J. Hirnforsch. 28 (1987) 6, 633–639

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Synaptic Density on Non-spiny Dendrites in the Cerebral Cortex of the House Mouse. A Phosphotungstic acid study.*

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With 3 Figures and 1 Table

(Received September 13, 1986)

Summary: A modification of the phosphotungstic acid method was used to investigate long segments of non-spiny dendrites in the electron microscope. The number of synapses on these dendrites was counted. The density was 1.9 synapses per micron of dendritic length. Taking into account the synapses not contained in the sections, (which are thinner than the dendrites) one gets a real density of 3.3 synapses per micron. This is more than the average density of synapses along spiny dendrites. It demonstrates that spines are not necessary for large numbers of synaptic contacts.

Introduction

The phosphotungstic acid stain (Bloom and Aghajanian, 1966) is selective for synaptic junctions and is therefore particularly useful in quantitative electron microscopic investigations of the neuropil. We have been using it for several years in our laboratory, and it has turned out to have various applications. It can be used, for example, for the visualization of synaptic junctions in the light microscope with dark field illumination (Braitenberg, 1981; Braitenberg and Schüz, 1983; Van Der Want et al., 1984).

Although it is not difficult to stain synaptic junctions with phosphotungstic acid (PTA), it is not so easy to achieve the desired degree of selectivity; the mitochondria also tend to accept PTA quite readily. The selectivity of the stain is influenced, for example, by the temperature and the water-content of the staining solution (Vrensen and De Groot, 1973a).

For the purpose of light microscopic visualization of synaptic junctions a high selectivity of the stain was crucial. We therefore applied an acid fixative and exposed the tissue to ultrasound during fixation in order to destroy the mitochondria. The result, as visualized on thin sections in the electron microscope, was, indeed, a beautiful dark stain of synaptic junctions on a pale background.

On the electron microscopic level, this treatment showed an interesting side effect: in some cells a darkening of the cytoplasm occurred. The cells differed considerably in their sensitivity to the harsh treatment: some did not seem to be affected at all, while others showed various degrees of darkening. In the neuropil, dark processes could be found. Interestingly, most of the dark segments which could be classified as dendrites, were of the non-spiny type.

One is relatively well informed about the synaptic density on spiny dendrites. Their synapses can be roughly equated with the spines and are thus detectable in the light microscope. Much less is known about the synapses on non-spiny neurons, for these synapses can only be recognized in electron micrographs. There, pieces of dendrite long enough to be identified as non-spiny are not very frequent. There are several reasons for this: 1. electron microscopic sections are very thin compared to the thickness of the dendrites. 2. in the cerebral cortex non-spiny neurons are much less frequent than spiny ones and 3. their dendrites have no preferred orientation in the neuropil, which means that encountering them sectioned along their longitudinal axis is merely a matter of chance.

The use of PTA enables us to investigate much thicker sections (up to 2000 Å) in the electron microscope than is the case in osmium-stained material. Long dendritic segments are, therefore, found much more often. Because of this and because of the selectivity mentioned above, this method seemed appropriate for an investigation of the synaptic density on non-spiny dendrites.

This is interesting for two reasons:

1) It is still an open question as to why some cells in the cortex have spines and others do not. A comparison of the synaptic density on their dendrites may contribute to the discussion on the role of dendritic spines.

2) There are strong indications that non-spiny cells in the cerebral cortex are inhibitory (e.g. Peters and Fairen, 1978; Somogyi and Cowey, 1981). The knowledge of their synaptology is therefore interesting for an understanding of the cortical network.

* Dedicated to Prof. Valentin Braitenberg on the occasion of his 60th birthday.

Method

a) Histological procedure

The method is largely in keeping with that of BLOOM and AGHAJANIAN (1968). Our modification consists mainly in a lower pH of the fixative, in the treatment of the tissue with ultrasound and in the use of a higher concentration of PTA.

