Functional Unity of the Ponto-Cerebellum: Evidence That Intrapontine Communication Is Mediated by a Reciprocal Loop With the Cerebellar Nuclei

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Möck, Martin; Sergejus Butovas, and Cornelius Schwarz. Functional unity of the ponto-cerebellum: evidence that intrapontine communication is mediated by a reciprocal loop with the cerebellar nuclei. J Neurophysiol 95: 3414–3425, 2006; doi:10.1152/jn.01060.2005. The majority of cerebral signals destined for the cerebellum are handed over by the pontine nuclei (PN), which thoroughly reorganize the neocortical topography. The PN maps neocortical signals of wide-spread origins into adjacent compartments delineated by spatially precise distribution of cortical terminals and postsynaptic dendrites. We asked whether and how signals interact on the level of the PN. Intracellular fillings of rat PN cells in vitro did not reveal any intrinsic axonal branching neither within the range of the cells’ dendrites nor farther away. Furthermore, double whole cell patch recordings did not show any signs of interaction between neighboring pontine cells. Using simultaneous unit recording in the PN and cerebellar nuclei (CN) in rats in vivo, we investigated whether PN compartments interact via extrinsic reciprocal connections with the CN. Repetitive electrical stimulation of the cerebral peduncle of ≈40 Hz readily evoked rapid sequential activation of PN and CN, demonstrating a direct connection between the structures. Stimulation of the PN gray matter led to responses in neurons ≤600 μm away from the stimulation site at latencies compatible with di- or polysynaptic pathways via the CN. Importantly, these interactions were spatially discontinuous around the stimulation electrode suggesting that reciprocal PN-CN loops in addition reflect the compartmentalized organization of the PN. These findings are in line with the idea that the cerebellum makes use of the compartmentalized map in the PN to orchestrate the composition of its own neocortical input.

INTRODUCTION

The cerebro-cerebellar projection consists of massive fiber bundles connecting neocortex and the cerebellum via an intercalated structure in the brain stem, the pontine nuclei (PN). A particular feature of this circuit is that the mapping of signals represented on the surface of the neocortex is reorganized on the level of the PN (Schwarz and Möck 2001; Schwarz and Thier 1999, 1999). Each neocortical site is mapped onto several separate compartments in three-dimensional (3D) pontine space one to several hundreds of micrometers wide. The total volume of pontine compartments subserving one spot in the neocortex has been estimated to cover a volume of 0.011 mm3 (Schwarz and Möck 2001). The compartments do not overlap with neighboring ones receiving terminals from different sites, a characteristic which we called “nonoverlapping distribution” (Schwarz and Möck 2001). There are notable exceptions from this rule as has been demonstrated for related sites in somatotopic maps of primary and secondary somatosensory cortices (Leergaard et al. 2004), and it has been shown that somatotopy is a principle that still governs distribution of compartments in 3D space, albeit on a larger spatial scale than compartmentalization (Brodal 1968a,b; Leergaard et al. 2000; Schwarz and Möck 2001). Nevertheless, the intriguing feature of compartmentalization in the PN is that it anticipates characteristic features of the representation found in one main target structure, the granular layer in the cerebellum (called there “fractured map,”) (Joseph et al. 1978; Shambes et al. 1978a,b). It has been shown that tactile signals on the two-dimensional (2D) map found in the granular layer are mapped according to rules reminiscent to the ones described in the preceding text for the PN. Signals are represented by nonoverlapping patches with sharp borders, and spatial relationship of patches cannot be predicted by the somatotopic map as found in the afferent neocortical areas (Bower et al. 1981). Presumably then the spatial reorganization of neocortical signals in the PN qualitatively reflects the specific topographical organization of signals adequate for cerebellar processing (Nelson and Bower 1990; Schwarz and Thier 1999). However, topographical reorganization by itself lends a poor raison d’être for a nucleus holding complexly organized neuronal elements and inputs from varied sources (Brodal and Bjaalie 1992; Mihailoff et al. 1989, 1992; Schwarz and Thier 1999). Pure topographical reorganization is achieved without an intervening synaptic relay in many central pathways. As an instructive example the reshuffling of retinal ganglion fibers from an organization dictated by retinal organization (macula representation on the nasal side) to one demanded by thalamo-cortical processing (macula representation in the center) within millimeters of the ocular nerve’s course may be mentioned. To elucidate the PN’s computational role beyond remapping of neocortical signals, it must be demonstrated if and how signals on the pontine map interact. Basically, two possibilities are to be considered: intrinsic versus extrinsic interaction. Morphological evidence in favor of intrinsic interaction is the finding that retrograde degeneration of pontine axons affected terminals and synaptic structures within the PN (Mihailoff 1978). Furthermore, communication via dendro-dendritic chemical synapses has been suggested from electron microscopic studies (Mihailoff and Border 1990; Mihailoff and McArdle 1981). Functional evidence has been more indirect. First, compound excitatory postsynaptic potentials (EPSPs) evoked from the cerebral pe-
duncle have been elicited in vivo (Allen et al. 1975) and in vitro (Möck et al. 1997). Second, antidromic stimulation of pontine axons from the cerebellum evoked repetitive firing in PN neurons (Sasaki et al. 1970). Both findings have been interpreted as evidence for intrinsic pontine connections. On the other hand, intrinsic axonal branching of projecting fibers has not been detected in seven cases of successful, retrograde axonal fillings from the middle cerebellar peduncle (Shinoda et al. 1992). Notwithstanding the rather unsettled question whether intrinsic processing exists, the alternative possibility of extrinsic processing has been clearly elucidated using morphological methods. All pontine signals flow to the cerebellar nuclei (CN), either directly (Mihailoff 1993; Shinoda et al. 1992) or via the cerebellar cortex. In turn, the CN send an excitatory feedback projection that shows a patchy divergence (Brodal and Siklins 1972; Brodal et al. 1972b; Kitai et al. 1976; Schwarz and Schmitz 1997b; Tsukahara et al. 1971; Verveer et al. 1997; Watt and Mihailoff 1983), and thus seems optimally suited to modulate subsets of pontine compartments in conjunction.

