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Kappa Opioid Receptor Activation Blocks Progressive Neurodegeneration After Kainic Acid Injection

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ABSTRACT: We recently demonstrated that endogenous prodynorphin-derived peptides mediate anticonvulsant, antiepileptogenic and neuroprotective effects via kappa opioid receptors (KOP). Here we show acute and delayed neurodegeneration and its pharmacology after local kainic acid injection in prodynorphin knockout and wild-type mice and neuroprotective effect(s) of KOP activation in wild-type mice. Prodynorphin knockout and wild-type mice were injected with kainic acid (3 nmoles in 50 nl saline) into the stratum radiatum of CA1 of the right dorsal hippocampus. Knockout mice displayed significantly more neurodegeneration of pyramidal cells and interneurons than wild-type mice 2 days after treatment. This phenotype could be mimicked in wild-type animals by treatment with the KOP antagonist GNTI and rescued in knockout animals by the KOP agonist U-50488. Minor differences in neurodegeneration remained 3 weeks after treatment, mostly because of higher progressive neurodegeneration in wild-type mice compared with prodynorphin-deficient animals. In wild-type mice progressive neurodegeneration, but not acute neuronal loss, could be mostly blocked by U-50488 treatment. Our data suggest that endogenous prodynorphin-derived peptides sufficiently activate KOP receptors during acute seizures, and importantly in situations of reduced dynorphinergic signaling—like in epilepsy—the exogenous activation of KOP receptors might also have strong neuroprotective effects during excitotoxic events.

KEY WORDS: excitotoxicity; glutamate; opioid system; neuropeptide; seizures; temporal lobe epilepsy

INTRODUCTION

With a prevalence of 1–2%, epilepsies are one of the most frequent neurological diseases (McNamara, 1999). About 70% of all epilepsy patients suffer from focal seizures arising from a distinct brain region. Mesial temporal lobe epilepsy (mTLE) is to our knowledge the most frequent type of epilepsy. Hippocampal sclerosis is a key feature of mTLE (for review see Engel, 2001). One main factor responsible for these neuronal losses is the excitotoxicity of the vast amounts of glutamate set free during seizures and hypoxia accompanying seizures (Meldrum, 2002). Besides the inhibitory neurotransmission through the GABAergic system, neuropeptides also contribute to the containment of glutamate overflow and thereby act as anticonvulsants.

Since the early 1980s, there has been evidence that opioids, namely dynorphin, act as modulators of neuronal excitability in vitro (Henriksen et al., 1982; Siggins et al., 1986; Wagner et al., 1993; Weisskopf et al., 1993). Dynorphin can act via delta (DOP), kappa (KOP), and mu opioid receptors (MOP), but displays highest affinity to KOP. The potentiation of endogenous antiictal mechanisms by opioids has been shown in animal models of epilepsy (Tortella and Long, 1988; Tortella, 1988; Takechi et al., 1998; Terman et al., 2000) including viral-induced seizures (Solbrig et al., 2006). In line with this, the deletion of prodynorphin in mice (Loacker et al., 2007) and low dynorphin expression in humans (Stogmann et al., 2002; Gambardella et al., 2003) is associated with increased epilepsy vulnerability. In contrast to several other peptides [i.e., neurokinin B, neuropeptide Y (NPY)], which are expressed in increased levels and even found in novel areas in epilepsy (Schwarzer and Sperk, 1995; Wasterlain et al., 2002), prodynorphin expression is reduced after an initial increase in most epilepsy models (for review see Simonato and Romualdi, 1996; Schwarzer, 2009). By contrast, KOP are present under epileptic conditions and the application of kappa agonists can suppress seizures (Solbrig et al., 2006). We recently demonstrated that endogenous dynorphin acting on KOP mediates anticonvulsant, antiepileptogenic and neuroprotective effects 3 weeks after local kainic acid (KA) injection (Loacker et al., 2007). However, the suitability of KOP agonists as neuroprotective agents could not be answered so far. We now used the model of unilateral intrahippocampal KA treatment, which produces a well described sequel of acute and delayed neurodegeneration (Suzuki et al., 1995; Bouilleret et al., 1999), to investigate neuropathological changes in wild-type ($dyn^{(+/+)}$) and prodynorphin knockout ($dyn^{(-/-)}$) mice and the pharmacology of the progression of neurodegeneration. We applied neuronal counts from

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Nissl-stained sections and somatostatin immunohistochemistry for a subgroup of highly vulnerable neurons and the hippocampal activity-dependent expression of NPY in mossy fibers. In addition, we investigated possible neuroprotective effects of KOP agonist treatment on acute and progressing neurodegeneration in dyn$^{(+/+)}$ mice.

MATERIALS AND METHODS

Animals

The generation of the prodynorphin knockout model was described earlier (Loacker et al., 2007). Mice were backcrossed onto the C57Bl/6N background for at least eight generations and littermates were used as controls. No obvious differences in fertility, body weight, or fur condition were noted, and only decent alterations in overall behavior were observed between dyn$^{(+/+)}$ and dyn$^{(-/-)}$ animals (Wittmann et al., 2009). For breeding and maintenance mice were group-housed with free access to food and water. Temperature was fixed at 23°C and 60% humidity with a 12 h light-dark cycle (lights on 7 am to 7 pm). Male mice at 12 to 16 weeks were tested in all experiments. All procedures involving animals were approved by the Austrian Animal Experimentation Ethics Board in compliance with the European convention for the protection of vertebrate animals used for experimental and other scientific purposes ETS no.: 123. Every effort was taken to minimize the number of animals used.

Intrahippocampal Injections of Kainic Acid

To study neuronal degeneration and hippocampal reorganization, KA or saline was injected into the dorsal hippocampus of deeply anesthetized (initial: ketamine 2 mg kg^{-1} i.p., maintenance: inhalation of sevoflurane through anesthesia machine) mice. Mice were immobilized in a stereotaxic instrument (David Kopf Inst.; Bilaney, Germany) with the nose bar adjusted to equal heights of lambda and bregma. Coordinates from bregma (1.8-mm anterior, 1.6-mm lateral, and 1.8-mm below skull) were chosen to target the stratum radiatum of CA1 in the right dorsal hippocampus (Paxinos and Franklin, 2001). KA (3 nmoles in 50 nl saline, pH 7.2) or the same volume of saline was applied with a 1 μl Hamilton syringe over a period of 2 min. The needle was kept in place for 5 min and retracted stepwise (0.3 mm min^{-1}) to minimize backflux. Animals were killed 1, 2, or 7 days or 3 or 5 weeks after KA injection by an overdose of thiopental (150 mg kg^{-1}) and brains were fixed by transcardial perfusion with 4% paraformaldehyde. Age-matched untreated and saline-injected animals of both genotypes were used as controls.

