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Research Report

Leukemia inhibitory factor is a key regulator of astrocytic, microglial and neuronal responses in a low-dose pilocarpine injury model

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ABSTRACT

Insult to the central nervous system (CNS) induces many changes, including altered neurotransmitter expression, activation of astrocytes and microglia, neurogenesis and cell death. Cytokines and growth factors are candidates to be involved in astrocyte and microglial activation, and the up-regulation of glial fibrillary acidic protein (GFAP) is associated with brain damage. One of these candidates is leukemia inhibitory factor (LIF), a pro-inflammatory cytokine that is induced in astrocytes by brain damage or seizure. LIF also regulates expression of both neuropeptide Y (NPY) and galanin following peripheral nerve injury. To test the hypothesis that LIF regulates astrocyte, microglial and neuropeptide responses to a mild insult, we used a low-dose pilocarpine model to induce a brief seizure in LIF knock-out (KO) mice. Compared to wild type mice, the LIF KO mouse displays reduced astrocyte and microglial activation in the hippocampus. In addition, LIF KO mice display dramatically altered NPY, but not galanin, expression in response to injury. Thus, LIF is required for normal glial responses to brain damage, and, as in the periphery, LIF regulates NPY expression in the CNS.

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1. Introduction

Cytokines and growth factors are key candidates for mediating the changes induced by damage to the brain as they can affect astrocyte proliferation, migration, morphology and adhesion (Jankowsky and Patterson, 2001; Merrill and Benveniste, 1996). Activation of astrocytes and up-regulation of glial fibrillary acidic protein (GFAP) are often associated with local tissue damage and neuron loss (Banner et al., 1997; Kragh et al., 1993; Steward et al., 1991, 1992a). A member of the interleukin-6-type cytokine family, leukemia inhibitory factor (LIF), can regulate astrocyte activation. The expression of GFAP is promoted in astrocyte progenitors by administration of LIF in vitro (Murphy et al., 1997), and pilocarpine-induced seizure increases LIF expression in the hippocampus (Minami et al., 1991, 2002), primarily in astrocytes (Jankowsky and Patterson, 1999). Furthermore, endogenous LIF is required for normal GFAP expression in the hippocampus during development and in adulthood (Bugga et al., 1998; Koblar et al., 1998), as well as for astrocyte and microglial activation following cortical injury (Sugiura et al., 2000).

As with cultured sympathetic neurons (Fann and Patterson, 1994), neuropeptide expression in sympathetic and sensory neurons following injury in vivo is also regulated by LIF

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(Corness et al., 1996; Rao et al., 1993; Sun et al., 1996; Sun and Zigmond, 1996a,b). One of these neuropeptides is neuropeptide Y (NPY), which is expressed in neurons of the CNS (Chronwall et al., 1985), including the hippocampus (Köhler et al., 1986). Expression of NPY is increased by seizures in both humans and rodents (Borges et al., 2003; de Lanerolle et al., 2003; Furtinger et al., 2001; Gall et al., 1990; Gruber et al., 1994; Husum et al., 1998; Lurton and Cavalheiro, 1997; Sundstrom et al., 2001). These changes in NPY expression are functionally relevant as studies in rodents have shown an anti-convulsive role for NPY (Baraban et al., 1997; Bijak, 1995; Colmers et al., 1991; Erickson et al., 1996; Schauwecker and Steward, 1997; Schwarzer et al., 1995).

To test the hypothesis that LIF is required for normal responses to brain damage, we compared the responses of LIF knock-out (KO) and wild type (WT) mice to a low dose of pilocarpine, focusing on the regulation of glial activation and neuropeptide expression.

2. Results

2.1. *Survival rate and seizure score*

Summarized in Table 1 are the survival rate and number of mice in each seizure stage. The data show a higher survival rate for LIF KO (85.2%) than for WT mice (66.7%), but no obvious difference in seizure stage distribution.

2.2. *Pilocarpine-induced activation of astrocytes and microglia is diminished in LIF KO mice*

GFAP-positive astrocytes are apparent in untreated WT and LIF KO hippocampus (Figs. 1A and B, respectively). Twenty-four hours after pilocarpine treatment, we find a strong up-regulation of GFAP-like immunoreactivity (LI) in the hippocampus of WT mice (Fig. 1C). However, in LIF KO mice, there are fewer and less intense GFAP-positive cells, particularly in the female dentate gyrus (DG) (Fig. 1D). One week post-seizure, GFAP-LI is less intense in both WT and KO mice (Figs. 1E and F, respectively), with less intense GFAP-LI in the KO (Figs. 1E and F).

To detect microglia, an antibody against Mac-1 was used, which labels the CD11b antigen present in activated microglia and macrophages. Staining for Mac-1 in sections from untreated WT (Fig. 2A) and LIF KO (Fig. 2D) animals reveals weakly stained thin processes throughout the hippocampus. At 24 h following pilocarpine treatment, activated microglia with round cell bodies and short processes are present in WT and KO mice (Figs. 2B and E, respectively). Measurements of optical density over individual activated cells show a statistically significant difference in the OD 1.21–1.40 interval ($P < 0.019$), with a corresponding opposite difference in the 0.21–0.40 interval that does not reach significance. However, the overall difference is minor. Seven days following pilocarpine injection, only a few cells show strong Mac-1-LI in WT (Fig. 2C) and LIF KO (Fig. 2F) mice, and no clear difference is seen between the two groups. Both groups show a similar decrease in Mac-1-LI at 7 days post-treatment compared to the 24-h time point. A sexual dimorphism is present in that both WT and KO male mice have smaller Mac-1-positive cell bodies and more extended processes compared to their respective female mice (data not shown).

