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CLIMBING FIBRE COLLATERALS CONTACT NEURONS IN THE CEREBELLAR NUCLEI THAT PROVIDE A GABAERGIC FEEDBACK TO THE INFERIOR OLIVE

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The inferior olive provides climbing fibres to Purkinje cells in the cerebellar cortex and gives off axon collaterals to the cerebellar nuclei. The cerebellar nuclei contain GABAergic neurons that provide an inhibitory projection to the inferior olive and excitatory neurons that influence behaviour through various other premotor nuclei in the brainstem and diencephalon. Whether the olivary axon collaterals innervate the GABAergic neurons in the cerebellar nuclei is unknown. In the present study we investigated this projection in mice at the ultrastructural level using post-embedding GABA immunocytochemistry and anterograde and retrograde tracing of biotinylated dextrane amine and gold–lectin. It is demonstrated that the olivary axon collaterals do not only innervate non-GABAergic neurons in the cerebellar nuclei, but also GABAergic nucleo-olivary cells, thus establishing a direct feedback loop to the inferior olive. © 1997 IBRO. Published by Elsevier Science Ltd.

Climbing fibres originate in the contralateral inferior olive of the medulla oblongata and synapse on the dendritic stems of Purkinje cells in the cerebellar cortex.[7,8,21] The climbing fibre projections are organised such that all olivary subdivisions project to one or two particular zones of Purkinje cells.[9,10] The Purkinje cells of one sagittal zone in turn project to a particular set of cerebellar or vestibular nuclei.[6,23,26] The motor control unit composed of an olivary subdivision, a cerebellar sagittal zone and a cerebellar subnucleus has been referred to as a module.[26] Purkinje cell axons innervate two types of neurons in the cerebellar and/or vestibular nuclei:[3] the inhibitory neurons that provide a GABAergic projection to the inferior olive and the excitatory neurons that influence (pre)motor centres in other areas of the brainstem and diencephalon. The GABAergic fibres in the inferior olive that are derived from the cerebellar nuclei innervate, for the most part, dendritic spines that are connected by gap junctions.[5] The function of this projection is probably to regulate the electrotonic coupling of olivary neurons.[13,16,27]

Olivary neurons not only provide climbing fibres to the cerebellar cortex, but also give off collaterals to the cerebellar nuclei.[24] These collateral projections are topographically organized with respect to the boundaries set by the different modules.[26] It was unknown whether the climbing fibre collaterals innervate both the inhibitory and excitatory neurons in the cerebellar nuclei. To address this issue we investigated the olivonuclear projection in mice using a new electron microscopic triple labelling method combining post-embedding GABA immunocytochemistry, anterograde tracing of biotinylated dextran amine (BDA), and retrograde tracing of a gold–lectin tracer.

Following injection of BDA in the inferior olive anterograde labelling was observed in the restiform body, cerebellar nuclei, and climbing fibre zones in the cerebellar cortex (Fig. 1). The BDA injections made in different sets of olivary subnuclei resulted in labelling in different climbing fibre zones and contralateral cerebellar nuclei as described previously for the mouse.[1] The anterogradely BDA-labelled collaterals of the olivary axons derived from the principal olive, rostral medial accessory olive, and rostral dorsal accessory olive gave off boutons in the lateral cerebellar nucleus, posterior interposed cerebellar nucleus, and anterior interposed nucleus, respectively. The most prominent collateral projection was that from the ventrolateral outgrowth to the ventromedial cerebellar nucleus. In addition, we observed some varicosities in the vestibular nuclei. Because no

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Abbreviations: BDA, biotinylated dextran amine; CF, climbing fibre; DAO, dorsal accesory olive; DMCC, dorsomedial cell column; MAO, medial accesory olive; P-cell, Purkinje cell; PO, principal olive; VLO, ventrolateral outgrowth.

