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DNA in Squid Synaptosomes

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Abstract
The synthesis of brain metabolic DNA (BMD) is modulated by learning and circadian oscillations and is not involved in cell division or DNA repair. Data from rats have highlighted its prevalent association with the mitochondrial fraction and its lack of identity with mtDNA. These features suggested that BMD could be localized in synaptosomes that are the major contaminants of brain mitochondrial fractions. The hypothesis has been examined by immunochemical analyses of the large synaptosomes of squid optic lobes that are readily prepared and identified. Optic lobe slices were incubated with 5-bromo-2-deoxyuridine (BrdU) and the isolated synaptosomal fraction was exposed to the green fluorescent anti-BrdU antibody. This procedure revealed that newly synthesized BrdU-labeled BMD is present in a significant percent of the large synaptosomes derived from the nerve terminals of retinal photoreceptor neurons and in synaptosomal bodies of smaller size. Synaptosomal BMD synthesis was strongly inhibited by actinomycin D. In addition, treatment of the synaptosomal fraction with Hoechst 33258, a blue fluorescent dye specific for dsDNA, indicated that native DNA was present in all synaptosomes. The possible role of synaptic BMD is briefly discussed.

Keywords Brain metabolic DNA · Synaptosomes · Presynaptic terminals · DNA synthesis

Introduction

DNA synthesis is generally considered to occur during cell division and repair of damaged DNA. Nonetheless, several studies have demonstrated that DNA synthesis is also taking place during periods of enhanced cell activity. The latter data have been interpreted to indicate that newly synthesized DNA might replace DNA segments concurrently lost under those conditions ([1–5], for reviews, see [6–8]). In addition, comparable data from the adult rodent brain have shown that newly synthesized DNA undergoes a marked turnover [9] and is modulated by learning [10–14], post-trial sleep [15, 16], and circadian oscillations ([17–19], for a recent review, see [20]).

In rats learning a reverse handedness task, the newly synthesized brain DNA (brain metabolic DNA or BMD) was present in nuclear and mitochondrial fractions and in neuronal perikaryal fractions [14]. At variance with avoidance learning, training for the reverse handedness task induced a marked decrease in BMD synthesis in learning rats with respect to control rats. Notably, the specific activity of mitochondrial BMD was considerably lower than nuclear BMD in the hippocampus (− 58% versus − 28%) and visual cortex (− 59% versus no modification). In addition, the content of mitochondrial DNA markedly increased in these regions of learning rats (by 2.4-fold and 3.7-fold) while they remained essentially unchanged in the nuclear fraction (by 0.21-fold and 0.19-fold).

These results could not be attributed to nuclear fragments contaminating the mitochondrial fraction since the specific activity of mitochondrial BMD was 4-fold higher than that of nuclear BMD [14]. Moreover, previous data had shown that mitochondrial BMD differed from mtDNA with respect to its considerably higher rate of synthesis and different subcellular distribution from cytochrome oxidase, a reliable mitochondrial marker [21, 22]. These considerations and the well-known major presence of synaptosomes in brain mitochondrial fractions suggested that BMD could be associated with synaptosomes. The hypothesis appeared in agreement with the BMD behavior in neuronal perikarya of learning and control rats that was similar to that of mitochondrial

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BMD and was attributed to nerve terminals known to remain attached to perikaryal plasma membranes [23].

Accordingly, the presence of newly synthesized BMD in synaptosomes was examined in immunochemical analyses of the synaptosomal fraction from squid optic lobes that was known to contain unusually large and readily identifiable synaptosomes [24, 25]. A brief report of these experiments has appeared [26].

Methods

Squid (*Loligo vulgaris*) were obtained from the Zoological Station Anton Dohrn, Naples, Italy. Dissected optic lobes were immersed in cold artificial sea water (ASW: 460 mM NaCl, 10 mM KCl, 11 mM CaCl2, 55 mM MgCl2, 0.6 mM KHCO3, 10 mM Tris-Cl pH 7.8) and used to prepare 300-μm thick slices with a tissue slicer equipped with a vibrating razor blade. Slices were incubated for 2 h at room temperature (about 18°) in Petri dishes containing 2 ml ASW and 20 μM 5-bromo-2′-deoxyuridine (BrdU, Merck); in separate experiments, they contained 20 μM BrdU plus 25 μg/ml actinomycin D; control slices were incubated without BrdU. Following incubation, slices were homogenized in 1.5 ml 0.7 M sucrose, 20 mM Tris-Cl pH 7.4 (homogenizing medium or HM) and the homogenate was centrifuged in an Eppendorf table centrifuge (13,000 rpm, 30 min). The synaptosomal fraction floating as a thin layer over the centrifuged homogenate [24, 25] was recovered and washed twice with HM. Following its gentle resuspension in 0.25 ml HM, it was fixed with 4% paraformaldeide in 0.5 M NaCl, 10 mM PBS (15 min; room temperature) and washed several times with 0.5 M NaCl, 10 mM PBS. Aliquots of the latter suspension were smeared on glass slides and treated with 0.1 M Tris-glycine buffer pH 7.4 to block free aldehydic groups. Samples were then exposed to the green fluorescent anti-BrdU antibody Alexafluor 488 conjugate (Merck) diluted 1:5 with 1% BSA in PB (1 h, room temperature).

