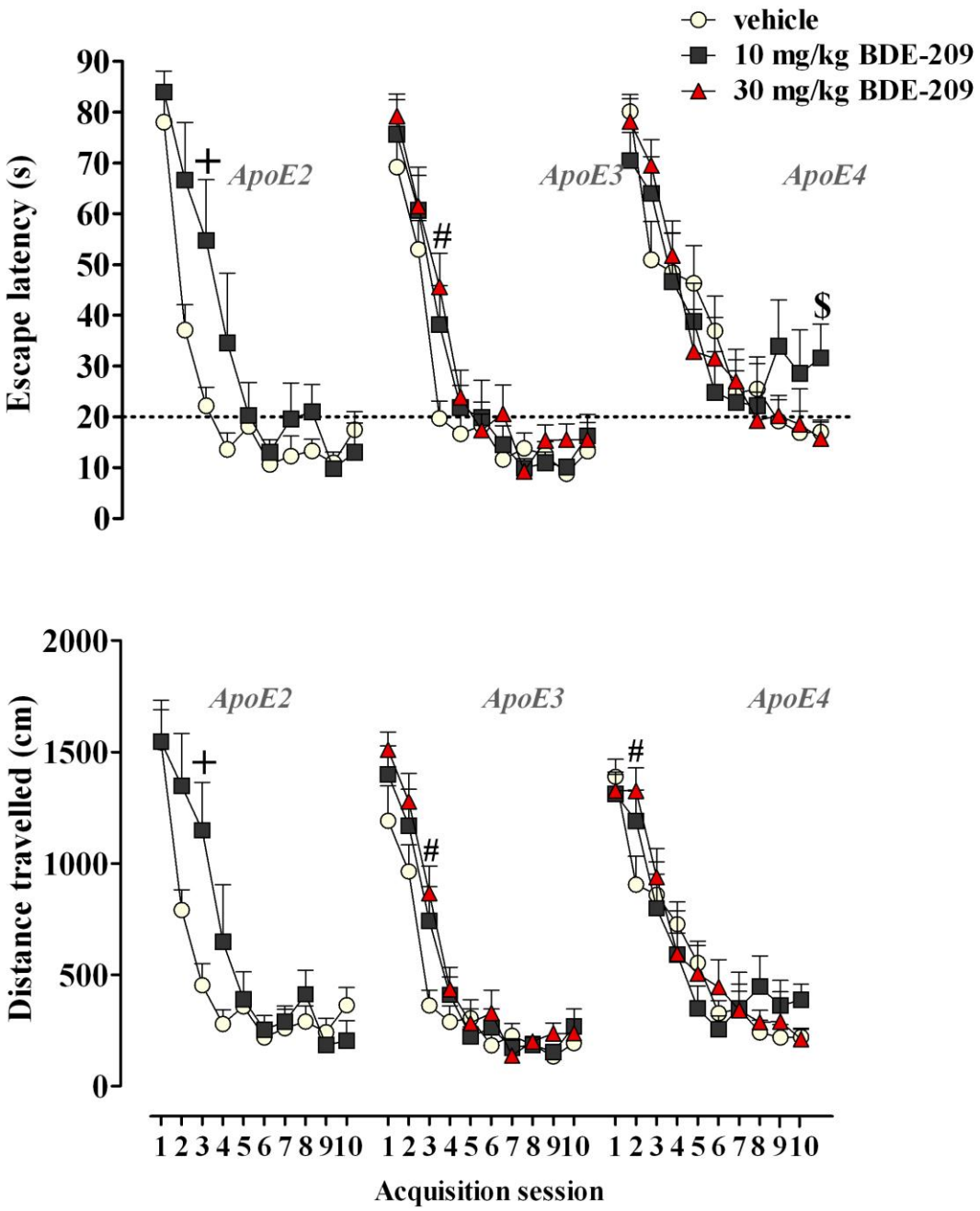

Figure III.12. MWM acquisition by genotype and treatment at 12 months

Escape latency and distance travelled through the 10 days of acquisition. Data are expressed as mean and S.E.M. The symbols indicate differences between: the control and low dose groups (+), the control and the high dose groups (#) and the the low dose and both the control and high dose groups ($). $p < 0.05$.

Retention of the MWM

We analysed the retention ability in the four probe trials performed during the acquisition period. A two-way ANOVA (genotype x treatment) for repeated measures was performed, using the session at the probe trial as a within-subject factor and the time spent (s) in the target quadrant (Tgq) and the distance travelled (cm) in the Tgq as dependent variables.

An effect of the session was significant on both the time spent in the Tgq [$F(3,89)=9.225, p<0.001$] and the distance travelled in the Tgq [$F(3,89)=6.705, p<0.001$] indicating an improvement through sessions. An overall effect of the genotype was observed in the distance travelled in the Tgq [$F(2,89)=4.888, p=0.010$], showing differences between ApoE3 and ApoE4 mice (Figure III.13). There was no observed effect of the treatment in the whole group.

Retention ability was additionally analysed in each genotype. A change was observed through the sessions on the time and the distance travelled in the Tgq in all the genotypes ($p<0.001$), as previously observed. An effect of the treatment was found between the control and the high dose exposed ApoE3 mice in the fifth session (Figures III.14 and III.15). ApoE3 mice exposed to the high dose showed worse retention in the fifth session compared to their control counterparts.

FIGURE III.13

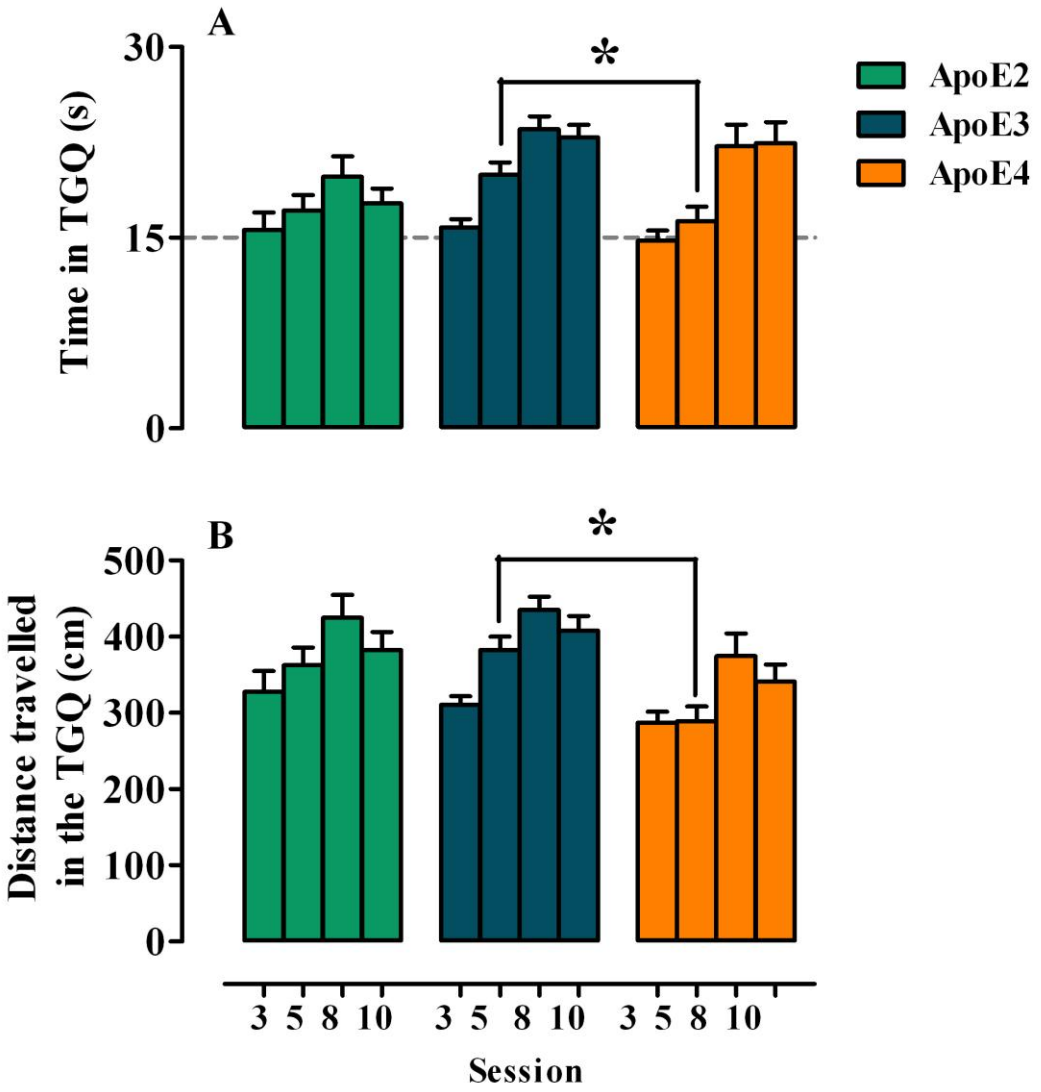

Figure III.13. Retention in the MWM by genotype at 12 months of age

Time spent (A) and distance travelled (B) in the Tgq. Data are expressed as mean and S.E.M. The asterisk indicates differences between groups at $p < 0.05$. The dashed line indicates execution by chance.

FIGURE III.14

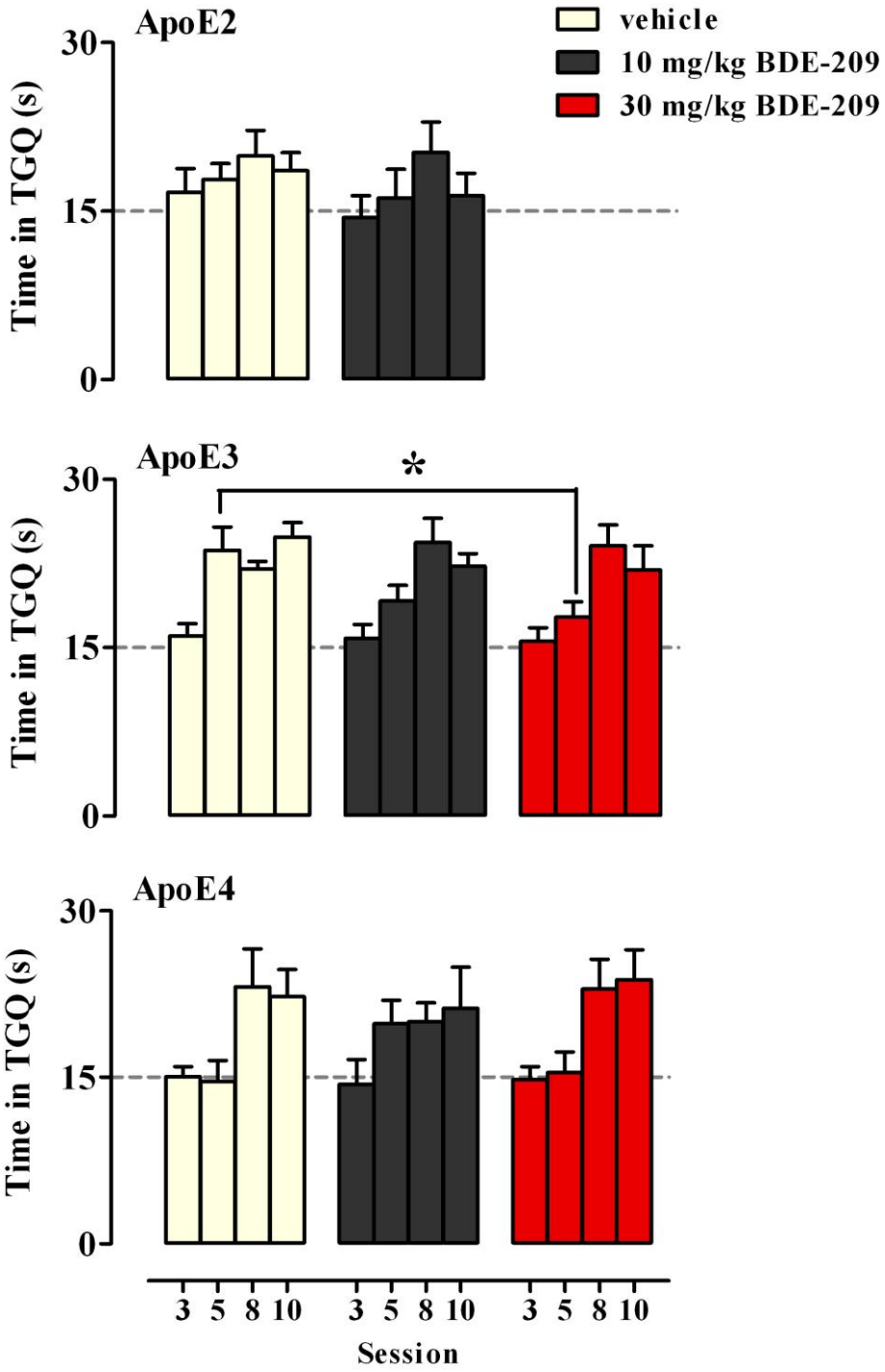

Figure III.14. Retention in the MWM by genotype and treatment at 12 months of age

Time spent in the Tgq. Data are expressed as mean and S.E.M. The asterisk indicates differences between groups at $p < 0.05$.