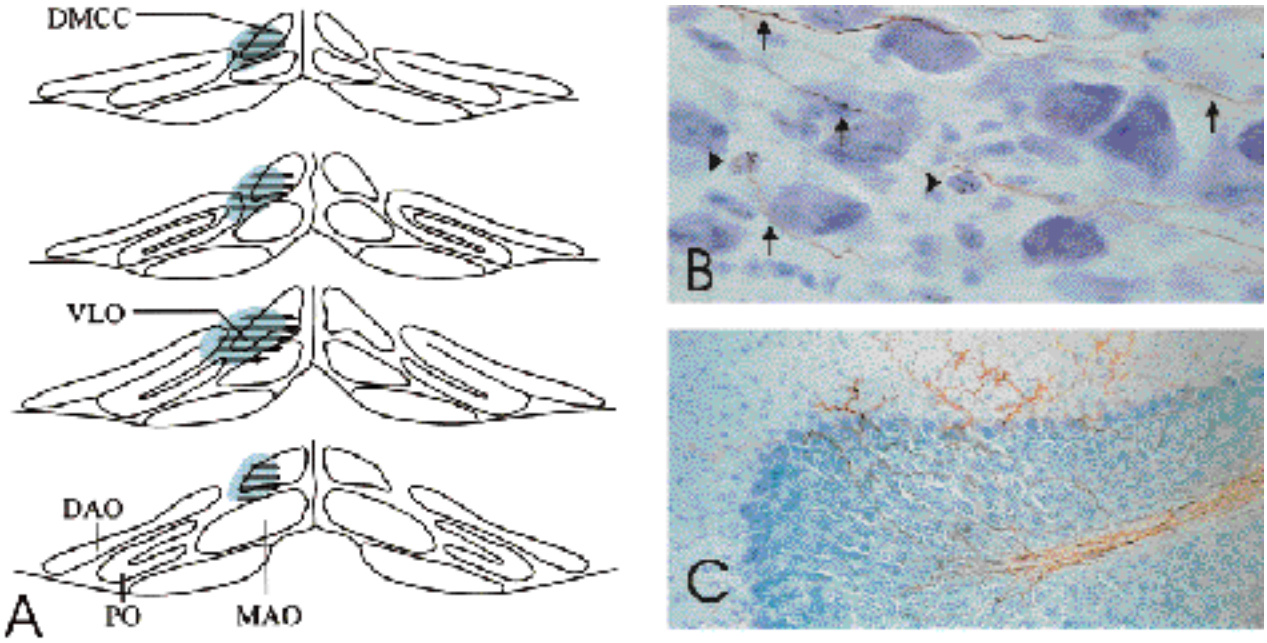

Fig. 1. Light microscopic demonstration of BDA and gold–lectin injection and projection sites. A) An example of a BDA (grey) and a gold–lectin (hatched area) injection site centred in the ventrolateral outgrowth (VLO) of the inferior olive in a mouse. Note that the injections overlap. (DAO, dorsal accesory olive; PO, principal olive; MAO, rostral medial accesory olive; DMCC, dorsomedial cell column.) B) Anterogradely BDA-labelled fibres (arrows) and retrogradely gold–lectin-labelled neurons (arrowheads) in the lateral cerebellar nucleus. C) BDA-labelled climbing fibres in the flocculus derived from the VLO. Note the absence of mossy fibres in the granular layer. The inferior olives of six mice (B6CBACa strain; Jackson Laboratories) were either unilaterally or bilaterally injected with both BDA (Molecular Probes, Inc.) and gold–lectin.[19] General anaesthesia was induced and maintained with a mixture of Ketamine (50 mg/ml) and Xylazine (2.8 mg/ml) in sodium chloride (3.6 mg/ml) according to the instructions of the Erasmus Animal Care Committee; the initial dose was 0.07 ml i.p. supplemented with 0.03 ml i.p. every 35 min. The animals were mounted in a stereotactic device, and access to the brainstem was obtained by a midline incision and by spreading the neck muscles laterally. The injections were made with double-barrel glass micropipettes with two tip diameters of 15 µm; one barrel was filled with 10% BDA in 0.1 M phosphate buffer and one barrel was filled with 250 nl gold–lectin.[19] The location of the inferior olive was verified by recording through the BDA-filled pipette the characteristic shape and rhythm of the action potentials of its neurons.[19] The BDA was injected iontophoretically (4 µAmp positive current, 8 min, 50% duty cycle) after gold–lectin had been injected useing pressure pulses. Subsequently, the dura and neck muscles were repositioned, and the skin was sutured. Five days after surgery, the animals were deeply re-anaesthetized with ketamine and xylazine, and transcardially perfused with 10 ml NaCl and 50 ml 2.5% glutaraldehyde and 0.5% paraformaldehyde in 0.1 M cacodylate buffer. The brainstem and cerebellum of these animals were removed, cryoprotected in sucrose, embedded in gelatin, and cut transversely on a cryotome in 40 µm sections. Subsequently, the sections were reacted with the ABC complex and diaminobenzidine to visualize the BDA, and silver intensified to enhance the gold labelling.[6,19]

labelled mossy fibres were found, the BDA-labelled collaterals in the cerebellar and vestibular nuclei cannot be mossy fibre collaterals originating from neurons in the reticular formation surrounding the inferior olive.

