Nuclear factor kappa B in the dorsal raphe of macaques: an anatomical link for steroids, cytokines and serotonin

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Objective: Nuclear factor kappa B (NFκB) is a transcription factor that activates gene expression in response to proinflammatory cytokines, and elevated cytokines are associated with depression, which has a serotonergic component. We questioned (1) whether serotonin neurons contain NFκB, (2) whether NFκB detection with immunocytochemistry is changed in the dorsal raphe nucleus (DRN) by ovarian hormone treatment and (3) whether ovarian hormones regulate midbrain NFκB gene or protein expression. **Methods:** Monkeys were spayed and treated with placebo, estrogen (E), progesterone (P) or E+P for 1 month ($n = 4$ animals/treatment group), and the mid-brain was harvested for immunocytochemistry and stereology. An antibody that detects nuclear location-specific (NLS)-NFκB p65 was applied, and the numbers of NLS-NFκB-immunopositive cells were counted in 9 sections of the DRN. Additional monkeys were used for Western blot analysis and quantitative reverse transcription-polymerase chain reaction (RT-PCR) for NFκB p65. **Results:** In placebo-treated macaques, neurons were double-immunostained for serotonin and nuclear NFκB p65 throughout the DRN. The mean total number of NFκB-positive cells equalled 2178 (and standard error of the mean [SEM] 129) in the placebo group, 1631 (SEM 221) in the E-treated group, 2314 (SEM 186) in the P-treated group and 1162 (SEM 100) in the E+P-treated group (analysis of variance $p = 0.003$). The E-treated and E+P-treated groups had a significantly lower density of cells stained positive for NFκB than the placebo or P-treated groups (post hoc). Unmasking of NLS-NFκB immunostaining in the DRN revealed dense immunostaining in the cytoplasm of large dorsal raphe neurons. There was no difference between treatment groups in the amount of NFκB p65 detected by Western blot or in the relative expression of NFκB p65 mRNA with quantitative RT-PCR. **Conclusions:** These observations are consistent with the notion that gene and protein expression of NFκB are constitutive but that ovarian hormones can decrease the nuclear location of NFκB in dorsal raphe neurons and, thereby, decrease the ability of NFκB to drive gene expression in response to cytokines.

Objectif : Le facteur nucléaire kappa B (NFκB) est un facteur de transcription qui active l'expression génique en réponse à des cytokines pro-inflammatoires, et l'élévation des cytokines est associée à la dépression, qui comporte un élément constituant sérotoninergique. Nous avons cherché à savoir (1) si les neurones à sérotoninergiques contiennent du NFκB, (2) si un traitement aux hormones ovariennes modifie la détection du NFκB par immunocytochimie dans le noyau du raphé dorsal (NRD) et (3) si les hormones ovariennes assurent la régulation de l'expression génique ou protéique du NFκB dans le mésencéphale. **Méthodes :** On a pratiqué une ovariectomie sur des guenons auxquelles on a administré un placebo, de l'oestrogène (E), de la progestérone (P) ou une combinaison E+P pendant un mois ($n = 4$ animaux/groupe de traitement). On a prélevé ensuite le mésencéphale pour effectuer des tests d'immunocytochimie et de stéréologie. On a appliqué un anticorps qui détecte le NFκB p65 nucléaire à localisation spécifique (NLS) et compté le nombre de cellules immunopositives pour le NLS-NFκB dans 9 coupes du NRD. On a utilisé d'autres sujets pour procéder à une analyse quantitative par transfert de Western et réaction en chaîne de la polymérase par transcription inverse (RT-PCR) pour le NFκB p65. **Résultats :** Chez les macaques traitées par placebo, on a soumis les neurones à une immunocoloration double pour la sérotonine et le NFκB p65 nucléaire dans tout le NRD. Le nombre total moyen de cellules positives pour le NFκB a atteint 2178 (et l'erreur type de la moyenne [ETM], 129) chez les sujets traités par placebo, 1631 (ETM 221) chez ceux qui ont reçu de l'oestrogène, 2314 (ETM 186) chez ceux qui ont reçu de la progestérone et 1162 (ETM 100) chez les sujets traités par la combinaison E+P (analyse d'écart $p = 0,003$). Les groupes qui ont

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reçu de l'œstrogène et les sujets traités par la combinaison E+P avaient une densité significativement moindre de cellules colorées positivement pour le NFκB que le groupe traité par placebo ou les groupes traités par progestérone (a posteriori). Le démasquage de l'immunocoloration NLS-NFκB dans le NRD a révélé une immunocoloration dense dans le cytoplasme des gros neurones du raphé. On n'a constaté aucune différence entre les groupes quant à la quantité de NFκB p65 détecté par transfert de Western ou quant à l'expression relative de l'ARNM du NFκB p65 par la méthode RT-PCR quantitative. **Conclusions** : Ces observations concordent avec le concept selon lequel l'expression génique et protéique du NFκB est constitutive, mais les hormones ovariennes peuvent réduire la localisation nucléaire du NFκB dans les neurones du raphé dorsal et, par conséquent, réduire la capacité du NFκB de catalyser l'expression génique en réponse aux cytokines.