The answer to the problem whether and how intrinsic and/or extrinsic interaction of pontine signals modulates PN activity offers important clues to pontine function. The present study therefore revisited the question of intrinsic pontine circuitry using intracellular fillings and extended it to functional investigation using double patch-clamp recordings in vitro. The extrinsic pathway via the CN was studied using multielectrode recording and stimulation in vivo. While no supporting evidence for intrinsic pontine circuitry was found, we elucidate functional properties of a reciprocal ponto-nuclear connection.

**Methods**

All experimental procedures were performed in accordance with the policy on the use of animals in neuroscience research of the Society for Neuroscience and German national law.

**Preparation of slices and intracellular staining**

The intrapontine course of axons of ponto-cerebellar projection neurons was studied in PN neurons stained by intracellular dye injection via conventional sharp microelectrodes. The neurons were either filled with Lucifer yellow (Aldrich, Milwaukee, WI) in slices of slightly fixed tissue or with neurobiotin (Sigma, St. Louis, MO) in acute slices.

The staining procedure and analysis of PN neurons filled with Lucifer yellow has been reported in detail elsewhere (Schwarz and Thier 1995, 1996). Briefly, deeply anesthetized adult Lister hooded rats in which ponto-cerebellar projection neurons were prelabeled by retrograde tracers (Fluorogold, Fluorochrome, Englewood, CO; rhodamine labeled latex microspheres, Lumafluor) into the injection of retrograde tracers (Fluorogold, Fluorochrome, Englewood, CO; rhodamine labeled latex microspheres, Lumafluor) into the injection of retrograde tracers (Fluorogold, Fluorochrome, Englewood, CO; rhodamine labeled latex microspheres, Lumafluor) into the injection of retrograde tracers (Fluorogold, Fluorochrome, Englewood, CO; rhodamine labeled latex microspheres, Lumafluor).

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For double patch recording in vitro, parasagittal slices of the pontine brain stem were prepared and maintained as described in the preceding text. The thickness of the slices, however, was reduced to 275 μm. Somatic whole cell patch-clamp recordings of pairs of PN neurons were performed with glass microelectrodes (resistance: 5–7 MΩ) filled with a solution containing (in mM) 131 K-glucurate, 5 NaCl, 5 K⁺ HEPES, 5 EGTA, 4 K⁺-ATP, 0.3 Na⁺-GTP, and 0.5 CaCl₂ adjusted to pH 7.3 with KOH. The patch procedure was visualized using a motorized (Luigs and Neumann, Ratingen, Germany) microscope (Axioskop, Zeiss, Germany) with a water-immersion objective (×40, Zeiss numerical aperture: 0.75), infrared illumination, Normarski optics, and an infrared-sensitive CCD camera (Newvicon C2400-07-C, Hamamatsu, Japan). Current-clamp recordings were done at room temperature using two NPI BA-1S amplifiers (NPI electronic, Tanner, Germany) in the bridge mode. Voltage recordings were digitized at a sampling rate of 20 kHz using a PC with a 1401plus interface and Spike2 software (Cambridge Electronic Design, Cambridge, UK). For simultaneous recordings from two PN neurons, pairs of cells were selected that laid in close proximity (both somas visible on the monitor screen, i.e., located

The preparation and maintenance procedures of acute slices for neurobiotin fillings were similar to those used for intracellular recordings of PN neurons (Möck et al. 1997; Schwarz et al. 1997). Deeply anesthetized Lister hooded rats (18–24 days old) were decapitated, and their brains were carefully removed and immediately cooled in artificial cerebrospinal fluid (ACSF, containing (in mM) 124 NaCl, 5 KCl, 1.2 KH₂PO₄, 1.3 MgSO₄, 26 NaHCO₃, 2.4 CaCl₂, and 10 d-glucose bubbled with 95% O₂-5% CO₂, 4°C). After isolating the pontine brain stem parasagittal slices were cut on a vibrating microtome (Leica, Wetzlar, Germany) to a thickness of 400 μm. For recovery, the slices were stored in ACSF at room temperature for 2 h. Subsequently, they were transferred to a submerged recording chamber and superfused with carbogenated ACSF at 35°C. Standard intracellular current clamp recordings were performed with glass microelectrodes filled with 2% neurobiotin in 3 M potassium acetate (50–100 MΩ) using an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA) in the bridge mode. Once a cell was successfully penetrated and had developed a stable somatic membrane potential, neurobiotin was iontophoretically applied by positive square wave current pulses (0.5–0.8 nA, 0.5-s duration at 1.5 Hz) for two periods of 15 min. Thereafter the slices were kept in the recording chamber for 30 min to allow dispersion of the dye, subsequently fixed by immersion in 4% paraformaldehyde in PB overnight, cryoprotected in 30% sucrose in PB, and cut on a freezing microtome to a thickness of 60 μm. Only one cell was filled per slice. To visualize filled cells, the sections were first treated with 3% H₂O₂ for 30 min to block endogenous peroxidases. After several rinses in PB, the sections were processed through an ascending series of dimethylsulfoxide (5–40% in PB) and 0.5% Triton X-100 in PB (30 min each) to facilitate the penetration of the detection system (Lübke et al. 1996). For detection of neurobiotin, the sections were incubated with an avidin-biotin-peroxidase complex (Vectastain, Vector Laboratories, Burlingame, CA) overnight at 4°C. Next, they were incubated in 0.05% diaminobenzidine (Sigma) for 10 min and then stained with 0.05% diaminobenzidine and 0.005% H₂O₂ in PB under visual control (2–3 min). The reaction was stopped by several rinses in PB (4°C), and the sections were mounted on subbed slide, dried and dehydrated, and coverslipped with Entellan (Merck, Darmstadt, Germany). Stained PN neurons were examined with a light microscope (Leica Diaplan, Wetzlar, Germany), and those cases in which an axon was unequivocally discernable were reconstructed from camera lucida drawings.

