SPECIFIC BINDING OF DHEA TO THE N-TERMINAL OF THE MICROTUBULE-ASSOCIATED PROTEIN MAP2

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**Summary**

The effect of neurosteroids is mediated through their membrane or nuclear receptors. However, no DHEA-specific receptors have been evidenced so far in the brain. In this paper, we showed by isothermal titration calorimetry that the dehydroepiandrosterone (DHEA) specifically binds to the dendritic brain microtubule-associated protein MAP2C with an association constant of $2.7 \times 10^7 \text{M}^{-1}$ and at a molar ratio of 1:1. By partial tryptic digestions and mass spectrometry analysis, we found that the binding involved the N-terminal region of MAP2C. Interestingly, MAP2C displays homologies with $17\beta$-hydroxysteroid dehydrogenase 1 ($17\beta$-HSD1), an enzyme required for estrogen synthesis. Based on these sequence homologies and on the X-ray structure of the DHEA-binding pocket of $17\beta$-HSD1, we modeled the complex of DHEA with MAP2C. The binding of DHEA to MAP2C involved specific hydrogen bonds that orientate the steroid into the pocket. This work suggests that DHEA can directly influence brain plasticity via MAP2C binding. It opens interesting ways for understanding the role of DHEA in the brain.
Introduction

The microtubule-associated proteins (MAPs$^1$) are characterized by their ability to promote tubulin polymerization and to stabilize microtubules. MAP2 is one of the most abundant MAPs in the brain. A single gene containing 20 exons encodes multiple MAP2 isoforms, which are produced by alternative splicing of a pre-mRNA (1). MAP2 isoforms have been divided into two groups depending on their molecular weight. High molecular weight MAP2 include MAP2A and MAP2B, whereas low molecular weight MAP2 include MAP2C and MAP2D. Expression of MAP2 isoforms is regulated during development. MAP2A is mostly expressed in adult brain, while MAP2B is present all along the development of the nervous system (2). Conversely, MAP2C is expressed at early developmental stages but is also found in adult retina and olfactory bulb, tissues in which neurogenesis persists in the adult stage (3). MAP2 belongs to a family of cytoskeletal proteins and is predominantly expressed in dendrites of neurons (4). It regulates neurite extension (5) and is associated with the development of neuronal polarity (6). All in all, these observations suggest that MAP2 plays a significant role in neuronal plasticity.

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1 The abbreviations used are: 17$\beta$-HSD1, 17$\beta$-hydroxysteroid dehydrogenase 1; CD, circular dichroism; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone-sulfate; ESI-MS, electrospray mass spectrometry; GABA, $\gamma$-aminobutyric acid; IPTG, isopropyl $\beta$-thiogalactopyranoside; ITC, isothermal titration calorimetry; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight spectrometry; MAP, microtubule-associated protein; NMDA, N-Methyl-D-Aspartate; PDB, Protein Data Bank; PHD, Profile network prediction HeiDelberg; PKA, Protein kinase A.
Regulation of MAP2 involves steroids such as estradiol or progesterone (7). Interestingly, these steroids are also synthesized in the brain for its own use and therefore called neurosteroids. In pioneering work, Baulieu and coworkers showed *in situ* synthesis of DHEA and its sulfate form (DHEA-S) in the rat brain, independently of the peripheral endocrine glands (8). Moreover, they showed that major steroids, such as pregnenolone, pregnenolone sulfate, DHEA, and DHEA-S, are synthesized *de novo* from cholesterol in different brain cells such as glial cells or Purkinje cells (for reviews, see (9), (10), (11)). As a consequence, these works opened an emerging field in the study of neurosteroid effects. Among these studies, many focused on the role of DHEA in the brain. Some concluded that DHEA displays neuroprotective effect against anoxia (12), NMDA-induced injury (13), and amyloid-β toxicity (14), or even that DHEA stimulates neurogenesis in rat hippocampus (15).

Steroid hormones act mostly via binding to specific nuclear receptors, but they also modulate neurotransmitter receptor function at the membrane level. However, the mechanism of action of neurosteroids in the brain is not fully understood. Another recent breakthrough came from equilibrium binding studies, which evidenced that pregnenolone was able to bind MAP2 and to stimulate microtubule assembly ((16), (17)). These observations clearly suggested that neurosteroids could directly bind on neuronal cytoskeleton and influence its dynamics with potential implication for brain plasticity.

