Levels of Ionotropic Glutamate and Muscarinic Receptors in Three Animal Models of Schizophrenia

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Abstract: There are well validated rodent paradigms of schizophrenia which are based on environmental manipulation (e.g. altered rearing conditions) or drug challenges. These manipulations induce behavioural changes in rodents that are thought to involve neuronal circuitry similar to the ones that are affected by the pathophysiology of the disorder. This study has investigated whether three such rodent paradigms (isolation rearing, neonatal PCP treatment or sub-chronic PCP treatment) are associated with changes in muscarinic receptors (CHRMs) or ionotropic glutamate receptors, some of which have been reported to be altered in the CNS of subjects with schizophrenia. [^{3}H]pirenzepine (CHRM1), [^{3}H]4DAMP (CHRM1/CHRM3), [^{3}H]MK801 (NMDA receptors) and [^{3}H]kainate (kainate receptors; KAR) binding were measured using *in situ* radioligand binding and autoradiography. Isolation rearing caused widespread decreases in [^{3}H]4DAMP ($p = 0.01$) and [^{3}H]kainate binding ($p = 0.03$). Neonatal PCP caused widespread increases in [^{3}H]4DAMP binding ($p < 0.0001$), whereas sub-chronic PCP treatment caused widespread decreases in the binding of that radioligand ($p < 0.002$) and widespread increases in [3H]MK801 binding ($p < 0.0001$). There were no changes in [^{3}H]pirenzepine binding to CHRM1 receptors in any paradigm or no significant within region changes in the binding of any radioligand. In conclusion, in the absence of any changes in CHRM1 receptors, our [^{3}H]4DAMP and the binding of [3H]MK801 data would suggest that different rodent paradigms cause variable changes in levels of CHRM3 and KAR in the rat CNS. Our data raises the possibility that such changes may, in part, modulate the behavioural differences that have been observed after isolation rearing, neonatal PCP treatment or sub-chronic PCP treatment.

Keywords: Neonatal PCP, chronic PCP, neonatal depravation, muscarinic receptors, NMDA, AMPA, kainate.

INTRODUCTION

Schizophrenia appears to manifest in individuals with a genetic predisposition after exposure to environmental factors which trigger the onset of the disorder [1]. This has resulted in the hypothesis that the disorder is an outcome of complex gene x environment interactions. Importantly, it has been noted that both pre- or peri-natal insults are associated with an increased risk of schizophrenia [2]. However, it is not clear how insults at such an early stage in human neurodevelopment can result in a disorder with a peak onset in late adolescence or early adulthood in males and with a 5 year delay in females [3]. The relatively late onset of the disorder has lead to speculation that either early life insults cause changes in neurodevelopment that do not result in functional abnormalities until late in CNS maturation or that schizophrenia results from abnormalities in late stage CNS maturation processes [4]. Whichever hypotheses may be correct, it is now widely accepted that changes in CNS gene expression is an endpoint of the complex gene x environmental interactions that underpin the onset of the symptoms of schizophrenia [1, 5]. At the level of gene expression, there are now considerable bodies of data showing changes in levels of ionotropic glutamate receptors [6-13] and muscarinic receptors (CHRMs) [7, 14-22] in the CNS of subjects with the schizophrenia. The possibility that these changes occur because of gene x environment interactions is supported by the demonstration that changing environment can affect levels of ionotropic receptors and CHRMs in the CNS of genetically modified, but not wild type, mice [23, 24]. Given the well documented changes in ionotropic receptors and CHRMs in the CNS from subjects with schizophrenia, knowing how gene x environment interactions can affect the expression of these receptors would be a significant advance in understanding the pathophysiology of the disorder. In this respect, it is significant that N-methyl-D-aspartic acid (NMDA) receptor antagonists such as phencyclidine (PCP) and ketamine

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1876-5238/11

induce schizophrenia-like symptoms in humans and worsen symptoms in subjects with schizophrenia [25]. These data, along with that from other lines of research such as postmortem studies [26], underpin the hypotheses that glutamatergic pathways are involve in the pathophysiology of the disorder [27]. Subsequently, the need to better understand the role of the glutamatergic pathways in the mammalian CNS and how they may be involved in the genesis of schizophrenia lead to the study of the effects of PCP treatment in rodents [28]. Such studies have shown that the acute administration of PCP produces transient behavioural effects thought to be analogous to positive, negative and cognitive symptom domains of schizophrenia (reviewed in [29]). More recently, longer term PCP treatments (e.g. sub-chronic PCP treatment of adult rats [30] or repeated PCP treatment of neonatal rats (a single administration on post-natal days 7, 9 and 11) [31] has been shown to be advantageous because such treatments produce long term behavioural changes in the absence of the overt neurodegeneration observed after high dose acute PCP treatment [32]. These differences are important because neurodegeneration does not seem to occur in the CNS of subjects with schizophrenia [33]. Moreover, repeated PCP treatment regimes cause long term robust deficits in cognitive function [34, 35], a heightened response to psycho-stimulants [36] and deficits in social behaviours [37-39] along with neuroanatomical changes such as hippocampal and cortical parvalbumin deficits [40, 41], regional specific changes in N-acetylaspartate [42], reductions in spine density in pre-frontal cortex [29] and regionally specific changes in BDNF expression [43], all of which have been reported in the CNS of subjects with schizophrenia. Thus, neonatal and sub-chronic PCP treatment would appear to offer the opportunity to study the impact of a controlled environmental factor on brain development in a rodent model that appears to have relevance to schizophrenia.