**In vitro electrophysiological procedures**

For double patch recording in vitro, parasagittal slices of the pontine brain stem were prepared and maintained as described in the preceding text. The thickness of the slices, however, was reduced to 275 μm. Somatic whole cell patch-clamp recordings of pairs of PN neurons were performed with glass microelectrodes (resistance: 5–7 MΩ) filled with a solution containing (in mM) 131 K-glucurate, 5 NaCl, 5 K⁺ HEPES, 5 EGTA, 4 K⁺-ATP, 0.3 Na⁺-GTP, and 0.5 CaCl₂ adjusted to pH 7.3 with KOH. The patch procedure was visualized using a motorized (Luigs and Neumann, Ratingen, Germany) microscope (Axioskop, Zeiss, Germany) with a water-immersion objective (×40, Zeiss numerical aperture: 0.75), infrared illumination, Normarski optics, and an infrared-sensitive CCD camera (Newvicon C2400-07-C, Hamamatsu, Japan). Current-clamp recordings were done at room temperature using two NPI BA-1S amplifiers (NPI electronic, Tanner, Germany) in the bridge mode. Voltage recordings were digitized at a sampling rate of 20 kHz using a PC with a 1401plus interface and Spike2 software (Cambridge Electronic Design, Cambridge, UK). For simultaneous recordings from two PN neurons, pairs of cells were selected that laid in close proximity (both somas visible on the monitor screen, i.e., located
within an area of 72 × 56 μm. To test whether these cells were interconnected by chemical or electrical synapses, we alternately applied clearly suprathreshold depolarizing current pulses (300 ms) to these cells while recording the membrane potential of both of them permanently for a period of 2.5 s. In 5 of 20 cases, we added 10 mM trimethylamine (Sigma) to the bath solution to facilitate the detection of possibly existing intercellular connections via gap junctions (Lee et al. 1996; Spray et al. 1981).

**Surgery and in vivo electrophysiological procedures**

Experiments were performed on 12 Sprague-Dawley albino rats (Charles River, Sulzfeld, Germany). The rats were anesthetized with a mixture of ketamine (175 mg/kg) and atropine (1 mg/kg) administered intraperitoneally. Anesthesia depth was maintained to ensure the absence of limb withdrawal and corneal reflexes. Additional injections of 25 mg/kg ketamine were given when needed. After mounting the head in a stereotaxic frame, the scalp was incised and small holes were drilled in the skull at B-7.5, L1 on the left side and at B-11.5, L3.2 (coordinates as given by Paxinos and Watson 1986) on the right side (coordinates as given by Paxinos and Watson 1986) to allow insertion of two multielectrode arrays into the PN (left hemisphere) and CN (right hemisphere). In some experiments, a third trepanation at B-5.5, L2.4 was performed to allow the insertion of a single etched and insulated tungsten microelectrode into the cerebral peduncle for electrical stimulation. While moving the electrode dorsoventrally into the brain, bursts of negative current pulses (100 μA at 300 Hz for 100 ms, 300-μs pulse duration, burst frequency: 1 Hz) were delivered to different sites of the electrode track until contralateral movements of the whole body were elicited. Typically, such a site was found at a depth of ca. 7–8 mm. The electrode was then fixed to the skull with dental acrylic. For the recording session, the cerebral peduncle was stimulated with single, 300-μs current pulses at a frequency <0.5 Hz. Stimulation amplitude was varied between the minimum current to evoke cerebellar neuronal responses and 100 μA. In the experiments using intra-pontine stimulation and recording, a ventral approach was used. The skin was cut at the neck and the trachea prepared, incised and intubated for artificial ventilation with oxygen (1 Hz, 1.2 ml; Small Animal Ventilation and Recording System; Harvard Instruments, Kent, UK). The neck muscles were dissected and moved laterally together with the trachea to lay open the base of the skull overlying the pontine brain stem. After trepanation above the brain stem, the PN were accessed directly from the ventral surface of the brain stem.

Multielectrode arrays were custom made from electrolytically etched (tip angle: 8°), 100-μm-diam tungsten rod (7190, A-M Systems) as reported previously (Butovas and Schwarz 2003). Electrodes were loaded into polyimide tubing and assembled into arrays consisting in two rows of four microelectrodes (tip distance: 250 μm) for simultaneous recordings in PN and CN and one row of seven electrodes (tip distance: 200 μm) for intra-pontine stimulation and recordings. The impedance of the electrically insulated electrodes was >2 MΩ. The connection to the head stage of the amplifiers was realized by a standard micro connector (pin to plug, Bürklin, München, Germany) that was modified by computer-aided drilling of small holes (200-μm diam, 1.5 mm depth) into the gold pins for insertion of the tungsten rods. The electrical connection between electrodes and connector was made by conductive glue (E-Solder 3021, Epoxy Produkte, Fühn/Odw, Germany).

Recording was performed by a multichannel extracellular amplifier (MultiChannelSystems, Reutlingen, Germany). The extracellular potentials were AC-coupled, amplified by a magnitude of 5,000, and band-pass filtered between 200 Hz and 5 kHz. For each channel, a threshold for spike detection was adjusted. Data were digitized at 20-kHz sampling rate and stored on a PC’s disk as spike cutouts (length: 2 ms, 1 ms before and 1 ms after the potential crossed the threshold) or continuous raw data. Multielectrode recordings were performed while the electrode arrays were lowered into the brain using a microdrive. For PN recordings, the electrode array was lowered through visual cortex, superior colliculus and pontine reticular formation. Characteristic 1-Hz bursts of PN action potentials were typically encountered at a depth ~8 mm and could be easily discriminated from the continuous spontaneous firing of reticular cells dorsal to the PN. In many cases, penetration of the cerebral peduncle was recognized by small amplitude neuronal noise which was modulated at 1 Hz as well, most probably reflecting the activity of peduncular fibers. All pontine recordings were carried out in ventral portions of the PN. The long axis of the array was oriented in laterolateral direction. For CN recordings, the electrode was lowered through the cerebellar cortex. Spike trains of Purkinje cells recorded were checked for the occurrence of complex spikes. The CN was reached at about 4 mm depth after a silent period of different length while the electrode passed through the cerebellar white matter. A distinctive feature of CN was the 1-Hz activity within its spike trains and the lack of complex spikes encountered. CN recordings were done in the lateral and interposed nucleus. The long axis of the array was oriented in laterolateral direction.

