Neuropeptide Y gene therapy decreases chronic spontaneous seizures in a rat model of temporal lobe epilepsy

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Temporal lobe epilepsy remains amongst the most common and drug refractory of neurological disorders. Gene therapy may provide a realistic therapeutic approach alternative to surgery for intractable focal epilepsies. To test this hypothesis, we applied here a gene therapy approach, using a recombinant adeno-associated viral (rAAV) vector expressing the human neuropeptide Y (NPY) gene, to a progressive and spontaneous seizure model of temporal lobe epilepsy induced by electrical stimulation of the temporal pole of the hippocampus, which replicates many features of the human condition. rAAV-NPY or a control vector lacking the expression cassette (rAAV-Empty) was delivered into the epileptic rat hippocampi at an early progressive stage of the disease. Chronic epileptic rats were video-EEG monitored to establish pre-injection baseline recordings of spontaneous seizures and the effect of rAAV-NPY versus rAAV-Empty vector injection. Both non-injected stimulated controls and rAAV-empty injected rats showed a similar progressive increase of spontaneous seizure frequency consistent with epileptogenesis. The delivery of rAAV-NPY in epileptic rat brain leads to a remarkable decrease in the progression of seizures as compared to both control groups and this effect was correlated with the NPY over-expression in the hippocampus. Moreover, spontaneous seizure frequency was significantly reduced in 40% of treated animals as compared to their pre-injection baseline. Our data show that this gene therapy strategy decreases spontaneous seizures and suppresses their progression in chronic epileptic rats, thus representing a promising new therapeutic strategy.

Keywords: adeno-associated viral vectors; anti-convulsant; EEG; hippocampus; neuropeptides

Abbreviations: AEDs = anti-epileptic drugs; CMV = cytomegalovirus; NPY = neuropeptide Y; rAAV = recombinant adeno-associated viral

Introduction

Epilepsy, the third most common neurological disease, is a chronic CNS disorder characterized by recurrent spontaneous seizures. Current anti-epileptic drugs (AEDs) mainly target transmitter receptors and ion channels (Rogawski and Loscher, 2004), and are effective in only 60–70% of individuals. Thus, approximately one-third of epileptic patients do not respond adequately to these treatments. Moreover, AEDs suppress seizures without influencing the underlying biologic process of epileptogenesis (Pitkanen and Sutula, 2002; Duncan et al., 2006). Patients that remain refractory to two or more AEDs are considered for surgical resection of the epileptic focus as a final therapeutic option. However, this invasive approach is associated with
functional impairments and is suitable for <10% of this population group (Foldvary et al., 2001; Duncan et al., 2006). Gene therapy may provide a realistic alternative to resective surgery (Perucca, 1998) since it offers the possibility to target the ‘therapeutic’ gene to the area of seizure generation without requiring tissue ablation. We have previously demonstrated that injection of the neurotropic recombinant adeno-associated viral (rAAV) vector containing the human recombinant neuropeptide Y (NPY) gene into the rat hippocampus induces a long-lasting expression of NPY in specific neuronal pathways, powerfully reduces acutely induced seizures and status epilepticus and delays kindling acquisition (Richichi et al., 2004). To date, no attempts have been made to evaluate the anti-epileptic efficacy of gene transfer in pathological chronic epileptic tissue which is characterized by morphological, molecular and functional changes that can alter the targets or cellular pathways required for transgene actions (Holmes and Ben-Ari, 2003). Here we report that intra-hippocampal injection of rAAV-NPY in a rat model of chronic epilepsy, ensuing from electrically induced status epilepticus, results in long-lasting NPY over-expression in neurons, induces a decrease of spontaneous seizure frequency and reduces the symptomatic progression of the disease. Our study provides proof-of-principle evidence that transfer of a gene expressing an endogenous anti-convulsant peptide may offer a new opportunity for therapeutic intervention of patients with drug-refractory epilepsy.

Methods

Rats were housed at a constant temperature (23°C) and humidity (60%) with a fixed 12 h light/dark cycle and ad libitum free access to food and water. Procedures involving animals and their care were conducted in conformity with the institutions guidelines that are in compliance with national (D.L.n.116, G.U., Suppl. 40, February 18, 1992) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996).