Isolation rearing is a rodent paradigm that also alters behaviours such as locomotor activity, pre-pulse inhibition (PPI), responsiveness to psycho-stimulants and cognitive functioning [44, 45]. In addition, rats subjected to isolation rearing have alterations in dopamine and serotonin neurochemistry, volume loss in the prefrontal cortex, reduced N-acetylaspartate in the temporal cortex, reduced hippocampal parvalbumin, reduced prefrontal GAT-1, increased prefrontal NR2A subunit expression, and reduced cortical and hippocampal spine density [43, 45-48], all of which have been reported as present in the CNS from subjects with schizophrenia. Importantly, a comparison of results from PCP treatment and isolation rearing suggest that different behavioural phenotypes result from each manipulation (e.g. the reproducible PPI deficit seen in isolation reared rats [45] is not a robust observation following sub-chronic or neonatal PCP administration [49, 50]). Hence, we postulated that the study of changes in CNS molecular cytoarchitecture across rodent models could reveal different outcomes from gene x environment interactions on the molecular cytoarchitecture that could have relevance to changes that occur in the CNS of subjects with schizophrenia. As the initial step in testing this hypothesis we measured levels of ionotropic glutamate receptors and CHRMs in the CNS of adult rats following either isolation rearing, neonatal or sub-chronic exposure to PCP because levels of these receptors appear to be altered in the CNS from subjects with schizophrenia.

MATERIALS AND METHODS

Materials

[^{3}H]pirenzepine, [^{3}H]kainic acid, [^{3}H]dizocilpine ([^{3}H]MK-801), and [^{3}H]4-diphenylacetoxy-N-methyl-piperidine methiodide ([^{3}H]4DAMP) were sourced from Perkin Elmer. [^{3}H]microscales were obtained from Amersham. All other laboratory grade chemicals were sourced from Sigma-Aldrich, Pty., Ltd. *Animal Models* Studies were conducted in compliance with the Home Office Guidance on the operation of the UK Animals (Scientific Procedures) Act 1986, and were approved by the GlaxoSmithKline Animal Procedures Review Panel.

Isolation Reared Rats

Male Lister Hooded rats (Charles River, UK) arrived at the facility on post-natal day (PND) 8–9 with foster mothers [11 pups per female, fostered at birth (PND 0)]. The rat pups used in the isolation rearing paradigm were cross-fostered at PND0 at the breeding facility. Cross-fostering at this age is not known to have any effect on either maternal interaction or overall development. On PND 28, the rats were rehoused singly (isolates) or in groups of five (grouped). In the case of group housing, animals were maintained in large "P2000" cages from Techniplast UK and there is no evidence that these conditions, which exceed stringent minimum requirements outlined by UK home office legislation, causes any stress to the animals. Animals raised under both conditions were housed in the same holding room for a period of 8 weeks before PPI testing. For a full description of isolation rearing and PPI procedure (see [51]; notably there was significantly impaired PPI in the animals that had undergone isolated rearing (Group housed = 61.8 +/- 3.1 %PPI *vs*. Isolation reared = 5.2 +/- 9.2 % PPI; $p<0.01$, n = 10-12,). Brains were collected for neurochemical analyses close to PND 133.

Neonatal PCP-Treated Rats

Pregnant time-mated Lister Hooded dams were obtained 7 days before parturition (Charles River, UK). In order to allow the dosing on PND7, the rat pups used in the neonatal PCP paradigm were not cross-fostered and disturbances to the litters were minimised prior to the PCP dosing starting on PND 7. Male pups were injected with PCP (10 mg/kg s.c.) on PNDs 7, 9 and 11 which is a well established dosing regimen [31]. Pups were weaned on PND 21 and grown to adulthood (PND 56 onwards). As described previously [52], the motor activity response to a novel environment was shown to be increased in the PCP treated rats when measured around PND 60 (Box crossings; Vehicle = 76.1 +/- 3.2, PCP = 97.3 +/- 5.0, n = 45, $p<0.01$) in animals from which brains were collected for neurochemical analyses on PND 75 for this study.