Histology and Cell Counts

For immunohistochemistry and Nissl staining the entire hippocampal region of paraformaldehyde-fixed tissue (Paxinos and Franklin, 2001) was cut to 40 μm free-floating sections. Series containing every sixth section were stained applying specific antibodies (provided by Prof. Guenther Sperk, Dept. Pharmacology, Medical Univ. Innsbruck, Austria) for prosomatostatin (SST) or NPY using a horseradish peroxidase-conjugated secondary antibody (P0448; Dako, Vienna, Austria) and 3,3′-diaminobenzidine for detection (Schwarzer et al., 1996, 2001). One series of sections was used for Nissl staining applying cresyl violet (Paxinos and Franklin, 2001). Cell counts were done from sections of the dorsal hippocampus, assigned to levels from 1.3 to 2.3 mm from bregma according to Paxinos and Franklin (2001). Cell numbers of principal cells (CA1, CA3a, CA3c) were assessed over a length of 125 μm for CA3c and 250 μm for CA1 and CA3a, counting the whole width of the layer. These counts were performed at 400× magnifications and included all cells except small darkly-stained cells, which most probably represent microglia. To control for correctness of counting, randomly chosen sections were stained with DAPI (2 μg ml^{-1}; overnight at 4°C) and nuclei were counted under a fluorescence microscope using an optical dissector device (Nikon Eclipse Microscope, Nikon GmbH, Vienna; Microfire Camera, Optronics, Goleta, CA; and Stereo Investigator 7 Software, MBF Bioscience, Magdeburg, Germany). The evaluation of the same areas of CA1 and CA3 of DAPI-stained sections revealed essentially the same cell numbers as obtained from Nissl-stained sections in controls and early after KA, when the number of glial cells was very low in the dense pyramidal cell layer. However, the differentiation between glial and neuronal DAPI-stained nuclei proved difficult and could have resulted in an overestimation of surviving neurons, especially at stages with strong gliosis. Therefore, data from Nissl-stained sections are presented in the results section. Numbers of hilar nonprincipal cells, representing mossy cells and distinct groups on interneurons, were assessed from Nissl-stained sections at 200× magnifications. Somatostatin immunoreactive (SST-ir) neurons were counted in the entire hippocampal subfields of the hilus, CA1 and CA3, respectively. Granule cell dispersion was evaluated from digital images obtained at 25× magnifications. The entire granule cell layer was outlined and the area was measured as pixels using ImageJ software (NIH, available from http://rsb.info.nih.gov/ij). Transformation from pixel to μm^2 was performed using a microscopy ruler. For each animal cell counts or granule cell area from five to seven sections within the area of interest were combined to yield average cell numbers applying the area under curve (AUC) calculation. The mean and standard deviations of the AUC values from five to seven brains were evaluated for each group and used for statistical analysis. All data indicate mean of AUC ± standard deviation (number of animals).

Opioid Receptor Pharmacology

The KOP agonist *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl] benzeneacetamide hydrochloride (U-50488) and the KOP antagonist 5′-guanidinyl-17-(cyclopropylmethyl)-6,7-dehydro-4,5α-epoxy-3,14-dihydroxy-6,7–2′,3′-

TABLE 1.

Time Course of Neurodegeneration in Wild-Type (WT) and Prodynorphin-Deficient (KO) Mice

	Nissl							
	Hilar non-principal neurons		CA1 pyramidal cells		CA3c pyramidal cells		CA3a pyramidal cells	
	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra
WT untreat.	30 ± 2.1 (3)		98 ± 5.8 (3)		45 ± 2.7 (4)		127 ± 11 (4)	
KO untreat.	29 ± 1.3 (4)		97 ± 2.7 (4)		44 ± 2.2 (4)		139 ± 9.9 (3)	
pooled untr.	30 ± 1.6 (7)		97 ± 3.9 (7)		45 ± 2.3 (8)		132 ± 12 (7)	
WT-KA-2d	2.8 ± 2.4 (6)**	23 ± 1.5 (6)*	5.4 ± 3.3 (5)**	90 ± 9 (6)	6.4 ± 2.9 (6)**	36 ± 6.6 (6)	94 ± 11 (6)**	122 ± 17 (6)
KO-KA-2d	2.0 ± 2.1 (7)**	17 ± 6.6 (7)**##	3.5 ± 2.5 (7)**	42 ± 34 (7)**##	5.8 ± 5.9 (7)**	33 ± 12 (7)**	57 ± 24 (7)**##	97 ± 36 (7)**
WT-KA-3w	0.6 ± 0.6 (5)**	18 ± 4.8 (5)**	2.0 ± 1.4 (5)**	71 ± 17 (5)	4.5 ± 1.6 (5)**	26 ± 3.6 (5)**	52 ± 15 (5)**	95 ± 8.8 (5)*
KO-KA-3w	0.5 ± 0.5 (6)**	18 ± 3.3 (6)**	3.3 ± 2.3 (6)**	45 ± 35 (6)**	1.5 ± 1.6 (6)**#	27 ± 3.5 (6)**	64 ± 12 (6)**	96 ± 15 (6)**
WT-NaCl-3w	28 ± 2.4 (6)	29 ± 2.6 (6)	103 ± 16 (6)	105 ± 12 (6)	44 ± 3.4 (6)	45 ± 4.1 (6)	125 ± 7 (6)	128 ± 8 (6)
KO-NaCl-3w	31 ± 3.1 (6)	32 ± 4.1 (6)	99 ± 7 (6)	102 ± 8 (6)	47 ± 5.7 (6)	50 ± 7.5 (6)	120 ± 4 (6)	127 ± 11 (6)

	Somatostatin-ir					
	Hilar interneurons		CA1 interneurons		CA3 interneurons	
	ipsi	contra	ipsi	contra	ipsi	contra
WT untreat.	15 ± 1.4 (6)		20 ± 2.9 (6)		32 ± 3.7 (6)	
KO untreat	15 ± 1.5 (7)		25 ± 2.0 (7)**		30 ± 2.7 (7)	
WT-KA-2d	1.2 ± 0.9 (6)**	12 ± 2.1 (6)	2.1 ± 0.7 (6)**	16 ± 5.6 (6)	13 ± 4.1 (6)**	18 ± 5.4 (6)**
KO-KA-2d	0.1 ± 0.2 (7)**#	5.0 ± 2.9 (7)**###	0.9 ± 0.7 (7)**#	15 ± 4.6 (7)**	4.4 ± 2.6 (7)**##	10 ± 5.0 (7)**#
WT-KA-3w	0.1 ± 0.2 (7)**	7.1 ± 1.0 (7)**	2.1 ± 1.3 (7)**	19 ± 4.5 (7)	5.9 ± 1.8 (6)**	16 ± 5.1 (6)**
KO-KA-3w	.01 ± .02 (6)**	6.4 ± 1.0 (6)**	0.7 ± 0.4 (6)**#	9.4 ± 2.4 (6)**###	6.1 ± 2.9 (6)**	18 ± 2.8 (6)**
WT-NaCl-3w	15 ± 1.4 (6)	16 ± 0.7 (6)	22 ± 3.6 (6)	24 ± 2.4 (6)	34 ± 3.7 (6)	32 ± 4.0 (6)
KO-NaCl-3w	13 ± 1.7 (6)	13 ± 2.4 (6)	23 ± 3.0 (6)	23 ± 3.8 (6)	29 ± 5.1 (6)	31 ± 5.5 (6)

