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Prostaglandins play a role in memory consolidation in the chick

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Abstract

Previous studies showed that inhibitors of cyclooxygenases have amnesic effects in chicks in a passive avoidance task. The onset of amnesia has a delay of 2 h post-training. To investigate if this effect is due to the inhibition of induction of the enzyme during learning, the release of cyclooxygenase products into the extracellular fluid was measured at 1, 2 and 3 h post-training. A cyclooxygenase inhibitor, ibuprofen, inhibited the training-dependent increase of cyclooxygenase products only 2 h and 3 h after learning when injected pre-training, as did dexamethasone which prevents cyclooxygenase induction, and SC58125 (1,2-diarylcyclopentene), an inhibitor of inducible cyclooxygenase. Injections 30 min post-training showed the same effect with the exception of dexamethasone. Injecting SC58125, ibuprofen, indomethacin, or dexamethasone i.c. before training showed amnesic effects for training on a one-trial passive avoidance task at 2 h but not 1 h after training. Injections 30 min post-training produced the same effects with the exception of dexamethasone. I conclude that cyclooxygenases are induced during training and that cyclooxygenase products are of importance in memory formation of the chick.

Keywords: Learning; Memory; Prostaglandin; Cyclooxygenase; Neurotransmitter; Retrograde messenger; Synaptic plasticity

1. Introduction

Memory formation is assumed to involve the modulation of synaptic connectivity as a result of coordinated pre- and postsynaptic events. According to this theory presynaptic glutamate release triggers up-regulation of the postsynaptic NMDA receptor, enhances Ca^{2+} flux and the production of a postsynaptic factor called a retrograde messenger, which then feeds back to the presynaptic side as the signal initiating an increase of transmitter release and subsequent synaptic reconstruction (see Bailey and Kandel, 1993, for a discussion). Most recently nitric oxide has been the prime candidate as such a retrograde messenger (Snyder, 1992; Bon et al., 1992). An earlier suggestion was that arachidonic acid might serve in this context, and its role in long-term potentiation has been extensively studied (Bliss et al., 1990; Bliss and Collingridge, 1993).

We have previously shown that nitric oxide fulfills some of the criteria for a retrograde messenger in a one trial passive avoidance learning task in the day-old chick. Injections of the nitric synthase inhibitors before training resulted in amnesia for the avoidance task (Hölscher and Rose, 1992, 1993; Hölscher, 1995b). From there, we went on to investigate the role of arachidonic acid. In the same passive avoidance task, blockade of phospholipase A_2 dependent arachidonic acid release produced amnesic effects (Hölscher and Rose, 1994). High performance liquid chromatography measurements of the release of arachidonic acid in chick brain slices after training showed an increase of release (Clements and Rose, 1994). Therefore, we assume that arachidonic acid plays an important role in learning and memory formation and in synaptic plasticity.

Since arachidonic acid is a precursor for many endogenous transmitter molecules, we investigated the role of enzymes which metabolize arachidonic acid in learning and memory consolidation. Recently, evidence has emerged that cyclooxygenases are expressed in neurons (Yamagata et al., 1993; Breder et al., 1995). These enzymes synthesize arachidonic acid metabolites such as prostaglandins and prostacyclins. Cyclooxygenase activity appears to be of importance in synaptic

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plastic changes that are related to memory formation. In one study inhibition of cyclooxygenases prevented the decreased release of glutamate that accompanies long-term depression in the cerebellar slice (Dickie et al., 1994). If cyclooxygenase products play a role as retrograde messengers, a change of transmitter release dependent on cyclooxygenase-activity-dependent way is to be expected. Furthermore, products of cyclooxygenases should be released by neurons into the extracellular medium to act as retrograde messengers. Indeed, an in vitro dialysis study showed an increase in the release of prostaglandins after stimulation of the *N*-methyl-D-aspartate glutamate receptor in the hippocampus (Lazarewicz and Salinska, 1995).