Treatment of the synaptosomal fraction with 5 μg/ml Hoechst 33258 (Invitrogen) lasted 10 min. Fluorescent signals were analyzed with a Zeiss Axioskop microscope, and images acquired with an AxioCam MRc5 camera and analyzed with AxioVision 4.7 software from Axioskop System (Zeiss).

Results

The synaptosomal fraction from optic lobe slices incubated with 20 μM BrdU showed several round bodies of about 5-μm diameter bearing a peripheral green fluorescent ring after it was treated with anti-BrdU antibody Alexafluor 488 conjugate (Fig. 1a). The fluorescent ring clearly indicated the presence of newly synthesized BrdU-labeled DNA. The circular shape and the size of these bodies corresponded to the well-known features of large synaptosomes derived from the nerve terminals of retinal photoreceptor neurons [24, 25]. The fluorescent ring was not present in some of the same bodies, as it may be seen from the similar sized shadows visualized in other fields of the same fraction (Fig. 1b). The results implied that DNA synthesis only occurred in a fraction of the large synaptosomes that electron microscopic analyses had shown to belong to different classes [27]. Green fluorescent signals were also present in smaller bodies of variable diameters

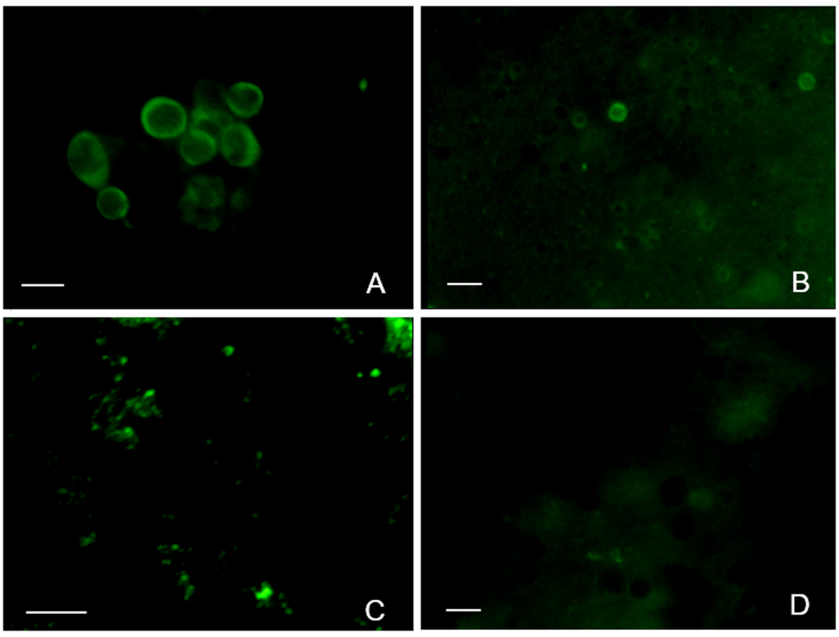

Fig. 1 Newly synthesized DNA is present in squid synaptosomes. In the synaptosomal fraction from slices incubated with 20 μM BrdU, some round bodies of about 5 μm bear peripheral green fluorescent rings (**a**) while other comparable bodies do not (**b**); in addition, green fluorescence is present in bodies of smaller size (**c**). No green fluorescence is present in the synaptosomal fraction from slices incubated without BrdU (**d**). Scale bars, 5 μm

down to 1 μm (Fig. 1c) that might correspond to smaller synaptosomes [27]. On the other hand, fluorescent signals were completely absent in the synaptosomal fraction of control slices incubated without BrdU (Fig. 1d), thus confirming the identification of the green fluorescent signal as newly synthesized BrdU-labeled DNA.