FIGURE III.15

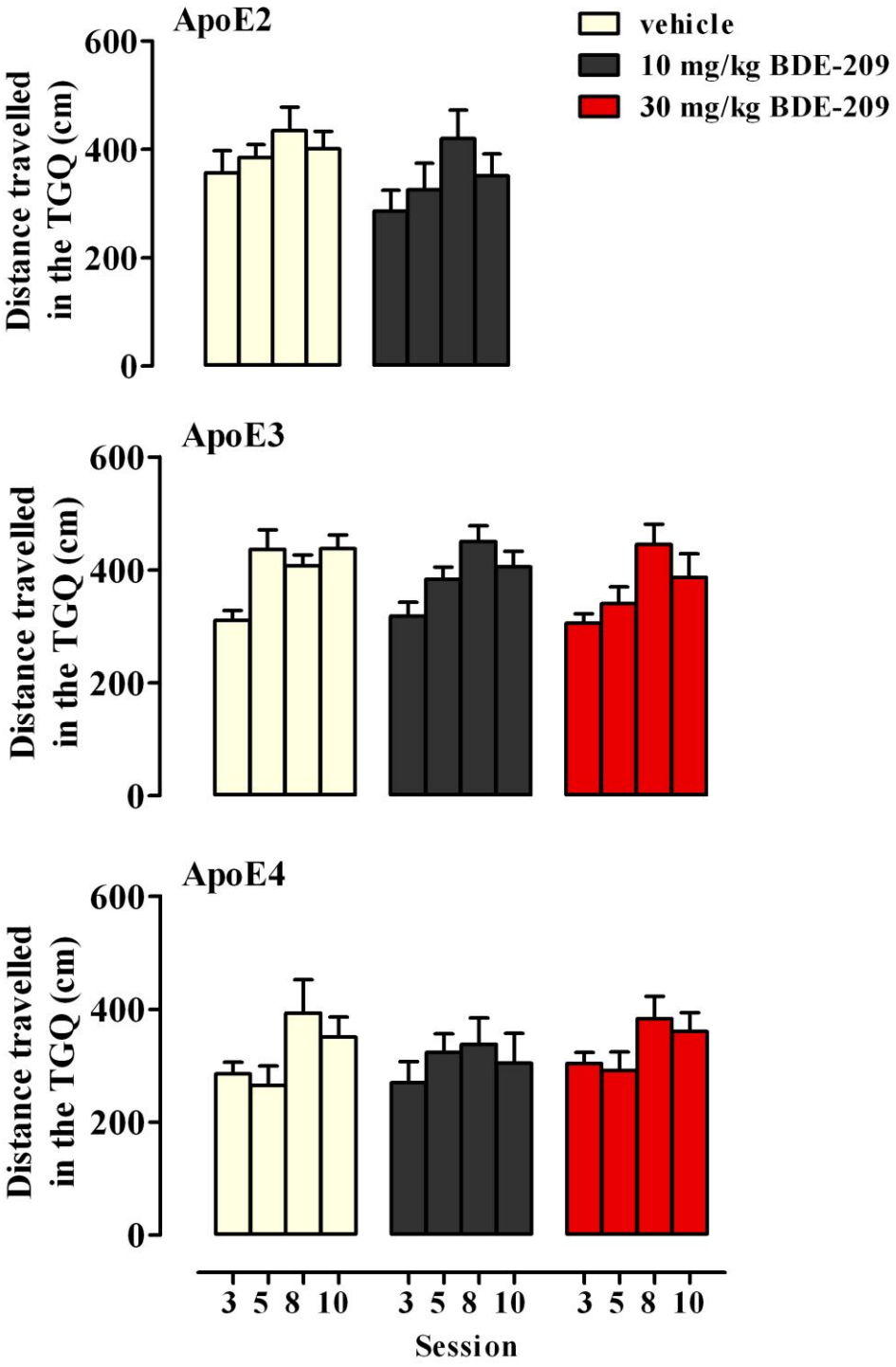

Figure III.15. Retention in the MWM by genotype and treatment at 12 months of age

Distance travelled in the Tgq. Data are expressed as mean and S.E.M.

Long-term retention

The time (s) spent in the target quadrant (Tgq) and the distance travelled (cm) in the Tgq during the 72h probe trial were both analysed as long-term retention variables. A two-way ANOVA (genotype x treatment) was performed.

Results showed an overall effect of the genotype in the time spent in the Tgq [$F(2,89)=3.328$, $p=0.041$], but there were no significant, observable differences between groups. Likewise, a one-way ANOVA performed by genotype, using the treatment as a between-subject factor, only points to a possible trend effect of the treatment in the time spent in the Tgq in ApoE3 group [$F(2,36)=2.826$,$p=0.073$]. Additionally, we compared control animals with exposed animals (taking both the low and the high dose groups) and an effect of treatment emerged in ApoE3 [$F(1,36)=5.448$,$p=0.025$] (Figure III.16). The interpretation of these results should be made taking into account the low scores reached in the long-term retention by ApoE3 females exposed to BDE-209, which obtained scores similar to the chance level.

Briefly, the results on the retention of the MWM reveal old ApoE2 females performed above the chance level during the acquisition, although they never reached the recall levels that the ApoE4 and ApoE3 reached. ApoE4 females need more training trials to show a recall comparable to ApoE3. ApoE3 females were affected by the treatment, since those exposed to the high dose showed a poor performance in the second probe trial and in long retention sessions compared to control ApoE3 females.

FIGURE III.16

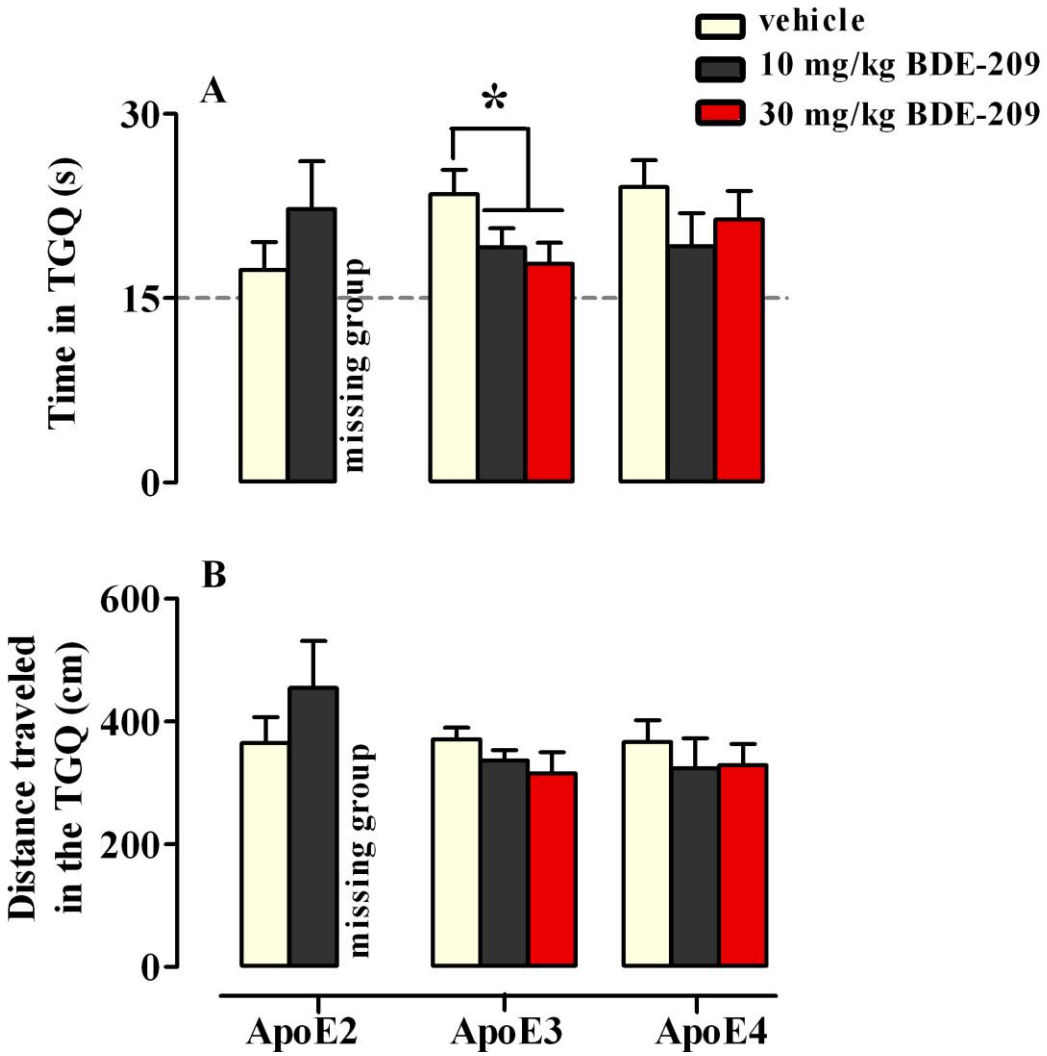

Figure III.16. Long-term retention in the MWM by genotype and treatment at 12 months of age

Time spent (A) and distance travelled (B) in the Tgq. Data are expressed as mean and S.E.M. The dashed line indicates execution by chance.

3.2 Discussion of the Experimental Phase III: 12 months old

ApoE female mice showed behavioural differences among genotypes and differential effects to postnatal exposure to BDE-209 at 12 months of age.

In this study, differences in body weight in old females were found regarding the ApoE genotype. ApoE4 female mice had a lower body weight compared with ApoE3 females. It is well known that ApoE influences metabolism and body weight, and human ApoE isoforms are associated with increasing body mass index in the order: ApoE2>ApoE3>ApoE4 (Arbones-Mainar et al. 2008; Pendse et al. 2009; Ferreira et al. 2011). Supporting this relationship, a recent study found higher body mass index in menopausal Korean woman homozygote for the ApoE3 allele (Lee et al. 2011). On the other hand, Bour and colleagues found increased body weight in male and female ApoE4 mice at 15 months old (Bour et al. 2008). Discrepancies between these studies could be related to age or stressful conditions during the testing procedure, such as the individual housing in Bour's study.