Cell counts from sections obtained from WT and KO animals killed at different times after KA and control animals are shown. The upper panel represents numbers obtained from Nissl-stained sections, the lower panel displays somatostatin immunoreactive cells. All data are given as mean AUC ± SD (n).
*$P < 0.05$;
**$P < 0.01$ vs. untreated control;
#$P < 0.05$;
##$P < 0.01$;
###$P < 0.001$ vs. WT with same treatment.

indolomorphinan dihydrochloride (GNTI) were purchased from Tocris Cookson. Both were dissolved in saline and pH was adjusted to 7.2. U-50488H (20 mg kg^{-1}) was applied intraperitoneally either 30 min before KA for acute effects or every other day starting from day 6 for the investigation of effects during the progressive stage. GNTI (3 nmoles) was given intracisternally under mild sevoflurane anesthesia 20 h before testing. Drug doses and application times were chosen according to recent studies in mice (Yokoyama et al., 1992; Jewett et al., 2001; Khavandgar et al., 2002; Manocha et al., 2003; Solbrig et al., 2006). GNTI was preferred over nor-binaltorphimine (nor-BNI) for its higher KOP selectivity and antagonist potency (Jones and Portoghese, 2000).

Statistical Analysis

Nissl data obtained from untreated mice of both genotypes were pooled for comparison with saline- or KA-treated animals. Statistical analysis of more than two groups (time course of neurodegeneration) was done by ANOVA and the Dunnett post hoc test. For statistical comparison of two groups, the Mann-Whitney U test was applied. *P*-values of <0.05 were considered significant.

RESULTS

Neuropathological and Morphological Changes After KA Injection

Wild-type ($dyn^{(+/+)}$) mice displayed significant neurodegeneration, mainly in the ipsilateral hippocampi, as soon as 2 days after KA treatment (Table 1, Fig. 1e). Thus, pyramidal cell loss was prominent in ipsilateral CA1 and CA3c where only 5 and 15% of neurons survived, respectively, whereas pyramidal neurons of CA3a were less affected (about a 25% loss). About 10% of hilar nonprincipal neurons survived the acute phase

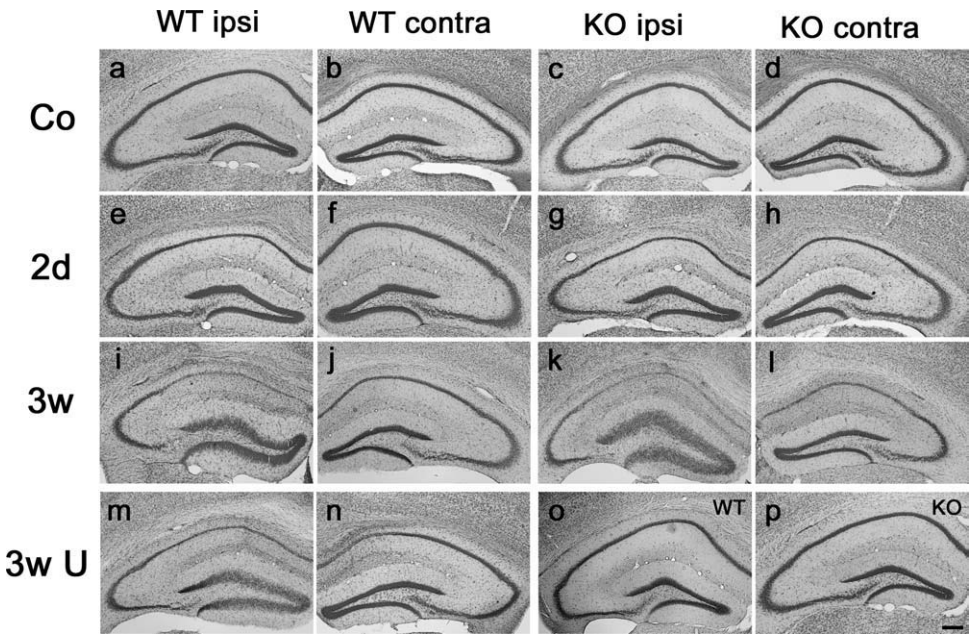

FIGURE 1. Acute and delayed neurodegeneration after the unilateral injection of kainic acid (KA) into the dorsal hippocampus is depicted for dyn$^{(+/+)}$ (a,b,e,f,i,j,m,n,o) and dyn$^{(-/-)}$ mice (c,d,g,h,k,l,p). Shown are the images from Nissl-stained sections obtained from untreated mice (a–d), 2 days (e–h) or 3 weeks (i–l) after KA, 3 weeks after KA with the administration of the specific kappa opioid receptors agonist U-50488H every other day starting from Day 6 (m,n) or 3 weeks after saline injection (o,p). Note the marked loss of CA1 neurons as soon as after 2 days. Bar in p (for a–p) represents 200 μm.

(Table 1, Figs. 1e and 2e). In the contralateral hippocampi of dyn$^{(+/+)}$ mice, a reduction in cell numbers by about 25% was observed in the hilus (Fig. 2f). Pyramidal cell numbers were not altered significantly in the contralateral hippocampi of dyn$^{(+/+)}$ mice (Table 1, Fig. 1f). In the ipsilateral hippocampi of dyn$^{(-/-)}$ mice, the loss of hilar nonprincipal neurons and CA1 and CA3c pyramidal neurons was comparable to dyn$^{(+/+)}$ mice (Figs. 1g and 2g); however, significantly greater neurode-

