Kappa opioid control of seizures produced by a virus in an animal model

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Epilepsy remains a major medical problem of unknown aetiology. Potentially, viruses can be environmental triggers for development of seizures in genetically vulnerable individuals. An estimated half of encephalitis patients experience seizures and ~4% develop status epilepticus. Epilepsy vulnerability has been associated with a dynorphin promoter region polymorphism or low dynorphin expression genotype, in man. In animals, the dynorphin system in the hippocampus is known to regulate excitability. The present study was designed to test the hypothesis that reduced dynorphin expression in the dentate gyrus of hippocampus due to periadolescent virus exposure leads to epileptic responses. Encephalitis produced by the neurotropic Borna disease virus in the rat caused epileptic responses and dynorphin to disappear via dentate granule cell loss, failed neurogenesis and poor survival of new neurons. Kappa opioid (dynorphin) agonists prevented the behavioural and electroencephalographic seizures produced by convulsant compounds, and these effects were associated with an absence of dynorphin from the dentate gyrus granule cell layer and upregulation of enkephalin in CA1 interneurons, thus reproducing a neurochemical marker of epilepsy, namely low dynorphin tone. A key role for kappa opioids in anticonvulsant protection provides a framework for exploration of viral and other insults that increase seizure vulnerability and may provide insights into potential interventions for treatment of epilepsy.

Keywords: seizure; encephalitis; Borna disease virus; hippocampus; dynorphin

Abbreviations: BDNF = brain derived neurotrophic factor; BDV = Borna disease virus; BrdU = bromodeoxyuridine; DCX = doublecortin; GFAP = glial fibrillary acidic protein; IR = immunoreactivity; KOR = kappa opioid receptor; NLX = naloxone; nor-BNI = nor-binaltorphimine; NeuN = neuronal nuclei


Introduction

Seizures and status epilepticus increase encephalitis morbidity and mortality. Despite advances in diagnostic techniques, no causal agent is identified in the majority of encephalitis cases (Koskiniemi et al., 2001; Glaser et al., 2003), which limits agent-specific therapies. The opioid system in the hippocampus is known to regulate excitability (Henriksen et al., 1982; Wagner et al., 1993; Weisskopf et al., 1993; Madamba et al., 1999). Dynorphin distribution and regulation are consistent with endogenous anticonvulsant function in experimental models (Kanamatsu et al., 1986; Tortella, 1988; Gall et al., 1990; Douglass et al., 1991; Hong et al., 1992; Romualdi et al., 1995; Simonato and Romualdi, 1996; Pierce et al., 1999) and a low dynorphin expression genotype in man is associated with epilepsy vulnerability (Stogmann et al., 2002; Gambardella et al., 2003). Recent hypotheses have also addressed the role of dynorphin in viral seizures (Solbrig and Koob, 2004).

In man, Borna disease virus (BDV) has been associated with hippocampal sclerosis (de la Torre et al., 1996; Czygan et al., 1999) and neuropsychiatric disorders (Lipkin et al., 2001; Ikuta et al., 2002). In rats, BDV causes a persistent infection with time-based neurological sequelae (Narayan et al., 1983; Solbrig et al., 1994), one of which has an epileptic phenotype (Solbrig and Koob, 2004; Solbrig et al., 2005). Given dynorphin expression can be an important determinant of seizure vulnerability, we tested the hypothesis that reduced dynorphin expression in the dentate gyrus of hippocampus owing to periadolescent virus exposure leads to epileptic responses. Using neuropharmacological,
Materials and methods

Animals

Subjects were male Lewis rats (Charles River Labs, Wilmington, MA, USA) group housed on a 12 h light–dark cycle with ad libitum access to food and water. All experimental procedures were performed in compliance with institutional (University of California-Irvine Institutional Animal Care and Use Committee; Animal Welfare Assurance no. A3416-01) and National Institutes of Health guidelines.

Infection of animals

Anaesthesia was induced with inhaled methoxyflurane administered in a closed bell jar containing gauze soaked with anaesthetic to achieve 0.22% minimal alveolar concentration (Committee on Pain and Distress in Laboratory Animals, ILAR, 1992). Under methoxyflurane anaesthesia, 4-week-old males were infected intra-cerebrally with BDV (BD rats) by injection of 1.6 × 10⁴ tissue culture infectious dose units, strain He/80-1, or sham infected with sterile saline. Analyses were at different times after infection, as indicated.

Infection of animals

BD and NL rats were anaesthetized with ketamine (Sigma, St Louis, MO, USA) group housed on a 12 h light–dark cycle with ad libitum access to food and water. All experimental procedures were performed in compliance with institutional (University of California-Irvine Institutional Animal Care and Use Committee; Animal Welfare Assurance no. A3416-01) and National Institutes of Health guidelines.

