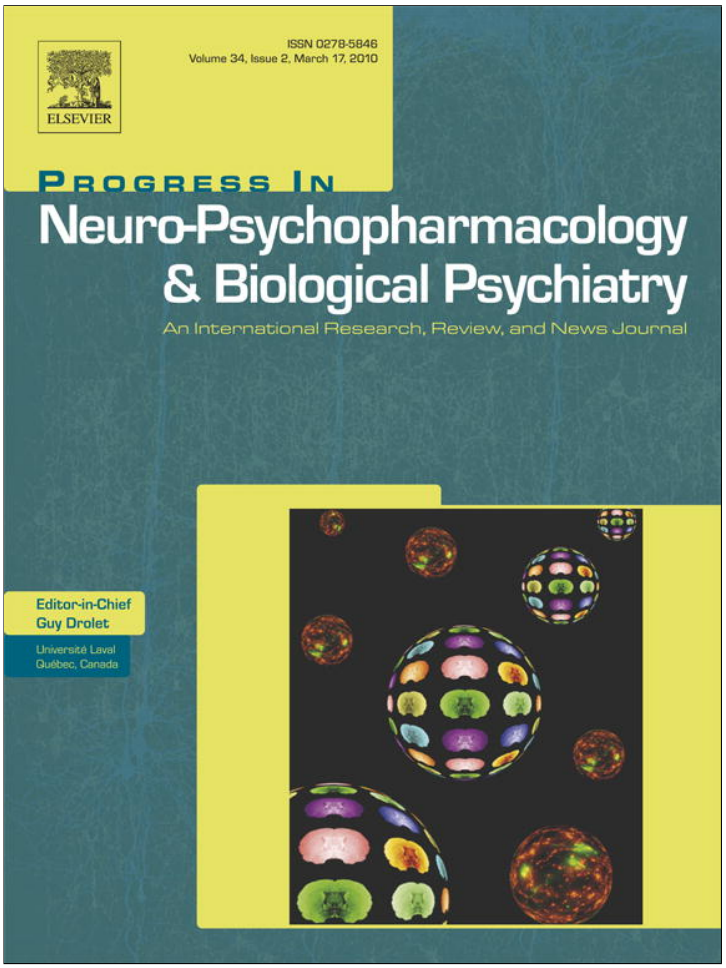

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Decreased kainate receptors in the hippocampus of apolipoprotein D knockout mice

Simone Boer [a,b], Diego Sanchez [c], Ivo Reinieren [a,d], Tom van den Boom [a,d], Madhara Udawela [a], Elizabeth Scarr [a,b], Maria D. Ganfornina [c], Brian Dean [a,e,f,*]

[a] The Rebecca L. Cooper Research Laboratories, The Mental Health Research Institute, Australia
[b] Centre for Neuroscience, The University of Melbourne, Parkville, Australia
[c] Instituto de Biología y Genética Molecular, Departamento de Bioquímica y Biología Molecular y Fisiología, Universidad de Valladolid-CSIC, Valladolid, Spain
[d] Department of Health and Environment Technology, Avans University, The Netherlands
[e] Department of Psychiatry, The University of Melbourne, Parkville, Australia
[f] Department of Psychological Medicine, Monash University, Clayton, Victoria, Australia

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ABSTRACT

Apolipoprotein D (ApoD) has many actions critical to maintaining mammalian CNS function. It is therefore significant that levels of ApoD have been shown to be altered in the CNS of subjects with schizophrenia, suggesting a role for ApoD in the pathophysiology of the disorder. There is also a large body of evidence that cortical and hippocampal glutamatergic, serotonergic and cholinergic systems are affected by the pathophysiology of schizophrenia. Thus, we decided to use in vitro radioligand binding and autoradiography to measure levels of ionotropic glutamate, some muscarinic and serotonin 2A receptors in the CNS of ApoD$^{-/-}$ and isogenic wild-type mice. These studies revealed a 20% decrease (mean ± SEM: 104 ± 10.2 vs. 130 ± 10.4 fmol/mg ETE) in the density of kainate receptors in the CA 2–3 of the ApoD$^{-/-}$ mice. In addition there was a global decrease in AMPA receptors ($F_{1,214} = 4.67$, $p < 0.05$) and a global increase in muscarinic M2/M4 receptors ($F_{1,208} = 22.77$, $p < 0.0001$) in the ApoD$^{-/-}$ mice that did not reach significance in any single cytoarchitectural region. We conclude that glutamatergic pathways seem to be particularly affected in ApoD$^{-/-}$ mice and this may contribute to the changes in learning and memory, motor tasks and orientation-based tasks observed in these animals, all of which involve glutamatergic neurotransmission.

1. Introduction

The discovery that levels of apolipoprotein D (ApoD) were increased in the CNS from subjects with schizophrenia (Thomas et al., 2001b, 2003a), bipolar disorder (Thomas et al., 2001b, 2003a), Parkinson's disease (Ordonez et al., 2006) and Alzheimer's disease (Terrisse et al., 1998; Thomas et al., 2003d) suggested a role in the pathophysiology of disorders of the human CNS. ApoD belongs to the lipocalin family of proteins, is synthesized and secreted by oligodendrocytes and astrocytes (Rassart et al., 2000), and is thought to have many functions. These functions include transportation of hydrophobic ligands (Rassart et al., 2000), the binding of arachidonic acid (Vogt and Skerra, 2001), signaling through arachidonic acid pathways (Thomas et al., 2003c), neuro-protection (Ganfornina et al., 2008; He et al., 2009) as well as acting as a neurotrophic factor and to increase synaptic density (Kosacka et al., 2009). Clearly increased levels of ApoD could affect any or all of these important functions, having either beneficial or detrimental effects in the CNS of subjects with any of these disorders.

