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A quantitative autoradiographic comparison of binding to glutamate receptor sub-types in hippocampus and forebrain regions of a food-storing and a non-food-storing bird

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Abstract

In two species of birds, food-storing marsh tits, *P. palustris*, and non-storing blue tits, *P. caeruleus*, quantitative receptor autoradiography was used to localize NMDA (*N*-methyl-D-aspartate)-sensitive [^{3}H]glutamate, [^{3}H]MK801, and [^{3}H]AMPA binding sites, in six regions of the forebrain: hippocampus and parahippocampus, hyperstriatum accessorium (vision) and ventrale (sensory integration), neostriatum (auditory), and lobus parolfactorius (basal ganglia). In both species high levels of labelling to both NMDA and AMPA receptors were observed throughout the forebrain. However, a marked difference in receptor labelling was apparent between the two species, with levels of binding to NMDA ion channel sites being significantly lower (20%) in both the hippocampus and parahippocampus, in food storers compared to non-food storers. The levels of binding to other forebrain regions were remarkably similar in the two species. No differences were seen in the binding to AMPA receptors in forebrain regions of either species. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Avian hippocampus; Food storing; Glutamate receptor sub-types

Relative to brain and body size, birds that store and retrieve large quantities of food, such as marsh tits, *Parus palustris*, have a larger hippocampal formation, with greater numbers of neurons (but not greater neuronal density), than non-storers such as blue tits, *P. caeruleus* [7,8,11,16]. Hippocampal lesions impair memory-based retrieval of stored food [17] and may also disrupt other forms of spatial memory in food-storing species [6,16]. The avian hippocampus is homologous to the mammalian hippocampus on the basis of embryology, connectivity and immunohistochemistry [3,5,14], although it is morphologically quite different.

Glutamate receptors, and in particular the NMDA (*N*-methyl-D-aspartic acid) sub-type, have been shown to play a crucial role in mammalian learning models, including retention of maze performance [23] and spatial learning [4,15]. Non-NMDA receptors may also play a role in learning; in classical conditioning of the rabbit nictitating membrane an autoradiographic approach was used to demonstrate selective increases in [^{3}H]AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) binding (to the quisqualate receptor) in the hippocampus [21].

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Glutamate receptors also play a role in non-mammalian learning systems. In the domestic chick there is a large increase in binding to NMDA-sensitive glutamate receptors in the intermediate and medial hyperstriatum ventrale (IMHV) 8 h after imprinting on a flashing red light [9], whilst 30 min after one trial passive avoidance training of the chick binding to NMDA-sensitive [^{3}H]glutamate receptors is markedly increased in two specific forebrain regions previously shown to be involved with memory formation and storage, and is coupled with alterations in binding of MK801 to the NMDA ion channel complex [19]. A marked increase in AMPA binding was observed 5.5 h downstream from the initial training time [18].

In the zebra finch Aamodt et al. [1] have examined the distribution and developmental changes in [^{3}H]MK801 binding within the song nuclei. However, no information exists on glutamate receptors in the brains of food-storing birds. Given the apparent importance of the hippocampus to food-storers, and the role of the hippocampus and glutamate receptors in the process of spatial learning in mammalian hippocampus, the present study utilized quantitative receptor autoradiography to address the localization and distribution of glutamate receptor subtypes (NMDA, AMPA) in the hippocampus of a food-storing bird, the marsh tit, *Parus palustris*, and a non-storer, the blue tit, *P. caeruleus*. Glutamate receptor subtypes were also localized in other forebrain regions contained within coronal sections through the tit hippocampus and, in addition, using sections from the same brains, we examined binding to the NMDA ion channel complex using [^{3}H]MK801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo [*a*,*d*]cyclohepten-5,10-imine).