Drugs

Drugs used were the general opioid antagonist naloxone (NLX) (1 mg/kg s.c.) (Sigma, St Louis, MO, USA), the kappa opioid receptor (KOR) agonist U-50488 (trans–[3-4–dichloro-N-methyl-N-[2-(pyrrolidinyl)cyclohexyl] benzeneacetamide] (10, 20, 40 mg/kg s.c.) (Sigma) (Tortella et al., 1986), morphine sulphate (5 mg/kg s.c.) (NIDA, Bethesda, MD, USA), nor-binaltorphimine (nor-BNI) (10 mg/kg s.c.) (Tocris, Ellisville, MO, USA) dissolved in saline. Naloxone dose dependently produces seizures in BD rats (Solbrig et al., 1996) and 1 mg/kg s.c. is always convulsant in BD animals. A 10 mg/kg s.c. nor-BNI is a mid-range dose that modifies analgesic or hyperthermic responding (Endoh et al., 1992). Morphine 5 mg/kg s.c. is a mid-range dose that initiates the development of dependence (Britton et al., 1985; Schulteis et al., 1997). U-50488 40 mg/kg s.c. is the lowest dose reported to fully protect against supramaximal electroshock seizures (Tortella et al., 1986).

Electroencephalography

BD and NL rats were anaesthetized with ketamine + xylazine (87 mg/kg + 13 mg/kg, i.p.) (Western Medical Supply, Arcadia, CA, USA). Stainless steel screw electrodes (Plastics One, Roanoke, VA, USA) were implanted in the cranium over the right and left retrosplenial cortices (overlaying hippocampus), fixed to the skull with dental cement. Ground electrodes were placed in the frontal bones over the prefrontal cortex. EEG signals were recorded continuously for up to 3 h after injections of naloxone (1 mg/kg s.c.), nor-BNI (10 mg/kg s.c.) and morphine (5 mg/kg s.c.). EEG signals were obtained from unrestrained rats using computers with flexible recording cables (Plastics One, Roanoke, VA, USA), and amplified and displayed in a Grass Polygraph (Model PS11) using manufacturer’s settings, data acquisition (PolyVIEW/XL) and analysis software (Astro-Med, Inc./Grass Telefactor, W. Warwick, RI, USA). After surgery, animals were allowed to recover for 1 week before study. Seizures were characterized by rhythmic spike or sharp wave discharges on the EEG tracings, with amplitudes at least two times higher than baseline, and accompanied by epileptic-like behaviours (staring spells, behaviour arrest, twitches, chowing, wet dog shakes, clonus, rearing with loss of balance) (Racine, 1972). Neuropharmacological experimental groups contained 4–8 BD rats and age-matched controls. EEGs also were recorded from younger animals where indicated.

Data analysis

Numbers of animals observed with epileptic behaviours were analysed using the Information Statistic for non-parametric independent samples (Kullback, 1968). Observations of epileptic-like behaviours were verified with recorded EEGs.

Histochemistry

The hippocampal formation was chosen for morphological study, based on localization of seizures to hippocampal areas (Solbrig et al., 2005) and tropism of virus to this region (Narayan et al., 1983). Dynorphin and enkephalin immunoreactivity (IR) in hippocampal formation were examined 6 weeks after infection, processing free-floating 50 mm frozen sections as described (Solbrig et al., 2005) with primary antibodies to dynorphin A (1–17) (1:150, Serotec, Oxford, UK) or met³-enkephalin (1:500, Chemicon, Temecula, CA, USA) (n = 4 per group, BD or NL). Antiserum against dynorphin A(1–17) has <0.001% cross reactivity to Dyn A (1–8), leu³-enkephalin, a-neo-endorphin and Dyn B (1–13). Met³-enkephalin antibody has 5.8% cross reactivity to leu³-enkephalin and <0.1% to other endorphins.

Next, viral effects on dynorphin expression in hippocampal formation were examined, measuring transcript levels in hippocampus and tracking development and maturation of granule cells, the only dynorphin-expressing cells of hippocampal formation.

Northern blot analyses

Effect of BDV infection on preprodynorphin (PPD) transcription in hippocampus was examined by northern hybridization. An aliquot of 15 µg total RNA from hippocampal tissue of BD rats 6 weeks after infection, extracted in Tri-Reagent (Molecular Research Centre Inc, Cincinnati, OH, USA) was size-fractionated in 2.2 M formamide/1% agarose gels, transferred to nylon membranes, UV crosslinked and hybridized to random primed ²⁵PDNA fragments generated from cloned DNA representing dynorphin sequence (Civelli et al., 1985) with GAPD transcripts as control for RNA loaded. Autoradiographic signals were quantified by phosphorimaging (Storm 840 Phosphorimagor; Molecular Dynamics, Sunnyvale, CA, USA) (n = 8 per group).