Table 1 – Survival rate and seizure grade distribution

	Total	Died	Survival (%)	Stage ≤3	Stage 3.5	Stage 4	Stage 5
WT	39	13	66.7	7	8	7	5
LIF KO	27	4	85.2	4	8	8	4

Survival rate and distribution of mice in the different seizure stages are summarized. Animals showing a stage 3 or less are put into one group since they were not analyzed.

2.3. *NPY expression increases strongly in pilocarpine-treated LIF KO mice*

Untreated WT and LIF KO mice display similar levels of NPY mRNA in DG granule cells and hilar neurons (Figs. 3A and D, respectively). Following pilocarpine treatment, the LIF KO (Fig. 3E) displays a striking increase in NPY mRNA expression in DG granule cells (Fig. 3G; $P < 0.001$) compared to WT (Fig. 3B). This difference is still apparent at 24 h (Figs. 3F, G; $P < 0.05$). In male LIF KO mice, NPY mRNA expression in DG granule cells is weaker compared to female KO mice (data not shown). In the hilus, WT neurons show comparable mRNA levels at all time points (Fig. 3H). In the LIF KO, however, there is increased NPY mRNA expression in hilar neurons at 24 h (compare Fig. 3C with F, H; $P < 0.05$).

Control WT and LIF KO mice show a fine network of NPY-IR fibers throughout the hippocampus, and no clear difference is seen between them (Figs. 4A and B). In WT mice, pilocarpine increases the number of NPY-IR DG hilar cells at 24 h (Fig. 4C, arrowheads). Although the fine network of NPY-IR fibers is reduced at 24 h, it recovers to normal levels in hippocampus and cortex at 7 and 14 days (C′ in Figs. 4E and G, respectively). By 24 h, a low dose of pilocarpine induces a much higher level of NPY-LI in mossy fibers in the in the LIF KO DG than in WT (compare Figs. 4C with 4D; Fig. 4I; $P < 0.001$). At 7 days, some mossy fiber NPY staining (Figs. 4F, J; $P < 0.01$) is still present in KO mice, while at 14 days NPY-LI has returned to control levels (Fig. 4H). In WT but not LIF KO mice, NPY-IR is present in the cortex at 7 days (compare Fig. 4E with F), but staining in the KO reappears by 14 days post-seizure (C′ in Fig. 4H).

2.4. *No difference in galanin-LI between WT and KO mice*

The difference in NPY expression between WT and KO is specific in that no obvious difference between the genotypes is observed in galanin staining, before or after pilocarpine treatment. A fine network of galanin-positive fibers is seen in both control WT and LIF KO mice (Figs. 5A and D, respectively). At 24 h following pilocarpine, only a few galanin-positive fibers are seen in WT (Fig. 5B) and KO (Fig. 5E) hippocampus, and, at 7 days, weak galanin-positive fibers are seen in both WT and KO hippocampus (Figs. 5C

