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Passive avoidance learning involves α2-noradrenergic receptors in a day old chick

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DAY-OLD chicks were trained on one-trial passive avoidance task, using methyl-anthranilate (MeA) as an aversive substance. Bilateral pharmacological manipulation of the intermediate hyperstriatum ventrale was performed by intracerebral application of an α2-noradrenergic agonist, clonidine (5 μM), or an antagonist, rauwolscine (300 μM). Only rauwolscine application (pre- or post-training) induced significant memory impairment. Quantitative receptor autoradiography was used to determine the kinetic properties of the binding sites for [^{3}H]clonidine or [^{3}H]rauwolscine in MeA-trained or water-trained (control) chicks, in forebrain areas known to be involved in avoidance learning. Scatchard analysis revealed that MeA-training resulted in a significant bilateral upregulation in the number of [^{3}H]rauwolscine binding sites (B_{max}) in the area of hyperstriatum ventrale. These findings suggest the importance of activation of α2-noradrenergic receptors in aversive learning in chicks. *NeuroReport* **9: 1679–1683**

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Introduction

Avian learning provides an important model for the study of vertebrate memory mechanisms and particularly in the chick, ontogenetically driven learning.[1] Chicks learn quickly to suppress their natural tendency to peck spontaneously at small objects in their field of view after initially pecking at a bead coated with a bitter tasting substance such as methyl anthranilate (MeA). This one-trial passive avoidance training paradigm, first described by Cherkin,[2] initiates a cascade of cellular events that lead to a modification of synaptic function in specific regions of the chick forebrain,[3] notably the intermediate medial hyperstriatum ventrale (IMHV) and lobus parolfactorious (LPO).

Many neurotransmitter receptors have been shown to be of importance in avoidance learning, including acetylcholine, serotonin, GABA, opioid, glutamate and dopamine[4–6] The noradrenergic system has also been implicated in enhancing learning and memory processes in mammals.[7] In birds, depletion of forebrain noradrenaline prevents imprinting,[8] while systemic application of β adrenergic antagonists impairs memory retention in passive avoidance training.[9] Although regions of the chick forebrain, especially hyperstriatum ventrale (HV), are rich in α2-adrenoceptors,[10] there is no clear evidence of the role of these receptors in the learning processes. To address this issue, we investigated the effects on passive avoidance training of intracerebral injections of an α2-agonist, clonidine and an α2-antagonist, rauwolscine. In addition we used quantitative receptor autoradiography *in vitro* to examine changes in binding characteristics to α2-receptors in forebrain regions following training.

Materials and Methods

Day-old chicks (Ross Chunky) avoid pecking at a bead coated with an unpleasant tasting substance, methyl anthranilate (MeA; one-trial passive avoidance paradigm). The training procedure was exactly as described previously.[11] Briefly, one day after hatching chicks were placed in pairs in aluminium pens illuminated with red light, allowed to settle for 1 h and pre-trained by presentation of a small white bead for 10 s, three times at 5 min intervals. After 30 min, chicks that pecked on all three presentations were trained on a chrome bead coated either with methyl-anthranilate (MeA) or water (W). One hour following training, all groups were tested for recall of the task, and the effect of intracerebral injections (into IMHV) of α2-agonist or antagonist, and the levels of α2-adrenoceptors were determined.

Bilateral pharmacological manipulation of IMHV: Intracerebral injections of 5 μl clonidine or rauwolscine or saline were made using Hamilton microsyringes and a stereotaxic Plexiglass headholder which precisely directed the injections to IMHV as described in Ref. 12. The skull of the day-old chick is unossified so injections can be performed quickly without the need for anesthesia. Animals show little discomfort and behave normally after the injection. Preliminary bilateral intra-IMHV injections of clonidine (0.1 μM–10 mM) or rauwolscine (12 μM–1.2 mM) determined the drug doses suitable for use in the behavioural pharmacological experiments. Abnormal behaviour (appearance of lethargy or inability to peck) was observed when chicks were injected with 10 μM clonidine or 600 μM rauwolscine or higher. Bilateral intra-IMHV application of saline or clonidine (5 μM) or rauwolscine (300 μM) was made either 30 min before (after pre-training) or immediately after training. During testing, chicks ($n = 137$) were scored as peck or avoid. For each drug application, a separate group of saline-injected (control) chicks was tested in parallel. This gave four pairs of numbers for pre- or post-training injections: clonidine chicks which avoid or peck, rauwolscine chicks which avoid or peck and control chicks for each drug injection. Each pair of groups (drug-injected, saline-injected) from each experimental protocol was analysed statistically using χ^2 tests with a null hypothesis that drugs had no effect on learning and memory and therefore all chicks should avoid the test bead. In order to exclude any non-specific, side effects of the injected drugs on the animal's pecking behaviour, a visual discrimination test was performed in parallel. Thirty minutes after training alternative presentations were made of a yellow-followed by a chrome, then yellow bead during the memory testing procedure. Animals ($n = 68$) were scored separately for pecking the yellow and the chrome bead and there was no statistically significant effect of the adrenergic agents on the ability of the animals to peck or discriminate between visually distinct objects. For the visual discrimination trials, χ^2 tests compared the number of chicks pecking or avoiding the chrome and the yellow beads (data not shown).