Introduction

The nuclear factor kappa B (NFκB) transcription factor was originally identified as a protein complex consisting of a 65-kd DNA-binding subunit and an associated 50-kd protein. NFκB can exist in both cytoplasmic and nuclear compartments.¹ Upon cytokine receptor activation, NFκB translocates to the nucleus, binds to NFκB response elements and promotes gene transcription. Cytokines have been strongly implicated in depression. Administration of cytokines to patients can precipitate depression, and depressed subjects have elevated levels of cytokines.^{2,3} These and similar correlations have led to a cytokine hypothesis of depression. However, the central nervous system targets of cytokines are not well defined.

Serotonin plays an important role in affective regulation, and dysfunction of the serotonin system is found in depressed patients.^{4,5} Work from this laboratory indicates that the ovarian steroids, estrogen and progesterone, regulate gene and protein expression in serotonin neurons.⁶ Estrogens bind with high affinity to nuclear transcription factors called ERα and ERβ,⁷ which in turn bind to estrogen response elements in the promoter region of target genes and/or interact with other transcription factors in a manner so as to either increase or decrease gene expression in a tissue-specific fashion.⁸

Serotonin neurons express the β form of the estrogen receptor, ERβ,^{9,10} and estrogen, with or without progesterone supplementation, acts in serotonin neurons to alter gene expression. In brief, estrogen treatment of spayed macaques increased progestin receptor protein¹¹ and increased expression of the gene that controls brain serotonin synthesis, called tryptophan hydroxylase-2 (*TPH-2*).¹² However, estrogen decreased expression of the serotonin reuptake transporter (*SERT*), serotonin-1A (*5-HT1A*) and monoamine oxidase-A (*MAOA*) genes in the dorsal raphe nucleus (DRN).^{13–15} The *SERT*, *5-HT1A* and *MAOA* genes lack any known form of an estrogen response element;^{16–18} however, the *SERT* and *5-HT1A* genes do contain NFκB response elements in their promoter regions.^{16,19}

Ligand-activated steroid receptors sequester NFκB and thereby decrease gene transcription that is dependent upon this factor.²⁰ In transfection studies, ERα markedly repressed NFκB-driven gene expression in an estrogen-dependent manner.²¹ Moreover, estrogen inhibited cytokine production by decreasing NFκB binding activity.²² Treatment of cells in culture with cytokines leads to I kappa B α (IκBα) serine

phosphorylation and degradation and, in turn, NFκB translocation to the nucleus.¹ However, the expression of NFκB is thought to be constitutive in immortalized cell lines.

We questioned whether serotonin neurons express NFκB and whether steroid receptors could sequester NFκB in serotonin neurons, thereby decreasing NFκB response element-driven gene expression, and whether NFκB gene or protein expression is regulated or constitutive in the macaque midbrain. We presented preliminary data in a review article,⁶ which indicated that treatment of monkeys with estrogen or estrogen plus progesterone decreased the number of raphe neurons with NFκB immunostaining in the nucleus. However, only 3 anatomical levels were examined, the antibody was not generated against nuclear location-specific (NLS)-NFκB, and the positive neurons were hand marked with the aid of camera lucida equipment and then counted.

Therefore, in this study we (1) determined whether serotonin neurons in the dorsal raphe of rhesus macaques contain NFκB, (2) extended the preliminary analysis with an antibody to NLS-NFκB and counted NFκB-positive neurons at 9 anatomical levels of the DRN with a computer-assisted stereology workstation, (3) used an antigen-retrieval buffer to unmask all of the intracellular NFκB for comparison with NLS-NFκB and (4) determined whether gene or protein expression of NFκB in the midbrain raphe region was altered by hormone therapy.

Methods

Animals

The Oregon National Primate Research Center (ONPRC) Animal Care and Use Committee approved this study. The monkeys were euthanized at the end of the treatment period according to the procedures recommended by the Panel on Euthanasia of the American Veterinary Association.

Adult female rhesus monkeys (*Macaca mulatta*) were ovariectomized and hysterectomized (spayed) in other research programs according to standard veterinary procedures by the surgical personnel of the ONPRC. About 3–6 months after finishing other protocols, the animals were assigned to this study. The animals were born in China and were aged 5–14 years according to dental examination. They weighed between 4.5 and 7.5 kg and were in good health.