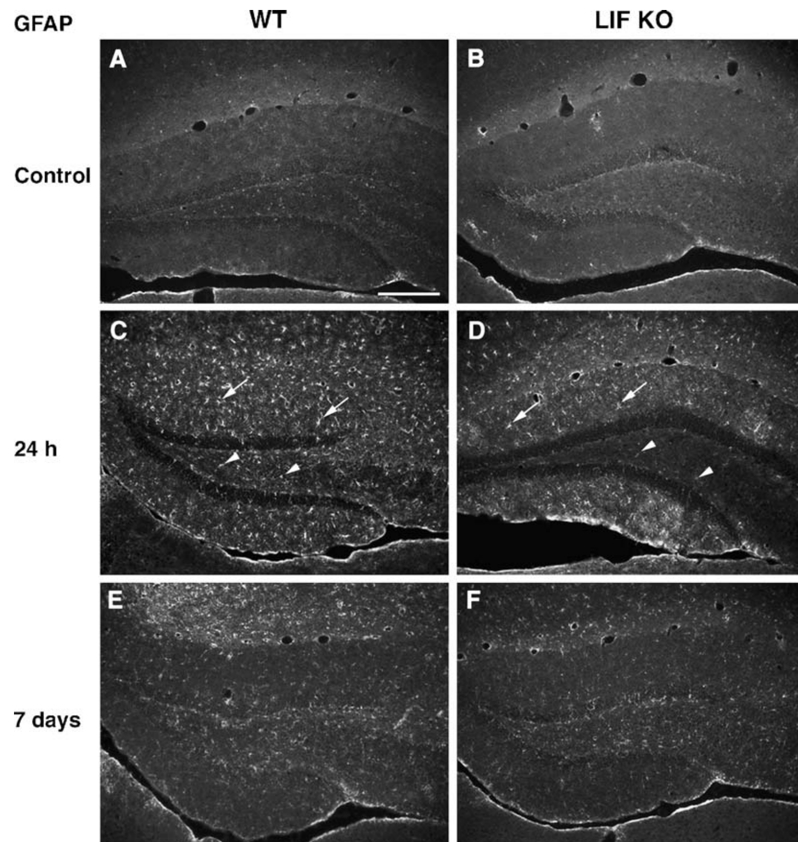

Fig. 1 – GFAP-LI in the female WT (A, C, E) and LIF KO (B, D, F) mouse hippocampus. In untreated WT (A) and KO (B) hippocampus, only a few GFAP-positive astrocytes are present. Twenty-four hours after seizure, activated GFAP-positive astrocytes are seen in the WT hippocampus (C) in both the DG (arrowheads) and molecular layers (arrows). Induction of seizure in KO mice (D) results in a few GFAP-positive astrocytes in the DG hilar area (arrowheads), but in other parts of the hippocampus, GFAP-IR astrocytes are present (arrows). Seven days post-seizure, only a few positive cells are seen in the either WT (E) or LIF KO (F) hippocampus.

and F, respectively), which is comparable to the 24-h time point.

3. Discussion

The aim of this study was to examine the functional role of LIF in the responses of hippocampal neurons and glia to a low-dose pilocarpine injury that induces seizures. We demonstrate that, in the absence of LIF, a low dose of pilocarpine has a reduced effect on astrocyte and microglial activation, but a more rapid and dramatic up-regulation of NPY expression. Furthermore, absence of LIF increases the survival rate following seizure. These differences between the genotypes are not due to a lack of seizure in the KO mice as seizures were monitored behaviorally in each animal. Moreover, at this dose of pilocarpine, LIF KO mice show strongly increased NPY mRNA and protein levels while WT mice show only small changes. Thus, the different activation levels of astrocytes and microglia apparent in the KO compared to WT are likely not due to a reduced pilocarpine response rather to the absence of LIF.

We used a low dose, peripherally administrated pilocarpine model for several reasons. First, the advantage of using a non-invasive method is that even small injuries to the brain, e.g. insertion of an electrode, cause activation of pro-inflammatory cytokines (Jankowsky et al., 2000). It is particularly important to avoid such reactions when studying the role of a cytokine such as LIF, which is pro-inflammatory in the nervous system (Gadient and Patterson, 1999). Second, high doses of convulsive drugs, or electrically induced seizures, are known to induce NPY expression in WT rodents (Colmers et al., 1991; Gall et al., 1990; Gruber et al., 1994; Lurton and Cavalheiro, 1997; Schwarzer et al., 1996). Third, a high dose of pilocarpine induces recurrent seizures resulting in extensive cell death in the hippocampus, amygdala and various thalamic areas (Gwinn et al., 2002; Humphrey et al., 2002; Scharfman et al., 2002). Therefore, a low dose of pilocarpine is more likely to unmask differences in expression of this neuropeptide caused by the absence of LIF. This pilocarpine paradigm does, in fact, induce brief seizures, and we carefully monitored the behavior of each animal and chose only those mice that displayed stage 3.5 seizures for further analysis. It has previously been shown that there are gender differences in response to pilocarpine-induced seizure, where males are more susceptible to develop temporal-lobe-like seizures than females (Mejias-Aponte et al., 2002). Due to limited number of animals in the present study, we did not