We report here that DHEA tightly binds to MAP2C. This binding involved polar and hydrophobic interactions and was essentially localized in the N-terminal extremity. Note that this N-terminal extremity is specific of MAP2 isoforms. It is absent in other MAPs, notably Tau, involved in neurodegenerative diseases. The direct interaction of DHEA to MAP2C raises the possibility that, in addition to the well-known steroid hormone-receptor interactions, direct regulation by neurosteroids at the cytoskeleton level may participate in the plasticity of the brain.
EXPERIMENTAL PROCEDURES

DNA Construct. A full-length cDNA of rat MAP2C was constructed as previously described (18). In brief, the 5’ Nde1-Pst1 fragment of pCG2b33 (19) was ligated into the 3’ Pst1-BamH1-digested fragment of pJBMap2c (20). This new construct was then digested by Nde1 and BamH1 and subcloned into the Nde1-BamH1-digested pET3a expression vector (Novagen, Madison, WI). The nucleotide sequence was checked by direct sequencing of double-stranded plasmid DNA (Biofidal, Vaux-en-Velin, France). We called the new plasmid construct pMap2C.

Expression and purification of MAP2C. E. coli strain BL21(DE3) (Novagen) was transformed with pMap2C and grown in LB supplemented with ampicillin (100 µg/mL) to OD₆₀₀ = 0.6-0.8. Then protein expression was induced with 0.4 mM IPTG for 2 hours. Bacteria were harvested by centrifugation at 6000 g. The pellet was resuspended in 50 mM phosphate buffer pH 7.5, and bacteria were broken with a French press. The lysate was then centrifuged for 45 min at 20,000 g to remove bacterial debris. Overnight ammonium sulfate fractionation (35% final) clarified the supernatant. The pellet was finally recovered by centrifugation at 25,000 g for 1 hour, dissolved in 50 mM phosphate buffer pH 7.5, and dialyzed against the same buffer. Proteins were first separated on HiTrap SP column (Amersham Biosciences AB, Uppsala, Sweden) previously equilibrated with 50 mM phosphate buffer pH 7.5, with a linear gradient of NaCl (0-100% in 100 min with a flow rate of 1mL.min⁻¹). The presence of MAP2C in each fraction was quickly checked by SDS-PAGE and MALDI-MS (Voyager Elite, Perseptive Biosystem Inc., Framingham, MA). Further purification was then done by reverse phase chromatography on Resource 15 RPC column (Amersham Biosciences AB). In brief, the reverse phase column was equilibrated in H₂O with 0.065% TFA, and proteins were separated with a linear gradient of acetonitrile in 0.05% TFA.
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(0-100% in 100 min with a flow rate of 1mL.min⁻¹). One-milliliter fractions were collected, loaded on an SDS-PAGE, and transferred onto nitrocellulose membranes for Western blot with antibodies to MAP2C. Purity of the sample was then checked by ESI-MS (ESI QTOF, Micromass, Manchester, UK). The fractions of interest were frozen in liquid nitrogen, freeze-dried, and kept at -80°C until use. For spectroscopic and calorimetric studies, the protein was resuspended in the appropriate buffer, and concentrations were determined by amino acid analysis.

**Peptide synthesis.** The N-terminal fragment of MAP2C (84-120), also present in MAP2A and MAP2B, was assembled in solid phase synthesis according to the method of Barany and Merrifield (21) with an automated synthesizer (ABI 433A, Perkin-Elmer, Applied Biosystem Inc., Forster City, CA). Purification was carried out on a Beckman high-pressure liquid chromatography apparatus with a C8 reverse phase column (Merck). All steps of purification were carried out at pH 4.5. Success of the synthesis was verified by electrospray mass spectrometry on a single quad PE-SCIEX API 150ex (Perkin-Elmer). Amino acid analyses were performed on a Beckman model 6300 analyzer.

**Polyacrylamide gel electrophoresis and Western blot experiments.** SDS-PAGE was performed on 15% polyacrylamide slab gels by standard procedures. For quick control of purification steps, the gels were stained with 0.1% Coomassie Brilliant Blue R-250 and analyzed for the presence of MAP2C. Alternatively, to check the purity of MAP2C, the gels were transferred onto nitrocellulose membranes and submitted to Western blot experiments with home-made polyclonal antibodies to MAP2C.

**Mass spectrometry.** Molecular mass of the protein was determined by ESI-MS (ESI QTOF, Micromass), and tryptic digestions were analyzed by MALDI (Voyager Elite, Perseptive Biosystem Inc.). In brief, for ESI-MS, samples were mixed with acetonitrile, 1% formic acid (1:1 v/v). For MALDI experiments, 0.5 µl of sample was crystallized with a
saturated solution of sinapinic acid (3,5-dihydroxybenzoic acid) in 0.1% aqueous TFA. Spectra were recorded in linear mode with a voyager MALDI-TOF (Applied Biosystems, Forster City, CA).

