The Activity of Spinal Commissural Interneurons During Fictive Locomotion in the Lamprey

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Biro Z, Hill RH, Grillner S. The activity of spinal commissural interneurons during fictive locomotion in the lamprey. J Neurophysiol 100: 716–722, 2008. First published May 28, 2008; doi:10.1152/jn.90206.2008. Commissural interneurons in the lamprey coordinate activity of the hemisegmental oscillators to ensure proper left–right alternation during swimming. The activity of interneuronal axons at the ventral commissure was studied together with potential target motoneurons during fictive locomotion in the isolated lamprey spinal cord. To estimate the unperturbed activity of the interneurons, axonal recordings were chosen because soma recordings inevitably will affect the level of membrane depolarization and thereby spike initiation. Of 227 commissural axons recorded during locomotor activity, 14 produced inhibitory and 3 produced excitatory postsynaptic potentials (PSPs) in target motoneurons. The axons typically fired multiple spikes per locomotor cycle, with ~10 Hz sustained frequency. The average shortest spike interval in a burst corresponded to an instantaneous frequency of ~50 Hz for both the excitatory and inhibitory axons. The maximum number of spikes per locomotor cycle was inversely related to the locomotor frequency, in accordance with previous observations in the spinal hemicord preparation. In axons that fired multiple spikes per cycle, the mean interspike intervals were in the range in which the amplitude of the slow afterhyperpolarization (sAHP) is large, providing further support for the role of the sAHP in spike timing. One hundred ninety-five axons (86%) fired rhythmically during fictive locomotion, with preferred phase of firing distributed over either the segmental locomotor burst phase (50% of axons) or the transitional phase (50% of axons). The discharge rate of a neuron while recording from the cell soma can be influenced by a variety of factors that affect the membrane potential level and the spike initiation zone. These difficulties can be avoided by performing intra-axonal recordings. A proportion of the axons could be identified by their synaptic effects on segmental motoneurons.

METH O DS

Electrophysiology

All experimental procedures were carried out in accordance with institutional guidelines and the regulations of the local ethical committee (Stockholms norra djurforsöksnämnd). Pieces of spinal cord spanning 10–15 segments were removed from the trunk region just caudal to the last Gill opening in 53 adult river lampreys (Lampetra fluviatilis) and superfused with cold (8–10°C) physiological solution with the following composition (in mM): 138 NaCl, 2.1 KCl, 2.6 CaCl₂, 1.2 MgCl₂, 4 glucose, and 2 HEPES. The commissural interneuronal axons identified by monosynaptic postsynaptic potentials (PSPs) in motoneurons were recorded from 18 preparations. D-glutamate (750 μM) was added to the perfusate to induce fictive locomotion with a mean frequency of 2.8 ± 0.6 Hz (range, 1.7–4.6 Hz). Microelectrode solutions consisted of 3 M KAc in 0.1 M KCl. The general experimental arrangement is shown in Fig. 1A. Once stable intracellular recording of a motoneuron (MN) has been obtained, the ventral midline was impaled blindly within the same segment using a second microelectrode to find putative premotor interneuron axons. Commissural axons are easily accessible for recording because the majority of them pass in the ventral commissure (Tang and Selzer 1979). Paired intracellular recordings were made in passive bridge mode from MNs and interneuron (IN) axons or potent...
significant phasic activity (performed on these 227 axons, and 195 of these (86%) had a activity with regard to the motoneuronal locomotor oscillations was further considered. Analysis of the phase relations of the axonal spike potential (AP) in the presynaptic IN axon.

0.53) with the postsynaptic MN oscillation cycle (top trace; at –74 mV). This IN fired multiple spikes per cycle on a flat membrane potential (indicating the lack of oscillation and synaptic activity).

A flat membrane potential span (passively recorded or during current injection) was also taken as evidence for an axonal recording. MNs were identified as producing PSPs in a segmental MN.

identification

Different qualitative and quantitative techniques were used to aid classification. In 127 cases, midline recording sites were filled with Lucifer yellow and Neurobiotin (Vector Laboratories, Burlingame, CA) during recording to confirm the axonal recording, with 58 being classified as axons (46%), 67 as dendrites (54%), and 2 as somata. The remaining axons were classified using electrophysiological methods. All recordings were examined for the presence of synaptic activity or oscillations, in which case they were regarded as being caused by recording from dendrites of neurons crossing in the ventral commissure. These were excluded from further analysis. A flat membrane potential and a constant phasing and number of spikes over a large membrane potential span (passively recorded or during current injection) was also taken as evidence for an axonal recording. MNs were identified by intracellular stimulation, which produced a one for one spike with constant latency in a segmental ventral root (Russell and Wallén 1980).