In vitro autoradiographic study of α2-adrenergic receptors: Chicks were trained by presenting a chrome bead coated with methylanthranilate (MeA), or water and 1 h later were tested using a dry chrome bead. Animals avoiding ($n = 9$) or pecking ($n = 9$) the chrome bead following MeA-training or water (W)-training respectively, were subsequently used for receptor autoradiography, killed by decapitation under ether anesthesia and their brains were rapidly removed. Forebrains were frozen at –40°C in dry-ice cooled isopentane, coded, placed in a cryostat, allowed to come to –15°C and were cut coronally (10 μm) at seven levels in the rostral-caudal plane (16 series of sections from the level of nucleus basalis to the caudal part of hyperstriatum ventrale). The sections were processed using standard autoradiographic procedures.[13,14] Codes were broken after all experimental procedures and analysis were performed. Briefly, kinetic analysis of α2-adrenergic receptors was performed using [^{3}H]clonidine (30 Ci/mmol, Amersham) and [^{3}H]rauwolscine (80 Ci/mmol, Amersham) and non-specific binding was determined in the presence of 100 μM phentolamine. Sections were lightly fixed for 10 min (0.5% paraformaldehyde in 50 mM Tris–HCl, pH 7.7), pre-incubated in Tris buffer, 4°C for 20 min and incubated with [^{3}H]clonidine (60 min) or [^{3}H]rauwolscine (30 min) at concentrations ranging from 0.2 to 8 nM. Free radioligand was removed by washes in 4°C Tris-buffer (5 and 10 min for [^{3}H]clonidine, 3×20 s for [^{3}H]rauwolscine). Tritium-sensitive film (Hyperfilm, Amersham) was exposed to labeled dried sections, along with plastic [^{3}H]standards ([^{3}H]Microscales, Amersham) for 3–4 months, developed in Kodak D-19, fixed with Agfa Acidofix and cleared in running water. Quantitative image analysis of the autoradiograms was performed using NIH-Image on a Power Macintosh 7100 and each hemisphere was measured separately. Specific binding, > 95% of the total binding, was expressed as fmol/mg tissue. Values for the equilibrium dissociation constant (K_d) and the maximum binding sites (B_{max}) for [^{3}H]clonidine and [^{3}H]rauwolscine in W- and MeA-trained animals were determined using EBDA-LIGAND (BIOSOFT), an iterative fitting program based on a one-site binding model.[15] Data were statistically analysed using a two-way analysis of variance (ANOVA) with split-plot design (SuperANOVA, Abacus Concepts Inc.), with the independent factor (whole plot) being the training substance (MeA or water), and split factor the side of the brain (left or right hemisphere). Variations in the total radioactivity of the sections due to the individuality of the animals, were normalized using a standardization procedure for each experiment. That is, the measured regional specific binding value per animal was multiplied by a correction factor (F), F being the ratio of the average specific binding of whole brain of all animals studied ($n = 18$) divided by the individual animal's whole brain average specific binding.

Results

Effects of bilateral application of α2-adrenergic agents on avoidance task: Intra- IMHV application of clonidine had no effect on acquisition or recall of

memory for the avoidance task employed (Fig. 1). When chicks were injected 30 min before training, 82% of clonidine-injected animals avoided the chrome bead, similar to saline-injected chicks. When injections were made after training, there was a slight increase in the number of chicks avoiding compared to the control group. In contrast, rauwolscine injections 30 min before or immediately after training resulted in a statistically significant decrease (Fig. 1) in the number of chicks avoiding the chrome bead (48 or 54% of rauwolscine-treated compared to 80 or 73% of saline-treated birds, pre- or post-training respectively, $p < 0.05$).