*Tubulin polymerization.* Tubulin was purified from porcine brain as previously described (22). Tubulin was equilibrated in polymerization buffer (3.4 M glycerol, 20 mM NaPi, 10 mM MgCl₂, 1 mM EGTA, 0.1 mM GTP, pH 6.5) by two successive runs on a Sephadex-G25 column (Amersham Biosciences). We used 15 µM tubulin for assembly with and without 3 µM MAP2C. The reaction components were mixed at 4°C and transferred into a pre-cooled cuvette at 4°C. Assembly was induced by increasing the temperature to 37°C. Polymerization was then monitored by recording the increase in absorbance at 350 nm through turbidimetry with a spectrophotometer (DU 7400, Beckman Coulter Inc., Fullerton, CA). Depolymerization was induced by decreasing temperature to 4°C.

*Circular dichroism measurements.* Circular dichroism (CD) spectra were recorded in the 260-178 nm range on a Jobin-Yvon UV CD spectrophotometer (MARK VI, Long-Jumeau, France). The instrument was calibrated with (+)-10-camphorsulfonic acid. A ratio of 2:1 was found between the positive CD band at 290.5 nm and the negative band at 192.5 nm. The measurements were carried out at 20°C using 0.005 cm path length cells. Spectra were recorded in 20 mM phosphate buffer pH 7, and the base line signal was removed from the final spectrum. Data were collected at 0.5 nm intervals with a scan rate of 3 nm/min. Circular dichroism spectra was reported as per amide. Protein concentration was determined by the Beckman Model 6300 amino acid analyzer (Beckman Coulter Inc.). Data were analyzed with the method of Manavalan and Johnson (23). This method uses 32 reference proteins with known secondary structures determined from high resolution X-ray crystallography data to deduce the secondary structure.
**Isothermal Titration Calorimetry (ITC).** For ITC experiments, MAP2C was resuspended in 50 mM MES buffer, 1mM DTT, pH 6.8, or in 50 mM phosphate buffer pH 7.5, and dialyzed against the same buffer for 2 hours to remove any remaining TFA counterions. Protein concentration was determined by amino acid analysis. DHEA was dissolved in 100% isopropanol or in 100% methanol and then diluted with the buffer to ensure a final concentration of 10% isopropanol or 20% methanol. For the corresponding ITC experiments, 10% isopropanol or 20% methanol was added to the protein solution. Binding of MAP2C to DHEA was carried out at 25°C using a MicroCal MCS titration calorimeter (MicroCal LLC, Northampton, MA). The enthalpy of binding ($\Delta H$), the affinity constant ($K_a$), and the molar binding stoichiometry ($N$) were obtained with the following procedure: 10 µL aliquots of DHEA ($2 \times 10^{-4}$ M) were injected with a 250 µL microsyringe into the 1.34 mL calorimeter cell containing the protein solution (at about $2 \times 10^{-5}$ M) to achieve a complete binding isotherm. The heat of dilution was measured by injecting the ligand into the buffer solution. The value obtained was subtracted from the heat of reaction to obtain the effective heat of binding. Titration curves were fitted using the MicroCal Origin software, assuming one set of sites. Changes in free energy $\Delta G$ and entropy $\Delta S$ were calculated from the relationship:

$$\Delta G = -RT\ln K_a = \Delta H - T\Delta S$$

**Tryptic digests of MAP2C.** MAP2C (50 µg) with and without DHEA was mixed with 0.5 ng of trypsin and incubated at 37°C. Digestions were stopped after 15 to 45 min by addition of 5% formic acid. Samples were then analyzed by matrix-assisted laser desorption ionization spectrometry.

**Secondary structure predictions.** “PHD” (Profile network prediction HeiDelberg) method was used to predict MAP2C secondary structure. This software developed by Rost and Sander ((24), (25)) predicts the secondary structure of proteins from multiple sequence alignments (http://cubic.bioc.columbia.edu/predictprotein/). Secondary structure is predicted through
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neural networks, based on 126 known three-dimensional protein structures, rating at an expected average accuracy > 72% for the three states: helix, strand and loop. In our alignments, however, we used only predictions with an average accuracy > 82% to be more confident with our results.

*Sequence alignment and molecular modeling.* The DHEA-binding pocket of the 17-β hydroxysteroid dehydrogenase 1 (17β-HSD1) crystal structure (Protein Data Bank code: 3DHE) is made of six regions. These six regions were well aligned with the MAP2C sequence by the ClustalX program (26). Five of them aligned with the N-terminal sequence of MAP2C: 1-14, 47-53, 58-61, 67-71, and 108-119; whereas only one region aligned with the C-terminal sequence: 295-310. We then used these six aligned regions to build the DHEA-binding pocket of MAP2C by structural homology with the 17β-HSD1 structure, according to the main principles outlined by Greer (27). For this, we first built the MAP2C backbone of each of these 6 regions with the software TITO (Tool for Incremental Threading Optimisation, (28), (http://bioserv.cbs.cnrs.fr/). This software allows one to generate the backbone of a protein of unknown structure that displays sequence homologies with a protein of known structure. However, at this step, only the backbone and not the lateral chains was obtained. Therefore, in a second step, we used the MaxSprout software ((29), http://www.ebi.ac.uk/maxsprout/) for generating the amino acid side chains. Third, to construct the final DHEA binding site of MAP2C, we superimposed the six regions of MAP2C with the homologous regions of the DHEA-binding pocket of 17β-HSD1. For that purpose, we used the Accelrys software *InsightII*, and *Builder modules* (San Diego, CA), run on a Silicon Graphics O2 workstation (SGI, Mountain View, CA). Then, we positioned the DHEA molecule in the pocket of MAP2C as it appears in 17β-HSD1. All these steps allowed us to build a model of the DHEA-binding pocket of MAP2C.