To test if cyclooxygenase activity is of importance for learning, drugs that inhibit these enzymes were tested in the passive avoidance task. Injection of cyclooxygenase inhibitors (ibuprofen, indomethacin, or naproxen) caused amnesia for the task. The onset of amnesia was 2 h after training, and no amnesic effects were visible before this time (Hölscher, 1995a). This delay of 2 h after training could be due to the induction of cyclooxygenases during training. Two isoforms of cyclooxygenases have been identified, a constitutive form (cyclooxygenase-1) and an inducible form (cyclooxygenase-2). Both isoforms have been isolated from neurons (Yamagata et al., 1993). The kinetics of cyclooxygenase-2 induction have been investigated in detail in various cell types. The cyclooxygenase inhibitor, salicylic acid, for example was found to block cyclooxygenase activity with a delay of 2 h by preventing de novo cyclooxygenase synthesis. Furthermore, cyclooxygenase-2 induction in fibroblasts by platelet-derived growth factor was detectable after 2 h (De Witt, 1991). A delay of cyclooxygenase-2 expression after induction by interleukin-1β was also found in A549 cells (Newman et al., 1994).

Therefore, I postulate that cyclooxygenases are induced by neuronal activity during learning and that their activity becomes essential for memory consolidation 2 h post-training. If products of cyclooxygenases play a role in memory consolidation at that time point, and if these products acts as retrograde messengers in neuronal communication, an increase of release of prostaglandins into the extracellular fluid is to be expected. I therefore measured the release of prostaglandins from brain tissue at different time points after injecting cyclooxygenase inhibitors pre- or post-training. The effect of the novel cyclooxygenase-2 inhibitor, SC58125 (Seibert et al., 1994), on prostaglandin release and on memory consolidation was tested. If cyclooxygenase-2 is responsible for the amnesic effects, a selective inhibitor should inhibit a learning-related increase of prostaglandin release and should also cause amnesic effects in a learning task. For the same reason the effect of dexamethasone on learning-related prostaglandin release and on learning and memory was tested. Dexamethasone prevents the induction of enzymes such as nitric oxide synthase or cyclooxygenase-2 (Kunz et al., 1994; Mitchell et al., 1994). To test if an amnesic effect observed after injection of dexamethasone or SC58125 follows the same time course as the effect of ibuprofen or indomethacin (Hölscher, 1995a), all four drugs were tested in the passive avoidance task at times 1 h or 2 h after training for direct comparison. If dexamethasone produces amnesic effects by inhibiting the induction of cyclooxygenase-2, it should not have an effect when injected post-training, because the drug does not inhibit the induction process when it is already started (Hall et al., 1994). All other drugs should inhibit cyclooxygenases when injected before or after training. Therefore, the effects of pre- or post-training drug injections were tested.

2. Materials and methods

2.1. Passive avoidance training

Ross Chunky chicks of both sexes, hatched in our own laboratory, were held in a communal incubator in a 12 h light/12 h dark cycle at 38–40°C until they were 26 ± 6 h old. As in our standard procedure (see Lössner and Rose, 1983), the animals were placed in pairs in $20 \times 20 \times 25$ cm aluminium pens each illuminated with a red 25 W light bulb and maintained at around 30°C. The chicks were allowed settle down in the experimental environment for about 1 h before pretraining with three presentations of a 2.5 mm diameter white bead mounted on a perspex rod. 10 min after the last pretraining trial each bird was presented for 10 s with a 4 mm chrome bead dipped in methylanthranilate. Birds which did not peck the white bead at least twice during the pretraining trials or did not show a clear disgust response (shaking the head) on the training trial with the chrome bead ($\sim 10\%$) were discarded from the experiment. At various times after training each bird was tested by a person blind to the treatment of the chicks, by a 15 s presentation of a dry chrome bead, and scored for pecking or avoidance. The normal response observed in trained chicks when tested is avoidance behavior by either shaking the head, backing actively away or (rarely) wiping the beak on the ground (~ 70–80%). Chicks which peck the bead on testing are regarded as amnesic for the training. Groups were compared using the χ^2 test.

2.2. Drugs and injection procedure

Ibuprofen was obtained from ICN (USA), indomethacin from Sigma (USA), and dexamethasone from Biomol (USA). Drugs were dissolved by sonica-

tion in 0.9% saline that contained 1% ethanol for injection. SC58125 (1,2-diarylcyclopentene) was a gift from Glaxo (UK). It was dissolved by sonication in warm saline which contained 1% ethanol shortly before injection. Solutions were prepared freshly every day.