The biological origin of synaptosomal BrdU-labeled DNA was confirmed by incubating optic lobe slices with 20 μM BrdU in presence of 25 μg/ml actinomycin D, an inhibitor of DNA polymerase. Under these conditions, the fluorescent signal was almost completely absent (Fig. 2b) while slices incubated with 20 μM BrdU exhibited the synaptosomal green fluorescent ring (Fig. 2a).

Additional support to the DNA localization in squid large synaptosomes was provided by treatment of the synaptosomal fraction with Hoechst 33258, a blue fluorescent dye selectively staining dsDNA. As shown in Fig. 3, all round bodies of about 5-μm diameter displayed blue fluorescent rings, indicating the presence of dsDNA. Since the size and shape of these bodies closely corresponded to those of the large synaptosomes [24, 25, 28], the results indicated that all large synaptosomes contain dsDNA. When compared to the presence of newly synthesized DNA only in a fraction of the large synaptosomes (Fig. 1), they further suggested that synaptosomal DNA synthesis could only occur under appropriate physiological conditions, in agreement with the presence of two main categories of large optic lobe synaptosomes differing in cytoplasmic density and number of clear vesicles [27].

Treatment of the synaptosomal fraction with Hoechst 33258 also visualized smaller bodies exhibiting blue fluorescent signals (Fig. 3). In view of their size reaching down to 1 μm, they might correspond to synaptosomal classes with diameter of 1.5–5.0 μm and 0.3–0.7 μm, as identified by electron microscopy [27].

Discussion

The blue fluorescence of the large round bodies stained by Hoechst 33258 in the synaptosomal fraction of squid optic lobes indicates that presynaptic terminals contain DNA. Indeed, they display the same size and shape of the synaptosomes derived from the large, carrot-like nerve terminals of retinal photoreceptor neurons [24, 25]. The same synaptosomes have previously been used to demonstrate (i) the presence of an active system of protein synthesis in the presynaptic domain [24, 28]; and (ii) the synthesis of presynaptic RNA by perisynaptic glial cells [25]. The latter result rested on the presence of newly synthesized RNA in the large optic lobe synaptosomes after incubation of optic lobe slices with radiolabeled uridine. These synaptosomes exclusively derive from the nerve terminals of retinal photoreceptor neurons. This localization excluded any perikaryal contribution to the synthesis of presynaptic RNA.

The blue fluorescent ring of the large synaptosomes is not likely to be due to mtDNA in view of its diffused appearance clearly at odds with the punctuated pattern expected from mtDNA. It should also be noted that the identification of BMD with mtDNA suggested by its prevalent presence in the mitochondrial fraction of rat brain [14, 22] was excluded by its markedly high rate of synthesis and by the divergent subcellular distribution with mitochondrial cytochrome oxidase [21].

The peripheral blue fluorescence ring of the large synaptosomes is strikingly similar to the peripheral green fluorescence ring signaling the presence of newly synthesizd BrdU-labeled DNA. This similarity indicates that either feature belongs to the same subcellular structure and, consequently, that synaptosomal dsDNA is likely to originate from the local synthesis of DNA. This conclusion is in agreement with the presence of dsDNA in all large synaptosomes but it may appear in contrast with the synthesis of DNA in only a fraction of the large synaptosomes. However, BMD synthesis is triggered by cell activity [20] and conditions triggering BMD synthesis in squid presynaptic terminals are not known. On the other hand, kinetic studies of rat cytoplasmic BMD have shown its persistent presence in the synaptosomal fraction for 1 month after its synthesis [22]. A comparable survival may be expected in the squid.

Since newly synthesized RNA of the large squid synaptosomes originated from the nuclei of perisynaptic glial cells ([25], for reviews, see [29, 30]), it may be tempting to assume

