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SHORT COMMUNICATION
Key role for enkephalinergic tone in cortico–striatal–thalamic function

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Abstract

Whereas the role of dopaminergic tone in the cortico-striatal-thalamic system is well-established, the role of endogenous opioids in the function of this system is less understood. We show that Borna disease virus infection of adult rats results in an increase in preproenkephalin transcripts in the striatum of Borna-infected rats, a region important for forming coordinated sequential motor actions and in developing programmes of thought and motivation. Stereotypic behaviours and dyskinesias, the clinical hallmarks of infection in adult Lewis rats (BD rats), are accompanied by a disrupted pattern of immediate early gene *c-fos* activation in the motor thalamus, with significance for the breakdown in coordinated sequential motor actions. We also find increased preproenkephalin in infected cultured neuroblastoma and rat foetal glial cells. The expression pattern of enkephalin mRNA *in vivo* and *in vitro* suggest that increased enkephalin function is one of the neuropharmacological means by which Borna disease virus causes motor disease of animals and possibly cognitive and affective disease in man, and further suggest that enkephalins play a critical role in the maintenance of a balanced tone of activity in the cortico-basal ganglia-thalamo-cortical loops.

Introduction

Borna disease virus (BDV) is a neurotropic negative-strand RNA virus, worldwide in distribution, that causes disturbances of movement and behaviour in a wide range of animal species (Narayan *et al*., 1983; Ludwig *et al*., 1988; Solbrig *et al*., 1994). The virus is a natural pathogen of several domestic mammalian and bird species, and has been linked by serology, detection of viral nucleic acid and virus isolation to neuropsychiatric disorders of man (de la Torre, 2001; Lipkin *et al*., 2001). Increased central nervous system enkephalin expression was a reported feature of early infection in rats, within 3 weeks of infection (Fu *et al*., 1993). The purpose of the present study was to characterize the effect of BDV infection on CNS enkephalin expression in specific components of the cortico-striatal-thalamic system in rats at the time their movement and behaviour disorder appeared.

Materials and methods

Adult male Lewis rats infected experimentally with this virus (BD rats) develop a movement and behaviour disorder characterized by stereotypic patterns of grooming, sniffing, rearing, gnawing, self-biting and dyskinesias (vacuous chewing and retrocollis) 6 weeks after infection (Solbrig *et al*., 1994). Preproenkephalin (PPE) mRNA expression in brain was examined by *in situ* hybridization after 6 weeks of infection, using [^{35}S]cRNA probes synthesized from PPE cDNA clones (Yoshikawa *et al*., 1984) following published methods (Solbrig *et al*., 1994). All procedures were performed on animals anaesthetized deeply with inhaled methoxyflurane in compliance with institutional (UCI IACUC–University of California-Irvine Institutional Animal Care and Use Committee) and National Institutes of Health guidelines.

Results

The PPE mRNA signal was higher in the caudate putamen of BD rats than in uninfected rats (Fig. 1A). Autoradiograms were analysed using a computer-based image analysis system (MCID; MicroComputer Imaging Device, Imaging Research Inc., St. Catharines, Ontario, Canada) with calibration curves constructed using [^{14}C]-polymer standards (American Radiolabelled Chemicals, St. Louis, MO, USA). The PPE signal, expressed as d.p.m./mg, was increased significantly in the caudate putamen of BD rats relative to uninfected rats (BD 6633.87 $\pm$ 876.81 vs. normal 3775.98 $\pm$ 743.26; values in d.p.m./mg, mean $\pm$ SEM; $t = 2.486$, d.f. = 10, $P < 0.05$, two-tailed *t*-test, $n = 6$ per group). Increases in signal were also found in nucleus accumbens, and central and basolateral amygdala. Increased caudate putamen signal was attributed to increased *in situ* hybridization signal per cell. Numbers of cells expressing enkephalin mRNA per high power (280 $\times$ 176 μm) field were similar in BD and normal rats (approximately 35–40 per field); however, numbers of silver grains per cell were increased throughout the caudate putamen in BD rats (Fig. 1B).