To measure viral effects on hippocampal cell proliferation, bromodeoxyuridine (BrdU) labelling was performed. To identify cell types exhibiting BrdU or BDV immunostaining, double-label studies were performed. To characterize viral effects on development of neurochemical phenotype, double label studies were performed.
BrdU injections
BrdU (Sigma) dissolved in saline was administered to rats at 5 weeks of age (1 week after BD infection) at a dose of 50 mg/kg i.p. ×3 (3 times on day 0) to label dividing cells (Kuhn et al., 1996). To evaluate the effect of BDV on cell proliferation, rats were sacrificed the following day. To evaluate effects of BDV on survival of newly born cells, rats were sacrificed 1, 2.5 or 4 weeks after last BrdU injection (n = 4 per experimental group). Brains of all rats were processed immunohistochemically for combined BrdU and markers of several cell types using peroxidase or fluorescent methods to assess phenotype of newly born cells (Kuhn et al., 1996).

Histological procedures
A one-in-six series of sections from control and BD animals surviving 1 day, 1, 2.5 and 4 weeks after the injection of BrdU were processed for BrdU (1:400 for DAB, Chemicon; 1:100 for fluorescence, Accurate, Westbury, NY, USA) and several cell type markers—neuronal nuclei (NeuN) (1:1000, Chemicon) anti-NeuN for mature neurons, glial fibrillary acidic protein (GFAP) (1:2000, Dako, Glostrup, Denmark) for astroglia, OX42 (1:500, Serotec) for microglia and mouse monoclonal antibody (38/15 H76) against BDV nucleoprotein (1:500, gift of L. Stitz). Other primary antibodies used were dynorphin A (Serotec 1:500), met-enkephalin (1:500, Chemicon), MASH1 (mammalian achaete-scute homologue) (1:250, Chemicon) and doublecortin (DCX) (1:500, Chemicon). BrdU labelling required the following pretreatment: DNA denaturation (50% formamide in 2× SSC, 65°C, 2 h), acidification (2N HCl 37°C, 30 min), rinse (0.1 M boric acid pH 8.5, 10 min) to reduce reactive aldehydes, and membrane permeabilization (0.25% Triton, 3% normal horse serum in PBS, 1 h). Because of reports of BrdU promoting neuronal apoptosis (Sekerkova et al., 2004), histology was evaluated in additional animals age matched to neuropharmacology animal subjects. MASH1 labelling required wash in 0.3% H2O2 in methanol for 15 min, PBS washes and overnight incubation in 6% normal goat serum with 0.2% Tween-20. Other antibodies used included 3% NGS and 0.2% Triton X-100, except dynorphin A and met-enkephalin which excluded Triton-X. Antigens were incubations in 3% NGS and 0.2% Triton X-100, except dynorphin A and met-enkephalin, which excluded Triton-X. Antigens were visualized with Alexa-488 or Alexa-546 secondary antibodies (1:1000, Molecular Probes, Carlsbad, CA, USA), or biotinylated secondary antibodies (Vector, Burlingame, CA, USA) processed by ABC histochemical method (Hsu et al., 1981) and developed with 3,3′-diaminobenzidine. Control sections were processed with omission of primary antisera. Nissl staining was performed by Cresyl violet. Timm staining to label zinc in mossy fibre synaptic vesicles and evaluate mossy fibre sprouting was performed on a separate group of BD animals 6 weeks after infection and age-matched NL uninfected animals according to the procedure of Vaidya et al. (1999). Brightfield and fluorescence signals were detected with a Nikon E800 epifluorescence microscope equipped with Spot2 CCD Digital Camera and Spot and Spot Advanced imaging software. Photomicrograph images were transferred into Photoshop and assembled using Adobe Photoshop CS.

BrdU quantification
Sections of 50 μm thickness were collected on a freezing microtome through the entire rostrocaudal hippocampal formation with every sixth section slide mounted for BrdU counting. Slides were examined for BrdU-positive cells in the granule cell layer of dentate gyrus. BrdU positive cells within the subgranular zone that were within 2-cell body widths of the granule cell layer were considered part of the granule cell layer (Kuhn et al., 1996). All BrdU-positive cells within the granule cell layer, regardless of size or shape, were counted under ×400 magnification throughout the rostral-caudal extent of the granule cell layer. Total numbers of BrdU-stained cells in the granule cell layer were multiplied by six and reported as total numbers of cells in the granule layer. Raw data for cell counts were statistically analysed using Student’s t-tests (P < 0.05). To control for differences in bioavailability or cell uptake of BrdU between uninfected and BD rats, BrdU-labelled cells in corpus callosum were counted in selected sections of age-matched NL and BD rats. The subventricular proliferative zone was not used as a control because animals were infected by intracerebroventricular (ICV) inoculation.