Given the notion that ApoD could have either beneficial or detrimental effects in the human CNS, it is significant that the antipsychotic drug clozapine increases ApoD levels in the rodent CNS (Thomas et al., 2001a). Subsequently, it has been shown that increasing levels of ApoD may be a common effect of atypical antipsychotic drugs (Khan et al., 2003). It is not yet clear whether typical antipsychotic drugs, as a class, act to increase (Thomas et al., 2003b) or decrease (Khan et al., 2003) levels of ApoD in the CNS.

Abbreviations: [^{3}H]AF-DX 384, [2,3-Dipropylamino-^{3}H]-5,11-dihydro-11-[2-[2-[(N,N-dipropylaminomethyl)piperidin-1-yl]ethylamino]-carbonyl]-6H-pyrido[2,3-b][1,4]benzodiazepin-6-o; [^{3}H]AMPA, [^{3}H]amino-3-hydroxy-5-methylisoxazole-4-proprionic acid; [^{3}H]MK-801, [^{3}H]dizocilpine; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; ApoD, apolipoprotein D; CA, Cornu Ammonis; Chrm, muscarinic receptors; DG, dentate gyrus; ETE, Estimated Tissue Equivalent; Gria1, AMPA ionotropic receptor sub-unit 1; Gria2, AMPA ionotropic receptor sub-unit 2; Gria3, AMPA ionotropic receptor sub-unit 3; Gria4, AMPA ionotropic receptor sub-unit 4; Grik1, Kainate ionotropic receptor sub-unit 1; Grik2, Kainate ionotropic receptor sub-unit 2; Grik3, Kainate ionotropic receptor sub-unit 3; Grik4, Kainate ionotropic receptor sub-unit 4; Grik5, Kainate ionotropic receptor sub-unit 5; Grin1, NMDA ionotropic receptor sub-unit 1; Grin2a, NMDA ionotropic receptor sub-unit 2a; Grin2b, NMDA ionotropic receptor sub-unit 2b; Grin2c, NMDA ionotropic receptor sub-unit 2c; Grin2d, NMDA ionotropic receptor sub-unit 2d; Grin3a, NMDA ionotropic receptor sub-unit 3a; Grin3b, NMDA ionotropic receptor sub-unit 3b; GR, Stratum granulosum; Htr2A, serotonin 2A receptor; KAR, kainate receptor; LA, Stratum lacunosum; MO, Stratum molecular; NMDA, N-methyl-D-aspartic acid receptor; OR, Stratum oriens; PY, Stratum pyramidale; RA, Stratum radiatum; W/T, isogenic wild-type mice.

* Corresponding author. The Rebecca L. Cooper Research Laboratories, The Mental Health Research Institute, Locked Bag 11, Parkville, Victoria 3052, Australia. Tel.: +61 3 9389 2990; fax: +61 3 9387 5061.
E-mail address: anddali@unimelb.edu.au (B. Dean).

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However, it is possible that some of the therapeutic benefits achieved with atypical antipsychotic drugs (Bilder et al., 2002) may be via central effects on ApoD.

The potential to understand the role of ApoD in the mammalian CNS has been advanced by the generation of an ApoD$^{-/-}$ mouse (Ganfornina et al., 2008). Early findings from the study of these mice show that they have increased sensitivity to oxidative stress, increased levels of brain lipid peroxidation, impaired locomotor abilities and deficits in learning (Ganfornina et al., 2008). In addition, compared to isogenic wild-type mice (W/T), ApoD$^{-/-}$ mice have been shown to have changes in arachidonic acid pathways after receiving clozapine (Thomas and Yao, 2007). This supports the suggestion that ApoD may be involved in the mechanisms by which clozapine reduces the symptoms of schizophrenia. As clozapine is likely to achieve its therapeutic benefits by targeting pathways affected by the pathophysiology of schizophrenia, these data also suggest that schizophrenia-related pathways may be affected in the CNS of ApoD$^{-/-}$ mice. If this proves to be the case then altered levels of ApoD in the CNS of subjects with schizophrenia could drive at least part of the pathophysiology of the disorder.

We postulated that if ApoD targets systems affected by the pathophysiology of schizophrenia, markers of those systems could be altered in the CNS of ApoD$^{-/-}$ mice. There is now a large body of evidence suggesting that cortical and hippocampal glutamatergic (Paz et al., 2008), serotonergic (Dean, 2003) and cholinergic (Raedler et al., 2007) systems are affected by the pathophysiology of schizophrenia. We have, therefore, measured levels of ionotropic glutamate, some muscarinic receptors (Chrm) and the serotonin 2A receptor (Htr2a), all of which are key components of cortical and hippocampal glutamatergic, serotonergic and cholinergic systems that have been shown to be altered in the CNS of subjects with schizophrenia (Dean, 2003; Raedler et al., 2007; Scarr et al., 2005), in the CNS from ApoD$^{-/-}$ and isogenic wild-type mice (W/T).

2. Methods

2.1. Materials

[^{3}H]pirenzepine, [2,3-Dipropylamino-^{3}H]-5,11-dihydro-11-[2-[2-[(N,N-dipropylaminomethyl)piperidin-1-yl]ethylamino]-carbonyl]-6H-pyrido[2,3-b][1,4]benzodiazepin-6-o ([^{3}H]AF-DX 384), [^{3}H]4-diphenylacetoxy-N-methylpiperidine ([^{3}H]4-DAMP), [^{3}H]kainic acid, [^{3}H]dizocilpine ([^{3}H]MK-801), [^{3}H]amino-3-hydroxy-5-methylisoxazole-4-proprionic acid ([^{3}H]AMPA), and [^{3}H]ketanserin were sourced from Perkin Elmer. [^{3}H]micro-scales were obtained from Amersham. All other laboratory grade chemicals were sourced from Sigma.

