Neuroscience 133 (2005) 193–207

INTRINSIC CONNECTIONS OF THE CINGULATE CORTEX IN THE RAT SUGGEST THE EXISTENCE OF MULTIPLE FUNCTIONALLY SEGREGATED NETWORKS

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Abstract—The cingulate cortex is a functionally and morphologically heterogeneous cortical area comprising a number of interconnected subregions. To date, the exact anatomy of intracingulate connections has not been studied in detail. In the present study we aimed to determine the topographical and laminar characteristics of intrinsic cingulate connections in the rat, using the anterograde tracers *Phaseolus vulgaris*-leucoagglutinin and biotinylated dextran amine. For assessment of these data we further refined and compared the existing cytoarchitectonic descriptions of the two major cingulate constituents, the anterior cingulate and retrosplenial cortices. The results of this study demonstrate that rostral areas, i.e. the infralimbic and prelimbic cortices and the rostral one third of the dorsal anterior cingulate cortex are primarily interconnected with each other and not with other cingulate areas. The caudal two thirds of the dorsal anterior cingulate cortex project to the caudal part of the ventral anterior cingulate cortex, whereas the entire ventral anterior cingulate cortex projects to only the mid-rostro-caudal part of the dorsal anterior cingulate cortex. Dense reciprocal connections exist between the remaining, i.e. the supracallosal parts of the anterior cingulate and retrosplenial cortices with a general rostro-caudal topography, in the sense that the rostral part of the anterior cingulate cortex and caudal part of the retrosplenial cortex are interconnected and the same holds true for the caudal part of the anterior cingulate cortex and rostral part of the retrosplenial cortex.

This topographical pattern of intracingulate connections relates to the results of several functional studies, suggesting that specific cingulate functions depend on a number of interconnected cingulate subregions. Through their intricate associational connections, these subregions form functionally segregated networks. © 2005 Published by Elsevier Ltd on behalf of IBRO.

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Abbreviations: ac, anterior commissure; AC, anterior cingulate cortex; ACd, dorsal anterior cingulate cortex; AChE, acetylcholinesterase; ACv, ventral anterior cingulate cortex; BDA, biotinylated dextran amine; DAB, diaminobenzine; DP, dorsal peduncular cortex; IL, infralimbic cortex; OC2-mm, occipital cortex area 2 mediomedial part; PHA-L, *Phaseolus vulgaris*-leucoagglutinin; PL, prelimbic cortex; RS, retrosplenial cortex; RSd, dorsal retrosplenial cortex; RSv, ventral retrosplenial cortex; RSv-a, ventral retrosplenial cortex ventral part; RSv-b, ventral retrosplenial cortex dorsal part; TBS-Tx, Tris-buffered saline (pH 8.6) with 0.5% Triton X-100.

0306-4522/05$30.00+0.00 © 2005 Published by Elsevier Ltd on behalf of IBRO.
doi:10.1016/j.neuroscience.2005.01.063

Key words: anterograde tracing, avoidance learning, prefrontal cortex, retrosplenial cortex, spatial memory.

The cingulate cortex is a structurally and functionally heterogeneous region, which arches around the corpus callosum on the medial surface of the brain. Its major subdivision is between a rostrally located anterior cingulate cortex (AC) and a caudally positioned retrosplenial cortex (RS; Baleydier and Mauguiere, 1980; Vogt et al., 1992). Functional studies examining the cingulate cortex provide support for the increasing recognition that cognitive processes cannot be localized to one structure but rather depend on interactions between interconnected areas of the brain, forming dynamic networks (Pastor et al., 2000; Friston, 2002; Horwitz, 2003). Thus, rather than being involved in one function, the two cingulate regions are involved in many different cognitive processes. For example, AC is involved in working memory, attention, maternal behavior, visceromotor and skeletomotor control and in processing affective components of pain, whereas RS plays a role in episodic memory and in the monitoring of visual events and eye movements (for reviews see: Vogt et al., 1992; Devinsky et al., 1995; Desgranges et al., 1998; Maddock, 1999; Maguire, 2001; Paus, 2001; Whishaw and Wallace, 2003). In addition, these complex cognitive functions themselves are not solely localized in the cingulate cortex but appear to be subserved by distributed networks of the brain that include the cingulate cortex (Desgranges et al., 1998; Vann et al., 2000; Aggleton et al., 2000; Sewards and Sewards, 2002; Whishaw and Wallace, 2003). The importance of interactions between the different subregions of the cingulate cortex for normal cognitive functioning has also been demonstrated. For example, functional studies in rodents have shown that AC and RS are involved in different stages of discriminative approach and avoidance learning and that communication between these two regions is necessary for a normal course of these processes (Gabriel and Sparenborg, 1987; Gabriel et al., 1991; Bussey et al., 1996; Freeman et al., 1996; Takenouchi et al., 1999).

