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REGULATION OF BRAIN-DERIVED NEUROTROPHIC FACTOR MESSENGER RNA LEVELS IN AVIAN HYPOTHALAMIC SLICE CULTURES

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Abstract—Mechanisms regulating the expression of brain-derived neurotrophic factor, a member of the neurotrophin family, have been extensively studied in the rat cerebral cortex, hippocampus and cerebellum. In contrast, little is known regarding the regulation of this growth factor in the hypothalamus. Here we present an analysis of the regulation of brain-derived neurotrophic factor messenger RNA levels in chick embryo hypothalamic slice cultures following exposure to potassium chloride, glutamate agonists and sex steroids. Following a week in chemically-defined media the tissue was depolarized by exposure to 50 mM potassium chloride for 6 h, resulting in a significant 4.2-fold increase in the level of brain-derived neurotrophic factor messenger RNA. This result is consistent with studies of other brain regions. Similar 6-h acute exposures of the hypothalamic cultures to 25 μM *N*-methyl-D-aspartic acid, 25 μM kainic acid and 25 μM α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid also significantly increased messenger RNA levels 2.5-, 2.1- and 1.4-fold, respectively. It was previously reported that brain-derived neurotrophic factor levels within the rat cerebral cortex, olfactory bulb and hippocampus are altered by exposure to 17β-estradiol. Here we show that in hypothalamic slice cultures neither acute nor chronic treatments with 10 and 100 nM 17β-estradiol and 10 nM testosterone significantly altered the steady-state level of this growth factor.

These findings show that neuronal activity, induced by glutamate agonists and potassium chloride, can regulate brain-derived neurotrophic factor messenger RNA levels within embryonic hypothalamic slice cultures. This regulation could play a critical role in the modulation of programmed cell death and synaptic maturation during development of the hypothalamus. © 2000 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: BDNF, chick embryo, estradiol, gene regulation, glutamate agonist, hypothalamus.

Mechanisms regulating the expression of brain-derived neurotrophic factor (BDNF) include an extensively studied Ca^{2+} signaling pathway[30,36] and the action of estradiol on a putative estrogen response element (ERE) in the BDNF gene.[34] During the past decade numerous studies have been reported characterizing several aspects of BDNF transcription, almost all of which have employed cortical, hippocampal or cerebellar neurons obtained from embryonic rats.[2,5,8,10,36,41] Tao *et al.* demonstrated that Ca^{2+} influx into embryonic rat cortical neurons activates BDNF transcription through interaction with a critical Ca^{2+} response element in the BDNF gene.[36] The level of intracellular Ca^{2+} ($[Ca^{2+}]_i$) depends upon Ca^{2+} influx via voltage-sensitive calcium channels (VSCC) and glutamate ionotropic receptors, and Ca^{2+} release from intracellular stores. Potassium chloride (KCl) can induce the activation of L-type VSCCs and hence increase $[Ca^{2+}]_i$. Meanwhile, glutamate, a major excitatory amino acid, regulates $[Ca^{2+}]_i$ by activation of two classes of ionotropic receptor, *N*-methyl-D-aspartic acid (NMDA) and non-NMDA. The non-NMDA class is comprised of α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) and kainic acid receptors. All three receptor subtypes are distinguished by their preferential responses to synthetic glutamate agonists.[23] In light of these mechanisms, the abilities of KCl,[7,8,10,28,36,41] glutamate,[8,10,28] NMDA,[2,28] AMPA[5] or kainic acid[5,15,28,41] to regulate BDNF transcription can be rationalized.

The existence and distribution of BDNF mRNA within the adult rat hypothalamus has been well-established.[21] Additionally, BDNF has recently been demonstrated to significantly increase the survival rate of neurons cultured from the hypothalamic paraventricular nucleus (PVN) and supraoptic nucleus.[18] Nevertheless, in light of the importance of this growth factor in the hypothalamus, there are surprisingly few reports describing its regulation. Both an osmotic stimulus[6] and a depletion of thyroid hormone[16] have been shown to increase BDNF mRNA within the rat PVN. Additionally, rats subject to immobilization stress exhibit an up-regulation of BDNF transcription in both the PVN and lateral hypothalamus.[32] Although glutamate plays a major role in the control of neuroendocrine neurons in the hypothalamus,[40] and exerts important trophic influences on the development of neurons,[19] until now there has been no previous characterization of hypothalamic BDNF mRNA expression in response to glutamate agonists.