2.2. Tissue preparation

The CNS were collected from 2 month old ApoD$^{-/-}$ female mice ($n = 10$), with a targeted deletion at exon 6 (Ganfornina et al., 2008), and ten isogenic W/T mice and frozen at −70 °C until required. Subsequently, 35 sequential frozen sections (20 μm thick; 3 total binding, 2 non-specific binding for each radioligand) were cut beginning at approximately 1.5 mm posterior to Bregma.

Mice were F1 progeny from homozygous crosses of the corresponding genotype to avoid maternal effects. The parental cohort consisted of littermates from crosses between heterozygous ApoD$^{+/-}$ from an isogenic line with homogeneous genetic background (backcrossed for 11 generations into C57BL/6 strain).

2.3. Radioligand binding with in situ autoradiography

Radioligand binding for this study was carried out using single-point saturation analyses; because the radioligand was present at concentrations at least three times that of the Kd for each radioligand this approach gives a good estimate of total receptor density (Scarr et al., 2003).

The binding of [^{3}H]kainic acid, [^{3}H]MK-801[^{3}H]AMPA (Scarr et al., 2005), [^{3}H]pirenzepine (Dean et al., 1996), [^{3}H]AF-DX 384 (Crook et al., 1999), [^{3}H]4-DAMP (Dean et al., 2008) and [^{3}H]ketanserin (Dean and Hayes, 1996) were all measured as described previously (conditions summarized in Table 1). Following completion of the radioligand binding protocols washed, dried and partially fixed sections were opposed to BAS-TR2025 phospho-imaging plates with [^{3}H]micro-scales until a quantifiable image was obtained and then scanned using a BAS5000 high resolution phosphoimager (Fuji Photo Film Co.) (Pavey et al., 2002). Images were analysed using AIS image analysis software (Imaging Research Inc.). Measurements from [^{3}H] high micro-scales were recorded and a standard curve was generated from the values. Total binding and non-specific binding values were estimated from the standard curve, density values were read at dpm/mg ETE (Estimated Tissue Equivalent) and converted to fmol/mg ETE, using the specific activities of the individual radioligands. Specific binding measurements were calculated by subtracting non-specific binding from total values.

2.4. Cresyl violet staining

The binding of each radioligand to each section was compared to the same section stained with cresyl violet to allow the intensity of binding in cytoarchitectural distinct regions to be measured. Cresyl violet staining was performed as described previously following imaging of radioligand bound sections (Dean et al., 2008). Specifically,

Table 1
The conditions used to determine receptor binding in ApoD$^{-/-}$ mice and wild-type littermates.

Receptor	Radioligand	Competing compound	Buffer	Conditions
Muscarinic M1/M4	15 nM [^{3}H]pirenzepine	1 μM QNX (3-quinuclidinyl xanthene-9-carboxylate hemioxalate salt)	10 mM KH_2PO_4, 10 mMNa_2HPO_4, pH 7.4	Pre-incubation: Nil Incubation: 30 min, RT
Muscarinic M2/M4	7 nM [^{3}H]AF-DX 384	1 μM tropacamide	10 mM KH_2PO_4, 10 mMNa_2HPO_4, pH 7.4	Pre-incubation: 30 min, RT Incubation: 60 min, RT
Muscarinic M3	3 nM [^{3}H]4-DAMP	10 μM 4-DAMP mustard	50 mM Tris–HCl, pH 7.4	Pre-incubation: 15 min, RT Incubation: 60 min, RT
Kainate	40 nM [^{3}H]kainic acid	1 mM L-glutamate	50 mM Tris-Acetate (pH 7.4)	Pre-incubation: 30 min, 4 °C Incubation: 60 min, 4 °C
NMDA	20 nM [^{3}H]MK-801	100 μM MK-801	50 mM Tris-Acetate (pH 7.4), 100 μM glutamate, 50 μM glycine, 50 μM spermidine	Pre-incubation: 30 min, 4 °C Incubation: 60 min, RT
AMPA	100 nM [^{3}H]AMPA	100 μM Quisqualic acid	50 mM Tris–HCl (pH 7.4), 2.5 mM$CaCl_2$, 0.1 M potassium thiocyanate	Pre-incubation: 30 min, 4 °C Incubation: 45 min, 4 °C
Serotonin2A	10 nM [^{3}H]ketanserin	10 μM Spiperone	170 mm Tris–HCl, pH 7.7	Pre-incubation: Nil Incubation: 60 min, RT

NMDA = N-methyl-D-aspartate, AMPA = Alpha-amino-3-hydroxy-5-methyl-4-isoxazoleproprionate.

sections were fixed in 10% formalin in phosphate buffered saline for 1 h at RT. Sections were immersed in 0.1% cresyl violet in 1% acetic acid for 15 min at 37 °C and then rinsed in dH_2O. Slides were immersed for 1 min twice in ethanol and then twice in xylene substitute for 1 min at RT. Sections were mounted in DPX and imaged using a light microscope with a digital camera attached.

2.5. Analysis

All experimental data were first interrogated with a Grubb's test to identify any statistically significant outliers and then the distribution of each data set was assessed using D'Agostino and Pearson omnibus normality. Two-way ANOVAs, with genotype and CNS regions as variables were used to identify any variance in radioligand binding. Bonferroni post-hoc tests were then used to identify the source of the variance.