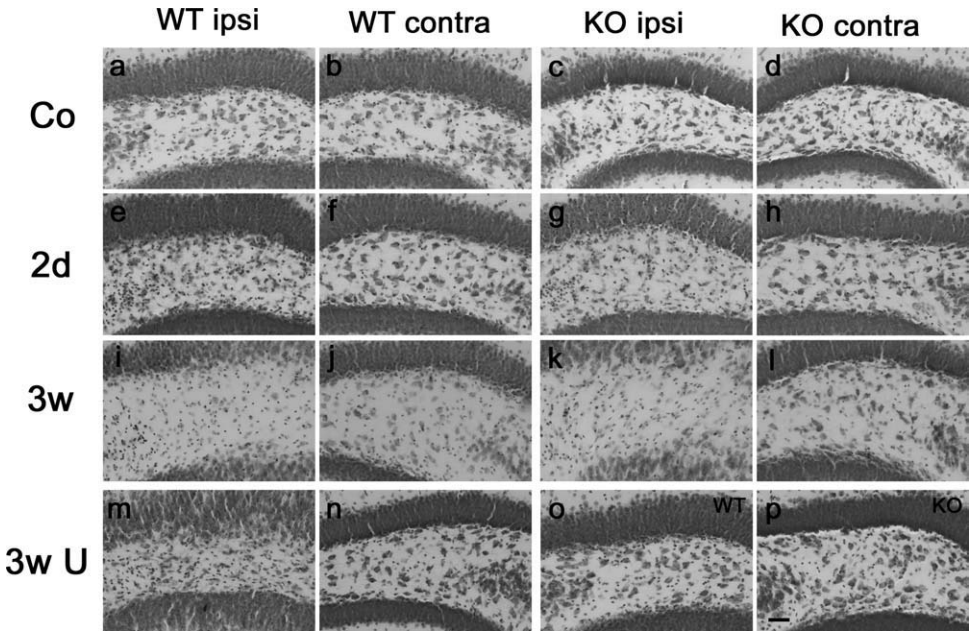

FIGURE 2. High magnification images of the hilus after unilateral injection of kainic acid (KA) into the dorsal hippocampus are depicted for dyn$^{(+/+)}$ (a,b,e,f,i,j,m,n,o) and dyn$^{(-/-)}$ mice (c,d,g,h,k,l,p). Shown are the images from Nissl-stained sections obtained from untreated mice (a–d), 2 days (e–h) or 3 weeks (i–l) after KA, 3 weeks after KA with the administration of the specific kappa opioid receptors agonist U-50488H every other day starting from Day 6 (m,n) or 3 weeks after saline injection (o,p). Note the marked loss of hilar nonprincipal and CA3c pyramidal neurons in the ipsilateral hippocampus as soon as after 2 days and the progression of neurodegeneration from 2 days to 3 weeks especially in dyn$^{(+/+)}$ mice. Bar in p (for a–p) represents 50 μm.

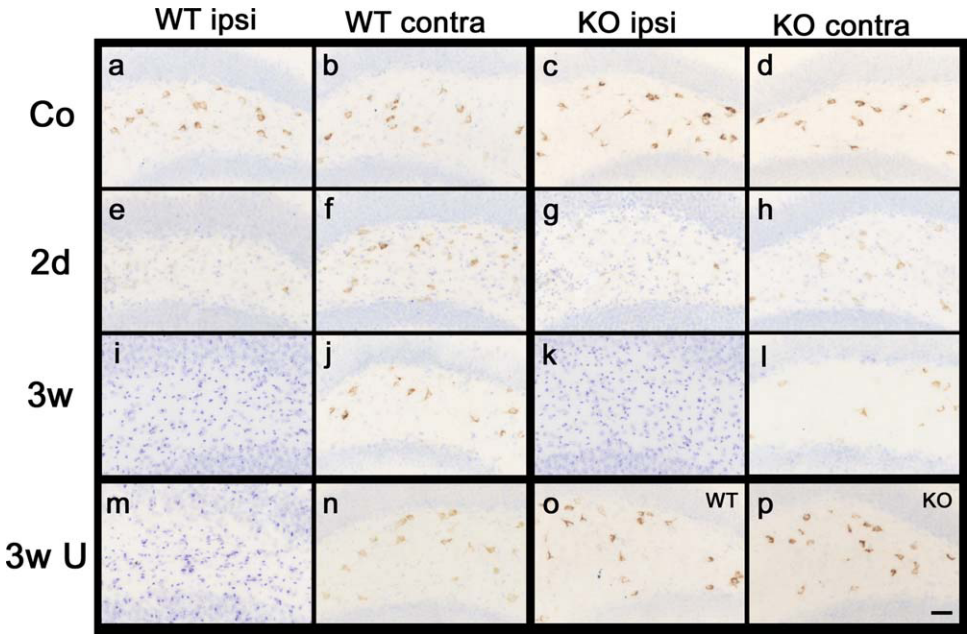

FIGURE 3. Somatostatin (SST) immunoreactivity after the unilateral injection of kainic acid (KA) into the dorsal hippocampus is depicted for dyn$^{(+/+)}$ (a,b,e,f,i,j,m,n,o) and dyn$^{(-/-)}$ mice (c,d,g,h,k,l,p). Shown are the images from sections labeled for SST immunoreactivity obtained from untreated mice (a–d), 2 days (e–h), 3 weeks (i–l) after KA or 3 weeks after KA with the administration of the specific kappa opioid receptors agonist U-50488H every other day starting from day 6 (m,n) or 3 weeks after saline injection (o,p). Note the reduction in SST immunoreactivity in the hilus, which was detected in the ipsilateral hippocampi of both genotypes (e,g,i,k,m), but also transiently in the contralateral hippocampus of dyn$^{(-/-)}$ mice (h) 2 days after treatment. Bar in p (for a–p) represents 50 µm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

generation was observed in the ipsilateral pyramidal layer of CA3a (about 55%). In addition, significant neuronal damage was observed in all subfields of the contralateral hippocampi of dyn$^{(-/-)}$ mice (Table 1, Figs. 1h and 2h). The loss of hilar nonprincipal neurons and CA1 pyramidal neurons was significantly higher in dyn$^{(-/-)}$ than dyn$^{(+/+)}$ mice.

Progressing neurodegeneration was evaluated from animals killed 3 weeks after treatment. At this stage, little further neurodegeneration in the pyramidal cell layer and in hilar nonprincipal neurons was evident in dyn$^{(-/-)}$ mice (Table 1, Figs. 2k,l). By contrast, neurodegeneration progressed in dyn$^{(+/+)}$ animals markedly (Table 1, Figs. 1i,j). At this late time point, the differences between the genotypes regarding neuronal counts were less pronounced than after 2 days. Significant divergences were observed in the number of Nissl-stained ipsilateral CA3c neurons (Table 1).

Alterations in Somatostatin-Immunoreactive Interneurons

SST-ir interneurons were investigated for two main reasons: (i) they are highly vulnerable to KA-induced damage (Magloczky and Freund, 1993); and (ii) they express KOP (Racz and Halasy, 2002). Thus, this group of interneurons is likely to react differently in the presence or absence of prodynorphin-derived peptides.

In the ipsilateral hippocampi, significant reductions in the numbers of SST-ir interneurons were observed in all KA-treated animals (Table 1; Fig. 3). In dyn$^{(+/+)}$ animals, the reductions in hilus and CA1 reached about 90% in CA3 about 60% after 2 days and 80% 3 weeks after KA treatment. Dyn$^{(-/-)}$ mice displayed significantly lower numbers of SST-ir neurons at both time points than dyn$^{(+/+)}$ animals. Thus, in the ipsilateral hilus (Fig. 3) and in CA1 barely any SST-ir cells were observed, and in CA3 their number was reduced by 80–90% as soon as after 2 days. In the contralateral hippocampi, dyn$^{(+/+)}$ animals displayed reduced numbers of SST-ir interneurons only in CA3 after 2 days. By contrast, 3 weeks after treatment, a significant reduction of SST-ir interneurons was also detected in the hilus (Fig. 3). Dyn$^{(-/-)}$ mice displayed reduced numbers of SST-ir interneurons in all subfields of the contralateral hippocampi as soon as 2 days after KA treatment. After 3 weeks, the numbers of SST-ir neurons was similar to those observed after 2 days in hilus (Fig. 3), but further reduced in CA1 (Table 1). The tendency of increased cell numbers in CA3 was not significant (Table 1).