The brains of adult albino house mice *(mus musculus)* were perfused with a mixture of glutaraldehyde and formaldehyde in cacodylate buffer, similar to that of PETERS (1970) but with 2% formaldehyde instead of 1%, and the pH of the fixative buffered at pH = 3 instead of 7.3. After perfusion, the brains were removed from the skulls and cut into slices of about 1 mm thickness with a razor blade. These were put into vials containing fixative, placed into an ultrasound device (Bransonic 12) and treated for 10—15 minutes at 50 kHz. Small blocks were then cut out of the cortex and dehydrated in 50%, 70%, 80% and 95% ethanol, 7—10 min per step. Then the blocks were stained with phosphotungstic acid for three hours at 60 °C in a well closed vial (according to the modification of VRENSEN and DE GROOT, 1973a and b). The phosphotungstic acid was a 2% solution in absolute ethanol to which 1 ml of distilled water per 100 ml of ethanol was added. PTA was removed with cold propylene oxide according to the procedure of BLOOM and AGHAJANIAN (1968). (Their instructions must be followed carefully in order to prevent explosive reactions.) The blocks were embedded in Epon-Araldite.

After polymerization, sections of a thickness ranging between 1400 Å and 2000 Å as judged by interference colours were cut on an ultramicrotome. Measurements were taken on electron micrographs of 24 dendrites from three different mice.

b) Measurements

The thickness of the sections is less than the thickness of the dendrites investigated. The real number of synapses along a piece of dendrite is, thus, higher than that counted on the section. In order to correct for the synapses not appearing in the sections the following approach was taken:

Only pieces of dendrites which had been cut axially were considered (fig. 1). They could be recognized by the fact that the synaptic junctions on both sides of the dendrite were cut more or less perpendicularly, with both the pre- and postsynaptic membrane specializations clearly visible, such as in Fig. 2b. In such a section, the two strips of dendritic membrane contained in the section are at their narrowest; their width being only slightly larger than the thickness of the section (see below). The number of synapses on such dendrites was counted. As the thickness of the sections is known, the number (N) of synapses counted can be attributed not only to a dendritic length (l) but also to an area of dendritic membrane. As is obvious from fig. 1, for this step it is also necessary to know the size of the synaptic junctions in order to establish the range in which they may be located and still enter the section. In analogy to the Abercrombie method (1946), the areal density (a) of synaptic junctions on the dendritic membrane is then

$$a = \frac{N}{2l(t + 2r_s)} \qquad (1)$$

where t is the average thickness of the sections and r_s the average radius of the synaptic junctions, which we approximate as circular discs bent around the roughly cylindrical dendrite.

The thickness of the sections was 1700 Å on average. In the calculations we used a somewhat lower value (t = 1500 Å) in view of the fact that it is not possible to identify synapses merely touching the section. The curvature of the membrane within the section was ignored. As long as the longitudinal axis of the dendrite is contained in the section, the curvature increases the value for (t) by only a factor between 1.04 and 1.07 (at the given thickness of the sections and of the dendrites investigated).

The diameter of the synaptic junctions was measured on those dendritic segments which had been grazed by the section and were thus seen from their surface. There the synaptic junctions show their more or less circular shape and their real size without any need for a correction. With the measured values (see results), formula (1) yields

$$a = 0.98\,N \qquad (2)$$

The areal density (a) on the dendritic membrane can be converted into the real synaptic density per dendritic length, taking into account the average thickness of the dendrites. The thickness was measured on the dendritic segments cut axially. In this case, a correction of the measured values is not required either.

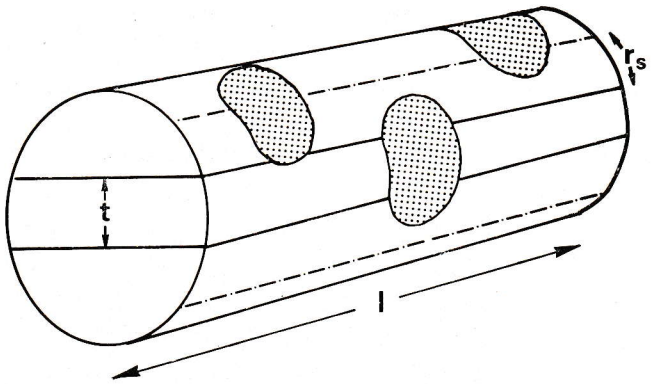

Fig. 1. Diagram of a dendritic segment cut in such a way that its longitudinal axis is contained in the section. Dotted areas indicate synaptic junctions; l, length of the segment; r_s, average radius of the junctions and distance from the section within which the centre of a synaptic junction must be located in order to appear in the section; t, thickness of the section.