In all animals the retrograde tracer gold–lectin was injected in the same set of olivary subdivision(s) as the anterograde tracer BDA (Fig. 1A). In all these cases the distribution of retrogradely-labelled neurons in the cerebellar nuclei overlapped with that of the BDA-labelled boutons (Fig. 1B); thus, the pattern of labelled cells was in line with the modular organisation of the olivocerebellar system. Generally, the retrogradely-labelled neurons in the cerebellar nuclei were smaller than the surrounding non-labelled neurons; neurons with a diameter larger than 30 µm were not labelled. In all animals at least 100 gold–lectin-labelled neurons appeared to be contacted by BDA-labelled fibres.

Electron microscopic analysis of the BDA-labelled boutons in the cerebellar nuclei of three mice demonstrated that the olivary climbing fibre collaterals indeed made direct synaptic contacts with the gold–lectin-labelled neurons (Fig. 2). The BDA-labelled terminals were packed with clear spherical vesicles and they established asymmetric synapses with distal and proximal dendrites, and also with cell bodies. In total, we analysed 316 BDA-labelled, synapse containing terminals; 265 were obtained from the ventromedial cerebellar nucleus while the remaining 51 terminals were obtained from the interposed nuclei. Fourteen of all terminals (obtained from three

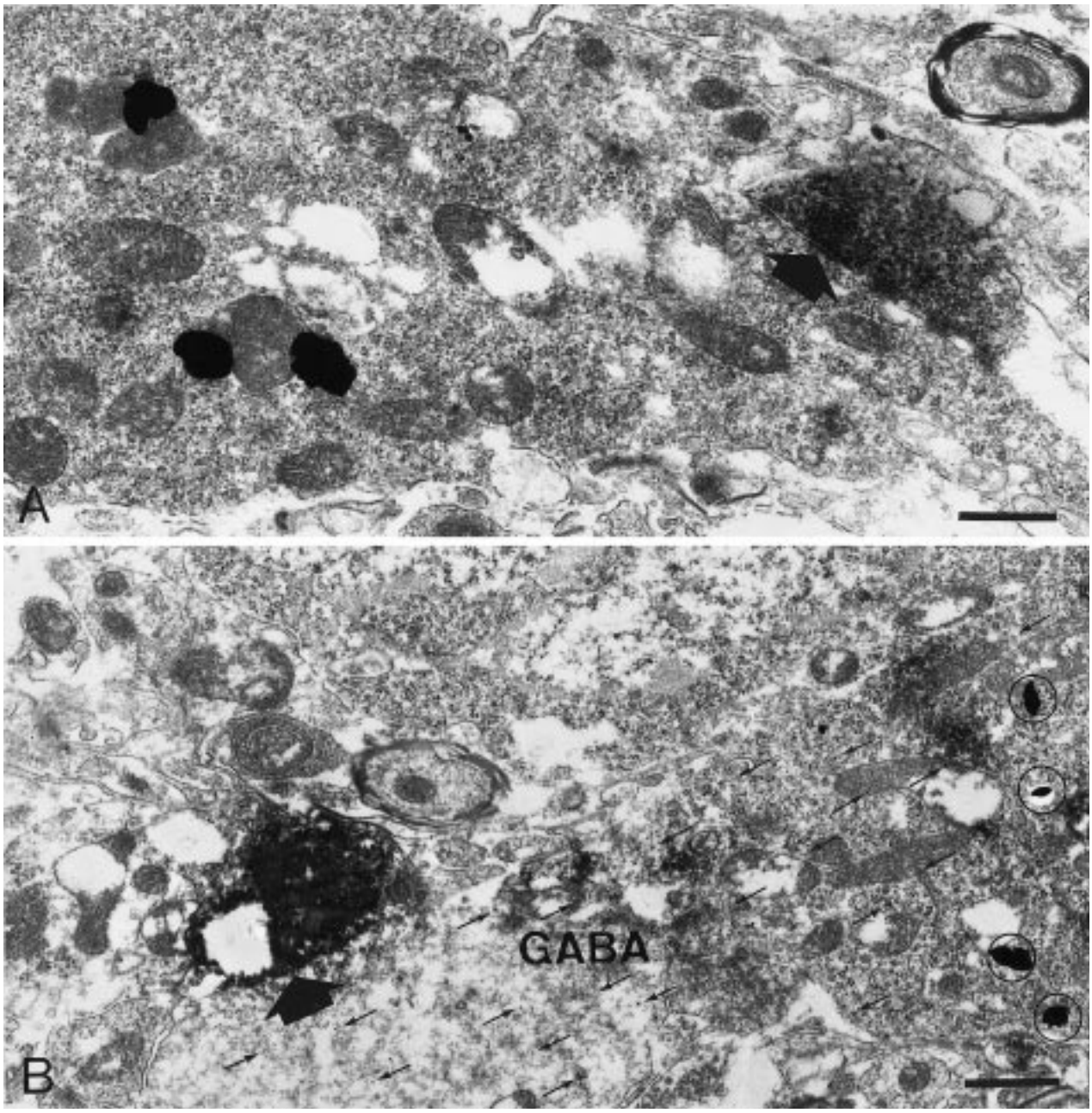

Fig. 2. Electron microscopic demonstration of direct contacts between climbing fibres collaterals and GABAergic cerebellar neurons that project to the inferior olive. A) BDA-labelled climbing fibre collateral (right) establishing a synapse (arrow) with a cerebellar neuron retrogradely labelled from the inferior olive with silver intensified gold–lectin (big black dots on the left). This micrograph was taken from an ultrathin section that was not processed for GABA immunocytochemistry. B) Ultrathin section of same tissue as in A processed for post-embedding GABA immunocytochemistry (gold particles, 15 nm in diameter, are indicated by small arrows). The silver intensified gold–lectin deposits on the right are indicated by a surrounding circle. Arrow indicates synapse. Scale bars in A and B=0.69 μm and 1.01 μm, respectively. Three mice (B6CBACa strain; Jackson Laboratories) were processed for electron microscopy; their brainstem and cerebellum were cut on a Vibratome in 70 μm sections. The sections were reacted with the ABC complex, osmicated in 1% OsO_4 in an 8% glucose solution (allowing light microscopic examination of the Vibratome sections after the osmication, and reducing the occurrence of potentially artificial silver reaction products), silver intensified, and embedded in Durcupan.