Few studies have investigated the action of estrogen on BDNF mRNA expression. Sohrabji *et al.* reported an up-regulation of BDNF mRNA in ovariectomized rat cerebral cortex and olfactory bulb 4 h after injection of estradiol benzoate.[34] Additionally, Singh *et al.* observed elevated BDNF transcript levels within the hippocampus of 17β-estradiol (E_2) treated ovariectomized rats.[31] Recently a chronic study was reported in which E_2 and soy phytoestrogens were fed to ovariectomized rats for eight weeks, resulting in

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid; BDNF, brain-derived neurotrophic factor; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; d_3H_2O, triply distilled water; DIV, day *in vitro*; E, embryonic day; E_2, 17β-estradiol; ERE, estrogen response element; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KA, kainic acid; KCl, potassium chloride; MEM, Minimum Essential Medium; NMDA, *N*-methyl-D-aspartic acid; PVN, hypothalamic paraventricular nucleus; RPA, ribonuclease protection assay; T, testosterone; VSCC, voltage-sensitive calcium channels.

increased BDNF mRNA expression in the frontal cortex.[25] To date, however, no research has been conducted on the regulation of BDNF by sex steroids in the embryonic hypothalamus. The ability of the sex steroids to induce sexually dimorphic changes within the hypothalamus, including enhanced neuritic outgrowth from newborn mouse preoptic area explants, has been well studied.[37–39] Furthermore, testosterone (T) administered chronically to postnatal male chickens induced alterations in the distributional pattern and in the number of synapses in the PVN.[26] We hypothesized that the trophic effects of E_2 and T could be mediated by BDNF. Two recent studies on the sexual differentiation of the song system in songbirds, in which the involvement of sex steroids is well established,[29] lend support to this hypothesis. First, based on the temporal coincidence between the transient expression of BDNF in the song control nuclei of male zebra finches and the development of singing behavior, Akutagawa and Konishi implicated this protein in the sexual differentiation of the song system.[1] Second, chronic testosterone exposure was shown to increase the expression of BDNF in the high vocal center of a female adult canary, a brain nucleus involved in the acquisition and production of song.[27]

Here we present results on the effects of sex steroids, KCl and glutamate agonists on the regulation of BDNF mRNA levels in avian hypothalamic slice cultures. A series of experimental protocols have been used, including acute treatments of KCl (to optimize the ribonuclease protection assay), NMDA, kainic acid, AMPA, E_2 and T, a combined acute treatment of E_2 and kainic acid, and chronic treatment of E_2.

EXPERIMENTAL PROCEDURES

The following methods are based partly upon previously reported techniques.[14,35] In summary, organotypic slice cultures of chick embryo hypothalamus were exposed to sex steroids, KCl and glutamate agonists according to either an acute or chronic treatment protocol. The total RNA was extracted and then assayed for BDNF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Organotypic tissue culture

Fertile chicken eggs (*Gallus domesticus*, layer type) on their 11th (E11) or 14th (E14) day of incubation were obtained from the Department of Animal Science's hatchery. Under sterile conditions the embryos were isolated, and each brain was dissected out and submerged in a sterile 1.5% agarose solution (Sigma, electrophoresis reagent) at 42°C; this is slightly above the body temperature of a chicken. The agarose solution was immediately cooled in an ice-bath for 2 min, during which time the sex of the embryo was determined. The resulting agarose block was mounted on the stage of a Vibratome Series 1000 Sectioning System using cyanoacrylic glue, such that the brain lay on its side. This block was submerged in ice-cold dissecting buffer consisting of Dulbecco's phosphate-buffered saline (Gibco BRL) supplemented with D-glucose (Sigma, 6 mg/ml). Two incisions were made into the brain to delineate the preoptic area and hypothalamus, the first, a frontal slice, running from the pineal gland to the ventral caudal limit of the hypothalamus, and the second, a horizontal slice, from the base of the tractus septomesencephalicus to the ventral edge of the cerebellum, along the dorsal border of the hypothalamus.[17] The brain was then sliced in a parasagittal plane with a section thickness of 400 μm. The five or six hypothalamic tissue slices centered about the midline of the brain were collected in either ice-cold Gey's or Hank's balanced salt solution (Gibco BRL), each containing D-glucose (6 mg/ml). This region of the developing chick brain is known to express a high density of estrogen receptors.[22] To minimize cell death the above procedure was performed as rapidly as possible, typically taking 12 min per embryo. The slices were then incubated for 45–90 min at 4°C to allow endogenous proteases, released during the slicing, to diffuse out of the tissue. Next, using a wide-bore pipette, the slices from each embryo were transferred onto a Millicell-CM culture plate insert (Millipore, 0.4 μm pore size, 30 mm diameter) that was placed into a tissue culture dish containing media; the media formulations are described below. The hypothalamic slices were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Treatment protocols and media formulations

Two treatment protocols were employed. In the acute treatment protocol the hypothalamic slices, obtained from E14 chick embryos, were maintained in Neurobasal-based media (see below) until the sixth day *in vitro* (DIV), and then replaced by Phenol Red-free Minimum Essential Medium (MEM)-based media (see below). Six-hour acute treatments of neurotransmitters or sex steroids occurred on DIV 7, after which the tissue was collected and processed. Note that the five or six hypothalamic slices obtained from a single embryo constitute a single sample. In the chronic treatment protocol the hypothalamic slices, obtained from E11 embryos, were maintained in MEM-based media throughout the whole experiment. The tissue was collected and processed on DIV 1, 2, 3 and 4.