Autoradiographic analysis of α2-adrenergic receptors following passive avoidance training: Both ligands studied showed a single high affinity binding site and exhibited a similar distribution pattern in a day-old chick forebrain, although rauwolscine binding levels were higher (35–55%) than those for clonidine. High densities of α2 receptors were observed in ventral and dorsal subregions of hyperstriatum ventrale (HVv, HVd), hyperstriatum intercallatum supremum, n. basalis, paleostriatum primitivum (PP), lateral septum (SL), and ectostriatal belt (Ebelt). Moderate binding was exhibited in lobus parolfactorius (LPO), hyperstriatum accessorium, area parahippocampalis (APH), paleostriatum augmentum (PA), neostriatum, archistriatum (A) and medial septum, whilst, ectostriatum (E) and hippocampus (Hp) were almost devoid of α2-receptors (Fig. 2). The B_{max} and Kd values of [^{3}H]clonidine and [^{3}H]rauwolscine binding sites in the areas known to be involved in memory processes[3,4] (HV, LPO and A) were determined by Scatchard analysis using LIGAND software (Tables 1, 2). B_{max} values ranged from background levels to 53 fmol/mg tissue for clonidine and 35–82 fmol/mg tissue for rauwolscine, while K_d ranged from 1.6 to 4.5 nM for both ligands. Moreover K_d for [^{3}H]rauwolscine binding was lower in HV, compared

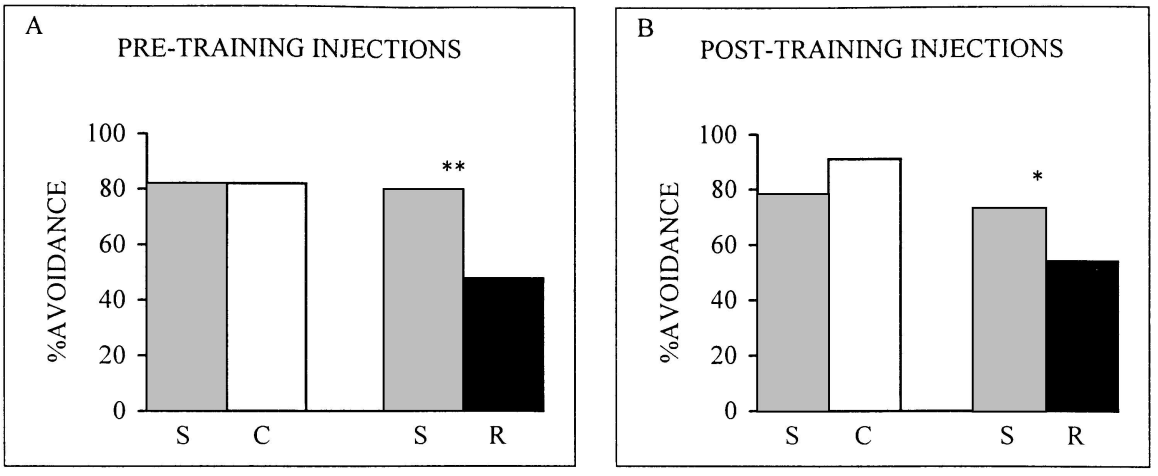

FIG. 1. Effect of pre-training (**A**) and post-training (**B**) bilateral intra-IMHV application of saline (S), clonidine (C) and rauwolscine (R) on the percentage of chicks avoiding the chrome bead. Significant difference, determined by χ^2 test is shown between saline and rauwolscine treatment: $*p < 0.5$, $**p < 0.01$.

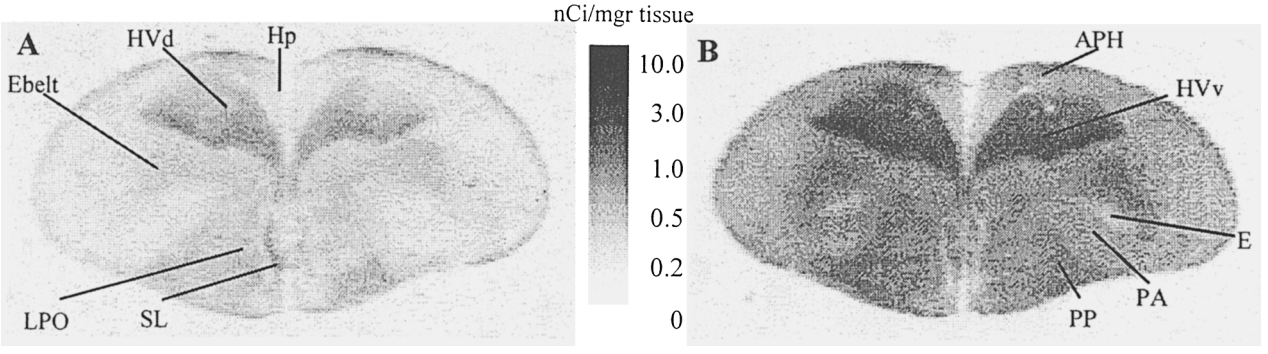

FIG. 2. Quantitative autoradiograms of chick forebrain coronal sections at the level of IMHV, showing the distribution of [^{3}H]clonidine (**A**) and [^{3}H]rauwolscine (**B**) specific binding. APH, area parahippocampalis, E ectostriatum, Ebelt ectostriatal belt, Hp hippocampus, HVv and HVd hyperstriatum ventrale ventral and dorsal part, respectively, LPO lobus parolfactorious, PA paleostriatum augmentum, PP paleostriatum primitivum, SL *n. septalis lateralis*.

