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STRONG EXPRESSION OF *NETRIN-G2* IN THE MONKEY CLAUSTRUM

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Abstract—The claustrum is a phylogenetically conserved structure, with extensive reciprocal connections with cortical regions, and has thus been considered important for sensory, motor, emotional, and mnemonic coordination or integration. Here, we show by *in situ* hybridization that the adult monkey claustrum is strongly positive for *NETRIN-G2*, a gene encoding a glycosyl phosphatidyl-inositol-linked membrane protein, which constitutes a subfamily with *NETRIN-G1* within the netrin/UNC6 family. There is a conspicuous dorsal/ventral differentiation, where the label is stronger in the ventral claustrum. *NETRIN-G2* positive neurons are not GABAergic, but rather correspond to claustrocortical projection neurons, as demonstrated by retrograde transport of Fast Blue from cortical injections and by double *in situ* hybridization for *NETRIN-G2* and *GAD67*. Since *NETRIN-G2* is known to be preferentially expressed in cortex, in contrast with the thalamically expressed *NETRIN-G1*, these results raise the possibility of some functional similarity in regulation of excitatory neural transmission in the claustrum and cortex. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: *in situ* hybridization, claustrocortical projection neuron, claustral subdivision, schizophrenia, visual attention.

The claustrum is phylogenetically conserved in mammals (Kowianski et al., 1999; Ashwell et al., 2004) but remains an enigmatic structure. Its best characterized feature is its reciprocal connectivity with widespread cortical regions, which has led to the suggestion that the claustrum is involved in sensory, motor, emotional, and mnemonic coordination or integration (LeVay and Sherk, 1981; Mufson and Mesulam, 1982; Pearson et al., 1982; Markowitsch et al., 1984; Arikuni and Kubota, 1985; Sherk, 1986; Minciacchi et al., 1991; Baizer et al., 1993; Baizer, 2001; Beneyto and Prieto, 2001; Tanné-Gariépy et al., 2002). The highly networked architecture of the claustrum has further led to the speculation that it may be engaged in cooperative activity and importantly related to integrated conscious percepts (Crick and Koch, 2005).

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Abbreviations: AChE, acetylcholinesterase; DAB, 3,3′-diaminobenzidine tetrahydrochloride; dFA, deionized formamide; DIG, digoxigenin; FB, Fast Blue; PB, phosphate buffer; PBS, phosphate-buffered saline; PBS-TG, phosphate-buffered saline containing 0.5% Triton X-100 and 5% normal goat serum; PFA, paraformaldehyde.

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In cat, electrical stimulation of dorsocaudal claustrum elicits excitatory responses in the striate cortex (Tsumoto and Suda, 1982). In monkey, neuronal activity was recorded from dorsal claustrum during both visually guided and memory guided arm movements (Shima et al., 1996). These findings suggest some form of functional specialization in different areas of claustrum. The claustrum has also been implicated in the kindling of generalized seizures from limbic sites (Mohapel et al., 2001; Zhang et al., 2001; Majak et al., 2002; Sheerin et al., 2004).

Several recent reports have re-addressed the connectional, chemical, or molecular features of the claustrum. In rodent gene expression profiles support the view that the claustrum along with the amygdala derives from pallial territory. That is, expression of the T-box containing transcription factor *Tbr1*, which is known to be expressed through the entire pallium and its derivatives, is observed in the claustrum and the endopiriform and basal amygdala nuclei. Expression of *Dlx5*, a marker of subpallium and its derivatives, is not observed in these areas (Medina et al., 2004).

In rodents, the claustrum is relatively small, closely adjoining the adjacent insular cortex (Swanson and Petrovich, 1998; Obst-Pernberg et al., 2001). There is a dorsal and ventral part. The dorsal part is called claustrum proper (also dorsal or insular claustrum), and the ventral part is the dorsal endopiriform nucleus (also called ventral or piriform claustrum). These subdivisions can be identified by specific gene expression patterns. That is, the claustrum proper strongly expresses *Cadherin-8* and *Emx1*. On the other hand, the ventral or endopiriform part expresses *neurogenin2* and/or *Semaphorin5A* but not *Cadherin-8* and *Emx1* (Medina et al., 2004). The claustrum proper can further be divided into three subdivisions: a superior, an intermediate and an inferior, based on the differential expression of *Cadherins* (Obst-Pernberg et al., 2001). In mouse, the central region of the dorsal claustrum is distinguished as a *Cadherin-8*-positive area with relatively low calretinin immunoreactivity, and is surrounded by calretinin immunoreactive fibers (Real et al., 2003; Dàvila et al., 2005). In monkey, no comparable subdivisions have been reported. But this may be because there are only a few detailed molecular and chemical investigations in this species (Reynhout and Baizer, 1999).