Induction of spontaneous seizures

Thirty-eight male Sprague-Dawley rats (~350 g) [26 experimental (rAAV-NPY and rAAV-Empty) and 12 non-injected stimulated controls] were deeply anaesthetized with Equithesin (1% pentobarbital + 4% chloral hydrate; 3.5 ml/kg, i.p.) and placed into a stereotoxic apparatus. Thereafter, two bipolar Teflon-insulated depth electrodes (wire Ø 0.127 mm, dorso-ventral tip separation 0.5 mm) were implanted into the hippocampus (from bregma, mm: AP –4.7; L± 5.0; –5.0 below dura) for stimulus delivery and monitoring of spontaneous seizures. Two screw electrodes (PlasticOne Inc., USA) were positioned over the nasal sinus and the left fronto-parietal cortex and used as ground and reference electrodes, respectively. One week after surgery, SE was induced by unilateral 90-min electrical stimulation of CA3 subfield of the temporal hippocampus. We applied a constant current stimuli (50 Hz, 400 μA, 1 ms monophasic square wave) for 10 min (10 s trains delivered every 11 s) followed by 1 min stimulus-off period. During and after stimulation, the rats were continuously video-EEG monitored to follow the development of SE. All 38 rats experienced SE and ~90% of these rats (n = 35) showed spontaneous recurrent seizures; three rats were excluded since they did not develop spontaneous seizures. Moreover, four out of 35 rats exhibiting spontaneous seizures were excluded from the final analysis because of severe gastrointestinal swelling, likely related to the injection of anaesthetics before the surgery. An additional group of non-injected sham control rats (n = 4) was implanted with electrodes and handled as experimental rats but received no stimulus; these rats were used in immunohistochemical experiments.

rAAV-vector production and injection

rAAV-vectors were prepared and purified by R. Bland (Neurologix Inc., U.S.A.) as described previously (During et al., 2003). Briefly, in the rAAV-NPY vector the human prepro-NPY (ppNPY) cDNA was subcloned into an expression cassette consisting of the cytomegalovirus (CMV)-chicken β-actin recombinant promoter (CBA), the woodchuck hepatitis virus post-translational regulatory element and a bovine growth hormone poly(A) signal. The same expression cassette, without the transgene was used as a control (rAAV-Empty) since it has been previously demonstrated that rAAV-Empty does not affect seizures or the related neuropathology when compared to rats injected with saline or with vectors carrying reporter genes (such as GFP) (Lin et al., 2003; Richichi et al., 2004).

Expression cassettes were subcloned into the rAAV backbone, which was flanked by rAAV2 inverted terminal repeats. rAAV capsid helper plasmid was added during the packaging process in a 1:1 ratio with the rAAV2 helper, in order to obtain high titre of chimeric vector expressing both serotype 1 and 2 capsid proteins.

At the time of electrode implantation, four 22-gauge stainless-steel cannulae were placed on the dural surface of rats to guide the vector injections into the dorsal and ventral hippocampus bilaterally (Fig. 1A). rAAV-vectors were injected using a 30-gauge needle protruding 3.2 mm (septal) or 4.8 mm (temporal) below the dura surface. Fourteen weeks after SE induction (Fig. 1), rats were selected for vector injection. Three microlitres of a stock solution of 5.2 × 1015 viral particles/ml was infused into all four hippocampal locations together with 0.5 ml of heparin (Sigma–Aldrich) to enhance vector distribution inside the brain parenchyma. Vector was injected at a flow rate of 1 μl/min using a 10 μl Hamilton syringe mounted on an infusion pump controlled by a microprocessor. One week on average was required for completing vector injections in all rats.

Previous studies showed that rAAV-NPY-mediated over-expression begins 3–7 days after vector injection, reaches its plateau after 2 weeks and is maintained for at least 6 months (Richichi et al., 2004), therefore experimental rats and their controls were used for subsequent analysis 4 weeks after vector injection.

Video-EEG recordings

Freely-moving rats were placed in Plexiglas cages (one rat per cage) and video-EEG recorded as previously described (Nissinen et al., 2000), during the first 24 h after SE induction, and 3 months later (for chronic recordings of spontaneous seizures; Fig. 1). Electrographic (EEG) activity was monitored using the Nervus EEG Recording System connected with a Nervus
VCR. A type WFL-II/LED15W infrared light (Videor Technical, camera positioned above the cages and connected to a Time Lapse was recorded using an infrared wide-angle lens analogical video hard drive, and later burnt on CDs. The behaviour of the animals