For intra-pontine stimulation and recording, the one-dimensional electrode array was introduced into the pontine nuclei from the ventral surface at an angle of 45° such that the electrodes penetrated the ventral PN in caudorostral direction. The first electrode in the row (located close to the ventral surface i.e., the brachium pontis) was then used for electrical stimulation while the others recorded the stimulus effects on neuronal firing rates (they spanned the dorsoventral extent of the ventral PN such that the 1 farthest away from the stimulus electrode was close to the cerebral peduncle). Very small stimulus intensities covering the threshold to evoke responses were used (8 μA, pulse durations from 100 to 600 μs resulting in charge transfer of 0.8–4.8 nC). All electrode positions for recording and stimulation were confirmed by locating electrolytic lesions (25 μA cathodal, 5 s) in Nissl-stained sections.

**Analysis of spike trains**

Spike sorting of single units was performed using a principal component clustering algorithm (Egert et al. 2002). Units were classified as single units if the interval between 2 SD from the mean voltage before the spike (1st bin of the cutout) and 2 SD from the mean voltage at the peak of the spike was ≥2 SD (a value that is determined by the 1st bin); the spike train showed an absolute refractory period of 1 ms and the SD of the latency of the peak of the spike after passing the threshold was <0.3 ms. All others were classified as multiunit data. The present sample comprises a total of 106 single units and 124 multiunits. Because results were comparable with the two classes of spikes, they were pooled for the analyses presented in this study.

Firing rate of neurons with respect to an event (i.e., electrical stimulation) was computed as spike renewal function (abbreviated here as perievent time histogram, PETH) at a resolution of 0.1 ms (bin width) and a moving window of 1 ms duration as described by (Abeles 1982b). This algorithm yields a measure of firing rate over time and allows the assignment of lower and upper confidence limits (in this study P = 0.05 and 0.95, respectively) to assess statistical significant deviation of firing rate as compared with a reference period. In the present study, fast trains of electrical stimuli (≤40 Hz) were delivered to the brain, thus conditions within the perievent time of subsequent PETHs in a train of stimuli cannot be considered as independent. Therefore firing rates in response to successive electrical stimuli were related to a common point of reference. To this end, the algorithm applied here deviated from that of (Abeles 1982b) in taking the reference firing rate for all PETHs in one stimulus train from a 1-s period of spontaneous firing immediately before the first stimuli within trains were delivered. The confidence limits offered by the renewal function were used to extract three parameters from the PETHs (see Fig. 4). First, the excess spikes were defined as the integral of the firing rate above/below the confidence limits. It signi-
ifies the significant part of the average additional (excess of the upper limit, positive sign) or missing spikes (excess of the lower limit, negative sign) in response to a stimulus. Second, the latency after the stimulus event to reach the confidence limit for the response in question was computed. Finally, the response width was computed as the duration for which the firing rate exceeded the respective confidence limit.

Statistics

Population data are depicted in median [5% percentile, 95% percentile] or if normally distributed in means ± SD. As nonparametric test for nonnormally distributed variables, the Mann-Whitney U test was used.

RESULTS

We approached the question of possible communication between pontine compartments in three different ways. We first sought to find evidence for direct intrinsic connectivity between PN neurons. Toward this goal we searched axons of intracellularly filled cells in vitro for intrinsic branching. In a second step, we searched for electrical and/or chemical synapses between PN neurons using double patch recordings in vitro. In a final section, we report results gained from stimulation of activity in PN and CN using stimulation in the cerebral peduncle or from within the PN.

Do ponto-cerebellar axons give off intrapontine axon collaterals?

In the present study, a total of 218 pontine projection neurons filled with either Lucifer yellow (n = 155) in fixed slices or with neurobiotin (n = 63) in living slices where used to analyze whether their axons branch inside the PN. Within this sample, 59 axons were clearly distinguishable from dendrites by means of a small and constant caliber and the lack of any kind of appendages (Schwarz and Thier 1996). As projection neurons were cut during the slicing of the tissue, the axons were contained to varying lengths inside the slice (apparent length). This apparent length varied between 11 and 738 μm (median: 150 μm). Often the course of the axon was found to be meandering within the PN at seemingly arbitrary paths before approaching the brachium pontis. The actual entry into the brachium was observed in two cases. Many axons, however, could be followed for a considerable stretch within the PN outside the range of the parent cell’s dendritic tree (52 of 59). A previous study indicated that the mean volume of a pontine compartment in rats is about 0.011 mm³ (Schwarz and Möck 2001). It, therefore appears that about half of the axons studied here displayed an apparent length that extended the radius of a typical compartment assuming a radial shape and therefore have high probability to show axonal parts that passed neighboring compartments (the median apparent length of axons: 150 μm; radius of a sphere with a volume of 0.011 mm³: 139 μm; indicated in Fig. 1A). Figure 1B shows the distribution of apparent axonal length within our sample. Gray columns are axons that display lengths under 150 μm and thus may be included within their parent compartment; black columns represent axonal length that had a high chance to display parts outside the parent compartment. However, the search for branching points on all stained axons using close microscopic inspection did not reveal a single collateral branching. Based on this material, we conclude that 59 axons do not branch within the parent compartment and roughly half of them do not do so in the neighboring compartment. Therefore we can exclude at a fairly high certainty that intra-compartment connections and inter-compartment connections between neighboring compartments exist. Our data set provides a less reassuring base to judge possible branching at remote sites and within the brachium pontis.

