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Effects of intra-hippocampal D-AP5 injections on one trial passive avoidance learning in adult laying hens (*Gallus gallus domesticus*)

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Domestic chickens are an established model organism for studies on learning and memory. Commonly, the chicks are used as subjects in several different learning tests, including one trial learning tests. However, for adult laying hens no such one trial learning tests have been established. In particular, there is no test established which focuses on the role of the hippocampus, a brain region, which is often critically involved in learning and memory consolidation. In this study we investigated the inhibitory effects of intra-hippocampal D-AP5 injections on a specific one trial passive avoidance learning test in adult laying hens (*Gallus gallus domesticus*). We used a step down avoidance learning paradigm (SDA) which is frequently used in rodents. Intra-hippocampal injections of D-AP5 impaired the learning abilities of adult laying hens compared to sham-injected control subjects. Thus, the experiments revealed that the hippocampus is critically involved in learning the inhibitory SDA task. Our results further indicate that the step down avoidance paradigm is suitable to examine learning and memory processes in adult laying hens.

Key words: hippocampus, step down avoidance learning inhibition, long-term memory, behavior, passive avoidance, chickens, APV

INTRODUCTION

Passive avoidance learning tests provide established tasks in research of learning and memory. These tests often involve specific brain areas such as the hippocampal region. Especially for laboratory rodents a number of different one trial avoidance tests are used for studying learning processes (e.g. Izquierdo et al. 1999, Jafari-Sabet 2006). To test the involvement of specific brain regions in behavioral tests, drug applications are a common tool to inhibit synaptic responses. One of these drugs is D-AP5 (D-(-)-2-Amino-5-phosphonopentanic acid) (e.g. Chen and Wyllie 2006, Ng et al. 1997) and inhibitory effects of D-AP5 injections on learning and memory are well investigated (e.g. Morris et al. 1986, Roesler et al. 1998, Steele and Morris 1999). D-AP5 represents an N-methyl-D-aspartate (NMDA) receptor antagonist which blocks the ionotropic glutamate NMDA receptor (NMDA-R). NMDA-receptors are also located in the hippocampus and are important for consolidation of information into long-term memory (e.g. Steele and Morris 1999), as shown, for instance, in rodents (e.g. Eichenbaum et al. 1992, Izquierdo and Medina 1997). Thus, the hippocampus can be considered to be a critical component of a functional system in long-term memory formation.

The domestic chicken is a widely used model organism in research of learning and memory processes (e.g. Rose, 2000). Especially in young chickens several tests have been used to investigate learning and memory tasks (e.g. Andrew 1991, Rogers 1995). Many tests, such as maze learning (Krause et al. 2006), require a prolonged training period. This prolonged training is not required in one trial learning tests, such as taste

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aversion learning (e.g. Rose and Stewart 1999, Nikolakopoulou et al. 2006). The advantage of these one trial learning tests is that the incident of learning can be determined with high precision. Such one trial passive avoidance learning tests have been established predominantly for young chickens (Rose and Stewart 1999, Edwards and Rickard 2005). Yet, no appropriate one trial learning test has been established for adult laying hens which otherwise have provided an important model system for studying learning processes (Krause et al. 2006, Nicol 2006).

The avian hippocampus is assumed to be homologous in morphology to the mammalian hippocampus (Atoji and Wild 2006). Also its functions are similar to those in mammals. Therefore it can be readily assumed that the hippocampus in adult laying hens is involved in learning a one trial passive avoidance task, such as the SDA, as known from mammals (Pawlak et al. 2002).

Here, we tested whether adult laying hens are capable to learn a one trial learning task (SDA) and whether the hippocampus is critically involved in such learning. We predicted that bilaterally intra-hippocampal D-AP5 administration inhibits learning. Sham-injections, in contrast, should have no such effects.