magnus 32/8 Amplifier (Nervus System, Iceland; sampling rate 256 Hz, high-pass filter 0.3 Hz, low-pass filter 100 Hz, sensitivity 1000 µV/cm). The digitized EEG data were stored on computer hard drive, and later burnt on CDs. The behaviour of the animals was recorded using an infrared wide-angle lens analogue video camera positioned above the cages and connected to a Time Lapse VCR. A type WFL-II/LED15W infrared light (Videor Technical, GmbH, Germany) was used at night to allow continuous 24 h/day video monitoring. Severity of SE was assessed by counting the total number of spikes during the first 24 h after SE induction (i.e. discontinuation of stimulation) using Clampfit 9.0 program (Axon Instruments, Union City, CA, USA). An epileptic spike was defined as a spike with a duration of <100 ms or as a spike-and-wave with a duration of <200 ms.

EEG seizures were manually identified by two independent observers blinded to the treatment; EEG seizures were defined by the occurrence of discrete episodes of high-frequency and high-voltage synchronous spike activity and/or multi-spike complexes (Fig. 1C). The minimal time period of synchronous activity required before the event was declared as an electrographic seizure was 10 s. EEG seizures were always associated with behavioural seizure manifestations verified from videotapes that were time-locked with the EEG (stages 2–5). In each rat, seizure frequency was counted by dividing the total number of EEG seizures by the total number of recording days (i.e. 14 days both at baseline and at post-injection period). The total time spent in seizures was defined as a sum of the duration of all seizures recorded during the baseline or post-injection periods. The values obtained separately for each rat were used to calculate the mean±standard error (SEM) in each experimental group. The behavioural severity of EEG seizures was scored from the corresponding video recording according to a modified Racine’s scale (Racine, 1972). Only score 4–5 (bilateral forelimb clonus and rearing with or without loss of balance) seizures were included in the analysis.

**NPY immunohistochemistry**

At the end of the 2-week post-injection video-EEG monitoring, all rats were treated for 3 consecutive days with a sedative dose of phenobarbital (30 mg/kg, i.p. every 12 h) to attenuate spontaneous recurrent seizures that are known to trigger NPY over-expression (Vezzani et al., 1999). Thus, we could better evaluate the increase in hippocampal NPY that was related to vector injection by decreasing the effect due to spontaneous seizures. At completion of phenobarbital treatment, chronic epileptic rats (n = 32) and non-injected sham control rats (n = 4) rats were deeply anaesthetized, perfused intra-cardially with 4% paraformaldehyde and their brains processed for immunohistochemistry as described before (Vezzani et al., 2002). Frozen coronal sections (1-in-5 series) were cut at a thickness of 30 µm with a sliding microtome. The first series of sections was stained with thionin and the adjacent series immunohistochemically with polyclonal antibody (T-4070, Bachem Holding ag, Swiss) raised against NPY.

One series of sections (section interval 150 µm) throughout the entire septotemporal axis of the hippocampus was stained with thionin (Nissl staining) and used for the semi-quantitative analysis of the severity of neurodegeneration in epileptic rats in subfields of the dentate gyrus (granule cell layer, hilus) and hippocampus proper (CA3 and CA1 pyramidal cell layers). The investigator was blinded to the treatment. To assess cell loss we used a scoring system (from 0 to 3) as described by Freund and colleagues (1992); Score 0, no cell loss; Score 1, 1–20% cell loss; Score 2, 21–50% cell loss; Score 3, over 50% cell loss. There was no difference in the severity of neurodegeneration between the rAAV-NPY and rAAV-Empty groups except in the CA3 where the damage was slightly more severe in the rAAV-Empty group (severity score: rAAV-Empty, 1.21 ± 0.18; rAAV-NPY, 0.61 ± 0.31, P < 0.05 by Tukey’s test).
NPY immunoreactive fibre and terminal density was assessed semi-quantitatively in a blinded manner throughout the entire septotemporal axis (section interval 150 µm). Quantification included subfields of the dentate gyrus (outer, mid and inner molecular layers; granular cell layer and hilus) and hippocampus proper (stratum radiatum, oriens and pyramidal in the CA1 and CA3; stratum lacunosum-molecular CA1 and stratum lucidum CA3). The scoring was as follows: Score 0, no immunopositive fibres and terminals; Score 1, a few individual immunopositive fibres and terminals (for a representative example, see the outer molecular layer in Fig. 3l); Score 2, a clear plexus of immunopositive fibres and terminals (see stratum oriens in Fig. 3g); Score 3, a dense plexus of labelled fibres and terminals (see inner molecular layer in Fig. 3l). For final analysis, the sum score of bilateral NPY expression in each subfield was calculated for each rat (13 areas analysed in both hippocampi, maximum score 78).