The most frequently used composition of organotypic slice culture media is 50% basal media, 25% balanced salt solution, and 25% horse serum, enriched with glucose.[9] However, we used a chemically defined medium with a composition more typical of that used in dissociated cell culture experiments. One problem with many commercial media is the inclusion of Phenol Red, a weakly estrogenic compound, as a pH indicator. Hence, for maintenance of the hypothalamic slices up to 24 h prior to any treatments we used Neurobasal medium containing Phenol Red, 2% B-27 serum-free supplement, 0.5 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all purchased from Gibco BRL). At all other times we used Phenol Red-free MEM with Earle's salts (Gibco BRL Life Technologies, Rockville, MD), 2% B-27 supplement, 0.5 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and an additional 4 mg/ml D-glucose. The sex steroid and neurotransmitter treatments were undertaken by supplementing the MEM-based media with the following solutions. Potassium chloride (KCl, Fisher), *N*-methyl-D-aspartic acid (NMDA, Sigma), kainic acid (KA, Sigma), and α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA, Sigma) were first dissolved in triply distilled water (d_3H_2O), and were then added to the media. Both 17β-estradiol (E_2, Sigma) and testosterone (T, Sigma) were first dissolved in ethanol to a concentration of 1 mg/ml. These solutions were then further diluted in d_3H_2O before being added to the media. An appropriate volume of ethanol diluted in d_3H_2O was added to control experiments in order to account for possible effects of the ethanol.

Throughout all experiments the culture medium was observed to remain at normal pH. Furthermore, before tissue processing, all slices were analysed using a phase-contrast microscope. Every tissue slice was both attached to the culture plate insert and displayed neuritic outgrowth, suggesting that the slices were in healthy condition.

Total RNA extraction and assessment

Due to the high lipid content of brain tissue we employed a phenol-based RNA extraction method. The extraction was performed using the ToTALLY RNA Isolation Kit (Ambion) according to the manufacturer's instructions. Briefly, the tissue was first sonicated in a guanidine thiocyanate denaturation solution using an Ultrasonic Processor (Vibracell). This was followed by a phenol:chloroform:isoamyl alcohol extraction, an acid-phenol:chloroform extraction, and isopropanol precipitation of the RNA. UV spectroscopy (260 and 280 nm) was then employed to determine the quantity and purity of the total RNA extracted from the hypothalamic slices. Finally, the quality of the total RNA was assessed by electrophoresis on a native 1% agarose gel. The RNA bands were stained with ethidium bromide and scanned using a PhosphorImager.

Ribonuclease protection assay

The ribonuclease protection assay (RPA) was performed using the RPA III Ribonuclease Protection Assay Kit (Ambion) according to the manufacturer's instructions. BDNF and GAPDH RNA probes were synthesized from cloned DNA plasmids,[13] kindly supplied by Dr Finn Hallböök (Uppsala University, Sweden), using the MAXIscript *In vitro* Transcription Kit (Ambion). Specifically, after linearization with *Eco*RI, a 180 nucleotide antisense chicken-BDNF RNA probe

