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LOCALIZATION OF IMMUNOREACTIVITY FOR DELETED IN COLORECTAL CANCER (DCC), THE RECEPTOR FOR THE GUIDANCE FACTOR NETRIN-1, IN VENTRAL TIER DOPAMINE PROJECTION PATHWAYS IN ADULT RODENTS

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Abstract—DCC (deleted in colorectal cancer)—the receptor of the netrin-1 neuronal guidance factor—is expressed and is active in the central nervous system (CNS) during development, but is down-regulated during maturation. The substantia nigra contains the highest level of netrin-1 mRNA in the adult rodent brain, and corresponding mRNA for DCC has also been detected in this region but has not been localized to any particular neuron type. In this study, an antibody raised against DCC was used to determine if the protein was expressed by adult dopamine neurons, and identify their distribution and projections. Significant DCC-immunoreactivity was detected in midbrain, where it was localized to ventrally displaced A9 dopamine neurons in the substantia nigra, and ventromedial A10 dopamine neurons predominantly situated in and around the interfascicular nucleus. Strong immunoreactivity was not detected in dopamine neurons found elsewhere, or in non-dopamine-containing neurons in the midbrain. Terminal fields selectively labeled with DCC antibody corresponded to known nigrostriatal projections to the dorsolateral striatal patches and dorsomedial shell of the accumbens, and were also detected in prefrontal cortex, septum, lateral habenular and ventral pallidum. The unique distribution of DCC-immunoreactivity in adult ventral midbrain dopamine neurons suggests that netrin-1/DCC signaling could function in plasticity and remodeling previously identified in dopamine projection pathways. In particular, a recent report that DCC is regulated through the ubiquitin–proteosome system via Siah/Sina proteins, is consistent with a potential involvement in genetic and sporadic forms of Parkinson's disease.

Key words: guidance factor, substantia nigra, interfascicular nucleus, striatonigral, mesolimbic, accumbens.

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Abbreviations: DAT, dopamine transporter; DCC, deleted in colorectal cancer; IR, immunoreactivity; MOR, μ-opioid receptor; Nurr1, Nur-related factor 1; PB, phosphate buffer; PD, Parkinson's disease; SNl, substantia nigra pars lateralis; SNv, substantia nigra, ventral tier in pars compacta; SNvd, substantia nigra, ventrally displaced tier in pars reticulata; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

Parkinson's disease (PD) is a progressively disabling, incurable movement disorder caused by the gradual death of dopamine neurons in the substantia nigra pars compacta (Fearnley and Lees, 1991). Recent genetic studies have identified two main cellular systems that appear to be important for this selective degeneration of midbrain dopamine neurons: the ubiquitin proteosome system (Gwinn-Hardy, 2002) and dopamine cell growth and survival systems (Le et al., 2003). Although a majority of genetic mutations identified to date occur in the less common early-onset forms of PD and influence the ubiquitin–proteosome system, the recent identification of mutations in Nur-related factor 1 (Nurr1)—a nuclear receptor critical for the development and maturation of midbrain dopamine neurons—in 10 familial cases of late-onset PD (Le et al., 2003), has focused attention on molecular mechanisms necessary for the development and support of mature dopamine neurons. During development, successive waves of nuclear transcription factors differentiate stem cells into dopamine neurons, and at least two transcription factors contribute to the full maturation of dopamine neurons (Burbach et al., 2003). Nurr1 is one of these, and regulates several genes involved in dopamine synthesis, transport, release, and reuptake. Also expressed in dopamine neurons at the same time as Nurr1 and tyrosine hydroxylase, is the homeobox protein Pitx3. Binding of Pitx3 to the response element of the tyrosine hydroxylase gene results in pronounced up-regulation of its transcription (Cazorla et al., 2000). In contrast to Nurr1, Pitx3 is only found in midbrain dopamine neurons and is reduced in PD (Smidt et al., 1997).

Netrin-1 is part of a family of recently identified guidance factors important for directing axons to their targets (Manitt and Kennedy, 2002). Netrin-1 plays an important role in the development and separation of the patch and matrix compartments in the striatum (Hamasaki et al., 2001), as well as in the development of other neural projections (Metin et al., 1997; Braisted et al., 2000; Schwarting et al., 2001). Deleted in colorectal cancer (DCC) is the high affinity cell surface receptor for netrin-1 and is widely expressed in the developing brain but is down-regulated prior to maturation. However, in adult rodents DCC mRNA has been located in the substantia nigra, striatum and cerebellum (Livesey and Hunt, 1997; Volenec et al., 1997). To determine if DCC is co-localized in dopamine neurons or is selectively expressed by particular types of neuronal projections in these areas, we used an antiserum against DCC in conjunction with markers

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of dopamine neurons (tyrosine hydroxylase [TH], calbindin and dopamine transporter [DAT]) and μ-opioid receptor (MOR), to examine the midbrain and forebrain of adult rats and mice.

EXPERIMENTAL PROCEDURES

Tissue removal

All experiments on animals were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th edn., 2004; http://www.nhmrc.gov.au) and approved by the Animal Ethics Committee of the University of New South Wales. All efforts were made to minimize animal suffering and the number of animals used. Adult male outbred Wistar rats (seven) and C57/Bl6 mice (two) were used in the study. Animals were anesthetized (rats: sodium pentobarbitone 45 mg/kg i.p.; mice: ketamine/xylazine 60 and 10 mg/kg i.p.) and perfused intracardially with freshly made buffered 4% formaldehyde.