Analysis of phenotype
A one-in-six series of sections from control and BD animals surviving 1 day, 1, week, 4 weeks after injection of BrdU were double-labelled for NeuN, GFAP, BDV, OX42, dynorphin A or met-enkephalin using fluorescent methods. At least 25 BrdU cells in the granule cell layer per rat were examined for colocalization and analysed using Spot Advanced Image Analysis or Bio-Rad MRC-600 laser confocal microscope. Percentages of colabelled BrdU-positive cells were determined by manual counts of digitally recorded images. Differences in treatment groups, set in 2 × 2 tables, were assessed by Chi-square analysis.

Other chemistries
Host soluble factors with regulatory actions on neurogenesis or dynorphin were assessed.

Corticosterone levels
Circulating corticosterone from whole blood collected at 6 p.m. from BD rats 6 weeks after infection and age-matched controls, were assayed by radioimmunoassay (ICN-rat corticosterone Immunochem Double Antibody 125I RIA kit, Linco Diagnostic Services, St Louis, MO, USA) with the sensitivity or detection limit approximately 5.7 ng/ml. Duplicate samples were analysed and expressed as ng/ml. Group differences were analysed by Student’s t-tests with significance set at P < 0.05 (n = 8–10 per experimental group).

BDNF (brain-derived neurotrophic factor) analyses
BD rats from separate groups of animals, 6 weeks after infection and age-matched controls, were euthanized by inhaled methoxyflurane, administered in a closed bell jar containing gauze soaked with anaesthetic to achieve 0.22% minimal alveolar concentration, followed by decapitation. Brains were rapidly removed, cut into coronal sections by razor blades using an ice-cold aluminum alloy mould, and the entire hippocampal formation (dentate gyrus and CA regions) dissected from these sections. Tissue was manually homogenized in lysis buffer (137 mM NaCl, 20 mM Tris, 10% glycerol, 1 mM PMSF, 10 mg/ml aprotinin, 1 mg/ml leupeptin, 0.5 mM Na vanadate, 1% NP-40), as described (Lauterborn et al., 2000). Total BDNF protein content for each sample was measured using the BDNF Emax Immunoassay System (Promega, Madison, WI, USA) with absorbance at 450 nm determined using a plate reader. Data analysis—differences between BD and NL groups in BDNF were analysed by Student’s t-tests with significance set at P < 0.05 (n = 8 per group).
Results
Seizures in BD rats
ICV infection of 4-week-old periadolescent Lewis rats is an established model of encephalitis (Narayan et al., 1983). To assess effects of infection on electroencephalographic tracings, serial EEGs of BD rats were recorded over the course of infection in freely moving rats from screw electrodes overlaying the hippocampus. After 3 weeks of infection, the earliest EEG changes were frequent sharp waves. Disorganized EEG patterns, with complex polyphasic waves containing an admixture of frequencies, were obtained 4 weeks after infection from BD rats showing startle myoclonus and hyperactivity. More encephalopathic EEGs with burst suppression patterns—high voltage bursts of slow waves intermingled with sharp transients or spikes against depressed background—were obtained 6 weeks after infection from BD rats showing stereotypical behaviours, rearing, falling or wild running. Furthermore, within another 2–4 weeks, repetitive sharp waves on EEG were captured, time-locked to spontaneous seizures as periods of behavioural arrest, staring spells, automatisms such as lip smacking, blinking, head nodding or retrocollis (dorsal head and neck extensions), falls or clonic limb movements (Fig. 1 lower tracings). Epileptiform activity was not observed in NL uninfected rats (Fig. 1 upper tracings).

A kappa opioid blocks seizures
The general opiate antagonist NLX is a dose-dependent convulsant in BD rats (Solbrig et al., 1996), producing periodic sharp waves on hippocampal EEGs together with staring spells, behaviour arrest, eye blinks or clonic movements, and c-fos elevations in hippocampus and amygdala (Solbrig et al., 2005). To further evaluate a role for opioids in brain excitability, the effect of a kappa opioid on seizures induced by naloxone was examined. NLX (1 mg/kg s.c.) rapidly and consistently caused seizures with a transition from background...
burst activity to rhythmic spike or sharp wave discharges within 5 min of drug administration (Fig. 2A). Administration of the selective KOR agonist U-50488 (40 mg/kg s.c.) 10 min before NLX (1 mg/kg s.c.) prevented NLX-induced seizures in all BD rats tested (BD NLX 8/8 seizures versus BD U50488 + NLX 0/8 seizures; 2I = 22.18 df = 1, P < 0.001, n = 8 per group) (Fig. 2A, lower tracing). Breakthrough seizures, as isolated EEG spikes and runs of sharp waves were recorded from BD animals receiving lower doses of U-50488 (10 and 20 mg/kg s.c.) (not pictured).