Paired whole cell patch-clamp recordings of pontine nuclei neurons in vitro

To approach the question of intrapontine connections on a functional level, we performed double patch-clamp recordings of neighboring neurons. This method, in addition to unveil axonic synaptic interconnections missed by the morphological analysis, would in addition allow us to detect dendro-dendritic connections via electrical or chemical synapses. The possibility of dendro-dendritic connections is suggested by the membrane specializations and dendritic appendages of atypical (nonspinous) morphology (Schwarz and Thier 1996). Although there is no evidence for gap junctions in rat PN so far, serial chemical synapses have been suggested based on electron microscopic observations (Mihailoff and McArdle 1981). To test whether one or more of these types of connections exist, we performed simultaneous whole cell patch-clamp recordings from proximate PN neurons. Twenty pairs of neurons were accepted for this test according to the following criteria: they

![Image](http://jn.physiology.org/10.1152/jn.00831.2005)

**FIG. 1.** Absence of intra-pontine axonal branching of pontine nuclei (PN) neurons. A: camera lucida drawing of a neurobiotin-filled PN neuron in a parasagittal slice of the PN. In this example, the detectable part of the axon (arrow) had a length of 738 μm almost reaching the ventral edge of the PN. The dashed circle surrounding soma and dendrites indicates the average diameter of a pontine compartment as defined by its afferent terminations (for details see text). B: distribution of the length of axons of PN neurons filled with either Lucifer yellow or neurobiotin (n = 59). The axon length was measured from camera lucida drawings. The median of the distribution was 150 μm, i.e., the gray bars represent those cases below the median (n = 28). c, caudal; d, dorsal; ped, cerebral peduncle; r, rostral; v, ventral.)
developed a stable somatic membrane potential without spontaneous firing, had an apparent steady-state input resistance, and clearly overshooting action potentials in response to suprathreshold depolarizing current pulses. On average, these cells had a somatic membrane potential of 60.8 ± 4.9 mV, a firing threshold of 38.4 ± 5.5 mV, and an apparent steady-state input resistance of 357 ± 133.6 MΩ (when tested with -10 pA pulses). Furthermore, these cells displayed a marked firing rate adaptation when depolarized across threshold and a rapid inward rectification in response to negative current pulses (-10 to -100 pA). Therefore their membrane properties were comparable to those observed with sharp electrode recordings at physiological temperature (Schwarz et al. 1997).

A typical observation made during simultaneous recordings of two neighboring PN neurons is shown in Fig. 2. Both cells were alternatingly stimulated by intracellular current application. Suprathreshold depolarizing current pulses evoked a spike train in the cell receiving the current pulse. However, during the time period in which one of the cells was activated, we did not detect any corresponding changes in the membrane potential of the other cell: neither postsynaptic potentials evoked via chemical synapses nor spikelets transmitted via gap junctions. The same negative result was obtained in the remaining 19 pairs of cells. Because the junctional conductance of electrical synapses is controlled by the internal pH, i.e., acidification reduces the conductance and vice versa (Spray et al. 1981), we applied 10 mM trimethylamine to the bath solution in five cases to increase the chance to detect electrical coupling. Alkalization of the internal pH, however, did not result in the disclosure of electrical coupling. Therefore we conclude that synaptic communication between proximate neurons is not a prevailing feature in rat PN.

Responses of PN and CN units to stimulation of cerebral peduncle

An alternative way how communication between pontine compartments could be established is via extrinsic pathways—in particular the reciprocal connection to the CN. We therefore extended our investigations to in vivo firing of PN and CN neurons using simultaneous recordings in the two structures with multielectrode arrays. Electrical stimulation in the cerebral peduncle, the afferents to the PN, evoked PN excitation at a mean latency of 4.0 ms and CN excitation at a latency of around 5.6 ms (single cathodal pulse, duration: 0.3 ms, amplitude: 100 µA, statistics given in the following text; Figs. 3 and 4). This pattern is consistent with a direct route of activation via excitatory synapses between PN and CN (Mihailoff 1993) but is not compatible with a route of activation via the cerebellar cortex. The reason is that Purkinje cells—the only output of the cerebellar cortex—would be expected to evoke an early inhibitory response in CN neurons (Ito et al. 1970). Following the fast excitatory response, CN firing was strongly suppressed for a period of time. Considering the activation latency of Purkinje cells found earlier (Schwarz and Welsh 2001), this phenomenon could in principle be based on Purkinje cell inhibition. Figure 3 plots examples of Purkinje cell responses of the earlier study together with the CN recordings of the present study. Purkinje cell activation evoked from deep layers of motor cortex displays a latency of...
10–11 ms (Schwarz and Welsh 2001), fitting well with the suppression of CN neurons observed after peduncular stimulation (even taking account of some extra run time of action potentials from motor cortex to the site in the peduncle where the stimulation was performed in the present study—the latency difference is not expected to exceed 2 ms). In view of the restriction of targets of the PN to the cerebellar cortex and the CN, we conclude that the fast CN excitation reflects direct activation via collaterals of excitatory mossy or climbing fibers originating in the PN and inferior olive and possibly other precerebellar nuclei. Finally the period of suppressed firing rate was then interrupted by a rebound excitation that was without counterpart in PC firing and therefore was possibly based on intrinsic properties of CN neurons (Jahnsen 1986; Kitai et al. 1977; Llinás and Mühlethaler 1988; McCrea et al. 1977).

Figure 4 demonstrates the responses of four PN and four CN units recorded simultaneously to the same stimulation in the cerebral peduncle. After the sharp early excitation (astersisk: 80 of 125 PETHs exceeded the confidence interval of 95%), PN neurons typically showed a weaker second excitatory response at a latency of ~15 ms which reached significance in 76 of 125 cases (Fig. 4, ●). In addition, all PN trains showed a very long suppression of firing rate lasting beyond the period of PN excitation and the fast CN excitation. The mean latency difference between PN and CN responses of 1.6 ms is well consistent with a monosynaptic conveyance of activation (PN: 4.0 ± 1.0 ms, n = 80; CN: mean 5.6 ± 1.6 ms, n = 28, Student’s t-test, P < 0.01). The short-latency CN response was typically followed by two more robust features of neuronal response: a suppression of firing rate (50 of 64 at an average latency of 6.6 ± 1.0 ms, Fig. 4, ●) and a second excitation at a latency of 17.1 ± 2.1 ms (53 of 64, Fig. 4, ●).