Results in the Zero Maze and the Open Field tests showed that ApoE4 is clearly hypoactive compared to the other genotypes. Decreased locomotor activity was previously reported in ApoE4 males at 15 months old (Bour et al. 2008), and in ApoE4 females from 10 to 22 months old (Siegel et al. 2010). Referring to the habituation to a new environment, all the animals in our study decreased their activity over time, independently of the genotype, but ApoE2 females showed higher levels of activity throughout the test. ApoE4 females showed more freezing and explored for less time the open areas of the EZM, indicating higher levels of anxiety compared to ApoE2

and ApoE3 mice. In the OF, ApoE4 female mice also made fewer entries to the centre of the maze compared to the other genotypes, supporting anxiety data obtained from the EZM. The ApoE3 mice explored for more time and were more active in the centre of the Open Field compared to the other genotypes, showing the least anxious phenotype at 12 months old. Increased measures of anxiety in ApoE4 mice were previously reported, and differences among ApoE genotypes become more evident with age (Raber 2007; Siegel et al. 2010). Studies in humans also support the association of the ApoE4 genotype with high anxiety levels. ApoE4 homozygous allele carriers showed higher anxiety scores compared with the homozygous for the ApoE3 allele, in subjects with probable AD (Robertson et al. 2005), and increased stress markers were found in carriers of the ApoE4 and a variant of the butyrylcholinesterase gene (Fiocco et al. 2009).

Spatial navigation and learning was influenced by the genotype at 12 months of age in the current study. ApoE4 spent more time and travelled more distances to reach the platform in comparison with ApoE2 and ApoE3 females. However, all the groups achieved good learning scores at the end of the acquisition period. Accordingly, Bour and colleagues found a lower global performance in the MWM in ApoE4 female mice (Bour et al. 2008) On the contrary, improved spatial learning in ApoE4 mice was found in 10-13 and 14-22 months-old females (Siegel et al. 2010). These discrepancies among studies might be due to differences in the protocol used in the MWM spatial reference task. In the current study, ApoE4 females at 12 months also showed worse retention ability in the second probe trial compared with ApoE3 females, although they improved their retention with training. Instead, retention ability in ApoE2 females was

poor during the whole training period, though they performed over the chance level. The Mathis group observed in ApoE4 females no preference for the target quadrant in a probe trial performed 24 hours after the last training session (Bour et al. 2008). In contrast, Siegel and colleagues did not find any differences among genotypes in retention of the MWM task at 10-13 or 14-22 months of age (Siegel et al. 2010). A possible explanation is that in Siegel's study probe trials were carried out one hour after the training session, and the ApoE4 genotype possibly preserves short-term memory. Instead, when probe trials take place 24 hours after the training session, detrimental memory effects appear. There were no differences in long-term retention (72h probe trial) among genotypes at 12 months of age in the present study, indicating that long-term retention is not affected. The poor retention exhibited by ApoE2 females is unexpected since their acquisition is as good as that observed in ApoE3 females. A workable explanation might be that the high swim velocity shown by this genotype causes longer trajectories not restrained to the target quadrant. Another possibility could be related to higher cognitive flexibility in ApoE2, which makes these animals search for the platform across all the quadrants when they do not find it in the last position. Nonetheless, these are just considerations and ApoE2 memory capacity should be studied further.

Exposure to BDE-209 during the postnatal period was shown to affect behaviour and learning of female mice at 12 months of age, depending on the genotype.

Body weight was lower in ApoE4 females exposed to BDE-209. Generally, studies do not report changes in body weight after PBDE exposure (Eriksson et al. 2002; Viberg et al. 2002; Viberg et al. 2003; Viberg et al.

2003; Viberg et al. 2004; Viberg et al. 2007; Johansson et al. 2008), so it is plausible the BDE-209 interacts with the metabolic susceptibility of the ApoE4 genotype.

BDE-209 exposure did not affect behaviour in the EZM or general activity in the OF test at 12 months of age, but triggered a decrease in the exploration activity in the centre of the maze in ApoE3 female mice. ApoE3 female mice showed high exploratory activity in the centre of the OF, indicating low levels of anxiety, which were increased in treated animals. No differences in the time spent in open arms in an EPM were found in NMRI mice at 2 or 4 months of age, after being exposed to BDE-209 on PND3 (Johansson et al. 2008). In the other hand, CD-1 Swiss female mice spent more time in the centre of the OF 2 months after postnatal exposure to BDE-99 (Branchi et al. 2002). The differences between strain, age and testing approach may be explanations for these differences.

Learning and memory were also affected by BDE-209 exposure since both acquisition and retention in the MWM task were impaired by the treatment. At 12 months of age, the escape latency and the distance travelled to reach the platform were higher during the first days of training, in the animals exposed at PND10 to the low and high dose of BDE-209. Those impairments during the acquisition period were more evident in ApoE2 and ApoE3 female mice. ApoE3 female mice exposed to BDE-209 also required more training trials to show good retention scores in comparison with ApoE3 control females. Furthermore, long-term retention was reduced in BDE-209 exposed ApoE3 females. Learning and memory derangements in rodents after PBDE exposure have been shown in spatial tasks (Eriksson

et al. 2001; Viberg et al. 2003; Viberg et al. 2006; Cheng et al. 2009; He et al. 2009) as well in non-spatial tasks (Dufault et al. 2005). Spatial learning and memory in the MWM after administration of BDE-209 have not been studied previously, but neonatal exposure to BDE-209 impaired learning in a light-dark discrimination task in 16-months-old mice (Rice et al. 2009). Rice and co-workers were the first to report such long-term effects after neonatal exposure to PBDEs. Regarding that, our study provides additional evidence of long lasting effects of neonatal exposure to BDE-209, which is able to disrupt behaviour, learning and memory twelve months after exposure, and interact with the genotype suppressing the cognitive advantage that shows at advanced ages in ApoE3.

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Chapter V: General Discussion and Future Perspectives

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In the present study the effects of an acute postnatal exposure to BDE-209 were evaluated at three major moments of life: the developmental period, young adulthood and old age, in ApoE2, ApoE3 and ApoE4 mice. Some of the effects observed are transient, while others are present in all the experimental phases. In this chapter, the results are discussed as a whole, offering a lifecycle perspective on the neurobehavioural performance of ApoE mice and the short and long term effects of the postnatal exposure to BDE-209 in this animal model.

Physical and behavioural differences among genotypes were observed in all the experimental phases, providing support for the idea that the ApoE genotype is not only a risk factor for disease in the elderly, but it provides different cognitive and behavioural phenotypes from birth. During development, ApoE4 mice showed a delay in the acquisition of some neuromotor abilities compared to the other genotypes, which was no longer observed in adults. Additionally, our study clearly points to a diminished

reproductive outcome in ApoE4 genotype as well as a delayed maturation in male mice. Human ApoE is the most important supplier of the cholesterol precursor for steroid production and could play an important role in the regulation of steroid hormone function, sexual maturation or reproduction (Corbo et al. 2004). However, there have been few studies on this topic, and there are only a few references on the relationship between ApoE polymorphisms and fertility.

Furthermore, mice showing different ApoE isoforms were differentially affected by the exposure to BDE-209, showing that ApoE2 mice are more vulnerable to toxic effects during development. ApoE2 mice exposed to BDE-209, especially those exposed to the high dose, showed a delay in physical and neuromotor development. Sexual maturation was delayed in BDE-209 exposed males independently of the genotype, and in ApoE2 females exposed to BDE-209. Previously, lower brominated congeners have been proven to delay puberty in exposed male and female rodents (Stoker et al. 2005; Lilienthal et al. 2006) and this effect could be mediated by the ability of PBDEs to interact with sexual hormones (Costa et al. 2007; Talsness 2008). In this study, sexual or reproductive features were not examined in adult ApoE mice, so we do not know if the effects observed in exposed males and ApoE2 females remain in adults and/or turn into an impaired reproductive capacity. The effects of PBDEs in adult reproductive organs are reported after exposure to low doses of tetra and penta-BDEs, but BDE-209 does not generally effect reproduction in adults ((EFSA) 2011). The myelin pattern was also affected by BDE-209 treatment in ApoE2, which could account for developmental retardation observed in exposed animals of this genotype. The myelination process is essential for a proper CNS function and is suitable to be disrupted by

environmental conditions such as nutritional deficits or toxic exposure during critical stages (Di Biase et al. 1997; Georgieff 2007; Pott et al. 2009). The mechanisms by which BDE-209 alters myelin compaction in ApoE2 genotype might be diverse; one of them could be mediated by thyroid hormones. Thyroid hormone deficiency alters myelin sheath compaction (Ferreira et al. 2007), and there is a lot of evidence of thyroid hormone disruption after PBDE exposure (Zhou et al. 2002; Branchi et al. 2003; Stoker et al. 2004; Ellis-Hutchings et al. 2006; Darnerud et al. 2007; Kuriyama et al. 2007; Richardson et al. 2008; Driscoll et al. 2009). In this study, the status of the thyroid hormone assessed at four months of age revealed an effect of BDE-209 exposure over FT4 levels in females. In addition, T3 was lower in the ApoE2 genotype, independently of the treatment. The inherent low T3 levels in ApoE2 may be worsened by BDE-209 exposure, which may then lead to a delayed myelin compaction pattern. Since BDE-209 does not modify thyroid levels in adult males, a more detailed study is needed of the thyroidal status during development after acute exposure to BDE-209 in order to better characterize the relationship between thyroid status in male ApoE2 mice and myelination. Another explanation for the myelination differences observed between the control and the BDE-209 exposed ApoE2 males is the difference in cholesterol metabolism in brain among genotypes (Dietschy 2009). ApoE2 mice were the most affected by the treatment during development and this might be related to the high cholesterol demands due to the rapid growth during this period. The absorption of cholesterol by the intestine is lower in ApoE2 phenotypes compared to ApoE4 (Scacchi et al. 1997) and in turn ApoE2 have an enhanced endogenous synthesis of cholesterol to compensate poor absorption. We speculate that the delay observed in treated mice could be related to interferences produced by BDE-209 on the

cholesterol endogenous production; however, further research is needed in order to corroborate these results and to explore possible mechanisms of action. In this study, endpoints such as thyroid function, myelination patterns or BDNF and TrkB levels are assessed at discrete points in the life cycle and it would be interesting to assess these endpoints at various points in time to characterize the temporal pattern of these measures.

Body weight is also affected by the genotype and the BDE-209 treatment, and those factors interact with gender and age. ApoE4 mice showed lower body weight after weaning compared with other genotypes, although litter size in ApoE4 genotype was smaller. At a young age, ApoE4 females showed a markedly lower body weight and, although differences among genotypes shortened with age, a lower body weight remained in old ApoE4 females. ApoE2 body weight gain was affected by BDE-209 exposure after weaning, but these effects disappeared in young and old adults. In contrast, BDE-209 effects in ApoE4 body weight were not observed during development but appeared in adults. ApoE4 young males exposed to BDE-209 weighed significantly less and the treatment effect became evident with age in ApoE4 females. In general terms effects related to BDE-209 observed in ApoE2 during development were no longer present in adults.