Results

Fig. 2a—e shows some examples of non-spiny dendrites, stained with PTA. For comparison, a spiny dendrite is shown in Fig. 2g.

The number of synapses counted on axially cut, non-spiny dendrites was 1.9 per micron of dendritic length.

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Fig. 2a)—e). Electron micrographs of non-spiny dendrites, showing many synapses, stained with PTA. Some of the synapses are indicated by arrows. f) Cross-section of a dendrite, showing four synapses cut transversally (right). On the left, two synapses cut tangentially can be seen. g) Dendrite with spines (s). Bars: 1 μm.

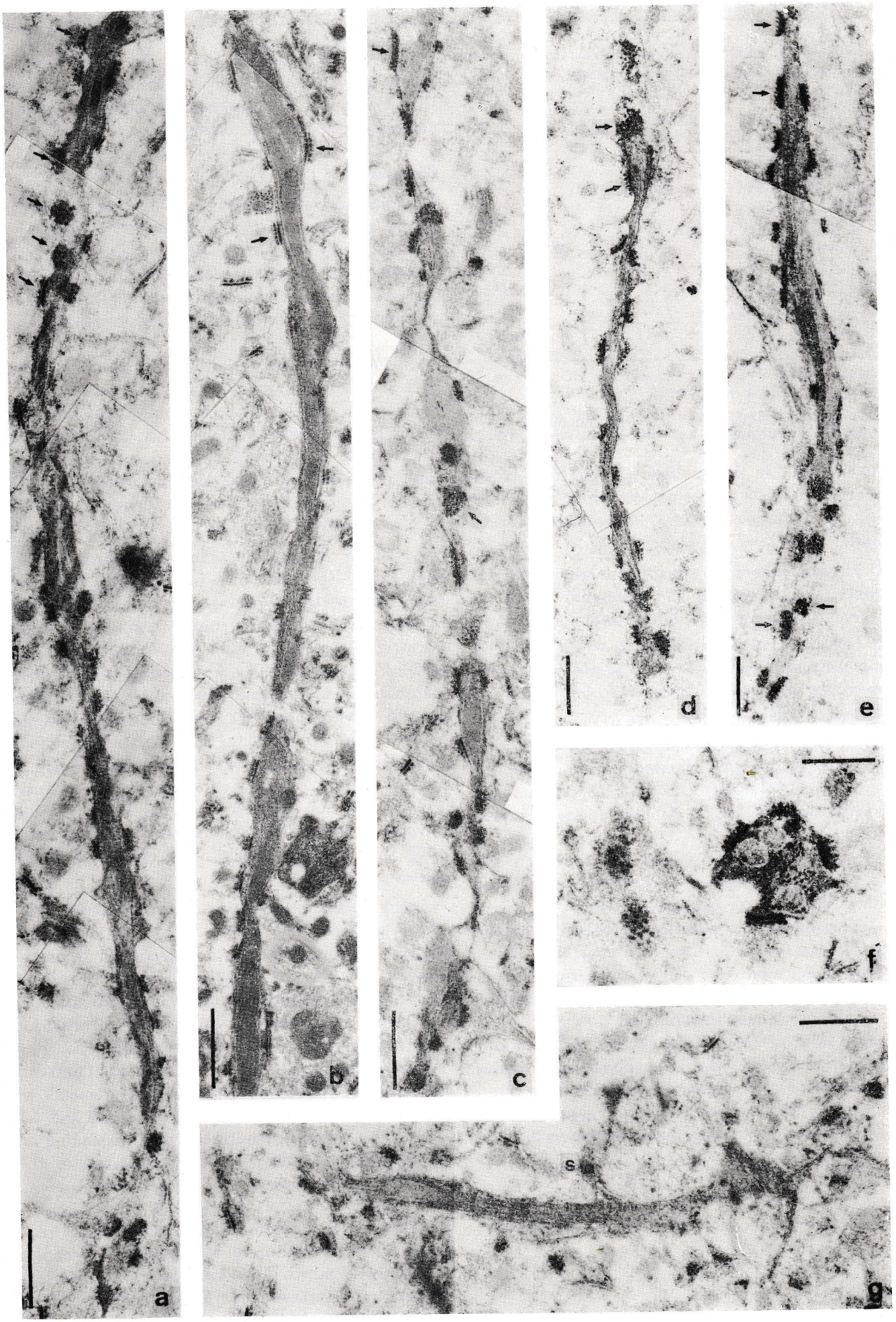

Fig. 2

Table I. Survey of the measured and corrected synaptic density and of the number and total length of dendritic segments used.

Synaptic density, measured on axially cut dendrites	1.9/µm
Standard deviation	0.6/µm
Number of dendritic pieces investigated	34
Total dendritic length investigated	201µm
Synaptic density on dendritic membrane*	1.86/µm²
Real synaptic density per length**	3.3/µm

* assuming a synaptic diameter of 0.36µm and a section thickness of 0.15µm.

**assuming a dendritic thickness of 0.57µm, which corresponds to the measured average.

As explained in the methods section, for the consideration of the synapses not contained in the sections, the following further measurements are required:

1) The diameter of the synaptic junctions. This was measured on 91 synapses and amounted to 0.36 µm on average, with a standard deviation of 0.1 µm.

2) The thickness of the dendrites, which varied between 0.4 and 0.9 µm and was 0.57 µm on average. This corresponds to a surface area of 1.79 µm² per micron of dendritic length.

These values lead to an areal density of 1.86 synapses per µm² of dendritic membrane (formula 2), and thus to a real synaptic density of 3.3 synapses per micron of dendritic length. Table I summarizes these data.

