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CONNECTIONS OF THE CAUDAL ANTERIOR CINGULATE CORTEX IN RABBIT: NEURAL CIRCUITRY PARTICIPATING IN THE ACQUISITION OF TRACE EYEBLINK CONDITIONING

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Abstract—The caudal anterior cingulate cortex (cAC) is an essential component of the circuitry involved in acquisition of forebrain-dependent trace eyeblink conditioning. Lesions of the cAC prevent trace eyeblink conditioning [Weible AP, McEchron MD, Disterhoft JF (2000) Cortical involvement in acquisition and extinction of trace eyeblink conditioning. Behav Neurosci 114(6):1058–1067]. The patterns of activation of cAC neurons recorded *in vivo* suggest an attentional role for this structure early in training [Weible AP, Weiss C, Disterhoft JF (2003) Activity profiles of single neurons in caudal anterior cingulate cortex during trace eyeblink conditioning in the rabbit. J Neurophysiol 90(2):599–612]. The goal of the present study was to identify connections of the portion of the rabbit cAC previously demonstrated to be involved in trace eyeblink conditioning, using the neuronal tract tracer wheat germ agglutinin conjugated to horseradish peroxidase, to better understand how the cAC contributes to the process of associative learning. Reciprocal connections with the claustrum provide a route for the transfer of sensory information between the cAC and neocortical and allocortical regions also involved in learning. Connections with components of the basal forebrain cholinergic system are described, with relevance to the proposed attentional role of the cAC. Reciprocal and unidirectional connections were in evidence in multiple thalamic regions, including the medial dorsal nucleus, which have been implicated in a variety of conditioning paradigms. Anterograde connections with the caudate and lateral pontine nuclei provide access to forebrain motor and brainstem sensory circuitry, respectively. The relevance of these connections to acquisition of the trace conditioned reflex is discussed.

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Abbreviations: AM, anterior medial thalamic nucleus; AP, anterior–posterior; BFCS, basal forebrain cholinergic system; cAC, caudal anterior cingulated; CeM, central medial thalamic nucleus; CR, conditioned response; CS, conditioned stimulus; DMSO, dimethylsulfate; EBC, eyeblink conditioning; EC, entorhinal cortex; GP, globus pallidus; Ha, precommissural hippocampus; HDB, horizontal limb, diagonal band of Broca; LP, lateral posterior thalamic nucleus; LPN, lateral pontine nucleus; MD, medial dorsal thalamic nucleus; mPFC, medial prefrontal cortex; MPN, medial pontine nucleus; nAcc, nucleus accumbens; NBM, nucleus basalis magnocellularis; PBS, phosphate-buffered saline; PL, prelimbic cortex; rAC, rostral anterior cingulate cortex; Re, reuniens thalamic nucleus; RSC, retrosplenial cortex; SM, submedial thalamic nucleus; TT, taenia tecta; US, unconditioned stimulus; VA, ventral anterior thalamic nucleus; VL, ventral lateral thalamic nucleus; VM, ventral medial thalamic nucleus; vPut, ventral putamen; WGA-HRP, wheat germ agglutinin conjugated to horseradish peroxidase.

Key words: limbic, claustrum, lateral pontine, associative learning, eyeblink conditioning.

The caudal aspect of the anterior cingulate cortex (cAC) is critically involved in the acquisition of trace eye-blink conditioning (EBC), an associative learning task in which paired presentations of a neutral conditioned stimulus (CS) and a behaviorally salient unconditioned stimulus (US) separated by a stimulus free trace interval, ultimately result in the elicitation of a conditioned response (CR). Damage to this region severely impairs acquisition of the task (Kronforst-Collins and Disterhoft, 1998; Weible et al., 2000). Single neuron activity recorded during training suggests that the rabbit cAC is part of an attentional mechanism detecting coincidence between temporally-related environmental stimuli (Weible et al., 2003).

The patterns of cAC neuronal activity observed during trace EBC are reminiscent of changes exhibited by neurons of the hippocampus (Solomon et al., 1986; McEchron and Disterhoft, 1997; Munera et al., 2001), another structure critically involved in acquisition of the conditioned reflex (Solomon et al., 1986; Moyer et al., 1990; Kim et al., 1995; Weiss et al., 1999). However, whereas the learning-related activity observed in the hippocampus develops over the initial days of training, the pattern of activity exhibited by cAC neurons during trace conditioning is evident during the earliest paired CS–US presentations. Robust CS-elicited increases in cAC neuronal firing rate are observed at the onset of training, prior to behavioral evidence of learning (Weible et al., 2003). This CS-elicited response is maintained during CS–US associated trace conditioning, but declines rapidly to baseline levels during pseudoconditioning, in which CS and US presentations are unpaired. In contrast, McEchron and Disterhoft (1997) described robust learning-related increases in CS-elicited excitatory responses immediately preceding initial CR expression during trace conditioning, and CS-elicited excitatory responses described by Munera and colleagues (2001) did not appear to differ significantly between neurons of trace and pseudoconditioned subjects during the first day of training. The maintained response of cAC neurons to the CS appears to represent the acquired salience of an otherwise behaviorally neutral stimulus imparted by the CS–US association, and may reflect an attentional role of the cAC during learning. Maintenance of the CS-elicited response by cAC neurons at the onset of trace conditioning could then facilitate the development of the CS–US association in the hippocampus, which is in turn required

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for successful acquisition of the task (McEchron et al., 2001). However, no direct connections between the rabbit cAC and the hippocampus have previously been described.