General activity assessed in the EZM and the OF tests diminished with increasing age as expected, but it was clearly affected by the genotype in both young and old mice. ApoE4 was hypoactive compared to the other genotypes, while ApoE2 showed a similar exploratory behaviour to the ApoE3 in EZM, but an increased locomotor activity in the OF. It is worthy to note that the increased activity observed in the ApoE2 mice, throughout the different ages tested, was not observed in a previous study carried out

by Raber's group that included ApoE2 mice. BDE-209 exposure affected the general activity at 4 months of age, depending on the genotype and sex. At a young age, the treatment increased activity in ApoE2 males, while decreased activity in ApoE3 and ApoE4 females, the latter being the most affected group. In aged mice, the effects of BDE-209 on activity disappear, likely because, indeed, the general activity was lower.

Throughout life, ApoE3 mice showed the least anxious profile followed by ApoE2 mice. Instead, ApoE4 explored less the open areas (EZM) and the centre of the OF, and showed higher levels of freezing at 4 and 12 months of age. In aged female mice, BDE-209 increased anxiety levels in ApoE3 genotype, equating their anxiety levels to those exhibited by the other groups.

Spatial learning ability was clearly different among genotypes at both young and old ages. ApoE3 displayed the best acquisition performance in the MWM reference memory task followed by ApoE2 in young mice, while ApoE4 showed higher latencies throughout the training period. Aged female mice showed slight worsening in acquiring the task, compared to young mice, but all groups achieved good learning scores at the end of the training. ApoE3 and ApoE2 females showed comparable acquisition performance at 12 months of age, while ApoE4 females continued requiring higher time and longer distances to get the platform compared to their counterparts. No differences were found between males and females in spatial navigation at 4 months of age; however, the BDE-209 treatment effect was only evident in ApoE3 males at 4 months of age whose treated animals showed increased latency and distance travelled to reach the platform. These detrimental effects of BDE-209 also appeared in females

with age, indicating that BDE-209 effects increased with age after a postnatal exposure. Other authors have also been reported this in habituation patterns and operant behaviour (Rice et al., 2009; Viberg et al. 2003) suggesting delayed toxicity.

Retention was also different among genotypes. As expected, the best retention during the training period was shown in ApoE3, though all the genotypes improved over time and they showed similar retention at the end of the acquisition period. At 4 months of age, males outscored females in ApoE2 and ApoE4 groups, however sex differences were only statistically significant for control ApoE4 mice in the last probe trial. BDE-209 treatment affected the retention only in young ApoE3 males; while this effect also appears in ApoE3 females with age. Aged ApoE3 females exposed to BDE-209 also showed poorer long-term memory.

Some of the effects produced by postnatal exposure to BDE-209 are observed in groups showing good scores for learning or low scores for anxiety. The ApoE3 genotype is particularly more vulnerable to the effects induced by BDE-209 on learning and memory. Similar scores obtained in ApoE3 males and females and the low variability observed in this genotype can increase statistical power to find the differences between control and exposed animals. In the ApoE4 group, the worsening effects of the genotype are probably stronger than toxic exposure. There is a common limitation of behavioural testing, which is when base levels are too low (ground effect) or too high (ceiling effect), so external manipulations, including pharmacological approaches, are not able to show any effect. In our opinion, some of the lack of effects on activity levels in aged ApoE4 mice could be related to this issue. At the same line, it is difficult to find

detrimental effects of the treatment in subjects whose learning baseline is poor. The different effects dependant on the genotype might ultimately mask the toxics effects in studies carried out in the human population when genotype differences are not taken into account. It is worthy of note that, according to the results obtained in this study, the vast majority of the population, who are ApoE3 carriers according to ApoE allele frequencies, would be at more risk of being affected by BDE-209 exposure. However, the effects will not be evident in epidemiological studies because the effect of BDE-209 is to diminish the ability of the ApoE3 genotype to the level of that observed in the other genotypes, which makes the population more uniform.

This study has shown that the ApoE genotype plays an important role in behaviour and learning, not only in old ages but also in late development and young adulthood. Furthermore, the ApoE genotype determines different vulnerabilities to the toxicity produced by postnatal exposure to BDE-209, which affects different functional targets within each genotype and at different stages of life. Interestingly effects are mainly observed in those subjects (ApoE3) obtaining the better learning scores, normalizing the scores to those of mice from the other genotypes. Thus, this effect could pass absolutely imperceptibly in human populations. In general, some sex differences exist during development, such as body weight, activity levels, anxiety or learning and memory, but these differences are small. More interestingly, sex interacts with the genotype and the treatment, which shows that the effects of BDE-209 exposure are dependent on these factors.

It is relevant to point that BDE-209 exposure during the postnatal period is able to affect development and to produce effects in adults, which last and

worsen with age. Furthermore, it should be highlighted that, despite the disruptive effects of BDE-209 not causing severe impairments, those effects are, however, triggered by a single exposure early in life and affect subjects with a distinct vulnerability in a different way. In the human population, those exposures may be responsible for subclinical deficits in children and adults and an increased or accelerated decline in the elderly. In addition, it should be taken into account that there are vast number of chemicals present in the environment that are able to trigger neurobehavioural derangements and may interact or add power to the adverse effects caused by exposure to BDE-209.

Future directions

Our results highlight the importance of studying genetic vulnerability to toxic agents and the use of longitudinal approaches for detecting the long-lasting effects of toxic substances, but raise a number of issues for further studies. The scope for further research mainly concerns the areas of:

1) Providing more face-validity to the exposure to BDE-209 compared to a human exposure,
2) Further exploring the neurobehavioural effects observed,
3) Aiming to elucidate the mechanisms through which BDE-209 acts.

The following considerations offer some direction for the development of future research that will reinforce and extend the findings of this thesis.

- In the current study, a single exposure to BDE-209 at a growth peak during development has caused long-term derangements. We

propose a longer period of exposure at lower doses, which would better mimic the continued exposure in human infants and would provide a wider period of development during which more critical events take place.

- It would be also interesting to explore the hyperactivity found in ApoE2 females. As higher levels of anxiety did not accompany that greater activity, we suggest evaluating ApoE mice in a 5-choice serial reaction time task in order to assess attention deficits or impulsive behaviours, which are characteristic of ADHD.

- In this study we have found differences among genotypes and effects of BDE-209 on learning and memory in the MWM task. We observed that, despite the worst acquisition being in the ApoE4 group, their retention was good and the males of that group tended to increase learning in the later sessions, while other groups, and especially females, showed a ceiling effect. For this reason, it would be interesting to assess learning for longer periods to determine when asymptotic learning occurs for all the genotypes. Additionally, as we found higher levels of anxiety in ApoE4 mice, it would be useful to employ less stressful spatial tasks, such as the Barnes Maze. Furthermore, the evaluation of other types of learning, such as Contextual and Cued Fear Conditioning, would provide very valuable information. This task is able to discriminate learning impairments associated to amydala or dependant on the hippocampus, which is the aim of a study currently underway.

- Our findings showed that age is an important factor in determining the effects of early exposure to BDE-209, since some effects emerge punctually at certain life stages and the manifestation of other effects remain or worsen with age. Regarding this, and taking into account that cognitive deterioration was not observed in mice at 12 months of age, it would be of great interest to evaluate that at very advanced ages. In fact, we have a study in progress in which ApoE mice have been subject to postnatal exposure to BDE-209 and are being evaluated at 18 months of age.

- The differences in thyroid hormone status found among genotypes, and the disruption in thyroid hormones observed after postnatal BDE-209 exposure make it interesting to better characterize the hormonal status by analysing TRH and TSH levels in order to detect the level at the hormonal axes interference.

- Another study of interest is the determination of BDNF and TrkB levels in other brain structures such as the frontal cortex and at different time-points in the lifecycle.

- The effects of exposure to BDE-209 on the myelin pattern are considered a key point of the study. Nevertheless, these results require corroboration by the use of semi-quantitative methods and a follow-up study at different stages of development.

- Further on, it would be of interest to explore epigenetics as a mechanism for the effects observed on the myelin pattern by exposure to BDE-209, elucidating whether those effects observed are

a consequence of a direct action on MBP or other myelin-related proteins or whether it is an indirect effect mediated by thyroid hormone dysfunction.

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Chapter VI: Conclusions

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1. **A**poE4 mice showed a delay in neuromotor maturation and sexual maturation only in males.

2. **A**poE4 mice showed a lower body weight than the other ApoE genotypes from weaning to adulthood.

3. **A**poE4 mice are less active and more anxious than the other ApoE genotypes at 4 and 12 months of age.

4. **S**patial learning is different among ApoE transgenic mice at 4 months of age. ApoE4 showed the worst acquisition of the task, ApoE3 showed the best acquisition performance and ApoE2 showed an intermediate learning phenotype.

5. **A**t 12 months of age ApoE4 performed worse in spatial learning than ApoE2 and ApoE3 mice.

6. **A**dult ApoE2 mice showed lower levels of thyroid hormones, especially in T3 levels.

7. **B**DNF levels in the hippocampus of adult ApoE2 mice are higher than those in ApoE4 mice.

8. **T**rkB receptor levels in the hippocampus of ApoE transgenic mice differ among the three genotypes tested. The ApoE2 showed the highest levels and the ApoE3 showed the lowest levels.

9. **P**ostnatal exposure to BDE-209 produces a delay in physical and neuromotor development in ApoE2 mice.

10. **P**ostnatal exposure to BDE-209 produces long lasting effects on body weight in ApoE4 males at 4 months of age and ApoE4 females at 12 months of age.

11. **P**ostnatal exposure to BDE-209 decreases activity in ApoE4 females at 4 months of age and increases anxiety in ApoE3 females at 12 months of age.

12. **P**ostnatal exposure to BDE-209 impairs spatial learning in ApoE3 males at 4 months of age and long-term memory in ApoE3 females at 12 months of age.

13. **E**ffects of postnatal exposure to BDE-209 are observed in developing ApoE2 mice and in young adult ApoE4 mice. The most consistent effects across the lifespan are observed in ApoE3 mice and consist of impaired learning at 4 months of age, and impaired learning and memory and increased anxiety at 12 months of age.

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(EFSA), E. F. S. A. (2011). "Scientific Opinion on Polybrominated Diphenyl Ethers (PBDEs) in Food." EFSA Journal 9 (15): 2156. [274 pp.] doi:10.2903/j.efsa.2011.2156.

(EPA), U. S. E. P. A. (2008). Toxicological review of Decabromodiphenil ether (BDE-209).

Acevedo, S. F., B. J. Piper, M. J. Craytor, T. S. Benice and J. Raber (2010). "Apolipoprotein E4 and sex affect neurobehavioral performance in primary school children." Pediatr Res 67(3): 293-9.

Ahmed, O. M., A. W. El-Gareib, A. M. El-Bakry, S. M. Abd El-Tawab and R. G. Ahmed (2008). "Thyroid hormones states and brain development interactions." Int J Dev Neurosci 26(2): 147-209.

Alexander, D. M., L. M. Williams, J. M. Gatt, C. Dobson-Stone, S. A. Kuan, E. G. Todd, P. R. Schofield, N. J. Cooper and E. Gordon (2007). "The contribution of apolipoprotein E alleles on cognitive performance and dynamic neural activity over six decades." Biol Psychol 75(3): 229-38.

Alonso, V., V. Linares, M. Belles, M. L. Albina, A. Pujol, J. L. Domingo and D. J. Sanchez (2010). "Effects of BDE-99 on hormone homeostasis and biochemical parameters in adult male rats." Food Chem Toxicol 48(8-9): 2206-11.

Arbones-Mainar, J. M., L. A. Johnson, M. K. Altenburg and N. Maeda (2008). "Differential modulation of diet-induced obesity and adipocyte functionality by human apolipoprotein E3 and E4 in mice." Int J Obes (Lond) 32(10): 1595-605.

