Short communication

Peptidergic input to immunohistochemically-identified Renshaw cells

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Abstract

Peptidergic influences on Renshaw cells were assessed in rat using gephyrin-immunoreactivity, as a Renshaw cell specific marker, in combination with substance P, calcitonin gene-related peptide- and nicotinic acetylcholine receptor-immunolabelling. An average of 3.9 substance P-, and 8.1 calcitonin gene-related peptide-, and 16.3 nicotinic acetylcholine receptor-immunoreactive close contacts or puncta were observed per Renshaw cell. Most appositions were somatic. These results provide neuroanatomical support for the peptidergic modulation of Renshaw cells.

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The control of Renshaw cell activity involves a complex interaction of numerous excitatory and inhibitory influences utilizing cholinergic, glycinergic, and serotonergic signalling molecules [1,2,8,9]. Evidence suggests that peptidergic modulation of this classical neurotransmitter input may also play a significant role in the neurochemical signal transduced by Renshaw cells. There is abundant pharmacological evidence describing the modulation of cholinergic-mediated Renshaw cell activity via substance P (SP)/nicotinic receptor interactions [6,16–18]. In addition, the localization of calcitonin gene-related peptide (CGRP) in α-motoneurons and its release at the neuromuscular junction [3] suggests that CGRP is a motoneuronal neuro-signalling molecule in the periphery. Centrally, there is electron microscopic evidence to suggest that CGRP may be present in recurrent axon collateral terminals presynaptic to putative Renshaw cells [10].

Here, we examined the abundance and localization of peptidergic input to, and the organization of nicotinic acetylcholine receptors (nAChR) upon, immunohistochemically-identified Renshaw cells [1,2,8,9]. A portion of these results have been reported in abstract form [7].

Adult, male Sprague–Dawley rats (n = 9) were perfused with 0.9% saline containing 0.1% sodium nitrite followed by either 4% paraformaldehyde alone or 4% paraformaldehyde and 0.16% picric acid in 0.1 M phosphate buffer (pH 7.4). The spinal cords were quickly removed and placed in the same fixative for 2 h followed by immersion, for at least 2 days, in cold (4°C) 25% sucrose and 10% glycerol in 50 mM phosphate buffer. Twenty micron transverse sections of midlumbar spinal cord were then cut on a freezing sliding microtome and collected into 0.1 M phosphate buffer with 0.9% saline (PBS). The tissue was then incubated for 48–72 h at 4°C in a primary antibody cocktail consisting of anti-gephyrin (mouse; 1:400; Cedarlane Labs) primary antibody combined with either anti-SP (rabbit; 1:1000; Chemicon), anti-CGRP (rabbit; 1:10 000; Peninsula) or anti-nAChR (rat; 1:10; Developmental Hybridoma Bank; see Refs. [20,21] for complete characterization) primary antibody diluted in 0.1 M phosphate buffer with 0.9% saline and 0.3% Triton X-100 (PBS–T). Following the primary incubation, sections were washed twice in PBS–T and incubated 1.5 h in FITC-conjugated donkey anti-mouse IgG (for gephyrin visualization) and CY3-conjugated donkey anti-rabbit (for SP and CGRP visualization). All secondary incubations were conducted at 1:100 dilution in PBS–T using Jackson
ImmunoResearch immunochemicals. The tissue was then washed for 20 min in PBS-T followed by a 20 min wash in 50 mM Tris–HCl (pH 7.4), mounted onto gel-coated slides from 50 mM Tris–HCl (pH 7.4), dried, and coverslipped with Vectashield (Vector) anti-fade mounting media.

Neurons displaying characteristic gephyrin-immunofluorescence were identified as Renshaw cells based on the previous demonstration that intracellularly-labeled, electrophysiologically-identified Renshaw cells display a unique staining pattern of gephyrin [1,2,8,9]. Qualitative and quantitative analyses of the presence and distribution of SP-immunoreactive (-IR) or CGRP-IR close contacts, and nAChR-IR puncta, on immunohistochemically identified Renshaw cells were conducted on neuronal profiles identified in single spinal cord sections using standard fluorescence microscopy (Olympus BX–60) and video images captured at 8 bits per pixel (framegrabber;) with a Dage-MTI CCD-300-RC video camera or laser scanning confocal microscopy (Olympus Fluoview). In order to resolve discrete puncta, all quantification of immunofluorescent puncta or close appositions on Renshaw cell profiles were conducted using a 100 × oil immersion UPlanFl iris-equipped objective. Figures were generated using Image Pro Plus (Media Cybernetics) and CorelDRAW (Corel). Statistical analysis of the data was conducted using SigmaStat (Jandel).

