Direct cortico-motoneuronal synaptic contacts are present in the adult rat cervical spinal cord and are first established at postnatal day 7

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Abstract

In order to demonstrate direct cortico-motoneuronal synaptic contacts in the cervical spinal cord of the rat and to determine at which postnatal age these contacts are established, an electron microscopic study using double labelling was performed. Corticospinal axons were anterogradely labelled after horseradish peroxidase (HRP)-gel implantation into the cerebral motor cortex and motoneurons were retrogradely labelled after cholera toxin subunit B conjugated to HRP (CTB-HRP) injections into the distal forelimb flexor muscle. With the histochemical procedures used, both tracers yield similar needle-like crystalline deposits. Labelled axons, however, can be well differentiated from labelled motoneuronal dendrites and somata on morphological grounds. In adult rats, direct cortico-motoneuronal contacts were encountered. Experiments in developing postnatal rats demonstrated that these synapses are first present on postnatal day 7.

Keywords: Corticospinal tract; Motoneurons; Synaptic contacts; Cervical spinal cord; Double labelling; Electron microscopy; Rat

The corticospinal tract (CST) is the latest central fibre system to develop in mammals. In the rat, its outgrowth into the spinal cord, its subsequent target finding and synaptogenesis even comes about postnatally (e.g. [8,19,21]). It thus provides a suitable model for studying developmental events. Understanding of the events occurring during central nervous system development might provide strategies to increase the regenerative capacities of the brain and spinal cord after damage. Previously, we have investigated the outgrowth of the CST into the cervical spinal grey. It appeared that the CST fibres overgrow their target during the first postnatal week, whereas later on redundant fibres are eliminated [4]. Because of the involvement of the CST in especially the voluntary flexion movements of the distal forelimb (e.g. [1,2,15,20]), we also examined the postnatal development of motoneurons (MNs) innervating the muscles involved [3]. It was shown that distal flexor MN dendrites increase in number and extension in the first postnatal week, followed by a decrease later on. From the two studies it was further concluded that the CST projection area largely overlaps these dendrites.

In order to investigate the influence of the target upon the development of the CST, it needs to be determined whether flexor MNs are a direct or an indirect target of the CST, and thus, whether direct synaptic contacts exist between the CST and flexor MNs. Although indications of the existence of direct synaptic cortico-motoneuronal contacts were presented electrophysiologically [6] and light microscopically [12], evidence at the electron microscopic level is still lacking. In the present study a method is presented to demonstrate these contacts in the adult rat and, in addition, we investigated at what age these contacts are formed. Ideally, such a technique needs to fulfil four criteria. Firstly, CST axons and flexor MN somata and dendrites need to be labelled massively, making it easier to find synapses in ultrathin sections. Secondly, survival times need to be short, especially when a developmental study is performed. Thirdly, the ultrastructure needs to be sufficiently conserved to recognize synaptic structures. Fourthly, the labelling should allow discriminating between labelled axons and labelled somata and dendrites, respectively. Pilot studies revealed that only the combination of anterograde horseradish peroxidase (HRP) labelling in conjunction with retrograde cholera
toxin subunit B conjugated to HRP (CTB-HRP) labelling, with the histochemical detection of HRP, fulfilled the first three criteria. Several other tracers proved to be less suitable, such as Phaseolus vulgaris leucoagglutinin (long survival times, bad ultrastructure), neurobiotin, wheat germ agglutinin, and dextran amines (all of them resulting in a small number of labelled fibres). A disadvantage of the method employed is that CST axons cannot be discriminated from MN somata and dendrites on the basis of the morphology of the reaction product, which in both cases consists of needle-like crystalline deposits. We demonstrate in this study, however, that this differentiation can easily be made on the basis of the ultrastructure of axons versus somata and dendrites, respectively.

Postnatal Wistar rats (Central Animal Laboratory, University of Nijmegen) of either sex, varying in age from the day of birth (postnatal day 0, P0) to young adult (P60) were used. At least three animals per age group were examined; the ages of the rats in this paper are the ages at their respective days of sacrifice.

Corticospinal axons were labelled by HRP-gel implantation, as previously described [4]. In brief, rats were anaesthetized with sodium pentobarbital (increasing with age: 18–60 mg per kg body weight, i.p.), the skin over the skull was incised and using a fine needle three to six small holes were made into the skull and the underlying cerebral cortex encompassing the entire left sensorimotor cortex. HRP (Boehringer Mannheim, grade I)-gels [9] were implanted effectuating a gradual release of the tracer. Flexor MNs were labelled by CTB-HRP injection, as previously described [3]. In brief, the skin of the forepaw was incised ventrally to expose the right flexor (FLEX)-muscles. Using a 5 μl Hamilton syringe fitted with a glass micropipette, 0.5 μl of a 0.1% CTB-HRP solution (List Biological) was pressure injected into the FLEX-muscles.

Since recurrent collaterals and sensory afferents from the injected muscle synapsing directly on the labelled MNs would lead to false-positive direct cortico-motoneuronal synaptic contacts, in each age-group animals in which the HRP-gel implantation into the cortex was omitted were also analyzed. Further processing was identical to the double-labelled material.

