Formation and adaptation of memory

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Chapter 5

Training and reversal training in the Y maze do not affect striatal PKA and calcineurin protein levels or AMPA receptor phosphorylation at the GluR1-S845 site

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Abstract

It has been suggested that both hippocampal and striatal systems are important for spatially-organized behavior. The dorsal striatum has been implicated in response learning (e.g. learning to always take the same direction, independent of spatial cues). However, it is unclear whether the striatum is also involved in learning and reversal learning in a Y-maze reference paradigm. Therefore, in this study, we investigated the effects of Y-maze learning and reversal learning on neuronal plasticity in the striatum. Mice were trained to locate a food reward in one of two accessible arms of a Y-shaped maze. When the animals had mastered the task, the food reward was relocated, after which they had to learn that the previously non-baited arm was now the baited one (reversal training). Protein levels of the cAMP-dependent protein kinase (PKA), the protein phosphatase calcineurin (CaN), as well as alpha-amino-3-hydroxy-5-methyl-4 isoxazolepropionic acid (AMPA) receptor phosphorylation at the serine 845 site of the glutamate receptor 1 (GluR1) subunit were measured during various phases of training and reversal training in the Y maze. Although mice learned to locate the baited arm during training and reversal training, we did not find any changes for PKA and CaN protein levels or GluR1-S845 phosphorylation in the striatum indicative for the use of a response learning strategy. Therefore these findings suggest that mice do not use their striatal system during learning and reversal learning in the Y maze.

Introduction

It is postulated that three dissociable neuronal systems exist, each subserving a particular form of memory (McDonald and White, 1993; Salinas and White, 1998). Every system refers to different anatomical structures that have a specialized role in coordinating behavior based on experiences (Salinas and White, 1998). Each of these systems is crucially dependent on one specific brain region: the hippocampus, striatum and amygdala. While the first two systems are important for spatially-organized behavior (although in different ways), the latter is required for processing relationships between stimuli and their significance (e.g. the emotional value) to the organism (for review, White and McDonald, 2002). The existence of three neuronal systems is supported by many studies in various species including rats (o'Keefe and Nadel, 1978; Packard et al., 1989; Packard and McGaugh, 1992), monkeys (Zola-Morgan et al., 1982; Zola-Morgan and Squire, 1984) and humans (Cohen and Squire, 1980; Knowlton et al., 1996; Hartley et al., 2003).

The hippocampal system is essential for processing information about and flexible utilization of the relationships between cues and events (Eichenbaum, 1992; Compton et al., 1997; Compton, 2004). Temporal or permanent inactivation of the hippocampal system results in impaired place learning (e.g. locating a particular goal in space based on distal cues, also known as allocentric learning) in many paradigms including the spatial version of the Morris water maze (Morris et al., 1982) and a modified version of the T maze (Packard and McGaugh, 1996; Compton, 2004).

Based on lesion studies, it has become apparent that the dorsal striatum is required for response learning (e.g. egocentric learning; making responses based on their own body orientation in space). For instance, rats with an inactivated striatum were impaired in a right-left discrimination paradigm (Cook and Kesner, 1998), as well as non-spatial versions of the Morris water maze and modified version of the a T maze (Packard McGaugh, 1996; Compton, 2004).

Depending on the nature of the task, one or more of these different neuronal systems can be activated in parallel in an intact animal, although activation of only one system is sufficient to solve the task. In case of reference learning in a symmetrical Y maze, mice are trained to locate a food reward. Only one of the two accessible arms is baited. The response of the mouse can be based on a particular turn response or it may have learned that food is located at a specific spatial location. As described above, the turn response strategy is based on the dorsal striatal system, while using a spatial strategy based on distal cues requires the hippocampal system.

We previously showed that learning and reversal learning in the Y maze changed protein levels of the protein phosphatases calcineurin (CaN) (Havekes et al., 2006) and the phosphorylation of one of its targets, the serine 845 (S845) site of the alpha-amino-3-hydroxy-5-methyl-4 isoxazoleproprionic acid (AMPA) receptor glutamate receptor 1 subunit (GluR1) (Havekes et al., 2007). Together, these data imply that mice used a place-learning strategy that is dependent on the hippocampus. However, our setup does not exclude the use of a striatum-dependent response strategy (e.g. always take the same direction, independent of spatial cues). Therefore, the aim of this study was to investigate whether training and reversal training in

the Y maze affected striatal plasticity, indicative for the use of a response strategy.

