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Rats with physically disconnected hypothalamo-pituitary tracts no longer contain vasopressin-oxytocin gene transcripts in the posterior pituitary lobe

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In rats, vasopressin- and oxytocin-encoding mRNAs are present in the posterior but absent in the anterior lobe of the pituitary gland. RNase protection experiments indicate that in the posterior pituitary and hypothalamus identical transcriptional start points are used. Furthermore, the two transcripts from posterior pituitary and hypothalamus show identical nucleotide sequences. Animals operated by paired electrical lesions in such a way that connections between the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the hypothalamus and the posterior pituitary lobe are destroyed continue to express the vasopressin and oxytocin gene in the hypothalamus but not in the posterior pituitary. Operated animals subjected to chronic intermittent salt loading for 6 days similarly contain vasopressin and oxytocin encoding transcripts in the hypothalamus but not in the posterior pituitary.

Vasopressin gene; Oxytocin gene; Posterior pituitary; Electrically lesioned animal; Pituicyte

1. INTRODUCTION

Expression of the oxytocin-vasopressin gene family has been observed in many extrahypothalamic tissues such as cerebellum, adrenal gland and reproductive organs [1], a list that recently has been extended by the unexpected addition of the rat pituitary [2]. Poly(A)$^+$ RNA extracted from the posterior but not from the anterior pituitary contains vasopressin-encoding transcripts [2]. These data have been confirmed and extended not only for vasopressin- but also for oxytocin-encoding mRNA from rat posterior pituitary using Northern blot and in situ hybridization techniques [3,4].

It is presently not known whether the oxytocin and vasopressin genes are expressed in pituicytes, a modified subtype of astrocytes of the posterior pituitary or whether the observed transcripts are present in the nerve-endings of the hypothalamic magnocellular neurons. In the latter case the respective mRNAs would have to travel axonally from the cell body, localized in the hypothalamus to the nerve endings in the posterior pituitary. Unfortunately, because of the special morphology of the posterior pituitary in situ hybridization at the light microscopy level does not allow one to distinguish between the two cell types.

In this report we show that rat posterior pituitary derived vasopressin- and oxytocin-encoding mRNAs are identical in sequence to their hypothalamic counterparts and that after physical disconnection of the hypothalamo-pituitary tracts by paired electrical lesions, vasopressin and oxytocin encoding mRNAs can no longer be detected in the posterior pituitary.

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2. MATERIALS AND METHODS

2.1. *Specific DNA fragments used*

A 199 bp *PstI/KpnI* DNA fragment, corresponding to nucleotide residues −36 to +163 (+1 = transcriptional start point) from the rat vasopressin gene [5] was cloned into the *SmaI/PstI* sites of pSP 65 after converting the 3′ cohesive ends generated by digestion with *KpnI* into blunt ends with T4 DNA polymerase according to standard procedures [6]. This clone was designated pSP AVP 5′. Similarly, a 204 bp *ApaI/MstI* DNA fragment, corresponding to nucleotide residues −47 to +157 of the rat oxytocin gene [7] was cloned into the *SmaI* site of pSP 65, after converting the 3′ cohesive ends generated by digestion with *ApaI* to blunt ends (clone pSP OT5′). The genomic fragments are oriented in the vector in a 3′ to 5′ direction relative to the position of the SP6 promoter. In vitro transcription yielded cRNAs representing the anti sense strand of the respective mRNAs.

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Clones representing the nonhomologous 3′ ends of the rat vasopressin and oxytocin genes were constructed by excising a 638 bp *PstI/Hin*dIII fragment from the vasopressin gene [5] or a 700 bp *AvaI/Eco*RI fragment from the oxytocin gene [7], these being subsequently inserted into the appropriate sites of the vector pUC 8. Chicken β actin cDNA was a kind gift of H.H. Arnold (Hamburg).

2.2. *Electrical lesion experiments*

Male Wistar rats (~200 g) were used in all experiments. Where indicated, chronic intermittent salt loading with 2% NaCl in the drinking water was performed for 6 days as recently described [8].