For single immunocytochemistry with an NLS antibody to NFκB p65 and for stereological analysis, 16 spayed rhesus macaques were assigned to a placebo group (Ovx), a 1-month

estrogen-treated group (E), a 1-month progesterone-treated group (P) or a 1-month E+P-treated group (E+P). Each treatment group consisted of 4 animals.

For double immunocytochemistry with an antibody to NF κ B p65 and serotonin, sections were used from 3 spayed macaques treated with placebo.

For Western blot analysis of NF κ B p65, 24 spayed rhesus macaques were assigned to a placebo group (Ovx), a 1-month E-treated group (E1), a 1-month E+P-treated group (EP1), a 5-month E-treated group (E5), a 5-month E+P-treated group (EP5) or a 5-month raloxifene-treated group (Ral5). Each treatment group consisted of 4 animals.

For RNA extraction and quantitative reverse transcription–polymerase chain reaction (RT-PCR) analyses of NF κ B p65, 9 spayed rhesus macaques were assigned to a placebo group (Ovx), a 1-month E-treated group (E) or a 1-month E+P-treated group (EP). Each treatment group contained 3 animals.

Hormone levels

Hormone replacement with placebo, estrogen, progesterone or estrogen plus progesterone was achieved with Silastic capsules, and raloxifene was administered in a daily food treat, as previously described.²³

Assays to confirm serum concentrations of estrogen and progesterone were performed using a Roche Diagnostics 2010 Elecsys assay instrument in the Endocrine Services Core of the ONPRC. Each assay had been validated for macaque plasma in comparison with traditional radioimmunoassays, as previously described.²⁴ Interassay coefficients of variation were less than 10% for each assay.

Tissue preparation

Sections for NLS-NF κ B p65 immunocytochemistry and stereology were obtained from animals in which the brain was perfused intracranially with 4% paraformaldehyde, blocked and frozen. Sections (25 μ m thick) were obtained with a sliding microtome, as previously described.¹⁰

Sections for double immunocytochemistry were obtained from 3 spayed placebo-treated animals, in which the brain was perfused intracranially with 20% sucrose, then blocked and frozen. Sections (10 μ m thick) were obtained on a cryostat, as previously described.²⁵

Tissue preparation for Western blots involved the dissected block of midbrain being wrapped in foil and immediately dropped in liquid nitrogen. Later, homogenates of the raphe region were prepared, as described by Smith et al.²³ Immediately before loading the gel, protein assays were performed on the supernatant with the Bio-Rad protein determination reagent, according to the Bradford method.²⁶

RNA preparations were obtained as described in Reddy and Bethea.²⁷

Single immunocytochemistry for NLS-NF κ B

Nine sections at 250- μ m intervals from the rostral to the

caudal extent of the DRN were removed from -80°C storage and submerged in 85% ethanol. The slides were then washed with phosphate-buffered saline (PBS, 0.05 mol/L phosphate, 0.14 mol/L NaCl, pH 7.3), blocked with normal goat serum and incubated with a rabbit antibody to NLS-NF κ B (primary rabbit anti-human-NLS-NF κ B [Rockland Immunochemicals, Gilbertsville, Pa.] at a titre of 1/50) in PBS for 48 hours at 4°C . The slides were then washed with PBS, blocked with normal goat serum and incubated with biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, Calif.). The slides were washed with PBS, treated with the avidin–biotin complex/horseradish peroxidase reagent ([HRP] Vector ABC Kit), washed with PBS followed by Tris (0.05 mol/L, pH 7.6) and then exposed to a diaminobenzidine (DAB)/hydrogen peroxide mix (200 mL of 0.05 mol/L Tris, 40 μ L of hydrogen peroxide and 50 mg of DAB [Dojindo Laboratories, Kumamoto, Japan]) for 10 minutes, revealing the brown NF κ B p65–positive nuclei. The slides were dehydrated using increasing concentrations of ethanol and xylene and were mounted using De-Pe-X (Fisher, Fair Lawn, NJ). A control reaction was also performed that omitted the primary antibody.

Unmasking of NF κ B

To obtain a visualization of all NF κ B p65, we used an antigen-retrieval protocol followed by immunostaining with the NLS-NF κ B antibody. Sections were removed from storage and placed in Antigen Unmasking Solution according to the manufacturer's directions (Vector Laboratories). Sections were microwaved in the buffer for 3, 6 or 9 minutes followed by incubation with NLS-NF κ B at a titre of 1/200. This titre is 4 times more dilute than the titre needed to detect NLS-NF κ B without antigen retrieval.