Arendt, T. (2001). "Alzheimer's disease as a disorder of mechanisms underlying structural brain self-organization." Neuroscience 102(4): 723-65.

Barres, B. A. and M. C. Raff (1994). "Control of oligodendrocyte number in the developing rat optic nerve." Neuron 12(5): 935-42.

Bartzokis, G., P. H. Lu, D. H. Geschwind, K. Tingus, D. Huang, M. F. Mendez, N. Edwards and J. Mintz (2007). "Apolipoprotein E affects both myelin breakdown and cognition: implications for age-related trajectories of decline into dementia." Biol Psychiatry 62(12): 1380-7.

Belles, M., V. Alonso, V. Linares, M. L. Albina, J. J. Sirvent, J. L. Domingo and D. J. Sanchez (2010). "Behavioral effects and oxidative status in brain regions of adult rats exposed to BDE-99." Toxicol Lett 194(1-2): 1-7.

Benvenga, S. (1997). "A thyroid hormone binding motif is evolutionarily conserved in apolipoproteins." Thyroid 7(4): 605-11.

Benvenga, S. and J. Robbins (1999). "Altered thyroid hormone binding to plasma lipoproteins in the syndrome of resistance to thyroid hormones." Biochimie 81(5): 545-8.

Berteau-Pavy, F., B. Park and J. Raber (2007). "Effects of sex and APOE epsilon4 on object recognition and spatial navigation in the elderly." Neuroscience 147(1): 6-17.

Birdsall, R. E., M. P. Kiley, Z. M. Segu, C. D. Palmer, M. Madera, B. B. Gump, J. A. MacKenzie, P. J. Parsons, Y. Mechref, M. V. Novotny and K. G. Bendinskas (2010). "Effects of lead and mercury on the blood proteome of children." J Proteome Res 9(9): 4443-53.

Bloss, C. S., D. C. Delis, D. P. Salmon and M. W. Bondi (2008). "Decreased cognition in children with risk factors for Alzheimer's disease." Biol Psychiatry 64(10): 904-6.

Bloss, C. S., D. C. Delis, D. P. Salmon and M. W. Bondi (2010). "APOE genotype is associated with left-handedness and visuospatial skills in children." Neurobiol Aging 31(5): 787-95.

Bour, A., J. Grootendorst, E. Vogel, C. Kelche, J. C. Dodart, K. Bales, P. H. Moreau, P. M. Sullivan and C. Mathis (2008). "Middle-aged human apoE4 targeted-replacement mice show retention deficits on a wide range of spatial memory tasks." Behav Brain Res 193(2): 174-82.

Branchi, I., E. Alleva and L. G. Costa (2002). "Effects of perinatal exposure to a polybrominated diphenyl ether (PBDE 99) on mouse neurobehavioural development." Neurotoxicology 23(3): 375-84.

Branchi, I., F. Capone, E. Alleva and L. G. Costa (2003). "Polybrominated diphenyl ethers: neurobehavioral effects following developmental exposure." Neurotoxicology 24(3): 449-62.

Branchi, I., F. Capone, A. Vitalone, F. Madia, D. Santucci, E. Alleva and L. G. Costa (2005). "Early developmental exposure to BDE 99 or Aroclor 1254 affects neurobehavioural profile: interference from the administration route." Neurotoxicology 26(2): 183-92.

Branchi, I. and L. Ricceri (2004). "Refining learning and memory assessment in laboratory rodents. An ethological perspective." Ann Ist Super Sanita 40(2): 231-6.

Brichtova, E. and L. Kozak (2008). "Apolipoprotein E genotype and traumatic brain injury in children--association with neurological outcome." Childs Nerv Syst 24(3): 349-56.

Burnham, N., R. F. Ittenbach, V. A. Stallings, M. Gerdes, E. Zackai, J. Bernbaum, R. R. Clancy and J. W. Gaynor (2010). "Genetic factors are important determinants of impaired growth after infant cardiac surgery." J Thorac Cardiovasc Surg 140(1): 144-9.

Cellerino, A., P. Carroll, H. Thoenen and Y. A. Barde (1997). "Reduced size of retinal ganglion cell axons and hypomyelination in mice lacking brain-derived neurotrophic factor." Mol Cell Neurosci 9(5-6): 397-408.

Cheng, J., J. Gu, J. Ma, X. Chen, M. Zhang and W. Wang (2009). "Neurobehavioural effects, redox responses and tissue distribution in rat offspring developmental exposure to BDE-99." Chemosphere 75(7): 963-8.

Chevrier, J., K. G. Harley, A. Bradman, M. Gharbi, A. Sjodin and B. Eskenazi (2010). "Polybrominated diphenyl ether (PBDE) flame retardants and thyroid hormone during pregnancy." Environ Health Perspect 118(10): 1444-9.

Chi, Y., H. Xia, M. Su, P. Song, X. Qi, Y. Cui, Y. Cao, T. Chen, Y. Qiu, A. Zhao, X. Ma, X. Zheng and W. Jia (2011). "Metabonomic Phenotyping Reveals an Embryotoxicity of Deca-Brominated Diphenyl Ether in Mice." Chem Res Toxicol.

Ciomartan, T., T. Asami and M. Uchiyama (1999). "Serum lipoproteins and apolipoprotein E in infants with congenital hypothyroidism." Pediatr Int 41(3): 249-52.

Cirulli, F., N. Francia, A. Berry, L. Aloe, E. Alleva and S. J. Suomi (2009). "Early life stress as a risk factor for mental health: role of neurotrophins from rodents to non-human primates." Neurosci Biobehav Rev 33(4): 573-85.

Coburn, C. G., M. C. Curras-Collazo and P. R. Kodavanti (2007). "Polybrominated diphenyl ethers and ortho-substituted polychlorinated biphenyls as neuroendocrine disruptors of vasopressin release: effects during physiological

activation in vitro and structure-activity relationships." Toxicol Sci 98(1): 178-86.

Colton, C. A., C. M. Brown and M. P. Vitek (2005). "Sex steroids, APOE genotype and the innate immune system." Neurobiol Aging 26(3): 363-72.

Corbo, R. M., R. Scacchi and M. Cresta (2004). "Differential reproductive efficiency associated with common apolipoprotein e alleles in postreproductive-aged subjects." Fertil Steril 81(1): 104-7.

Costa, L. G. and G. Giordano (2007). "Developmental neurotoxicity of polybrominated diphenyl ether (PBDE) flame retardants." Neurotoxicology 28(6): 1047-67.

D'Hooge, R. and P. P. De Deyn (2001). "Applications of the Morris water maze in the study of learning and memory." Brain Res Brain Res Rev 36(1): 60-90.

Darnerud, P. O. (2003). "Toxic effects of brominated flame retardants in man and in wildlife." Environ Int 29(6): 841-53.

Darnerud, P. O., M. Aune, L. Larsson and S. Hallgren (2007). "Plasma PBDE and thyroxine levels in rats exposed to Bromkal or BDE-47." Chemosphere 67(9): S386-92.

Darnerud, P. O., G. S. Eriksen, T. Johannesson, P. B. Larsen and M. Viluksela (2001). "Polybrominated diphenyl ethers: occurrence, dietary exposure, and toxicology." Environ Health Perspect 109 Suppl 1: 49-68.

De Blasi, S., A. Montesanto, C. Martino, S. Dato, F. De Rango, A. C. Bruni, V. Mari, E. Feraco and G. Passarino (2009). "APOE polymorphism affects episodic memory among non demented elderly subjects." Exp Gerontol 44(3): 224-7.

Dennis, N. A., J. N. Browndyke, J. Stokes, A. Need, J. R. Burke, K. A. Welsh-Bohmer and R. Cabeza (2009). "Temporal lobe functional activity and connectivity in young adult APOE varepsilon4 carriers." Alzheimers Dement 6(4): 303-11.

Di Biase, A. and S. Salvati (1997). "Exogenous lipids in myelination and myelination." Kaohsiung J Med Sci 13(1): 19-29.

Dietschy, J. M. (2009). "Central nervous system: cholesterol turnover, brain development and neurodegeneration." Biol Chem 390(4): 287-93.

Dingemans, M. M., G. M. Ramakers, F. Gardoni, R. G. van Kleef, A. Bergman, M. Di Luca, M. van den Berg, R. H. Westerink and H. P. Vijverberg (2007). "Neonatal exposure to brominated flame retardant BDE-47 reduces long-term potentiation and postsynaptic protein levels in mouse hippocampus." Environ Health Perspect 115(6): 865-70.

Djalali, S., M. Holtje, G. Grosse, T. Rothe, T. Stroh, J. Grosse, D. R. Deng, R. Hellweg, R. Grantyn, H. Hortnagl and G. Ahnert-Hilger (2005). "Effects of brain-derived neurotrophic factor (BDNF) on glial cells and serotonergic neurones during development." J Neurochem 92(3): 616-27.

Domingo, J. L., R. Marti-Cid, V. Castell and J. M. Llobet (2008). "Human exposure to PBDEs through the diet in Catalonia, Spain: temporal trend. A review of recent literature on dietary PBDE intake." Toxicology 248(1): 25-32.

Driscoll, L. L., A. M. Gibson and A. Hieb (2009). "Chronic postnatal DE-71 exposure: effects on learning, attention and thyroxine levels." Neurotoxicol Teratol 31(2): 76-84.

Du, J., J. Chang, S. Guo, Q. Zhang and Z. Wang (2009). "ApoE 4 reduces the expression of Abeta degrading enzyme IDE by activating the NMDA receptor in hippocampal neurons." Neurosci Lett 464(2): 140-5.

Dufault, C., G. Poles and L. L. Driscoll (2005). "Brief postnatal PBDE exposure alters learning and the cholinergic modulation of attention in rats." Toxicol Sci 88(1): 172-80.

Eggesbo, M., C. Thomsen, J. V. Jorgensen, G. Becher, J. O. Odland and M. P. Longnecker (2011). "Associations between brominated flame retardants in human milk and thyroid-stimulating hormone (TSH) in neonates." Environ Res 111(6): 737-43.

Eichenbaum, H., P. Dudchenko, E. Wood, M. Shapiro and H. Tanila (1999). "The hippocampus, memory, and place cells: is it spatial memory or a memory space?" Neuron 23(2): 209-26.

Ellis-Hutchings, R. G., G. N. Cherr, L. A. Hanna and C. L. Keen (2006). "Polybrominated diphenyl ether (PBDE)-induced alterations in vitamin A and thyroid hormone concentrations in the rat during lactation and early postnatal development." Toxicol Appl Pharmacol 215(2): 135-45.

Eriksson, P., E. Jakobsson and A. Fredriksson (2001). "Brominated flame retardants: a novel class of developmental neurotoxicants in our environment?" Environ Health Perspect 109(9): 903-8.

Eriksson, P., H. Viberg, E. Jakobsson, U. Orn and A. Fredriksson (2002). "A brominated flame retardant, 2,2',4,4',5-pentabromodiphenyl ether: uptake, retention, and induction of neurobehavioral alterations in mice during a critical phase of neonatal brain development." Toxicol Sci 67(1): 98-103.

Fernandez, R. (2008). Brominated flame retardants and their metabolites: Novel insights into endocrine disruptive propertives. Institute of Risk Assessment Sciences (IRAS), Utrecht University.

Ferreira, A. A., M. J. Pereira, A. C. Manhaes and P. C. Barradas (2007). "Ultrastructural identification of oligodendrocyte/myelin proteins in corpus callosum of hypothyroid animals." Int J Dev Neurosci 25(2): 87-94.