In untreated animals, a significant difference in SST-ir interneurons was observed in area CA1 (Table 1). However, no such difference was observed 3 weeks after saline injection, suggesting that this difference is because of SST expression levels around the detection limit and not different numbers in SST expressing cells.

Alterations in NPY Expression Patterns

NPY expression in hippocampal granule cells and interneurons is stimulated by the increased release of glutamate during

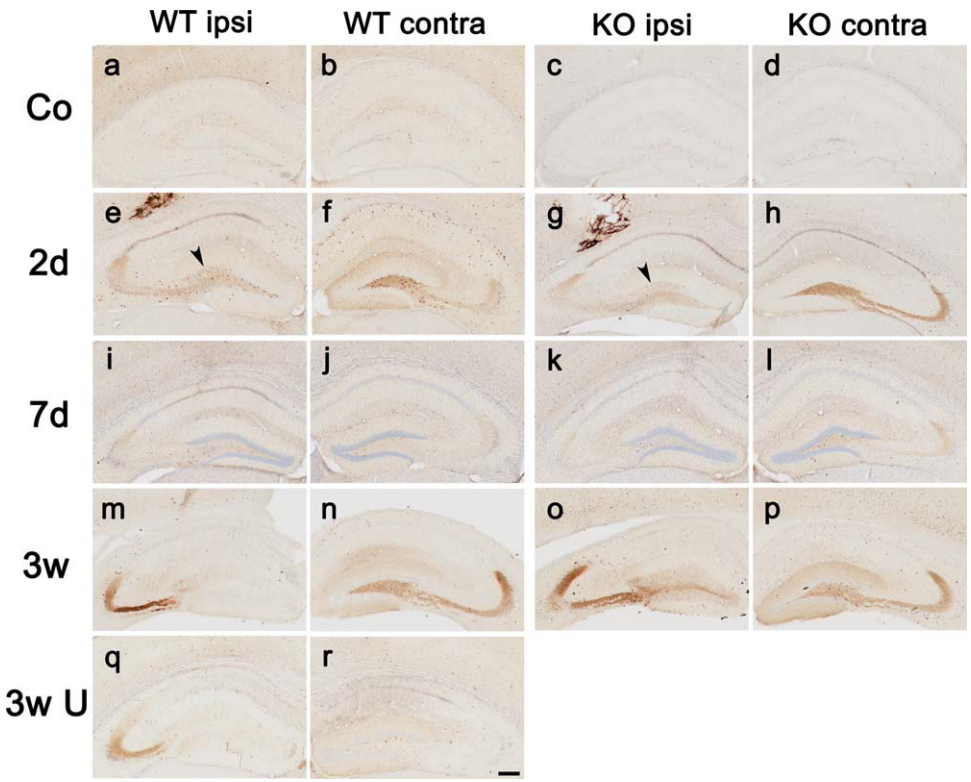

FIGURE 4. Neuropeptide Y (NPY) immunoreactivity after the unilateral injection of kainic acid (KA) into the dorsal hippocampus is depicted for dyn$^{(+/+)}$(a,b,e,f,i,j,m,n,q,r) and dyn$^{(-/-)}$ mice (c,d,g,h,k,l,o,p). Shown are the images from sections labeled for NPY immunoreactivity obtained from untreated mice (a–d), 2 days (e–h), 7 days (i–l), or 3 weeks (m–p) after KA and 3 weeks after KA with the administration of the specific kappa opioid receptors agonist U-50488H every other day starting from Day 6 (q,r). Arrowheads in (e) and (g) indicate NPY-ir in granule cells. Note the NPY immunoreactivity in the CA1 pyramidal cell layer, which was detected in the ipsilateral hippocampi of both genotypes (e.g.), but also in the contralateral hippocampus of dyn$^{(-/-)}$ mice (h). Bar in r (for a–r) represents 200 µm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

seizures (Gruber et al., 1994; Schwarzer and Sperk, 1998). Therefore, we used alterations in NPY immunoreactivity as a marker for increased hippocampal excitation after KA injection. Two days after KA injection, dyn$^{(+/+)}$ animals displayed moderate NPY-ir in mossy fibers of both hemispheres and strong activation of NPY-ir in interneurons (Figs. 4e–h). Granule cells were mostly unlabeled, except those in the lateral part of the upper blade in the ipsilateral hippocampus, which displayed faint NPY-ir [Figs. 4e,g (arrowheads) and 5a,c]. NPY labeling in mossy fibers appeared more pronounced in those of dyn$^{(-/-)}$ mice, especially contralateral to the injection site. Two days after KA, hippocampal interneurons displayed strong NPY-ir, although their number was markedly reduced in the ipsilateral hilus and CA1 area of dyn$^{(+/+)}$ animals (Fig. 4e) and in all subfields of both hippocampi in dyn$^{(-/-)}$ mice (Figs. 4g,h and 5a–d). In line with this, dyn$^{(+/+)}$ animals displayed increased NPY-ir in the outer molecular layer of the contralateral dentate gyrus (Fig. 4e), where the axons of hilar SST/NPY interneurons terminate. In dyn$^{(-/-)}$ mice, NPY-ir was markedly reduced in this area (Fig. 4h). Increased NPY-ir mostly returned to basal levels within 1 week of KA treatment in both genotypes (Figs. 4i–l). Three weeks after KA, all animals displayed strong NPY-ir in mossy fibers of both hippocampi (Figs. 4m–p). Mossy fibers showed markedly reduced NPY-ir in those parts of the ipsilateral hippocampi affected by severe granule cell dispersion (Figs. 4m,o and 5e,g).

Pharmacology of Neuroprotective Effects of Endogenous Dynorphin

Dynorphins preferentially bind to KOP; however, they can also activate the other classical opioid receptors mu and delta, as well as NMDA receptors (for review see Schwarzer, 2009). Therefore, we applied the KOP-specific antagonist GNTI before KA to dyn$^{(+/+)}$ animals to reproduce the phenotype of dyn$^{(-/-)}$ mice, and the KOP-specific agonist U-50488H before KA to dyn$^{(-/-)}$ mice to rescue their increased acute neurodegeneration. GNTI treatment of dyn$^{(+/+)}$ animals caused increased neurodegeneration of ipsilateral CA3a neurons and contralateral hilar nonprincipal neurons and CA1 pyramidal cells (Table 2) in response to KA treatment, thereby resembling the dyn$^{(-/-)}$ phenotype. By contrast, the treatment of dyn$^{(-/-)}$ mice with the KOP agonist U-50488H reduced the KA-induced neurodegeneration in the same areas (Table 2), indicating the rescue of the phenotype.