3. Results

All radioligands showed different binding patterns in the cortex and hippocampus (Fig. 1) but exhibited homogenous binding across the striatum (data not shown). Hence, an integrated measure of radioligand binding across the striatum was made. Radioligand binding in the cortex showed variation with [^{3}H]AMPA (Fig. 1C) showing homogeneous across the cortex, [^{3}H]kainic acid binding and [^{3}H]pirenzepine binding being present in two discrete layers (Fig. 1B and D) and [^{3}H]MK801 (Fig. 1A), [^{3}H]AF-DX 384, [^{3}H]4-DAMP and [^{3}H]ketanserin binding (Fig. 1D–G) forming three discrete layers. For

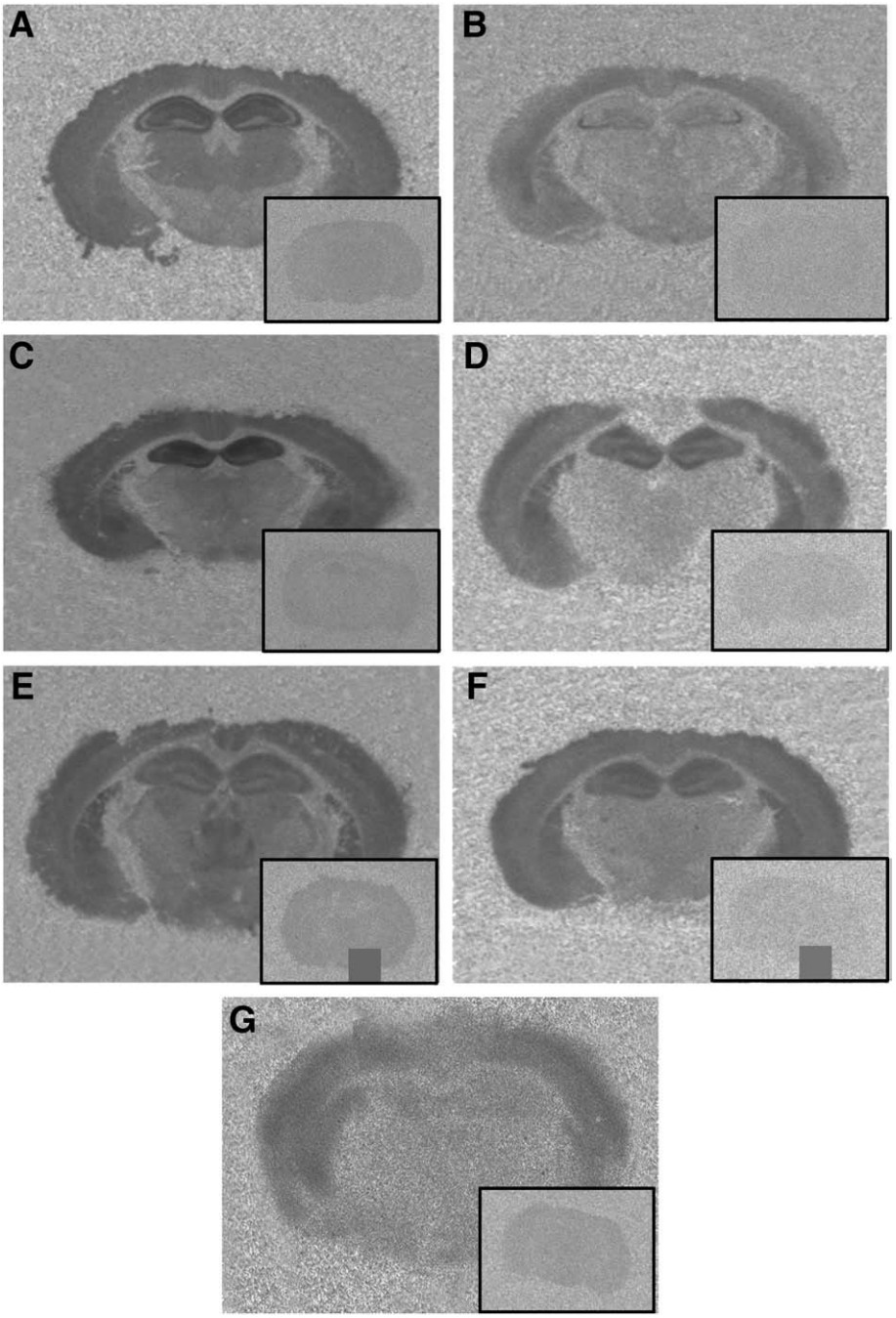

Fig. 1. Typical autoradiographs showing the binding of [^{3}H]MK-801 (A), [^{3}H]kainic acid (B), [^{3}H]AMPA (C), [^{3}H]pirenzepine (D), [^{3}H]AF-DX 384 (E), [^{3}H]4-DAMP (F) and [^{3}H] ketanserin (G) to a section of isogenic wild-type mice CNS. The non-specific binding for each radioligand is shown as an insert.

[^{3}H]kainic acid, the outermost layer of binding encompassed cortical laminae I–III and the innermost layer corresponding to laminae IV–VI. In the case of [^{3}H]pirenzepine, the outer layer contained laminae I–II whilst the innermost layer contained laminae III–VI. [^{3}H]MK801, [^{3}H]AMPA, [^{3}H]AF-DX 384, [^{3}H]4-DAMP and [^{3}H]ketanserin binding could be separated into three layers; for [^{3}H]MK801 the outer layer contained laminae I–II, the middle layer contained lamina III whilst the innermost layer incorporated laminae IV–VI. For the remaining radioligands, the outer radioligand binding layer contained laminae I–II, the middle radioligand binding layer contained laminae III–IV whilst innermost radioligand binding layer contained laminae V–VI.

Due to the different binding patterns within the cortex we decided to standardize our approach across radioligands by measuring binding within each cortical laminae. To allow levels of radioligand binding to be measured within each laminae, we stained each section with cresyl violet so we could define each laminae in the cortex. We then compared the stained section to the autoradiograph generated from that section to define each laminae on the autoradiograph along cytoarchitectural grounds. The different radioligands seem to bind to similar regions of the hippocampal formation. Thus, all radioligands showed specific binding in the Cornu Ammonis (CA) 1and dentate gyrus (DG) but only [^{3}H]kainate and [^{3}H]AMPA binding could be detected in CA 2–3. Within CA1 differing levels of binding of some radioligand could be detected in Stratum oriens (OR), Stratum pyramidale (PY), Stratum radiatum (RA) and Stratum lacunosum (LA) whilst in the DG differential levels of binding could be detected in the Stratum granulosum (GR) and the Stratum molecular (MO). For consistency, the binding of each radioligand was measured in each of these anatomically defined regions of the hippocampal formation.