One half of the ventral commissure recordings were classified as axons (n = 227), whereas the remaining showed synaptic activity typical of somatic or dendritic recordings (n = 205), which were not further considered. Analysis of the phase relations of the axonal spike activity with regard to the motoneuronal locomotor oscillations was performed on these 227 axons, and 195 of these (86%) had a significant phasic activity (P < 0.05). Many axons were firing throughout the oscillation cycle, some of which had a significant phasic modulation. In these axons, the probability of firing was therefore significantly higher in certain phases than in others, and in such cases, the firing pattern is referred to as being phase modulated.

A total of 185 intra-axonal recordings with the number of spikes exceeding 300 were spike trigger analyzed off-line for the presence of a PSP in a segmental MN. Traces were excluded from the spike-triggered average when a postsynaptic (MN) action potential occurred within a time window of –10 to +60 ms with respect to the time of the peak presynaptic (IN) action potential. Using these criteria, 17 IN axons were identified as producing PSPs in a segmental MN.

Phases (φ) are expressed as a fraction of the postsynaptic MN oscillation cycle phase, with φ = 0.0 (equal to φ = 1.0) defining the midtrough phases. The midtrough phase of the intracellularly recorded trunk MN oscillation cycle was measured using the following procedure: action potentials were removed from the intracellular signal, which was subsequently midpass filtered to remove both high-frequency (synaptic) and low-frequency events (changes in membrane potential). Midtrough phases were defined as being the midpoint (time) between the decreasing and increasing slopes crossing the baseline (zero). Such analysis assumes symmetrical (sineoidal) membrane potential oscillations in trunk MNs.

The MN oscillation cycle was divided into four quadrants (cf. Wheatley et al. 1994). IN activity is referred to as occurring in phase for φ = 0.375–0.625, antiphase for φ = 0.875–1.0, rising phase for φ = 0.125–0.375, and falling phase for φ = 0.625–0.875, the latter two being transitional phases. Peak depolarization in the MN oscillation cycle thus occurs at approximately φ = 0.5.

In previous studies (Buchanan 1982; Buchanan and Cohen 1982; Kahn 1982), interneuronal firing phases were normalized to the onset of the ipsilateral ventral root (VR) burst (φVR), and a conversion factor is necessary to allow comparison with the findings presented here. During fictive swimming in the isolated spinal cord preparation, the mean ipsilateral MN peak depolarization phase occurs at the
midipsilateral VR burst at approximately $\phi_{\text{MN}} = \phi_{\text{VR}} + 0.2$. The conversion ratio for a contralateral MN is thus approximately $\phi_{\text{MN}} = \phi_{\text{VR}} + 0.7$. It follows that peak activity at onset of the ipsilateral ventral root burst (observed in CCINs; Buchanan 1982; Buchanan and Cohen 1982) corresponds to the falling phase (0.7) of the postsynaptic (contralateral) MN oscillation cycle.

While for an IN–MN pair, $\phi$ will be an accurate measure for the mean IN firing phase with respect to the postsynaptic MN, some degree of uncertainty remains for all calculated phases when trying to compare them with the ventral root burst activity. This uncertainty stems from the variability between the firing times of individual motoneurons within the same segment. All identified axons ($n = 17$) had significant phasic activity ($P < 0.05$) with synaptic latencies of $3.5 \pm 1.1$ ms (range: $1.5–5.5$ ms). They were classified as monosynaptic by previously established criteria of having a constant latency within the range commonly observed (Buchanan 1982; Buchanan and Grillner 1987). Based on this, and the assumption that axons as a rule cross at the same segment at the soma (Tang and Selzer 1979), we consider INs producing excitatory PSPs (EPSPs) and inhibitory PSPs (IPSPs) in the postsynaptic MN as segmental and denote them as commissural excitatory (CEIN) and inhibitory interneurons (CIIN), respectively (Biró et al. 2006).