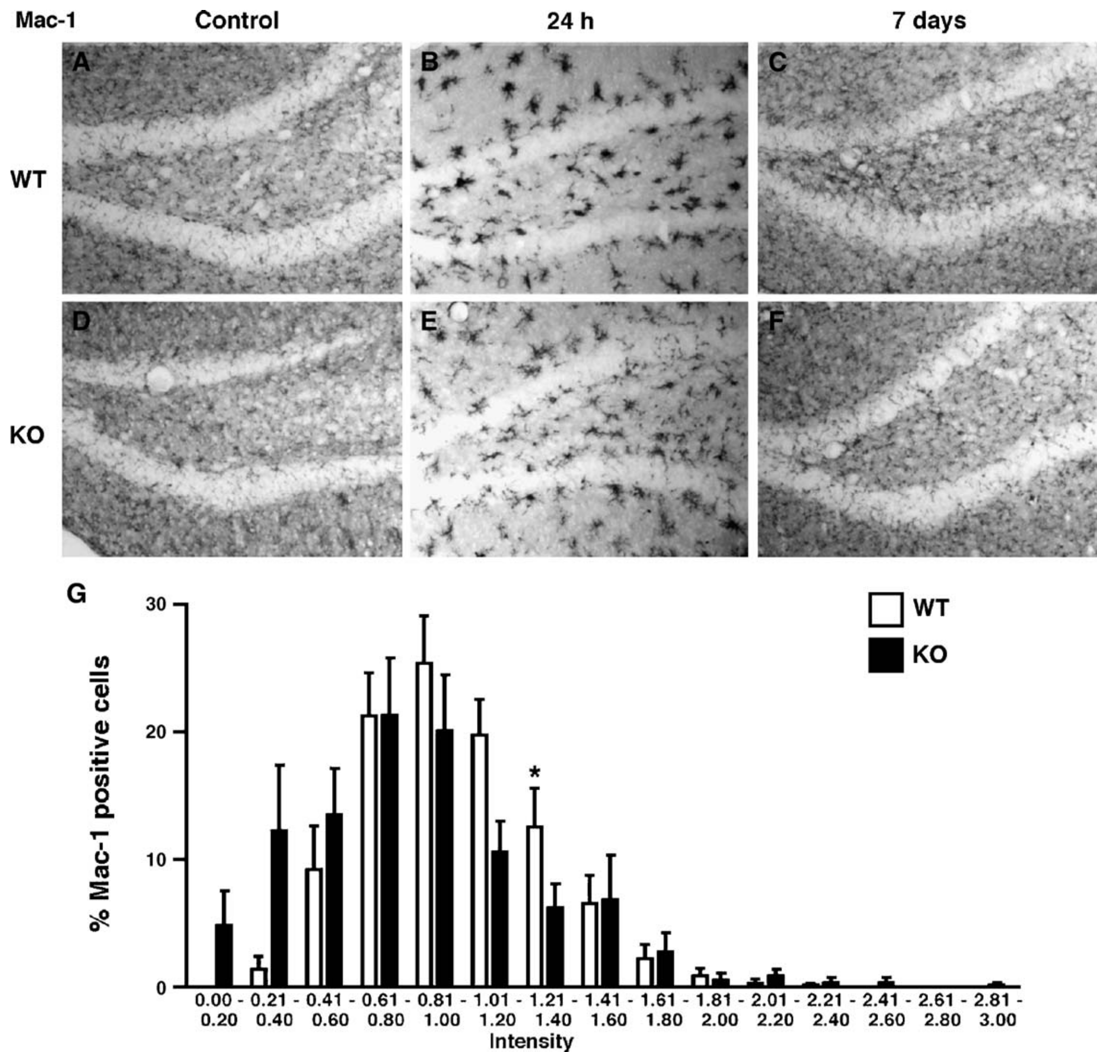

Fig. 2 – Mac-1-LI in female WT (A–C) and LIF KO (D–F) dentate gyrus. Thin Mac-1-positive processes are seen in both WT (A) and LIF KO (D) untreated mice. At 24 h following seizure, activated microglia, with round cell bodies and short processes, express strong Mac-1-LI in both WT (B) and KO (E) mice. Seven days after pilocarpine treatment, both WT (C) and KO (F) mice show weak Mac-1 staining, with small cell bodies and long processes. Optical density (OD) measurement of Mac-1-LI (G) in individual cells shows trend toward higher levels in WT mice (open bars) than in KO mice (black bars). This is statistically significant at the OD 1.21–1.40 interval (G, *$P < 0.019$).

divide the results from female and male mice when statistical analyses were made. However, we did notice some gender differences in glial activation (weaker in males) and NPY up-regulation (weaker in males). We did not observe any gender or genotype differences in galanin expression.

Astrocyte activation occurs in response to many types of damage to the CNS (Banner et al., 1997; Kragh et al., 1993; Steward et al., 1991, 1992a). Studies in rats (Lehtimäki et al., 2003; Minami et al., 1991, 2002) and mice (Jankowsky and Patterson, 1999) have shown that LIF expression is induced following seizure, predominantly in GFAP-positive astrocytes (Jankowsky and Patterson, 1999). Physical injury to the rat cortex also induces LIF expression in astrocytes (Banner et al., 1997). Importantly, compared to WT, cortical injury in LIF KO mice results in a diminished astrocytic and microglial response (Sugiura et al., 2000). In addition, previous studies have shown that induction of seizure induces a rapid expression of pro-inflammatory cytokines and related receptors in a time-dependent manner (Plata-Salaman et al., 2000; Turrin and Rivest, 2004). Activation of astrocytes is mediated via glutamate-dependent increase in $[Ca^{2+}]_i$ (Charles et al., 1992; Finkbeiner, 1992) and leads to increased levels of ATP, which further activate microglia (Guthrie et al., 1999; Verderio and Matteoli, 2001). There is also evidence that glia signal to other cell types such as neurons (Moller et al., 2000; Nedergaard, 1994; Pasti et al., 1997). In addition, uninjured female LIF KO mice display a lower level of GFAP staining than male LIF KO mice, despite having an equivalent number of astrocytes (Bugga et al., 1998).

In the present study, NPY mRNA and protein levels were dramatically up-regulated by pilocarpine in LIF KO mice. While this is the first demonstration of neuropeptide control by LIF in the CNS in vivo, the result is consistent with prior results in the PNS, where LIF suppresses NPY expression in