To confirm a role of kappa opioids in protecting the brain from overstimulation, behavioural effects of the kappa opioid antagonist nor-BNI were examined. Administration of the KOR antagonist nor-BNI (10 mg/kg s.c.) had convulsant effects, producing recurrent hippocampal spike or sharp wave discharges accompanied by staring spells, lip trembling or smacking, rhythmic trunk flexions or hindlimb extensor spasms. Ictal episodes occurred 45 min to 2 h after drug administration (BD nor-BNI 7/7 seizures versus NL nor-BNI 0/4 seizures; 2I = 9.87, df = 1, P < 0.01, n = 4–7 per group), consistent with the peak KOR blocking effect (Endoh et al., 1997).

To evaluate a mu opioid receptor component to the seizures, BD rat behaviours were probing used the mu opioid receptor agonist morphine. Morphine (5 mg/kg s.c.) induced epileptic behaviours: staring spells or myoclonic jerks of trunk or forepaws time-locked to periodic spike or wave discharges on EEG (BD morphine 5/5 seizures versus NL morphine 0/5 seizures 2I = 13.86, df = 1, P < 0.001, n = 5 per group). Ictal episodes, beginning 15–20 min after drug administration, were frequent at 30 min (Fig. 2C), approximating the kinetics of binding and receptor-induced internalization of opioids with their receptors in mammalian cells (Gaudriault et al., 1997).

Specificity of the KOR system for anticonvulsant effects was assessed by U-50488 treatment prior to morphine. Administration of the KOR agonist U-50488 (40 mg/kg s.c.) to BD rats 10 min before morphine (5 mg/kg s.c.) prevented seizures in all rats tested (BD U50488 + morphine 0/5 seizures versus BD morphine 8/8 seizures, 2I = 17.32, df = 1, P < 0.001, n = 5–8 per group), but the KOR agonist-morphine combination depressed both consciousness and EEG tracings (Fig. 2C, lower tracing).

**Encephalitis induces dynorphin loss in hippocampus**

To assess neuropathological effects, BD rat brains were examined. BD rats 6 weeks after infection showed an atrophic, disorganized hippocampal formation with laminar disruption and apparent increased density of cells in hilus (Fig. 3A). The granule layer was preserved as a rim of NeuN-positive cells (Fig. 3B). A proportion of surviving neurons were BDV positive, recognized by BD nucleoprotein antibody (Fig. 3A, top right).

Coincident with granule cell loss, mossy fibre labelling was not seen in BD rats. Timm staining was attenuated in the hilus and granule cell layer, and absent from the molecular layer (Fig. 3C). In Fig. 3D and E, neither dynorphin nor enkephalin labelling in cell bodies is discernible in BD rats.

**Encephalitis induces enkephalin increase in CA1**

In contrast to the dentate gyrus, met-enkephalin immunostaining was faint in cell bodies that were sparsely scattered in stratum radiatum of hippocampal field CA1 of NL rats (Fig. 3F) but prominent in CA1 interneurons of BD rats (Fig. 3F arrows). Cell bodies labelled in CA1 were: (regio superior and inferior) stratum pyramidale cells that extend apical dendrites into stratum radiatum of hippocampal field CA1, stellate and bipolar stratum radiatum neurons with a dendritic process radiating to pyramidal layer and another to hippocampal fissure. The cells correspond in localization and morphology with previously described populations of enkephalin-like immunoreactive interneurons of the stratum pyramidale, stratum radiatum, and stratum radiatum–stratum lacunosum-molecular interface (Gall et al., 1981). BD rat met-enkephalin-like IR cells also immunostained with BDV nucleoprotein antibody (Fig. 3F, right).

**Failed neurogenesis and survival of new neurons contribute to dynorphin loss in hippocampal formation**

To study the developmental aspect of dynorphin expression in hippocampal formation, the development and maturation of granule cells, the only dynorphin-expressing cells of the...
hippocampal formation (McGinty et al., 1983) were examined following injections of BrdU (50 mg/kg i.p. ×3) on day 7 of infection. BD and control rats were given BrdU to label dividing cells and sacrificed at selected time points to track proliferating cells. Differences in numbers and distribution of proliferating cells were apparent at all time points. BD rats showed fewer BrdU-positive cells in the granule cell layer at 1 day (BD 6974 ± 330 versus NL 12511 ± 672, P <
antibodies to BDV and Mash-1, a beta helix-loop-helix transcription factor expressed in neuronal progenitors (Ross et al., 2003). Proliferating cells committed to a neural fate were infected and expressed BDV nucleoprotein one week after infection (Fig. 5B). However, later in infection, neuroblasts or DCX positive cells (Gleeson et al., 1999; Brown et al., 2003) could not be recognized (Fig. 5D). Clearly, BDV infection is damaging to developing neurons. At which stage virus is toxic to developing neurons will be the subject of further study.