Production of diabetes insipidus in rats was carried out as described in Thorn et al. [9]. In the experimental group (5 rats each), in dormicum-hypnorm or brietal anaesthesia, a unipolar electrode was introduced through 2 holes made in the skull, at the bregma and as close to the midline as possible. The tip was sited midway between the optic chiasma and the ventromedial nuclei, and paired lesions were produced by 3–4 mA/20 s. The procedure was repeated after 7–10 days. A stable polyuria occurred approximately 7 days after the second operation. Daily water consumption was monitored and diuresis and urine osmolality were measured.

2.3. *RNA isolation and Northern blot analysis*

Total RNA from hypothalamus, pituitary gland, anterior and posterior pituitary was prepared as published [10]. RNA was denatured with glyoxal [6], electrophoresed in 1.45% agarose gels and transferred to Zeta Probe membranes (Bio Rad, Munich, FRG). Hybridization was carried out with labelled [11] DNA probes ($3–5 \times 10^6$ cpm/ml) specific for the vasopressin (638 bp *PstI/Hin*dIII in pUC 8; [5]) or oxytocin (700 bp *AvaI/Eco*RI in pUC 8; [7]) genes.

2.4. *PCR amplification of vasopressin and oxytocin mRNA*

Poly(A)$^+$ RNA from rat hypothalami and pituitary glands was converted into single stranded cDNAs [12]. 100 ng (hypothalamus) or 200 ng (pituitary gland) ss cDNA was amplified by the polymerase chain reaction in 100 μl of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.01% gelatine, 0.05% Nonidet P40, 0.05% Tween 20, 1 mM $MgCl_2$, 200 μM each of dATP, dTTP, dGTP and dCTP, 1 μM of each primer (see legend of Fig. 1) and 2.5 units Taq polymerase (Cetus Norwalk, USA). First cycle (1 loop): 5 min, 96°C, 4.5 min, 50°C, 3 min, 72°C; second cycle (29 loops): 1.5 min, 96°C, 1.5 min, 50°C, 3 min, 72°C; third cycle (1 loop): 1.5 min, 96°C, 1.5 min, 50°C, 5 min, 72°C. For reamplification, a 10 μl aliquot of the first reaction was used under identical conditions.

Amplified products were resolved on preparative agarose gels, electrophoretically eluted and cloned into the *Sma*I site of the vector M13 mp18. Sequence determination of template DNAs was performed according to [13].

2.5. *Southern blot analysis*

PCR products were run on 1.0% agarose gels and transferred to nylon membranes (Bio Rad, Munich, FRG) in 0.4 M NaOH [14]. Hybridization was carried out with labelled [11] DNA probes (5×10^5 cpm/ml) specific for the vasopressin or oxytocin gene (638 bp *PstI/Hin*dIII in pUC 8, 700 bp *AvaI/Eco*RI in pUC 8).

2.6. *RNase protection assays*

RNase protection experiments were performed essentially as described [15]. Briefly, 5 μg of hypothalamic and 30 μg pituitary RNA were dissolved in 30 μl 80% formamide, 40 mM Pipes, pH 6.4, 400 mM NaCl, 1 mM EDTA and hybridized overnight (>8 h) at 45°C to a ^{32}P labelled in vitro synthesized vasopressin (pSP AVP 5′) or oxytocin (pSP OT 5′) specific anti sense RNA (7×10^5 cpm/reaction) corresponding to the 5′ ends of the respective mRNAs. After digestion with RNase A and RNase T1, proteinase K treatment, phenol extraction and alcohol precipitation, protected bands were electrophoretically separated in 6% sequencing gels [16] and exposed to X-ray films (Fuji RX).

3. RESULTS

3.1. *Sequence analysis and mapping of transcriptional start points of neural lobe vasopressin and oxytocin mRNAs from rat posterior pituitary*