After the optimal survival time (48 h for P0–P21, and 72 h for P60 as previously determined) the rats were reanaesthetized (sodium pentobarbital, increasing with age: 25–90 mg per kg body weight, i.p.) and transectially perfused with ice-cold 0.1 M phosphate buffered saline (PBS; pH 7.4) followed by 1% paraformaldehyde, 2% glutaraldehyde and 5% sucrose in 0.1 M phosphate buffer (PB; pH 7.4). After perfusion the brain and spinal cord were dissected from the skull and spine, respectively, postfixed by immersion for 2 h in the above mentioned fixative and immersed in 5% sucrose in PB (pH 7.4). Using a vibratome, 50 μm sections were cut from cervical spinal segments 7 and 8 (C7 and C8, respectively). The sections were collected in PB (pH 7.4) and immediately processed according to the protocol of Joosten et al. [10]. In brief, the sections were rinsed in PB (pH 6.0), pre-incubated in tetramethylbenzidine-ammoniumhexamolybdate medium (250 mg AHM and 5 mg TMB (dissolved in absolute ethanol) in 100 ml PB, pH 6.0) for 20 min. The incubation was started by adding 50 μl 30% H2O2 per 100 ml pre-incubation medium every 5 min for a total of 20 min. The reaction was terminated by rinses in PB (pH 6.0). Thereafter the sections were osmificated in 1% OsO4 in PB (pH 5.0) for 4 h, rinsed in PB (pH 5.0), dehydrated in increasing concentrations of ethanol, rinsed in aceton, and finally embedded in Epon 812 on re-epoxyed slides. After light-microscopic examination, selected sections were photomicrographed under bright field illumination using an automatic Zeiss photomicroscope II and further processed for electron microscopy.

Semi-thin and ultra-thin sections were cut using a Reichert-Jung ultracut microtome. Semi-thin sections were counterstained with toluidine-blue and examined with a Zeiss light microscope to select the field of interest. Ultrathin sections were collected on 75-mesh formvar coated copper grids, contrasted with uranyl acetate for 20 min and lead citrate for 10 min, and examined in a Philips EM 301 or a Jeol EM 1010 at an accelerating voltage of 60 kV.

Light microscopic analysis of the vibratome sections revealed that both the CST and the flexor MNs and their dendrites were well labelled in the cervical spinal cord segments 7 and 8 (C7–C8) at all postnatal ages investigated including the adult. The area containing the MN somata and presumed dendrites was trimmed and further processed for electron microscopy (Fig. 1A).

Examination of ultrathin sections of single labelled material, i.e. from animals which received only CTB-HRP injections into the flexor muscles, never revealed double labelled synapses, i.e. synapses containing HRP reaction product in the pre- and post-synaptic element of a synaptic terminal, at any of the ages investigated. Up until P5, labelled axon terminals were found, probably belonging to primary afferents, but never in the area where labelled dendrites were encountered, as was also noted at the light microscopic level [3]. The anterograde transport rate was likely to be too slow to label these axons in the field of interest. It can thus be concluded that the double labelled synapses found after both anterograde and retrograde labelling reflect direct cortico-motoneuronal contacts.

In ultrathin sections axons could be well discriminated from dendrites and somata on the basis of their ultrastructural morphology. MN somata are characterized by, among others, clusters of ribosomes (Fig. 1B), axon terminals by synaptic vesicles and mitochondria (Fig. 1C,D), and dendrites by microtubular structures and a relatively low amount of electron-dense material (Fig. 1C,D) [18]. Besides, the HRP crystals in general tended to be smaller...
Double-labelled synapses could first be found at P6. Already present in the area of the labelled MN dendrites, double-labelled synapses showed a preference for MN dendrites. Examination of orthoantibody sections at P6 revealed no obvious synapses at the MN somata. In contrast, MN dendrites were already suggested from P1 as being contacted by round vesicles. Round vesicles (Fig. 1C) were widely scattered on MN dendrites and were in contact with MN dendrites and MN soma. The needle penetration here was able to locate round vesicles (Figs. 1C), and this was most likely due to the round vesicles (Fig. 1D) and the most likely MN dendrites in the CST and MN dendrites. The round vesicles were located in the CST and MN dendrites, which might suggest that those contacts were scattered on MN dendrites and were in contact with MN dendrites and MN soma and dendrites. This also indicates that the CST axons might be housed in MN somata and dendrites. This affects the restriction of the CST and MN dendrites.
In addition, we have demonstrated that corticomotoneuronal contacts are generated between P5 and P7, being the ages at which CST axons start to overlap with flexor MN dendrites [3,4]. It is reasonable to assume that at later ages more synapses are added since the terminal field of the CST still increases thereafter [4]. It would be very interesting to know if any transient contacts are formed between the CST and flexor MNs, in analogy to other fibre systems with their respective targets (e.g. [7, 11, 13, 14, 16, 17]). Using the expression of the immediate early gene c-fos after kainate stimulation of the motor cortex, transient contacts were found between the CST and spinal interneurons [5]. The technique described in the present paper is suited for a detailed quantitative electron microscopic study in order to tackle this question.