Immunofluorescence

Frozen sections (50 μm) were processed as a 1:4 series for dual-labeling immunofluorescence. Sections were treated with 0.1 M phosphate buffer (PB) containing 5% non-immune horse serum, and then incubated for 48 h at room temperature with combinations of primary antisera diluted in PB containing 2% horse serum and 0.2% Triton X-100. The anti-DCC antibody was raised in rabbit against a C-terminal peptide (antiserum 2473), as characterized previously (Seaman et al., 2001). Dilutions from 1:3000 to 1:5000 were optimal for observing bright DCC-immunoreactivity (IR) and low background staining in sections containing midbrain dopamine neurons. This staining decreased in intensity with an antibody dilution of 1:10,000, and could not be reliably detected using a dilution of 1:30,000. A dilution of 1:1000 revealed numerous very weakly stained neuronal cell bodies that were distributed relatively homogenously throughout the neocortex, forebrain and midbrain. The DCC antiserum was used in combination with antiserum raised against TH (host species sheep; Chemicon International, Temecula, CA, USA AB1542; 1:1500), calbindin (mouse monoclonal; Sigma C-8666; 1:7000), DAT (host species rat; Chemicon MAB369; 1:1000) or MOR (host species guinea-pig; Chemicon AB 1774; 1:2000). Secondary antisera conjugated with Cy3 (rabbit) or FITC (other species; Jackson Immunoresearch Laboratories, West Grove, PA, USA) were then applied for 4 h, before washing and coverslipping. Preadsorption testing of the DCC antiserum was performed by mixing various dilutions (1:5000, 1;10,000 or 1:20,000) with 7.8–50 μM of the C-terminal peptide antigen (SEESHKPTEDPASV: Seaman et al., 2001) for 0.5–24 h prior to incubating midbrain and cerebellum sections. This caused a detectable reduction of weak DCC-IR in neurons in the cerebellum but failed to cause a consistent reduction of the bright DCC-IR in the substantia nigra and ventral tegmental area (VTA).

Microscopy and image documentation

Sections were assessed for cellular location of DCC-IR, and expression compared with TH, DAT, calbindin or MOR. Monochrome eight-bit digital images were acquired with a SpotRT camera and were processed only by adjusting saturation and contrast to best resemble the native immunostaining signal. Representative images from key areas have been shown as single or merged pairs (Cy3 and FITC). All observations were made on brain tissue from three to seven rats and two mice. Results from the two species were essentially identical so have been illustrated in detail only for rats.

RESULTS

DCC-IR is preferentially localized in dopamine neurons situated in the ventral tier of the rodent substantia nigra

To establish if DCC-IR is expressed in mesencephalic dopamine neurons, we performed an immunohistochemical analysis using antibodies raised against TH and a peptide fragment of the C-terminal sequence of DCC (Seaman et al., 2001). Fig. 1 illustrates the pattern of DCC-IR seen in a series of coronal sections of adult rat midbrain. Throughout this region, strong DCC-IR was almost completely restricted to dopamine neurons immunoreactive for TH (Figs. 1, 2 and 3). We analyzed rostral-caudal series of midbrain sections in rat and mouse and determined the localization of these neurons within subregions of the A8, A9 and A10 dopamine cell groups, using the atlas of Paxinos and Watson (1997) and the nomenclature of Gerfen and colleagues (Gerfen, 1985, 1992; Gerfen et al., 1987a,b).

In the rostral midbrain, DCC-IR first appeared as a contiguous layer lateral to the midline in A9 dopamine neurons situated in the ventral tier of the substantia nigra pars compacta (SNv) (Fig. 1). DCC-IR was rarely detected in dopamine neurons in the dorsal tier of the SNv, in the substantia nigra pars lateralis (SNl), or in the adjacent retrorubral fields. This basic pattern of localization observed in the rostral midbrain was maintained in the medial and caudal midbrain (Fig. 1c, d), except at these levels, DCC-IR was also localized to ventrally displaced dopamine neurons in the substantia nigra pars reticulata (SNvd).

Calbindin-IR was used to further investigate the apparent preferential localization of DCC-IR in ventral tier dopamine neurons (Gerfen et al., 1987a; Gerfen, 1985; McRitchie et al., 1996). This staining revealed that most of the DCC-IR neurons in the pars compacta were situated ventral to the mass of calbindin-IR neurons in the dorsal tier, and ventral to the adjacent dense zone of calbindin-IR fibers in the dorsomedial pars reticulata (Fig. 2e–h). Although co-localization of DCC- and calbindin-IR was rare in the substantia nigra, significant numbers of double-immunostained neurons were identified in medial aspects of the pars compacta (Fig. 2d) that corresponded to a region identified as the pars medialis by some reports (McRitchie et al., 1996).

In the A10 cell group, DCC-IR was predominantly localized in small dopamine neurons situated in the interfascicular nucleus (Figs. 1b, c and 3a, c). DCC-IR dopamine neurons were rarely observed outside of this subregion of the A10 cell group, and these were mostly situated adjacent to the interfascicular nucleus in the rostral or caudal linear nuclei or along the medial margin of the ventral tegmental area (VTA). As was found in the A9 cell group, calbindin-IR also rarely co-localized with DCC-IR A10 dopamine neurons (Fig. 3d–f). No DCC-IR neurons were present in the extension of the A10 cell group in the supramammillary nucleus, nor were they detected in any of the more rostral dopamine cell groups in the hypothalamus (Fig. 4a, c). However, some DCC-IR