Poly(A)$^+$ RNA from rat hypothalamus and the entire pituitary gland were converted into single stranded cDNA and amplified by the polymerase chain reaction (PCR) using primers (20-mers) corresponding to the extreme 5′ and 3′ ends of either rat hypothalamic vasopressin or oxytocin mRNAs [5,7]. Amplified products of the pituitary cDNAs were identical in size to those of the hypothalamus and hybridized strongly to ^{32}P labelled probes specific for either vasopressin- or oxytocin-encoding transcripts (Fig. 1). The bands corresponding to the largest of the hybridizing fragments were subcloned and sequenced. This revealed that vasopressin- and oxytocin-encoding mRNAs from posterior pituitary and hypothalamus show identical nucleotide sequences (Fig. 1). In the hypothalamus, several nearby transcriptional start points were shown to exist in the vasopressin and oxytocin genes [5,7]. RNase protection experiments demonstrate that in posterior pituitary and hypothalamus RNAs identical patterns of protected bands are observed, occurring in the same relative abundance. Moreover, anterior lobe RNA is devoid of both transcripts as indicated by the complete absence of protected fragments (Fig. 2).

3.2. *Northern blot analysis after disconnection of the hypothalamo-pituitary tracts*

In a series of experiments the connection between the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the hypothalamus and the posterior pituitary lobe was destroyed by paired electrical lesions. Whereas the urine osmolality of the sham operated group was 1984 ± 102 mOsm, the operated animals exhibited a severe (urine osmolality 319.8 ± 46.0 mOsm) or a mild (urine osmolality 854.0 ± 18.2 mOsm) form of diabetes insipidus. Sham operated, mildly or severely diabetic animals were subjected to chronic intermittent salt loading for 6 days. This has previously been shown to result in an increase in vasopressin and oxytocin mRNA content in the hypothalamus [8]. Each group was paralleled by a control group which received tap water ad lib.

Posterior pituitary and hypothalamus RNAs were subsequently analyzed by Northern blot hybridization to radiolabelled vasopressin- and oxytocin-specific probes. The data shown in Fig. 3 reveal several interesting features:

(i) In the posterior pituitary vasopressin- and oxytocin-encoding transcripts are virtually absent in all groups of lesioned animals (Fig. 3, lanes 5–8). In sham operated animals both mRNA species are easily detected and accumulate after salt loading (lanes 3, 4), a result that is in agreement with recent reports [3,4].