Subjects were three female and four male blue tits (*Parus caeruleus*) and three female and eight male marsh tits (*Parus palustris*). All birds were wild caught in Oxfordshire, UK, and were sexed by post-mortem examination of gonads. Subjects had been housed in one room in 0.77 × 0.44 × 0.44 cages (length × width × height), and were maintained on a 11:13-h light:dark cycle. From 19 August through 15 November 1994, all subjects were trained or tested every other day on a one-trial associative memory task in a large test room (3.9 × 3.9 × 2.5 m) adjacent to their home cages. This behavioural test, which has been described in detail elsewhere [2], measures accuracy of recovery of a stored food item, but can be used with both food-storing and non-storing species. The purpose of this experimental treatment was to ensure that all birds had experienced some food retrieval experience prior to being sacrificed. To this end, each subject was given two trials of the memory task at retention intervals of 0.25, 2, 4, 6 and 24 h. On 15 November, each subject was given the first phase of a test (in which the location of a stored food-item is learned), and then sacrificed by decapitation 93–119 min later. All but one marsh tit had been observed to store numerous seeds in their home cages during the month prior to sacrifice, while no blue tit was observed to have stored food in its home cage, despite identical diet and housing conditions.

Birds were killed by decapitation and the brains removed from the skull, and cut into forebrain and hind brain. Forebrains were then frozen in an isopentane/CO_2 mixture, coded and wrapped in foil and were transferred to the Open University where they were stored at −70°C until required. All subsequent procedures were performed 'blind' with the experimenters not knowing which brains came from storing or non-storing species. Autoradiographic procedures were essentially similar to those described previously [18–20]. Ten μm coronal sections were cut on a Reichert cryostat at −18°C, from the mid-hippocampal region of the brains, collected onto subbed coverslips and brought to room temperature before being processed and pre-incubated in 50 mmol l^{-1} Tris–citrate buffer (pH 7.4) at 4°C for 1 h to remove endogenous glutamate.

Glutamate receptor sites were labelled, respectively, with: [^{3}H]glutamate (70.4 nmol l^{-1}; specific activity 46 Ci mmol^{-1}); [3H]AMPA (77.8 nmol l^{-1}; specific activity 29.2 Ci mmol^{-1}), and [^{3}H]MK801 (3.1 nmol l^{-1}; specific activity 28.8 Ci mmol^{-1}), each in 50 mmol l^{-1} Tris–citrate buffer (pH 7.4). The radioligands were supplied by New England Nuclear. The solutions of each ligand were applied as a drop to cover the sections, and incubated (in triplicate) for 20 min at 4°C in a humid chamber for [^{3}H]glutamate, [^{3}H]AMPA, and 20 min at room temperature for [^{3}H]MK801 (preliminary experiments indicated that binding equilibrium is established by this time, similar to that used by Kavanagh et al. [10]). NMDA-displaceable binding of [^{3}H]glutamate was estimated in further triplicate sections incubated with 100 μmol l^{-1} unlabelled NMDA, whilst non-specific binding of [^{3}H]AMPA was measured in the presence of 1 mmol l^{-1} unlabelled glutamate, and that of [^{3}H]MK801 binding in the presence of 100 μmol l^{-1} MK801.

To terminate the incubation, all sections were washed three times in ice-cold buffer (5 s for glutamate, 2 s for AMPA and 30 s for MK801), dipped in ice cold distilled water to remove buffer salts, and rapidly air-dried. The processed sections on coverslips were glued onto card, apposed to LKB [^{3}H]Ultrofilm secured between aluminium sheets, and exposed in the dark for up to 60 days. Films were developed in Agfa G150 developer for 4 min at 20°C, fixed in Ilford Hypam fixer for 3 min and washed in running water for at least 1 h. Densitometry of the film autoradiograms was performed on an MCID image analysis system, essentially as described previously [18–20], and a standard curve was generated from a series of bird brain paste tritium