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RESULTS

Purification procedures respected the functional integrity of MAP2C. Procedures described in the literature generally use heat to purify microtubule-associated proteins. To avoid such treatment for preventing protein denaturation, we employed milder conditions in our purification scheme. As shown by SDS-PAGE, ion-exchange chromatography produced an enriched fraction of MAP2C with an apparent molecular weight of 70 kDa (Fig. 1A, lane 1). This observation was confirmed by Western blot experiments with polyclonal antibodies to MAP2C (Fig. 1A, lane 2). This fraction was further purified by HPLC to a high level of purity (Fig. 1A, lane 3). Electrospray mass spectrometry analysis (Fig. 1B) showed a MAP2C molecular mass of 49,172 ± 5 Da. This molecular mass corresponds to the full-length protein without the N-terminal methionine, which is often cleaved by bacteria. Nonetheless, this result indicated a discrepancy with the apparent molecular size observed by SDS-PAGE. This behavior suggested a non globular three-dimensional pattern of microtubule-associated proteins, as previously reported (19). Circular dichroism (CD) spectrum was characterized by a negative band at 200 nm, which is usually associated with random coil structures (Fig. 2). However, that the intensity of this band was low relative to CD spectra of peptides in full random coil (30) indicated that other secondary structures exist in this protein. Analysis of this spectrum according to the method of Manavalan and Johnson (23) indicated 31% of \( \beta \)-turns, 21% of \( \beta \)-sheet or extended structures, and 4% of \( \alpha \)-helices (Table 1). The circular dichroism spectrum of an \( \alpha \)-helix is characterized by three bands, a positive and a negative contribution respectively at 190 and 207 nm due to \( \pi \)-* transitions, and a negative contribution at 222 nm due to \( n\)-* transitions (30). The MAP2C spectrum did not show a positive contribution at 190 nm, indicating a very low content in \( \alpha \)-helices. However, the high content of \( \beta \)-turns and the negative contribution at 185 nm observed with type II \( \beta \)-turn might explain the absence of an \( \alpha \)-helix positive contribution at 190 nm. Finally, to check the
functionality of our purified preparation of MAP2C, we performed tubulin polymerization assays. Indeed, MAPs are characterized by their ability to promote tubulin polymerization and to stabilize microtubules. Therefore, we performed tests with and without MAP2C. As expected, with MAP2C, we observed a left shift of the spectrum, indicating that MAP2c increased microtubule nucleation and polymerization (Fig. 3). The increase in plateau and the slower decrease induced by lowering temperature to 4°C showed that MAP2C increased the stabilization of microtubules. These results indicated that purified MAP2c was functional. We therefore investigated the possible binding of DHEA to this functional MAP2C.

**DHEA binds to MAP2C.** Binding of DHEA to MAP2C was studied by isothermal titration calorimetry (ITC). ITC is the method of choice for measuring interactions between proteins and small ligands. This method gives direct access to all thermodynamic parameters of the interaction: $K_a$ (affinity constant), $\Delta G$ (free energy), $\Delta H$ (enthalpy), $\Delta S$ (entropy), and $n$ (number of sites). As a consequence, we can deduce the information about the type of interactions between the ligand and the protein. A negative $\Delta H$ value reflects polar interactions and Van der Waals contacts, and a positive $\Delta S$ reflects hydrophobic contacts. A typical set of data in 50 mM MES pH 6.8 is shown in Figure 4A. The upper plot represents the raw calorimetric data for the ligand-into-protein titration, and the lower plot represents the binding isotherm. DHEA bound to MAP2C with a strong affinity ($K_a = 2.7 \times 10^7 \text{ M}^{-1}$), which revealed the specificity of interaction, and with a stoichiometric of 1:1. In addition, DHEA binding resulted in a strong exothermic effect ($\Delta H = -34 \pm 5 \text{ kJ.M}^{-1}$). This high enthalpy was due to a combination of the binding itself, mainly via polar or Van der Waals interactions, and to the buffer ionization. Therefore, to determine the contribution of buffer ionization to the thermodynamic values and to calculate the entropy ($\Delta S$), we performed the same experiment in 50 mM phosphate buffer pH 7.5, a buffer with no heat of ionization ($\Delta S$ for MES). With phosphate buffer, the difference between values measured in MES and phosphate
buffers were attributable solely to proton exchange occurring during the binding of DHEA. As illustrated in Figure 4B, the enthalpy changed sharply for the binding of DHEA to MAP2C \((\Delta H = -10 \pm 8 \text{ kJ.M}^{-1})\), whereas the affinity remained constant \((K_a = 9.7 \times 10^6 \text{ M}^{-1})\). We therefore concluded that several protons were exchanged during the binding of DHEA. However, their exact number was difficult to determine because the signal in phosphate buffer was extremely low and because it gave an inaccurate \(\Delta H\) value. \(\Delta H \) \((\Delta H = -10 \pm 8 \text{ kJ.M}^{-1})\) and \(\Delta S \) \((\Delta S = 0.101 \pm 0.01 \text{ kJ.M}^{-1})\) values showed that the DHEA binding was both enthalpically and entropically driven, indicating that the number of polar and hydrophobic interactions increased due to DHEA binding. We therefore wanted to look at the region in which DHEA binds to MAP2C. We then looked for DHEA binding domain of MAP2C by tryptic digestion experiments (see below).