To study actual dynorphin expression in hippocampal formation, the effect of BDV infection on dynorphin mRNA in hippocampal formation was examined by northern blotting. PPD transcripts in BD rat hippocampus were decreased but not absent (BD 1165 ± 800 versus NL 15255 + 1037, mean optical density, arbitrary units, t(1,14) = 10.749, P < 0.0001, n = 8 per group). Low corticosterone (Thai et al., 1992) or elevated BDNF (Croll et al., 1994) can downregulate dynorphin at the level of transcription. To examine whether host soluble factors could be linked to decreased transcription or stability of a dynorphin precursor mRNA in surviving dentate granule cells, serum corticosterone and hippocampal BDNF levels were measured. No group differences in BDNF levels in hippocampal formation were observed [BD 1.064 ± 0.122 versus NL 1.011 ± 0.125 pg/100 µg, t(1,15) = 0.299, P = 0.7689 n = 8–9 per group]. Corticosterone levels were not reduced, but instead were increased in BD rats [BD 282.00 ± 30.027 versus NL 212.20 ± 11.563 ng/ml, t(1,16) = 2.354, P < 0.05, n = 8–10 per group], to suggest a host role in inhibition of neurogenesis (Cameron and Gould, 1994).

Discussion

The present study supports the hypothesis of a critical role of opioids in the maintenance of a balanced tone of activity in hippocampal circuits related to seizure induction, and a key role for the KOR in anticonvulsant protection in viral seizures in rodents. Our results show a rodent model of viral encephalitis based on BDV reproduces a functional neurochemical change, low dynorphin tone, important in epilepsy vulnerability. Genotypes producing low dynorphin tone are associated with temporal lobe epilepsy and febrile seizures in man (Stogmann et al., 2002; Gambardella et al., 2003). BD rats lack hippocampal dynorphin owing to

Fig. 3 Viral-induced opioid pathology in coronal sections through hippocampus. (A) Nissl-stained sections show contraction of hippocampus with laminar disruption and nerve cell loss in BD rats. Some dentate granule cells are labelled by BDV antibody (arrow, top right). (B) Robust NeuN IR in dentate layers, hilus and CA3 in NL rats contrasts with residual granule layer preserved as rim of NeuN-positive cells in BD rat (right). (C) Higher magnification shows Timm staining of hilus, portions of granule cell layer and molecular layer near tip of dentate gyrus in NL rats. Such staining is attenuated in BD rats, and absent from supragranular regions (right). (D) A network of dynorphin A-like IR cells and the processes of granule cells are visible in the NL rat but absent in the BD rat. (E) The overlapping dentate network of met-enkephalin-like IR cells and processes in the NL rat is reduced in the BD rat to scattered IR particles through granule and subgranule layers. (F) In CA1, met-enkephalin-like IR interneurons are faint in NL rat, but they are much more intensely stained in the BD rat (arrows). Met-enkephalin-like IR cells also expressed BDV nucleoprotein antigen (arrows). Scale bars = 200 µm (A, B and F, photographed at 100×; C, D and E, photographed at 200×). NL, normal uninfected, BD, Borna disease rat, g, granule cell, hf, hippocampal fissure, aBD, anti-BD, BDV nucleoprotein antibody.
depopulation of the granule layer and loss of competence of surviving granule cells to express dynorphin. Mechanisms of dentate gyrus dynorphin depletion as well as upregulation of enkephalin elsewhere in the hippocampus are demonstrated.

The non-selective opioid receptor antagonist NLX has been shown to produce seizures in BD rats which map to hippocampal and limbic circuits (Solbrig et al., 1996, 2005). Their seizures have a periodicity of 1–2 per second, within the dynamic range for frequency-dependent facilitation of hippocampus (Nadler, 2003). In this study, administration of the kappa agonist U-50488 controls NLX seizures, while the selective kappa antagonist nor-BNI reproduces the convulsive phenotype. NLX seizures, with latencies of <5 min, were distinct from morphine seizures, with onsets 15–20 min after drug administration, and were frequent at 30 min, matching the internalization kinetics of binding and receptor-induced internalization of opioids with their receptors (Gaudriault et al., 1997).

Specificity of kappa effect also is supported by the finding that the kappa agonist U-50488 blocks convulsant effects of morphine (5 mg/kg s.c.) in BD rats. The results suggest that a resting, non-seizing hippocampus requires balanced kappa and mu opioid tone. Once endogenous opioid systems are destabilized, seizures result.

A role for hippocampal opioid systems in seizure vulnerability is in agreement with previous studies of animal models of seizure risk following in utero morphine exposure (Schindler et al., 2004). In male rats prenatally exposed to morphine, epileptiform activity is more easily induced in entorhinal cortex (Velisek et al., 2000) and systemic naloxone increases seizure susceptibility (Schindler et al., 2004). In utero morphine exposure reduces dynorphin-derived peptides in CA3 and increases mu opioid receptors in dentate gyrus, CA3, CA1 in adult male rats, as well as decreasing pro-enkephalin mRNA/met-enkephalin peptide and increasing prodynorphin mRNA/dynorphin B peptide in granule layer of dentate gyrus. Naloxone seizures, measured as decreased latency to bicuculline seizures after naloxone administration, are associated with reduction in dynorphin peptides in CA3 in these animals (Schindler et al., 2004). After prenatal morphine exposure, ovariectomy and hormone replacement, female adult rats have similarly increased MOR binding in CA3 and increased susceptibility to flurothyl seizures (Slamberova et al., 2003).