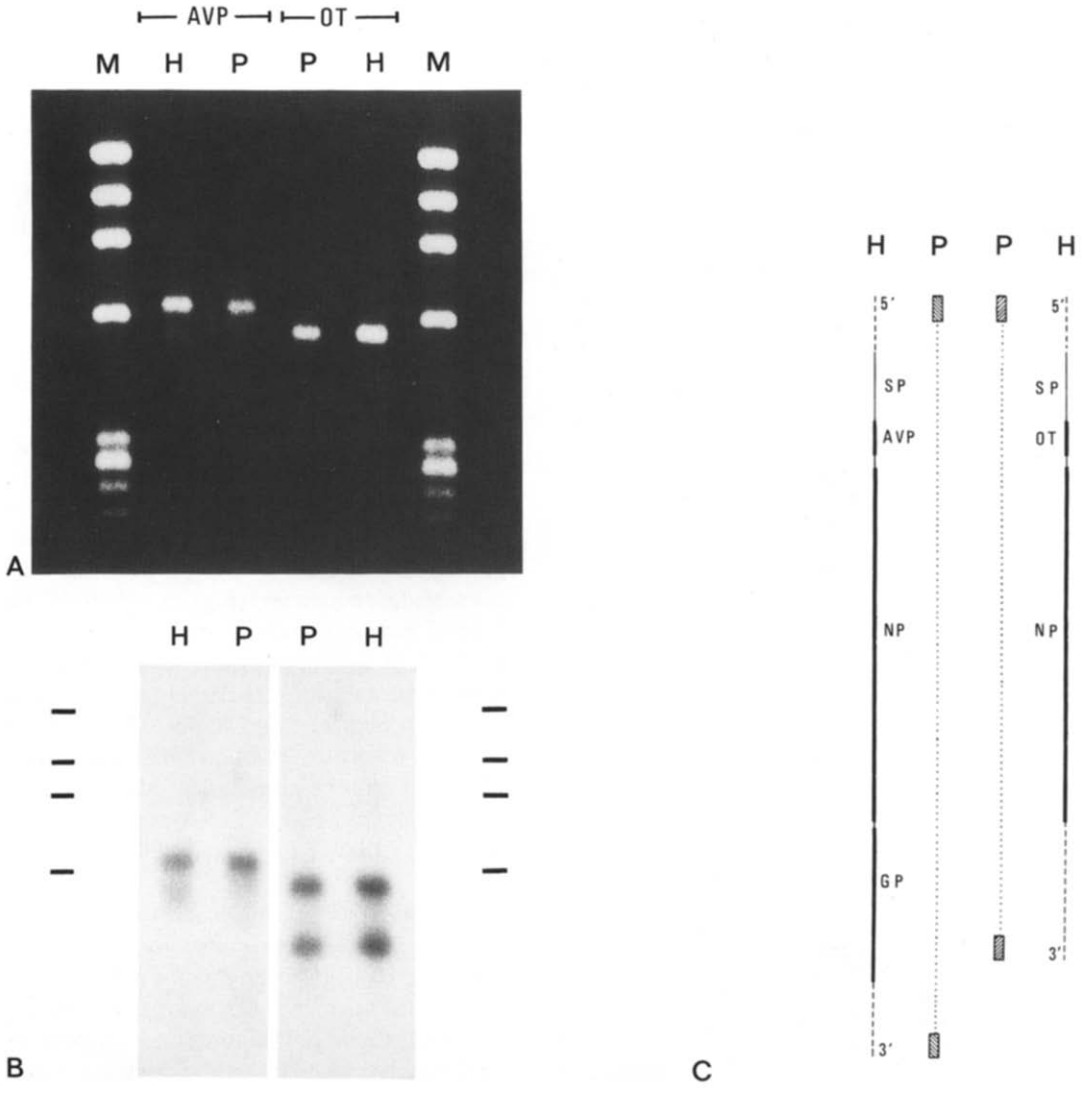

Fig. 1. Agarose gel electrophoresis and Southern blot analysis of products generated by PCR amplification of single stranded cDNAs derived from rat hypothalamus (H) or total pituitary gland mRNA (P). Primers used for amplification of single stranded vasopressin-encoding cDNAs: 5′ AGCAGAGCAGAGCTGCACGC 3′ (forward primer) and 5′ TTGCATTTTTTAAAAGTTTA 3′ (reverse primer), primers used for amplification of single stranded oxytocin-encoding cDNAs: 5′ ACCATCACCGACGGT GGATC 3′ (forward primer) and 5′ TTGAAGAGG AAATCTGCTTT 3′ (reverse primer). (A) Agarose gel electrophoresis of amplified products. (B) Southern blot analysis of PCR amplified products using a ^{32}P-labelled vasopressin- or oxytocin-specific probe. (C) Schematic representation of the identity of hypothalamic and pituitary mRNAs encoding the vasopressin and oxytocin precursor, respectively. AVP, vasopressin, GP, glycopeptide; H, hypothalamus; M, DNA size markers (*Hae*III cut *Phi*X174 DNA); NP, neurophysin; OT, oxytocin. The hatched boxes indicate the primer sequences. The bars shown on the left and right side indicate the positions of the four largest DNA fragments of *Hae*III cut *Phi*X174 DNA. The dotted lines indicate that pituitary vasopressin and oxytocin mRNA are identical in nucleotide sequence to their hypothalamic counterparts [5,7].

(ii) Compared to their hypothalamic counterparts, posterior pituitary RNAs are shorter due to a different degree of polyadenylation.

(iii) In the hypothalamus of sham-operated animals, a strong hybridization signal is observed for both vasopressin- and oxytocin-encoding mRNA (lanes 9, 10). In contrast, hypothalamic vasopressin encoding transcripts are drastically reduced in lesioned animals showing a severe diabetes insipidus (lanes 11, 12) and are moderately reduced in those showing a mild diabetes insipidus (lanes 13, 14). This reduction is less significant for the oxytocin-encoding mRNA (lanes 11–14).