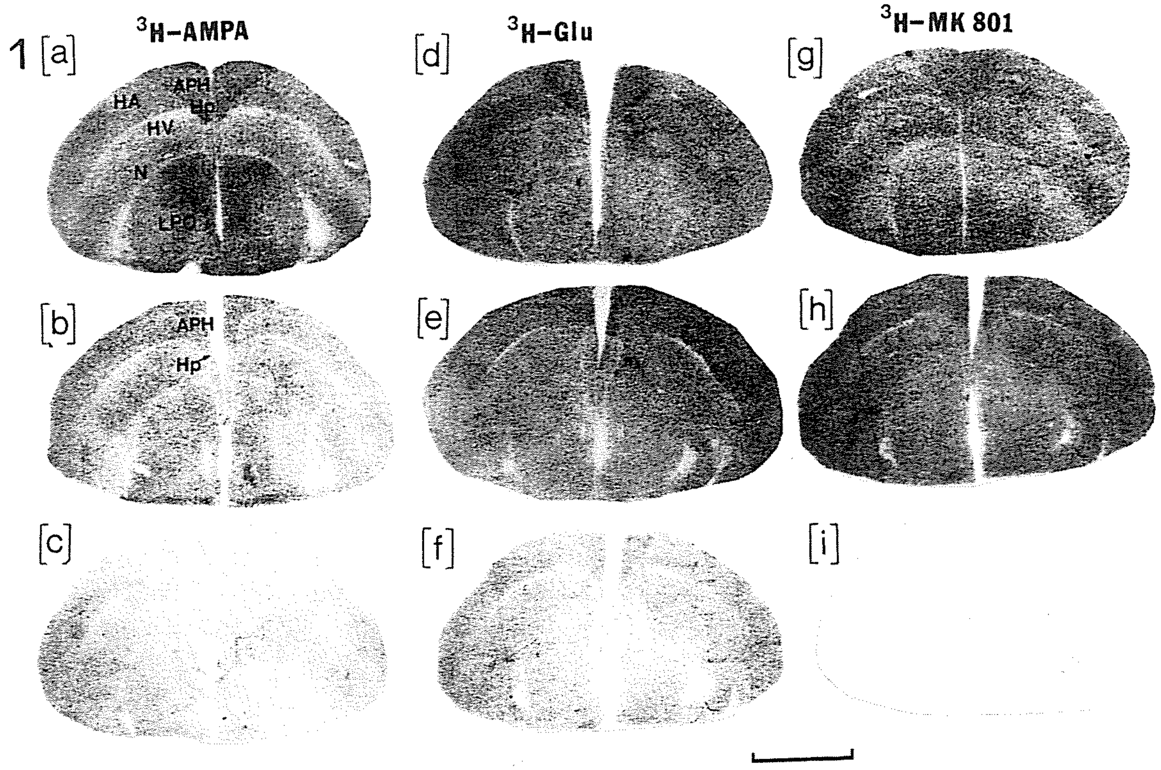

Fig. 1. (a–i) Photographs from tritium film autoradiograms apposed to coronal sections from the brains of marsh and blue tits. These illustrate the distribution of [^{3}H]AMPA binding for marsh tit (a); blue tit (b); and non-specific binding in blue tit (c); total [^{3}H]glutamate for marsh tit (d); blue tit (e); NMDA-displaceable [^{3}H]glutamate binding in blue tit (f); [^{3}H]MK801 binding for marsh tit (g); blue tit (h); and non-specific binding in blue tit (i). Qualitatively, no major differences are readily apparent in binding levels between the two species but densitometric analyses (see Table 1) show that [^{3}H]MK801 binding levels are higher in the hippocampus and hippocampal region of the blue tit. Abbreviations: hippocampus (Hp); parahippocampus (APH); hyperstriatum ventrale (HV); hyperstriatum accessorium (HA); lobus parolfactorius (LPO); neostriatum (N). Scale bar = 2 mm.

standards, prepared according to the method of Unnerstall et al. [22] and apposed on the same tritium film sheets as the ^{3}H-labelled brain sections. Six forebrain regions (both left and right hemispheres separately) were measured, in each of the birds and the binding levels were determined. Because of the use of brain paste standards, the fact that sections were only 10-μm thick, and that myelin is sparse in the avian forebrain regions examined, it is unlikely that quenching is a factor in the data obtained. As labelled in the autoradiogram (Fig. 1a) the regions measured were (with abbreviations): hippocampus (Hp); parahippocampus (APH); hyperstriatum ventrale (HV); hyperstriatum accessorium (HA); lobus parolfactorius (LPO); and neostriatum (N).