Glutamate injections into the striatum has shown to facilitate response learning (Packard, 1999; Packard and Teather, 1999, while intervention of N-methyl-d-aspartate (NMDA) receptors in the striatum deteriorated response learning, leaving place learning unaffected (Packard and Teather, 1997; Leonibus et al., 2005). These data indicate that calcium-requiring signaling cascades are essential for striatum-dependent response learning. Since both PKA and CaN are at least partly dependent on NMDA-receptor activation and calcium influxes into the post-synaptic neuron, we investigated protein levels of both PKA and CaN during various phases of learning and reversal learning in the Y-maze. Since the phosphorylation state of the GluR1-S845 is controled by opposing actions of PKA and CaN, we also measured the S845 phosphorylation levels after learning and reversal learning in the Y maze.

Materials and Methods

Animals and housing conditions

A total of 56 male C57BL/6j mice (Harlan, Horst, Netherlands), 12 to 16 weeks old, were individually housed in standard macrolon cages equipped with a removable slot which could be locked on to a Y maze. Subjects were maintained on a 12 hour light/dark cycle (lights on at 7.30 a.m.) with ad libitum food (hopefarm® standard rodent pellets) and water. A layer of sawdust served as bedding. Four days before the beginning of the experiment, subjects were food deprived to 90 % of their individual body weight under ad libitum feeding conditions. Animals were weighed and fed after the last training session of each day. The procedures described in the present study were approved by the Dutch Animal Experiment Committee of the University of Groningen in compliance with Dutch law and internal regulations.

Y maze

Behavioral testing was conducted in a tubular plexiglass Y maze. Both start arm (27.5 cm long) and the two arms forming the Y (both 27.5 cm long and diverged at a 120° angle from the stem arm) were 5 cm in diameter. The home cage was connected to the start arm of the Y maze. Perforations at the endings of the two arms forming the Y allowed odors from food crumbs placed next to the perforations to prevent animals to discriminate between baited and non-baited arms by olfactory cues. Each arm was equipped with a guillotine door halfway the arm which could be operated manually from the experimenters position. Small grey plastic blocks (1 cm high) were placed 4 cm from the end of the arms to prevent visual inspection for food presence from a distance. The experimental room contained visual cues, which served as distal spatial cues.

Habituation procedure

Habituation was performed as previously described. During the first day, three habituation trials were performed (3 hour interval). The first habituation trial consisted of placing the mice in the centre of the Y maze and mice were allowed to explore the maze for 4 minutes. During the subsequent habituation, the home cage was connected to the start arm of the Y maze. Mice were given the opportunity to freely enter the maze without handling. Starting from the home cage, subjects could explore one of the two arms (the other arm was closed). The open arm was baited with small crumbs of food (0.05-0.1 g) placed at the end of the arm. When the reward was consumed and the mouse retreated to the home cage, the home cage was removed from the Y maze. The

third habituation was similar to the second habituation, but now the previously blocked arm was accessible and baited, and the previously accessible arm was blocked. The second and third habituations were given to prevent the development of a preference for either of the two arms.

Test procedure

After habituation, testing procedure was executed as described previously (Havekes et al., 2006, 2007). In short, mice received a training and reversal training in a Y-maze reference paradigm. Every training session consisted of 6 trials. During the entire training, either right or left arm was baited. When a subject visited one of the two accessible arms, the non-visited arm was closed. A visit to the baited arm was recorded as a correct trial. After the subject retreated to the home cage, the maze was cleaned with damped paper cloth and rebaited. The subject was then again allowed to explore the maze and visit either of the two accessible arms. During the reversal training, the food reward was relocated to the previously unrewarded arm. The T7, RT1, RT3 and RT7 group received 7 sessions of training, divided over 4 days, while the T1 and T3 group received respectively 1 and 3 sessions of training in case of the T3 group divided over two days. After the training, the RT1, RT3 and RT7 group received respectively 1, 3 and 7 additional sessions in 1, 2 and 3 days, but now with the food reward located in the previously unrewarded arm (reversal training). Mice were assigned to one of the following groups: home cage controls (HCC, n=9); training (T1, n=7; T3, n=8; T7, n=7); reversal training (RT1, n=9; RT3, n=8; RT7, n=8). These time points were chosen to study the dynamics of various markers of neuronal plasticity during both training and reversal training: acquisition phase of training (T1 and T3), end of training (T7), reversal effect (RT1), acquisition phase of reversal training (RT3), and end of reversal training (RT7). Directly after the last session, animals were deeply anesthetized with CO2/O2 followed by a quick dissection (within 2 minutes) of the brain. The striatum of both hemispheres was collected and processed for biochemical analysis.