(iv) Chronic intermittent salt loading of lesioned and sham-operated animals leads to an increase in the length of the hypothalamus-derived vasopressin- and oxytocin-encoding mRNAs, most likely due to increased polyadenylation; but little if any increase has been observed in the level of the respective hypothalamic transcripts (lanes 9–14). This is quite in contrast to osmotically stressed but not operated animals which accumulate hypothalamic vasopressin- and oxytocin-

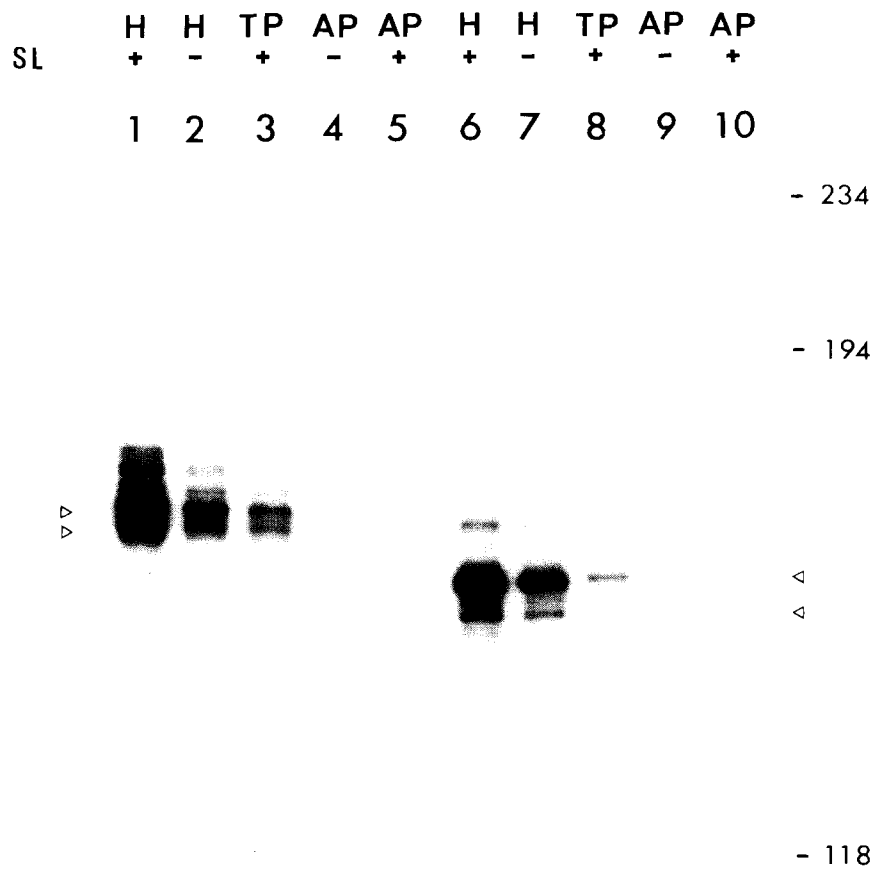

Fig. 2. RNase protection experiment. Total cellular RNAs from rat hypothalamus (lanes 1, 2, 6, 7), total pituitary gland (lanes 3, 8) and anterior lobe of the pituitary gland (lanes 4, 5, 9, 10) were hybridized to ^{32}P labelled anti sense RNAs corresponding to a portion of the 5′ untranscribed region, the 5′ transcribed region as well as exon A of the rat vasopressin (AVP) or oxytocin (OT) gene. The open arrowheads point to the position of the major protected bands. DNA size markers (in bases) are shown on the right. H, hypothalamus; AP, anterior pituitary; TP, total pituitary gland; SL, salt loading for 7 days.