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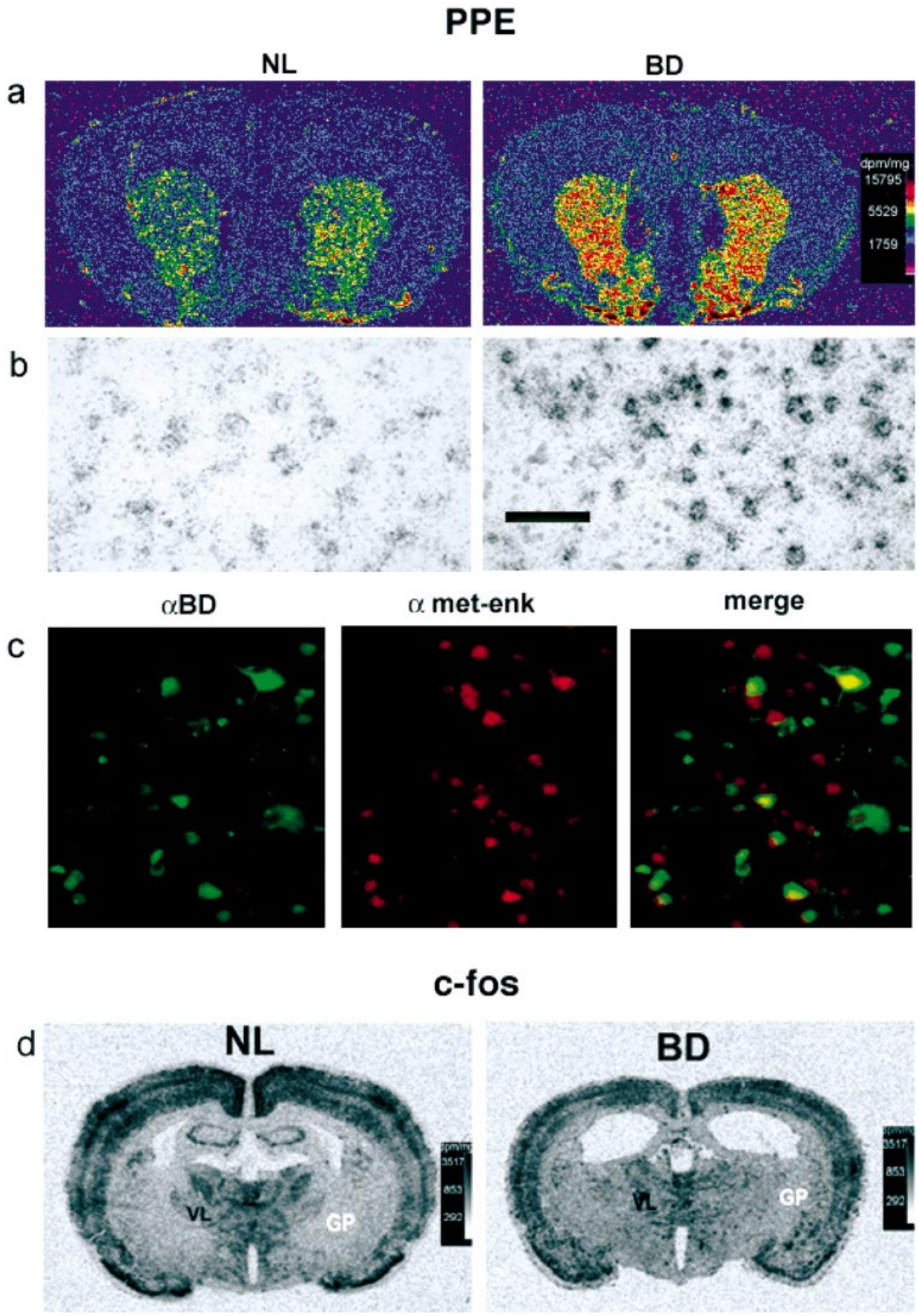
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Double-labelling immunofluorescence histochemistry showed co-localization of virus and methionine (met)-enkephalin, a biologically active product of the PPE gene, in caudate putamen (Fig. 1C). A total of 63 ± 4% of cells expressing BDV phosphoprotein also expressed met-enkephalin; 37 ± 4% of met-enkephalin-expressing cells also expressed BDV phosphoprotein (mean ± SEM of counts obtained from 306 BDV-infected cells, 544 met-enkephalin cells in three animals). The increase in PPE expression in BD rat brain (Fig. 1A and B) and coincidence of BDV and met-enkephalin immunoreactivity (Fig. 1C) is consistent with the hypothesis that infection directly influences met-enkephalin expression.

The effect of infection on *c-fos* expression was examined by *in situ* hybridization as an index to integrity of circuitry. Instead of the well-circumscribed ventrolateral nuclear signal in the thalamus of uninfected rats, BD rats had a chaotic pattern of gene activation in the ventrolateral region, with irregular clustering of *c-fos* signal in rostral and central thalamic nuclei, and increased pallidal signal (Fig. 1D). Although cortico-basal ganglia–thalamo–cortical loops participate in the learning and maintenance of sequential motor actions dependent on sensorimotor integration (Graybiel & Rauch, 2000a), our results are consistent with enkephalin overexpression effecting a desynchronization of signal through the motor thalamus, breaking up motor programmes of coordinated sequential actions.