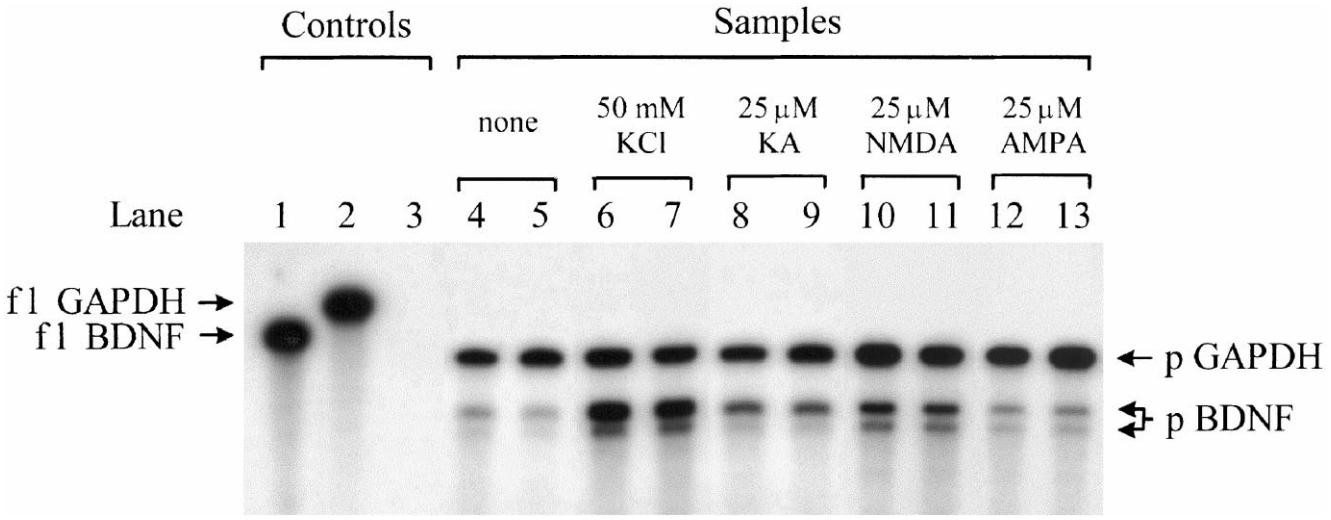

Fig. 1. Autoradiograph of ribonuclease protection assay for brain-derived neurotrophic factor and glyceraldehyde-3-phosphate dehydrogenase mRNA levels in cultured hypothalamic slices following acute exposure to KCl and glutamate agonists. Following 6-h exposures to the substances indicated, the total RNA was extracted and assayed (40 μg). The protected RNA fragments were analysed on a denaturing 5% polyacrylamide gel and then exposed to X-ray film for two days. The gel includes two probe-only positive control assays showing single bands that correspond to full length (f l) BDNF (180 nt, lane 1) and GAPDH (202 nt, lane 2) RNA probes. In the negative control assay (lane 3) the RNA probes and yeast RNA have been digested by RNase A/T1. Each of the sample assays (lanes 4–13) shows both protected (p) BDNF (doublet centered near 140 nt) and GAPDH (single band near 170 nt) probe fragments.

was transcribed from the T3 promoter, which corresponded to amino acid residues 182–228 of prepro-BDNF.[13] This probe was labeled with UTP α-^{32}P (ICN Biomedicals) to a specific activity of 1×10^9 c.p.m./μg. The 202 nucleotide antisense chicken-GAPDH RNA probe was first linearized with *Hind*III, and then transcribed from the T7 promoter. This probe was labeled with UTP α-^{32}P to a specific activity of only 5×10^6 c.p.m./μg. The RNA probes were prepared and used on the same day.

Quantities of BDNF RNA probe corresponding to approximately 1000 c.p.m. and GAPDH RNA probe corresponding to approximately 800 c.p.m. were hybridized to 40 μg of total RNA extracted from the hypothalamic slices. This step proceeded overnight at an optimized temperature of 56°C in Hybridization III Buffer (RPA III Kit). Next the samples were treated with a mixture of 0.4 units RNase A and 15 units RNase T1 for 30 min at 37°C, corresponding to the recommended 1:100 dilution of RNase A/T1 Mix (RPA III Kit). Following precipitation in RNase Inactivation/Precipitation III Solution (RPA III Kit) the protected RNA fragments were analysed by electrophoresis on a denaturing 5% polyacrylamide gel. Finally, X-OMAT AR X-ray film (Kodak) with a single intensifying screen was exposed to the gel for two days at −20°C. This film was developed, read by a Lumiscan 75 Scanner, and quantified using Scion Image software (Scion Corporation).

Data analysis

All experiments for a given treatment group were repeated typically three to six times. Levels of BDNF mRNA were normalized to the intensities of the GAPDH mRNA internal standard to control for differences in the amount of mRNA extracted and analysed. Treatment group means were then calculated. ANOVA tests using EXCEL 97 (Microsoft) were used to determine the significance of differences among the various control and treatment groups. Statistical significance was set at $P < 0.05$ for all experiments.

RESULTS

Effect of potassium chloride on the level of brain-derived neurotrophic factor mRNA

Our protocol was based upon other *in vitro* studies[7,10,28,36] that have reported the up-regulation of BDNF transcript following chronic depolarization, typically employing a 50 mM KCl solution with peak induction 6 h after the start of treatment.[8,41] Using an identical protocol we observed a 4.2-fold increase in BDNF mRNA levels, versus control data, as described below. This experiment served as a positive control allowing optimization of the RPA.

Following the 6-h, 50 mM KCl treatment on DIV 7, the total RNA was extracted and then assessed both quantitatively and qualitatively; yields of 2 μg total RNA per 1 mg tissue were typically achieved. A_{260}/A_{280} ratios of between 1.7 and 2.0 were recorded for all the samples, confirming the RNA to be of sufficient purity for the RPA. Furthermore, analysis of the samples by gel electrophoresis showed distinct 28S and 18S rRNA bands, consistent with intact RNA. Next the total RNA samples were assayed for BDNF mRNA. Figure 1 shows the results of representative assays analysed on a denaturing polyacrylamide gel. The appearance of the protected BDNF fragments as a doublet results from a mismatch between the actual BDNF mRNA sequence and one end of the RNA probe. This mismatch originated in the degenerate primers that were used to synthesize the BDNF DNA template,[13] and was then transcribed from the DNA template to the RNA probe. In further experiments an increased or decreased RNase concentration resulted in a shift of intensity within the doublet towards the shorter (RNase cleaves at mismatch) or longer (no cleavage at mismatch) probe fragment, respectively, hence confirming our explanation. The entire doublet was included in the quantitation of the BDNF mRNA signal that was in turn normalized to the intensity of the GAPDH mRNA internal standard. Analysis of the gel yielded normalized BDNF mRNA levels of KCl-treated cultures compared to controls, as shown in Table 1. The 50 mM KCl depolarization induced a very highly significant 4.2-fold increase in the level of BDNF mRNA ($P < 0.001$). The treatment and control experiments were both performed using three independent samples, where each sample comprises five or six slices derived from a single embryo. Finally, to determine the reproducibility of the RPA a single RNA sample was assayed in triplicate. After normalization the GAPDH signal intensity was equal to 1.000 ± 0.052 (or $\pm 5.2\%$), and the BDNF signal equal to 0.290 ± 0.016 (or $\pm 5.4\%$), where the ranges correspond to 3σ. This high reproducibility allowed us to assay each sample only once.