METHODS

Subjects and housing

We used 96 adult laying hens (Lohmann Selected Leghorn), 30 to 40 weeks old, as subjects. Laying hens are regarded as adult from the age of 20 weeks on (Jensen 2006). Prior the experiments, subjects were kept in standard litter floor compartments with commercial food and water provided *ad libitum*. From two to three days before the experiments, subjects were transferred into single cages (35 × 65 × 70 cm) and placed in an adjacent compartment with identical conditions. The research was carried out according to the German laws for experimentation with animals and permission to conduct the experiments was granted by the local authority (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit – LAVES).

Experimental procedures

The SDA apparatus consisted of an experimental platform (50 cm × 30 cm) which was placed 3 cm above a grid (50 cm × 70 cm) and was surrounded by an acrylic glass barrier which was covered with wire mesh (Fig. 1). We observed the subjects directly *via* a monitor and recorded all experiments on video. Foot shocks (duration 1 s; 920 V 50 Hz; max 1.98 mA) were controlled using an oscilloscope and could be triggered by the observer once the subjects had stepped onto the grid. The SDA test was performed in two stages: In a training trial subjects were placed on the platform. When subjects of the experimental group stepped on the grid, a foot shock was triggered, whereas no such shock was given for subjects from the control groups

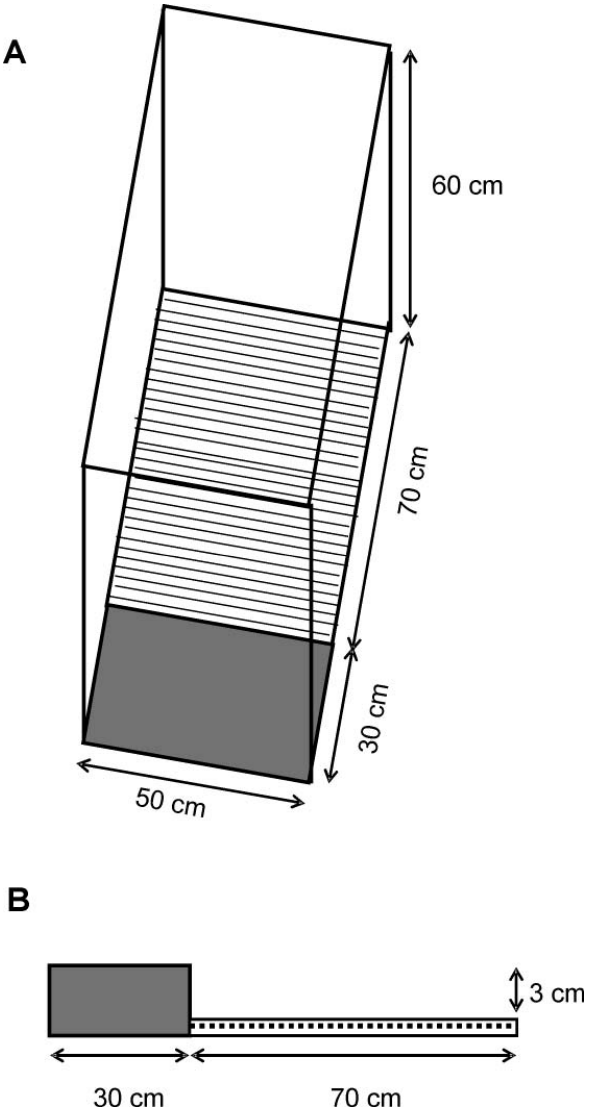

Fig. 1. Schematic drawing of the step down avoidance test (SDA) (a) top view and (b) profile. The SDA apparatus consisted of a platform (grey areas), a grid and was surrounded by an acrylic glass barrier which was covered with wire mesh.