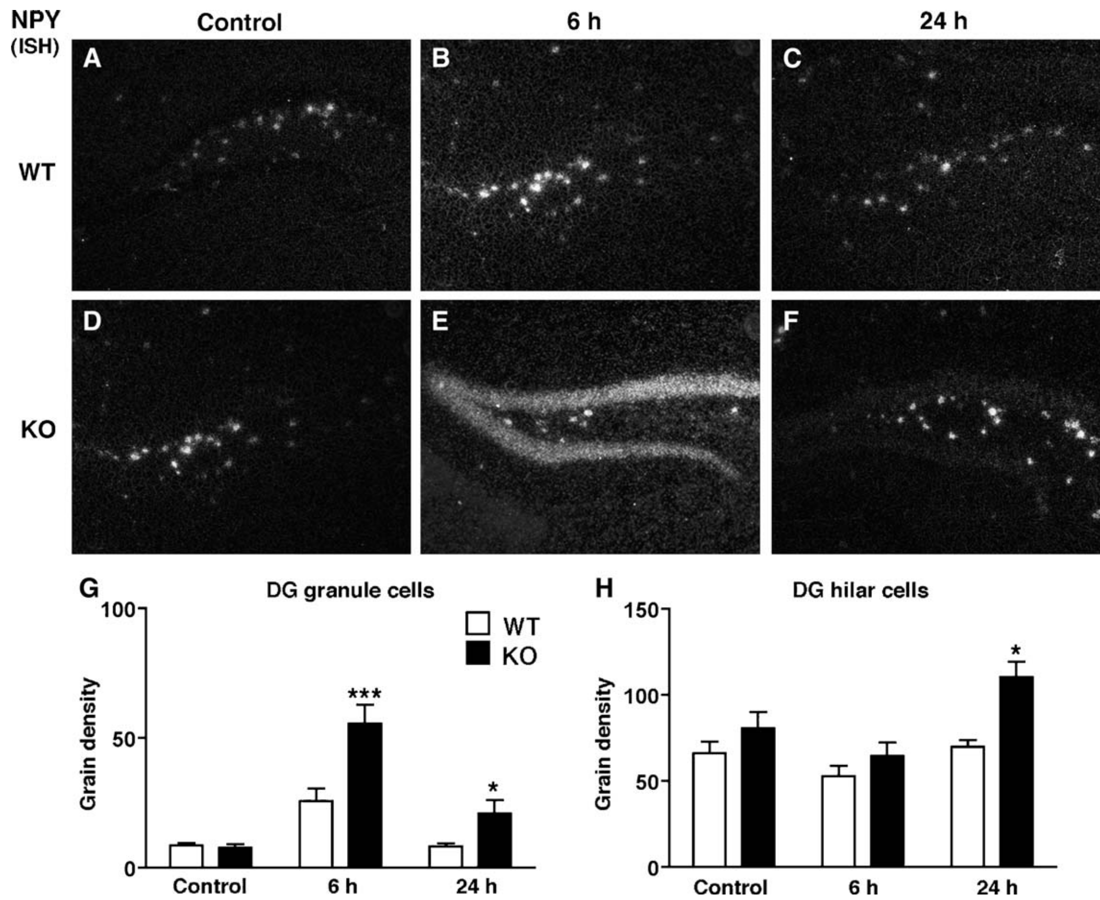

Fig. 3 – In situ hybridization (ISH) detecting NPY mRNA in female WT (A–C) and LIF KO (D–F) dentate gyrus and grain density quantification (G, H). In untreated WT (A) and KO (D) mice, NPY mRNA is only detected in DG hilar cells. At 6 h post-seizure, however, a significant increase (G; ***$P < 0.001$) in mRNA levels is seen in the KO (E) granule cell layer. No difference is apparent between WT (B) and KO (E) mice at 6 h in the hilar cells (H; $P > 0.05$). At 24 h, NPY mRNA levels are still elevated in the KO DG granule and hilar cells compared to WT (C) (G and H; *$P < 0.05$).

culture and in vivo (Corness et al., 1996; Fann and Patterson, 1994; Rao et al., 1993; Sun et al., 1996; Sun and Zigmond, 1996a,b). The lack of effect of pilocarpine on NPY expression in WT mice is likely due to the low dose used. Following stronger types of seizure, NPY expression is up-regulated in the hippocampus of both humans (de Lanerolle et al., 2003; Furtinger et al., 2001; Sundstrom et al., 2001) and rodents (Bellmann et al., 1991; Gall et al., 1990; Lurton and Cavalheiro, 1997).

Like NPY, galanin has anti-convulsive effects (Gall et al., 1990; Kokaia et al., 2001; Mazarati et al., 1992, 1998, 2000, 2001). However, we see no difference in galanin expression following seizure between WT and LIF KO mice. It is interesting that the seizure survival rate is higher in LIF KO (85.2%) than in WT mice (66.7%). This raises the possibility that the rapid increase of both NPY mRNA and protein expression could play a positive role in surviving the initial phase of seizure induction. This hypothesis is supported by a previous study in rats showing that the anti-convulsive effects of NPY and galanin are different, where NPY has a rapid effect and galanin a more long-lasting effect (Mazarati and Wasterlain, 2002). Further experiments are required to explore the effect of LIF on seizure survival.

In summary, endogenous LIF is a key coordinator of neuronal, microglial, astrocytic and inflammatory cell responses to injury in the nervous system. In the KO mouse, diminished activation of both astrocytes and microglia suggests that these mice are less prone to an inflammatory response. This is consistent from results with other forms of injury. Since our results suggest that LIF is involved in NPY expression related to seizure induction, it will be important to further investigate the role of NPY in the LIF KO mice. The difference in NPY expression between male and female LIF KO mice, in particular, needs further investigation.