As part of a more extended study, we here report that the primate claustrum is strongly positive for *NETRIN-G2*, one of the genes in the *NETRIN-G* subfamily (*NETRIN-G1*, *-G2*, also called *laminet-1*, *-2*; Nakashiba et al., 2000, 2002; Yin et al., 2002) within the netrin/UNC6 family. The netrins are secreted molecules, known to regulate axon guidance in early development in diverse species (Ishii et al., 1992;

Serafini et al., 1994). Unlike classical netrins, NETRIN-Gs are localized to the cell membrane through a carboxyl-terminal glycosyl phosphatidyl-inositol (GPI) anchor. In rodents, there is a complementary distribution, such that netrin-G1 is predominantly in thalamo-cortical axons, whereas netrin-G2 is in cortico-cortical axons (Nakashiba et al., 2002). In mouse, *netrin-G2*, but not *netrin-G1*, is strongly expressed in the claustrum, as well as in cortical neurons (Yin et al., 2002). Preliminary results in monkey support a comparable dissociated expression (Miyashita et al., 2004).

EXPERIMENTAL PROCEDURES

Eight adult macaque monkeys (*1 Macaca mulatta* and *7 Macaca fuscata*) were used in this study for *in situ* hybridization alone ($n=4$) or for *in situ* hybridization in combination with fluorescent retrograde tracing after injection of Fast Blue (FB) (see below; results are identical for both species). All experimental protocols were approved by the Experimental Animal Committee of the RIKEN Institute, and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), revised 1996. Every effort was made to minimize the number of animals used and any pain or discomfort. As a terminal procedure, animals were anesthetized with ketamine (11 mg/kg, i.m.) and Nembutal (overdose, 75 mg/kg, i.p.), and were perfused transcardially, in sequence, with saline containing 0.5% sodium nitrite, 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.4) for 30 min, and chilled RNaseA-free 0.1 M PB with 10%, 20% and 30% sucrose. Brains were removed from the skull, and were immersed into 30% sucrose in 0.1 M PB. After the brains sank, blocks were trimmed and cut serially in the coronal plane by frozen microtomy for Nissl staining, acetylcholinesterase (AChE) histochemistry, Anti-Neuronal Nuclei (NeuN) immunohistochemistry (50 μm thickness), and *in situ* hybridization for *NETRIN-G2* (30 μm thickness). For brains injected with FB (see below), sections were collected for FB observation (50 μm thickness), *in situ* hybridization for *NETRIN-G1* and *NETRIN-G2* (30 μm thickness), double processing for FB and *in situ* hybridization for *NETRIN-G2* (20 μm thickness), and double *in situ* hybridization for *NETRIN-G2* and *GAD67* (20 μm thickness). Extra sections for *in situ* hybridization were transferred to 20% glycerol and 20% ethylene glycol solution in 0.1 M PB, and stored −20 °C until processing.

Nomenclature conventions

In this study, we use "*NETRIN-G1*" and "*NETRIN-G2*" for primate genes. For rodent, lower case is used ("*netrin-G2*"). For proteins, non-italic upper case for primate ("NETRIN-G2") and lower case for rodent ("netrin-G2").

Riboprobe preparation

The 846 bp fragments of human *NETRIN-G2* were amplified from KIAA1857 plasmid as a template (obtained from Kazusa DNA Institute, Chiba, Japan) by polymerase chain reaction (PCR) with a pair of primers (forward; 5′-GCGACATGTCATCCTCCA-3′, reverse; 5′-TTCTGGCACAGCAGCTGGT-3′). These amplified DNA fragments were subcloned into pGEM-T Easy Vector (Promega, Madison, WI, USA). This plasmid and plasmids which contain monkey *NETRIN-G1* (Miyashita et al., 2004) and *GAD67* cDNA (Komatsu et al., 2005; kind gift from Drs. Y. Komatsu, A. Watakabe and T. Yamamori, Okazaki, Japan) were used as templates for synthesizing the antisense and sense RNA probes. Antisense and sense RNA probes were synthesized using digoxigenin (DIG)-RNA labeling kit or fluorescein-RNA labeling kit with SP6 or T7 RNA polymerase (Roche Diagnostics, Indianapolis, IN, USA).