Regarding the number of SST-ir neurons, U-50488H treatment of dyn$^{(-/-)}$ mice reversed the phenotype in all subfields

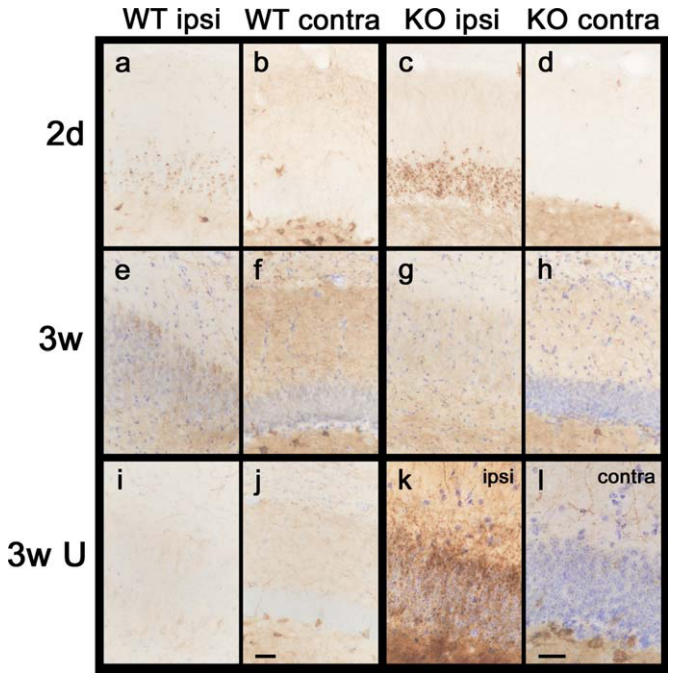

FIGURE 5. High magnification images depicting neuropeptide Y (NPY) immunoreactivity after unilateral KA injection into the dorsal hippocampus for dyn$^{(+/+)}$(a,b,e,f,i–l) and dyn$^{(-/-)}$ mice (c,d,g,h). Shown are the images from sections labeled for NPY immunoreactivity obtained from 2 days (a–d), or 3 weeks (e–h;k,l) after KA or 3 weeks after KA with the administration of the specific kappa opioid receptors agonist U-50488H every other day starting from day 6 (i,j). Note the strong NPY immunoreactivity of granule cell somata of the ipsilateral hippocampus 2 days after KA (a, c). After 3 weeks, granule cells are strongly dispersed in the ipsilateral hippocampus of all groups of mice (e,g,i). Numerous NPY immunoreactive mossy fibers were observed in the ipsilateral hippocampus of all groups of mice projecting through the granule cell layer, innervating the supergranular layer [depicted from a mouse with little dispersion close to the tip of the granule cell layer in high magnification in (k)]. No such mossy fiber sprouting was observed in the contralateral hippocampus (l). Bar in j (for a–j) and l (for k,l) represent 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

investigated (Table 2), resulting in numbers of SST-ir neurons indistinguishable from those observed in dyn$^{(+/+)}$ animals (Fig. 3). In line with this, GNTI pretreatment of dyn$^{(+/+)}$ animals decreased the number of SST-ir neurons significantly (Table 2).

The pharmacological data suggest that a major part of the neuroprotective function of endogenous prodynorphin-derived peptides is actually owing to KOP activation.

Potentials of the KOP Selective Agonist U-50488H as a Neuroprotective Agent

To evaluate the potential use of KOP-specific agonists as neuroprotective agents we treated dyn$^{(+/+)}$ animals with 20 mg kg^{-1} U-50488H. To study the effects in acute neurodegeneration U-50488H was applied 30 min before KA injection and mice were killed after 2 days. For effects during the progressive neurodegeneration phase, U-50488H was applied every other day starting from Day 6 after KA until mice were killed 3 weeks after treatment.

As shown in Table 3, no significant differences in cell counts obtained from Nissl-stained sections were observed when U-50488H was applied during the acute phase. The only difference, which reached significance, was a less pronounced reduction of SST-ir interneurons in CA1 of U-50488H-treated animals. By contrast, significant protective effects of the KOP agonist treatment were observed during the progressive phase. In fact, most of the progressive neurodegeneration was suppressed by the KOP agonist treatment (Table 3, Figs. 1m,n). Interestingly, NPY-ir appeared much less pronounced in mossy fibers of KOP agonist-treated animals than in nontreated (Figs. 4q,r and 5i,j).

The U-50488H treatment had much less effect on the number of SST-ir interneurons, with significantly increased numbers observed only in the contralateral hippocampus (Table 3). No such improvement was evident in the ipsilateral hippocampus. Besides neuronal degeneration, granule cell dispersion demarked strong morphological reorganization after KA injection in the ipsilateral hippocampus. The beginnings of dispersion in the upper blade of the granule cell layer was observed in some dyn$^{(-/-)}$ mice, but not in dyn$^{(+/+)}$ animals 7 days after KA. Three weeks after KA, strong granule cell dispersion was observed in both genotypes, mostly restricted to the ipsilateral side (Fig. 5).

The extent of granule cell dispersion was not influenced by the KOP agonist treatment (Figs. 5e,g,i). Thus, the area covered by the ipsilateral granule cell layer was 352,860 ± 30,000 μm^2 (5) for dyn$^{(+/+)}$ mice, 367,260 ± 107,200 μm^2 (5) for dyn$^{(-/-)}$ mice, and 351,100 ± 87,400 μm^2 (5) for U-50488H-treated dyn$^{(+/+)}$ animals. Sprouting of mossy fibers was clearly restricted to the ipsilateral hippocampus and observed in all groups of animals 3 weeks after treatment (Figs. 5e–l).

DISCUSSION

In this study we show effects of endogenous dynorphin and KOP agonist treatment on excitotoxic neurodegeneration, employing a model combining acute and progressing neuropathological and morphological alterations induced by an intrahippocampal injection of KA (Suzuki et al., 1995; Bouilleret et al., 1999). Although KA was injected into the stratum radiatum of area CA1 of the dorsal hippocampus, diffusion of KA into CA3 and the dentate gyrus cannot be excluded. In fact the expression of NPY-ir in the upper lateral part of the granule cell layer observed after 2 days (arrowheads in Figs. 4e,g), supports such a spread. KA, through stimulation of KA-receptors on glutamatergic neurons, induces strong glutamate release, which subsequently causes neuronal damage through Ca^{2+} influx via NMDA receptors (Fariello et al., 1989). These changes are not restricted to the injected hippocampus, but also affected the contralateral one at the dose of KA used in this study. Changes in the contralateral hippocampus are especially interesting because they arise from increased network activity and the direct effects of KA itself can be excluded.

TABLE 2.