Variation in the densities of the autoradiograms of the different brains (each species separately) was standardized in order to permit a comparison of labelling in the different brains. This involved expressing the mean measured value for each region as a percentage of the mean for the whole section in which it was located, multiplied by the mean value for all regions in the brains for each group. In subsequent statistical analysis these mean standardized values for binding to the NMDA, MK801 and AMPA receptors were used, and values given in tables and figures are expressed as mean ± standard error. Because of small and variable sample sizes within each species and sex, a non-parametric design was used to analyse all the results. Separate Mann–Whitney *U*-tests were performed to compare species for each of the regions listed above, except that APH was combined with Hp to allow analysis of the entire 'hippocampal region' (HR). (To compensate for the use of multiple statistical tests, alpha-level was recalculated using a sequential Bonferroni adjustment, with the appropriate alpha-level given below for each comparison.)

Examples of the localization of the quisqualate receptor with [^{3}H]AMPA, the NMDA-sensitive [^{3}H]glutamate receptor sub-type, and the NMDA ion channel with [^{3}H]MK801, by quantitative receptor autoradiography in the marsh and blue tit are shown in Fig. 1a–i. Since the displacement of binding to these receptors is essentially similar in both species NMDA-displaceable [^{3}H]glutamate binding, and non-specific binding (NSB) of [^{3}H]AMPA and [^{3}H]MK801 are shown only for blue tits. It should be noted that NSB is negligible for [^{3}H]MK801 binding (Fig. 1i). High levels

Table 1
Densitometric measurements of specific binding in vitro of: [^{3}H]glutamate to NMDA-sensitive glutamate receptors, and [^{3}H]MK801 and [^{3}H]AMPA to receptors in six forebrain of blue tits (B, non-food-storing birds), and marsh tits (M, food-storing birds)

		Hp		HR		HV		HA		LPO		N	
		B	M	B	M	B	M	B	M	B	M	B	M
MK801	X	1824	1476	1734	1460	1296	1213	1264	1179	935	947	1174	1182
	S.E.	71	26	56	23	39	19	62	31	28	22	37	19
NMDA	X	232	198	233	206	209	190	203	189	153	162	192	186
	S.E.	11	5	13	5	5	4	14	9	4	7	9	4
AMPA	X	418	473	422	456	363	331	355	345	398	358	357	349
	S.E.	22	25	17	29	13	8	11	29	33	10	12	9

Data are means ± standard errors, from seven blue tits and 11 marsh tits, and are expressed as standardized mean values (as described in the text). Abbreviations: hippocampus (Hp); parahippocampus (APH); hyperstriatum ventrale (HV); hyperstriatum accessorium (HA); lobus parolfactorius (LPO); neostriatum (N). Note that the measurement for HR (hippocampal region) comprises that for APH combined with that for Hp.

of labelling occur to both glutamate and AMPA receptors throughout the forebrain regions examined, and also of MK801 to the NMDA ion channel sites. The greater area of the hippocampus and parahippocampus of the food-storing marsh tits compared to the non-storing blue tits is notable in the autoradiograms of binding of [^{3}H]AMPA, total [^{3}H]glutamate and [^{3}H]MK801 (compare Fig. 1a, d, and g with b, e and h).

A preliminary analysis was carried out to examine the effects of sex and hemisphere on binding to NMDA-sensitive [^{3}H]glutamate, [^{3}H]MK801, or [^{3}H]AMPA receptors. Separate Mann–Whitney *U*-tests for each combination of sex, hemisphere, brain region and receptor type showed that there were no significant differences between left and right hemispheres, and males and females differed significantly in only one hemisphere of one region for one receptor type (left N, [^{3}H]AMPA, $P < 0.003$). Given the overall similarity of left and right hemispheres, and males and females, across all six regions examined and for all three receptor types, we pooled data from male and female birds and averaged data for each of the forebrain regions examined. The results are presented in Table 1. As described above, HR represents the hippocampal and parahippocampal regions. Binding of [^{3}H]MK801 is significantly lower in Hp of the food-storing marsh tit than in the non-storing blue tit (19%, $P = 0.001$, significant at adjusted alpha $= 0.007$), and lower similarly in HR (16%, $P < 0.002$, significant at adjusted alpha $=$ 0.008). Binding to NMDA-sensitive [^{3}H]glutamate receptors tended, as with [^{3}H]MK801 binding, to be lower similarly in marsh tit in both Hp (15%) and HR (12%) ($P < 0.012$ for Hp and 0.056 for HR), but these differences were not quite significant at adjusted alpha $= 0.007$ and 0.008, respectively. No significant differences occurred between species in the binding to NMDA-sensitive [^{3}H]glutamate receptors, or of [^{3}H]MK801, in any of the other four forebrain regions (all $P > 0.01$, all differences $< 10\%$). No significant differences were observed between species in the binding to AMPA receptors in any of the forebrain regions (all $P > 0.07$, all differences $< 10\%$, except in the hippocampus where binding in the marsh tit was 13% higher than in the blue tit, but the difference was also non-significant).