Double immunocytochemistry

To demonstrate the colocalization of NF κ B p65 and serotonin, double immunocytochemistry was performed on sections from 3 placebo-treated spayed animals. The midbrain sections were stained for NF κ B p65, as described above, with modifications to prevent inappropriate cross-reactions between antibodies. The NF κ B p65 primary antibody was changed to one that was made in goat in order to avoid cross-reactivity with the serotonin antibodies (primary goat anti-human-NF κ B p65 against the C-terminus; Santa Cruz Biotechnology, Santa Cruz, Calif. [SC372G]), but it was not nuclear location specific. It was used at a concentration of 5 $\mu\text{g/mL}$. The blocking serum was normal donkey serum from Chemicon (Temecula, Calif.). A donkey anti-goat biotinylated secondary antibody was applied (concentration 1/300; Rockland Immunochemicals), followed by the avidin–biotin HRP complex from Santa Cruz Biotechnology. NF κ B p65 immunostaining was developed in DAB plus nickel (pH 6.5, 180 mL of 0.15 mol/L sodium acetate, 20 mL of 2.5% nickel sulfate, 40 μ L of hydrogen peroxide and 140 mg of DAB) to produce a black reaction product, which occurred largely in the nucleus of the neurons. After DAB treatment,

the slides were washed in Tris and incubated overnight in 4% paraformaldehyde at 4°C.

On the third day, the slides were stained for serotonin. The slides were blocked with normal sheep serum and a solution of 3% bovine serum albumin in PBS for 1 hour, followed by overnight incubation in the primary anti-serotonin antibody. The serotonin antibody was generated in rabbit against serotonin conjugated to BSA (DiaSorin, Stillwater, Minn.). It was used at a concentration of 1/1500 in a solution of 0.3% Triton X-100 in PBS. A sheep anti-rabbit biotinylated secondary antibody from Chemicon was applied (1/500) followed by avidin-biotin HRP (Vector Laboratories). The slides were incubated in DAB for 20 minutes, yielding a brown reaction product in the cytoplasm. Once the serotonin staining protocol was completed, the slides were dehydrated and mounted as described above.

Data collection and analysis

The NLS-NFκB-positive neurons were counted at 9 anatomical levels of the DRN with a Marianas Imaging Workstation (Intelligent Imaging Innovations, Denver, Colo.). The station contains a Zeiss Axiovert 200M microscope with a Coolsnap HQ CCD camera and a motorized stage (Applied Scientific Instruments), all controlled by a dual processor Dell Precision 670. The workstation created and saved a montage of the region at each level. Then, using Slidebook software, the area of interest was outlined and the positive cells were scored. The number of cells was recorded, and the area outlined was measured in square micrometres. For each animal, the total number of cells from the 9 levels was calculated. The rostral-caudal length of the area was determined by addition of the section thicknesses ($9 \times 25 \mu\text{m}$) plus the intervals ($8 \times 250 \mu\text{m}$), which equalled $2225 \mu\text{m}$ or 2.225 mm. The average area in square micrometres of the 9 levels was converted to square millimetres and multiplied by the length to obtain the volume of the area analyzed in cubic millimetres. The total number of positive cells was divided by the volume to yield the density of NLS-NFκB-positive neurons in the dorsal raphe region of each animal.

The total numbers of positive cells and the density of positive cells/mm³ for each treatment group were compared using a 1-way analysis of variance (ANOVA) with Student-Newman-Keuls post hoc pairwise comparison ($p < 0.05$ was considered significantly different).

Western blot assay

Rabbit anti-NFκB p65 against the N-terminus (Santa Cruz Biotechnology [SC109]) was used at a dilution of 1/200 in buffer containing 50 mol/L Tris and 150 mol/L NaCl (pH 7.5). Goat anti-rabbit antibody conjugated with HRP (Chemicon) was used as a second antibody at a dilution of 1/7000 in 8% nonfat milk. Western blotting was performed with Tris-glycine precast 10% SDS gels ($16 \times 18 \text{ cm}$) with 10 lanes (Jule, Inc., Milford, Conn.), as previously described.²³ Initially, the linearity of the NFκB p65 signal was examined by loading increasing concentrations of protein from an extract of RN46

cells (25–150 μg protein), previously shown to contain NFκB,²⁸ and increasing aliquots of dorsal raphe extract (50–300 μg protein). For the experimental gels, monkey raphe extracts containing 200 μg of protein and 100 μg of RN46 cell extract protein were loaded on the 10% SDS polyacrylamide gel. The aliquot of the extract of RN46 cells was included on each gel as a positive control.

There were 4 animals in each treatment group. Comparisons of 6 treatments with a total of 24 samples required multiple gels. Therefore, the samples were loaded on each gel in a matched manner. That is, the order of loading the lanes from left to right was always a spayed animal and then an individual animal from each treatment group to be compared. This enabled a statistical covariance correction for gel-to-gel variation if needed. However, the gels were very consistent and a covariance analysis was not required.

Densitometric analysis of Western blotting results

The bands corresponding to NFκB p65 were analyzed with NIH Image, as previously described.²³ An overall comparison between the groups was obtained with 1-way ANOVA, and a difference between treatment groups was sought with 95% confidence. Post hoc pairwise comparisons were not obtained because of the lack of a significant difference in the ANOVA. All statistical analyses were conducted with the Prism Statistical Program (GraphPad, San Diego, Calif.).