In single, transverse sections of rat lumbar spinal cord, Renshaw cell profiles were identified by characteristic large, abundant and intense gephyrin-IR clusters on their perikarya and proximal dendrites. All putative Renshaw cell profiles were similar in appearance, abundance and location to that previously described [1,2,8,9]. The somata and proximal dendrites of 481 Renshaw cells were examined to determine the distribution and abundance of either close contacts arising from immunoreactive peptidergic terminal enlargements or anti-nicotinic receptor-immunofluorescent puncta.

In gephyrin and substance P immunoreacted tissue, 730 SP-IR axonal varicosities were localized in close apposition to 184 Renshaw cell somata or proximal dendrites (Fig. 1A, 1B; Table 1); 69% of the boutons made close contacts with Renshaw cell somata while the remainder were present adjacent to proximal processes. SP-IR close contacts ranged in size from 0.5 to 3 μm in diameter. Occasional SP-IR boutons were observed immediately apposed to large gephyrin clusters (Fig. 1B). In tissue simultaneously reacted with gephyrin and nicotinic acetylcholine receptor primary antibodies, 2314 nAChR-immunofluorescent puncta were observed on 142 Renshaw cell somata or proximal dendrites (Fig. 1C; Table 1). Most (96%) of the puncta were localized on Renshaw cell somata. All dendrite-associated puncta were located on those portions of the processes immediately adjacent to the perikarya. Unlike the punctate nAChR-immunoreactivity observed on Renshaw cells, numerous neighboring putative motoneurons and unidentified interneurons also displayed an intense, cytoplasmic nAChR-immunoreactivity (Fig. 1C). In gephyrin and calcitonin gene-related peptide immunoreacted tissue, 1261 CGRP-IR axonal varicosities were localized in close apposition to 155 Renshaw cell somata or proximal dendrites (Fig. 1D; Table 1); 89% of the boutons made close contacts with Renshaw cell somata while the remainder were present adjacent to proximal processes. CGRP-IR boutons were all small and appeared to be composed of clusters of multiple, distinct, very small (<0.5 μm) puncta.

As motoneuronal recurrent axon collateral input is a defining characteristic of Renshaw cells [13], numerous post- and pre-synaptic aspects of this cholinergic signalling mechanism have been investigated using electrophysiological, pharmacological and neuroanatomical methodologies. Evidence suggests that, in addition to motoneuron-Renshaw signalling via classic acetylcholine/cholinergic receptor interactions, neuropeptides could play important modulatory roles in this motor pathway. The presence of CGRP in motoneurons has been well described and it has been demonstrated to have both transmitter-like and discrete puncta, all quantification of immunofluorescent CGRP in motoneurons has been well described and it has been acknowledged.

Statistical analysis of the data was conducted using SigmaStat (Jandel). Collateral terminals in the ventral horn of the spinal cord is sparse. A brief report has described moderate CGRP immunoreactivity in axon collaterals impinging upon putatively identified Renshaw cells [10]. These ‘Renshaw elements’ were also found to be depleted of immunoreactive CGRP following supramaximal antidromic stimulation of sciatic nerve. Here, we provide quantitative evidence regarding the abundance and distribution of CGRP-IR varicosities closely apposed to Renshaw cells. Although these close contacts are consistent with both the presence of this peptide in α-motoneurons and the known motoneuron–Renshaw cell circuitry, it has been shown that the majority of cholinergic terminals, as labelled by vesicular acetylcholine transporter (VACHT), are apposed to Renshaw cell dendrites [2]. As 89% of the CGRP-IR appositions detailed here were localized on Renshaw cell somata, this suggests that CGRP- and VACHT-IR terminals may represent different axonal systems. Although a direct primary afferent input to Renshaw cells has not been described [5], non-motoneuronal sources for the CGRP-IR terminals, such as descending pathways [14], must be acknowledged.