In vitro effects of BDV on PPE transcription in BDV (strain V)-infected cell lines (Pauli & Ludwig, 1985) were examined by Northern hybridization. Fifteen micrograms total RNA extracted from cells in Tri-Reagent (Molecular Research Centre Inc., Cincinnati, OH, USA) were size-fractionated in 2.2 M formaldehyde/1% agarose gels, transferred to nylon membranes, UV cross-linked and hybridized to random primed [^{32}P]DNA fragments generated from cloned DNA representing enkephalin or BDV sequence. Levels of PPE mRNA, the enkephalin precursor, were increased in two neural cell lines infected with BDV: LAN (human neuroblastoma) and C6 (rat astroglia). The differential effect on enkephalin was more pronounced in LAN cells than C6 cells (Fig. 2A). Total RNA loaded per lane was standardized using a probe for the cellular gene glyceraldephyde-3-phosphate dehydrogenase (GAPDH) and autoradiographic signal quantified by phosphorimaging (Storm 840 Phosphorimager; Molecular Dynamics, Sunnyvale, CA, USA). Persistent infection of LAN cells caused an increase in PPE mRNA of 2.4-times control (uninfected) values (Fig. 2B).

Specificity of viral effects for PPE in striatum was assessed by measuring levels of dopamine D2 receptor, *c-fos* and preprodynorphin (PPD) mRNA in caudate putamen by *in situ* hybridization using [^{35}S]cRNA probes synthesized from dopamine D2 receptor (Bunzow *et al*., 1988), *c-fos* (Curran *et al*., 1987), and PPD (Civelli *et al*., 1985) clones. Levels were similar in BD and uninfected rats for D2 receptor (BD 3282.96 ± 362.53 d.p.m./mg vs. normal 3489.67 ± 556.24 p;d.p.m./mg; $t = 0.3113$, $P > 0.05$), *c-fos* (BD 84.14 ± 1.01 d.p.m./mg vs. 74.97 ± 11.94 d.p.m./mg, $t = 0.7653$, $P > 0.05$) and dynorphin (BD 2997.21 ± 44.78 d.p.m./mg vs. 3266.65 ± 254.61 d.p.m./mg, $t = 1.042$, $P > 0.05$).

Although the basis for enkephalin induction by BDV remains unknown, a potential mechanism is suggested by *in vitro* experiments indicating that BDV biases cells toward kinase reactions through mitogen-activated protein kinase pathway activation (Hans *et al*., 2001; Planz *et al*., 2001). These reactions, hypothesized to enhance viral replication and infectivity (Planz *et al*., 2001), would terminate with phosphoCREB (cyclic AMP response element binding protein) elevations, increasing transcription factors important for striatal enkephalin expression (Konradi *et al*., 1993), in turn increasing enkephalin mRNA. Induction could be restricted to enkephalin because there is limited infection of striatal dynorphin-expressing cells. Although partial dopamine denervation occurs in BD rats (Solbrig *et al*., 1994), increases in both striatal dopamine D2 receptor and PPE mRNA that accompany complete 6-hydroxydopamine deafferentation (Gerfen *et al*., 1990) are not seen. Thus, enkephalin