Sub-Chronic PCP-treated rats

Male Lister Hooded rats (Charles River, UK), approximately 200-220 g at the time of dosing, were administered PCP (1 mg/kg i.p.) or vehicle (saline solution),

twice daily (am and pm) for 7 days [30, 53] followed by 7-days washout. Two weeks later brains were collected for neurochemical analyses. Sub-chronic PCP-treated rats were not assessed for behavioural changes, as previous studies in our laboratory have demonstrated a heightened motor activity response to a challenge dose of PCP in every batch of animals tested (>10) [53].

Tissue Preparation

In all cases, rats were killed by decapitation after stunning and without anaesthesia. Brains were rapidly frozen and stored at -70^{o}C until required. Subsequently, 25 sequential frozen sections (20μm thick; 3 total binding, 2 non-specific binding for each radioligand) were cut, beginning approximately 4.0 mm posterior to Bregma.

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 [54].

The binding of [^{3}H]kainic acid and [^{3}H]MK-801 [9] as well as [^{3}H]pirenzepine [7] and [^{3}H]4DAMP [55] binding were measured as described previously (conditions summarised in Table **1**). Following completion of the radioligand binding protocols; tissue sections were washed, dried and partially fixed prior to being 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.) [56]. 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 (TB) and non-specific binding (NSB) values were obtained by comparing the intensity of the tissue section image to the [^{3}H]microscales based standard curve so that the radioligand binding could be calculated as d.p.m/mg ETE (Estimated Tissue Equivalent). These values were then converted to fmol/mg ETE using the specific activities of the individual radioligands and specific binding calculated by subtracting NSB from TB.

Cresyl Violet Staining

To determine the distribution of radioligand binding, discrete areas of binding on each autoradiograph were compared to the section from which the autoradiograph was generated after the section had been stained with cresyl violet. Cresyl violet staining was performed as described previously [57] after the autoradiographic image had been generated. Thus, sections were fixed in 10% formalin in phosphate buffered saline for 1 hour at RT. Sections were immersed in 0.1% cresyl violet in 1% acetic acid for 15 minutes at 37ºC and then rinsed in dH_2O. Slides were immersed for 1 minute twice in ethanol and then twice in xylene substitute for 1 minute at RT. Sections were mounted in DPX and imaged using a light microscope with a digital camera attached.

Analysis

In this study, measurements were taken based on discrete areas of radioligand binding and the cytoarchitectural regions included within these regions of binding determined. This approach minimised the number of measurements made and hence lessoned the likelihood of Type 1 errors [58]. All experimental data were interrogated with a Grubb's test to identify any statistically significant outliers, unless stated otherwise, no outlying data was found. The distribution of each data set was assessed using D'Agostino and Pearson omnibus normality and unless stated otherwise data distribution was parametric. Two-way ANOVAs, with either rearing condition or drug treatment 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. For transparency, where outlying data were found, statistically analyses without the outlying data are presented as the primary analyses and the analyses with the outlying data are presented within square brackets.

Results

Muscarinic Receptors: [^{3}H]Pirenzepine Binding

Specific [^{3}H]pirenzepine binding was apparent in the cortex, stratum oriens, stratum lacunosum, strata granulosum, molecular and the polymorphic layer of the dentate gyrus (Fig. **1A**). Cortical [^{3}H]pirenzepine binding was in three layers, Layer 1 containing cortical laminae I and II, Layer 2 containing cortical Laminae III and IV with Layer 3 containing cortical Laminae V and VI [59]. There was an outlying data point in Layer 1 and Layer 3 of one rat in the sub-chronic PCP treated rats (Table **2**).

Table 1. The Conditions Used to Determine Receptor Binding in Rats that had Undergone either Experienced or not Experience Maternal Deprivation or had Neonatal or Sub-Chronic Treatment with PCP or Vehicle

Receptor	Radioligand	Competing Compound	Buffer	Conditions
Muscarinic M1/M4	15nM [^{3}H]pirenzepine	1μM QNX (3-quinuclidinyl xanthene-9-carboxylate hemioxalate salt)	10mM KH_2PO_4, 10mMNa_2HPO_4, pH 7.4	Pre-Incubation: Nil Incubation: 30min, RT
Kainate	40nM [^{3}H]kainic acid	1mM L-glutamate	50mM TRIS-Acetate (pH7.4)	Pre-Incubation: 30min, 4^{0}C Incubation: 60min, 4^{0}C
NMDA	20nM [^{3}H]MK-801	100μM MK-801	50mM TRIS-Acetate (pH7.4), 100μM glutamate, 50μM glycine, 50μM spermidine	Pre-Incubation: 30min, 4^{0}C Incubation: 60min, RT

Abbreviations: 4DAMP = 4-diphenylacetoxy-N-methyl-piperidine methiodide, NMDA = N-methyl-D-aspartate, QNX = 3-quinuclidinyl xanthene-9-carboxylate hemioxalate salt, RT = room temperature.