Pharmacological evidence exists supporting a peptidergic influence on Renshaw cells. Iontophoretic application of substance P was found to abolish both cholinergic and motoneuron recurrent collateral induced [6,16–18] excitation of Renshaw cells. It was proposed that this effect was mediated by a substance P/nicotinic acetylcholine receptor interaction, as a nicotinic acetylcholine receptor antagonist (dihydro-β-erythroidine) interfered with the actions of...
Fig. 1. Peptidergic boutons forming close appositions to, and nAChR-IR puncta on, gephyrin labelled Renshaw cells in rat lumbar spinal cord. In all micrographs green (FITC) corresponds to gephyrin immunofluorescence and red (CY3) corresponds to SP, CGRP or nAChR immunofluorescence. (A) A gephyrin immunoreactive Renshaw cell somata (green) is apposed by a single SP-immunoreactive fiber (red) with multiple small en passant and terminal enlargements (arrows). (B) A gephyrin immunoreactive Renshaw cell somata (green) is apposed by numerous SP-immunoreactive puncta (red). Occasional SP-immunoreactive puncta appear directly apposed to gephyrin immunoreactive clusters (arrow). (C) A gephyrin immunoreactive Renshaw cell somata (green; cell in center of image) displays numerous nAChR-immunoreactive puncta (red). Adjacent, unidentified neurons (asterisks) display intense, cytoplasmic nAChR-immunoreactivity and no gephyrin immunofluorescence. The inset shows the Renshaw cell with nAChR labelling only. (D) A gephyrin immunoreactive Renshaw cell somata (green) is apposed by numerous CGRP-immunoreactive (red) appositions (arrows). The fine axon connecting these en passant enlargements cannot be seen in the image. Note that the CGRP-immunoreactive boutons appear to be composed of multiple small immunoreactive puncta. The inset, obtained at a different focal plane, shows the gephyrin immunoreactive clusters on the Renshaw cell. The CGRP-immunoreactive puncta remain visible at the upper edge of the cell. Scale bars in microns. All micrographs are of transverse sections from the L4 spinal segment. Dorsal is to top.
substance P on Renshaw cells [18]. Both nicotinic and muscarinic acetylcholine receptors have been shown to have powerful excitatory effects on Renshaw cells [11,12,15,19]. Our demonstration here of nAChR-immunoreactivity associated with Renshaw cells lends anatomical support for the presence of nicotinic receptors on these cells and suggests that they have a somatic localization. The apparent incongruity between the dendritic distribution of VACHT-labelled cholinergic terminals and the somatic distribution of nAChR-IR puncta may be a function of a differential distribution between muscarinic and nicotinic acetylcholine receptors or a selective difference in antibody recognition due to receptor subunit composition of the nicotinic receptors.

Renshaw cells appear to receive a higher density of substance P-IR close contacts (3.9 close contacts/RC) as compared to 5-HT-IR boutons (2.8 close contacts/RC) [9]. In the ventral spinal cord it has been demonstrated that not all substance P immunoreactive fibers contain 5-HT-immunoreactivity and vice versa [4,22]. This agrees with our observations that not all substance P immunoreactive fibers in the immediate vicinity of Renshaw cells were double labelled for 5-HT transporter (unpublished observations). This, in combination with the quantitative difference between the number of substance P and 5-HT close contacts on Renshaw cells, suggests that not all substance P-IR input to Renshaw cells is derived from descending serotonergic fibers. Efforts at labelling substance P receptors (NK-1) on Renshaw cells did not reveal labelled puncta or cytoplasmic/membrane labelling as observed in the dorsal horn (not shown). The complementary distribution between substance P and VACHT-IR close contacts on Renshaw cells may indicate that the pharmacologically-mediated substance P/cholinergic interactions occur via a presynaptic or paracrine influence. However, the correspondence between the somatic distribution of both substance P- and nAChR-IR suggests that peptidergic modulation of cholinergic neurotransmission could occur at somatically localized nicotinic receptors.

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