4. Experimental procedures

4.1. *Animals and experimental procedures*

The strain of LIF KO mice used was that of Stewart et al. (1992b), which has been intermittently backcrossed with the C57BL/6 strain for over 10 years to maintain fertility and viability, and, as controls, WT mice from the KO colony were used. A polymerase chain reaction (PCR)-based method was used to determine the genotype of the mice. Genomic DNA was isolated from tail biopsies using a DNA isolation kit (Qiagen, Chatsworth, CA), and 50–100 ng was

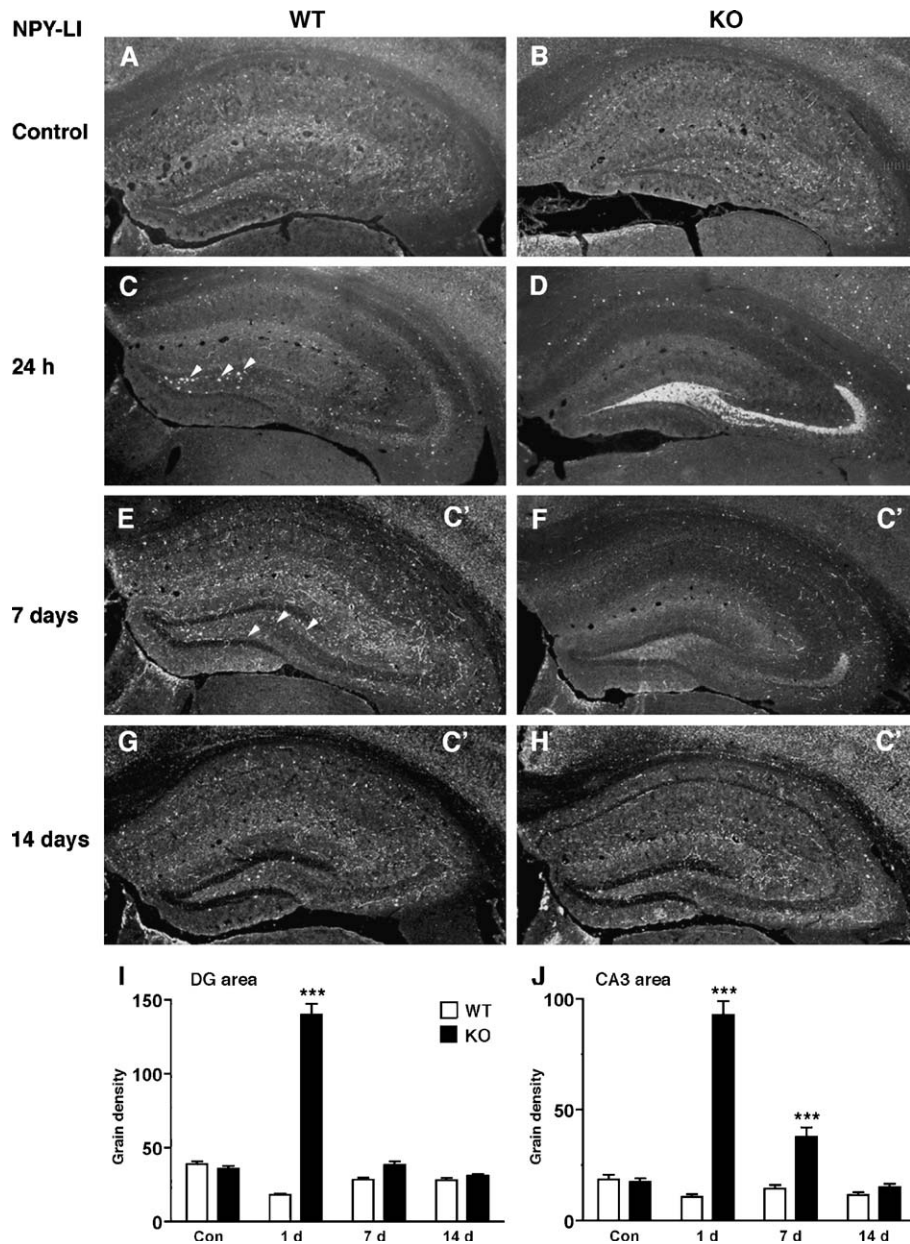

Fig. 4 – NPY-LI in female WT (A, C, E, G) and LIF KO (B, D, F, H) hippocampus. A similar NPY-IR fiber network is seen in WT (A) and KO (B) untreated mice. At 24 h post-seizure, strong NPY-LI is seen in mossy fibers in KO mice (D), but not in WT (C). Quantification reveals significant (*$P < 0.001$) increases in both DG (I) and CA3 (J) areas, compared to WT mice. At 7 days, NPY-positive mossy fibers are still present in KO mice (F). In addition, NPY-LI is seen in the WT mouse cortex (C′ in E), but not in the LIF KO mouse cortex (C′ in F). Furthermore, at 7 days, there is increased NPY-LI in the LIF KO CA3 area (J; ***$P < 0.001$), but not in DG (I). At 14 days, both WT (G) and KO (H) mice display similar NPY-IR fiber networks in the hippocampus. At this time point, there are also NPY-positive fibers in the LIF KO cortex (C′ in H) comparable to WT (C′ in G).**

