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COMPARABLE REDUCTION IN Zif268 LEVELS AND CYTOCHROME OXIDASE ACTIVITY IN THE RETROSPLENIAL CORTEX FOLLOWING MAMMILLOTHALAMIC TRACT LESIONS

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Abstract—Damage to the mammillothalamic tract (MTT) produces memory impairments in both humans and rats, yet it is still not clear why this diencephalic pathway is vital for memory. One suggestion is that it is an important route for midbrain inputs to reach a wider cortical and subcortical network that supports memory. Consistent with this idea, MTT lesions produce widespread hypoactivity in distal brain regions as measured by the immediate-early gene, c-*fos*. To determine whether these findings were selective to c-*fos* or reflected more general changes in neuronal function, we assessed the effects of MTT lesions on the expression of the immediate-early gene protein, Zif268 and the metabolic marker, cytochrome oxidase, in the retrosplenial cortex and hippocampus. The lesions decreased levels of both activity markers in the superficial and deep layers of the retrosplenial cortex in both its granular and dysgranular subregions. In contrast, no significant changes were observed in the hippocampus, despite the MTT-lesioned animals showing marked impairments on T-maze alternation. These findings are consistent with MTT lesions providing important, indirect inputs for normal retrosplenial cortex functioning. These distal functional changes may contribute to the memory impairments observed after MTT lesions.

Key words: diencephalic amnesia, hippocampus, immediate-early gene, mammillary bodies, memory, anterior thalamic nuclei.

INTRODUCTION

The mammillothalamic tract (MTT) is a white matter bundle, which carries unidirectional projections from the mammillary bodies to the anterior thalamic nuclei. Damage to the MTT appears to be a consistent feature of patients with diencephalic amnesia following stroke (e.g., Van der Werf et al., 2003; Yoneoka et al., 2004; Carlesimo et al., 2007). Furthermore, MTT lesions in rats produce impairments on a number of spatial memory tasks (Field et al., 1978; Thomas and Gash, 1985; Vann and Aggleton, 2003; Vann et al., 2003; Winter et al., 2011; Nelson and Vann, 2014). It is, however, still uncertain why damage to this structure has such notable effects on memory.

One possibility is that disconnecting the mammillary body projections to the anterior thalamic nuclei results in a loss of ascending midbrain inputs, e.g., from Gudden's tegmental nuclei (e.g., Vann, 2013; Vann and Nelson, 2015), which in turn causes distal hypoactivity in other connected memory structures. This account is consistent with previous findings, as lesions to the MTT or the anterior thalamic nuclei reduce the expression of the immediate early gene *c-fos* in both the retrosplenial cortex and the dorsal hippocampus (Jenkins et al., 2002a,b; Jenkins et al., 2004; Poirier and Aggleton, 2009; Vann and Albasser, 2009; Vann, 2013; but see Dupire et al., 2013, Loukavenko et al., 2015).

The present study investigated whether the MTT lesion-induced changes in the hippocampus and retrosplenial cortex are selective to the immediate-early gene c-*fos*, or reflect a more widespread dysfunction. Two different markers of neuronal activity were assessed: Zif268 is a transcription factor involved in the regulation of a number of synaptic proteins and, hence, is implicated in synaptic plasticity (e.g., Davis et al., 2003; Knapska and Kaczmarek, 2004); cytochrome oxidase is a component of the mitochondrial electron transport chain required for oxidative phosphorylation and can be used to map levels of neural metabolism (Wong-Riley, 1989; Poremba et al., 1998). Both markers are, therefore, implicated in neuronal activity but via different pathways. Furthermore, increased levels of c-Fos, Zif268 and cytochrome oxidase have been found in both the retrosplenial cortex and hippocampus of rats performing spatial memory tasks (Vann et al., 2000a,b; Soule et al., 2008; Pothuizen et al., 2009; Barbosa et al., 2013). If the projections carried via the MTT *are* important

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Abbreviations: ANOVA, Analysis of variance; AP, antero-posterior; DV, dorso-ventral; LM, lateral-medial; MTT, mammillothalamic tract; Rdg, retrosplenial dysgranular cortex; Rga, retrosplenial granular a cortex; Rgb, retrosplenial granular b cortex.

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for the optimal functionality of the retrosplenial cortex and hippocampus during spatial memory performance, it might be expected that MTT lesions would disrupt the expression of all three neuronal markers.

Two separate cohorts of rats with MTT lesions were examined: tissue from the first cohort was stained for the expression of Zif268 while tissue from the second cohort was used to quantify levels of cytochrome oxidase. Animals from both cohorts were tested on a forced-run version of the radial-arm maze task in a novel room prior to perfusion to increase neuronal activity above baseline. The lesions in the second cohort were additionally verified both behaviorally and immunohistochemically, i.e., rats were tested on a reinforced T-maze alternation task and the tissue was processed for calbindin-staining to visualize the dense fibrous stain in the ventrolateral part of the anteroventral thalamic nucleus attributed to mammillothalamic input (e.g., Rogers and Resibois, 1992).