In our model, NLX is convulsant by blocking KOR tone from an already weakened or attenuated dynorphin system, and morphine is convulsant by enhancing mu over kappa tone. However, one cannot exclude convergent downstream or ion channel responses to NLX and morphine as explanation for similarities in convulsant effects.

The chemical neuroanatomy of BD rat hippocampus is consistent with the neuropharmacological results. Dynorphin IR was absent in the dentate granule layer, and enkephalin IR increased in CA1 of BD rats. Owing to retention of a dentate layer of NeuN positive cells, loss of mature granule cells cannot fully account for dynorphin loss. BrdU studies revealed that the virus interfered with granule cell development and survival, such that young neurons were lost too early to repopulate the granule layer with mature dynorphin-positive phenotypes. The dentate granule layer was sporadically infected, thus limiting the direct role of virus in transmitter downregulation (Hans et al., 2001). Hippocampal BDNF levels were unchanged in BD rats and serum corticosterone levels were increased, and such changes have not been reported to downregulate dynorphin (Thai et al., 1992; Croll et al., 1994). Effects at gene-expression level are not excluded, but direct causes through viral interference or host soluble factors were not found. However, BD rats show early EEGs with high levels of bursting waves, and seizures have been shown to deplete hippocampal dynorphin at the mRNA and peptide levels (Xie et al., 1989; Douglass et al., 1991, reviewed in Simonato and Romualdi, 1996). Thus, a different host factor, subclinical seizures, could exhaust dynorphin from surviving granule cells, triggering an evolution to clinical seizures. These results add to previous studies of viral and immunological determinants of virulence, which have already demonstrated depopulation of the granule layer resulting from inflammatory/immune-based neuronal loss (Gosztonyi and Ludwig, 1995; Planz and Stitz, 1999). The chief damaging elements are cell loss, failure of neurogenesis, depletion of dynorphin with seizures and inability to restore dynorphin to meet demand. No doubt cell loss is important, but it is multiple factors, acting together, that expose key sites of vulnerability in the brain’s carefully regulated control of excitability. One could speculate based on the present study that a significant contribution to overall dynorphin pathology is depletion of dynorphin with seizures.

The classic hippocampal circuit is a trisynaptic circuit utilizing glutamatergic neurotransmission. At each step, excitatory tone is modulated by a diverse group of inhibitory and excitatory neurons (Freund and Buzsaki, 1996). In normal brain, the dentate granule cells serve as a high-resistance gate or filter, inhibiting propagation of hypersynchronous discharges from entorhinal cortex to hippocampus. Gating depends on several factors, including release of inhibitory neurotransmitters, such as dynorphin and others, and structural integrity. BDV-induced loss of the neuropharmacological and structural gate, as shown in this study by failed neurogenesis and infection of young neurons, may prove important in initiation and spread of temporal lobe seizures. Dynorphin or kappa opioid presynaptic inhibitory effects in hippocampus through Shaker type potassium channels on mossy fibres (Simmons and Chavkin, 1996) are presumed lost to the BD rats. However, dynorphin also decreases neuronal activity via post-synaptic actions on potassium M channels of principal CA3 and CA1 hippocampal neurons (Moore et al., 1994; Madamba et al., 1999). It is the anticonvulsant actions of KOR agonists on CA3 or CA1 principal neurons, neurons that survive in BD viral infection, that mediate the pharmacological effects observed in this study, which may be considered therapeutically relevant.
Fig. 4 Viral effects on neurogenesis, survival and phenotype. (A) Numbers of BrdU-labelled cells in granule layer of hippocampus at various time points after BrdU injection. BDV infection decreases numbers of BrdU labelled cells within granule cell layer and subgranule zone 1 day, 1, 2.5 and 4 weeks after BrdU injection, relative to normal uninfected rats. *P < 0.05, **P < 0.01 values are mean (± SEM) cell counts per animal for entire hippocampus (n = 4 per group for each time point). (B) Distribution of BrdU-labelled cells. Coronal sections through the hippocampus of NL and BD rats show proliferating cells stained by antibodies against BrdU at 1 day, 1, 2.5 and 4 weeks after BrdU injection. Arrow indicates area of inflammatory angiogenesis interrupting granule layer proliferation in BD rat. (C) Percentage of BrdU positive cells that colabelled with NeuN, GFAP, BDV antibody or OX42, measures neurons, glia, infected cells and microglia, respectively, among proliferating cells. During BD, there is a progression of BrdU-positive phenotypes, from NeuN and GFAP early in disease, to OX42 late in disease. Roughly 50% of BrdU-labelled cells are NeuN-positive at day 1, 82% of BrdU cells are OX42 immunoreactive at 4 weeks. BrdU-labelled cells were scored within granule cell layer and subgranule zone. NeuN, neuronal nuclei; GFAP, glial fibrillary acidic protein; OX42, monocyte microglia marker. (D) OX42 immunofluorescence staining shows the microglial response to BD infection 4 weeks after BrdU injection. The dentate gyrus, hilus, fissure and perivascular areas of BD rat hippocampus are heavily stained (lower panel) compared with NL rat hippocampus. Scale bars = 200 μm.
Beyond the dentate gate, interneurons showing increased enkephalin IR, which co-localized with BDV IR in interneurons of stratum radiatum and stratum pyramidale of CA1, are positioned to influence mu receptors over a wide distribution and strengthen hippocampal excitation. In rodents, mu opioid receptors are on a neurochemically heterogeneous subset of hippocampal interneurons, most frequently on interneurons that are specialized to inhibit pyramidal cells (Blasco-Ibanez et al., 1998). In this distribution, interneurons use mu opioids to limit their own activity as well as that of their targets (Drake and Milner, 2002; Drake et al., 2002). Thus, there is a net excitation, either by enkephalin upregulation arising as a direct effect of virus (Solbrig et al., 2002), or by overstimulation of stratum radiatum interneurons. Prominent enkephalin staining in BD rats may signify recurrent seizures, with CA1 enkephalin interneurons activated in feedforward manner by Schaffer collaterals, using the hippocampal intrinsic excitatory circuit of CA3 projections to CA1. Prominent enkephalin staining may also signify more interconnected networks, with reorganization of CA1 associational pathways into more excitatory networks (Esclapez et al., 1999; Lehmann et al., 2001).