Table 1. The effect of acute treatments of depolarizing agents and sex steroids on steady-state brain-derived neurotrophic factor mRNA levels in cultured hypothalamic slices

Acute treatment	BDNF mRNA level
None	1.0 ± 0.04
50 mM KCl	4.2 ± 0.3 ***
25 μM AMPA	1.4 ± 0.1 ***
25 μM KA	2.1 ± 0.2 ***
25 μM NMDA	2.5 ± 0.3 **
10 nM E_2	1.0 ± 0.1
10 nM T	1.2 ± 0.2
25 μM KA and 10 nM E_2	1.7 ± 0.2 *

Following six days *in vitro*, the hypothalamic slices were incubated for 6 h in the presence of the substances indicated, and then the total RNA was extracted and assayed for BDNF mRNA. The normalized values given are the mean ± S.E.M. of between three and nine experiments and were compared to control data using ANOVAs
*$P < 0.05$,
**$P < 0.01$,
***$P < 0.001$.

Effect of glutamate agonists on the level of brain-derived neurotrophic factor mRNA

An equivalent 6-h acute treatment protocol to that used in the KCl depolarization studies was employed here. Three glutamate agonists were tested, NMDA, KA and AMPA, all at a concentration of 25 μM in the media. This concentration has been shown to induce maximal expression of BDNF mRNA following kainic acid treatment of dissociated rat hippocampal neurons.[41] The treatment and control experiments were each performed using samples in triplicate, and then the entire experiment was repeated, giving a total sample size of six embryos. The raw results of the RPA are shown in Fig. 1. Following analysis, the normalized BDNF mRNA levels for each glutamate agonist are presented in Table 1. All three glutamate agonists increased the level of BDNF mRNA in the hypothalamus, but to varying degrees: NMDA is the most potent with a highly significant 2.5-fold increase ($P < 0.01$), KA with a very highly significant 2.1-fold increase ($P < 0.001$), and AMPA is the least potent with a very highly significant 1.4-fold increase ($P < 0.001$), all versus control data.

Effect of sex steroids on the level of brain-derived neurotrophic factor mRNA

A series of protocols were employed, including acute treatments of E_2 and T, a combined acute treatment of E_2 and KA, and chronic treatment of E_2. In the 6-h acute treatments using 10 nM E_2 and T, hypothalamic slices were prepared from both male and female E14 chick embryos and received a 6-h treatment on DIV 7. The duration of this treatment is also compatible with the protocol used to study the up-regulation of BDNF mRNA in the rat cerebral cortex and olfactory bulb.[34] Each sex steroid treatment was performed using samples in quadruplicate. The results of the assay were analysed, and normalized BDNF mRNA levels for each treatment are presented in Table 1. Whereas 10 nM E_2 did not alter the level of BDNF mRNA in either male or female embryos compared to control data, 10 nM T induced a small increase in the level of mRNA in female, but not male, embryos; however, this result was not statistically significant.

The justification for the 6 h combined treatment of 10 nM E_2 and 25 μM KA is based on recent evidence by Gu and Moss that E_2 can potentiate kainate-induced currents in hippocampal CA1 neurons.[12] Also, estrogen administration has been shown to potentiate glutamate-induced excitation in cerebellar Purkinje cells.[33] We hypothesized that if such a potentiation occurred within the hypothalamus then our previously observed increase in BDNF mRNA by 25 μM KA treatment might be further increased by the addition of E_2. The lowest concentration of E_2 at which Gu and Moss observed this effect was 10 nM; by employing the same concentration in our experiments we could compare the results to those obtained individually for both 10 nM E_2 and 25 μM KA. Surprisingly, following a 6-h acute treatment, the addition of E_2 slightly reduced the level of BDNF mRNA in both male and female embryos compared to 25 μM KA alone, as shown in Table 1; experiments were repeated in quadruplicate. However, this reduction was not statistically significant and hence we must conclude that E_2, at this concentration, had no effect on the KA-induced expression of BDNF mRNA.