(Table I). Immediately after these trials, subjects were transferred back into single cages. The test trial was performed 24 hours after the training trial and was identical to the training trial but without a foot shock. In case of successful learning, memory storage and retrieval, we expected experimental subjects in the test trials to leave the platform significant later than control subjects (e.g. Roy and Chapillon 2004). In order to standardize the exploration period on the platform, subjects that did not leave the platform within 450 s in the training trial were excluded from the experiments. Test trials were terminated when subject remained 900 s on the platform. All experiments were conducted between June and August 2006 in Celle, Germany.

Fifteen minutes prior to the training trial, subjects from the injection treatment groups A, B, and C (Table I) received an injection of either D-AP5 or ACF (Roesler et al. 1998). In total 72 subjects were successfully tested. Subjects from group A (n_A=12) received a D-AP5 injections. The sham injected subjects in group B (n_B=15) and group C (n_C=15) received an ACF injection to control for effects of anesthesia, surgery and injection on behavioral performance (Table I). Subjects from group D (n_D=15) and group E (n_E=15) represent non-operated control groups (Table I). Twenty-two additional subjects were excluded from then experiment and the analysis because of an exceedingly high latency to leave the platform in the training trial. Two more subjects were excluded because the cannulas were not placed correctly.

Table I

Treatment group overview

	injection treatment groups			non operated treatment groups	
	A	B	C	D	E
n	12	15	15	15	15
Footshock	yes	yes	no	yes	no
Injection	D-AP5	Sham	Sham	none	none

Subjects from groups A, B, and C received an injection before the training trial. For sham injections ACF was used. Subjects of groups A were expected not to learn the SDA because of the inhibitory effects of the D-AP5 administration. The sham injected subjects in group B and C represented a control for effects of anesthesia, surgery and injection procedure. Subjects of the non-operated group D were expected to learn the SDA task, whereas subjects in group E should not learn the SDA. See text for details.

Surgery procedure and drug application

Prior to injections, subjects from the injection treatment groups (A, B, and C; Table I) were anaesthetized (Midazolamchloride, 4.5 mg/kg body weight, Midazolam-Ratiopharm, Germany; Medetomidin, Dormitor, Pfizer, USA; 0.35 mg/kg; Fentanylcitrate, 6 µg/kg, Fentanyl – Ratiopharm, Germany) and then fixed in a stereotaxic frame (FMI-Föhr Medical Instruments, Seeheim Ober-Beerbach, Germany). We implanted a double guide cannula (Plastics One, Virginia, USA), which ended intra-hippocampally (head angle −12 mm; 1.0 mm posterior to the lambda, bilaterally 2.5 lateral the sagittal suture) (Fig. 2a). Cannulas were fixed using dental cement (Stoelting, USA). Subsequently, subjects were transferred into single cages and were allowed to recover for 24 to 36 hours. Subjects from the non-operated treatment groups D and E (Table I) were handled identically except that they received no surgery. When experiments were completed, subjects were sacrificed and brains were extracted and frozen at −80°C. Histological slices with a thickness of 20 µm were stained using haematoxylin. We controlled that intra-hippocampal injections were placed correctly (Fig. 2b, c).

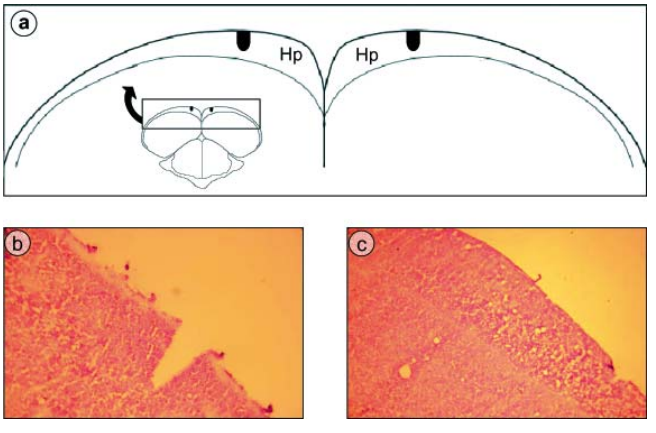

Fig. 2. Schematic drawing of the brain of an adult laying hen. (a) The areas where cannulas were implanted and intra-hippocampal injections took place are indicated by the black cylinders. (b) Histological slice of the hippocampal region with a lesion from intra-hippocampal injection; (c) histological slice of the hippocampal region with no implantations. (Hp) Hippocampus.