Ferreira, D. C., T. F. Costa, S. L. Aguiar, A. R. Marques, S. A. Ramos, K. B. Gomes and J. I. Alvarez-Leite (2011). "Association of apoliprotein E polymorphisms and metabolic syndrome in subjects with extreme obesity." Clin Chim Acta 412(17-18): 1559-62.

Findeis, M. A. (2007). "The role of amyloid beta peptide 42 in Alzheimer's disease." Pharmacol Ther 116(2): 266-86.

Fiocco, A. J., N. P. Nair, G. Schwartz, F. N. Kin, R. Joober, J. Poirier and S. J. Lupien (2009). "Influence of genetic polymorphisms in the apolipoprotein (APOE) and the butyrylcholinesterase (BCHE) gene on stress markers in older adults: a 3-year study." Neurobiol Aging 30(6): 1001-5.

Franco-Pons, N., C. Virgos, W. F. Vogel, J. M. Urena, E. Soriano, J. A. del Rio and E. Vilella (2006). "Expression of discoidin domain receptor 1 during mouse brain development follows the progress of myelination." Neuroscience 140(2): 463-75.

Fuentes, S., M. T. Colomina, P. Vicens, N. Franco-Pons and J. L. Domingo (2007). "Concurrent exposure to perfluorooctane sulfonate and restraint stress during pregnancy in mice: effects on postnatal development and behavior of the offspring." Toxicol Sci 98(2): 589-98.

Gascon, M., M. Vrijheid, D. Martinez, J. Forns, J. O. Grimalt, M. Torrent and J. Sunyer (2011). "Effects of pre and postnatal exposure to low levels of polybromodiphenyl ethers on neurodevelopment and thyroid hormone levels at 4 years of age." Environ Int 37(3): 605-11.

Gaynor, J. W., M. Gerdes, E. H. Zackai, J. Bernbaum, G. Wernovsky, R. R. Clancy, M. F. Newman, A. M. Saunders, P. J. Heagerty, J. A. D'Agostino, D. McDonald-McGinn, S. C. Nicolson, T. L. Spray and G. P. Jarvik (2003). "Apolipoprotein E genotype and neurodevelopmental sequelae of infant cardiac surgery." J Thorac Cardiovasc Surg 126(6): 1736-45.

Gaynor, J. W., A. S. Nord, G. Wernovsky, J. Bernbaum, C. B. Solot, N. Burnham, E. Zackai, P. J. Heagerty, R. R. Clancy, S. C. Nicolson, G. P. Jarvik and M. Gerdes (2009). "Apolipoprotein E genotype modifies the risk of behavior problems after infant cardiac surgery." Pediatrics 124(1): 241-50.

Gaynor, J. W., G. Wernovsky, G. P. Jarvik, J. Bernbaum, M. Gerdes, E. Zackai, A. S. Nord, R. R. Clancy, S. C. Nicolson and T. L. Spray (2007). "Patient characteristics are important determinants of neurodevelopmental outcome at one year of age after neonatal and infant cardiac surgery." J Thorac Cardiovasc Surg 133(5): 1344-53, 1353 e1-3.

Gee, J. R. and V. C. Moser (2008). "Acute postnatal exposure to brominated diphenylether 47 delays neuromotor ontogeny and alters motor activity in mice." Neurotoxicol Teratol 30(2): 79-87.

Georgieff, M. K. (2007). "Nutrition and the developing brain: nutrient priorities and measurement." Am J Clin Nutr 85(2): 614S-620S.

Gibson, R. A. (2011). "Milk fat and health consequences." Nestle Nutr Workshop Ser Pediatr Program 67: 197-207.

Godfrey, M. E., D. P. Wojcik and C. A. Krone (2003). "Apolipoprotein E genotyping as a potential biomarker for mercury neurotoxicity." J Alzheimers Dis 5(3): 189-95.

Graef, S., P. Schonknecht, O. Sabri and U. Hegerl (2011). "Cholinergic receptor subtypes and their role in cognition, emotion, and vigilance control: an overview of preclinical and clinical findings." Psychopharmacology (Berl) 215(2): 205-29.

Grandjean, P. and P. J. Landrigan (2006). "Developmental neurotoxicity of industrial chemicals." Lancet 368(9553): 2167-78.

Greenwood, P. M., C. Lambert, T. Sunderland and R. Parasuraman (2005). "Effects of apolipoprotein E genotype on spatial attention, working memory, and their interaction in healthy, middle-aged adults: results From the National Institute of Mental Health's BIOCARD study." Neuropsychology 19(2): 199-211.

Grootendorst, J., A. Bour, E. Vogel, C. Kelche, P. M. Sullivan, J. C. Dodart, K. Bales and C. Mathis (2005). "Human apoE targeted replacement mouse lines: h-apoE4 and h-apoE3 mice differ on spatial memory performance and avoidance behavior." Behav Brain Res 159(1): 1-14.

Hartman, R. E., D. F. Wozniak, A. Nardi, J. W. Olney, L. Sartorius and D. M. Holtzman (2001). "Behavioral phenotyping of GFAP-apoE3 and -apoE4 transgenic mice: apoE4 mice show profound working memory impairments in the absence of Alzheimer's-like neuropathology." Exp Neurol 170(2): 326-44.

Hatters, D. M., C. A. Peters-Libeu and K. H. Weisgraber (2006). "Apolipoprotein E structure: insights into function." Trends Biochem Sci 31(8): 445-54.

He, P., A. G. Wang, T. Xia, P. Gao, Q. Niu, L. J. Guo and X. M. Chen (2009). "Mechanisms underlying the developmental neurotoxic effect of PBDE-47 and the enhanced toxicity associated with its combination with PCB153 in rats." Neurotoxicology 30(6): 1088-95.

Heise, V., N. Filippini, K. P. Ebmeier and C. E. Mackay (2010). "The APOE varepsilon4 allele modulates brain white matter integrity in healthy adults." Mol Psychiatry 16(9): 908-16.

Henck, J. W. (2002). Handbook of Neurotoxicology. Totowa, New Jersey, Humana Press Inc.

Herbstman, J. B., A. Sjodin, M. Kurzon, S. A. Lederman, R. S. Jones, V. Rauh, L. L. Needham, D. Tang, M. Niedzwiecki, R. Y. Wang and F. Perera (2010). "Prenatal exposure to PBDEs and neurodevelopment." Environ Health Perspect 118(5): 712-9.

Higgins, G. A., C. H. Large, H. T. Rupniak and J. C. Barnes (1997). "Apolipoprotein E and Alzheimer's disease: a review of recent studies." Pharmacol Biochem Behav 56(4): 675-85.

Huang, Y. (2006). "Apolipoprotein E and Alzheimer disease." Neurology 66(2 Suppl 1): S79-85.

Jenner, A. M., W. L. Lim, M. P. Ng, M. R. Wenk, G. Shui, M. J. Sharman, S. E. Gandy and R. N. Martins (2010). "The effect of APOE genotype on brain levels of oxysterols in young and old human APOE epsilon2, epsilon3 and epsilon4 knock-in mice." Neuroscience 169(1): 109-15.

Johansson, N., H. Viberg, A. Fredriksson and P. Eriksson (2008). "Neonatal exposure to deca-brominated diphenyl ether (PBDE 209) causes dose-response changes in spontaneous behaviour and cholinergic susceptibility in adult mice." Neurotoxicology 29(6): 911-9.

Jordan, C., V. Friedrich, Jr. and M. Dubois-Dalcq (1989). "In situ hybridization analysis of myelin gene transcripts in developing mouse spinal cord." J Neurosci 9(1): 248-57.

Jordan, C. L. and L. Doncarlos (2008). "Androgens in health and disease: an overview." Horm Behav 53(5): 589-95.

Kalueff, A. V. (2007). "Neurobiology of memory and anxiety: from genes to behavior." Neural Plast 2007: 78171.

Kenneth L. Davis, D. C., Joseph T. Coyle, and and C. Nemeroff (2002). Neuropsychopharmacology: The Fifth Generation of Progress., American College of Neuropsychopharmacology.

Kim, T. H., Y. J. Lee, E. Lee, M. S. Kim, S. J. Kwack, K. B. Kim, K. K. Chung, T. S. Kang, S. Y. Han, J. Lee, B. M. Lee and H. S. Kim (2009). "Effects of gestational exposure to decabromodiphenyl ether on reproductive parameters, thyroid hormone levels, and neuronal development in Sprague-Dawley rats offspring." J Toxicol Environ Health A 72(21-22): 1296-303.

Klein, A. B., R. Williamson, M. A. Santini, C. Clemmensen, A. Ettrup, M. Rios, G. M. Knudsen and S. Aznar (2011). "Blood BDNF concentrations reflect brain-tissue BDNF levels across species." Int J Neuropsychopharmacol 14(3): 347-53.

Kodama, Y., T. Kikusui, Y. Takeuchi and Y. Mori (2008). "Effects of early weaning on anxiety and prefrontal cortical and hippocampal myelination in male and female Wistar rats." Dev Psychobiol 50(4): 332-42.

Kodavanti, P. R., C. G. Coburn, V. C. Moser, R. C. MacPhail, S. E. Fenton, T. E. Stoker, J. L. Rayner, K. Kannan and L. S. Birnbaum (2011). "Developmental exposure to a commercial PBDE mixture, DE-71: neurobehavioral, hormonal, and reproductive effects." Toxicol Sci 116(1): 297-312.

Kodavanti, P. R. and E. C. Derr-Yellin (2002). "Differential effects of polybrominated diphenyl ethers and polychlorinated biphenyls on [3H]arachidonic acid release in rat cerebellar granule neurons." Toxicol Sci 68(2): 451-7.

Kodavanti, P. R. and T. R. Ward (2005). "Differential effects of commercial polybrominated diphenyl ether and polychlorinated biphenyl mixtures on intracellular signaling in rat brain in vitro." Toxicol Sci 85(2): 952-62.

Kononen, J., S. Soinila, H. Persson, J. Honkaniemi, T. Hokfelt and M. Pelto-Huikko (1994). "Neurotrophins and their receptors in the rat pituitary gland: regulation of BDNF and trkB mRNA levels by adrenal hormones." Brain Res Mol Brain Res 27(2): 347-54.

Kukolja, J., C. M. Thiel, T. Eggermann, K. Zerres and G. R. Fink (2010). "Medial temporal lobe dysfunction during encoding and retrieval of episodic memory in non-demented APOE epsilon4 carriers." Neuroscience 168(2): 487-97.

Kuriyama, S. N., A. Wanner, A. A. Fidalgo-Neto, C. E. Talsness, W. Koerner and I. Chahoud (2007). "Developmental exposure to low-dose PBDE-99: tissue distribution and thyroid hormone levels." Toxicology 242(1-3): 80-90.

Lambrinoudaki, I., G. Kaparos, D. Rizos, F. Galapi, A. Alexandrou, T. N. Sergentanis, M. Creatsa, G. Christodoulakos, E. Kouskouni and D. Botsis (2009). "Apolipoprotein E and paraoxonase 1 polymorphisms are associated with lower serum thyroid hormones in postmenopausal women." Clin Endocrinol (Oxf) 71(2): 284-90.

Lanterna, L. A. and F. Biroli (2009). "Significance of apolipoprotein E in subarachnoid hemorrhage: neuronal injury, repair, and therapeutic perspectives--a review." J Stroke Cerebrovasc Dis 18(2): 116-23.

Lee, D. J., K. M. Kim, B. T. Kim, K. N. Kim and N. S. Joo (2011). "ApoE polymorphism may determine low-density lipoprotein cholesterol level in association with obesity and metabolic syndrome in postmenopausal Korean women." Yonsei Med J 52(3): 429-34.