[5] Ultrathin sections obtained from all three animals were cut on a Reichert ultratome in 50 nm sections, alternatingly processed for post-embedding GABA immunocytochemistry, counterstained with uranyl acetate and lead citrate, and analysed in a Philips electron microscope (CM 100).[4]

animals) contacted directly gold–lectin-labelled proximal dendrites and/or cell bodies. Twelve of these 14 direct synaptic contacts were present in the ventromedial cerebellar nucleus, while two terminals were observed in the posterior interposed nucleus and anterior interposed nucleus. In the sections that were processed for post-embedding GABA immunocytochemistry with the use of gold particles[4] we observed that all retrogradely gold–lectin-labelled neurons (n=176), including those that were contacted by BDA-labelled terminals, were GABAergic (Fig. 2B). Since all olivary projecting neurons in the cerebellar nuclei are known to be GABAergic,[4] this observation confirms our conclusion that the retrogradely gold–lectin-labelled neurons were derived from the inferior olive and not the surrounding reticular formation. On the other hand, it should be noted that BDA-labelled terminals also frequently showed synaptic contacts with GABAergic profiles that were not retrogradely labelled with gold–lectin (n=37). This indicates that not all olivary projecting neurons in the cerebellar nuclei were retrogradely labelled with gold–lectin and/or that climbing fibre collaterals also contact GABAergic interneurons in the cerebellar nuclei.[3]

Apart from their synaptic contacts with GABAergic and gold–lectin-labelled structures, many BDA-labelled terminals contacted non-labelled dendrites and cell bodies (n=131). Presumably, many of the non-labelled distal dendrites of this category have been interpreted falsely as being part of neurons that do not project to the inferior olive because peripheral dendrites are difficult to label with post-embedding GABA immunocytochemistry and/or retrograde gold–lectin tracing. However, at least some of the non-labelled neurons (n=8) contacted by the BDA-labelled fibres must have been neurons that do not project to the inferior olive, because their cell body had a diameter larger than 30 μm, indicating that they belong to the category of excitatory premotor neurons that project to non-olivary brainstem nuclei or the diencephalon.[22]