The N-terminal extremity of MAP2 binds DHEA. To look at the DHEA binding region of MAP2C, we performed limited proteolytic digestions with trypsin, with and without DHEA, and we analyzed the fragments by mass spectrometry (Table 2). We found that, with DHEA, the entire N-terminal \((1-120)\) was more resistant to cleavage. With DHEA, K10 and R93 were transiently resistant to cleavage, whereas K112 and K117 remained uncleaved even after 45 min of digestion. Because three of these amino acids \((R93, K112, K117)\) were very close in the sequence, we looked more precisely around this region. Using PHD software that predicts protein secondary structure, we observed the presence of scarce \(\alpha\)-helices and \(\beta\)-sheets localized at both extremities of MAP2C (Fig. 5). In addition, the region including the three amino acids \(R93, K112, K117\), and encompassing the 84-120 region was predicted to be structured with \(\alpha\)-helices. Interestingly, this 84-120 sequence is absent from Tau, which is unable to bind pregnenolone, a precursor of DHEA (16). We then synthesized the 84-120 N-terminal peptide to confirm these results by monitoring DHEA binding to MAP2C in this N-terminal region.
An additional region is required for the binding of DHEA. Circular dichroism experiments showed the 84-120 N-terminal peptide adopted a random coil structure in phosphate buffer. However, in the presence of moderate TFE concentrations (up to 23%), it adopted an $\alpha$-helix structure (Fig. 6). This result indicated its propensity to fold in $\alpha$-helix as predicted by PHD method. Isothermal titration calorimetry experiments with the 84-120 N-terminal peptide did not reveal any signal of DHEA binding (not shown). We therefore concluded that additional regions of MAP2C were necessary for DHEA binding. Most probably, the binding is ensured by the whole N-terminal and also other regions of MAP2C that confer a three-dimensional structural motif. To identify these regions, we performed a sequence homology comparison between MAP2C and DHEA-binding proteins.

MAP2C shared sequence homologies with steroid binding proteins. To localize the DHEA binding site of MAP2C, we looked at the structure of steroid-binding proteins available in the Protein Data Bank. Interestingly, we found that 17$\beta$-hydroxysteroid dehydrogenase 1 (17$\beta$-HSD1) was co-crystallized with DHEA (31). 17$\beta$-HSD1 belongs to a family of enzymes required for the synthesis of active androgens and estrogens. Surprisingly, MAP2C showed sequence homologies with 17$\beta$-HSD1, especially with the DHEA binding site of 17$\beta$-HSD1 (Fig. 7). These homologies included five N-terminal regions of MAP2C (M1-W14, G47-E53, F58-H61, Y67-K71, G108-D119), which are localized in the 1-120 N-terminal sequence (Fig. 7A), and one additional C-terminal region of MAP2C (A295-L310) (Fig. 7B). Indeed, M1-W14 and G108-D119 regions contain amino acids previously identified by tryptic digestion (K10 and R112/R117, respectively). These data suggested that these five N-terminal regions and one additional C-terminal region should together form the DHEA binding pocket of MAP2C. We therefore used the structure of 17$\beta$-HSD1 to build a model of the DHEA-binding site of MAP2C.
Reconstruction of the hydrophobic MAP2C DHEA binding pocket by homology modeling. DHEA displays the common scaffold of steroids, i.e. the cholesterol nucleus (Fig. 8). In addition, DHEA is able to form hydrogen bonds through its hydroxyl radical of the A ring and through the ketone radical of the D ring. In 17β-HSD1, DHEA binds to the hydrophobic pocket and is orientated in this pocket by hydrogen bonds involving A and D rings (31). The O-3 atom of the A ring of DHEA can form two possible hydrogen bonds, one with H221 or one with E282 of 17β-HSD1. The O-17 atom of the D ring can make one possible hydrogen bond with Y155 (Fig. 9A). By homology with 17β-HSD1, we built a model of the DHEA-binding pocket of MAP2C bound to DHEA (Fig. 9B). As for 17β-HSD1, the binding of DHEA in the pocket of MAP2C involved hydrophobic interactions and hydrogen bonds. Hydrogen bonds were established through O-3 and O-17 atoms from A and D rings respectively. First, H116 or D118 could form hydrogen bonds with the O-3 atom of the A ring. Second, on the opposite side of DHEA, the O-17 atom could form one hydrogen bond with the H13 of MAP2C. All these hydrogen bonds were found in the N-terminal region of MAP2C. In addition, H13, H116, and D118 were localized in the regions M1-W14 and G108-D119 involved in the binding, as suggested by tryptic digestion. This model is supported by the isothermal titration calorimetry experiments that showed proton exchanges during the binding of DHEA. At the experimental pH of isothermal titration calorimetry experiments, only histidine residues were able to exchange protons, suggesting the presence of histidine residues in the binding site. In conclusion, the binding of DHEA to MAP2C involved hydrophobic residues that formed a hydrophobic pocket with highly specific hydrogen bonds that orientated DHEA into the pocket.