subjected to PCR amplification (5 min at 95 °C, hold at 4 °C; 38 cycles of 30 s at 95 °C, 45 s at 68 °C, 45 s at 72 °C; and 10 min at 72 °C, then hold at 4 °C). The reaction mixture contained [1× PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl), 2.5 mM $MgCl_2$, 0.1 mM dNTPs, 0.1 U/μl Taq-polymerase] (Promega, Madison, WI) and 0.4 pmol/μl primers (each) in a total volume of 25 μl. Two specific DNA fragments were co-amplified: a 192-bp LIF gene fragment (using a LIF sense primer, 5′-cgcctaacatgacagacttcccat-3′, and an LIF antisense primer, 5′-aggcccctcatgacgtctatagta-3′) and a 541-bp neomycin gene fragment (using a Neo-sense primer, 5′-ccagctcttcagcaatatcacggg-3′, and a Neo-antisense primer, 5′-cctgtccggtgccctgaatgaact-3′). LIF WT mice contained only the LIF product (192 bp); the heterozygotes had both bands (192 and 541 bp); and the LIF-deficient mice had only the larger fragment (541 bp).

Animals of both genders were pretreated with atropine (0.4 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO) to minimize the peripheral effect of pilocarpine, 15 min prior to administration of pilocarpine (160–170 mg/kg, i.p.; Sigma). Seizures were scored using the scales of Racine (1972) and Schauwecker and Steward (1997) modified as follows: normal activity (stage 0), immobility (stage 1), stiffened and extended tail (stage 2), partial body clones or head bobbing (stage 3), whole body clonic seizure while

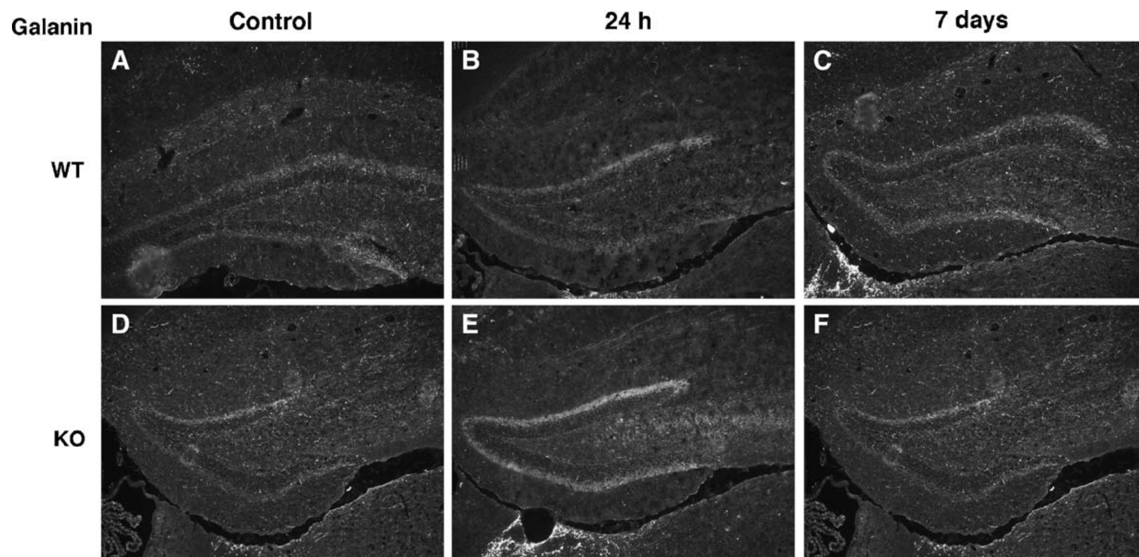

Fig. 5 – Galanin-LI is shown in WT (A–C) and LIF KO (D–F) mice. There is no difference in galanin expression between WT and LIF KO mice. Control WT (A) and KO (D) mice show disperse galanin-positive fibers in the hippocampus. At 24 h, weak galanin-positive fibers are still present in both WT (B) and KO (E) mice, and, at 7 days, galanin-LI has returned to normal intensity level in both WT (C) and KO (F) mice.

retaining posture (stage 3.5), rearing and falling (stage 4) and tonic–clonic seizure with loss of posture or jumping (stage 5). Two hours after the first grade 3.5 seizure, animals were treated with Diazepam (10 mg/kg, i.p.; Abbott Laboratories, North Chicago, IL) to stop seizures and monitored until they were fully recovered. During this 2-h time period, the mice did not experience constant seizures. Most animals that experienced grade 3.5 or higher and had 2–4 4- to 6-min long, stage 4 events and occasionally reached grade 5. Mice that did not reach grade 3.5 following pilocarpine administration mice were not included in the study since their GFAP expression did not differ from controls. Control animals were treated, as above, but with 0.1 ml 0.9% saline instead of pilocarpine, and untreated naive mice were also included as a separate group. Control and naive mice showed the same expression patterns and therefore served as controls for statistical purposes.

After a survival time of 6 h (5 WT + 5 KO) or 24 h (2 WT + 2 KO) and naive control mice (3 WT + 3 KO), animals used for in situ hybridization were anesthetized with an overdose of sodium-pentobarbital (Nembutal; Abbott Laboratories), and brains were rapidly dissected and frozen. For immunohistochemical experiments, survival times were 24 h (6 WT + 6 KO), 7 days (3 WT + 3 KO) or 14 days (4 WT + 4 KO), and naive control (6 WT + 6 KO) animals were anesthetized as above and perfused with 0.9% saline followed by 4% (w/v) paraformaldehyde and 14% (v/v) saturated picric acid in 0.16 M phosphate buffer. Brains were dissected, post-fixed in the same fixative for 2 h at 4 °C, transferred to phosphate-buffered saline (PBS) containing 15% sucrose (w/v), stored overnight at 4 °C and then frozen. Fourteen-micrometer-thick sections were cut on a cryostat, mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) and stored in −20 °C until use.