Drugs injected were D-AP5 (Tocris, Missouri, USA) and ACF (artificial cerebrospinal fluid; Harvard apparatus, Massachusetts, USA). For drug injection subjects received a short term anesthesia with Isoflurane (Isoflo, Abbott, Quennsborough Kent, GB). A volume of 0.25 µl with 25 nmol of D-AP5 (e.g. Bock et al. 1996, Bock and Braun 1999) per hippocampal side was injected (group A; Table I) after dissolving in ACF, and gently warmed in a water bath at 55°C. As a neutral control, we applied ACF as sham injection (e.g. Manteuffel et al. 2002). Gently warmed ACF were injected into both hippocampal sides (groups B, C; Table I), in the same volumes as D-AP5. All Injections were performed over a 60 s period. Thereafter, the double injection cannula was kept in the tissue for at least 30 s. Intra-hippocampal injections were made using a micro dialysis pump (Harvard Apparatus, Massachusetts, USA) and Hamilton micro liter syringes. Only one dose of D-AP5 was used, because our objective was to examine in general if the hippocampus is involved in learning this task. Dose-dependent learning inhibition to D-AP5 administration in adult laying hens needs to be investigated in further studies.

Data collection and statistical analysis

In each trial we measured (1) the latency to leave the platform as indicator of learning and (2) the number of calls as an indicator for arousal (Krause et al. 2006). Normal distribution of the residuals was tested using the Kolomogorov–Smirnov test with Lilliefors correction (KS-L test). As the data were not normally distributed, we used square route transformation. After transformation, the latencies in the injection groups did not deviate significantly from a normal distribution in test trials (KS-L for test trials P=0.11) and marginally deviated from a normal distribution in the training trials (KS-L test for training trials P=0.04). However, the visual inspection of the Q-Q-plots revealed no systematic deviations from normal distribution. In addition, as the criteria for homogeneity of variances of latencies of the injection treatment groups in both, the training and the test, trials were met (Levene's test , all P>0.05), we decided to use an one-way ANOVA to test differences between groups in training and in test trials, and therefore have the opportunity to use adequate *post-hoc* tests. ANOVA's are relatively robust against mild violations of the normal distribution criteria as given in our data (Sokal and Rohlf 1995, Quinn and Keough 2002). However, results of the one-way ANOVA's did not differ from the non-parametric Kruskall-Wallis test. Latencies from non-operated treatment groups, even after square route transformation, differed strongly from normal distribution (KS-L, all P<0.008) and also did not reach a homogeneity in variances. We therefore analyzed latencies in the non operated treatment group with Mann-Whitney *U*-tests. Differences between groups A, B, C in the injection treatment were analysed with least-significant differences (LSD) *post-hoc* tests after the one-way ANOVA.

Afterwards, for each group (A to E) we tested whether the subjects' latencies were increased in the test trial compared to the training trial [paired *t*-test for parametric data (A, B, C) or Wilcoxon test for non parametric data (D, E)].

Vocalizations were analyzed for the injection treatment groups (A, B, C) and the non operated treatment groups (D, E) using Mann-Whitney *U*-tests to compare groups in the training and the test trials with each other. Thereafter for each group a Wilcoxon test was done to compare vocalization in the training trial with vocalization in the test trials. All tests were performed two-tailed. Statistical analysis was done using SPSS 15.0.1.