From the perspective of the network theory of cognitive functioning, cognitive deficits should not only be attributed to a dysfunction in one brain region, but may also result from disconnections between different cerebral areas (Delbeuck et al., 2003). This notion is supported by lesion studies examining spatial learning and memory processes in rats, in which both AC and RS cortices are involved (Warburton et al., 1998; Vann et al., 2000, 2003). Disruption

of the communication between these two regions, through lesioning of the cingulum bundle, leads to a severe impairment in spatial learning and memory tasks (Markowska et al., 1989; Aggleton et al., 1995; Warburton et al., 1998). It seems somewhat contradictory that disruption of the network through combined lesions of the AC and RS, which spare the underlying white matter, only leads to a slight impairment on such tasks (Neave et al., 1994; Aggleton et al., 1995; Warburton et al., 1998). However, these studies have very rarely lesioned the entire cingulate cortex and generally spared either the midcingulate regions or the rostral and caudal extremes of the cingulate cortex. A possibility exists that the spared cingulate regions in those studies are themselves interconnected, forming functional networks that are each capable of subserving spatial learning and memory processes.

However, the detailed organization of intracingulate connections has not been studied in a single comprehensive study. To date, most studies have focused on a single cingulate region, or employed older tracing techniques that lack currently available anatomical specificity (Beckstead, 1979; Bassett and Berger, 1982; Vogt and Miller, 1983; van Groen and Wyss, 1990, 1992, 2003; Fisk and Wyss, 1999). Moreover, the cytoarchitectonic maps used in these studies reveal a number of discrepancies, which complicates a comparison between them (Brodmann, 1909; Rose, 1931; Krieg, 1946; Rose and Woolsey, 1948; Caviness, 1975; Krettek and Price, 1977; Zilles et al., 1980; Vogt and Peters, 1981).

In the present study, we examined the organization of intracingulate connections in rats using sensitive anterograde tracers, deployed in both the AC and RS. We sought to ascertain in particular the topographical and laminar characteristics of intracingulate networks. In view of the discrepancies between previously proposed cingulate maps, the subdivision of the cingulate cortex was examined first with the use of different staining methods in coronal, sagittal as well as horizontal sections.

EXPERIMENTAL PROCEDURES

Fifty-five female Wistar rats (weight: 180–220 g; Harlan Central Proefdier Bedrijf, Zeist, the Netherlands) were used in this study. All experiments were approved by the Animal Ethical Committee of the Vrije Universiteit concerned with ethical aspects of experiments and care for laboratory animals and were in accordance with national and EC guidelines. Care was taken to minimize the number of animals and their suffering as much as possible.

Cytoarchitectonic evaluation and flat map construction

Five animals were anesthetized with sodium pentobarbital (Nembutal; 0.1 ml/100 g body weight intraperitoneally; Ceva, Paris, France) and transcardially perfused with 400 ml 4% formalin solution. The brains were removed from the skull and stored overnight in a mixture of 20% glycerol and 2% dimethyl sulfoxide in distilled water. One brain was photographed in all directions and served as a standard brain surface map for the cingulate cortex. Three other brains were sectioned on a freezing microtome (30 μm), one in the coronal, one in the sagittal and one in the horizontal plane. Sections from each brain were collected as six equally spaced series in separate vials and stored for further processing. One series of each of the three brains was stained with Cresyl Violet (Nissl-stain), one with an antibody against parvalbumin (protocol as described by Wouterlood et al., 1995) and one series was histochemically reacted for the presence of acetylcholinesterase (AChE; protocol as described by Tago et al., 1986). The fifth brain was a coronally cut brain (30 μm) from laboratory stock, with one in six sections stained for myelinated fibers (Loyez iron–hematoxylin method).