3.1. Glutamate receptors

Analysis showed outlying [^{3}H]kainic acid binding data (Table 2: results in italics) from one mouse in the three regions of the hippocampus and in the striatum. Outlying [^{3}H]AMPA binding data was also detected in the LA. Notably, the analyses of the complete data set showed a non-parametric distribution but the analysed without the outlying data showed a parametric distribution. More importantly, analyses of variance using two-way ANOVA gave the same outcome whether or not the outlying data was included. Thus, for completeness and brevity, we have presented the analyses of the complete datasets.

3.1.1. [^{3}H]MK-801 binding

[^{3}H]MK-801 binding did not vary with genotype ($F_{1,207}=1.03$, $p=0.31$) but varied between across regions ($F_{12,207}=136.3$, $p<0.0001$) (Table 2); there was no interaction between variables ($F_{12,207}=0.42$, $p=0.95$). Under the conditions used in our study [^{3}H]MK801 would bind to the N-methyl-D-aspartic acid receptor (NMDAR) (Reynolds and Miller, 1988) where the drug binding site was available because its ion channel was open (Scarr et al., 2005). Thus our binding data suggests there is no change in open NMDA receptors in the CNS of ApoD$^{-/-}$ mice. The rank order of [^{3}H]MK-801 binding was RA>MO>OR=LA>cortical laminae I–II>cortical lamina III>cortical laminae IV–VI>GR=striatum, $p<0.0001$.

3.1.2. [^{3}H]kainic acid binding

There was significant variance in [^{3}H]kainic acid binding with genotype ($F_{1,223}=17.22$, $p<0.0001$) and region ($F_{13,223}=116.4$, $p<0.0001$) (Table 2, Fig. 2); there was no interaction between these variables ($F_{13,223}=1.18$, $p=0.30$).

The variation in [^{3}H]kainic acid binding with genotype was due to a significant decrease (20%, $p<0.001$) in [^{3}H]kainic acid binding in the CA 2–3 of ApoD$^{-/-}$ mice compared to W/T littermates (Fig. 2A). As [^{3}H]kainate would predominantly bind to the kainate receptor (KAR) these data suggest there is a decrease in KAR in the CA 2–3 of ApoD$^{-/-}$ mice. There was variation in [^{3}H]kainic acid binding with region; the rank order of binding was CA 2–3=cortical laminae IV–VI>striatum=MO>GR=LA=cortical laminae I–III>OR=PY=RA ($p<0.001$).

3.1.3. [^{3}H]AMPA binding

[^{3}H]AMPA binding varied with genotype ($F_{1,214}=4.67$, $p<0.05$) and region ($F_{13,214}=34.6$, $p<0.0001$) (Table 2, Fig. 2) but there was no interaction between variables ($F_{13,214}=0.43$, $p=0.96$).

Post-hoc tests failed to identify any within regional differences in [^{3}H]AMPA binding with genotype; a further examination of the data showed that the omnibus difference in [^{3}H]AMPA binding identified by the two-way ANOVA was due to the cumulative effect of non-significant

Table 2
The binding (mean ± SEM) of [^{3}H]MK-801, [^{3}H]AMPA, [^{3}H]kainic acid, [^{3}H]pirenzepine, [^{3}H]AF-DX 384, [^{3}H]4-DAMP and [^{3}H]ketanserin to the sections of CNS from APOD$^{-/-}$ mice and isogenic wild-type mice.

Radioligand	[^{3}H]MK-801		[^{3}H]AMPA*		[^{3}H] Kainate***		[^{3}H]Pirenzepine		[^{3}H]AF-DX 384***		[^{3}H]4-DAMP		[^{3}H]Ketanserin	
Region	ApoD$^{-/-}$	WT	ApoD$^{-/-}$	WT	ApoD$^{-/-}$	WT	ApoD$^{-/-}$	WT	ApoD$^{-/-}$	WT	ApoD$^{-/-}$	WT	ApoD$^{-/-}$	WT
Cortex														
Lamina 1	311±8.9	307±11	219±18	225±17	38±2.7	44±3.0	232±16	238±5.7	159±6.2	145±6.4	190±12	180±8.2	25±1.9	30±2.6
Lamina 2	311±8.9	307±11	219±18	225±17	38±2.7	44±3.0	232±16	238±5.7	159±6.2	145±6.4	190±12	180±8.2	25±1.9	30±2.6
Lamina 3	285±5.9	286±6.7	193±17	194±15	38±2.7	44±3.0	160±15	177±8.8	113±4.8	106±4.3	139±10	137±7.7	66±5.0	64±4.0
Lamina 4	222±6.6	225±9.4	222±23	226±14	102±4.0	108±5.0	160±15	177±8.8	113±5.0	106±4.7	139±10	137±7.7	66±5.0	64±4.0
Lamina 5	222±6.6	225±9.4	222±23	226±14	102±4.0	108±5.0	162±11	178±6.3	125±5.0	118±4.7	145±9.6	144±8.7	46±2.6	45±2.2
Lamina 6	222±6.6	225±9.4	222±23	226±14	102±4.0	108±5.0	162±11	178±6.3	125±5.0	118±4.7	145±9.6	144±8.7	46±2.6	45±2.2
Hippocampus														
CA1–OR	475±21	460±27	629±57	733±103	*24±3.2*	26±1.1	295±25	291±16	143±9.1	125±6.9	186±16	167±22	17±0.7	18±0.9
CA1–PY	276±18	274±30	629±57	733±103	*24±3.2*	26±1.1	295±25	291±16	143±9.1	125±6.9	222±17	200±23	17±0.7	18±0.9
CA1–RA	608±23	561±23	629±57	733±103	*25±4.3*	27±1.6	295±25	291±16	143±9.1	125±6.7	228±19	206±26	17±0.7	18±0.9
CA1–LA	453±24	442±30	*539±64*	637±82	41±5.2	44±3.0	261±28	281±18	113±7.2	104±5.1	219±14	211±15	17±0.7	18±0.9
CA 2–3	–	–	374±42	451±38	104±10.2	130±9.4***	–	–	–	–	–	–	–	–
DG–MO	530±13	511±18	539±61	552±57	50±5.5	59±3.1	297±15	290±10	134±6.0	119±4.8	213±18	196±16	17±0.7	18±0.9
DG–GR	184±4.0	195±7.5	392±45	423±37	42±5.4	42±2.1	217±24	248±18	98±3.6	87±5.4	174±9.4	167±9.2	17±0.7	18±0.9
Striatum	184±9.1	181±13	191±31	*194±22*	*62±3.1*	78±4.2	236±15	248±11	173±9.7	158±13	196±12	184±14	52±4.4	46±3.8