In previous studies of the hypothalamus a 100 nM (or higher) estradiol concentration has successfully enhanced both neuritic and dendritic outgrowth from mouse hypothalamic explants.[37–39] Here we exposed hypothalamic slices to 100 nM E_2 from the start of the culture, and collected tissue on DIV 1, 2, 3 and 4. The results, showing the relative amounts of BDNF mRNA in E_2-exposed and untreated cultures throughout the duration of the experiment, are shown in Fig. 2 (raw data) and Fig. 3 (normalized data). At no point in time were the transcript levels in the two groups significantly different. Furthermore, after grouping all of the samples from every time-point together, there was still no significant difference between the E_2-exposed and untreated cultures. The highest level of transcript recorded in both treated and untreated cultures was 24 h after culturing. We observed a similar transient peak in the level of BDNF mRNA in an experiment employing untreated E13 embryos; an approximately 1.5-fold increase in BDNF mRNA level was observed 6 h after culturing relative to transcript levels at the start of the experiment, which decreased to a 1.2-fold increase after 24 h, and returned to basal levels after 48 h. We believe that this transient peak in expression, discussed below, is a survival response of the tissue following damage inflicted during slicing.

DISCUSSION

Brain-derived neurotrophic factor mRNA turnover in vitro

The BDNF gene has a complex structure with four short 5′ exons (I, II, III and IV), each with a promoter upstream, and one 3′ exon (V) encoding the mature BDNF protein. Alternative use of these promoters, in addition to two possible sites for polyadenylation, results in a total of eight distinct BDNF mRNAs.[36] The probe used to measure BDNF mRNA in this study hybridizes to a region of the 3′ exon. Consequently, this work does not approach the issue of differential expression of multiple transcripts, but measures the pool of all BDNF mRNAs.

Two studies have investigated the decay kinetics of BDNF mRNA *in vitro*. After stimulating hippocampal neurons with KCl, Sano *et al.*[28] added actinomycin-D to stop mRNA synthesis, and from the decay of BDNF mRNA calculated a

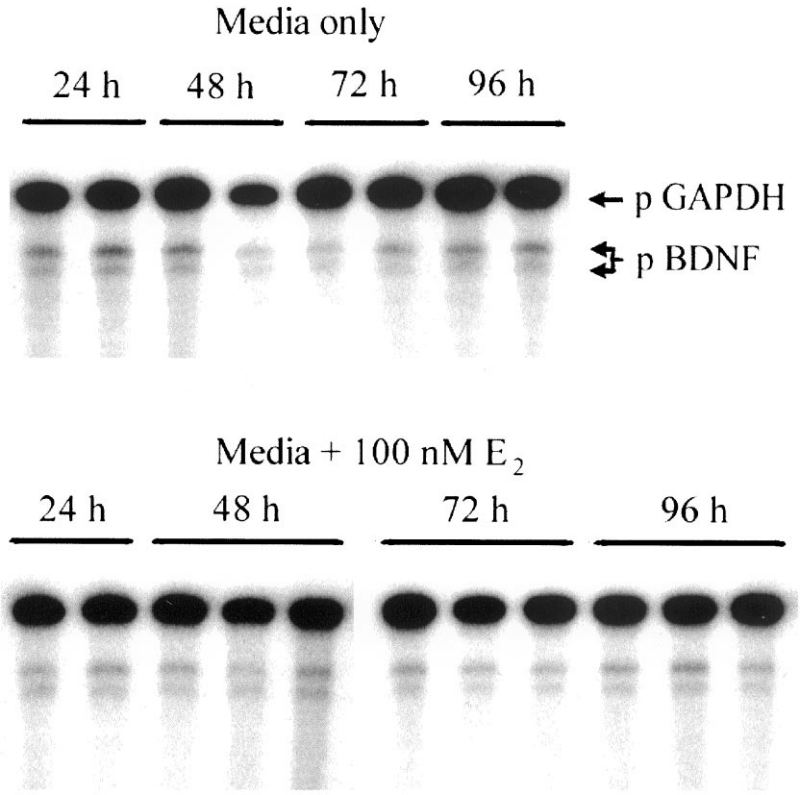

Fig. 2. Autoradiograph of ribonuclease protection assay for brain-derived neurotrophic factor and glyceraldehyde-3-phosphate dehydrogenase mRNA levels in cultured hypothalamic slices following chronic exposure to estrogen. Hypothalamic slices were cultured both with and without 100 nM E_2 included in the media. After 24, 48, 72 and 96 h the total RNA was extracted and assayed. The protected RNA fragments were analysed on a denaturing 5% polyacrylamide gel and then exposed to X-ray film for two days. Each of the gel lanes shows both protected (p) BDNF and GAPDH probe fragments.