In situ hybridization

In situ hybridization was performed according to standard procedures with minor modifications (Liang et al., 2000). Free-floating sections were rinsed with 0.1 M PB and fixed with 4% PFA overnight in the cold room. Sections were rinsed twice with 0.1 M PB and twice with 0.75% glycine in 0.1 M PB. Then, sections were permeabilized with 0.3% Triton X-100 in 0.1 M PB for 15 min. They were washed with 0.1 M PB for 5 min and then digested with Proteinase K (10 μg/ml in 0.1 M Tris–HCl; 50 mM EDTA, pH 8.0) for 15 min at 37 °C. Then, they were acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min and rinsed twice with 0.1 M PB. Before hybridization, they were incubated in hybridization buffer [50% deionized formamide (dFA), 2% Blocking Reagent (Roche Diagnostics), 5× SSC (1× SSC consists of 0.88% NaCl and 0.44% $Na_3C_6H_5O_3 \cdot 2H_2O$), 0.1% *N*-lauroylsarcosine, and 0.1% sodium lauryl sulfate] at 60 °C for 2 h. Sections were then transferred to new hybridization buffer containing DIG-labeled antisense RNA probes and incubated at 60 °C overnight. After incubation, sections were washed in 2× SSC, 50% dFA, 0.1% *N*-lauroylsarcosine at 50 °C for 15 min, twice. They were rinsed with RNase A buffer (10 mM Tris–HCl; 10 mM EDTA; 0.5 M NaCl pH 8.0) and then incubated with 20 μg/ml RNase A in RNase A buffer at 37 °C for 30 min. Then, sections were washed in 2× SSC; 0.1% *N*-lauroylsarcosine, twice, 15 min each, and in 0.2× SSC; 0.1% *N*-lauroylsarcosine, twice, 15 min each at 37 °C. After sequential washing, sections were rinsed with TS7.5 buffer (0.1 M Tris–HCl; 0.15 M NaCl pH 7.5) and then incubated in 1% Blocking Reagent in TS7.5 for 2 h at room temperature. After blocking, sections were transferred to anti-DIG antibody (1:1000, Roche Diagnostics) in 1% Blocking Reagent. After overnight antibody incubation, sections were washed three times with TS7.5 containing 0.1% Tween20 for 15 min. They were rinsed in TS9.5 buffer (0.1 M Tris–HCl; 0.1 M NaCl; 50 mM $MgCl_2$ pH 9.5) and were incubated in NBT/BCIP (Roche Diagnostics) solution until adequate color development.

Control experiments were carried out, and no signals were observed when sections were processed with both DIG- and fluorescein-labeled sense RNA probes.

Double fluorescent *in situ* hybridization for *NETRIN-G2* and *GAD67*

Sections (20 μm thickness) were used for double fluorescent *in situ* hybridization. Pre-hybridization and washing were the same as the single hybridization protocol. Hybridization was performed in buffer containing both DIG-labeled *NETRIN-G2* and fluorescein-labeled *GAD67* antisense RNA probes at 60 °C overnight. The DIG-labeled probe was detected by anti-DIG antibody conjugated with horseradish peroxidase (Roche Diagnostics) and enhanced with TSA Plus DNP System (PerkinElmer LifeSciences, Boston, MA, USA). The fluorodetection was done by anti-DNP antibody conjugated with Alexa 488 (Amersham Biosciences, Piscataway, NJ, USA). The fluorescein-labeled probe was detected by anti-fluorescein antibody conjugated with alkaline phosphatase. The fluorodetection was done by using HNPP Fluorescent Detection Set (Roche Diagnostics) after immunolabeling.

Table 1. Location of cortical injections

Monkey	FB injection	Survival time (days)	Body weight (kg)
295	V4, TEO	18	4.9
299	Area 4, TEp	15	5.0
263	TEp	17	4.7
301	TEp	15	6.0