RESULTS

Injection treatment (groups A, B, C)

Latencies from subjects in the three injection treatment groups did not differ significantly in the training trials (one-way ANOVA, $F_{2,41}$=2.23, P=0.12; Fig. 3). Post-hoc test reveal no significant difference between any of the three groups (A vs. B; A vs. C, B vs. C, LSD-*post hoc* tests: all P>0.05).

Latencies from subjects of the injection groups differed significantly in the test trials (one-way ANOVA, $F_{2,41}$=10.91, P=0.0002; Fig. 3). *Post-hoc* comparisons showed that subjects from group A had significantly shorter latencies in the test trials than subjects from group B (A<B, LSD-*post hoc* test, P=0.025). Subjects from group C also had significantly shorter latencies in the test trial than subject from group B (C<B, LSD-*post-hoc* test, P<0.001). Also groups A and C differed in their latencies in the test trial (LSD-*post hoc* test, P=0.045).

Latencies from subject from group A and C were not significantly increased in the test trials compared to the training trials (paired *t*-tests, both P>0.15; Fig. 3). Subjects from group B had a significantly

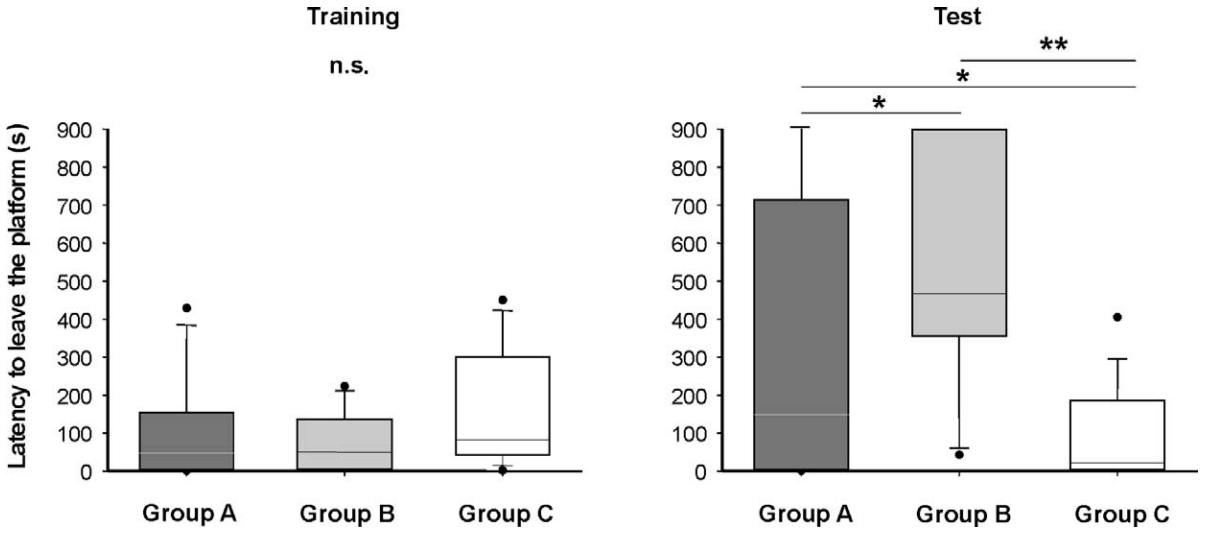

Fig 3. Latencies (median, quartile, range) to leave the platform in the training trial and the test trial for the injection-treatment. Group A – D-AP5 Injection with foot-shock; Group B – Sham Injection (ACF) with foot-shock; Group C – Sham Injection (ACF) no foot-shock. See text for details.

higher latency in the test trials compared to the training trials (paired *t*-test, $t=-5.9$, $P<0.0001$; Fig. 3)

Non operated treatment (groups D, E)

Latencies in the non operated groups did not differ significantly in the training trial between group D and group E (Mann-Whitney *U*-test, $n=30$, $Z=-1.67$, $P=0.095$, Fig. 4). Latencies between groups D an E differed significantly in the test trials. Subjects from group D had significant higher latencies in the test trials compared to subjects of group E (Mann-Whitney *U*-test, $n=30$, $Z=-2.41$, $P=0.016$; Fig. 4).