**DISCUSSION**
Our work provides evidence that MAP2C is able to bind DHEA. One molecule of DHEA bound to one molecule of MAP2C within a hydrophobic pocket resembling those of steroid-binding dehydrogenases. This binding involved both the N-terminal and the C-terminal, which closes the pocket, through hydrophobic interactions and hydrogen bonds that orientate the steroid into the pocket. These hydrogen bonds are necessary for the binding to be specific and for the hormones to be discriminated (31).

Historically, MAP2 has been described as a flexible protein with few secondary structures that displays an extended structure with a majority of random coils ((32), (33)). However, random coil does not mean unwinding proteins. Indeed, true random coil structures do not exist, even under strongly denaturing conditions (34). These observations may explain why MAP2 obtained by boiling purification procedures still retains its tubulin-polymerization and steroid binding activity (16). In addition, very recently, Malmendal and coworkers (35) showed by partial tryptic digestion and NMR studies that there is a nascent structure in the N-terminal region of MAP2C. They suggested this nascent structure could be stabilized by cofactor. Further support of these observations is that preincubation of MAP2 with tubulin increases more than eight times the number of steroid binding sites present in a calf purified MAP2 preparation (16). These experiments suggest that the fixation of MAP2 to tubulin increases its own structuring. Like Baulieu and coworkers (16), we believe neurosteroids operate through a novel mode of action. Neurosteroids are thought to exert their effects through binding to nuclear receptors in neurons, which triggers the regulation of gene transcription, and through binding at the membrane level to neurotransmitter receptors, like NMDA or GABA receptors. In spite of continuous research, no specific DHEA receptors have been identified in the central nervous system (for review see (36)). The hypothesis that DHEA interacts directly with cytoskeleton components is supported by the observation that, in ovariectomized rat, MAP2 protein content is induced by exogenous estradiol or
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progesterone at physiological doses (7). All in all, these results suggest that steroids could specifically stabilize MAP2 via direct binding, and thereby modulate MAP2 content in neurons.

Interestingly, MAP2 also interacts with other members of signal transduction pathways such as Src and Grb2 (37) or protein kinase A (PKA) (38). Interaction with PKA, which modulates MAP2 phosphorylation state, results in profound effects on microtubule dynamics (39) and cellular morphology. Furthermore, such binding allows PKA to be anchored in dendrites and the PKA signal transduction pathway to be activated (40). Because the MAP2 PKA-binding domain (82-113) and the MAP2 DHEA-binding pocket (108-119) partially overlap, DHEA binding may interfere with PKA binding and may block its activity. Note, however, that none of the phosphorylated Ser/Thr in MAP2 isoforms belong to the DHEA binding site. Most of these sites are localized in the central or in the C-terminal domain of MAP2. Only one phosphorylation site was found in the N-terminal domain (S136). These observations suggest that these known phosphorylation sites on Ser/Thr should not affect DHEA binding. By contrast, the DHEA-binding pocket of MAP2C contains a tyrosine (Y67) that is a potential phosphorylation site as predicted by the NetPhos software ((41), http://www.cbs.dtu.dk/services/NetPhos/). Hence, if Y67 is phosphorylated in vivo, it should avoid the binding of DHEA by introducing a negative charge in the hydrophobic pocket.