The robustness of the ratio between in-phase/antiphase versus transitional phase firing was assessed by incrementally increasing the threshold value for the mean resultant length ($r$) and calculating the percentage of firing in each category. Thus at a threshold level set to 0.5, only axons with $r$ values exceeding 0.5 from the center gives the degree of rhythmicity ($r$), and the angular position the relative phase of the firing relative to the swim cycle. The analyses were performed only on IN recordings with significant rhythmic activity ($P < 0.05$).

RESULTS

CIINs

The CIINs were identified by the short-latency, small-amplitude IPSPs (0.18 $\pm$ 0.11 mV; 0.05–0.42 mV; $n = 14$) they evoked in the postsynaptic motoneuron. CIINs fired on average 2.9 $\pm$ 1.2 (1.3–7; $n = 14$) spikes per cycle. Figure 1B shows the activity of a CIIN (bottom trace) and its target MN (top trace) during fictive locomotion. The flat membrane potential (thus lack of discernible oscillation or synaptic activity; Fig. 1B) indicates an intra-axonal recording, which is confirmed by the axon filled at the recording site (Fig. 1C). This CIIN axon fired on average 4.2 spikes per cycle producing a 0.2-mV IPSP in a MN in the same segment (Fig. 1D). All CIINs displayed a phase locked or phase modulated activity (Fig. 2A). Seven CIINs fired in the falling phase (D, F, M, S, Ty, W; Fig. 2A) of the MN oscillation: five in antiphase (A, B, E, J, R), one in the rising phase (Cs; Fig. 2A), and one in phase (O; Fig. 2A).

The mean value of the shortest interspike interval (ISI) within a cycle for all CIINs was 19.8 $\pm$ 5.5 ms (range, 12–31 ms; $n = 14$), whereas the mean value of the ISI for the CIIN population was 98.3 $\pm$ 35.3 ms (range, 45–168 ms; $n = 14$). Three types of ISI–phase relationships were observed for the CIINs: type 1, in which the ISI increased during the preferred firing phase, did not exhibit a relatively constant minimal ISI (Fig. 2C). In type 2, short intervals could occur throughout the burst and gradually increased the range until an abrupt cessation of activity (Fig. 2D). Type 3 had full-cycle phase-independent tonic-like firing (Fig. 2E). Five CIINs (type 1) fired within a narrow phase range with clear (J, S) or weak (D, M, O) phase-dependent ISI, with exclusively and progressively longer ISIs at the end of the preferred phase of firing (Fig. 2, A and C). Nine CIINs (type 2) showed an ISI pattern in which short intervals could be present throughout the preferred phase of firing, whereas the probability of longer intervals increased. In four of these CIINs (A, B, Cs, E), firing was delimited to a narrow ISI range, three of which (A, B, E) had an abrupt cessation at the end of the preferred firing phase (Fig. 2, A and D). The remaining five CIINs (type 3; F, Ly, R, Ty, W) fired throughout the entire cycle (see Fig. 2, A and E).

CEINs

The CEINs ($n = 3$) were identified as producing short-latency, small-amplitude EPSPs (Fig. 1F) in the postsynaptic MN (0.03, 0.08, and 0.10 mV; average, 0.05 $\pm$ 0.04 mV). Figure 1F shows the averaged postsynaptic (MN) response to the presynaptic axonal action potential (IN) for the IN axon–MN pair in Fig. 1E. The axon fired in phase with its postsynaptic target MN (top trace) during fictive locomotion.

The CEINs displayed a clear phase-locked activity as shown in the phase histograms in Fig. 2B. Two CEINs peaked in the transitional phases (one in the rising phase, one in the falling phase), whereas the remaining CEIN peaked in phase (midcycle). The three CEINs fired on average 3.1 $\pm$ 0.4 (range, 2.6–3.5) spikes/cycle. The mean shortest ISI for the CEINs

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**Histochemistry and visualization**

The microelectrode solutions (0.1 M LiCl) were supplemented with 5% Lucifer yellow and 4% Neurobiotin. Midlines processes were visualized during the experiment by using an IR-sensitive CCD camera (Hamamatsu Photonics) mounted on a DIC microscope (Nikon). For whole mount histochemistry, the spinal cords were fixed for 2 h at 5°C in 4% paraformaldehyde and rinsed three times for 15 min in 0.2 M PBS solution and treated with 10% normal goat serum (Jackson ImmunoResearch) for 30 min followed by 12-h incubation in carbocyanine 5 (Cy5)-conjugated streptavidin (1:600; Jackson ImmunoResearch) in PBS at 5°C. After additional rinsing, the preparations were dehydrated in a graded alcohol series (50, 70, 90, 99.5; 15 min each), cleared in methyl salicylate, and mounted in DPX on slides. The slides were subsequently scanned using a Zeiss LSM 510 META (Carl Zeiss) confocal microscope.