Latencies from subjects from group D were significantly increased in the test trial compared the training trial (Wilcoxon match-pair test, $n=15$, $Z=-3.35$, $P=0.001$, Fig. 4). Latencies from subjects from group E had no significant increase in latencies in the test trial compared to training trials (Wilcoxon match-pair test, $n=15$, $Z=-1.36$, $P=0.17$, Fig. 4).

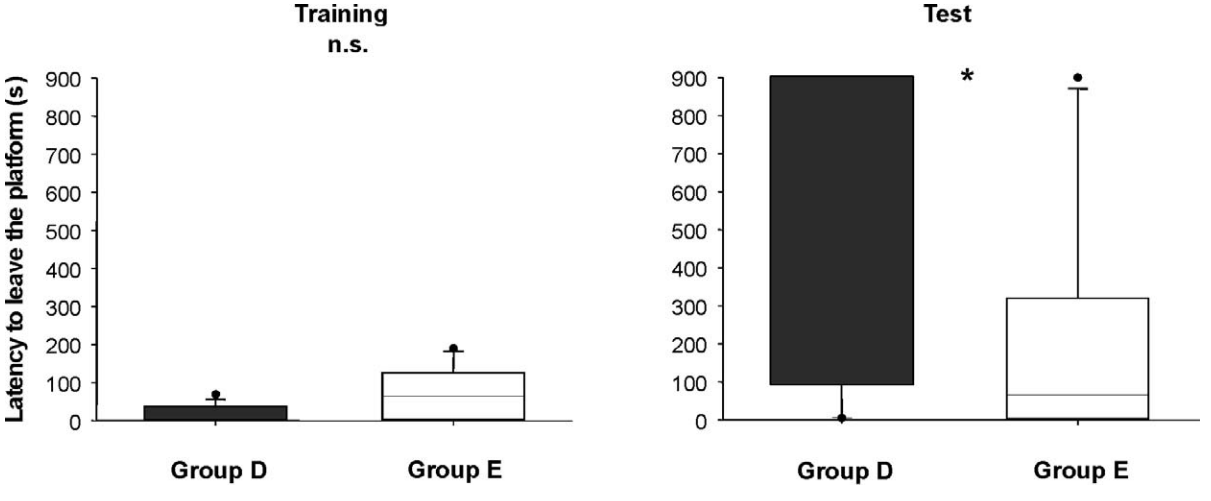

Fig. 4. Latencies to leave the platform in the training trial and the test trial for non operated treatment. Group D – with foot-shock; Group E (no foot-shock). Medians (midlines), interquartile ranges (boxes) and full ranges (vertical lines) are shown in the box plot. See text for details.

Table II

Results for vocalization from the injection treatment and the non operated treatment groups

		injection Treatment			non operated Treatment	
Groups:		A	B	C	D	E
		Vocalization			Vocalization	
Training trial	Median	0.5	0	1	0	1
	1st Quart.	0	0	0	0	0
	3rd Quart.	2.25	0.5	2	2.5	3.5
Test trial	Median	1.5	8	0	11	0
	1st Quart.	0	1.5	0	2.5	0
	3rd Quart.	4.5	21	1.5	82.5	4
Training vs. Test	*P*	0.18	0.002	0.16	0.0015	0.97
Wilcoxon test	*Z*	−1.34	−3.06	−1.40	−3.18	−0.04
	n	8	13	11	13	14