Lenroot, R. K. and J. N. Giedd (2011). "Annual Research Review: Developmental considerations of gene by environment interactions." J Child Psychol Psychiatry 52(4): 429-41.

Li, F. Q., K. A. Fowler, J. E. Neil, C. A. Colton and M. P. Vitek (2010). "An apolipoprotein E-mimetic stimulates axonal regeneration and remyelination after peripheral nerve injury." J Pharmacol Exp Ther 334(1): 106-15.

Lilienthal, H., A. Hack, A. Roth-Harer, S. W. Grande and C. E. Talsness (2006). "Effects of developmental exposure to 2,2 ,4,4 ,5-pentabromodiphenyl ether (PBDE-99) on sex steroids, sexual development, and sexually dimorphic behavior in rats." Environ Health Perspect 114(2): 194-201.

Lombroso, P. (2004). "[Learning and memory]." Rev Bras Psiquiatr 26(3): 207-10.

Long, J. R., P. Y. Liu, Y. J. Liu, Y. Lu, D. H. Xiong, L. Elze, R. R. Recker and H. W. Deng (2003). "APOE and TGF-beta1 genes are associated with obesity phenotypes." J Med Genet 40(12): 918-24.

Luciano, M., A. J. Gow, S. E. Harris, C. Hayward, M. Allerhand, J. M. Starr, P. M. Visscher and I. J. Deary (2009). "Cognitive ability at age 11 and 70 years, information processing speed, and APOE variation: the Lothian Birth Cohort 1936 study." Psychol Aging 24(1): 129-38.

Martin, M. G., S. Perga, L. Trovo, A. Rasola, P. Holm, T. Rantamaki, T. Harkany, E. Castren, F. Chiara and C. G. Dotti (2008). "Cholesterol loss enhances TrkB signaling in hippocampal neurons aging in vitro." Mol Biol Cell 19(5): 2101-12.

Masuo, Y. and M. Ishido (2011). "Neurotoxicity of endocrine disruptors: possible involvement in brain development and neurodegeneration." J Toxicol Environ Health B Crit Rev 14(5-7): 346-69.

Mattson, M. P. (2008). "Glutamate and neurotrophic factors in neuronal plasticity and disease." Ann N Y Acad Sci 1144: 97-112.

Meerts, I. A., J. J. van Zanden, E. A. Luijks, I. van Leeuwen-Bol, G. Marsh, E. Jakobsson, A. Bergman and A. Brouwer (2000). "Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin in vitro." Toxicol Sci 56(1): 95-104.

Miller, R. H. (2002). "Regulation of oligodendrocyte development in the vertebrate CNS." Prog Neurobiol 67(6): 451-67.

Miodovnik, A. (2011). "Environmental neurotoxicants and developing brain." Mt Sinai J Med 78(1): 58-77.

Mondadori, C. R., D. J. de Quervain, A. Buchmann, H. Mustovic, M. A. Wollmer, C. F. Schmidt, P. Boesiger, C. Hock, R. M. Nitsch, A. Papassotiropoulos and K. Henke (2007). "Better memory and neural efficiency in young apolipoprotein E epsilon4 carriers." Cereb Cortex 17(8): 1934-47.

Morck, A., H. Hakk, U. Orn and E. Klasson Wehler (2003). "Decabromodiphenyl ether in the rat: absorption, distribution, metabolism, and excretion." Drug Metab Dispos 31(7): 900-7.

Morgado, I. (2005). "[The psychobiology of learning and memory: fundamentals and recent advances]." Rev Neurol 40(5): 289-97.

Morris, R. G. (2003). "Long-term potentiation and memory." Philos Trans R Soc Lond B Biol Sci 358(1432): 643-7.

Moser, V. C. (2000). "The functional observational battery in adult and developing rats." Neurotoxicology 21(6): 989-96.

Mutter, J., J. Naumann, C. Sadaghiani, H. Walach and G. Drasch (2004). "Amalgam studies: disregarding basic principles of mercury toxicity." Int J Hyg Environ Health 207(4): 391-7.

Nichol, K., S. P. Deeny, J. Seif, K. Camaclang and C. W. Cotman (2009). "Exercise improves cognition and hippocampal plasticity in APOE epsilon4 mice." Alzheimers Dement 5(4): 287-94.

Nierenberg, J., N. Pomara, M. J. Hoptman, J. J. Sidtis, B. A. Ardekani and K. O. Lim (2005). "Abnormal white matter integrity in healthy apolipoprotein E epsilon4 carriers." Neuroreport 16(12): 1369-72.

Paratcha, G. and C. F. Ibanez (2002). "Lipid rafts and the control of neurotrophic factor signaling in the nervous system: variations on a theme." Curr Opin Neurobiol 12(5): 542-9.

Park, J. S., J. She, A. Holden, M. Sharp, R. Gephart, G. Souders-Mason, V. Zhang, J. Chow, B. Leslie and K. Hooper (2011). "High postnatal exposures to polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) via breast milk in California: does BDE-209 transfer to breast milk?" Environ Sci Technol 45(10): 4579-85.

Pasquini, J. M. and A. M. Adamo (1994). "Thyroid hormones and the central nervous system." Dev Neurosci 16(1-2): 1-8.

Patil, S. S., B. Sunyer, H. Hoger and G. Lubec (2009). "Evaluation of spatial memory of C57BL/6J and CD1 mice in the Barnes maze, the Multiple T-maze and in the Morris water maze." Behav Brain Res 198(1): 58-68.

Pendse, A. A., J. M. Arbones-Mainar, L. A. Johnson, M. K. Altenburg and N. Maeda (2009). "Apolipoprotein E knock-out and knock-in mice: atherosclerosis, metabolic syndrome, and beyond." J Lipid Res 50 Suppl: S178-82.

Pfankuch, T., A. Rizk, R. Olsen, C. Poage and J. Raber (2005). "Role of circulating androgen levels in effects of apoE4 on cognitive function." Brain Res 1053(1-2): 88-96.

Pott, F., S. Gingele, T. Clarner, J. Dang, W. Baumgartner, C. Beyer and M. Kipp (2009). "Cuprizone effect on myelination, astrogliosis and microglia attraction in the mouse basal ganglia." Brain Res 1305: 137-49.

Querfurth, H. W. and F. M. LaFerla (2010). "Alzheimer's disease." N Engl J Med 362(4): 329-44.

Raber, J. (2007). "Role of apolipoprotein E in anxiety." Neural Plast 2007: 91236.

Raber, J. (2008). "AR, apoE, and cognitive function." Horm Behav 53(5): 706-15.

Raber, J., Y. Huang and J. W. Ashford (2004). "ApoE genotype accounts for the vast majority of AD risk and AD pathology." Neurobiol Aging 25(5): 641-50.

Raber, J., D. Wong, M. Buttini, M. Orth, S. Bellosta, R. E. Pitas, R. W. Mahley and L. Mucke (1998). "Isoform-specific effects of human apolipoprotein E on brain function revealed in ApoE knockout mice: increased susceptibility of females." Proc Natl Acad Sci U S A 95(18): 10914-9.

Raber, J., D. Wong, G. Q. Yu, M. Buttini, R. W. Mahley, R. E. Pitas and L. Mucke (2000). "Apolipoprotein E and cognitive performance." Nature 404(6776): 352-4.

Ramos, A. (2008). "Animal models of anxiety: do I need multiple tests?" Trends Pharmacol Sci 29(10): 493-8.

Reichardt, L. F. (2006). "Neurotrophin-regulated signalling pathways." Philos Trans R Soc Lond B Biol Sci 361(1473): 1545-64.

Reiman, E. M., K. Chen, G. E. Alexander, R. J. Caselli, D. Bandy, D. Osborne, A. M. Saunders and J. Hardy (2004). "Functional brain abnormalities in young adults at genetic risk for late-onset Alzheimer's dementia." Proc Natl Acad Sci U S A 101(1): 284-9.

Reitz, C. and R. Mayeux (2009). "Endophenotypes in normal brain morphology and Alzheimer's disease: a review." Neuroscience 164(1): 174-90.

Ribes, D., M. T. Colomina, P. Vicens and J. L. Domingo (2008). "Effects of oral aluminum exposure on behavior and neurogenesis in a transgenic mouse model of Alzheimer's disease." Exp Neurol 214(2): 293-300.

Rice, D. C., E. A. Reeve, A. Herlihy, R. T. Zoeller, W. D. Thompson and V. P. Markowski (2007). "Developmental delays and locomotor activity in the C57BL6/J mouse following neonatal exposure to the fully-brominated PBDE, decabromodiphenyl ether." Neurotoxicol Teratol 29(4): 511-20.

Rice, D. C., W. D. Thompson, E. A. Reeve, K. D. Onos, M. Assadollahzadeh and V. P. Markowski (2009). "Behavioral changes in aging but not young mice after neonatal exposure to the polybrominated flame retardant decaBDE." Environ Health Perspect 117(12): 1903-11.

Richardson, V. M., D. F. Staskal, D. G. Ross, J. J. Diliberto, M. J. DeVito and L. S. Birnbaum (2008). "Possible mechanisms of thyroid hormone disruption in mice by BDE 47, a major polybrominated diphenyl ether congener." Toxicol Appl Pharmacol 226(3): 244-50.

Robertson, J., J. Curley, J. Kaye, J. Quinn, T. Pfankuch and J. Raber (2005). "apoE isoforms and measures of anxiety in probable AD patients and Apoe-/- mice." Neurobiol Aging 26(5): 637-43.

Rudy, J. W. (2009). "Context representations, context functions, and the parahippocampal-hippocampal system." Learn Mem 16(10): 573-85.

Ruiz-Medina, J. (2007). Facilitación del aprendizaje y la memoria de una tarea de referencia espacial en el LAM por AEIC en ratas wistar. Psicobiologia. Barcelona, Universidad Autónoma de Barcelona.

Ryan, L., K. Walther, B. B. Bendlin, L. F. Lue, D. G. Walker and E. L. Glisky (2011). "Age-related differences in white matter integrity and cognitive function are related to APOE status." Neuroimage 54(2): 1565-77.

Salehi, B., M. I. Cordero and C. Sandi (2010). "Learning under stress: the inverted-U-shape function revisited." Learn Mem 17(10): 522-30.

Santisteban, P. and J. Bernal (2005). "Thyroid development and effect on the nervous system." Rev Endocr Metab Disord 6(3): 217-28.

Sartori, S. B., R. Landgraf and N. Singewald (2011). "The clinical implications of mouse models of enhanced anxiety." Future Neurol 6(4): 531-571.

Savitz, J., L. van der Merwe, D. J. Stein, M. Solms and R. Ramesar (2007). "Genotype and childhood sexual trauma moderate neurocognitive performance: a possible role for brain-derived neurotrophic factor and apolipoprotein E variants." Biol Psychiatry 62(5): 391-9.

Scacchi, R., R. M. Corbo, O. Rickards, E. Mantuano, A. Guevara and G. F. De Stefano (1997). "Apolipoprotein B and E genetic polymorphisms in the Cayapa Indians of Ecuador." Hum Biol 69(3): 375-82.

Schmechel, D. E., J. Browndyke and A. Ghio (2006). "Strategies for dissecting genetic-environmental interactions in neurodegenerative disorders." Neurotoxicology 27(5): 637-57.

Schuhmacher, M., H. Kiviranta, P. Ruokojarvi, M. Nadal and J. L. Domingo (2009). "Concentrations of PCDD/Fs, PCBs and PBDEs in breast milk of women from Catalonia, Spain: a follow-up study." Environ Int 35(3): 607-13.