Table 1. Dissociation constants (K_d) of [^{3}H]clonidine and [^{3}H]rauwolscine binding in forebrain regions of water- and MeA-trained chicks

Brain area	Water		MeA		F-values		*p*	
	Left	Right	Left	Right	Training	Side	Training	Side
[^{3}H]Clonidine								
HVd	2.06 ± 0.16	2.27 ± 0.08	2.48 ± 0.13	2.44 ± 0.09	3.88	1.05	0.07	
HVv	2.11 ± 0.23	2.10 ± 0.18	2.04 ± 0.10	2.31 ± 0.12	0.12	1.94		
LPO	3.22 ± 0.49	3.77 ± 0.78	3.62 ± 1.04	3.42 ± 0.57	< 0.01	0.19		
A	3.31 ± 0.49	3.35 ± 0.91	3.20 ± 0.15	3.78 ± 0.73	0.05	0.31		
[^{3}H]Rauwolscine								
HVd	1.86 ± 0.04	1.85 ± 0.05	1.95 ± 0.08	1.96 ± 0.06	1.33	0.01		
HVv	1.62 ± 0.05	1.61 ± 0.06	1.63 ± 0.05	1.67 ± 0.06	0.25	0.24		
LPO	2.56 ± 0.28	2.56 ± 0.32	2.63 ± 0.23	2.72 ± 0.19	0.12	0.12		
E	4.35 ± 1.39	3.77 ± 0.73	4.47 ± 0.71	4.08 ± 0.96	0.04	0.80		
A	2.69 ± 0.43	2.12 ± 0.24	2.22 ± 0.24	2.23 ± 0.11	0.28	1.96		

Data are means ± s.e.m., expressed in nM, determined by Scatchard analysis ($n = 9$/group) and statistically analysed by two-way ANOVA with split-plot design (df = 1,1). HVd and HVv: Hyperstriatum ventrale dorsal and ventral part respectively, LPO: Lobus parolfactorius, A: Archistriatum and E: Ectostriatum.

Table 2. Maximum specific binding sites (B_{max}) of [^{3}H]clonidine and [^{3}H]rauwolscine in forebrain regions of water- and MeA-trained chicks

Brain area	Water		MeA		F-values		*p*	
	Left	Right	Left	Right	Training	Side	Training	Side
[^{3}H]Clonidine								
HVd	43.42 ± 1.10	43.18 ± 1.40	45.69 ± 1.79	44.90 ± 1.93	0.90	0.45		
HVv	52.43 ± 0.96	51.53 ± 1.79	52.02 ± 1.43	51.50 ± 1.63	0.01	0.59		
LPO	30.36 ± 1.72	35.58 ± 2.80	31.89 ± 3.14	32.96 ± 2.06	0.03	7.37		0.02
A	14.70 ± 1.52	14.13 ± 1.13	13.41 ± 0.89	15.76 ± 1.48	0.01	2.62		
[^{3}H]Rauwolscine								
HVd	67.43 ± 1.59	66.80 ± 2.20	73.47 ± 2.05	75.32 ± 2.20	6.51	0.65	0.02	
HVv	74.15 ± 1.53	73.94 ± 2.59	80.01 ± 1.81	81.96 ± 1.84	6.64	1.42	0.02	
LPO	65.52 ± 5.13	67.27 ± 6.69	62.79 ± 3.60	66.53 ± 2.89	0.07	3.28		
E	36.36 ± 4.47	36.96 ± 2.33	36.21 ± 2.84	36.67 ± 1.95	< 0.01	0.07		
A	47.14 ± 4.22	39.44 ± 1.38	43.30 ± 1.99	38.86 ± 1.45	0.86	5.13		0.05

Data are means ± s.e.m., expressed in fmol/mg tissue, determined by Scatchard analysis ($n = 9$/group). Two-way ANOVA with split-plot design (df = 1,1) showed significant differences (*p*-values shown) following MeA training and side differences independent to the aversive task. Abbreviations as in Table 1.

with that for [^{3}H]clonidine binding. There was no hemispheric difference in the affinity of the ligands used, but we observed laterality, independent of the avoidance training task, in [^{3}H]rauwolscine binding sites in archistriatum (A) and [^{3}H]clonidine binding sites in LPO (Table 2).