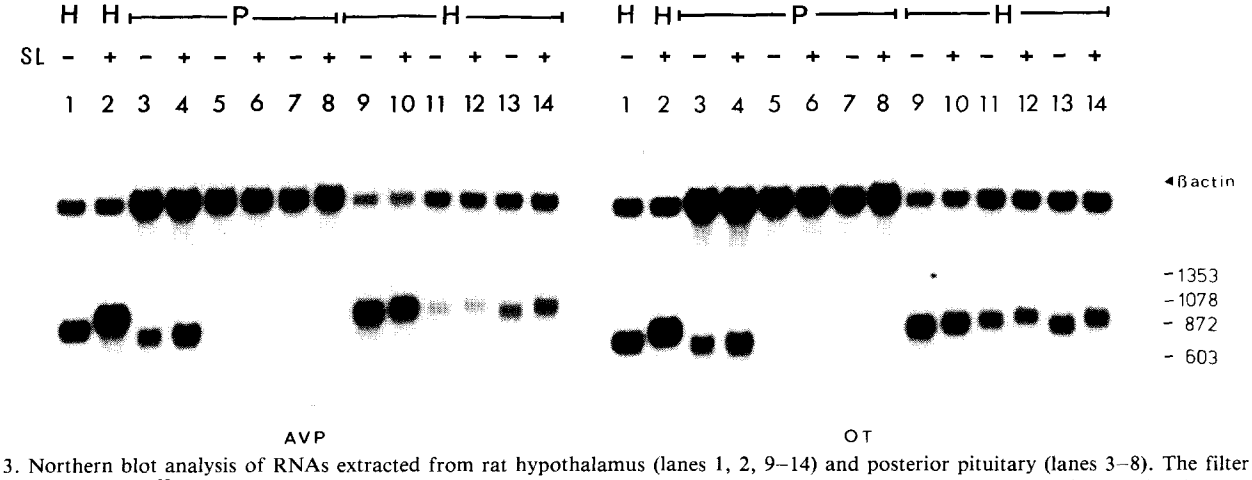

Fig. 3. Northern blot analysis of RNAs extracted from rat hypothalamus (lanes 1, 2, 9–14) and posterior pituitary (lanes 3–8). The filter was first hybridized to a ^{32}P labelled vasopressin (AVP)-specific probe and after removal of hybridized DNA to a labelled oxytocin (OT)-specific probe, and finally to a labelled chicken β actin cDNA. DNA size markers in bases are indicated on the right. Lanes 1 and 2: Hypothalamic RNA from control animals and rats treated with 2% saline for 7 days, respectively. Five rats each were sham operated (lanes 3, 4, 9, 10) or electrically lesioned which produced a severe (lanes 5, 6, 11, 12) or mild (lanes 7, 8, 13, 14) artificial diabetes insipidus. In addition, animals were subjected to intermittent salt loading for 6 days (lanes 4, 6, 8, 10, 12, 14). Hypothalamic RNA: 3 μg/lane (lanes 1, 2, 11–14), about 2 μg/lane (lanes 9, 10); in these animals RNAs were extracted from individually punched SONs and PVNs, explaining the slightly reduced signal observed for the actin mRNA. Posterior pituitary RNA: total RNA extracted from 5 lobes/lane. RNAs shown in lanes 3 and 9, 4 and 10, 5 and 11, 6 and 12, 7 and 13, 8 and 14, respectively, were extracted from hypothalami and posterior pituitary lobes of the same animals. H, hypothalamus; P, posterior pituitary; SL, salt loading.

encoding mRNAs that contain significantly longer poly(A) tails (Fig. 3, lanes 1, 2).

4. DISCUSSION

The results presented here show that vasopressin- and oxytocin-encoding mRNAs from rat hypothalamus and posterior pituitary are identical in their nucleotide sequence, this agreeing with the idea that vasopressin and oxytocin precursors are encoded by single copy genes [1]. The only difference observed is that posterior pituitary derived transcripts contain shorter poly(A) tails than those found in the hypothalamus. After destruction of the hypothalamo-pituitary axis, vasopressin- and oxytocin-encoding transcripts are no longer detectable in the posterior pituitary, even when the animals are subjected to chronic intermittent salt loading, which in the sham operated control group leads to an accumulation of both mRNA species. These findings might be interpreted to suggest that vasopressin- and oxytocin-encoding mRNAs are axonally transported from the hypothalamus to the posterior pituitary. However, this appears rather unlikely, since in order to obtain the shorter posterior pituitary mRNAs a precise trimming at the poly(A) tail of the hypothalamic transcripts would have to occur during axonal transport.

Alternatively the pituicytes of the posterior pituitary, a heterogenous group of cells [17], could be the site of vasopressin and oxytocin gene expression. This being the case, our data suggest that the pituicytes depend on contacts with axons of magnocellular neurons and/or on neural inputs deriving from other parts of the brain for their ability to express the vasopressin and oxytocin genes. It may also be that vasopressin and oxytocin gene expression is sharply down-regulated in response to the high activity of the pituicytes in phagocytosing cell debris resulting from the electrical lesion.

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