Sample sizes differ due to ties

The additional behavioral data support the results of the latencies to leave the platform. The injection treatment groups A, B and C did not differ in the number of calls in the training trials (Mann-Whitney *U*-tests, all $Z>-1.8$, all $P>0.07$). In the test trials subjects group A tended to vocalize less than subjects from group B (Mann-Whitney *U*-test, $n_A=12$, $n_B=15$, $Z=-19.5$, $P=0.052$), and subjects from group C vocalized significantly less than subjects from group B (Mann-Whitney *U*-test, *n* C=15, $n_B=15$, $Z=-3.1$, $P=0.002$). Groups A and C did not differ in the number of calls in the test trial (Mann-Whitney *U*-test, $n_A=12$, $n_C=15$, $Z=-1.2$, $P=0.23$). The comparison for each group between training and test trial are shown in Table II. In non operated groups D and E the number of calls in subjects were not different from each other in the training trials (Mann-Whitney *U*-test, $n_D=15$, $n_E=15$, $Z=-0.66$, $P=0.51$). In the test trials, subjects from group D vocalized significantly more than subjects from group E (Mann-Whitney *U*-test, $n_D=15$, $n_E=15$, $Z=-2.8$, $P=0.005$). The comparison for each group between training and test trial are shown in Table II.

DISCUSSION

The results reveal that in adult laying hens intra-hippocampal injections of D-AP5 impair the learning performance in the step down avoidance test (SDA test). Sham-injected and non-operated subjects learned the SDA task. Hence the surgery procedures had no noticeable effect on learning performance.

The finding that in the test trials the latencies of group A and group C both differed significantly from group B is a remarkable indicator that intra-hippocampal injections of D-AP5 have impairing effects on one trial learning in adult hens. That the latencies from subjects of group A are not significantly increased in the test trial compared to the training trial, as in group C and group E, reveals our assumption that D-AP5 injections impaired the hippocampus-dependent learning. The finding that the latencies of groups A and C differed from each other in the test trial was not as expected prior the experiment. This may suggest that D-AP5 injections in the hippocampal region of group A, may have more effects on adult hen's behavior addi-

tionally to the impairing effects on the learning performance. What these other effects may be or where they result from remains unclear and could be addressed in further studies. Nevertheless, intra-hippocampal D-AP5 injection can be assumed to have impairing effects on learning in adult laying hens. The experiments provide strong evidence that the hippocampus is critically involved in one trial avoidance learning in adult laying hens.

In day old chickens it had already be shown that the intra-hippocampal injection of D-AP5 blocks the NMDA-R (N-methyl-D-aspartate Receptor) (e.g. Steele and Morris 1999, Rose 2000). As a consequence, retrieval of learnt contents from long term memory is inhibited by the repressive effects of D-AP5 in the hippocampus. The same inhibitory effects of intra-hippocampal D-AP5 injections have also been shown in mammals in similar one trial passive avoidance tasks (e.g. Cammarota et al. 2000, Pawlak et al. 2002). Thus, our experiments provide further evidence that the underlying principles of learning one trial passive avoidance task are similar in birds and mammals. This conclusion also receives support from the homologous morphology of the hippocampal structures of birds and mammals (Atoji and Wild 2006).

Therefore, the SDA test offers a learning test for adult laying hens, which is expected to be relevant for further research on learning and memory. The SDA might be an appropriate tool for neurobiological and pharmacological research on learning. It could be useful in comparative studies on learning and memory in birds and mammals (Cammarota et al. 2000, Izquierdo et al. 2000). The SDA further offers the opportunity to test hypotheses in a more applied context of neurobiological and behavioral research such as testing qualities of housing systems and enrichment in adult hens. In mice it is known that different housing conditions and different levels of enrichment have profound effects on neuronal proliferation and learning performance (e.g. Kempermann et al. 1997, van Praag et al. 1999). Similar effects are known from chickens where even short-term environmental enrichment during ontogeny leads to an improved learning ability (Krause et al. 2006).

CONCLUSION

We found that one trail passive avoidance learning tasks can be learned by adult laying hens and critically involves the hippocampus. Our data give further evidence that learning a similar task involves similar brain regions in birds and mammals.

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