For intracerebral injections, the animals were placed in a stereotaxic plexiglas headholder (Davis et al., 1979) which contains bore holes that guide the injection cannula along defined anterior and lateral coordinates and which allows fast and precise injections into the intermediate medial hyperstriatum ventrale, the forebrain region of the chick brain known to be the site of the molecular cascade of processes required for memory formation (Rose, 1991). Solutions were injected bilaterally into the forebrain using a Hamilton syringe fitted with a plastic sleeve as a stop for an injection depth of 4 mm. All injections were 5 μl in volume. Because of the young chick's soft unossified skull, such injections take only a few seconds for each bird, no anaesthesia is needed and chicks show no signs of distress. For the dose-response tests, solutions of the appropriate concentrations were prepared. Control birds were injected with 0.9% saline which contained 1% ethanol. Each chick was injected and tested only once. Injection sites were verified by injecting ink in 50% of the chicks used. Animals were killed by decapitation. Location of injection sites varied little and was in no case outside the area of the intermediate hyperstriatum ventrale.

2.3. Control experiment

To test if prostaglandin release is linked to the learning of an aversive stimulus and not to motor activity or other parameters, six chicks were trained and tested as described in the training scheme for the passive avoidance task. The chrome bead used to train chicks was not dipped in an aversive substance but in water. The animals received 5 μl of saline per hemisphere i.c. 30 min pre-training. Brain tissue samples were taken at 1, 2, and 3 h after training. Prostaglandin analysis was done as described below.

2.4. Training scheme

2.4.1. Prostaglandin measurements

Groups of chicks (n = 6 per group) were injected i.c. bilaterally with either drug (three groups) or with saline (one group) 30 min before training, trained, and tested for avoidance/peck at either 1, 2, or 3 h after training. At each time point, one saline and three drug groups were tested. The same training scheme was used for the prostaglandin measurements after post-training injections. Drugs or saline were injected 30 min post-training.

2.4.2. Behavioral pharmacology

Groups (n = 15–16 per group) were injected bilaterally with either drug or saline. For pretraining injections, the animals were injected i.c. 30 min before training, and post-training injections, the animals were injected bilaterally 30 min after training. All animals were tested at 3 h post-training.

2.5. Brain tissue incubation

The animals were beheaded after testing, and tissue samples from the left and right hemispheres were dissected out, using a resin brain mould to cut tissue slabs, followed by location of regions under a stereo microscope, as described previously (Rose and Csillag, 1985). Samples of the intermediate medial hyperstriatum ventrale were obtained, as described by Bullock et al. (1987). The samples were stored in 1 ml test tubes on ice.

Samples were cross-chopped on a McIlwain tissue chopper set at 350 μm. These tissue prisms were suspended in 1 ml of incubation buffer (Krebs-Henseleit buffer containing 118 mM NaCl, 1.3 mM $CaCl_2$, 1.2 $MgSO_4$, 25 mM $NaHCO_3$, and 11.7 mM glucose), gently stirred, filtered, and put into in 10 ml vials that contained fresh buffer (1 ml). The buffer was gassed 15 min before use with 95% O_2-5% CO_2 and equilibrated to pH 7.4. The prisms were incubated in a water bath at 37°C for 30 min. After incubation, buffer aliquots were taken and frozen at −20°C for further analysis. Tissue samples were frozen at −20°C for protein analysis, using the Bradford method (Bradford, 1976). Samples were homogenized in 1 ml buffer and pipetted into a microwell plate in triplicate (three wells per sample), a Commassie brilliant blue solution in ethanol and H_3PO_4 (10 mg/5 ml/10 ml) was added. Samples were read at 595 nm on a Titretek plate reader. Absorbance of samples was compared to that of bovine serum albumin protein standards.