Sebastiao, A. M., N. Assaife-Lopes, M. J. Diogenes, S. H. Vaz and J. A. Ribeiro (2011). "Modulation of brain-derived neurotrophic factor (BDNF) actions in the nervous system by adenosine A(2A) receptors and the role of lipid rafts." Biochim Biophys Acta 1808(5): 1340-9.

Sharma, S., S. Rakoczy and H. Brown-Borg (2010). "Assessment of spatial memory in mice." Life Sci 87(17-18): 521-36.

Shepherd, J. K., S. S. Grewal, A. Fletcher, D. J. Bill and C. T. Dourish (1994). "Behavioural and pharmacological characterisation of the elevated "zero-maze" as an animal model of anxiety." Psychopharmacology (Berl) 116(1): 56-64.

Siegel, J. A., G. E. Haley and J. Raber (2010). "Apolipoprotein E isoform-dependent effects on anxiety and cognition in female TR mice." Neurobiol Aging.

Smith, M. A. (1996). "Hippocampal vulnerability to stress and aging: possible role of neurotrophic factors." Behav Brain Res 78(1): 25-36.

Sotres-Bayon, F., C. K. Cain and J. E. LeDoux (2006). "Brain mechanisms of fear extinction: historical perspectives on the contribution of prefrontal cortex." Biol Psychiatry 60(4): 329-36.

Staskal, D. F., J. J. Diliberto and L. S. Birnbaum (2006). "Disposition of BDE 47 in developing mice." Toxicol Sci 90(2): 309-16.

Staskal, D. F., H. Hakk, D. Bauer, J. J. Diliberto and L. S. Birnbaum (2006). "Toxicokinetics of polybrominated diphenyl ether congeners 47, 99, 100, and 153 in mice." Toxicol Sci 94(1): 28-37.

Stein, D. S., N. J. Blum and W. J. Barbaresi (2011). "Developmental and behavioral disorders through the life span." Pediatrics 128(2): 364-73.

Stewart, W. F., B. S. Schwartz, D. Simon, K. Kelsey and A. C. Todd (2002). "ApoE genotype, past adult lead exposure, and neurobehavioral function." Environ Health Perspect 110(5): 501-5.

Stoker, T. E., R. L. Cooper, C. S. Lambright, V. S. Wilson, J. Furr and L. E. Gray (2005). "In vivo and in vitro anti-androgenic effects of DE-71, a commercial polybrominated diphenyl ether (PBDE) mixture." Toxicol Appl Pharmacol 207(1): 78-88.

Stoker, T. E., S. C. Laws, K. M. Crofton, J. M. Hedge, J. M. Ferrell and R. L. Cooper (2004). "Assessment of DE-71, a commercial polybrominated diphenyl ether (PBDE) mixture, in the EDSP male and female pubertal protocols." Toxicol Sci 78(1): 144-55.

Strolin Benedetti, M., R. Whomsley and E. L. Baltes (2005). "Differences in absorption, distribution, metabolism and excretion of xenobiotics between the paediatric and adult populations." Expert Opinion on Drug Metabolism & Toxicology 1(3): 447-471.

Sullivan, P. M., B. Han, F. Liu, B. E. Mace, J. F. Ervin, S. Wu, D. Koger, S. Paul and K. R. Bales (2009). "Reduced levels of human apoE4 protein in an animal model of cognitive impairment." Neurobiol Aging 32(5): 791-801.

Sullivan, P. M., B. E. Mace, N. Maeda and D. E. Schmechel (2004). "Marked regional differences of brain human apolipoprotein E expression in targeted replacement mice." Neuroscience 124(4): 725-33.

Suzuki, S., K. Kiyosue, S. Hazama, A. Ogura, M. Kashihara, T. Hara, H. Koshimizu and M. Kojima (2007). "Brain-derived neurotrophic factor regulates cholesterol metabolism for synapse development." J Neurosci 27(24): 6417-27.

Talsness, C. E. (2008). "Overview of toxicological aspects of polybrominated diphenyl ethers: a flame-retardant additive in several consumer products." Environ Res 108(2): 158-67.

Talsness, C. E., S. N. Kuriyama, A. Sterner-Kock, P. Schnitker, S. W. Grande, M. Shakibaei, A. Andrade, K. Grote and I. Chahoud (2008). "In utero and lactational exposures to low doses of polybrominated diphenyl ether-47 alter the reproductive system and thyroid gland of female rat offspring." Environ Health Perspect 116(3): 308-14.

Tanaka, H., J. Ma, K. F. Tanaka, K. Takao, M. Komada, K. Tanda, A. Suzuki, T. Ishibashi, H. Baba, T. Isa, R. Shigemoto, K. Ono, T. Miyakawa and K. Ikenaka (2009). "Mice with altered myelin proteolipid protein gene expression display cognitive deficits accompanied by abnormal neuron-glia interactions and decreased conduction velocities." J Neurosci 29(26): 8363-71.

Tau, G. Z. and B. S. Peterson (2010). "Normal development of brain circuits." Neuropsychopharmacology 35(1): 147-68.

Taylor, A. E., P. A. Guthrie, G. D. Smith, J. Golding, N. Sattar, A. D. Hingorani, J. E. Deanfield and I. N. Day (2011). "IQ, educational attainment, memory and plasma lipids: associations with apolipoprotein E genotype in 5995 children." Biol Psychiatry 70(2): 152-8.

Thompson, C. C. and G. B. Potter (2000). "Thyroid hormone action in neural development." Cereb Cortex 10(10): 939-45.

Tseng, L. H., C. W. Lee, M. H. Pan, S. S. Tsai, M. H. Li, J. R. Chen, J. J. Lay and P. C. Hsu (2006). "Postnatal exposure of the male mouse to 2,2',3,3',4,4',5,5',6,6'-decabrominated diphenyl ether: decreased epididymal sperm functions without alterations in DNA content and histology in testis." Toxicology 224(1-2): 33-43.

Turyk, M. E., V. W. Persky, P. Imm, L. Knobeloch, R. Chatterton and H. A. Anderson (2008). "Hormone disruption by PBDEs in adult male sport fish consumers." Environ Health Perspect 116(12): 1635-41.

van Meer, P., S. Acevedo and J. Raber (2007). "Impairments in spatial memory retention of GFAP-apoE4 female mice." Behav Brain Res 176(2): 372-5.

Vandenbrouck, Y., B. Janvier, C. Loriette, G. Bereziat and M. Mangeney-Andreani (1994). "The modulation of apolipoprotein E gene expression by 3,3'-5-triiodothyronine in HepG2 cells occurs at transcriptional and post-transcriptional levels." Eur J Biochem 224(2): 463-71.

Viberg, H. (2009). "Neonatal ontogeny and neurotoxic effect of decabrominated diphenyl ether (PBDE 209) on levels of synaptophysin and tau." Int J Dev Neurosci 27(5): 423-9.

Viberg, H., A. Fredriksson and P. Eriksson (2002). "Neonatal exposure to the brominated flame retardant 2,2',4,4',5-pentabromodiphenyl ether causes altered susceptibility in the cholinergic transmitter system in the adult mouse." Toxicol Sci 67(1): 104-7.

Viberg, H., A. Fredriksson and P. Eriksson (2003). "Neonatal exposure to polybrominated diphenyl ether (PBDE 153) disrupts spontaneous behaviour, impairs learning and memory, and decreases hippocampal cholinergic receptors in adult mice." Toxicol Appl Pharmacol 192(2): 95-106.

Viberg, H., A. Fredriksson and P. Eriksson (2004). "Investigations of strain and/or gender differences in developmental neurotoxic effects of polybrominated diphenyl ethers in mice." Toxicol Sci 81(2): 344-53.

Viberg, H., A. Fredriksson and P. Eriksson (2007). "Changes in spontaneous behaviour and altered response to nicotine in the adult rat, after neonatal exposure to the brominated flame retardant, decabrominated diphenyl ether (PBDE 209)." Neurotoxicology 28(1): 136-42.

Viberg, H., A. Fredriksson, E. Jakobsson, U. Orn and P. Eriksson (2003). "Neurobehavioral derangements in adult mice receiving decabrominated diphenyl ether (PBDE 209) during a defined period of neonatal brain development." Toxicol Sci 76(1): 112-20.

Viberg, H., N. Johansson, A. Fredriksson, J. Eriksson, G. Marsh and P. Eriksson (2006). "Neonatal exposure to higher brominated diphenyl ethers, hepta-, octa-, or nonabromodiphenyl ether, impairs spontaneous behavior and learning and memory functions of adult mice." Toxicol Sci 92(1): 211-8.

Vila-Rodriguez, F., W. G. Honer, S. M. Innis, C. L. Wellington and C. L. Beasley "ApoE and cholesterol in schizophrenia and bipolar disorder: comparison of grey and white matter and relation with APOE genotype." J Psychiatry Neurosci 36(1): 47-55.

Vila-Rodriguez, F., W. G. Honer, S. M. Innis, C. L. Wellington and C. L. Beasley (2011). "ApoE and cholesterol in schizophrenia and bipolar disorder: comparison of grey and white matter and relation with APOE genotype." J Psychiatry Neurosci 36(1): 47-55.

Vizcaino, E., J. O. Grimalt, M. J. Lopez-Espinosa, S. Llop, M. Rebagliato and F. Ballester (2010). "Polybromodiphenyl ethers in mothers and their newborns from a non-occupationally exposed population (Valencia, Spain)." Environ Int 37(1): 152-7.

Vorhees, C. V. and M. T. Williams (2006). "Morris water maze: procedures for assessing spatial and related forms of learning and memory." Nat Protoc 1(2): 848-58.

Wang, Z. Y., T. Miki, Y. Ding, S. J. Wang, Y. H. Gao, X. L. Wang, Y. H. Wang, T. Yokoyama, K. Warita, K. Ohta, S. Suzuki, T. Ohnishi, T. Obama, K. S. Bedi, Y. Takeuchi and B. E. Shan (2011). "A high cholesterol diet given to apolipoprotein E-knockout mice has a differential effect on the various neurotrophin systems in the hippocampus." Metab Brain Dis 26(3): 185-94.

Watson, R. E., J. M. Desesso, M. E. Hurtt and G. D. Cappon (2006). "Postnatal growth and morphological development of the brain: a species comparison." Birth Defects Res B Dev Reprod Toxicol 77(5): 471-84.

Weiss, B. a. O. D., John L. (1994). Neurobehavioral Toxicity: Analysis and Interpretation. New York, Raven Press Ltd.

Wolk, D. A. and B. C. Dickerson (2010). "Apolipoprotein E (APOE) genotype has dissociable effects on memory and attentional-executive network function in Alzheimer's disease." Proc Natl Acad Sci U S A 107(22): 10256-61.

Wright, R. O., H. Hu, E. K. Silverman, S. W. Tsaih, J. Schwartz, D. Bellinger, E. Palazuelos, S. T. Weiss and M. Hernandez-Avila (2003). "Apolipoprotein E genotype predicts 24-month bayley scales infant development score." Pediatr Res 54(6): 819-25.

Xiao, J., T. J. Kilpatrick and S. S. Murray (2009). "The role of neurotrophins in the regulation of myelin development." Neurosignals 17(4): 265-76.

Zhou, T., M. M. Taylor, M. J. DeVito and K. M. Crofton (2002). "Developmental exposure to brominated diphenyl ethers results in thyroid hormone disruption." Toxicol Sci 66(1): 105-16.

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DL:T. 162-2012