Protein extraction

Striatum homogenates were lysated in ice-cold homogenization buffer (10mM Tris base pH 7.6, 320mM sucrose, 150mM NaCl, 5mM EDTA, 5mM EGTA, 1mM benzamidine, 1mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 50mM NaF) with an inhibitor cocktail (complete Mini EDTA free, Roche Diagnostics, Mannheim, Germany). Homogenates were centrifuged at 1000 x g for 10 minutes to remove nuclei and large debris. Protein concentrations of the cytosol fraction was determined using the method of Bradford (Bradford, 1976). Samples were diluted using homogenization buffer. Sodium dodecyl sulphate buffer (50% glycerin, 321.5mM Tris/HCl pH 6.8, 10% SDS, 25% β-mercaptoethanol, 0.1% bromophenol blue) was added followed by 5 min heat denaturation at 95°C. Subsequently, the samples were aliquoted and stored at -80°C until further processing.

Equal concentrations of protein were resolved in 10% SDS-polyacrylamide gels, blotted electrophoretically to Immobilon-P transfer membrane (Millipore, Bedford, MA, USA) and blocked in blocking buffer (0.1% Tween-20, 0.2% I-block, Tropix, Bedford, MA, in tris buffered saline (TBS, pH 7.4)) at 5°C. Membranes were incubated with combinations of primary antibodies overnight in buffer (containing 0.05% Tween-20, 0.1% I-block, Tropix, MA in PBS). Pilot experiments with single primary antibodies and combinations of primary antibodies did not reveal any signs of crossreactivity. After rinsing with blocking buffer, membranes were incubated with the proper horse radish peroxidase-conjugated-secondary antibodies (Santa Cruz, CA, USA; Amersham, NJ, USA) in TBS containing 0.5% tween (TBST) for one hour at room temperature. Afterwards membranes were rinsed with TBST. For chemoluminescent labeling, membranes were incubated with a mix of Pierce Detection reagent 1 and 2 (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The immunoreactive bands were captured on autoradiography film (Kodak X scientific image film, Rochester, NY, USA). Densitometric scans of the immunoreactive bands were digitized, grey levels and surface levels of each individual band were measured using a Quantimet 500 image analysis system (Leica, Cambridge, UK). Integrated

optical densities (IOD) were calculated by multiplication of the values for grey level and surface area. Blots were probed with antibody directed against actin (MP Biomedicals, Solon, OH, USA), which was used to correct for variation in protein levels, together with one of the following antibodies: phosphor-GluR1-Serine 845, GluR1, PKA-RIIα,β subunit (Upstate, Charlottesville, VA, USA) and CaN catalytic subunit (Sigma-Aldrich, St. Louis, MO, USA).

Statistical analysis

Analysis of the behavioral data was performed using repeated measures analysis. Protein levels of PKA, CaN and GluR1 as well as the phosphorylation levels of S845 were analyzed with one way analysis of variance (ANOVA). $P < 0.05$ was considered as significant.

Results

Behavioral performance in the Y maze during training and reversal training

During training in the Y maze, mice gradually learned to locate the baited arm ($F_{6,168} = 29.238$, $P < 0.001$; Fig.1), resulting in a final score ranging from 75.0 ± 4.5 % to 88.1 % ± 4.8 % after 7 sessions. No group or interaction effects were found. After training, the RT1, RT3 and RT7 groups were confronted with a relocation of the food reward to the previously non-baited arm (reversal training). The performance in all groups dropped initially, indicating mice still had a preference for the previously rewarded arm. Gradually, mice shifted their preference to the previously unrewarded arm (RT7 group, $F_{6.36} = 13.732$, $P < 0.001$; Fig.1). The RT7 group reached a final score of 73.8 % ± 3.37 %.