Pharmacology of Neuroprotective Effects of Endogenous Dynorphin

	Nissl							
	Hilar non-principal neurons		CA1 pyramidal cells		CA3c pyramidal cells		CA3a pyramidal cells	
	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra
WT-KA	2.8 ± 2.4 (6)	23 ± 1.5 (6)	5.4 ± 3.3 (5)	90 ± 9 (6)	6.4 ± 2.9 (6)	36 ± 6.6 (6)	94 ± 11 (6)	122 ± 17 (6)
WT-KA-GNTI	1.1 ± 0.9 (7)	19 ± 1.5 (7)***	4.0 ± 1.9 (7)	64 ± 20 (7)***	6.1 ± 1.4 (6)	33 ± 3.9 (7)	76 ± 12 (7)*	122 ± 16 (7)
KO-KA	2.0 ± 2.1 (7)	17 ± 6.6 (7)	3.5 ± 2.5 (7)	42 ± 34 (7)	5.8 ± 5.9 (7)	33 ± 12 (7)	57 ± 24 (7)	97 ± 36 (7)
KO-KA-U50488	2.4 ± 1.0 (7)	24 ± 3.3 (6)*	4.0 ± 1.7 (6)	80 ± 18 (6)*	8.3 ± 6.9 (6)	37 ± 2.9 (6)	81 ± 8.9 (6)*	114 ± 10 (6)

	Somatostatin-ir					
	Hilar interneurons		CA1 interneurons		CA3 interneurons	
	ipsi	contra	ipsi	contra	ipsi	contra
WT-KA	1.7 ± 0.7 (5)	12 ± 1.9 (5)	2.4 ± 0.7 (5)	20 ± 5.9 (5)	14 ± 4.7 (5)	20 ± 2.9 (5)
WT-KA-GNTI	0.7 ± 0.7 (6)*	7.4 ± 0.9 (6)***	1.3 ± 0.8 (6)*	13 ± 2.9 (6)*	8.0 ± 3.7 (6)*	16 ± 2.9 (6)*
KO-KA	0.1 ± 0.2 (7)	5.0 ± 2.9 (7)	0.9 ± 0.7 (7)	15 ± 4.6 (7)	4.4 ± 2.6 (7)	10 ± 5.0 (7)
KO-KA-U50488	1.5 ± 0.9 (7)**	11 ± 1.7(7)***	5.4 ± 2.1 (7)***	17 ± 6.2 (7)	12 ± 5.0 (7)**	22 ± 3.6 (7)***

Cell counts from sections obtained from wild-type (WT) and prodynorphin deficient (KO) animals treated with KOP specific antagonists or agonists killed two days after KA are shown. The upper panel represents numbers obtained from Nissl-stained sections, the lower panel displays somatostatin immunoreactive cells. All data are given as mean AUC ± SD (n).

*$P < 0.05$;

**$P < 0.01$;

***$P < 0.001$ KA + KOP agonist/antagonist treatment vs. KA treatment.

Our data clearly show the neuroprotective effects of endogenous prodynorphin-derived peptides early after KA injections. Pharmacological tests suggest that these neuroprotective effects of endogenous dynorphins are mostly mediated by KOP. Thus, the blockade of KOP in $dyn^{(+/+)}$ mice resulted in increased neurodegeneration after KA treatment, resembling the phenotype of $dyn^{(-/-)}$ mice. By contrast, the phenotype of $dyn^{(-/-)}$ mice was rescued by the application of the KOP-specific agonist U-50488H. However, the application of the KOP agonist U-50488H did not affect neurodegeneration in $dyn^{(+/+)}$ mice during the acute phase, suggesting the sufficient stimulation of KOP receptors through endogenous dynorphins. By contrast, U-50488H reduced neuronal loss and morphological reorganization during the later phases of this model, when progressive neurodegeneration and morphological adaptations were accompanied by reduced levels of endogenous dynorphins (Suzuki et al., 1995).

The correctness of the prodynorphin knockout in our animals was shown elsewhere. KOP expression, distribution and functioning appeared unchanged in our mice (Loacker et al., 2007). The local injection of KA into the stratum radiatum of CA1 caused acute and—after a silent phase of several days—progressing neurodegeneration and granule cell dispersion. Neurodegeneration is dependent on increased hippocampal activity subsequent to the initial injection and also spreads to the contralateral hippocampus at the KA dose used. The hippocampi of both hemispheres are interconnected through commissural fibers (Swanson et al., 1978; Frotscher et al., 1991; Finnerty and Jefferys, 1993). Although the two hippocampi appear well synchronized under physiological conditions (Soltesz et al., 1993; Kocsis et al., 1994), the contralateral hippocampus displays distinct EEG patterns and neurochemical adaptations after unilateral KA injection (Arabadzisz et al., 2005). The synchronization of the two hippocampi is mainly mediated by glutamatergic fibers and desynchronization can be induced by filtering such inputs. One candidate to be involved in such processes is dynorphin. Reduction of excitatory neurotransmission is seen as the main effect of dynorphin in the hippocampus (Rusin et al., 1997). Regulation of hippocampal excitability by dynorphin was shown over 20 yrs ago (Henriksen et al., 1982; Wagner et al., 1993; Weisskopf et al., 1993). Dynorphin release was shown in several seizure models and anticonvulsive actions were frequently proposed (for reviews see Bortolato and Solbrig, 2007; Schwarzer, 2009). KOP density is rather low in the hippocampus. By contrast, their distribution is strategically perfect in terms of dampening excitation in the limbic circuitry. Presynaptic KOP are located on terminals of perforant path fibers, mossy fibers and pyramidal neurons (Drake et al., 1994; Terman et al., 2000). CA1 and CA3 neurons also contain KOP mRNA (Mansour et al., 1994). Presynaptic KOP of perforant path fibers and mossy fibers, as well as postsynaptic KOP on CA3 pyramidal neurons, are potential targets for the dynorphin released from granule cell dendrites and mossy fibers, respectively.

TABLE 3.

Effects of KOP Stimulation of Acute and Progressing Neurodegeneration after KA Injection

	Nissl							
	Hilar non-principal neurons		CA1 pyramidal cells		CA3c pyramidal cells		CA3a pyramidal cells	
	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra
WT-KA-2d	2.8 ± 2.4 (6)	23 ± 1.5 (6)	5.4 ± 3.3 (5)	90 ± 9 (6)	6.4 ± 2.9 (6)	36 ± 6.6 (6)	94 ± 11 (6)	122 ± 17 (6)
WT-KA-U-2d	0.6 ± 0.6 (4)	22 ± 1.6 (4)	3.3 ± 1.5 (5)	75 ± 38 (5)	6.0 ± 3.3 (5)	37 ± 9.5 (5)	84 ± 35 (5)	122 ± 18 (5)
WT-KA-3w	0.6 ± 0.6 (5)	18 ± 4.8 (5)	2.0 ± 1.4 (5)	71 ± 17 (5)	4.5 ± 1.6 (5)	26 ± 3.6 (5)	52 ± 15 (5)	95 ± 8.8 (5)
WT-KA-U-3w	1.3 ± 0.7 (7)	24 ± 4.1 (7)*	5.6 ± 3.0 (7)*	49 ± 25 (7)	3.7 ± 2.8 (7)	36 ± 4.3 (7)**	77 ± 16 (7)*	119 ± 13 (7)**