A rough check of the result can be obtained as follows: On dendritic segments which have been grazed by the section, as for example in the lower part of fig. 2e, it is possible to measure the distance between neighbouring synapses. One may now ask: What average distance between neighbours corresponds to a packing density of 1.86 synapses/µm²? To answer this question, we must imagine that the synapses are arranged in a regular pattern, such as a square or a triangular array, in which neighbouring synapses maintain a constant distance from each other. In a square array, the relation between synaptic density (a) and distance (d) of immediate neighbours is:

$$d = \sqrt{\frac{1}{a}}.$$

In an array of equilateral triangles, it becomes:

$$d = \sqrt{\frac{2}{a\sqrt{3}}}$$

With a = 1.86/µm², d is in the first case 0.73 µm, in the second 0.79 µm. These values agree nicely with the distances between neighbouring synaptic patches on the electron micrographs.

Discussion

Dark neurons: The phenomenon of dark neurons is an old problem and has been treated in detail by Cammermeyer (1962). It is now generally accepted that they are caused by inadequate fixation and/or mechanical damage during preparation. This presumably also applies to the dark processes used in this study.

Cells with spineless dendrites vary considerably in their morphology and can be classified into different types (e.g. Jones, 1975; Herrmann and Schulz, 1978; Hedlich and Winkelmann, 1982, Farién et al., 1984). It could not be determined within this study whether the "dark neurons" constituted a random selection of non-spiny dendrites or whether they were restricted to a special type.

Synaptic size: Our value (3,600 Å) derived exclusively from the synapses on non-spiny dendrites, is higher than that reported by Aghajanian and Bloom (1967) in the neuropil of the rat and by Vrensen and De Groot (1973; 1974) and De Groot and Vrensen (1978) in the rabbit, also measured on PTA-stained material. The mean values reported by these authors range between 2,150 and 2,520 Å. However, it would be premature to speculate that synaptic junctions on non-spiny dendrites are larger than in the neuropil on average: from measurements on synaptic area in semi-thin sections by Vrensen et al. (1980) one can calculate a diameter of 3,670 Å. Jones et al. (1974) even report a synaptic size of 4,200 Å in the quinea-pig cortex.