One trial passive avoidance training resulted in a significant bilateral increase in B_{max} for rauwolscine binding in HV in both the dorsal (HVd) and ventral part (HVv). In contrast, the number of clonidine binding sites showed no changes following MeA-training, but we observed a decrease (close to significant) in the K_d for clonidine binding in the dorsal part of HV, following one-trial passive avoidance training.

Discussion

The present study was designed to examine the contribution of α2-adrenoceptors in the early and intermediate stages of learning in chick. An important issue is the role of adrenoceptors in the synaptic events in the IMHV during passive avoidance training. The behavioural data showed that the bilateral administration of the α2-antagonist rauwolscine, either pre- or immediately post-training caused amnesia probably, on the basis of the noradrenaline hypothesis of learning,[7] by inhibiting the function of α2 postsynaptic receptors in IMHV. Our results do not support a contribution by presynaptic α2-adrenoceptors because the α2-agonist clonidine, had no significant effect on acquisition of memory for the passive avoidance task. If a presynaptic α2 action were to be important, clonidine should also cause amnesia for the task, since activation of α2 presynaptic receptors inhibits noradrenaline release[16]. In addition support for the postsynaptic site of action comes from studies in aged primates, where clonidine improved learning via action on post-synaptic α2-adrenoceptors in prefrontal cortex, which was blocked specifically by the α2-antagonist yohimbine.[17]

The localization and biochemical properties of clonidine and rauwolscine binding in the forebrain of control and trained chicks provided additional information on synaptic changes in IMHV following learning. Quantitative autoradiographic saturation analysis of binding parameters in selected forebrain areas revealed significant upregulation in the number of [^{3}H]rauwolscine binding sites and a decrease in [^{3}H]clonidine affinity, specifically in the hyperstriatum ventrale (including the IMHV), following MeA-training. Whilst rauwolscine labels α2-adrenoceptors and binds to all three cloned human α2-adrenergic receptor subtypes,[18] clonidine is considered to be a partial agonist and may also detect a population of non-adrenergic imidazoline binding sites, although with at least 10-fold lower affinity[19] (K_d in the range of 57 nM). In the present study, a non-adrenergic component of [^{3}H]clonidine binding can be considered negligible, since we studied only the high affinity binding sites (K_d 2 nM). The observed range of K_d values for [^{3}H]rauwolscine and [^{3}H]clonidine binding are in agreement with previously reported values in human,[20] rat[21] and chick[22] brain for α2-adrenoceptors. In addition, studies in chick membrane preparations have shown that both clonidine and rauwolscine binding sites display similar kinetic and pharmacological characteristics to that of the α2A-adrenoceptor subtype described for mammalian brain: i.e. yohimbine and rauwolscine are about 10-fold more potent than prazosin and phentolamine.[14,22]

The cellular origin of these receptors is unclear since immunocytochemical[23] and anatomical[24] studies showed a paucity of noradrenergic fibers in the area of hyperstriatum ventrale. This indicates that they may possibly be heteroreceptors on different population of neurons in the hyperstriatum ventrale. Moreover, the changes of α2-adrenoceptor binding at the time point studied here (60 min), after passive avoidance training, appear to occur within the intermediate stages of memory formation, as defined by Ng and Gibbs.[25] Further studies are therefore necessary in order to elucidate the precise role of α2-adrenoceptors in chick avoidance training and to determine whether their activation is required specifically for memory acquisition or is a phenomenon common to several steps in the learning processes.

Conclusion

The present study provides evidence on the role of α2-adrenoceptors in the passive avoidance learning paradigm in a day-old chick. Specifically, the intermediate hyperstriatum ventrale, considered to be a key area for learning process in the chick forebrain, was shown to exhibit high densities of α2-adrenoceptors, by means of [^{3}H]clonidine and [^{3}H]rauwolscine autoradiography. Pre- or post- training blockade of these receptors, by bilateral intra-IMHV application of the α2-antagonist rauwolscine, caused significant memory impairment. Moreover, *in vitro* quantitative receptor autoradiography revealed significant bilateral upregulation of [^{3}H]rauwolscine binding sites (B_{max}) in both dorsal and ventral compartments of HV, following one-trial avoidance learning. Taken together the behavioural and autoradiographic data suggest that activation of α2 postsynaptic adrenoceptors in HV is essential for memory formation in the early and intermediate stages of aversive learning.

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