Quantitative RT-PCR

TriReagent-extracted RNA from the microdissected block of the 9 rhesus midbrains containing the DRN was subjected to quantitative RT-PCR for NFκB p65. Complementary DNA (cDNA) synthesis was performed using Oligo-dT 15 primer (Invitrogen, Carlsbad, Calif.) and M-MLV reverse transcriptase (100 U/ μg of RNA [Invitrogen]) at 42°C for 1 hour.

Quantitative RT-PCR was conducted with the Dynamo Sybr Green qPCR Mix F-400L, as previously described.²⁷ Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the reference gene. A standard curve was generated for *GAPDH* and NFκB p65 with Quantitative PCR Human Reference Total RNA from Stratagene (La Jolla, Calif.). The slope of the curve was used to calculate the relative weight (pg) of each transcript in the RNA extracted from the mid-brain tissue blocks. Then, the ratio of the NFκB p65 transcript to *GAPDH* was calculated.

Statistical comparisons between treatment groups were conducted with 1-way ANOVA. Post hoc pairwise comparisons were not conducted, because there was no difference between the groups with ANOVA. Differences were considered significant if $p < 0.05$.

Primer selection

The target area of NFκB p65 was selected in the 3' untranslated region. The target sequence was then loaded into Primer Express software, which chooses the primers for optimum quantitative RT-PCR. The primers were obtained from

Invitrogen Life Technologies. The primers used were the following:

NF κ B p65 — Accession # L19067

Forward — AGCCTCCAGCCCCAGTGAAG

Reverse — CTCGGTAAAGCTGAGTTTGCG

Amplified product = 151 bp

GAPDH — Accession # BC029340

Forward — CAT TGC CCT CAA CGA CCA C

Reverse — CCC CTC CCC TCT TCA AGG

Amplified product = 255 bp

Results

NLS-NF κ B-positive nuclei were counted in 9 sections at 250- μ m intervals of the DRN from all 16 animals. Figure 1 illustrates representative montages that were generated from 1 level of 1 animal in each treatment group. The area that was counted is outlined. The NLS-NF κ B-positive cells were marked and counted at a high magnification, as illustrated in

Fig. 1: Representative montages (left) from 1 level of 1 animal in each treatment group stained for NLS-NF κ B with an anti-human antibody from Rockland Immunochemicals (Gilbertsville, Pa.). The antibody was made in rabbit and used at a titre of 1/50. After generation of the montage, the area was outlined and then magnified for cell counting, as shown on the right. E = estrogen, NLS-NF κ B = nuclear location-specific nuclear factor kappa B, Ovx = ovariectomized, P = progesterone.

the left panels. Positive cells in the trochlear nucleus were not included. As illustrated in Figure 2 (top), there were significantly fewer NLS-NF κ B-positive cells in the DRN of E-treated and E+P-treated animals compared with the placebo-treated animals and animals treated with progesterone only

Fig. 2: Top: histogram illustrating the mean (and standard error of the mean [SEM]) total number of cells that stained positive for NLS-NF κ B in the DRN in each of the treatment groups. There was a significant difference between the groups (ANOVA, $F_{3,12} = 8.16$, $p = 0.003$). Post hoc pairwise comparisons with the Student–Newman–Keuls test indicated that the E- and E+P-treated groups had significantly fewer cells stained positive for NLS-NF κ B than the placebo group. The E+P group also had significantly fewer cells stained positive for NLS-NF κ B than the P-only treated group. **Bottom:** histogram illustrating the mean (and SEM) density of NLS-NF κ B-positive cells in the DRN of each treatment group. There was a significant difference between the groups (ANOVA, $F_{3,12} = 5.52$, $p = 0.013$). Post hoc pairwise comparisons with the Student–Newman–Keuls test indicated that the E- and E+P-treated groups had a significantly lower density of cells stained positive for NLS-NF κ B than the placebo group and the P-only treated group. ANOVA = analysis of variance, DRN = dorsal raphe nucleus, E = estrogen, NLS-NF κ B = nuclear location-specific nuclear factor kappa B, Ovx = ovariectomized, P = progesterone. a,d,f = $p < 0.05$, b,c,e,g = $p < 0.01$.

(ANOVA, $F_{3,15} = 8.16$, $p = 0.003$). The mean total number of NF κ B-positive cells equalled 2178 (and standard error of the mean [SEM] 129) in the placebo group, 1631 (SEM 221) in the E-treated group, 2314 (SEM 186) in the P-treated group and 1162 (SEM 100) in the E+P-treated group. In Figure 2 (bottom), the density of NLS-NF κ B-positive cells was also significantly reduced with estrogen and estrogen-plus-progesterone treatment compared with placebo or treatment with progesterone only (ANOVA, $F_{3,15} = 5.52$, $p = 0.013$).