In a next step, we investigated whether the neuronal responses of PN and CN just described were able to follow repetitive inputs from neocortical fibers by analyzing responses to bursts of five stimulation pulses at frequencies of 5, 10, 20, and 40 Hz applied to the cerebral peduncle (Fig. 5A). The general finding was that the short-latency excitatory peak in PN and CN survived repetitive stimulation at all frequencies. In some neurons, even an increment in the short-latency response could be observed (Fig. 5, B and C). The late excitation, however, was highly sensitive to repetitive stimulation. It was visible with burst stimuli delivered at the lowest frequency tested (5 Hz) but was significantly suppressed applying higher stimulus frequencies, particularly so in the CN.

For the quantification of responses to repetitive stimulation, only stronger responses (minimum 0.1 excess spikes) were selected. The rationale for this selection was that artifacts introduced by the detection of a response using a threshold are minimal if responses far exceeding the threshold are considered. Forty-six PN units showed short-latency responses exceeding 0.1 excess spikes. These units generated an average of 0.41 excess spikes at a latency of 4.1 ms. The duration of significant elevation of firing rate was 2.1 ms. The population data of excess spikes, latency, and response duration showed a robust—yet on average unchanging—excitatory response to pulses in bursts from 5 to 40 Hz (Fig. 6B). Statistical testing was performed by grouping the responses as follows. Group 1 included all responses to the first pulses in a stimulus train irrespective of stimulus frequency. Groups 2–5 included the responses to the remaining stimuli ordered with respect to frequency. Applying a one-way ANOVA, it was then tested if the responses to repetitive stimuli differed significantly from the responses to the first stimulus in a train. For the short-latency response of PN trains, the null hypothesis had to be accepted (P > 0.05) that responses to different stimulus frequencies were indistinguishable to the response after the first pulse testing three variables (excess spikes, latency, and response width). The PN response observed at longer latencies (14 ± 2.5 ms) exceeded in only 10 of 125 cases the criterion of 0.1 excess spikes. This response was sensitive to repetitive stimulation in a frequency-dependent fashion such that higher frequencies tended to evoke smaller late responses. Statistical testing reached significance in the strength of the response (1-way ANOVA, P = 0.03). The multiple comparisons (Scheffé), however, did not reach significant results. The closest to significance was the difference between the first
pulses and the group of 40-Hz pulses \((P = 0.06, \text{marked with a white circle in Fig. } 6)\). The latency and precision (response width) of the responses during repetitive stimulation were not significantly different (1-way ANOVA, \(P > 0.05\)).

Similar results were obtained for the CN units. Statistical testing of neuronal responses to the first pulse in a spike train and the ones evoked by the following pulses delivered at different frequencies were performed as has been described before for the PN. Again only trains were selected for the analysis if the response to be tested was stronger than 0.1 excess spikes following single pulses (short-latency response: 9 of 64; long-latency response: 28 of 64). For the short-latency excitatory response and the ensuing suppression, one-way ANOVA indicated no difference between the groups \((P > 0.05)\). However, a highly significant difference was found for the late excitatory response (1-way ANOVA, \(P < 10^{-5}\)). The post hoc test (Scheffé) revealed that the excess spikes and the response width of the late response were significantly lower during repetitive stimulation as compared with the first pulse (marked with asterisks for \(P < 0.05\) and double asterisks for \(P < 0.01\) in Fig. 6E). The latency showed a certain tendency to be longer with increasing stimulus frequency a trend that reached significance with 40-Hz trains.

In summary, PN and CN neurons are well able to respond and follow fast repetitive activation in the activity of the peduncular fibers for up to five periods without attenuation of the short-latency excitatory response. On the other hand, excitatory responses at latencies >10 ms in both structures are sensitive to repetitive stimulation. Higher stimulus frequencies are most effective to reduce these responses.

Responses to intra-pontine stimulation

To directly assess intra-pontine interaction, we used a dense linear seven-electrode array (tip distance: 200 \(\mu\m) inserted into the PN using a ventral approach. The first electrode in the row (located close to the ventral surface i.e., the brachium pontis) was then used for electrical stimulation while the others recorded the stimulus effects on neuronal firing rates (they spanned the dorsoventral extend of the ventral PN such that the one farthest away from the stimulus electrode was close to the cerebral peduncle). Stimulus intensities covered the threshold to evoke responses (0.8–4.8 nC). We performed recordings at 30 locations of the array within the PN in four animals. In 10 of these locations, the electrical stimulus evoked responses on one of the recording electrodes. In the remaining locations, no responses were obtained using this stimulus intensity. Surprisingly, the effects of intra-pontine stimulation differed from that of peduncular stimulation reported above in a markedly qualitative way. First of all, responses to intra-pontine stimulation occurred at a longer latency than those to stimulation in the cerebral peduncle (Fig. 7, A and B). Whereas the latter arrived on average at 4 ms, the ones obtained after intra-pontine stimulation showed a mean latency of 8.5 ± 1.4 ms. Second, responses after peduncular stimulation always showed a long-lasting inhibitory period (≤300 ms), whereas those evoked by intra-pontine stimulation did not. Importantly, assessment of threshold activation by varying the stimulus intensities revealed that this qualitative difference was not due to the difference in absolute stimulus intensities used. Compared with intrapontine stimulation, threshold intensities were much higher and variable from site to site for peduncular stimulation (7.5–30 nC)—most
probably due to varying spatial relationships of the stimulation site to the location of fibers that targeted the recorded units in the PN. However, using threshold activation as reference point rather than absolute intensities, the qualitative difference held. The inhibitory pattern evoked by peduncular stimulation appeared as soon as threshold activation was reached for all stimulation sites and all neurons studied (Fig. 7A), whereas such a pattern was never seen after intra-pontine stimulation (Fig. 7C). These observations allow the conclusion that the responses seen after intra-pontine stimulation were not mediated by peduncular fibers (or their branches) within the pontine gray matter (Fig. 7B).