2.6. Analysis of prostaglandin concentrations

Aliquots of samples were pipetted into a precoated ELISA microwell plate obtained from Cascade (UK). Aliquots were pipetted in triplicate. Intra-assay variation was less than 10%. Averages of readings of triplicates were taken. Each plate had its own standards against which samples were measured to normalize for variation between plates. The cross-reactivity of the antibody used was: prostaglandin E_2, A_1, A_2, B_1, B_2, E_2 = 100%, prostaglandin $F_{1\alpha}$ 10%, 6-keto-protaglandin E_1 80%, prostaglandin D_2 0.26%, 11β-protaglandin $F_{2\alpha}$ 0.1%, Thromboxans < 0.01%. The sensitivity of the assay was up to 0.1 ng/ml. A solution of prostaglandin E_2 horseradish peroxidase conjugate which competes with prostaglandins for antibody bind-

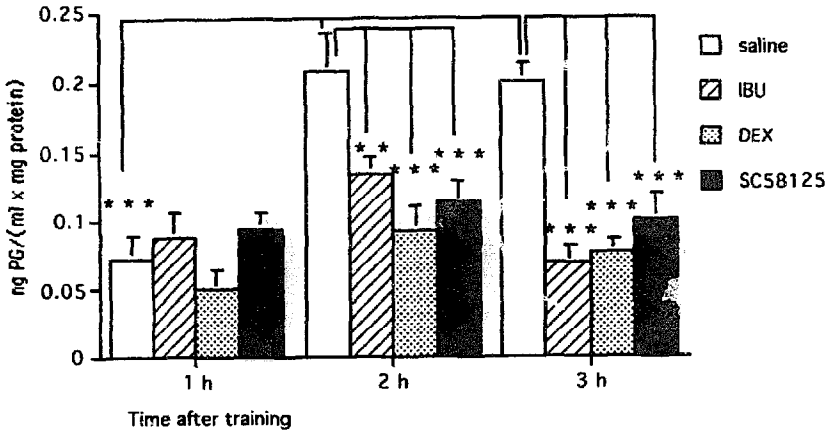

Fig. 1. Pre-training drug injections: release of prostaglandins (ng PG/ml per mg protein) from chick brain tissue slices taken after training on a passive avoidance task. Ibuprofen (5 μl/hemisphere of a 300 μM solution), or dexamethasone (5 μl/hemisphere of a 20 μM solution), or the cyclooxygenase-2 inhibitor, SC58125 (5 μl/hemisphere of a 500 μM solution), were injected 30 min pre-training, samples were taken 1, 2, or 3 h post-training. Samples were taken from six groups ($n = 6$ per group). See Results section for details. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

ing sites was added (50 μl/well). The plate was incubated at room temperature for 1 h. The plate was emptied then and washed with 200 μl of washing buffer 3 times (Na_2HPO_4 15 mM, KH_2PO_4 3.5 mM, NaCl 150 mM, TWEEN 20 0.05%); 150 μl of dye substrate (5,5'-tetramethylbenzidine plus H_2O_2) was added. After 30 min at room temperature, the reaction was stopped with 1 M HCl (100 μl/well). The plate was read at 450 nm, absorbance was calibrated against a prostaglandin E_2 standard, and prostaglandin release was expressed in ng/mg protein, using the results of the Bradford (1976) protein analysis.

2.7. Statistical analysis

Results were analyzed by a one-way analysis of variance (ANOVA) assuming normal distribution of the data (Bland, 1987). Bartlett's test for homogeneity of variance showed that data columns were from populations with equal standard deviations. A Tukey-Kramer Multiple Comparison Test was used post-hoc. A P value of less than 0.05 was considered statistically significant. The data from behavioral experiments were analyzed with the χ^2 test, assuming that each peck was an independent event.

3. Results

3.1. Pre-training injections

When the release of prostaglandins was measured 1 h post-training, test and saline groups showed a very low level of prostaglandins, at the limit of the detection by the ELISA test employed. At the 2 h time point, however, an increase of prostaglandins release in the