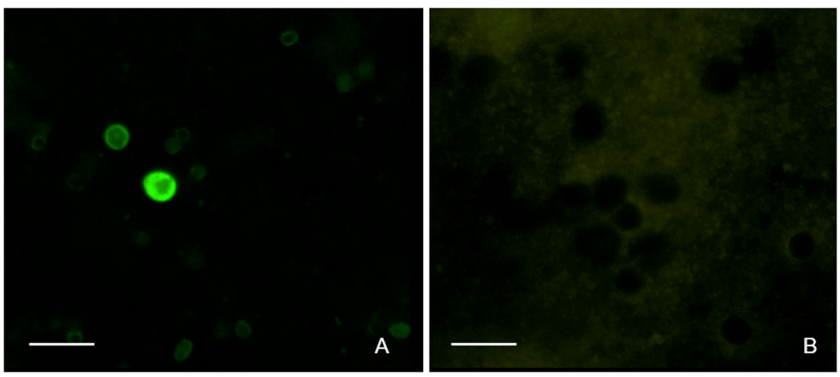

Fig. 2 Actinomycin D inhibits the synthesis of synaptosomal DNA. Round bodies of about 5 μm bearing peripheral green fluorescent rings are present in the synaptosomal fraction from slices incubated with 20 μM BrdU (**a**) but little if any fluorescence is present in the synaptosomal fraction from slices incubated with 20 μM BrdU plus 25 μg/ml actinomycin D (**b**). Scale bars, 10 μm

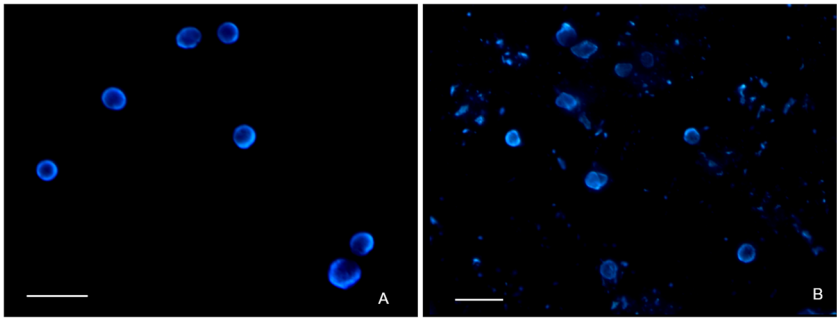

Fig. 3 Native DNA is present in squid synaptosomes. In the synaptosomal fraction stained by Hoechst 33258, all round bodies of about 5 μm bear peripheral blue fluorescent rings (**a**); blue fluorescence is also present in bodies of smaller size (**b**). Scale bars, 10 μm

that synaptosomal DNA might also be derived from the same glial cells. However, this interpretation contrasts with the demonstration that subcellular BMD is synthesized in rat cytoplasm by reverse transcription before acquiring the double stranded configuration and being transferred to nuclei [21, 22]. It follows that squid presynaptic BMD may also originate by reverse transcription in the same nerve terminals and be eventually transferred to perisynaptic glial nuclei. According to this interpretation, presynaptic terminals receiving newly transcribed RNA from perisynaptic glial nuclei [25] might be expected to deliver their reverse transcribed DNA to the same perisynaptic glial nuclei. Overall, an intriguing spiraling of nucleic acids.

The presence of DNA in synaptic regions raises the problem of its role in brain activity and calls attention to the cytoplasmic localization of extra-mitochondrial DNA in a wide variety of cells [31–37]. In mammalian brain, the problem has been highlighted by the identification of BMD in subcellular fractions [14, 21, 22] and by its modulation by learning, circadian oscillations and, more generally, brain activity [20]. It may be worth noting that in the optic lobes of *Octopus vulgaris* a significant amount of DNA was present in the floating synaptosomal fraction [38] but data were attributed to nuclear contamination.

As mentioned in the "Introduction," the synthesis of metabolic DNA in non-neural cells has been interpreted to reflect the needed replacement of DNA segments lost during periods of enhanced cell activity [1–5]. On the other hand, in the learning brain BMD might not only replace the lost DNA but might also be involved in the formation of the novel behavioral response to a changed environment [6–10]. Accordingly, since BMD is reverse transcribed from cytoplasmic template RNA [22, 39], learning rats should be expected to differ from control rats not only in BMD but also in template RNA possibly including mRNA and small RNA. An intriguing question may then be raised with regard to the origin of BMD difference. Are they going to be completely accounted by differences in template RNA in view of the latter common origin from the same DNA [14]? The question could be answered by comparing sequences of learning and control BMD with the corresponding sequences of template RNAs. If differences appear to be complementary, the novelty of learning BMD could be ascribed to template RNA. On the contrary, if learning BMD sequences could not be entirely attributed to corresponding differences in template RNA, the unaccounted BMD novelty might have a different origin related to other variables of the activated brain cells. Notably, it should not be forgotten that an appropriately modified electromagnetic field has been shown to behave as a DNA template [40] and that control and active brain cells are continuously exposed to a highly variable electromagnetic environment.

Compliance with Ethical Standards

Competing Interests All authors declare that they have no conflict of interests.

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