Abbreviations: CA = Cornu Ammonis, DG = dentate gyrus, GR = Stratum granulosum, LA = Stratum lacunosum, MO = Stratum molecular, OR = Stratum oriens, PY = Stratum pyramidale, RA = Stratum radiatum, W/T = isogenic wild-type mice.

* $p<0.05$.

*** $p<0.0001$.

decreases in radioligand binding across all hippocampal regions (7–17%) in the ApoD$^{-/-}$ mice (Fig. 2B). These data would be consistent small wide spread decreases in the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) in the CNS of ApoD$^{-/-}$ mice. The rank order of [^{3}H]AMPA binding was OR = PY = RA > LA = MO > CA 2–3 = GR > laminae IV–VI = laminae I–II > laminae III ($p<0.001$).

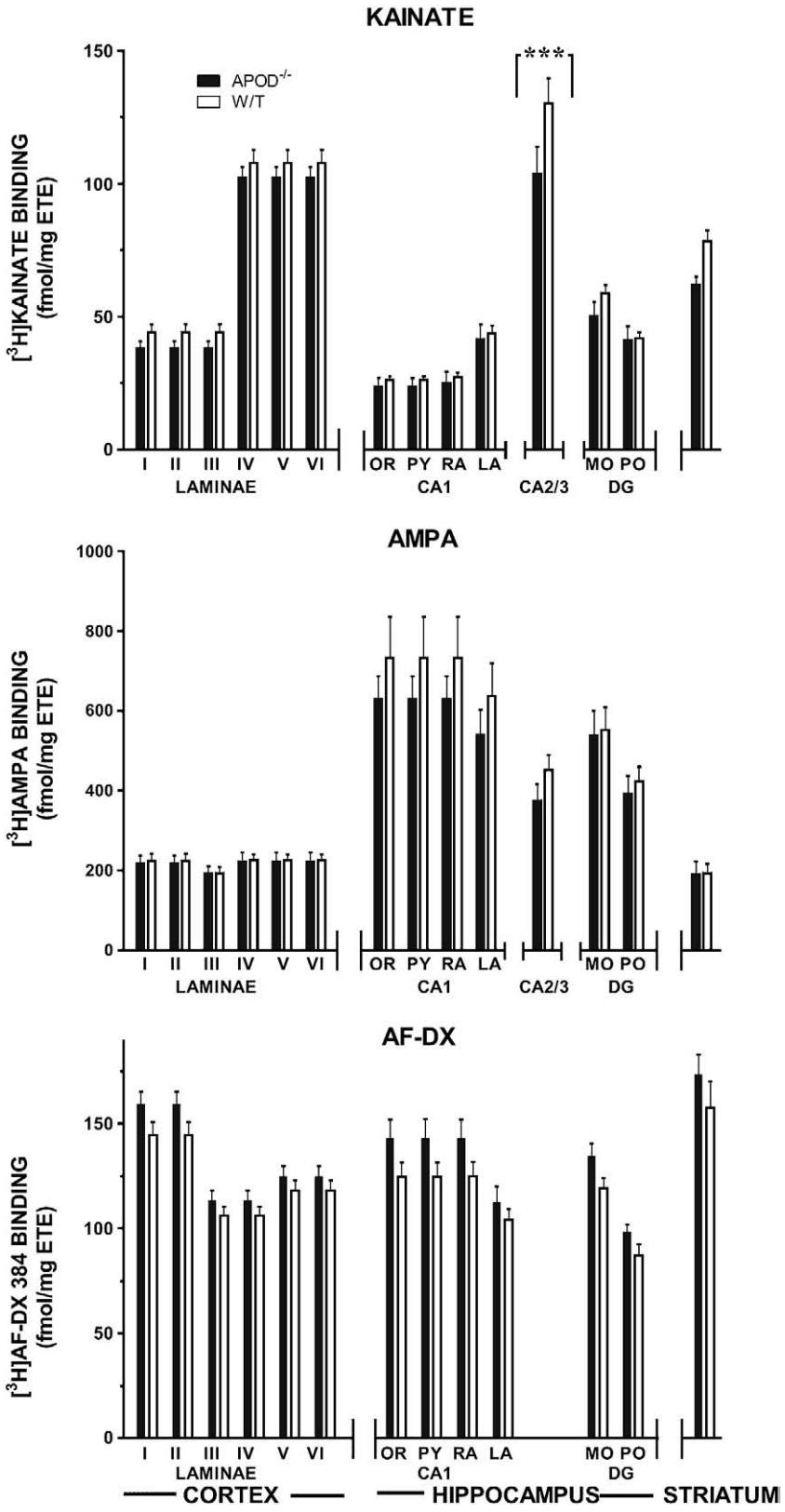

Fig. 2. The density (mean ± SEM) of kainate, AMPA and muscarinic M2/M4 (AF-DX 384) receptors in the cortex and hippocampus of ApoD$^{-/-}$ mice and wild-type littermates. Abbreviations: CA = Cornu Ammonis, DG = dentate gyrus, GR = Stratum granulosum, LA = Stratum lacunosum, MO = Stratum molecular, OR = Stratum oriens, PY = Stratum pyramidale, RA = Stratum radiatum, W/T = isogenic wild-type mice. *** = $p<0.001$.