**Statistical analysis**

Electrophysiological data were analyzed on a PowerMac G5 using AxoGraph 4.9.2 (Axon Instruments, Union City, CA) and StatView 5 (SAS Institute, Cary, NC). Additional graphical illustrations were done using KaleidaGraph 4.0.2 (Synergy Software, Reading, PA). Data are reported as mean $\pm$ SD unless otherwise stated, $n$ refers to the number of neurons, and value ranges are reported as (min – max). Circular statistics was performed to calculate the mean of the IN axon firing phase with respect to the motoneuron oscillation cycle, whereas the resultant length ($r$) and its significance ($P$ value) were calculated using the Rayleigh test of uniformity in R 2.3 (circular statistics package 0.3, The R Foundation for Statistical Computing, Vienna, Austria). This test is used to calculate the probability that a distribution of circular data are uniform (with regard to the IN axon firing phases, uniform distribution is tonic activity), and the mean resultant length is proportional to the degree of rhythmicity. The results from this test can be illustrated in a circular plot (as in Fig. 3F) with the full circle representing the swim cycle period, the distance
within a cycle was $21.0 \pm 11.5 \text{ ms}$ (range, 11–34 ms), and the mean ISI was $56.5 \pm 18.3 \text{ ms}$ (range, 35–69 ms). The ISI–phase relationships of the three CEINs was distributed with one axon in each subtype (types 1–3) shown in Fig. 2, C–E.

**Activity of phasically firing commissural axons**

To assess the representative inclusiveness of the identified commissural axonal population and to quantify the firing properties for a larger population, the analyses were extended to include all 227 commissural axons, i.e., also those with unidentified target cells (the side of the spinal cord at which the cell body is located is unknown). A majority of them (86%) displayed a phasic firing pattern ($P < 0.05$), and the mean number of spikes per cycle was $2.5 \pm 1.8$ (range, 0.1–11.0), with a mean cycle duration of $365 \pm 77 \text{ ms}$ ($n = 195$). The mean shortest ISI was $33 \pm 38 \text{ ms}$ (range, 7.5–288 ms; $n = 195$), yielding a highest instantaneous spike frequency of 133 Hz. The mean ISI was $105 \pm 62 \text{ ms}$ (range, 26–346 ms). The 32 axons that did not display a phasic firing pattern ($P > 0.05$) were excluded from further analysis.

The mean phase value for each axon (with regard to 1 cycle) is plotted along the circumference of the circle in Fig. 3A, whereas the distance from the origin represents the degree of rhythmicity as estimated by the Rayleigh test ($r$ values from 0 to 1). The identified inhibitory and excitatory axons represented in Fig. 3, A and F, are indicated with the same capital letters as used in Fig. 2A. The antiphase inhibitory axons (A, B, E, J, and R) represented in Fig. 2A display $r$ values $>0.4$, as do the other distinct groups in the falling phase (D, F, M, S), whereas, particularly, the W and Ly axons have a very low $r$ value.

Excluding eight axons that showed a biphasic firing pattern, the distribution of the mean firing phases with respect to the MN oscillation cycle was nonuniform, with 61% of the axons firing in the transitional phases ($n = 187$). To assess the robustness of this finding, the threshold value for the lowest degree of rhythmicity ($r$ value) included in the analysis was incrementally increased as shown in Fig. 3, B and C. The 60–40% ratio remained constant (i.e., not dependent on the degree of rhythmicity) for $r \leq 0.7$, but for the few values above $r \geq 0.8$, the in-phase/antiphase activity dominated.