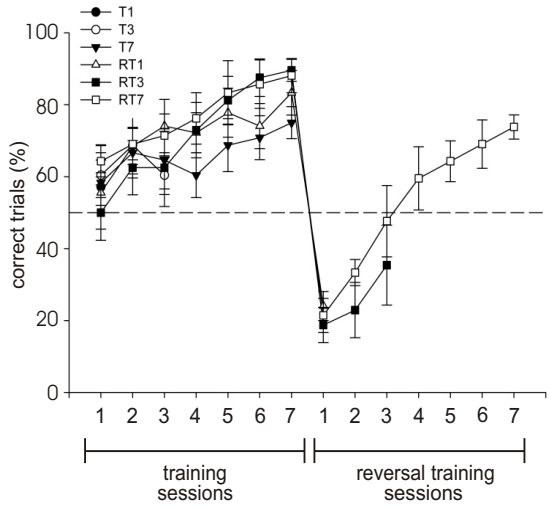

Figure 1. *Performance dung training and reversal training in the Y maze. The percentage of correct trials per session is shown for the T1 (n = 7), T3 (n = 8), T7 (n = 7), RT1 (n = 9), RT3 (n=8), and RT7 (n=8) group.*

PKA and calcineurin protein levels in the striatum

Western blot analysis was performed to determine whether training and reversal training altered protein levels of PKA RIIα,ß in the striatum. Figure 2A shows a representative blot for PKA RIIα,ß. The IODs of the immunoreactive bands are depicted in figure 2B. Training and reversal training induced a slight but not significant reduction in striatal PKA protein levels as compared to home cage controls.

Next, we assessed whether learning and reversal learning affected CaN protein levels in the striatum. Figure 3A shows representative bands for the catalytic subunit of CaN. The IODs of the immunoractive bands are shown in figure 3B. Although, there was a slight decrease in CaN in the T7-group, overall analysis did not reveal any significant effects.

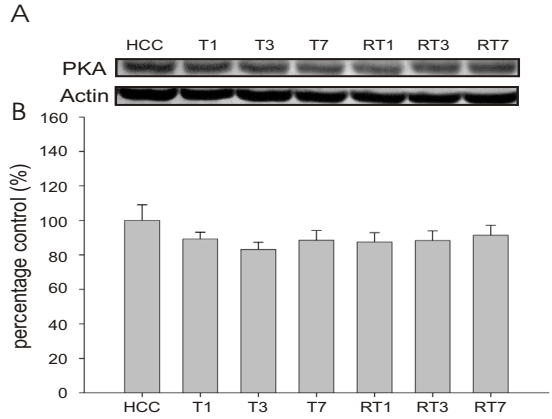

Figure 2. *PKA protein levels in the striatum are not changed during the course of learning and reversal learning in the Y maze. (A) Representative immunoreactive bands for PKA RIIα,ß and actin. (B) Quantification of striatal PKA RIIα,ß protein levels, relative to actin levels for all groups.*

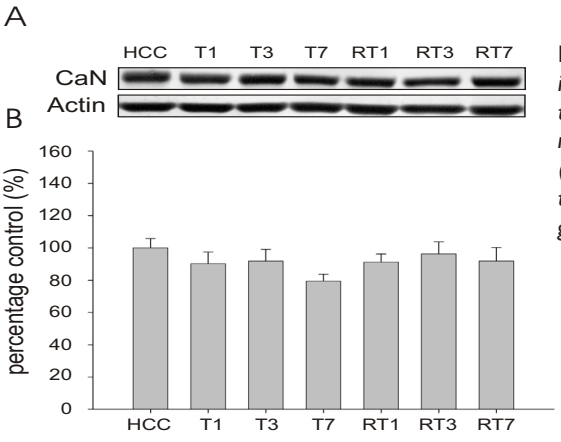

Figure 3. *Learning and reversal learning does not affect CaN protein levels in the striatum are (A) Representative immunoreactive bands for CaN and actin. (B) Quantification of striatal CaN protein levels, relative to actin levels for all groups.*