EXPERIMENTAL PROCEDURES

Animals

Subjects were 43 naïve male Dark Agouti rats (Harlan, Bicester, UK). The 20 rats in Cohort 1 (Zif268) weighed 215–250 g at the time of surgery; the 23 rats in Cohort 2 (cytochrome oxidase) weighed 226–252 g at the time of surgery.

Rats were housed in pairs under diurnal light conditions (14 h light/10 h dark) and behavioral testing was carried out during the light phase at a regular time of day. Rats were thoroughly handled before the study began and were given free access to water throughout the experiments. During the behavioral test period the animals were food deprived but their body weight did not fall below 85% of their free feeding weight. The rats from both cohorts had been part of previously published studies (Vann and Albasser, 2009; Nelson and Vann, 2014). The rats in Cohort 1 had been part of a study assessing the effects of MTT lesions on the expression of another immediate-early gene protein, c-Fos. The rats in Cohort 2 came from a separate behavioral study (Nelson and Vann, 2014) and, as a result, had been tested on several behavioral tasks in addition to those presented here, including a working memory task in the watermaze, an object-in-place task, a go-no-go place discrimination task, a passive placement task in the watermaze and a working memory task in the radial-arm maze. All experiments were carried out in accordance with UK Animals (Scientific Procedures) Act, 1986 and associated guidelines.

Stereotaxic surgery

Animals in each cohort were divided into two groups: one received bilateral MTT lesions (Cohort 1: MTTx1, $n = 10$; Cohort 2: MTTx2, $n = 13$), while the other group underwent control surgery (Cohort 1: Sham1, $n = 10$; Cohort 2: Sham2, $n = 10$). Before surgery, all animals were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/kg pentobarbital sodium salt; Sigma–Aldrich, United Kingdom) and then positioned in a stereotaxic head-holder (David Kopf Instruments, Tujunga, CA, USA). All rats were maintained on oxygen during surgery and given an analgesic (Meloxicam; Boehringer Ingelheim, Rhein, Germany). The position of the incisor bar of the stereotaxic frame was set at +5.0 mm to the interaural line. A midline incision was made on the top of the scalp to expose the dorsal skull, which was drilled at the point of the lesion.

For Cohort 1, an electrode (0.7 mm tip length, 0.25 mm diameter; Radionics TCZ, Radionics, Burlington, VT, USA) was lowered vertically and its tip temperature was raised to 60 °C for 15 s using a RFG4-A Lesion Maker (Radionics). The stereotaxic coordinates were: antero-posterior (AP) −1.2 mm (relative to bregma), lateral-medial (LM) ±0.9 mm (relative to bregma), and dorso-ventral (DV) −6.9 mm (from top of the cortex).

For Cohort 2, an electrode (0.7 mm tip length, 0.25 mm diameter; Diros Technology Inc., Toronto, Canada) was lowered vertically and its tip temperature was raised to 70 °C for 22 s using an OWL Universal RF System URF-3AP lesion maker (Diros Technology Inc.). The stereotaxic coordinates were: AP −2.0 mm (relative to bregma), LM ±0.9 mm (relative to bregma), and DV −6.2 mm (from top of the cortex).

For the surgical controls, the electrode was positioned at the same AP and LM coordinates but was only lowered to a DV position of +1.0 mm above the lesion site to avoid damaging the tract and left *in situ* without raising the temperature of the tip.

After surgery, the skin was sutured, an antibiotic powder applied (Acramide: Dales Pharmaceuticals, UK) and animals received 5 ml of glucose saline subcutaneously. They were then placed in a temperature-controlled recovery box until they awoke from the anesthetic. Animals were allowed 2–3 weeks to recover before starting any behavioral training during which time all animals had recovered their preoperative weight.

Behavioral testing

Standard T-maze task (Cohort 2). Apparatus. Testing was carried out in a modifiable four-arm (cross-shaped) maze. One of the arms could be blocked off to form a T-shaped maze. The floors of the T-maze were made of wood, which had been painted white. Each arm was 45.5 cm long and 12 cm wide. The sidewalls (32.5 cm high) were made of black Perspex. At the end of each arm was a sunken food-well (2 cm in diameter and 0.75 cm deep). Access to an arm could be prevented by placing an aluminum barrier at the entrance to the arm. The maze was placed on a table (74 cm high) for the duration of testing. Salient visual cues were hung on the walls of the test room and lighting was provided by overhead lights.

Pretraining and testing procedures. Pretraining began 2 months after surgery; prior to this the rats were tested on a watermaze task. Each animal was given 3 days of