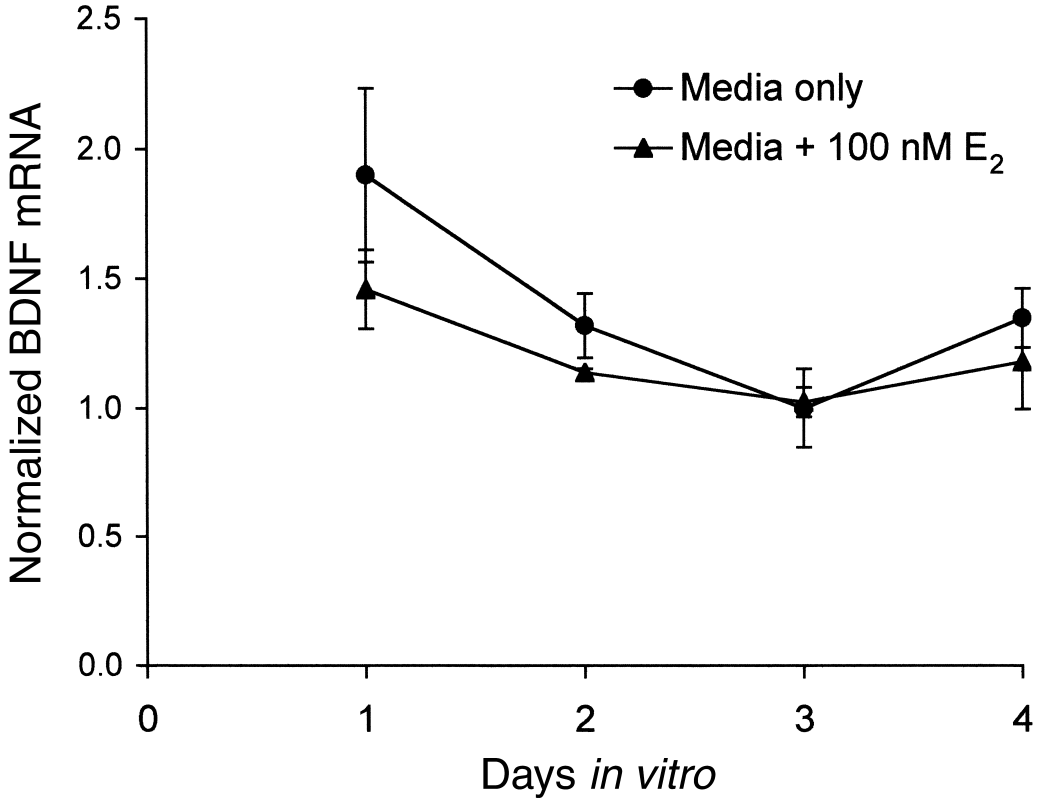

Fig. 3. Level of brain-derived neurotrophic factor mRNA in hypothalamic slice cultures for both untreated and 100 nM E_2-chronically exposed samples. The slices were harvested one, two, three and four days after culturing and then assayed for BDNF mRNA. Each time-point represents either two or three separate experiments. The data are arbitrarily referenced to the lowest media-only value and are expressed as the mean ± S.E.M.

half-life of 2.5 h. In the second study the KCl depolarizing stimulus was removed and the BDNF transcript returned to control levels within 6 h.[8] The transient increase in BDNF mRNA level we described in the final section of our results peaked 6 h after culturing, was still elevated after 24 h, and returned to basal levels after 48 h. This decay does not follow a 2.5-h half-life, and hence cannot be due to a sudden increase in BDNF transcript at the moment of slicing. The transient peak in expression most likely results from a prolonged recovery of the tissue following damage inflicted during the slicing. Knowing that this recovery takes up to 48 h could be a useful result for other experiments that culture brain slices.

Effect of potassium chloride-induced depolarization

The 4.2-fold increase in steady-state BDNF mRNA levels following 50 mM KCl depolarization serves as a positive control for the RPA. Furthermore, the magnitude of the observed KCl-induced increase is consistent with studies of other brain areas. For example, Elliott *et al.* reported a maximal six-fold increase in BDNF mRNA 6 h after stimulating cultured rat hippocampal neurons with 50 mM KCl.[8] To our knowledge, however, this is the first report that chronic depolarization by KCl can increase the level of this growth factor transcript within hypothalamic tissue.

Effect of glutamate agonist treatments

We have shown that in avian hypothalamic tissue 6-h acute exposures of the three glutamate agonists tested, each at a concentration of 25 μM, can increase the levels of BDNF mRNA as follows: NMDA (2.5-fold) > KA (2.1-fold) > AMPA (1.4-fold). We believe that this is the first demonstration of glutamate agonist-induced BDNF transcription in hypothalamic tissue. Similar studies have been reported for the rat hippocampus, cerebellum, neostriatum, cortex and mesencephalon, and for the chicken optic tectum and retina, as discussed below.

Using primary cultures of embryonic rat hippocampal neurons, both 25 μM KA[41,42] and 100 μM NMDA[28] treatments increase the expression of BDNF mRNA. In another study of hippocampal neurons, AMPA receptor modulators (ampakines) have been shown to induce an increase in this transcript.[20] Taken together, these studies implicate both NMDA and non-NMDA glutamate receptors in mechanisms that alter hippocampal BDNF transcription. The same conclusion can be drawn for cerebellar granule cells in which, using primary cultures of postnatal rat neurons, Bessho *et al.*[4] observed the following increases in BDNF transcription after 4-h treatments: 50 μM KA (8.0-fold) > 50 μM AMPA (7.6-fold) > 30 μM NMDA (3.9-fold). Furthermore, a similar pattern has been reported for cortical neurons. First, KA elevated levels of BDNF mRNA in an adult rat cortex *in vivo*,[41] and in a second study glutamate transiently induced BDNF expression via NMDA receptors in cultured embryonic cortical neurons.[10] However, a different situation has been reported in the neostriatum, where BDNF mRNA was up-regulated following injection of KA and AMPA, but was unaffected by either quinolinate or 1S,3R-1-aminocyclopentane-1,3-dicarboxylic acid, both NMDA receptor agonists.[5] Interestingly, primary cultures of fetal mesencephalic dopaminergic neurons exhibit the entirely opposite behavior to that of the neostriatum. Aliaga *et al.*[2] attempted to modulate BDNF mRNA expression following 6-h treatments of 25 μM KA and 100 μM NMDA; only the NMDA significantly increased the expression (specifically of exons I and III).