The sections were examined with a light microscope and analyzed with the use of Neurolucida imaging software (MicroBrightfield, Inc., Colchester, USA). This program enables the visual image under the microscope to be directly drawn into the computer, maintaining proportions between adjoining sections in each axis. Cingulate cortex outlines and (sub)divisions were drawn separately for each staining of the coronally ($n=2$), sagittally ($n=1$) and horizontally ($n=1$) cut brains. Subsequently, all drawings were exported to the Neuro-explorer program (MicroBrightfield, Inc.), which enables 3D-rotation of the stack of sections. The midline view of each stack of sections was exported to Corel Draw and projected onto the photograph of the standard brain. The rostral and caudal tip of the corpus callosum, the anterior commissure (ac) and the rostral tip of the hippocampus were used as markers to correct for size differences between brains. Once fitted onto the standard brain all the lines indicating cingulate subdivisions were visually averaged and used as a standard map of the cingulate cortex.

A two-dimensional unfolded map of the cingulate cortex was constructed using an adaptation of the method used by Suzuki and Amaral (1994). The outlines of the Neurolucida drawings of the coronally cut Nissl-, AChE- and parvalbumin-stained coronal sections were straightened and then plotted onto the standard map of the cingulate cortex in an Excel spreadsheet. Points indicating the cingulate subdivisions were connected and the resulting lines were smoothed.

Anterograde tracing experiments

Fifty animals received injections with the anterograde tracers *Phaseolus vulgaris*-leucoagglutinin (PHA-L; 2.5%; Vector, Burlingame, CA, USA; 25 mg/ml in 0.05 M Tris-buffered saline, pH 7.4) and/or biotinylated dextran amine (BDA; Molecular Probes, USA; 5% solution in 0.01 M phosphate buffer) to study the intracingulate projection patterns. Twenty-one animals had been used in previous studies (Berendse et al., 1992; Wright and Groenewegen, 1995) and using a protocol similar to that in the earlier studies, new injections were placed in 29 animals. Rats were anesthetized with an i.m. injection of a mixture of Aescoket (ketamine 100 mg/ml; Boxtel, the Netherlands; 9 mg/100 g body weight) and Rompun (xylazine 2 mg/ml; Bayer, Brussels, Belgium; 1.3 mg/100 mg body weight) and subsequently mounted in a stereotactic frame. A midline incision of the skin was made, and following additional local anesthesia with Xylocaine (lidocaine 10%) spray the periost was cleaved and moved aside. Burr holes were made with a dental drill above cingulate areas to be injected. A glass micropipette (7–13 μm inner diameter) filled with either PHA-L or BDA was lowered into the cingulate cortex, using coordinates derived from the atlas of Paxinos and Watson (1998). For the sake of reduction in the number of animals, most animals received one injection in the cingulate cortex and one injection with another tracer into other areas of the brain, in order to study afferent connections (not described in this study). Some animals received bilateral injections with BDA and PHA-L in the cingulate cortex. The tracers were iontophoretically injected by applying a positive, pulsed DC current (7 s on/7 s off, 7.5 mA for PHA-L, 6.5 mA for BDA) for 10–30 min. Injections were aimed such that each of the subareas along the entire rostro-caudal and ventro-dorsal extent of the cingulate cortex received at least one injection. After a survival period of 8–14 days, the animals were anesthetized with sodium pentobarbital (Nembutal intraperitoneally 60 mg/kg body weight; Ceva) and transcardially perfused with 750 ml 0.9% NaCl