To assess whether the slow afterhyperpolarization (sAHP) would affect the ISI, we compared the mean ISI in the axons with the sAHP recorded in the soma (Fig. 3D, data from Biró et al. 2006). The delay between the action potential and the peak sAHP was measured in a quiescent preparation for another population of identified last-order segmental premotor interneurons and found to be $36 \pm 13 \text{ ms}$ (range, 18–77 ms; $n = 28$; Fig. 3D, dark bars). The observed values for peak sAHP time were somewhat shorter than those reported for EINs in a previous study ($72 \pm 64 \text{ ms}$ from Buchanan et al. 1989). The time constant after the peak of the sAHP (Fig. 3D, inset) was $119 \pm 52 \text{ ms}$ (range, 36–272 ms; $n = 18$; falling to $1/e \approx 36.8\%$ of the peak value; Fig. 3i, light bars). The mean ISI for the whole IN population was $105 \pm 62 \text{ ms}$ (range, 26–346 ms; $n = 227$; Fig. 3E), thus in the approximate range in which the sAHP amplitude is relatively large, from onset to $1–1/e$ of the amplitude (Fig. 3i), supporting a role for the sAHP in spike timing during fictive locomotion.

The average number of spikes in the different axons is plotted versus frequency of swimming in Fig. 3F. There was no correlation between the degree of rhythmicity ($r$ value) and $f$ cycle duration ($R^2 = 0.02$; data not shown) or 2) the number of spikes per cycle ($R^2 = 0.04$). Although no correlation was found between swimming frequency and the mean number of spikes per cycle ($R^2 = 0.14$), the maximum number of spikes per cycle for each 0.25-Hz bin was inversely related to the swim frequency ($R^2 = 0.97$).
DISCUSSION

Pattern of activity in inhibitory and excitatory commissural axons

By performing paired microelectrode recording from the ventral commissure and segmental MNs during fictive locomotion, we identified commissural interneuron axons with a broad range of firing rates. Of the 14 inhibitory interneurons, 10 had a very clear modulation either in antiphase of the target motoneuron or in the falling phase of this activity. In the commissural inhibitory interneurons previously studied with soma recordings (Buchanan and Cohen 1982; Buchanan and Kasicki 1995; Kahn 1982), the activity pattern has been reciprocal, with a tendency to be phase advanced in relation to the ipsilateral ventral root activity (to the soma). This means that the activity would occur in the falling phase of contralateral motoneurons or in antiphase as found here. The remaining four INs displayed either very limited rhythmicity or had the overall activity in phase with the target MNs. Three excitatory commissural interneurons were phasically modulated in different parts of the cycle, but the population is too small to comment on further. It should be noted that in the caudal part of the spinal cord, commissural excitatory interneurons are known to monosynaptically excite fin MNs, which are typically active in phase with myotomal MNs on the contralateral side (Mentel et al. 2008). The role of the excitatory INs in the rostral part of the spinal cord, recorded in this study, remains unclear.

Activity in unidentified commissural axons

For the remaining unidentified 178 axons, the segmental locations of both the cell body and synaptic targets are unknown, and thus a proportion of these may not be local, possibly including some spinobulbar neurons (Einum and
Buchanan 2006; Vinay et al. 1998), but as much as 86% of the axons displayed significant locomotor-related activity. Consequently, one should anticipate that the phasic activity pattern should be distributed in all parts of the cycle, when considering the results with the inhibitory and excitatory INs. The mean phases of the commissural interneurons were nonuniformly distributed, with a majority of them (~60%) firing in the transitional phases. This 60–40 ratio remained constant when the threshold for the lowest level of rhythmicity included in the analysis was gradually increased from 0 to 0.75 (see Fig. 3C). A similar pattern, with dominant transitional phase activity (68%) was reported for rhythmically active neurons in the mudpuppy (Wheatley et al. 1994; see, however, Cheng et al. 2002).