Several reasons could explain this variation in BDNF transcription between different agonists and between different brain areas. First, the distribution of different glutamate receptor subtypes throughout the various brain regions will directly affect the ability of these regions to display altered levels of transcription. For example, injection of KA into rat striatum induces the highest increase in BDNF transcription, consistent with the extensive distribution of KA receptors in this area of the brain.[5] Similar reasoning can be used to rationalize the smaller increase in BDNF transcription we observed in the hypothalamus compared to studies on the hippocampus and cortex,[41] since the hypothalamus has a lower density of glutamate-binding sites than these other regions.[40] Second, the glutamate agonists have different affinities for their respective receptors. This could explain why NMDA was the most potent agonist at increasing BDNF mRNA levels in the present study, where all agonists were employed at 25 μM, since NMDA has an extremely high affinity for its receptor. Third, the four or more promoters in the BDNF gene allow multiple points of transcriptional regulation. Hence, the activation of different subtypes of glutamate receptors can differentially regulate the expression of BDNF exon-specific transcripts in the brain. Finally, the maturity of the neurons being investigated might affect the magnitude of BDNF induction. Evidence suggests that immature neurons are much less susceptible to the excitotoxic effects of glutamate than older ones.[10] For example, glutamate and KA have demonstrated neuroprotective actions on the embryonic chick retina, yet sustained activation of the AMPA and KA receptors later in development led to massive cell death.[3] This neuroprotective action almost certainly involves BNDF, since Karlsson and Hallböök have recently shown that KA increases the expression of BDNF mRNA in the E15 chick embryo optic tectum and retina.[15] Hence, it is plausible that immature neurons, during early development, are able to increase BDNF transcription to a greater degree than older neurons.

Effect of sex steroid treatments

Using the protocols described in this study, the level of BDNF transcript in hypothalamic slice cultures was not modulated by any E_2 treatments, including acute, chronic and combined with a glutamate agonist. Acute exposure to testosterone increased the level of expression in female embryos, but the result was not significant. This does not, however, exclude the possibility that sex steroids modulate BDNF expression within the hypothalamus, although based on several related studies we employed supraphysiological concentrations of sex steroids that could have led to down-regulation of the receptors. It is also possible that endogenous T or E_2 remained within the hypothalamic slices throughout the culture period and then masked the effects of the exogenously applied sex steroids. Alternatively, our experimental conditions for inducing BDNF transcription could be correct, but our choice of techniques to characterize this expression might not have been optimal. Unlike the more homogeneous distribution of BDNF mRNA in the cerebral cortex, for example, this transcript is dispersed within several discrete nuclei in the hypothalamus.[21] If we increased BDNF

transcription in just one or two of these nuclei then that response might have been "diluted out" to an undetectable level when the entire hypothalamus was assayed. A technique that allows the specific location of the transcript to be determined, such as *in situ* hybridization, may yet serve to support our hypothesis.

In contrast to the well-characterized regulation of BDNF by neurotransmitters, there are few studies investigating the effects of E_2 exposure on levels of BDNF mRNA and protein in the brain. Within the rat frontal cortex two independent experiments have observed an up-regulation of BDNF mRNA following acutely[34] and chronically[25] administered estrogen. However, the effect of estrogen on hippocampal neurons is less clear. Both Gibbs[11] and Singh *et al.*[31] observed an increase in hippocampal BDNF mRNA in rats exposed to estrogen, whereas estrogen-treated rat hippocampal neurons in culture exhibited a down-regulation of BDNF protein.[24] Although a direct comparison of these studies is difficult due to differences in experimental methods, clearly more work is required to elucidate the role of estrogen in the regulation of BDNF expression.

In conclusion, we have studied both neurotransmitter and sex steroid-induced regulation of BDNF mRNA levels in the avian hypothalamus using organotypic slice culture methods. Whereas all three glutamate agonists investigated, acting via NMDA and non-NMDA receptors, induced an increase in BDNF mRNA levels, no statistically significant changes in the level of this transcript were detected following exposure to estrogen and testosterone. To our knowledge this is the first study describing the effect of neuronal activity, induced by glutamate agonists and potassium chloride, on the regulation of BDNF mRNA levels within the hypothalamus.

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