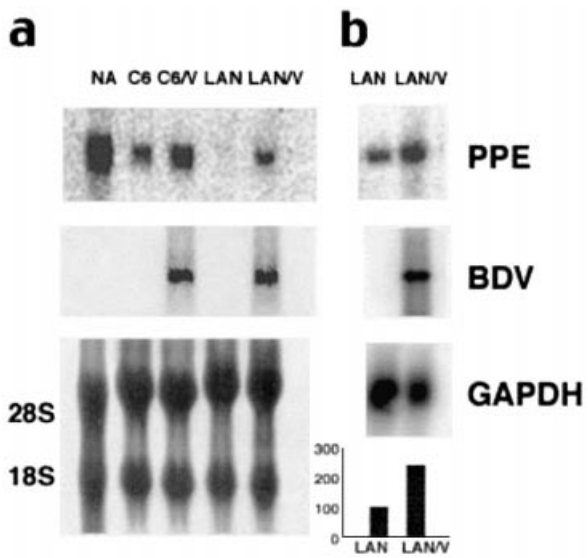

FIG. 2. Borna disease virus infection results in increased levels of PPE mRNA in neural cell lines. (A) Northern hybridization analysis of total RNA extracted from uninfected (C6) or infected (C6/V) rat glioma cells, uninfected (LAN) or infected (LAN/V) human neuroblastoma cells, or uninfected Lewis rat nucleus accumbens (NA). Membranes were hybridized with random primed [^{32}P]DNA probes for detection of transcripts encoding preproenkephalin (top panel), Borna disease virus phosphoprotein (middle panel), and then stained with Coomassie blue for detection of 28 S rRNA and 18 S rRNA (lower panel) as an index of RNA concentration and integrity. (B) Northern hybridization analysis of total RNA extracted from uninfected (LAN) or infected (LAN/V) cells for detection of transcripts encoding PPE (top panel), Borna disease virus phosphoprotein (second panel), or GAPDH (third panel). Hybridization signal for RNA encoding preproenkephalin in LAN/V and LAN cells was quantified following normalization to signal for GAPDH (lowest panel).

FIG. 1. Functional neuroanatomy of Borna disease virus infection in rat. (A) Borna disease virus infection results in increased levels of preproenkephalin mRNA in rat striatum. *In situ* hybridization analysis of coronal sections through uninfected or infected striatum. Sections were hybridized with [^{35}S]cRNA probes for detection of preproenkephalin transcripts, opposed to film, quantified using MicroComputer Imaging Device software and [^{14}C]-standards. (B) Light micrographs of emulsion-dipped, Nissl-stained sections employed in Fig. 1A. Scale bar, 50 μm. (C) Immunofluorescence labelling of striatal coronal sections, using mouse anti-BDV phosphoprotein (gift of L. Stitz) and rabbit anti met-enkephalin antibodies (Chemicon, Temecula, CA, USA), FITC-conjugated donkey anti-mouse IgG and Texas Red-conjugated goat anti-rabbit IgG (Rockland, Gilbertsville, PA, USA). Borna disease virus shown in green; met-enkephalin, red; colocalization of virus and met-enkephalin, yellow. (D) Borna disease virus infection results in a disorganized, dispersed pattern of immediate early gene *c-fos* mRNA expression in motor thalamus. Coronal sections were hybridized with a [^{35}S]cRNA probe for detection of *c-fos* transcripts, apposed to film and imaged by MCID. VL, ventrolateral thalamic nucleus; GP, globus pallidus.

upregulation as a result of pure dopamine lesion is excluded. Although manipulations that drive phosphorylating kinase reactions to phosphoCREB might induce both striatal PPE (Konradi *et al*., 1993) and PPD (Cole *et al*., 1995), only 14 ± 1% of cells expressing dynorphin B express BDV phosphoprotein (values represent mean ± SEM of counts obtained from 214 BDV-infected cells, 173 dynorphin B cells in three animals) whereas 37 ± 4% of met-enkephalin-expressing cells also express BDV phosphoprotein. Thus, virus-driven kinase reactions are more likely to influence striatal expression of met-enkephalin than dynorphin.

Our analyses of the BD rat model are consistent with a role for enkephalin upregulation in the production of stereotypic behaviours. Enkephalin analogues are known stimulants of motor behaviour (Stinus *et al*., 1980; Kalivas *et al*., 1983). Morphine sensitization produces oral stereotypies (Pollock & Kornetsky, 1996) and endomorphin-1 injected into the globus pallidus induces orofacial dyskinesias in rats (Mehta *et al*., 2001), behaviours similar to those observed in the context of BDV infection.

In the normal striatum, a greater percentage of neurons in patches/striosomes than matrix contain enkephalin (Gerfen & Young, 1988). Assuming a similar striosome : matrix distribution for enkephalin in BD rats, the most parsimonious interpretation of data presented here is that viral infection has resulted in increased activity of the striosomal compartment relative to the matrix. A striosome-dominant gene activation pattern is another hypothesized determinant of stereotypy (Graybiel *et al*., 2000b).

These results with a neurotropic virus demonstrate a key role for enkephalinergic tone in the highly sensitive and exquisitely balanced cortico-basal ganglia–thalamo–cortical loops. By increasing transcription or stability of an enkephalin precursor mRNA, BDV causes a behavioural disease characterized by repetitive or fragmented motor actions. Further evaluation of pathways and mechanisms of the viral effect on neuropeptide expression should lead to enhanced understanding of the role of neuropeptides in movement disorders, and of neuropeptides in the aetiopathology (Gillberg *et al*., 1985; Engel & Rocha, 1992) and treatment of neuropsychiatric diseases.

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Abbreviations

BDV, Borna disease virus; CREB, cyclic AMP response element binding protein; GAPDH, glyceraldephyde-3-phosphate dehydrogenase; PPD, preprodynorphin; PPE, preproenkephalin.

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