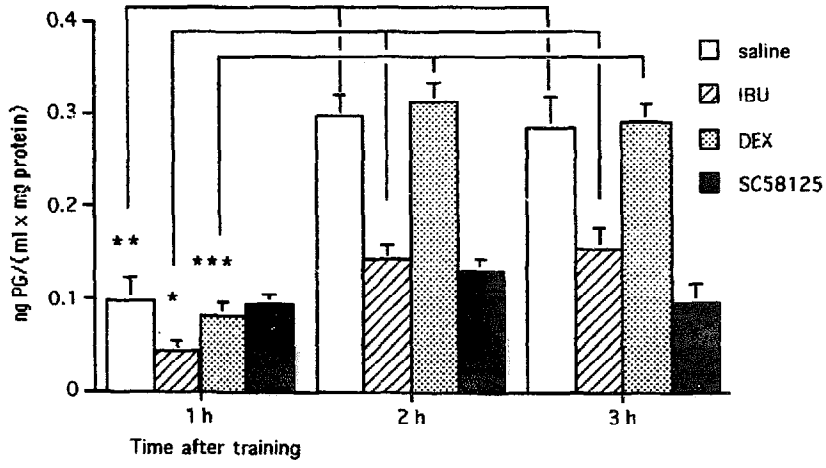

Fig. 2. Post-training drug injections: release of prostaglandins (ng PG/ml per mg protein) from chick brain tissue slices taken after training on a passive avoidance task. Ibuprofen (5 μl/hemisphere of a 300 μM solution), or dexamethasone (5 μl/hemisphere of a 20 μM solution), or the cyclooxygenase-2 inhibitor, SC58125 (5 μl/hemisphere of a 500 μM solution) were injected 30 min post-training, samples were taken 1, 2, or 3 h post-training. Samples were taken from six groups ($n = 6$ per group). See Results section for details. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

saline group was visible. Neither ibuprofen (5 μl/hemisphere of a 300 μM solution), dexamethasone (5 μl/hemisphere of a 20 μM solution), nor SC58125 (5 μl/hemisphere of a 500 μM solution) injection produced an increase of prostaglandin release 2 h or 3 h after training.

When looking at the differences within groups of each time point, at the 2 h time point, all drug groups showed less prostaglandin release than the saline group. The same was observed at the 3 h time point (Fig. 1).

3.2. Post-training injections

Injection of saline or drugs 30 min after training had a different effect. At the 1 h time point, again very little prostaglandin was released in all groups. Neither ibuprofen (5 μl/hemisphere of a 300 μM solution), dexamethasone (5 μl/hemisphere of a 20 μM solution), nor SC58125 (5 μl/hemisphere of a 500 μM solution) injection produced a difference in release compared to the saline group. At the 2 h time point, prostaglandin release increased in the saline and dexamethasone groups compared to the 1 h values. This increase remained visible at the 3 h time point. Ibuprofen and SC58125 abolished the increase of release 2 h after training.

Looking at the differences within groups of the 2 h and 3 h time point, the saline and dexamethasone groups were not different from each other, while a significant difference was measured between the saline and ibuprofen or SC58125 groups (Fig. 2).

3.3. Control experiment

Training saline injected chicks (5 μl/hemisphere i.c.) to peck a bead that was dipped in water and not in an aversive substance did not lead to an increase of prostaglandin after training. Samples taken at 1, 2, and 3 h showed release between 0.02 and 0.05 ng prostaglandin/ml per mg protein at any time point measured. There was no increase of prostaglandins release after 2 or 3 h as seen in Figs. 1 and 2 was seen (data not shown).

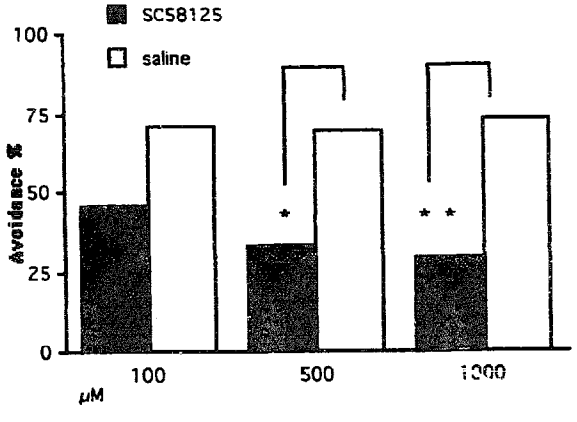

Fig. 3. Dose-response curve of the effect of the cyclooxygenase-2 inhibitor, SC58125, on memory consolidation in the chick. Injection of 5 μl/hemisphere containing 100, 500, or 1000 μM 30 min after training; testing was 3 h after training. $^{*}P < 0.05$, $^{**}P < 0.02$.