4.2. *In situ hybridization*

Oligonucleotides complementary to rat NPY mRNA nucleotides 1297–1344, 1581–1624 and 1671–1714 (Larhammar et al., 1987) were used. Probes were labeled with ^{35}S-α-dATP (Amersham Biosciences, Piscataway, NJ) at the 3′-end using terminal deoxynucleotidyltransferase (Amersham) and purified using ProbeQuant™ G-50 columns (Amersham). Probes were then diluted in a hybridization solution containing 50% deionized formamide (Ambion Inc., Austin, TX), 4× standard saline citrate (SSC; 1× SSC = 0.15 M NaCl, 0.15 M Na-Citrate), 1× Denhardt's solution [0.02% bovine serum albumin (Sigma), 0.02% Ficoll (Sigma), 0.02% polyvinylpyrrolidone (Sigma), 0.02 M $NaPO_4$ (pH 7.0)], 1% *N*-lauroylsarcosine (Sigma), 10% Dextran sulfate (Sigma)], 50 mg/l denatured salmon testis DNA (Ambion) and 200 mM dithiothreitol (Sigma). Sections were air-dried overnight and incubated with the labeled probe solution for 16–18 h at 42 °C. Sections were rinsed in 1× SSC four times 30 min at 55 °C followed by 1 h at room temperature then rapidly dehydrated through alcohol and air-dried. Slides were dipped in liquid photo emulsion NTB2 (Kodak, Rochester, NY) and, after exposure, developed using D19 (Kodak) and fixed in Kodak Rapid Fixer (Kodak).

4.3. *Immunohistochemistry*

All incubations were carried out at room temperature unless otherwise stated. Sections were incubated overnight at 4 °C with rat anti-Mac-1 (1:100; Biosource, Camarillo, CA) or rabbit anti-GFAP (1:1000; DAKO, Carpinteria, CA). Sections were rinsed in PBS then incubated with biotinylated goat anti-rat (1:400; Vector) or anti-rabbit (1:400; Vector) for 1.5 h. Sections stained for GFAP were incubated with fluorescein isothiocyanate (FITC)-conjugated streptavidin (1:500; Vector, Burlingame, CA) for 30 min, rinsed in PBS and mounted in PBS:glycerol containing 0.05% *p*-phenylenediamine (Johnson and Nogueria Araujo, 1981; Platt and Michael, 1983). Sections stained for Mac-1 were incubated using ABC kit (Vector) according to the manufacturer's directions and developed using 3,3-diaminobenzidiene (Vector), rinsed in PBS, air-dried and mounted with paramount (Fisher Scientific).

Sections incubated with antibodies raised in rabbit against NPY (1:4000; Sigma) or galanin (1:4000; Theodorsson and Rugarn, 2000) were processed using a modified tyramide signal amplification (TSA) (Perkin-Elmer, Boston, MA) protocol. Briefly, sections were pretreated with 0.01% H_2O_2 in PBS for 15 min, rinsed in PBS then incubated with antiserum overnight at 4 °C. After rinsing in PBS, sections were incubated with biotinylated goat anti-rabbit (1:400; Vector) for 2 h and then with the ABC kit (Vector). The signal was amplified using the TSA kit (1:100; Perkin-Elmer, Boston, MA) for

15 min then incubated with FITC-conjugated streptavidin (1:500; Vector), rinsed in PBS and mounted in PBS:glycerol as above.

4.4. *Analysis and statistics*

Sections from all animals in the study were analyzed. For in situ hybridization, 6–8 sections/animal were analyzed; for immunohistochemistry, 4–6 sections/animal (Mac-1) and 7–8 sections/animal (NPY) were analyzed. Sections were analyzed using a Nikon Diaphot 300 microscope, and pictures were captured using a SPOT-RT CCD camera and Spot 3.4 software (Diagnostic Instruments Inc, Sterling Heights, MI). Optical density (OD) measurements were performed using NIH Image software 1.62 (NHI; http://rsb.info.nih.gov/nih-image/Default.html). For Mac-1, optical density was measured over individual cell bodies and presented as OD/pixel, and 467 cells from WT mice and 475 cells from KO mice were measured. mRNA levels were measured using a fixed-size rectangle (DG area) or outline of DG cells using 20× magnification, and background levels from an unlabeled area in the hippocampus were subtracted. All mRNA results are presented as mean grain density per section for the specific brain area. Immunohistochemical staining density was captured in the same way as for grain density. The data are presented as mean OD per section for the specific brain area. All images were finalized using Photoshop 7.0 (Adobe Systems Inc., San Jose, CA).

Student's *t* test was used when analyzing Mac-1 staining. Results from mRNA grain density measurements were analyzed using the Kruskal–Wallis test followed by Dunn's post hoc test. One-way ANOVA followed by Tukey post hoc test was used for OD measurements of NPY-LI. Values were considered significant when $P < 0.05$. All data are presented as mean ± SEM.

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