3.2. Muscarinic receptors

3.2.1. [^{3}H]pirenzepine binding

There was no variation in [^{3}H]pirenzepine binding with genotype ($F_{1,208}=2.19$, $p=0.14$) but binding did vary between regions ($F_{12,208}=20.27$, $p<0.0001$) (Table 2), with no interaction between these two variables ($F_{12,208}=0.26$, $p=0.99$). Under the experimental conditions used over 80% of [^{3}H]pirenzepine binding would be to the muscarinic M1 receptor (Chrm1) (Scarr and Dean, 2008) suggesting that there is no change in the density of that receptor in the CNS of ApoD$^{-/-}$ mice. The rank order of [^{3}H]pirenzepine binding between regions was MO = OR = PY = RA > LA > Striatum = cortical laminae I–II > GR > cortical laminae III–VI ($p<0.001$).

3.2.2. [^{3}H]AF-DX 384 binding

[^{3}H]AF-DX 384 binding varied with both genotype ($F_{1,208}=22.77$, $p<0.0001$) and region ($F_{12,208}=18.86$, $p<0.0001$) (Table 2: Fig. 2) but there was no interaction between these two variables ($F_{12,208}=0.25$, $p=1.0$).

There was no significant within-region difference in [^{3}H]AF-DX 384 binding with genotype, however a closer examination of the data shows consistent increases (6–13%) in [^{3}H]AF-DX 384 binding across all brain regions in ApoD$^{-/-}$ mice compared with W/T littermates (Fig. 2C); the cumulative effect of these differences leading to the significant change in global binding identified with the two-way ANOVA. As, under the conditions used in these studies [^{3}H]AF-DX 384 would mainly bind to Chrm2 and Chrm4 (Miller et al., 1991), our data indicates there is an generalized increase in Chrm2, Chrm4 or the sum of Chrm2/4 in the CNS of ApoD$^{-/-}$ mice. The rank order of [^{3}H]AF-DX 384 binding with region was striatum = cortical laminae I–II = OR = PY = RA = MO > cortical laminae V–VI > LA = cortical laminae III–IV > GR ($p<0.001$).

3.2.3. [^{3}H]4-DAMP binding

[^{3}H]4-DAMP binding did not vary with genotype ($F_{1,207}=3.34$, $p=0.07$) but did vary between regions ($F_{12,207}=8.75$, $p<0.0001$); there was no interaction between variables ($F_{12,207}=0.15$, $p=1.0$) (Table 2). As [^{3}H]4-DAMP binding would mainly be to Chrm3 (Araujo et al., 1991), these data would suggest that Chrm3 are not altered in the CNS of the ApoD$^{-/-}$ mouse. There was variation in radioligand binding across region; the rank order of binding being LA = RA = PY = MO > striatum = cortical laminae I–II = OR = GR > cortical laminae V–VI = cortical laminae III–VI ($p<0.001$).

3.3. Serotonin receptors

3.3.1. [^{3}H]ketanserin binding

There was no significant variance in [^{3}H]ketanserin binding with genotype ($F_{1,204}=0.02$, $p=0.62$) but binding did vary across regions ($F_{12,204}=111.8$, $p<0.0001$) (Table 2); there was no interaction between variables ($F_{12,204}=0.61$, $p=0.83$). As [^{3}H]ketanserin would predominantly bind to the serotonin 2A receptor (Htr2A) these data suggest that receptor is not altered in the CNS of ApoD$^{-/-}$ mice. The rank order of [^{3}H]ketanserin binding across regions was cortical laminae III–VI > striatum = cortical laminae IV-V > cortical laminae cortical laminae I–II > OR = PY = RA = LA = MO = GR ($p<0.001$).

4. Discussion

The major finding from this study was a significant decrease (−20%) in the density of KAR in the CA 2–3 in ApoD$^{-/-}$ mice compared with W/T mice. Another finding from our study is that ApoD$^{-/-}$ had widespread decreases in AMPAR across the CNS that did not reach significance in any single CNS region. Importantly, ApoD$^{-/-}$ mice did not have deficits in all ionotropic glutamate receptors as levels of the NMDA receptor was not altered in these mice. Ionotropic glutamate receptors are formed from sub-units that define the pharmacological and physiological characteristics of each receptor (Kew and Kemp, 2005). The KAR is formed from Grik1, 2, 3, 4 and 5 sub-units; our data would suggest that there must be a decrease in the expression of one of those subjects in ApoD$^{-/-}$ mice. As Grik4 or 5 is required to form KAR high affinity binding sites it would also seem probable that changes in the expression of one of those sub-units must be occurring in the CNS of ApoD$^{-/-}$ mice. Similarly, our data on the AMPAR would suggest there would be decreases in the expression of the Gria1, 2, 3 or 4 sub-units that form this receptor. By contrast, our data would not suggest that there is changed expression of the NMDAR sub-units Grin1, 2a, 2b, 2c, 2d, 3a or 3b that form the NMDAR. Given our radioligand binding data, further experiments to determine the changes in sub-unit gene expression leading to changes in binding to KAR and AMPAR would be worthwhile. Another finding from our study is that ApoD$^{-/-}$ had widespread non-significant increases in Chrm2/4. By contrast, levels of Chrm1, Chrm3and Htr2A did not appear altered in individual CNS regions of ApoD$^{-/-}$ mice. Thus, our data would seem to support the hypotheses that ApoD may have a role in maintaining the glutamatergic function in the mammalian CNS. This hypothesis is consistent with previous data showing that exogenous ApoD can help protect against the neurotoxic effects of kainic acid (He et al., 2009), some of which are mediated by KAR (Garthwaite and Wilkin, 1982) and AMPAR (Tomita et al., 2007).