Table 2. The Binding (Mean ± SEM fmol / mg Estimated Tissue Equivalents) of [^{3}H]pirenzepine, [^{3}H]4DAMP, [^{3}H]kainate and [^{3}H]MK801 to the CNS from Three Paradigms that are Suggested to Precipitated Behavioural Outcomes Similar to those Displayed by Subjects with Schizophrenia

		Isolated Rearing		Neonatal PCP		Sub-Chronic PCP	
		Isolated	Non-Isolated	Treated	Vehicle	Treated	Vehicle
	Rats n	12	10	15	16	12	12
[^{3}H]PIRENZEPINE BINDING							
Cortex	Layer 1	274 ± 3.3	277 ± 3.7	300 ± 3.8	302 ± 3.8	***292 ± 4.9***	305 ± 5.4
	Layer 2	171 ± 2.9	170 ± 4.6	183 ± 3.4	179 ± 3.2	176 ± 4.1	180 ± 3.7
	Layer 3	193 ± 3.7	200 ± 4.7	215 ± 6.0	210 ± 5.3	***213 ± 3.7***	212 ± 3.2
Hippocampus	Stratum Oriens	360 ± 6.4	342 ± 5.4	368 ± 6.6	373 ± 5.2	270 ± 6.8	264 ± 5.9
	Stratum lacunosum	213 ± 8.8	237 ± 10	268 ± 8.3	253 ± 5.6	205 ± 5.8	202 ± 5.4
Dentate	Polymorphic layer	255 ± 8.7	211 ± 8.9	197 ± 11.0	196 ± 6.3	365 ± 6.7	374 ± 6.4
	Strata granulosum + moleculare	327 ± 6.8	324 ± 3.7	351 ± 7.0	352 ± 6.3	344 ± 5.5	350 ± 8.3
[^{3}H]4DAMP BINDING							
		n = 11	n = 10	n = 15	n = 15	n = 10	n = 9
Cortex	Layer 1	294 ± 3.6	317 ± 4.5	348 ± 8.8	332 ± 4.8	372 ± 17.4	387 ± 18.4
	Layer 2	***208 ± 1.8***	223 ± 4.3	237 ± 4.4	228 ± 2.4	250 ± 8.1	254 ± 7.8
Hippocampus	Stratum oriens layer 1	227 ± 9.5	232 ± 5.1	363 ± 12.7	358 ± 10.9	426 ± 25.1	434 ± 26.6
	Stratum oriens layer 2	134 ± 6.2	134 ± 5.5	209 ± 6.0	194 ± 5.5	220 ± 9.1	223 ± 9.7
	Stratum oriens layer 3	246 ± 11.2	247 ± 7.7	300 ± 8.0	278 ± 7.4	316 ± 19.7	322 ± 21.0
	Stratum radiatum layer 1	351 ± 9.9	355 ± 10.0	402 ± 12.8	395 ± 11.1	472 ± 28.8	483 ± 30
	Stratum radiatum layer 2	206 ± 6.1	207 ± 5.7	219 ± 7.2	204 ± 6.8	224 ± 8.7	226 ± 9.5
	Stratum radiatum layer 3	254 ± 6.6	266 ± 7.0	319 ± 7.8	292 ± 7.7	331 ± 21.2	337 ± 22.8
	Stratum lacunosum	322 ± 6.3	326 ± 6.2	272 ± 6.8	266 ± 4.4	300 ± 14.3	305 ± 14.8
Dentate	Strata granulosum + molecular	303 ± 6.5	***302 ± 2.3***	407 ± 8.3	379 ± 6.3	428 ± 26	436 ± 28.4
	Polymorphic layer	225 ± 3.9	235 ± 4.7	225 ± 4.9	214 ± 2.0	236 ± 11.8	239 ± 12.4
[^{3}H]KAINATE BINDING							
Cortex	Layer 1	10.1 ± 0.9	10.6 ± 1.1	3.8 ± 1.1	4.7 ± 1.1	5.3 ± 1.5	6.0 ± 1.0
	Layer 2	15.7 ± 1.0	17.6 ± 1.6	7.0 ± 1.2	8.4 ± 1.3	16.2 ± 1.2	14.5 ± 1.8
Hippocampus	CA3	31.6 ± 2.6	37.0 ± 2.7	13.6 ± 1.9	17.3 ± 2.3	25.7 ± 3.4	26.4 ± 3.5
	Other	9.3 ± 0.7	11.0 ± 1.0	4.5 ± 0.9	5.7 ± 0.9	11.2 ± 1.4	7.2 ± 0.8
[^{3}H]MK801 BINDING							
Cortex	Layer 1	258 ± 7.3	250 ± 11.9	282 ±6.3	283 ± 5.9	272 ± 12.1	288 ± 8.3
	Layer 2	184 ± 5.6	192 ± 9.9	216 ± 6.2	215 ± 4.4	216 ± 9.4	228 ± 6.3
Hippocampus	Alveus	144 ± 5.1	159 ± 3.7	***150 ± 5.5***	153 ± 3.4	142 ± 6.8	158 ± 5.3

Table 2. Contd….