Synaptic density: As mentioned in the introduction, one of the aims of this study was to compare synaptic density on spiny and non-spiny dendrites. In a previous Golgi-study (Schüz, 1976) the density of spines on pyramidal cells in the mouse was investigated. In that instance, the values counted were corrected for the spines which were not visible in the section (because of superposition of the dendritic shaft) and can therefore be compared with the measurements in this paper. The density ranged between 0.9 and 4.1 spines per micron of dendritic length, depending on the neuron and on the position of the sample on the dendritic tree. The average was 1.9 spines per micron.

Of course, since some synapses are situated on the dendritic shaft, the number of spines does not constitute an exact measure of the number of synapses on a spiny dendrite. In layer I of the rat cortex, Vaughan and Peters (1973) found a ratio of 2.1 : 1

Fig. 3a—d. The points indicate synapses along and around non-spiny dendrites. The position of the dendrite in each picture can be recognized because of the concentration of synapses on its membrane. e) for comparison, a corresponding picture of a spiny dendrite. Bar: 1 μm.

between synapses on spines and those on dendritic shafts of spiny dendrites. In layer IV the ratio was 3.5 : 1 (Peters and Feldman, 1977). An even higher ratio (4.9 : 1) can be deduced from investigations by White and Hersch (1981) on dendrites of pyramidal cells in the mouse cortex. Taking these ratios into account, one arrives at an average density between 2.3 to 2.8 synapses per micron.

Shrinkage: When comparing quantitative results obtained by different histological methods, possible differences in shrinkage have to be taken into account. This was done by comparing photographs taken at various stages of preparation, from the perfused brain to the embedded tissue. Shrinkage turned out to be practically the same in Golgi- and PTA-preparations, namely by a linear factor of 0.88, and therefore does not affect the quantitative comparison between the two kinds of preparations.

It is possible, of course, that the process resulting in "dark cells" implies an additional shrinkage of these cells. However, such shrinkage could only affect the thickness of the dendrites, not their length, since the continuity with the surrounding tissue is maintained. Shrinkage in thickness would not affect our results, since they are ultimately expressed in synaptic density per dendritic length.

In any case, the high density of synapses cannot be due merely to our technique since non-spiny dendrites with astonishingly high synaptic density have also been shown in normal osmium-stained material (Colonnier, 1968; Peters, 1971).

Colonnier (1968) has pointed out that the role of spines is *not* to enlarge the dendritic membrane, as there is enough space between the spines for more synapses. The present study proves that the dendritic surface may indeed accommodate large numbers of synapses without spines. Spines are no prerequisite for a high density of synapses; on the contrary, on spiny dendrites the density is lower than on smooth ones. A similar result has been reported by Meek (1981) in the optic tectum of the goldfish and by Müller et al. (1984) in the visual cortex of the rabbit. Although these authors find a lower density of synapses along dendrites than we — which can be explained in part by a lower degree of shrinkage in osmium-

containing preparations — they come to the same conclusion: more synapses are present on non-spiny than on spiny dendrites.

This speaks in favour of other hypotheses on dendritic spines, for example of that of their role as the substrate of synaptic plasticity (e.g. GLOBUS and SCHEIBEL, 1967; RALL, 1974; FIFKOVÁ et al., 1982; SCHÜZ, 1986).

In the cerebral cortex, the spiny (presumably excitatory) neurons outnumber by far the spineless (presumably inhibitory) ones (GLOBUS and SCHEIBEL, 1967; BRAITENBERG, 1978; WINFIELD et al., 1980). It is therefore interesting to know that the input to the latter is slightly more than proportional to their total dendritic length in the neuropil.

A further observation should be recorded. We put a transparent foil onto the photographs of several non-spiny dendrites and one spiny one and marked all the synapses, including those in the surrounding tissue (See Fig. 3). In the case of the non-spiny ones the position of the dendrites is still recognizable because of the concentration of synapses on them. One gains the impression that these dendrites are flanked by regions of lower synaptic density. This suggests that spineless dendrites attract presynaptic elements toward their own surface. Indeed, they must do so, for they would not encounter as many presynaptic elements as they ultimately do contact if the presynaptic elements were distributed at random in the neuropil.

Acknowledgement

We are very grateful to Prof. Valentin BRAITENBERG and Dr. Günther PALM for valuable advice and for the correction of the manuscript, and to Mrs. Shirley WÜRTH for linguistic assistance.

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