**Statistical analysis of data**

Data are presented as means ± SEM. Wilcoxon matched pairs test was used to analyse pre-injection with post-injection seizure parameters within the same experimental group. Differences in seizure frequency and duration and in the temporal profile of seizures between control and experimental groups were analysed by Mann–Whitney post hoc test. Difference in the number of rats with non-progressive epilepsy in the different treatment groups was calculated by χ²-test. Differences in the neurodegeneration severity score and in NPY immunoreactivity score were analysed by Tukey’s test. The least-square linear regression was used to analysed the correlation between the seizure progression index and the NPY immunoreactive score in each rat. A P-value <0.05 was considered to be significant.

**Results**

To demonstrate that the severity of SE was similar in all rats, we carried out analysis of the EEG spike counts and behavioural seizures during SE induction and for 24 h thereafter. No significant differences in any of these parameters were observed among the experimental groups (P > 0.05 by Kruskall–Wallis). Three months after electrically-induced SE, 21 rats were video-EEG recorded continuously for 2 weeks, 24 h/day (Fig. 1), to assess the baseline EEG seizure frequency and duration that were 0.5 ± 0.1 seizures/day and 48 ± 4 s, respectively. Thereafter, rats were randomized into two groups, one treated with rAAV 1/2-CBA-NPY and the other with the same vector lacking the NPY gene cassette (rAAV-Empty). Four weeks later, rats were continuously video-EEG recorded for additional 2 weeks. To assess the effect of gene transfer on seizure frequency and severity in individual rats, we compared Video-EEG recordings performed in the same animals before and after vector injection; recordings were also compared between the rAAV-NPY and rAAV-Empty groups. To assess whether the rAAV-Empty vector injection per se had any effect on the seizure parameters, 10 additional rats were stimulated and video-EEG recorded as above but received no injection (non-injected stimulated controls); SE in non-injected stimulated controls did not differ from the other experimental groups (P > 0.05 by Kruskall–Wallis).

**Analysis of spontaneous seizure progression**

Since a progressive increase in EEG seizure frequency over the time is a typical feature for epilepsy models caused by electrically-induced SE (Bertram and Cornett, 1994; Pitkanen and Sutula, 2002; van Vliet et al., 2004), we analysed the daily seizure frequency during 2-weeks of recording in chronic epileptic rats (Fig. 2A). The temporal analysis of seizure occurrence indeed showed a significant progression in the frequency of seizures both in non-injected stimulated controls (Stimulated-CTR, Fig. 2A) and rAAV-Empty groups (Fig. 2A). Since these two control groups did not significantly differ, we used only rAAV-Empty injected rats for subsequent seizure analysis. Differently from control rats, no progression was observed in rAAV-NPY injected rats (Fig. 2A).

The percentage of rats with secondarily generalized behavioural seizures (stages 4–5) (Racine, 1972) was similar in rAAV-Empty (10 out of 11 rats) and in rAAV-NPY (10 out of 10 rats) injected rats, however the progression in the number of stages 4–5 seizures differed in the two groups over the time (rAAV-Empty: 0.4 ± 0.1 stages 4–5 seizures/day at baseline to 0.8 ± 0.2 stages 4–5 seizures/day during the post-injection period (P < 0.05); rAAV-NPY: 0.3 ± 0.1 stages 4–5 seizures/day at baseline to 0.4 ± 0.1 stages 4–5 seizures/day during the post-injection period, P > 0.05, by two-tailed Wilcoxon test).

**Analysis of seizure frequency and total time in spontaneous seizures**

Subsequently, we analysed the mean seizure frequency and the total time spent in seizures over 2 weeks of recording in the rAAV-Empty and rAAV-NPY groups.