		Isolated Rearing		Neonatal PCP		Sub-Chronic PCP	
CA1	Stratum oriens	402 ± 11.3	393 ± 11.5	422 ± 13.6	432 ± 8.1	396 ± 16.3	422 ± 13.8
	Stratum pyramidale	263 ± 8.3	264 ± 9.4	282 ± 8.5	281 ± 8.6	270 ± 13.3	299 ± 12.9
	Stratum radiatum	414 ± 11.2	402 ± 10.0	445 ± 11.0	452 ± 7.8	413 ± 18.5	441 ± 16.4
CA2	Stratum oriens	265 ± 7.4	267 ± 5.9	295 ± 7.0	303 ± 6.7	271 ± 9.0	288 ± 9.0
	Stratum pyramidale	157 ± 6.2	162 ± 11.1	181 ± 9.1	196 ± 10.2	158 ± 8.1	171 ± 7.4
	Stratum radiatum	296 ± 7.4	302 ± 9.1	340 ± 8.8	348 ± 8.5	303 ± 11	329 ± 10.8
CA3	Stratum oriens	308 ± 9.5	299 ± 7.6	324 ± 9.8	326 ± 6.2	296 ± 12.4	313 ± 10.8
	Stratum pyramidale	171 ± 5.0	170 ± 7.0	188 ± 5.1	191 ± 5.1	171 ± 8.4	189 ± 8.3
	Stratum radiatum	332 ± 8.4	330 ± 8.2	361 ± 10.1	355 ± 6.9	324 ± 12.3	351 ± 13.0
	Stratum lacunosum	297 ± 7.3	303 ± 6.3	350 ± 7.0	345 ± 6.8	316 ± 12.2	344 ± 12.4
Dentate	Stratum molecular	426 ± 9.3	425 ± 9.4	446 ± 12.6	445 ± 7.6	***412 ± 15.3***	432 ± 14.4
	Stratum granulosum + polymorphic layer	154 ± 4.8	157 ± 8.2	185 ± 4.0	186 ± 4.3	169 ± 7.0	189 ± 7.8

[^{3}H]Pirenzepine Binding: Isolation Reared Rats

Isolated rearing was not associated with any significant variation in [^{3}H]pirenzepine binding ($F = 1.8$, d.f. = 1,6,140, $p = 0.18$). There was a significant variation in binding between CNS regions ($F = 220$, d.f. = 1,6,140, $p < 0.001$) (Table **2**). There was a significant interaction between the variables ($F = 5.7$, d.f. = 1,6,140, $p < 0.001$). The rank order of binding of [^{3}H]pirenzepine across regions showed a change in rank order between polymorphic layer of the dentate gyrus and stratum lacunosum between rearing conditions due to [^{3}H]pirenzepine binding being significantly increased ($p = 0.002$) in the polymorphic layer of the dentate gyrus and a trend to being decreased (not significant: $p = 0.09$) in the stratum lacunosum from the isolated reared rats (Table **2**; Fig. **1B**). Notably, these changes in [^{3}H]pirenzepine binding did not survive correction for repeated measures within the two-way ANOVA.

[^{3}H]Pirenzepine Binding: Neonatal PCP treated rats

There was no significant variation in [^{3}H]pirenzepine binding with drug treatment in the neonatal PCP treated rats ($F = 0.41$, d.f. 1,6,203, $p = 0.52$) but there was significant variation in binding between CNS regions ($F = 307$, d.f. = 6,1,203, $p < 0.001$) (Table **2**). There was no interaction between the variables ($F = 0.59$, d.f. = 6,1,203, $p = 0.74$). The rank order of [^{3}H]pirenzepine binding was the same for both the treated and vehicle group.

[^{3}H]Pirenzepine Binding: Sub-Chronic PCP Treated Rats

[^{3}H]pirenzepine binding did not vary with sub-chronic PCP treatment ($F = 1.01$, d.f. 1,6,152, $p = 0.32$ [$F = 0.63$, d.f. 1,6,154, , $p = 0.43$]) but there was significant variation in binding between CNS regions ($F = 298$, d.f. = 6,1,152, $p < 0.001$ [$F = 30$, d.f. = 6,1,154, $p < 0.001$]) (Table **2**). There was no interaction between the variables ($F = 0.39$, d.f. = 6,1,152, $p = 0.89$ [$F = 0.39$, d.f. = 6,1,154, $p = 0.89$]). The rank order of [^{3}H]pirenzepine binding was the same for both groups.