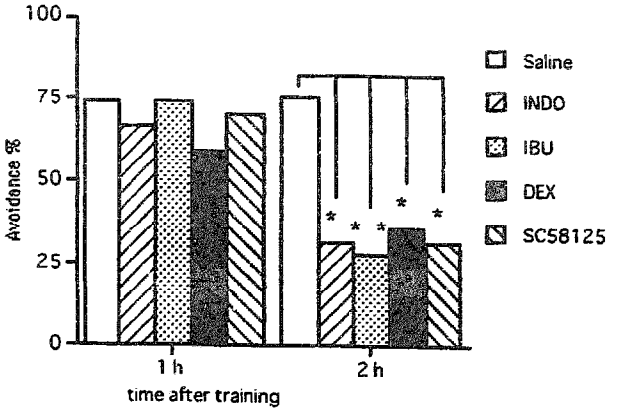

Fig. 4. Effect of cyclooxygenase inhibitors on memory consolidation in the chick: pre-training drug injection. Injection of 5 μl/hemisphere 30 min before training ($n = 15$–16 per group) of the cyclooxygenase-1 and cyclooxygenase-2 inhibitors, indomethacin (500 μM) or ibuprofen (300 μM), or dexamethasone (20 μM), or the cyclooxygenase-2 inhibitor, SC58125 (500 μM). Testing was at 1 h or at 2 h after training. $^{*}P < 0.05$, $^{**}P < 0.01$.

3.4. Behavioral pharmacology

Injecting the cyclooxygenase-2 inhibitor SC58125, (5 μl/hemisphere of 500 or 1000 μM), before training produced amnesic effects for the task 3 h post-training. The lower dose of 5 μl per hemisphere of a 100 μM solution did not produce a significant difference in performance between the drug and saline groups (Fig. 3).

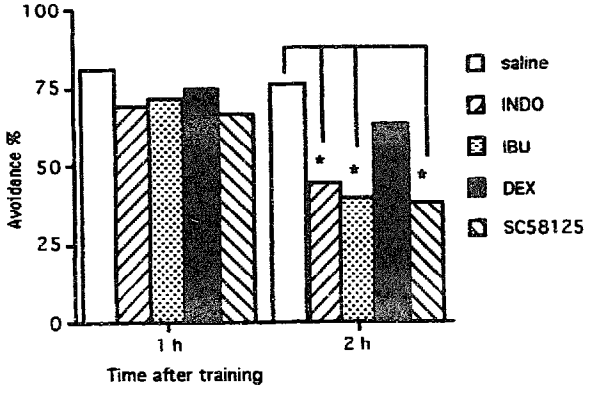

Fig. 5. Effect of cyclooxygenase inhibitors on memory consolidation in the chick: post-training drug injection. Injection of 5 μl/hemisphere 30 min after training ($n = 15$ per group) of the cyclooxygenase-1 and cyclooxygenase-2 inhibitors, indomethacin (500 μM) or ibuprofen (300 μM), or dexamethasone (20 μM), or the cyclooxygenase-2 inhibitor, SC58125 (500 μM). Testing was at 1 h or at 2 h after training. $^{*}P < 0.05$.

Injecting the cyclooxygenase-1 and cyclooxygenase-2 inhibitors, ibuprofen (5 μl/hemisphere of a 300 μM solution) or indomethacin (5 μl/hemisphere of a 500 μM solution), or the cyclooxygenase-2 inhibitor SC58125, (5 μl/hemisphere of a 500 μM solution), or dexamethasone (5 μl/hemisphere of a 20 μM solution) before training produced amnesic effects in chicks (Fig. 4). Injecting these drugs after training produced amnesic effects except in the dexamethasone group (Fig. 5).