In the rAAV-Empty control group (n = 11) the mean frequency of EEG seizures over the 2-weeks post-injection recording (i.e. 20 weeks post-SE) was 3-fold increased (1.7 ± 0.4 seizures/day) as compared to the respective baseline (i.e. 14 weeks post-SE) in the same rats (0.6 ± 0.1 seizures/day, < P < 0.01 by two-tailed Wilcoxon matched pairs test; Fig. 2B). Importantly, in the rAAV-NPY group (n = 10), no increase in the frequency of spontaneous seizures was observed as compared to respective baseline in the same rats (from 0.5 ± 0.1 seizures/day at baseline to 0.5 ± 0.1 seizures/day during post-injection recording; Fig. 2B). When comparing the EEG recording during the post-injection period in the two experimental groups, the seizure frequency in the rAAV-NPY group was lower than that in the rAAV-Empty group (0.5 ± 0.1 seizures/day in the rAAV-NPY group versus 1.7 ± 0.4 seizures/day in the AAV-Empty group, P < 0.01 by two-tailed Mann–Whitney test).

As another indicator of the severity of epilepsy, we calculated the total time spent in seizures that was 2.4-fold
increased in the rAAV-Empty group during the post-injection recording as compared to the pre-injection baseline in the same animals (7.2 ± 1.5 h at baseline versus 17.4 ± 4.1 h at post-injection period, \(P < 0.05\) by two-tailed Wilcoxon matched pairs test; Fig. 2B). In the rAAV-NPY group, no increase in the total time spent in seizures was observed after vector injection as compared to respective baseline in the same rats (from 5.1 ± 1.0 h at baseline to 6.5 ± 1.5 h during post-injection period; Fig. 2B). A 2.7-fold increase in the total time spent in seizures was found during the post-injection recording period in the rAAV-Empty group (17.4 ± 4.1 h; Fig. 2B) as compared to the corresponding recording period in the rAAV-NPY group (6.5 ± 1.5 h, \(P < 0.01\) by two-tailed Mann–Whitney test; Fig. 2B). No significant differences were observed in the mean duration of individual EEG seizures between the rAAV-NPY group (58 ± 4 s; number of seizures analysed was 66 in 10 rats) and rAAV-Empty group (51 ± 3 s; 259 seizures in 11 rats) in the post-injection period.

### Analysis of experimental group populations

In rats, as in humans, it is unlikely that every single animal will respond similarly to a given treatment, therefore we assessed the effect of rAAV-NPY injection on spontaneous seizures by carrying out a subgroup analysis of rats with progressive or non-progressive seizure frequency and compared them with the corresponding population of rats injected with the rAAV-Empty vector. In the rAAV-Empty group, 7 out of 11 rats (64%) showed progression in seizure frequency (i.e. >1.5-fold increase as compared to the baseline, ‘progressive’ in Fig. 2C) while in the rAAV-NPY group, progression was found in only 2 out of 10 rats (20%) (\(P < 0.05\) as compared to the rAAV-Empty group, \(\chi^2\)-test). We observed that in these two rAAV-NPY injected rats (‘progressive’ in Fig. 2C), the mean number of seizures over the 2 weeks recording period was ~3.0-fold higher than their baseline values while an average 5-fold increase was reckoned in the seven rats showing seizure progression in the rAAV-Empty group.
In the rAAV-NPY group, 80% of rats did not show seizure progression: four out of eight rats (40% of the total number of rAAV-NPY injected rats, \( n = 10 \)) showed a significant 50% decrease in both the frequency (0.7 ± 0.3 seizures/day at baseline versus 0.3 ± 0.2 seizures/day at post-injection period \( n = 4 \), \( P < 0.01 \) by two-tailed Wilcoxon matched pairs test) and total time spent in seizures (7.3 ± 2.6 h at baseline versus 4.0 ± 2.1 h at post-injection period \( n = 4 \), \( P < 0.01 \) by two-tailed Wilcoxon matched pairs test; Fig. 2C).

**Association of NPY expression with the anti-epileptic response**

Figure 3A shows representative patterns of expression of NPY in the hippocampus of rAAV-NPY, rAAV-Empty and non-injected stimulated control rats (Stimulated-CTR), 22 weeks on average after SE, as compared to non-injected sham controls (SHAM-CTR). In accordance with our previous findings (Richichi et al., 2004), the rAAV-NPY group showed an enhanced density of immunopositive terminals in the inner molecular layer of the dentate gyrus (panel 1). In the hippocampus proper, terminal labelling was prominently increased in the stratum oriens, pyramidale and radiatum of the CA1-CA3 subfields (d,h). Moreover, in the rAAV-NPY, rAAV-Empty and non-injected stimulated-control groups, we found a decrease in NPY positive interneurons in stratum oriens CA1 (b,c,d) and in the hilus (j,k,l), as previously reported (Vezzani et al., 1999; Vezzani and Sperk, 2004). Thus, the increased NPY in fibres denotes upregulation of this peptide in surviving neurons as well as ectopic expression in mossy fibres. The increased NPY in rAAV-Empty and non-injected stimulated control rats reflects the effect of spontaneous seizures on the endogenous peptide levels and it is clearly less than in rats injected with rAAV-NPY.