Muscarinic Receptors: [^{3}H]4DAMP binding

Specific [^{3}H]4DAMP binding could be measured in the cortex, stratum radiatum, dentate gyrus, stratum lacunosum and the stratum oriens (Fig. **1C**). Radioligand binding in the cortex was in two layers with the outer layer of binding encompassing cortical laminae I and II with the inner layer across the remaining laminae of the cortex. Notably, the binding of [^{3}H]4DAMP in the stratum oriens and stratum radiatum could be divided into three discrete layers (Table **2**). There was an outlying data point for the data from cortical Layer 2 from one rat in the isolated reared animals and in the strata granulosum + molecular from one of the group reared rats. To do

[^{3}H]4DAMP Binding: Isolation Reared rats

Isolated rearing was associated with a significant variation in [^{3}H]4DAMP binding ($F = 6.06$, d.f. = 1,10,207, $p = 0.01$ [$F = 7.01$, d.f. = 1,10,209, $p = 0.009$]) which also varied between CNS region ($F = 175$, d.f. = 1,10,207, $p < 0.0001$ [$F = 176$, d.f. = 1,10,209, $p < 0.001$]). There was no interaction between the variables ($F = 0.59$, d.f. = 1,10,207, $p = 0.82$ [$F = 0.59$, d.f. = 1,10,209, $p = 0.81$]). Further analyses failed to show any region specific changes in [^{3}H]4DAMP binding indicating the global variation was due to the summed effects of widespread non-significant decreases in radioligand binding in the isolated reared rats (Fig. **1D**). There was no change in the rank order of [^{3}H]4DAMP binding between treatments.

[^{3}H]4DAMP Binding: Neonatal PCP Treated Rats

[^{3}H]4DAMP binding varied with PCP treatment ($F = 21$, d.f. = 1,10,308, $p < 0.0001$) and between CNS regions ($F = 179$, d.f. = 1,10,308, $p < 0.0001$). There was no significant interaction between PCP treatment and CNS regions ($F = 0.57$, d.f. = 1,10,308, $p = 0.83$) . Post hoc analyses failed to

Fig. (2). A & B: Typical autoradiographs of total and non-specific (Insert) [^{3}H]kainate (A) and [^{3}H]MK801 (B) binding to rat CNS. **C & D**: The specific binding (mean ± SEM) of [^{3}H]kainate to CNS from rats after or combined rearing (C) and [^{3}H]MK801binding (D) to CNS from rats after sub-chronic PCP treatment

and the Stratum lacunosum which was uniformly labelled through the CA. In the neonatal PCP treated animals, an outlier was detected [^{3}H]MK 801 binding for one rat in the alveus of the PCP treated group (Table **2**) and an outlier was detected in the Stratum moleculare of the dentate gyrus of one rat receiving sub-chronic PCP treatment.

The distribution of data was shown not to be binomial in the CA1 Stratum oriens, CA2 Stratum pyramidale, CA1 Stratum radiatum and Dentate Gyrus Stratum molecular of the group reared rats in the isolated rearing paradigm. In the neonatal PCP treated rats data was not binomially distributed in Layer 1 of the cortex of the PCP treated group. The data from the entire sub-chronic PCP treated paradigm were normally distributed. Thus, because the majority of data was binomially distributed, the more conservative parametric analyses were completed for all comparisons.

[^{3}h]MK 801 Binding: Isolation Reared

Isolation rearing cause no variation in [^{3}H]MK 801 binding with rearing ($F = 0.02$, d.f. = 1,14,299, $p = 0.90$) but there was significant differences between CNS regions ($F = 247$, d.f. = 14,1,299, $p < 0.0001$). There were no interactions between the variables ($F = 0.38$, d.f. = 14,1,299, $p = 0.98$). The rank order of regional binding is shown in was not changed.

[^{3}H]MK 801 Binding: Neonatal PCP Treated Rats

[^{3}H]MK 801 binding did not vary after PCP drug treatment ($F = 1.06$, d.f. = 1,14,434, $p = 0.31$ [$F = 0.76$, d.f. = 1,14,435, $p = 0.38$]) but binding differed significantly between CNS regions ($F = 313$, d.f. = 14,1,434, $p < 0.001$ [$F = 307$, d.f. = 1,14,435, $p < 0.001$]). There were no interactions between the variables ($F = 0.26$, d.f. = 14,1,434, $p = 1.00$ [$F = 0.27$, d.f. = 1,14,435, $p = 1.00$]). The rank order of regional binding was not changed.

[^{3}H]MK 801 Binding: Sub-Chronic PCP Treated Rats

[^{3}H]MK 801 binding varied after PCP treatment ($F = 23.9$, d.f. = 1,14,329, $p < 0.001$ [$F = 24.8$, d.f. = 1,14,330, $p < 0.001$]) and between CNS regions ($F = 130$, d.f. = 14,1,329, $p < 0.001$ [$F = 130$, d.f. = 14,1,330, $p < 0.001$]). There were no interactions between the variables ($F = 0.15$, d.f. = 14,1,330, $p = 0.99$ [$F = 0.13$, d.f. = 14,1,330, $p = 1.00$]) Post hoc testing failed to identify a significant difference in [^{3}H]MK 801 binding with drug treatment and therefore the global difference in radioligand binding was the cumulative effect of non-significant decreases in binding in the sub-chronic treated group (Table **2**, Fig. **2D**).