It has been previously shown that ApoD$^{-/-}$ mice have complex, regionally-selective changes in somatostatin and somatostatin receptors (Rajput et al., 2009). These findings are of relevance to our study as it has been shown that somatostatin can regulate glutamate release (Grilli et al., 2004). Moreover, it has long been known that glutamate can regulate somatostatin secretion suggesting there are complex reciprocal interactions between the two neurotransmitters systems (Gardette et al., 1995). Thus, current studies in ApoD$^{-/-}$ are consistent with the notion that ApoD can modulate the interaction between somatostatin and glutamatergic pathways in the mammalian CNS.

This study also showed that there was a widespread increase in Chrm2, Chrm4 or Chrm2/4 across the CNS of the ApoD$^{-/-}$ mice. By contrast, our data suggests that central levels of Chrm1 and Chrm3 are not altered in these animals. The data on [^{3}H]AF-DX 384 binding are intriguing as both levels of ApoD (Thomas et al., 2001b) and [^{3}H]AF-DX 384 binding (Gibbons et al., 2008) have been reported as altered in the cortex of subjects with bipolar disorder. To our knowledge, there is no data showing functional links between the expression of Chrm2 or Chrm4 receptors and ApoD but given our data from APOD$^{-/-}$ mice determining if there are interactions between the two proteins in mammalian CNS would be valuable. Moreover, further experiments to determine if Chrm2 or Chrm4 are selectively altered in the ApoD$^{-/-}$ would be worthwhile as Chrm2 has been shown to be the cholinergic autoreceptor in the rat CNS (Lapchak et al., 1989). Thus, a selective decrease Chrm2 could indicate that there are widespread changes in cholinergic innervation in the ApoD$^{-/-}$ mouse.

The rationale for undertaking this study was that some of the changes in neurotransmitter receptors in the CNS of subjects with schizophrenia may be mediated, at least in part, by the increased levels of ApoD that have been reported in the CNS of subjects with the disorder (Thomas et al., 2001b, 2003a). It is therefore significant that there is a report of decreased levels of KAR in the hippocampus of subjects with schizophrenia (Kerwin et al., 1990) whilst our data shows increased levels of the receptor in the CA 1–2 of ApoD$^{-/-}$ mice. However, this early finding must be balanced against later studies that do not show KAR or AMPAR to be altered in the hippocampus from subjects with schizophrenia (Beneyto et al., 2007; Gao et al., 2000; Scarr et al., 2003). In addition, changes in cortical NMDA receptors, KAR, CHRM1 and HT2AR have been reported in subjects with schizophrenia (Dean and Hayes, 1996; Dean et al., 2002; Scarr et al., 2005) but the density of these receptors are unchanged in ApoD$^{-/-}$ mice. Thus, on balance our study does not suggest that changes in levels

of ApoD in the CNS of subjects with schizophrenia are likely to mediate the changes in neurotransmitter receptors observed in subjects with the disorder. However clozapine binds to most of the neurotransmitter receptors affected by the pathophysiology of schizophrenia (Ereshefsky et al., 1989) and results in altered expression of ApoD (Thomas et al., 2001a). These data would be consistent with the notion that the changes in ApoD in the CNS of subjects with schizophrenia are secondary to changes in neurotransmitter receptors.

Whilst our initial hypothesis that the absence of ApoD would affect the expression of neurotransmitter receptors that are changed by the pathophysiology of schizophrenia was not proven; our study has shown a 20% decrease in KAR in the CA 2–3 of ApoD$^{-/-}$ mice. KAR in the hippocampal formation have been shown to have roles in regulating glutamate (Malva et al., 1998) and GABA (Rodriguez-Moreno and Lerma, 1998) release, mediating slow postsynaptic currents (Castillo et al., 1997) and LTP (Lauri et al., 2001), regulating nitric oxide (Radenovic and Selakovic, 2005) and superoxide (Radenovic et al., 2004) production as well as modulating excitatory neurotransmission (Contractor et al., 2000) and neurogenesis (Bernabeu and Sharp, 2000). Significantly, hippocampal KAR are predominately heteromeric receptors and made up of GluR5 and Glur6 sub-units; however, pre-synaptic KAR seem to predominantly contain GluR6 sub-units (Mulle et al., 2000). This is significant because pre-synaptic KAR have been shown to control GABA release (Cossart et al., 2001), therefore it would be of interest to determine if the loss of KAR in ApoD$^{-/-}$ mice is associated with a loss of the GluR6 sub-unit which could indicate a dysregulation of GABA release.

5. Conclusions

From existing data, we would postulate that the loss of KAR we identified in the hippocampal formation would have physiological consequences for the ApoD$^{-/-}$ mouse. This proposal gains further credence from the observation that ApoD$^{-/-}$ mice have deficits in learning and memory, motor tasks and orientation-based tasks (Ganfornina et al., 2008) which are all functions that depend on hippocampal glutamatergic pathways (Holscher and Schmidt, 1994; Pisharodi and Nauta, 1985). Our current data could therefore indicate that at least some of these changes may result from decreased KAR in the CA 2–3 and generalized decreases in AMPAR throughout the hippocampus. This hypothesis sits well with a growing body of evidence supporting a role for KAR and AMPAR in the CA 2–3 region in learning and memory (Queiroz and Mello, 2007; Schiapparelli et al., 2006), motor neurons (Carriedo et al., 1996) and orientation tasks (Sutherland et al., 1983). Thus, further investigation of changes in glutamatergic pathways in the hippocampus of ApoD$^{-/-}$ are warranted to better understand the role of ApoD in maintaining hippocampal function. Finally, it will be intriguing to discover if there are widespread changes in cholinergic innervation in the CNS of these mice as the cholinergic system is also important in learning and memory (Raedler et al., 2007).

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