During development of the NLS-NF κ B immunocytochemistry protocol, several different buffer systems and antibody titres were examined. We found that the NLS-NF κ B antibody at a dilute titre (1/200) stained a large reservoir of NF κ B p65 in the cytoplasm of a large population of neurons in the raphe nucleus, if the section was processed with an antigen-retrieval buffer. However, if the sections were processed with PBS, then the antibody to NLS-NF κ B stained fewer neurons in a more restricted manner, and a more concentrated titre

Fig. 3: Photomicrographs illustrating NLS-NF κ B immunostaining in the DRN after antigen retrieval versus no antigen retrieval. Left panels: preincubation of sections with antigen-unmasking buffer and microwave treatment for 6 min yields widespread cytoplasmic staining with intense nucleolus staining even at a dilute titre of antibody (1/200). Right panels: without antigen retrieval, there is sparser immunostaining that is largely nuclear or perinuclear even at a concentrated titre of antibody (1/50). The antigen-retrieval buffer apparently reveals the large amount of NF κ B present in the cell, which is normally bound to I κ B. Omission of the primary antibody or further dilution of the primary antibody eliminated staining. DRN = dorsal raphe nucleus, NLS-NF κ B = nuclear location-specific nuclear factor kappa B.

(1/50) was required to obtain any staining. Smaller nuclear staining was also observed that may correspond to interneurons or glia. Figure 3 illustrates the difference in immunostaining obtained with the antibody to NLS-NF κ B when sections were processed with or without an antigen-retrieval buffer.

In order to verify that serotonin neurons contain NF κ B p65, a double immunostaining procedure was employed. However, both the antibody to NLS-NF κ B and the antibody to serotonin are made in rabbit, which precludes their use together in a double immunostaining protocol. Therefore, we

Fig. 4: Panels A–D illustrate cells in the DRN (A,B,C) and descending tract or medial longitudinal fasciculus (D) stained for NF κ B p65 with an antibody raised in goat from Santa Cruz Biotechnology (Santa Cruz, Calif.). The nuclei of cells that are positive are highlighted with a black arrow, and negative cells are highlighted with an orange arrow. The picture in panel A is magnified 200-fold, and the pictures in panels B–D are magnified 400-fold. Panels E–G illustrate cells from the DRN that were double-labelled for NF κ B p65 (nuclei) and serotonin (cytoplasm). The nuclei are stained black/dark brown and the cytoplasm is stained light brown in positive cells. The double-headed black arrows indicate double-labelled cells. The single-headed black arrow indicates a cell that only contains NF κ B p65-positive nuclei. The green arrow indicates a cell that appears to be positive for serotonin but lacks a nucleus. The orange arrow indicates a cell that is negative for both NF κ B p65 and serotonin. The pictures in panels E–G are magnified 600-fold. DRN = dorsal raphe nucleus, NF κ B = nuclear location-specific nuclear factor kappa B.

employed an antibody to NF κ B p65 that was made in goat (Santa Cruz Biotechnology) for the double immunostaining protocol. Although this antibody is not nuclear location specific, most of the immunostaining was located in the nucleus. This antibody was also sensitive to fixation, so the sections were obtained from spayed animals that had been intracranially perfused with 20% sucrose rather than formaldehyde. The serotonin immunostaining is largely cytoplasmic, but it requires fixation and cross-linkage to protein. Therefore, the sections were first immunostained for NF κ B p65, then fixed overnight with 4% paraformaldehyde, washed and then immunostained for serotonin. With the additional application of different colour development, we were able to demonstrate

Fig. 5: Panel A: linearity of NF κ B p65 signal is reflected by increased signal density with increasing amounts of protein loaded. The protein is an extract of RN46 cells. Similar results were obtained with 50, 100, 200 and 300 (not shown) μ g of protein from the dorsal raphe region. Panel B: a representative film from a Western blot for NF κ B p65 on extracts of monkey raphe region is shown. One animal from each treatment group was included, as well as an extra ovariectomized control animal and an extract of RN46 cells. Each monkey sample contained 200 μ g of protein, and the RN46 cell extract contained 100 μ g of protein. The same arrangement was repeated on 4 blots to assay the 4 animals in each treatment group. Panel C: Histogram illustrating the average optical density (and standard error of the mean [SEM]) of all of the treatment groups. There was no difference between the groups (analysis of variance, $p > 0.1$). E = estrogen, NLS-NF κ B = nuclear location-specific nuclear factor kappa B, OD = optical density, Ovx = ovariectomized, P = progesterone, Ral = raloxifene.

that subpopulations of serotonin neurons contain NF κ B p65. Figure 4 illustrates neurons that are double-labelled for NF κ B p65 in the nucleus (black) and for serotonin in the cytoplasm (brown), as well as neurons that are labelled for serotonin only and NF κ B p65 only.