Discussion

This study has shown that there are widespread decreases in [^{3}H]4DAMP binding in the CNS of rats after isolation rearing and sub-chronic PCP treatment. [^{3}H]kainate binding showed widespread decreases in rats which had experienced isolated rearing. By contrast, there were widespread increases in [^{3}H]4DAMP after neonatal PCP treatment and decreases in [^{3}H]MK 801 binding after sub-chronic PCP treatment. [^{3}H]pirenzepine binding was not altered in any of the paradigms studied.

Under the assay condition used in this study it has been shown that [^{3}H]kainate would predominantly bind to the kainate receptor (KAR) [9], [^{3}H]MK 801 to NMDAR [9],

[^{3}H]pirenzepine to CHRM1 [60] and [^{3}H]4DAMP to CHRM1/CHRM3 [61]. Importantly we have shown changes in [^{3}H]4DAMP binding in the absence of change in [^{3}H]pirenzepine binding to CHRM1. This suggests that any changes in [^{3}H]4DAMP binding are reflecting changes in CHRM3.

Based on our current understanding of the binding of radioligands used in our study, our data would be consistent with small widespread decreases in KAR after isolation rearing ranging from 3% in Layer 1 of the cortex to 15% in areas of the hippocampus. The data also are consistent with widespread decreases of between 5% (dentate gyrus stratum molecular) and 11% (dentate gyrus stratum granulosum + polymorphic layer) in NMDAR after sub-chronic PCP treatment. Decreases in CHRM3 were less than 7% in the cortex after isolation rearing to between 3.5% in the Stratum oriens layer 1 and 9.8% in the polymorphic layer of the dentate gyrus after sub-chronic PCP treatment. Finally, CHRM3 was increased between 1% in layer 1 of the stratum oriens and 9% in layer 3 of the stratum radiatum after neonatal PCP treatment. These latter two sets of data suggest that the adult and neonatal PCP treatment paradigms may have subtle effects on the expression of CHRM3 in different CNS regions.

In any study of receptor density, it is important to consider whether the magnitude of change is larger than the receptor reserve, which is the number of receptors present in a tissue in excess of that needed for a full physiological response [62]. This is because any change in receptor density which is smaller than the receptor reserve is unlikely to elicit change in receptor signalling of a magnitude that will have any physiologically significant consequence [63]. In this respect, current data suggest that the NMDAR has a large receptor reserve whereas the other ionotropic glutamate receptors have close to no receptor reserve [64]. This suggests that the decreases in NMDARs we report after sub-chronic PCP treatment are unlikely to be sufficient to cause a change in receptor signalling. By contrast, the absence of any KAR receptor reserve means that the small change in receptor density we report after isolated rearing would result in altered receptor signalling and therefore could be contributing to the behavioural changes observed in these animals. There is a more comprehensive literature on receptor reserve for CHRM function. Hence, in SH-SY5Y cells it has been reported that the overall receptor reserve for signalling by the CHRM agonist carbachol is 50% [65]. Studies using cloned receptors in CHO cells have subsequently suggested that the CHRM3 has a large receptor reserve [66]. In mouse hippocampus, CHRM1 has been reported to have a receptor reserve of 85% [67] whereas the CHRM2 appears to have little to no receptor reserve [68]. There appears to be little or no receptor reserve for CHRM-mediated acetylcholine release [68]. Finally, in the cortex, it has been reported that there is a very low receptor reserve with regards to CHRM1 signalling through phosphoinositide pathways whereas there was a high receptor reserve for CHRM2 signalling through cyclic AMP [69]. Hence, at least with CHRMs, it would appear that receptor reserve can vary significantly depending on the signalling pathway being activated.

The KAR, like other ionotropic glutamate receptors, is made up of sub-units [70] from two families of sub-units (GluR 5, 6 and 7 and KA 1 and 2) which have been differentiated on the basis of gene sequence homologies [71]. Notable for this study is that high affinity [^{3}H]kainate binding is to the KA 1 or 2 sub-units [70]. We are not aware of any previous studies examining levels of KAR in the CNS of rats following isolation rearing and therefore our findings of widespread decreases in KAR are novel. It has been reported that 24 hours after a single injection of PCP there is a dose-dependent (0.86, 4.3 or 8.8 mg / kg) increase in KAR in the Cornu Ammonis 3 and dentate gyrus [72] suggesting drugs which target the NMDAR can indirectly affect the density of KAR. Our data did not show any statistically significant changes in the density of KAR in the CNS of adult rats following neonatal or sub-chronic PCP which could suggest any effects of the PCP treatment on KAR is short lived.