4. Discussion

The data are in agreement with the results of previous behavioral pharmacological studies on the effect of non-specific cyclooxygenase inhibitors on memory consolidation. Injection of cyclooxygenase inhibitors pretraining produces amnesic effects 2 h but not 1.75 h after training (Hölscher, 1995a). The learning-related increase of release of prostaglandins observed in the saline group followed the same time course, i.e. a large increase of release after 2 h post-training compared to 1 h post-training values. Injecting ibuprofen, dexamethasone, or SC58125 before training, however, prevents this training-related increase of prostaglandin release. Injecting these four drugs before training also prevented memory consolidation of the passive avoidance task. I assume that the amnesic effect of dexamethasone is due to the prevention of induction of cyclooxygenase-2. Glucocorticoids have several modes of activity and also inhibit the induction of other enzymes e.g. nitric oxide synthase (Kunz et al., 1994; Mitchell et al., 1994). Also, in behavioral experiments with chick or rats, glucocorticoids are able to enhance memory formation. This is dependent on the dose: enhancement of memory consolidation in the chick was observed on injection of low doses (0.1–1 μg) of corticosterone while higher doses (2 μg) do not have an effect (Sandi and Rose, 1994). The same observation was made in in vitro studies of hippocampal neurotransmission. Low doses of corticosterone (0.5 nM) enhance long-term potentiation while a higher dose (5 μM) reduces it (Rey et al., 1994). This dose dependence suggests that the different effects of glucocorticoids have different sensitivities. It is to be assumed that the memory-enhancing low doses do not inhibit the induction of cyclooxygenase-2. In the study presented here the doses were very high and did not enhance memory formation. The time course of memory impairment, which was the same as for known cyclooxygenase inhibitors, and the ability of dexamethasone to prevent prostaglandin release suggest that the amnesic effect seen in the present study was primarily the result of inhibition of cyclooxygenase-2 induction.

On post-training injection of drugs, the selective cyclooxygenase-2 inhibitor, SC58125, prevented memory formation, supporting the theory that cyclooxygenase-2 is induced during training in the chick. Ibuprofen and indomethacin prevented memory formation when injected post-training as well. This confirms the result obtained with SC58125, since these drugs are not selective for cyclooxygenase subtypes and inhibit the inducible isoform as well. Dexamethasone did not prevent memory formation in the behavioral task nor did it prevent the increase of prostaglandins release 2 h post-training. Dexamethasone does not inhibit cyclooxygenase itself but only its induction, and once the induction process is activated it cannot be stopped by dexamethasone (Hall et al., 1994). This supports the hypothesis that learning induces cyclooxygenase expression which becomes of importance 2 h post-training. The injection of saline and the training of chicks on a bead dipped in water in the control experiment led to no increase in prostaglandin release. This shows that the increase was not due to the injection procedure, or to motor activity such as pecking the bead.

The results suggest that prostaglandins play a role in learning and memory formation of the chick. As has been shown before, nitric oxide (Hölscher and Rose, 1992; Hölscher 1995b) as well as arachidonic acid (Hölscher and Rose, 1994; Clements and Rose, 1994) play a role in learning and memory formation in the chick. The time courses of activation of the enzymes which synthesize the messengers are different: while nitric oxide synthase inhibitors act very quickly when injected pretraining, inhibitors of the arachidonic acid releasing enzyme, phospholipase A_2, are effective from 75 min post-training on only (Hölscher and Rose, 1994), the time point when release of arachidonic acid into the extracellular fluid was the highest (Clements and Rose, 1994).

It appears that nitric oxide, arachidonic acid, and arachidonic acid metabolites act together synergetically in neuronal transmission. Furthermore, induction of enzymes in neurons appears to be of importance for memory consolidation and synaptic plastic processes. This has been shown before with long-term potentiation of synaptic transmission in the hippocampus. Long-term potentiation caused the induction of several enzymes such as Ca^{2+}/calmodulin-dependent kinase II, and γ-protein kinase C (Thomas et al., 1994). A different study showed that consolidation of long-term potentiation required a critical period for gene transcription (Nguyen et al., 1994).

Taking the results together, it appears that learning and memory consolidation are dependent on the successive activity of different neurotransmitters which act to enhance or decrease synaptic transmission. These neurotransmitters may act in a cooperative way, modulating each other via positive or negative feedback and controlling the successive activation of transmitter sys-

tems by inducing the synthesis of the respective synthesizing enzyme.

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