The present investigation demonstrates a strong similarity between domestic chicks and wild adult tits of two species in the distribution and binding levels of glutamate receptor sub-types across the six forebrain regions examined [19] and the autoradiograms shown in Fig. 1 demonstrate the precision of localization of the ligands.

We had two predictions at the outset of this exploratory study. First, we expected differences between the food-storing and non-storing species in the hippocampus and hippocampal region, and not in the other four regions examined, as this is where functional neuroanatomical differences have been previously identified [6–8,11,12]. Also, we expected that, where differences existed, there would be higher levels of binding to glutamate-sensitive receptors in the food-storing species, as these species have greater demands placed on their spatial memories.

Our first prediction was supported, as we found significant differences (15–20%) in binding in both the hippocampus and hippocampal regions, but not other regions, for [^{3}H]MK801-sensitive receptors. A similar trend was found for NMDA-sensitive receptors, but differences between food-storers and non-storers were non-significant when corrections for multiple statistical tests were applied. In contrast, the levels of binding to [^{3}H]MK801- and NMDA-sensitive receptors in the remaining forebrain regions were remarkably similar ($<$ 10% different in all cases) between the two species. No significant species differences were seen in the binding to AMPA receptors in forebrain regions of either species; however, it is possible that such differences existed, but could not be detected by examination of binding of a fixed concentration as used in the present study.

Our second prediction, that binding levels would be higher in food-storing species, was not met, as, quite unexpectedly, it was the non-storers that had higher levels of binding per unit area of tissue. Although the total hippocampal volume is larger in storers than non-storers, the neuronal density is similar, at least in adult birds [7,11], so the receptor binding density differences cannot be explained on the basis of differing neuronal densities.

Because the presence or absence of food-storing behaviour is just one of many differences between marsh and blue tits, it might seem premature to conclude that differences in receptor binding in the forebrain have a functional relationship with food-storing behaviour. However, the finding that these differences occurred only in the hippocampal region, and not in the other forebrain areas examined, is suggestive of a link with food-storing behaviour because food-storing and retrieval are likely to place great demands on spatial memory, possibly requiring increased function from the hippocampus. Indeed, as discussed above, marsh tits and most food-storing birds have a proportionately larger hippocampus, relative to the rest of the telencephalon, than non-storers [9]. Therefore, given that NMDA and [^{3}H]MK801 receptors have been implicated in learning and spatial memory in mammals [4,13,23], this finding sets the scene for further investigation into the mechanism by which levels of binding are related to memory for spatial locations. Important further issues are whether a non-storing bird, with its smaller hippocampus, requires higher levels of glutamate binding to undertake routine spatial tasks? Moreover, does the higher level of receptor binding in non-storing birds indicate that memory for the locations of stored food does not require the involvement of glutamate receptors in the same way as other avian memory tasks such as imprinting [9] and passive avoidance training [18,19]? And, if so, why are levels higher in non-storers, rather than equal? It may be that the foraging activities of non-storers require enhanced spatial learning and memory at different temporal or spatial scales than food-storers. An important way in which progress could be made in answering some of these questions will be to undertake kinetic studies to determine whether there are differences in either receptor-binding affinity K_D, or maximal binding levels B_{max}, to the NMDA-sensitive glutamate and AMPA receptors.

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