For Western analysis, it was not necessary to use an antibody that was nuclear location specific. Therefore, we used an antibody to human NF κ B p65 that was made in rabbit (Santa Cruz Biotechnology). The Western blot analysis of NF κ B p65 was linear across a 6-fold range of protein (Fig. 5). Analysis of the raphe extracts indicated that there was no statistically significant difference in the amount of NF κ B p65 extracted from the raphe region of animals treated with placebo, estrogen for 1 month, estrogen plus progesterone for 1 month, estrogen for 5 months, estrogen plus progesterone for 5 months or raloxifene for 5 months (Fig. 5).

Quantitative RT-PCR for NF κ B p65 and *GAPDH* was conducted on RNA extracted from the raphe region of 3 placebo-treated, 3 E-treated and 3 E+P-treated monkeys. The primers amplified a segment of NF κ B p65 located in the 3' untranslated region that is unique to NF κ B p65. The concentration of NF κ B p65 was normalized by the concentration of *GAPDH* in each sample. There was no difference in the relative abundance of NF κ B p65 mRNA between the treatment groups (Fig. 6). The sequence of the monkey NF κ B p65 amplicon confirmed the identity of the product.

The serum levels of estrogen and progesterone achieved by implantation of Silastic capsules in the animals used to count NLS-NF κ B p65 neurons were reported in Gundlach et al.¹⁵ The serum levels of estrogen and progesterone in the animals used for Western blot analysis were reported in Smith et al.,²³

Fig. 6: Histogram illustrating the relative abundance (and standard error of the mean [SEM]) of NF κ B p65 in spayed macaques treated with placebo (ovx), estrogen for 28 days (E) or estrogen for 28 days supplemented with progesterone for the last 14 days (E+P). There was no difference in the ratio of NF κ B: *GAPDH* between the groups (analysis of variance, $p > 0.1$). *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase gene, NLS-NF κ B = nuclear location-specific nuclear factor kappa B, Ovx = ovariectomized.

and the levels in the animals for qRT-PCR were reported in Reddy and Bethea.²⁷ Serum estrogen and progesterone concentrations were significantly elevated by treatment, indicating that the treatments were efficacious. The levels of estrogen and progesterone attained during treatment are comparable to the levels observed during the midluteal phase of a normal macaque menstrual cycle.

Discussion

NFκB is a multiprotein complex that can activate a variety of genes involved in early defence reactions of higher organisms. In nonstimulated cells, NFκB resides in the cytoplasm in an inactive complex with the inhibitor IκB. Pathogenic stimuli release IκB via ubiquitination and phosphorylation. This allows NFκB to enter the nucleus, bind to DNA control elements and, thereby, induce the synthesis of mRNA. The activation of NFκB is triggered by a variety of agents including cytokines, interleukin-1, tumour necrosis factor, viruses, double-stranded RNA, endotoxins, phorbol esters, UV light, ionizing radiation and oxidative stress.²⁹

The central nervous system contains NFκB, and many of the agents that activate NFκB can cross the blood-brain barrier.³⁰ An interesting correlation has been observed between cytokines and depression in humans.^{31,32} Individuals with major depressive disorder have increased circulating cytokines, and administration of cytokines precipitates depressive feelings.^{2,3} Recently, proinflammatory cytokines have been found to affect the metabolism of brain serotonin through the enzyme indoleamine-2,3-dioxygenase (IDO) that metabolizes tryptophan (the precursor of 5-HT) to neurodegenerative quinolinate.³³

Steroid hormones, particularly estrogen, progesterone and glucocorticoids, interfere with the action of NFκB in different tissues,^{22,34-37} including the brain.³⁸ The mechanism by which steroid hormones block NFκB action appears to involve a protein-protein interaction between the ligand-activated steroid receptor and NFκB. NFκB is then unavailable to bind to the NFκB response element.^{20,39} In this indirect manner, steroid hormones can reduce gene expression that is supported by NFκB. Steroids may also increase IκB, the cytoplasmic binding protein of NFκB.⁴⁰