Spatially, responses evoked from intra-pontine sites were seen exclusively on the first three electrodes along the array (≤600 µm). Interestingly though, responding neurons were distributed unevenly along the electrode array. Figure 7C exemplifies this observation. PETHs based on single-unit recordings from three electrodes neighboring the stimulus site are shown. The neuron recorded from the electrode 400 µm away from the stimulation site responded to a 1.6-nC charge transfer, whereas the neurons at a distance of 200 and 600 µm did not. The nonresponsive neurons kept inactive even after application of the maximum intensity (4.8 nC). In fact, at only half of the recording sites we obtained responses in a continuous fashion. In these cases, either the first electrode or the two first electrodes (directly neighboring the stimulation site) picked up responding neurons (Fig. 8, rows 1–5). In the remaining five cases, there was always a gap (i.e., a nonresponding neuron) in the row of neurons recorded between the stimulation site and a responsive one further away (Fig. 8, rows 6–10). In other words, a response at a distant electrode did not predict that neurons located closer to the stimulation electrode would respond as well.
DISCUSSION

This study revealed interactions of sites within the PN that are as far apart as 600 μm using low-intensity intrapontine electrical stimulation. This interaction is most likely based on reciprocal connections between PN and CN because, first, neither axonal collaterals of PN neurons in this range nor dendro-dendritic coupling of adjacent PN neurons was found. Second, rapid direct signal transmission from PN to CN could be demonstrated. Third, the response latencies matched a possible di- or polysynaptic transmission between PN and CN. Studying the temporal characteristics of signal procession through PN and CN revealed that this circuit could follow high rate repetitive stimulation of its afferents (≤40 Hz).

Lack of intrapontine communication mediated by intrinsic connections

Evidence for or against the possibility that pontine sites interact via intrinsic collaterals has been partly contradictory and indirect. The study most to the point so far was the intra-axonal filling with horseradish peroxidase (HRP) from the medial cerebellar peduncle (Shinoda et al. 1992) (although the main goal of this study was the demonstration of collateral branches to the CN—not intrinsic branches in the PN). In a small subset of seven axons, Shinoda et al. were able to backfill the entire axon down to the pontine soma but did not observe any hint of intrinsic branching. However, the possible existence of dendro-dendritic interactions suggested by electron microscopic studies was left unresolved (Mihailoff and Border 1990; Mihailoff and McArdle 1981). Also the existence of possible electrical synapses remained unknown. Moreover, the existence of intrinsic connectivity was suggested by more indirect evidence—the finding of degenerating synaptic terminals after lesioning ponto-cerebellar fibers (Mihailoff 1978); repetitive firing after retrograde stimulation of PN neurons (Sasaki et al. 1970); and sequences of EPSPs evoked by stimulation of the cerebral peduncle in the pontine nuclei in cats in vivo (Allen et al. 1975) and in rats in vitro (Möck et al. 1997). We have revisited the problem by filling pontine neurons in vitro which yielded a large number of axons filled with either Lucifer yellow or neurobiotin, two intracellular tracers that have been readily used to visualize small axonal branching. Furthermore, we have intracellularly recorded from neighboring pairs of PN neurons. Neither of these approaches yielded positive evidence for the existence of short-range interactions. It has to be pointed out that our morphological and electrophysiological investigation did not resolve the issue of long range interactions (e.g., connections to the other hemisphere, etc.) which the study of Shinoda et al. readily covered. But, within the short range of up to ~0.7 mm, it confirmed and extended the finding of Shinoda et al. (1992) with large numbers of filled axons and with additional functional inves-

![Fig. 7](http://jn.physiology.org/)

**FIG. 7.** Intra-pontine stimulation close to threshold discloses long-latency responses in PN cells. A: representative response of a PN unit (multiunit) after stimulation of the cerebral peduncle (compare Figs. 4–6). Responses to pulses of different intensities are shown (7.5, 15, and 30 nC corresponding to 75, 150, and 100 μA at 300 μs). The example depicts the typical finding that the inhibition appears together with the excitatory peak at threshold intensities (denoted by arrows). B: comparison of effects of stimulation inside the PN (top) with the peduncular stimulation (bottom, single unit, note the 2 distinct excitatory peaks as shown before) at an expanded time scale. The fast excitatory response after peduncular stimulation occurs much earlier than that seen after intra-pontine stimulation. C: multielectrode recording of responses after intra-pontine stimulation. Each row depicts PETHs obtained from electrodes with distance of 200 μm (bottom), 400 μm (middle), and 600 μm (top) to the stimulation electrodes. The symbols on the far left indicate the location of the stimulation electrode (×, ventral PN close to the medial cerebellar peduncle) and recording electrodes (● and ○, located dorsally from the stimulation electrode in the ventral PN, ○: nonresponsive unit, ●: responsive unit). Along rows responses after different stimulus intensities are shown (charge transfer from 0.8 to 4.8 nC). Stimulus intensities were varied in pseudorandom way. The excitatory response recorded on the center electrode emerged at a charge transfer of 1.6 nC. Note that the units on the other 2 electrodes remained unresponsive to stimulation at all intensities. Bin width of histograms: 1 ms.
antidromic directions. While orthodromic activation of pontine tivity in neocortical fibers that runs in orthodromic as well as old stimulation intensities. Peduncular stimulation evokes ac-

excitation was visible in pontine recordings even with thresh-

long-lasting inhibition after the short-latency cortical activation is possible. In addition, a sequence of fast peduncular activity to the CN without the need of cerebello-

sequence of neuronal responses evoked from PN and CN after Watt and Mihailoff 1983). Our present finding of a fast se-

from the CN (Brodal et al. 1972a; Schwarz and Schmitz 1997; Shinoda et al. 1994; Mihailoff 1994; Shinoda et al. 1987, 2000; Sugihara et al. 1996) and, second, the well-known 10-Hz oscillations in the inferior olive evoked by neocortical stimulation under ketamine (Schwarz and Welsh 2001) were not present in the PN activity observed here. Finally, it should be noted that monosynaptic activation of inhibitory synapses in the pontine nuclei, albeit they have been found in vitro, are not sufficient to explain the entire inhibitory response because their action is much shorter (Möck et al. 1997).