	Somatostatin-ir					
	Hilar interneurons		CA1 interneurons		CA3 interneurons	
	ipsi	contra	ipsi	contra	ipsi	contra
WT-KA-2d	1.2 ± 0.9 (6)	12 ± 2.1 (6)	2.1 ± 0.7 (6)	16 ± 5.6 (6)	13 ± 4.1 (6)	18 ± 5.4 (6)
WT-KA-U-2d	2.2 ± 0.6 (4)	13 ± 1.8 (4)	6.2 ± 4.0 (4)**	13 ± 1.2 (4)	15 ± 1.8 (4)	24 ± 6.7 (4)
WT-KA-3w	0.1 ± 0.2 (7)	7.1 ± 1.0 (7)	2.1 ± 1.3 (7)	19 ± 4.5 (7)	5.9 ± 1.8 (6)	16 ± 5.1 (6)
WT-KA-U-3w	0.3 ± 0.4 (7)	11 ± 2.3 (7)**	1.2 ± 1.6 (7)	18 ± 6.7 (7)	7.8 ± 4.0 (7)	22 ± 4.1 (7)*

Cell counts from sections obtained from wild-type (WT) and prodynorphin deficient (KO) animals treated with the KOP specific agonists U-50488H either during the injection of KA or from days 6 to 21 are shown. The upper panel represents numbers obtained from Nissl-stained sections, the lower panel displays somatostatin immunoreactive cells. All data are given as mean AUC ± SD (n).

*$P < 0.05$;

**$P < 0.01$ KA + KOP agonist treatment vs. KA treatment.

The neuropathology of dyn$^{(-/-)}$ mice fits very well to the distribution of KOP receptors and their proposed functions in the hippocampus. Thus, hilar neurons and CA3 neurons are strongly affected. Both are innervated by mossy fibers, which arise from granule cells containing dynorphin and presynaptic KOP. Presynaptic KOP activation through dynorphin released from mossy fiber collaterals is seen as one important factor in controlling hippocampal excitation (Terman et al., 2000). Stimulation of postsynaptic KOP on GABAergic SST-ir interneurons and pyramidal cells as another. Markedly more neurodegeneration was observed in the contralateral CA1 pyramidal cell layer of dyn$^{(-/-)}$ mice than dyn$^{(+/+)}$ mice. CA1 neurons were shown to express KOP mRNA in the rat (Mansour et al., 1994). In mouse KOP immunoreactivity was observed mainly in the stratum lacunosum-moleculare and stratum oriens of CA1, but appeared markedly less than in CA3 (Loacker et al., 2007). Because area CA1 is the target area for KA injection, the ipsilateral region can be influenced by the injection itself. By contrast, the contralateral hemisphere should reflect network-induced effects only. Signaling through commissural fibers entering the CA1 area through the alveus or perforant path fibers innervating CA1 from the stratum lacunosum-moleculare might be dampened by KOP activation.

Even more impressive are the differences in the numbers of SST-ir interneurons. However, these differences do not necessarily reflect neurodegeneration. Reduced numbers of SST-ir neurons might also be caused by the markedly increased release of SST from such neurons, usually induced by strong excitation. This can lead to a situation where SST levels are temporarily below the detection limit in some neurons, but later recover. This might be the case in the contralateral area CA3, where SST-ir neurons appear less frequent after 2 days than after 3 weeks. However, in most cases SST-ir neuronal counts did not recover and might indeed reflect the loss of these neurons. The sum of both effects was markedly higher in dyn$^{(-/-)}$ mice compared with dyn$^{(+/+)}$ mice, suggesting that the dynorphinergic activation of KOP dampens the excitation of interneurons.

Although dynorphins bind preferentially to KOP, the other two classical opioid receptors (mu and delta) also show relatively high affinities to dynorphins (Toll et al., 1998). Therefore, it has to be considered that the phenotype of the prodynorphin-deficient mice might be influenced by the lack of stimulation of any of the three opioid receptors. As shown by the pharmacological experiments in this study, the dyn$^{(-/-)}$ phenotype can be well mimicked by blocking KOP receptors in dyn$^{(+/+)}$ mice with the specific antagonist GNTI. In addition, the dyn$^{(-/-)}$ phenotype can be entirely rescued by the stimulation of KOP receptors with the specific agonist U-50488H. Therefore, we suggest that the activation of receptors other than KOP plays only a minor role in the neuroprotective effects of endogenous prodynorphin-derived peptides. This is in line with the pharmacology of the anticonvulsant effects of dynorphin (Loacker et al., 2007).

Our data suggest that the neuroprotective activity of KOP agonists depends on the level of endogenous dynorphin. Thus, no beneficial effects of KOP agonist treatment could be observed in the acute phase in dyn$^{(+/+)}$ mice, suggesting that endogenous dynorphin is sufficient to stimulate the majority of KOP. By contrast, a lack of dynorphin leads to increased neurodegeneration. This is in

line with data on the threshold for pentylenetetrazole-induced seizure. This threshold is lower in $dyn^{(-/-)}$ mice than $dyn^{(+/+)}$ mice and could not be further increased in $dyn^{(+/+)}$ mice by U-50488H (Loacker et al., 2007). With decreasing dynorphin levels in the later stages of this model, progressing neurodegeneration can be observed (Suzuki et al., 1995). This progressing neuronal damage might be related to increased glutamate release because the main inhibitory effect of dynorphin acting on KOP in the hippocampus is the reduction of glutamate release (Rusin et al., 1997). This is supported by enhanced NPY expression in granule cells during the progressive stage, which is induced through the activation of group I metabotropic glutamate receptors (Schwarzer and Sperk, 1998). Treatment with the KOP agonist U-50488H not only blocked neurodegeneration, but also dramatically reduced NPY immunoreactivity in mossy fibers (Fig. 4). Therefore, we propose that the neuroprotective effects of U-50488H are indeed because of reduced glutamate release.

The potential beneficial effects of KOP agonists in human TLE are difficult to assess in an animal model. Irrespective of the alterations in dynorphin levels (de Lanerolle et al., 1997; Jeub et al., 1999; Pirker et al., 2001), KOP binding sites, representing the drug target, appear to be conserved in TLE patients (de Lanerolle et al., 1997). A point mutation in the prodynorphin promoter, which leads to reduced prodynorphin expression, was proposed to increase the risk for secondary generalized seizures (Stogmann et al., 2002; Gambardella et al., 2003). Thus, KOP agonists might represent a valuable drug for seizure and neurodegeneration control and might help overcome presently unsolved therapeutic problems in pharmacological refractory epilepsies (Nilsen and Cock, 2004).

In conclusion, our data clearly show that endogenous prodynorphin-derived peptides play an important role in the regulation of hippocampal excitability and excitotoxicity in seizure naive mice. Under naive conditions, the addition of KOP agonists has no beneficial effect on neurodegeneration. However, these peptides are decreased under epileptic conditions, and the supplemental stimulation of KOP is capable of blocking most of the progressive neurodegeneration and glutamate-induced NPY expression in granule cells. This, on one hand, suggests a lack in endogenous dynorphin leads to increased glutamate release, which would then be a driving force in progressive neurodegeneration. On the other hand, KOP agonists might be considered potential neuroprotective and anticonvulsant drugs, which would be active after the establishment of epilepsy. Therefore, they might be especially beneficial for those patients refractory to classical antiepileptic treatment.

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