The average shortest spike intervals for the inhibitory and excitatory axons were very similar (19.8 and 21 ms), corresponding to a spike rate of ~50 Hz, the average spike interval being 98.3 and 56.5 ms, respectively. For the entire large population of axons, the average shortest interval was somewhat longer (33 ms), and the average spike interval was 105 ms. This may suggest that other types of axons may also be included in this population. The number of spikes per activity cycle is a value that previously has been difficult to estimate because of the uncertainty with the soma recordings. A broad distribution was observed for the number of spikes per cycle for both identified and unidentified axons, with a range of 0.1–11, with 2.5 spikes per cycle on average. The maximum number of spikes per cycle at a given swimming frequency was inversely correlated to the swimming frequency ($R^2 = 0.97$) and at swimming frequencies >4 Hz, the number of spikes per cycle was ~2. Similarly, the high-frequency swimming bouts (>5 Hz) observed in the spinal hemicord preparation (lateral separation along the midline) were entirely dominated by single spikes in each cycle (Cangiano and Grillner 2005). Our findings further support the hypothesis that, by increasing the number of spikes per cycle, commissural inhibitory interneurons can slow down the locomotor frequency in the intact spinal cord. This finding and the relative dominance of commissural inhibition over excitation (based on impalement probability) with regard to the number of identified axons and the size of the postsynaptic response provide further indirect support for the hypothesis of predominant reciprocal inhibition and a weaker commissural excitation in lamprey (Cohen and Harris-Warrick 1984; Hagevik and McClellan 1994).

**Synaptic connectivity**

In accordance with previous reports (Biró et al. 2006; Buchanan and Grillner 1988), the recorded PSPs in the MNs were small (<0.6 mV). In one third of the Ins, the amplitude of the PSPs was <0.1 mV. The concurrent synaptic locomotor drive in the postsynaptic MNs may, however, have shunted the PSPs. Monosynaptic transmission can also be depressed as a result of a sustained high-frequency firing in the range observed here (Parker and Grillner 1999). In this limited dataset, we found, however, no correlation between the mean PSP amplitude and the mean number of spikes per cycle ($R^2 = 0.09$).

Although 86% (195/227) of the commissural axons fired rhythmically ($P < 0.05$), the degree of connectivity to segmental MNs was just 7% (17/227). In an early study, the inhibitory (trough) phase in a MN oscillation cycle was suggested to be caused by “a relatively small number of premotor elements” (Russell and Wallén 1983; see also Kahn 1982). Dual MN recordings have also shown a high correlation of synaptic activity in a proportion of the MNs in the same segment, suggesting a high degree of divergence from single premotor INs to motoneurons with similar function (Buchanan and Kasicki 1999; Wallén et al. 1985). A low degree of connectivity was found also when correlating IN activity with muscle response in the mudpuppy (18%) (Cheng et al. 2002). However, the broad distribution of firing across phases may indicate a larger number of premotor elements, perhaps with small IPSPs that were not detected in previous studies.

**Slow sAHP and distribution of ISI**

The mean ISI for the whole population was in the approximate range in which the mean sAHP amplitude is large (see Fig. 2, F, and G), supporting previous findings (El Manira et al. 1994; Wallén et al. 2007) that the sAHP is an important factor determining spike timing during fictive locomotion. INS undergoing spike-frequency adaptation are, however, expected to have characteristic phase-dependent ISIs (type 1; Fig. 2C). One third of the commissural INS showed clear phase-dependent ISIs and, for the others, short intervals could occur late in the activity period, although the number of cycles with long intervals increased (Fig. 2, D and E). The overall instantaneous frequency of a neuron is determined both by the trajectory of the synaptic drive potential (including synaptic noise) in each cycle and the intrinsic membrane properties, including sAHP summation.

**Concluding remarks**

This study has been designed to establish the discharge pattern of the commissural interneurons during fictive locomotion under conditions when the axon rather than the cell soma is impaled. In the subpopulation of identified inhibitory and excitatory neurons, the average highest instantaneous spike rate was as high as 50 Hz, whereas the overall burst rate was ~10 Hz during each burst. Two thirds of the inhibitory neurons fired as anticipated from previous studies in antiphase or somewhat phase advanced. The remainder had, however, a high level of activity sometimes throughout the cycle or in phase with the ventral root activity. The overall activity of the axonal population was distributed both in-phase/antiphase with regard to the motoneuronal activity but also in the transitional phases between the bursts of activity on the two sides of a segment. The variability may reflect a spread in the functionality of different types of interneurons that is necessary to shape an output controlling swimming but also including the postural adjustments needed to control body orientation, the end result of which would be goal-directed locomotion with efficient movement through the water (see Grillner and Kashin 1976; Williams et al. 1989).

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REFERENCES