The fast excitation and slow inhibition seen with peduncular stimulation was entirely absent after intra-pontine stimulation. This strongly suggests that the stimulation strategy applied did not stimulate a significant amount of pontine afferents. The following facts seem to be important to explain this finding. The electrodes were oriented in a dorsoventral direction with the stimulation electrode close to the brachium pontis (medial cerebellar peduncle) and the recording electrodes located toward the cerebral peduncle. It is therefore conceivable that the electrical field induced (which we chose to be small and close to activation threshold to keep the activated spot of pontine tissue as small as possible and to keep stimulus artifacts in the neighboring recording electrodes manageable) predominantly reached outgoing fibers entering the brachium and to a much lesser extent incoming fibers from the cerebral peduncle. These arguments also reduce the likelihood that other afferent fiber systems which are not part of the cerebral peduncle (and thus were not activated by the experiments with peduncular stimulation) were at the basis of the responses after intrapontine stimulation because, like peduncular fibers, they commonly enter the PN along a dorsoventral trajectory often penetrating through the cerebral peduncle. One prominent example is the feedback projection of the CN to the PN (Schwarz and Schmitz 1997). In summary, we conclude that intra-pontine stimulation (as applied in this study) selectively activates the pontine efferents and leaves the afferents largely unaffected. It, thus seems to be optimal to isolate the effect of activity in PN outputs from the activity related to diverse connections of the branches of PN input fibers. Consequently, if one recalls that the only pontine projection target is the cerebellum, the delayed pontine excitation observed with this type of stimulation...
must have been mediated by the cerebellum. The fact that it is a pure excitation excludes the involvement of Purkinje cells because the inhibitory action of Purkinje cells onto CN projection neurons (De Zeeuw and Berrebi 1996) would have been imposed on PN cells as a withdrawal of excitation (Schwarz and Schmitz 1997). This is not to say that Purkinje cell inhibition did not play a role in the responses observed. It only indicates that the first part of the response (which was always excitatory) was not determined by Purkinje cell activity. At a later time of the PN response, it is well conceivable that Purkinje cell inhibition overlays the excitatory response but does not impose as inhibitory response of its own.

**Functional considerations**

The question how sites in the pontine nuclei interact is fundamental for our understanding of pontine function. In case of pure intrinsic interconnections, the computational role of the PN would have to be considered as a feedforward adaptor of neocortical signals for the use of the cerebellum. The present study does not favor such a role (however, the existence of putative inhibitory interneurons, albeit seemingly exclusive to the PN in primates, should be mentioned at this point) (Mihailoff et al. 1992; Möck et al. 1999; Thier and Koehler 1987). Rather, our observations suggest that pontine sites uniquely communicate via a reciprocal ponto-nuclear feedback loop. In this setting, the role of the pontine nuclei appears to be situated more in the framework of a larger ponto-cerebellar loop. In this setting, the role of the pontine nuclei appears to be a feedback-controlled input stage performs a dynamic adaptation of incoming signals for the purposes of the receiving structure. It is an interesting fact that such dynamic adaptation seems to be distinctive for the mossy fibers originating in the pontine nuclei as other mossy fiber systems seem to be lacking cerebellar feedback (notably the classic ones originating in the vestibulare nucleus ganglion and spinal chord). What makes neocortical signals conveyed via the PN so different from other mossy fiber signals that they have to be adapted before they enter the cerebellum?

Two notions, one pertaining to the spatial, the other to the temporal aspects of coding of pontine signals seem worth considering and may guide future research. First, as laid out in the introduction, the precise and complex topographical rearrangement of neocortical signals in the PN may reflect specific demands of cerebellar signal processing. Dynamic feedback control could serve as a spatial filter that dynamically selects relevant signal combinations from local sets of pontine compartments. Second, temporal coding, based on synchronous and/or rhythmic firing among neuronal assemblies, thought to be present in neocortex (Abeles 1982a; Llinás 1988; Singer and Gray 1995) may need recoding of some sort before entering the cerebellum. The reason is that an assembly code that relies on divergent, reciprocal interconnectivity (Abeles 1982a; Brairtenberg and Schütz 1991) can hardly be upheld by the cerebellar cortex, which essentially is a feedforward throughput structure lacking reciprocal connections (Schwarz and Thier 1999). Indeed, oscillatory neuronal activity in the cerebellar cortex was found to be located in the molecular layer (Courtemanche and Lamarre 2005; Hartmann and Bower 1998), but little evidence has been gathered to date that cerebellar simple spikes of Purkinje cells are able to convey information using synchronous rhythmic activity (Jaeger 2003; Schwarz and Welsh 2001). The present study yielded evidence that rhythmic activity of ≤40 Hz readily gains access to the cerebellum via the PN-CN loop. Patterns of spontaneous activity in the CN, however, do not indicate a propensity of the CN to actively upregulate and uphold such rhythmic activity (Schwarz, unpublished observation). It is therefore conceivable that the cerebellum may extract information contained in a temporal code but may be inapt to passing it on. The necessity of recoding implied by this scenario may find its structural basis in the complex arrangement of pontine compartments under reciprocal feedback control of the cerebellum as described in the present study. There is the possibility to be studied in the future that a temporal code in neocortical signals could be converted to a spatial code by the dynamically adjustable spatial filter implemented by the PN-CN loop. In this view, the cerebellum could single out neocortical signals that are bound by a temporal code for further processing on the cerebro-cerebellar loop.

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**References**


De Zeeuw CI and Berrebi AS. Individual Purkinje cell axons terminate on both inhibitory and excitatory neurons in the cerebellar and vestibular nuclei. Ann NY Acad Sci 781: 607–610, 1996.


Spray DC, Harris AL, and Bennett MV. Gap junctional conductance is a simple and sensitive function of intracellular pH. Science 211: 712–715, 1981.