Our finding of global decreases in NMDAR after sub-chronic PCP treatment is in partial agreement with another study that reported a decrease in that receptor after 7 days administration of MK 801 (0.5 mg / kg) to adult rats [73]. In another study it was reported that NMDAR were decreased in 36 day old rats injected with PCP (5 mg / kg) from PND 5 to 15 [74]; this study also reported decreases in levels of NR2B, but not NR1, NR2A or NR2C, sub-unit mRNA. By contrast, PCP (single injection: 0.86, 4.3, 8.8 mg / kg) has been reported to cause a dose-dependent increase in hippocampal [^{3}H]glutamate binding to the NMDAR 24 hr after drug injection [72]. In other studies, using seven day old rats treated with MK 801 (1 mg / kg), there was an increase in mRNA for the NR1, NR2A, NR2B and NR2D NMDAR sub-units in the cortex, hippocampus and striatum 2 to 4 hours after the administration of MK801 [52, 75]. Thus it would seem that the effects of NMDAR antagonists on NMDAR levels may depend on the age of the rats and dosing regimen. This notion is supported by a study that compared the outcome of a single dose of PCP (10 mg / kg) on PN7 (rats sacrificed after 0, 4, 8 or 24 hr) to that of multiple injections of the same does of PCP on PND 7, 9 and 11 (rats sacrificed after 24 hr) [76]. The single injection of PCP increased the levels of cortical NR1 and NR2B, but not NR2A, sub-units of the NMDAR whereas repeated PCP injections increased cortical NR1 and NR2A, but not NR2B, sub-units of the NMDAR. Our study has shown that repeated injection of PCP during the neonatal period does not change levels of NMDAR in the adult CNS suggesting any effects of injecting PCP on NMDAR density is not long lasting.

This study has shown there are small widespread changes in levels of CHRM3 following isolation rearing (< -7.5 %), neonatal PCP administration (1 to 9%) and sub-chronic PCP treatment (4 to 10%). However, none of these changes in CHRM3 exceed the CHRM3 receptor reserve and thus are unlikely to be of a sufficient magnitude to have significant physiological outcomes. This hypothesis needs to be treated with some caution as it has been shown that apparent receptor reserves differ for different CHRM / signalling pathway interactions and thus it may be premature to totally exclude the possibility that such small changes in CHRM3 density are having effects on signalling pathways and hence cellular function. In this study, there was no change in

[^{3}H]pirenzepine binding in any paradigm which differs from a previous study that showed complex changes in [^{3}H]pirenzepine following neonatal PCP treatment, culminating in a 10% increase in binding in the cortex of adult rats after drug treatment [77].

In conclusion, this study shows that animal models developed to study the pathophysiology of schizophrenia cause differential changes in the molecular cytoarchitecture of the CNS. However, neither isolation rearing nor PCP treatment caused changes in the molecular cytoarchitecture of the CNS in rodent that have been reported in the CNS of subjects with schizophrenia. Significantly, ongoing studies of the CNS from subjects with schizophrenia are revealing that extensive changes in gene expression are associated with the disorder [78] and it is therefore likely that changes in the expression of some of these genes will occur in different animal models of the disorder. The challenge will therefore be to identify an animal model that has a change in CNS expression of a specific gene which mirrors what occurs in the CNS from subjects with schizophrenia. For example, our data shows a decrease in KAR after isolation rearing and some studies report decrease in KAR in schizophrenia [6, 9, 79, 80]. Hence, further study of KAR in rats following isolation rearing should allow a greater understanding as to how exposure to such an environmental factor earlier in life can cause changes in the expression of a gene similar to those observed in subjects with schizophrenia.

ABBREVIATIONS

QNX	=	3-quinuclidinyl xanthene-9-carboxylate hemioxalate salt
4DAMP	=	4-diphenylacetoxy-N-methyl-piperidine methiodide
ETE	=	Estimated Tissue Equivalent
KAR	=	Kainate receptor
CHRM1	=	Muscarinic M1 receptor
CHRM3	=	Muscarinic M3 receptor
CHRM4	=	Muscarinic M4 receptor
CHRMs	=	Muscarinic receptors
NMDA	=	*N-Methyl-D-aspartic* acid
NSB	=	Non-specific binding
PCP	=	Phencyclidine
PND	=	Post-natal day
PPI	=	Pre-pulse inhibition
RT	=	Room temperature
TB	=	Total binding

ACKNOWLEDGEMENTS

BD is an NH&MRC Senior Research Fellow (APP1002240) and ES is the ARH Royce Abbey Post-Doctoral Fellow. This research was supported in part by NHMRC Project grants 509333, 628669 and 566967. The study was supported by Operational Infrastructure Support (OIS) from the Victorian State Government and by the funding grants.

CONFLICTS OF INTEREST STATEMENT

The authors declare no conflict of interest.

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Received: January 20, 2011 Revised: May 06, 2011 Accepted: May 07, 2011