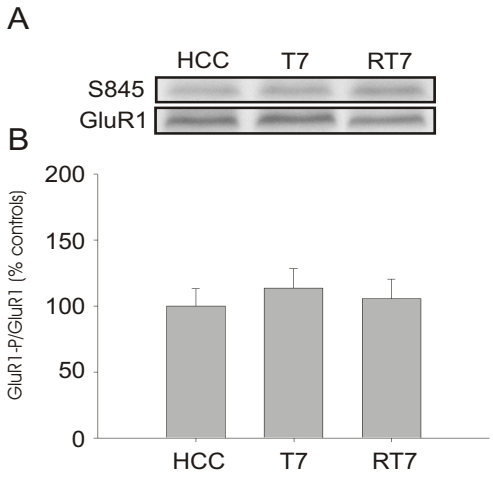

Figure 4. *GluR1-S845 phosphorylation is not changed at the end of training or reversal training. (A) Representative immunoreactive bands for S845 phosphorylation and GluR1. (B) Quantification of striatal S845 phosphorylation, relative to GluR1 levels for the HCC, T7 and RT7group.*

GluR1-S845 phosphorylation in the striatum

The GluR1-S845 site of the AMPA receptor can be dephosphorylated by CaN. Since there was a trend for a decrease in CaN protein levels at the end of training, we investigated whether this slight reduction of CaN affected AMPA receptor phosphorylation at the GluR1-S845 site. Immunoreactive bands for S845 and GluR1 are shown in figure 4A. S845 phosphorylation was not affected at the end of training or the end of reversal training (Fig 4B). GluR1 levels in the striatum were slightly enhanced at the end of reversal training, although the increase was not significant (data not shown).

Discussion

The aim of the present study was to investigate whether training and reversal training in a Y-maze reference paradigm changed protein levels of PKA and CaN, as well as GluR1-S845 phosphorylation in the striatum, indicative for response learning mediated by the striatum. Protein levels of PKA and CaN were not affected by training and reversal training. Likewise, the GluR1-S845 phosphorylation was not altered at the end of training or reversal training. These data show that training and reversal training do not change striatal plasticity at the level of CaN and PKA or their substrate the GluR1-S845 site and suggest that mice did not use a response learning strategy in our Y-maze learning paradigm.

The lack of changes for PKA, CaN and GluR1-S845 could be due to various reasons. One explanation is that in our setup, mice rather used a place learning strategy to locate the baited arm instead of a response strategy. In a paradigm comparable to the one we used, Packard and McGaugh (1996) showed that rats can use both the striatum and hippocampal system. During the initial phase of learning (e.g. after 7 days of training), rats tended to use an approach requiring the hippocampus, but with extended training (e.g. after 14 days

of training), they switched to a striatal strategy. Similar shifts from hippocampal to striatal strategies with extended training have been reported in rats (Colombo et al., 2003) and humans (Orban et al., 2005). In line with the suggestion that mice used a place learning strategy, we previously showed that CaN protein levels and GluR1-S845 phosphorylation in the hippocampus were altered after learning and reversal learning (Havekes et al., 2006; Havekes et al., 2007). Furthermore, in our paradigm performance did not reach a sealing effect within 7 sessions of training and 7 sessions of reversal training, which makes it unlikely that mice were overtrained.

Other studies have reported that a striatum-dependent approach is preferentially used in case of extended training or overtraining (Packard and McGaugh, 1996; Colombo et al., 2003). For instance, rats still tended to use a hippocampus-dependent strategy after 8 days of training. Only after 16 days of training, they switched to an approach requiring their striatum (Packard and McGaugh, 1996). Alternatively, it could very well be that other proteins that require calcium for their activation may play a more prominent role in striatum-dependent response learning. One candidate is the calcium/calmodulin-dependent kinase II, that has recently been implicated in striatum-dependent learning (Wiltgen et al., 2007).

Future experiments in which mice are subjected to overtraining in the same paradigm should further clarify whether CaN, PKA and GluR1 containing AMPA receptors may play a role in striatum dependent response learning.

Author contributions

R.H. and E.A.V.d.Z. generated the hypotheses and designed the experiments. R.H. and I.M.N. carried out the behavioral testing and tissue collection. J.N.K. carried out the biochemical analyses. R.H. wrote the manuscript with input from E.A.V.d.Z. and I.M.N.

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