We believe that high-molecular weight MAP2 isoforms also bind DHEA for the two following reasons. First, all the six regions forming the DHEA binding pocket are conserved in high-molecular-weight isoforms (Fig. 10). Second, Murakami et al. (16) showed that high-molecular-weight MAP2 isoforms purified from calf were able to bind pregnenolone, the direct precursor of DHEA. This result demonstrates that the steroid binding site is conserved in high-molecular-weight isoforms of MAP2. In conclusion, DHEA binding, and more
generally steroid binding to MAP2, is of importance for both fetal (MAP2C) and adult (MAP2A, MAP2B) brain isoforms.

In addition, Reyra-Neyra et al. showed that the expression of MAP2 was modified by estradiol or progesterone whereas Tau content was not (7). Tau is another related MAP preferentially expressed in axons, which does not bind steroids (16). Interestingly, Tau lacks the N-terminal region involved in the binding of DHEA, and is involved in several pathologies of the central nervous system known as tauopathies, like Alzheimer’s disease. In these diseases, Tau forms fibrillar deposits in the brain of patients. Conversely, so far, MAP2 has never been shown to form fibrillar deposits in these pathologies (42). The difference between the N-terminal sequence of MAP2 and Tau could account for the difference in their aggregative properties. This hypothesis is supported by recent observations that showed the importance of the N-terminus of Tau in the aggregation process (43). In this way, steroid binding to the N-terminal of MAP2 could be a protective event against fibrillar aggregation and may influence neuronal plasticity.

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REFERENCES


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TABLES

Table 1. Secondary structure analysis of MAP2C. Secondary structures (in %) were determined from circular dichroism data according to the method of Manavalan and Johnson (23).

<table>
<thead>
<tr>
<th>Helix</th>
<th>sheet or extended structure</th>
<th>Turn</th>
<th>Other</th>
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</thead>
<tbody>
<tr>
<td>4</td>
<td>21</td>
<td>31</td>
<td>44</td>
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Table 2. Variations of MAP2C tryptic digestion without and with DHEA. « + » indicates cleavage and « - » absence of cleavage.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>MAP2C 15 min</th>
<th>MAP2C 45 min</th>
<th>MAP2C + DHEA 15 min</th>
<th>MAP2C + DHEA 45 min</th>
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</thead>
<tbody>
<tr>
<td>K10</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>R93</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>K112</td>
<td>+</td>
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<td>-</td>
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<tr>
<td>K117</td>
<td>-</td>
<td>+</td>
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LEGEND OF FIGURES

**Figure 1.** *Purification of MAP2C.* (A) MAP2C purification after an ion exchange chromatography analyzed by SDS-PAGE (lane 1), followed by a Western blot with polyclonal antibodies to MAP2C (lane 2), and after a reverse phase chromatography analyzed by SDS-PAGE (lane 3). M: Low molecular weight markers (Amersham Pharmacia Biotech). (B) Electrospray mass spectrometry analysis of MAP2C (see text for details).

**Figure 2.** *Secondary structure of MAP2C.* CD spectrum of MAP2C (1.48 mg/mL) in 20 mM phosphate buffer pH 7.0.

**Figure 3.** *Tubulin polymerisation assay.* The tubulin assembly curve is given as a solid line, whereas tubulin polymerization with MAP2C is given as a dashed line. Polymerization is started by heating the samples to 37°C. At the time indicated by the arrow, depolymerization was induced by cooling the samples to 4°C.

**Figure 4.** *DHEA specifically binds MAP2C.* The upper plot shows the raw calorimetric data for DHEA binding. The lower plot represents the isotherm of binding. Experiments were done in 50 mM MES pH 6.8 (A) or in 50 mM phosphate buffer pH 7.5 (B). Molar ratio is DHEA/MAP2C.

**Figure 5.** *Secondary structure prediction of MAP2C.* Predictions were done with the PHD method (see “Experimental procedures”). Only predictions with a probability of 82% or more were retained. Helix predictions are represented by dark gray boxes, -sheets by light gray boxes.
Figure 6. The 84-120 N-terminal peptide of MAP2C can adopt an α-helical structure. Circular dichroism of the 84-120 N-terminal peptide of MAP2C (0.30 mg/mL) in 20 mM phosphate buffer pH 7.0 without TFE (black line), in 9% TFE (dots), or in 23% TFE (squares).

Figure 7. MAP2C displays block homologies with the DHEA binding site of 17β-HSD1. Human 17β-HSD1 (PDB code: 3DHE) is aligned with the (1-122) N-terminal (A) and with the (295-383) C-terminal (B) sequences of MAP2C showing homologies with 17β-HSD1. Conserved residues “*” and semi-conserved residues are represented in dark gray. Six regions in the DHEA binding pocket of 17β-HSD1 aligned well with MAP2C. Five of them corresponded to N-terminal sequences (1-14), (47-53), (58-61), (67-71), (108-119) and only one with the C-terminal (295-310) sequence (in gray). These regions were used to model the DHEA binding site of MAP2C. “®” corresponds to lysine and arginine residues identified by tryptic digestion and MALDI experiments. The N-terminal peptide (84-120) of MAP2C is underlined. Numbering corresponds to the MAP2C sequence.