In the present study the connections between the inferior olive and the cerebellar nuclei in mice were investigated at both the light microscopic and electron microscopic level. The topographic organisation of the nucleo-olivary connections and the olivocerebellar modules found in this study agree with those described for rat and cat.[10,20,24] We demonstrated for the first time that collaterals of olivary axons in mammals directly innervate the cerebellar neurons that provide a GABAergic projection to the inferior olive, thus creating a feedback loop. This finding contradicts the results by Borsello *et al.*[2] who did not observe a direct collateral innervation of the GABAergic neurons. The different results may have been due to the facts that most climbing fibre collaterals innervate the peripheral dendrites of neurons in the cerebellar nuclei,[25] and that these structures are technically difficult to label at the electron microscopic level with the use of double or triple labelling methods combining different tracing and/or immunocytochemical tools.

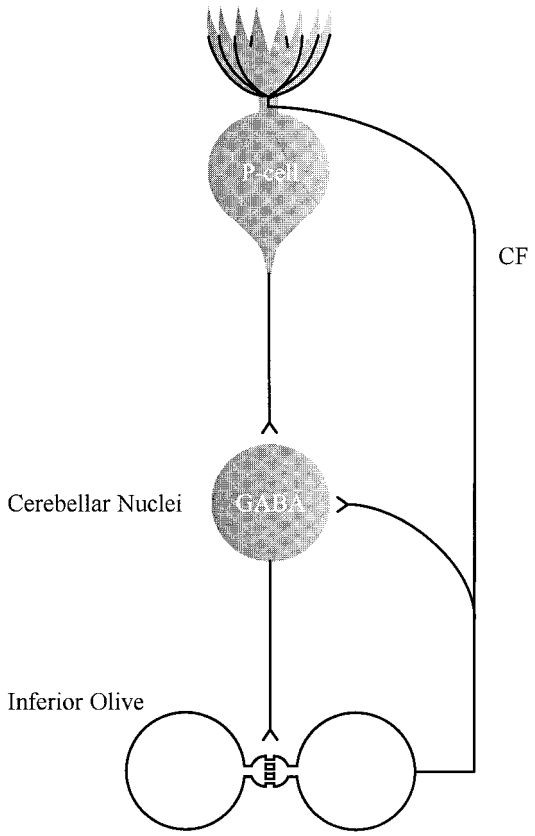

Fig. 3. Diagram showing all four synapses completing a module. Climbing fibres (CF) originate from olivary neurons; they innervate the dendritic tree of Purkinje cells (P-cell) and give off collaterals to the cerebellar nuclei. The GABAergic neurons in these nuclei receive an inhibitory (also GABAergic) input from the same Purkinje cells and they innervate the electrotonically-coupled dendrites of the same olivary neurons that in turn give rise to the climbing fibres.

The present investigation of the climbing fibre collateral innervation of the neurons in the cerebellar nuclei that project to the inferior olive completes the description of the olivocerebellar modules at the synaptological level (Fig. 3). All synaptic contacts of the modular loops between the olivary neurons, the Purkinje cells, and the GABAergic neurons in the cerebellar nuclei have now been identified.[26] The functional significance of this connection awaits further investigation. Collaterals of the olivocerebellar fibres as well as of the mossy fibre inputs to the cerebellar nuclei have been assumed to set a background excitation that is modified by the inhibitory action of the Purkinje cell input.[2] Both mossy fibre and climbing fibre collaterals have, indeed, been found to activate cerebellar nuclei neurons before the arrival of a complex spike.[11,12,14,15,17,18] However, none of these studies has distinguished conclusively the properties of the excitatory and inhibitory

neurons in the cerebellar nuclei at the electrophysiological level. Most likely, the vast majority of the recordings have been made from the large excitatory neurons, which are much more easy to record with a microelectrode than the smaller GABAergic neurons.

In summary, we conclude that the olivocerebellar collaterals directly innervate neurons that provide a GABAergic feedback to the inferior olive; the functional implications of this projection remain to be elucidated.

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