Thus, viral infection, by producing opioid system destabilizations, induces the same limbic opioid changes as would be anticipated during frequent hippocampal seizures (i.e. dentate dynorphin depletion by perforant path activity and CA1 enkephalin interneuron stimulation by feedforward Schaffer collateral excitatory activity). These opioid changes promote and sustain a proconvulsive state. The Borna model illustrates circumstances that deplete dynorphin from the granule cell layer and upregulate enkephalin elsewhere in the hippocampus. The BD rat overlaps with other epilepsy models with dynamic neuropeptide profiles that have established a role of dynorphin deficits in seizures. However, the BD model goes on to demarcate an increased, excitatory enkephalin network in CA1 and supports an additional role for CA1 enkephalin upregulation in seizure vulnerability. The model may apply to any condition with granule cell loss, silencing or failed neurogenesis and enhancement or redirection of excitation to CA1. Thus, the model may generalize to other epilepsies where dynorphin depletion due to recurrent seizures and enkephalin upregulation in CA1 interneurons due to repetitive hippocampal circuit stimulation occurs.

The epileptic syndrome produced by the BD rat is greatly simplified at the neuropharmacological level by the observation that restoration of dynorphin tone can prevent seizures. Normally, KOR receptors are found on mossy fibre terminals, principal neurons, perforant pathway and supramamillary afferents (reviewed in Solbrig and Koob, 2004). The dynorphin system, hypothesized to be a neuromodulatory homeostatic system, may be released on demand by excessive stimuli. KOR activation in hippocampus achieves effects desirable in anticonvulsants. The KOR effects include opening K+ channels (Simmons and Chavkin, 1996) or closing Ca2+ channels (Rusin et al., 1997), thereby controlling pre-synaptic transmitter release, increasing M type K+ currents to stabilize membranes and post-synaptically silence excitatory neurotransmission (Madamba et al., 1999), or acting on classes of interneurons (Racz and Halasy, 2002) to desynchronize the gamma aminobutyric acid inhibitory network. Due to preservation of some of these actions in BD rats, a single pharmacological manipulation, the use of a drug with narrow (KOR) specificity, appears to overcome the mix of neuropharmacological and lesion effects produced in BD rats.

A dominant role for dynorphin, trumping other transmitters, in defending against encephalitic seizures, may be an important part of the normal neuroadaptive response of the brain to overstimulation. A more radical view possibly important for the pathogenesis of seizures is that the phenotype is determined by the most prevalent break with homeostasis, which in this case is by dynorphin, an inhibitory/modulatory neurotransmitter, and such a view suggests the feasibility of exploring the use of kappa opioid agonists in refractory seizures.

Acknowledgements
The authors thank Lothar Stitz for the gift of BDV nucleoprotein antibody, Chris Gall for helpful discussions, Steve Henriksen and his lab members for advice on EEG recording, and Mike Arends for editorial assistance. This work was supported by National Institutes of Health grant NS042307 from the National Institute of Neurological Disorders and
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Stroke to MVS and UC Discovery BIO 03-10408 and Cortex Pharmaceuticals CP-360222 to JCL.

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Brain (2006), 129, 642–654 653