Figure 8. Chemical structure of DHEA (3-beta-Hydroxy-5-androsten-17-one). DHEA is formed by the hydrophobic cyclo-pentenophenanthrene scaffold common to steroid hormones. Four rings A, B, C, and D compose this scaffold. The two oxygen atoms O-3 (OH in position 3) and O-17 (C=O in position 17) can establish hydrogen bonds.

Figure 9. The DHEA binding site of MAP2C shows hydrophobic interactions and specific hydrogen bonds. DHEA is represented in red. The backbone is in white. Aromatic (Y, F, W, H) and basic (R, K) residues are in blue. Residues that form hydrogen bonds with DHEA are
in yellow (H, Y, D, E). The DHEA binding site of 17\[\beta\]-HSD1 (A) and MAP2C (B) is composed of hydrophobic and aromatic residues that bind the steroid ring of DHEA. In the 17\[\beta\]-HSD1 binding site, H221, E282, and Y155 form possible hydrogen bonds with the O-3 and the O-17 atoms of DHEA that orientate the steroid into the pocket. In the MAP2C binding site, these hydrogen bonds with DHEA are preserved, but they are those of H116, D118, and H13. K10, K112, and K117 identified by tryptic digestion and MALDI experiments are localized around the pocket.

**Figure 10. Structure of MAP2 isoforms and Tau.** (A). Map2 isoforms are divided in two groups: high-molecular weight isoforms (HMWMAP2) including MAP2A and MAP2B, and low-molecular weight isoforms (LMWMAP2) including MAP2C. LMWMAP2 isoforms lack the projection domain (PD). They are made of the N- and the C- terminal domains of HMWMAP2 isoforms linked together. The tubulin-binding domain (TBD) is formed by three or four repeated sequences (black boxes). The five N-terminal sequences and the C-terminal sequence that we identified as involved in the DHEA binding are in hatched boxes. (B). Tau C-terminal domain is highly homologous to MAP2 isoforms whereas its N-terminal domain is entirely different. Therefore the N-terminal sequences constituting the DHEA-binding pocket of MAP2C are not present in Tau.
Figure 1

A

B

% Intensity

Mass

49172.5

100%

50%

0%

49200 49300

49187.5

49200 49300

70 kDa
Figure 2
Figure 4

A

Time (min)

μcal/second

kcal/mole of injectant

Molar Ratio

Ka = 2.7 * 10^7 M^{-1}
N = 0.9 ± 0.1
\( \Delta H = -34 \pm 5 \) kJ M^{-1}
\( \Delta G = -42 \pm 1 \) kJ M^{-1}

B

Time (min)

μcal/second

kcal/mole of injectant

Molar Ratio

Ka = 9.7 * 10^6 M^{-1}
N = 1.1 ± 0.2
\( \Delta H = -10 \pm 8 \) kJ M^{-1}
\( \Delta G = -40 \pm 2 \) kJ M^{-1}
\( \Delta S = 30 \pm 10 \) kJ M^{-1}
Figure 5

MADERKDEGK APHwTSA3LT EAAAHPSPE MDQGGSIGE
LSRGANGPPT REEBEGAFGE HGSQGTTSDT KENGINGELT
SADRETTACEV SARIVQVVTQ FAVAVLKGEO EKEAQHCDP
AALPLAEET VNLPPSPPPS FASEQTAALE EATSGESAQA
PSFRKQAKDK VTDGITKSPNE KRSSLPRPSS ILPPRRGVSQ
DRENSPSLLN SISARRRTT RSEPIRRAGK SGTSTPTTPG
STAITPCTTP SYSSRTPCTP CTSSYTRTPG TTKSLILVPS
EKKVAILRTP PKSPATPKQL EKINQPLPDL KNVKSKIGST
DNIKYQPKGG QVQIVTKKID ISHVTSKCGS LKNIRHRPGG
GRVKIESVKL DFKEKAQAKV GSLDNAHHPG GGGNVKIDSQ
KLNPREHAKA RVDHGAEIIT QPSRSVSSAS PRRSVVSSS
GSINLLESPQ LATLAEDVTA ALAKQGL
Specific binding of DHEA to the N-terminal of the microtubule-associated protein MAP2
Emmanuelle Laurine, Daniel Lafitte, Catherine Grégoire, Eric Séréé, Erwann Loret, Soazig Douillard, Bernard Michel, Claudette Briand and Jean-Michel Verdier

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