Previous anatomical studies of the anterior cingulate in the rabbit have been useful in developing hypotheses of cAC involvement in trace EBC. However, these studies have either involved the more rostral prefrontal cortex (e.g. Arikuni and Ban, 1978; Buchanan et al., 1994) or have focused on specific subsets of connections (e.g. Benjamin et al., 1978; Buchanan et al., 1989). The goal of the present study was to identify the pathways afferent and efferent specifically to the cAC, the region previously examined using lesion (Weible et al., 2000) and *in vivo* electrophysiological recording (Weible et al., 2003) techniques during trace EBC. To achieve this goal, the anterograde and retrograde tracer wheat germ agglutinin conjugated to horseradish peroxidase (WGA-HRP) was injected into the cAC. Here, the identified connections and their functional implications are described, and a hypothetical circuit is proposed for cAC involvement in learning based on previous work from this laboratory and others (Berger and Bassett, 1992; Weiss and Disterhoft, 1996).

EXPERIMENTAL PROCEDURES

Subjects

We collected data from seven 2.5- to 3.0-month-old female New Zealand white albino rabbits. All subjects were treated and maintained in accordance with the guidelines of Northwestern University's Animal Care and Use Committee, as well as the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). All efforts were made to minimize the number of subjects utilized in the present study. All precautions available to minimize any pain and discomfort experienced by each subject were taken.

Surgical preparation

All subjects underwent the same surgical procedure. Ketamine (60 mg/kg) and xylazine (10 mg/kg) were administered intramuscularly to anesthetize subjects prior to surgery; supplements were given as needed. The scalp of each subject was shaved, cleaned of remaining hair with depilatory cream, and washed with Betadine. Subjects were then placed in a stereotaxic frame. An incision was made along the midline, and the scalp and fascia retracted. A standard dental burr was used to make a rectangular opening $\sim 2 \times 4$ mm in area immediately anterior to bregma (medial–lateral: 0.0–2.0 mm, anterior–posterior (AP): +1.0 to +5.0 mm) to expose the dura immediately above the cAC unilaterally. The orientation of the skull was then adjusted such that bregma was positioned 1.5 mm above lambda.

WGA-HRP injection

The tracer WGA-HRP was chosen for the present study for two reasons. First, the use of this tracer provided a means of identifying both anterograde and retrograde projections with a single injection and single histological protocol. Second, WGA-HRP is not taken up by fibers of passage (Grob et al., 1982; Steindler, 1982) thus avoiding the complication of transport resulting from fibers passing through the target region. WGA-HRP (2%, in 0.5 M NaCl) was injected using a pulled pipette fixed with epoxy to the tip of a 0.5 μl Hamilton syringe. The injection sites and volumes are summarized in Table 1.

Table 1. Injection sites and volumes

Subject	Volume (nl)	Coordinates (mm)		
		AP	ML	DV
1	20	2.0	0.8	3.0
2	20	3.0	0.8	2.3
3	20	3.5	0.8	3.0
4	10	2.0	0.6	2.5
5	10	1.6	0.6	2.5
6	20	3.0	0.7	3.0
7	20	2.5	0.8	3.0

Depth was measured from the dura. A small incision was made in the dura through which the pipette was passed. The pipette was lowered at increments of 0.5 mm, with a two minute pause between increments. The needle was maintained at the target depth for five minutes prior to injection. Injections of either 10 nl or 20 nl of WGA-HRP were made, with 20 nl injections occurring in two 10 nl pulses to minimize the incidence of tissue damage around the injection site. Following the completion of the injection, the needle remained at depth for an additional five minutes. The needle was then removed, and the hole in the skull packed with Gelfoam (Pharmacia and Upjohn, Kalamazoo, MI, USA). The scalp was sutured, and the subject was allowed to recover. Buprenex (0.02 mg/kg) was administered post-operatively at 12 h intervals for 36 h.

Tissue preparation and processing

Survival times for all subjects ranged from 46 to 50 h post-WGA-HRP injection. The perfusion and tissue processing protocols followed in the present study were adapted from the study by Weinberg and van Eyck (1991) which employed a tetramethylbenzidine/tungstate reaction. Briefly, each rabbit was killed with a lethal dose of sodium pentobarbital and then perfused transcardially with 1 l of 37 °C 0.9% NaCl (1 L/min), followed by 1 l of fixative [.033 L/min; 2.5% glutaraldehyde, 0.5% paraformaldehyde, and 0.1% picric acid in 0.1 M phosphate buffered saline (PBS), pH 7.4]. The brain was removed and stored at 4 °C in glycerol/dimethylsulfate (DMSO) solutions of increasing concentration to reduce the incidence of freezing artifact (10% glycerol/2% DMSO 1–3 days; 20% glycerol/2% DMSO 4 days; Rosene et al., 1986). The brains were then immersed in gelatin (10%; 300 Bloom). The gelatin was hardened by exposure to fumes from 10% formalin (4 °C) and then placed in a 20% glycerol/2% DMSO solution for three days at 4 °C. A notch was made in the gelatin along the longitudinal (horizontal) axis to serve as a histological marker for tissue mounting. The notched gelatin block was then placed in chilled (−75 °C) isopentane for 1–2 h, then stored at −80 °C until sectioning. For sectioning, the block was mounted on the stage of a sliding microtome with Tissue-Tek and surrounded with pulverized dry ice. The brain was cut at 50 μm thick coronal sections which were stored in chilled PBS (pH 7.4).

Every fifth section was stored separately for immediate reaction. The reaction followed was that described by Weinberg and van Eyck (1991) for light microscopic histological analysis. Following reaction, sections were mounted onto gelatinized slides, dried, and Nissl stained with Cresyl Violet for bright field viewing and an estimation of cell counts. Coverslips were applied using DPX mounting medium (Electron Microscopy Sciences, Ft. Washington, PA, USA).

Histological analysis and illustration generation

The methods described here were adopted to provide a semi-quantitative basis of label comparison between different injection