We found that estrogen or estrogen-plus-progesterone treatment of spayed macaques reduces expression of several pivotal genes in serotonin neurons.⁶ Upon examination of the promoter regions, NFκB response element(s) were detected. In this study, immunohistochemical examination of the macaque DRN indicates that serotonin neurons contain NFκB p65, although not all neurons contain sufficient quantities in the nucleus for immunodetection at one time. Moreover, in the DRN, where the majority of forebrain projecting serotonin cell bodies reside, there is a decrease in the number of NLS-NFκB-positive cells upon treatment with estrogen or estrogen plus progesterone. Treatment with estrogen alone reduced detection of NLS-NFκB, but the addition of progesterone added to the significance of the reduction. There was no reduction in the number of NLS-NFκB-positive cells with progesterone alone, suggesting that the facilitatory effect of

supplemental progesterone requires estrogen to induce progesterone receptors that in turn bind progesterone. The indication is that ligand-activated ERβ and progesterone receptors reduce NFκB translocation to the nucleus in neurons of the DRN. In turn, this could lead to a reduction in NFκB response element-driven gene expression, such as the 5-HT_{1A} autoreceptor. It is attractive to speculate that increases in cytokines activate NFκB and drive the expression of the 5-HT_{1A} gene, which in turn could decrease serotonin neural firing and serotonin neurotransmission, resulting in depressive affect. Moreover, the presence of ovarian hormones could block this cascade, which may underlie the ability of estrogen to ameliorate some depressive symptoms in women.

A body of literature suggests that gene and protein expression of NFκB is constitutive, although overexpression in cancer cells has been noted.⁴¹ Most studies focus on the translocation and activation of NFκB, concluding that gene and protein expression are relatively constant.⁴² Indeed, NFκB appears early in central nervous system development.⁴³ The detection of NFκB in serotonin neurons of nonhuman primates links the activity of the immune system and the serotonin system in a manner that subserves correlations between sickness and depression.

In this study, NFκB p65 protein in the midbrain raphe region was not altered by any treatment in a Western blot analysis, nor was NFκB p65 mRNA expression altered in a quantitative RT-PCR assay. In addition, there was a large amount of cytoplasmic immunostaining observed in DRN neurons after antigen retrieval, indicating that the NLS site on cytoplasmic NFκB was unmasked by antigen retrieval. These results are consistent with the notion that in the normal midbrain NFκB p65 is constitutively expressed and that the effects of steroids on NFκB, and in turn on gene expression, are largely the result of translocation. It is particularly noteworthy to compare the immunostaining obtained with an NLS-NFκB antibody in the DRN with and without antigen retrieval. One is struck by the large reservoir of NFκB in the cytoplasm of the large, probably serotonergic neurons of the DRN. This reservoir of NFκB is ready and waiting to activate, translocate and alter gene expression in a manner that may be negative for serotonin neurotransmission and perhaps even serotonin cell survival.

A significant role for NFκB in neuropathologies has recently been reviewed,⁴⁴ and therapies that target NFκB are receiving increased attention.⁴⁰ Our study showing that NFκB colocalizes in serotonin neurons suggests that one mechanism for cytokine-related depression could involve an action of cytokines on serotonin neurons and the NFκB signalling pathway. In addition, the observation that estrogen and estrogen plus progesterone prevent nuclear localization of NFκB in the DRN provides encouragement to seek alternative therapies for depression that may use the steroid receptors or NFκB.

SERMs, or selective estrogen receptor modulators, are ligands whose estrogenic properties vary from tissue to tissue, depending on the isoform of estrogen receptor present, as well as the coregulators recruited.⁴⁵ Raloxifene is a SERM that has estrogenic-like effects in bone, but it is a high-affinity

antagonist of ERα in other reproductive tissues.⁴⁶ Although raloxifene has poor penetration of the blood–brain barrier, studies are accumulating that indicate interesting estrogenic actions of raloxifene in the central nervous system. For example, raloxifene decreased fear and anxiety,⁴⁷ affected brain activation patterns during visual encoding⁴⁸ and had remarkable estrogen-like effects on neuroendocrine parameters in postmenopausal women.⁴⁹ We previously showed that TPH protein, TPH phosphorylation and SERT protein are increased after 5 months of raloxifene in a manner similar to the effect of estrogen.²³ Combined with our report that primate serotonin neurons contain ERβ, these data suggest that raloxifene acts as an estrogen agonist at ERβ in the context of a serotonin neuron and, in turn, has positive effects on anxiety and neuroendocrine parameters in postmenopausal women. This study shows that raloxifene, like estrogen, has no effect on the protein expression of NFκB. It will be of further interest to examine the effect of raloxifene on NFκB translocation.

In conclusion, NFκB p65 was detected in serotonin neurons in the DRN. This observation provides a biological scaffold for the ability of cytokines to induce negative affect. Moreover, the number and density of NLS-NFκB-positive cells was significantly lower in the DRN of animals treated for 1 month with estrogen or estrogen plus progesterone. However, neither protein levels nor gene expression for NFκB in the raphe region was altered by estrogen or estrogen-plus-progesterone treatment. These results are consistent with the body of literature showing that steroid hormones prevent translocation of NFκB to the nucleus via protein–protein interaction with steroid receptors, but they rarely change the expression of NFκB. Therefore, therapies that target ERβ, progesterin receptors and NFκB may be useful in the treatment of depression in women.

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