NPY over-expression in rAAV-NPY injected rats, was observed for about 3 mm around the injection site and never occurred outside the injected hippocampus. To assess if the anti-epileptic effect measured in the rAAV-NPY group was associated with the increased expression of NPY in the hippocampus, NPY positive fibres were scored as described in ‘Methods’ section. For final analysis, the sum score of bilateral NPY expression was calculated for each rat (immunoreactivity score, see Table 1). Figure 3B shows that the NPY expression in the hippocampus was significantly higher in rAAV-NPY versus rAAV-Empty injected rats \( P < 0.01 \), Tukey’s test). These rAAV-NPY injected rats showed reduced spontaneous seizures as compared to rAAV-Empty injected rats, as assessed starting one month after vector injection (Fig. 2B).

To investigate whether the level of NPY expression in the hippocampus axon terminals in the rAAV-NPY and rAAV-Empty groups was inversely correlated with the progression of seizures, we plotted the immunoreactive score in each epileptic rat against its seizure progression index (Fig. 4). A higher NPY expression was clearly associated with a milder seizure progression (Fig. 4B), thus resulting in a significant inverse correlation between these two parameters \( y = -17.70x + 45.96; r^2 = 0.25; P < 0.05 \).

The highest level of NPY expression was found in rats injected with the rAAV-NPY vector with all injections in optimal locations as assessed by microscopic analysis of thionin-stained sections (Fig. 4A). Accordingly, in three out of 10 rats in the rAAV-NPY group with at least two of the...
injections outside the hippocampus (lateral ventricle) the expression of NPY was similar to that in the rAAV-Empty group (Fig. 4A), thus confirming that increased expression of NPY in rAAV-NPY injected rats is associated with a precisely targeted vector injection.

Discussion

This study presents the novel finding that rAAV vector-mediated NPY gene expression in the hippocampus of chronic epileptic rats results in long-term strong attenuation of spontaneous recurrent seizures. Importantly, the treatment arrested the increase in the frequency of spontaneous seizures in the majority of epileptic rats. This evidence suggests that the transgene can have an effect on the progression of the disease. However, the unequivocal demonstration of this hypothesis requires a longer follow-up study of spontaneous seizures. Similar to animal models, progressive features are common in TLE patients who often do not adequately respond to AEDs, become drug-refractory, and eventually are evaluated for surgical resection of the seizure focus (Kwan and Brodie, 2000).

Some neuropeptides, like NPY, are considered good therapeutic candidates since they act as endogenous anti-convulsants as shown by pharmacological and genetic approaches (Baraban et al., 1997; Vezzani et al., 1999; Mazarati, 2004; Vezzani and Sperk, 2004). The initial experimental attempts to use neuropeptide gene therapy to suppress seizure activity established the proof-of-principle concept that AAV-mediated transgene expression has anti-ictal effects on acutely induced seizures (Haberman et al., 2003; Richichi et al., 2004; McCown, 2006; Foti et al., 2007). In these experiments, however, the vector carrying the ‘therapeutic’ gene (galanin or NPY) was infused into the ‘normal rat brain’ before the induction of acute seizures. In experimental models of chronic epilepsy and in patients with TLE, the brain has several abnormalities including neuronal loss as well as morphological and functional synaptic rearrangements which could affect the ability of the vector to transfect a sufficient number of neurons to provide the therapeutic effect. Moreover, reorganization of neuronal circuits in epileptic brain could change the target(s) of the transduced peptide and/or its release kinetics. This could result in the loss or reduction of the efficacy of gene therapy.

Here we used an epilepsy model, which mimics many of the neuropathological features of human TLE including hippocampal sclerosis (Bertram and Cornett, 1994). The observed anti-epileptic effect demonstrates that rAAV vector-mediated NPY gene expression functions also in a pathological condition, even when there is a partial loss of neurons normally expressing NPY.

The efficacy of the AAV-NPY vector in reducing spontaneous seizures in epileptic rats was highly dependent on the extent of NPY increase in fibres in the hippocampus proper and in the dentate gyrus. Increased NPY immunoreactivity was previously observed in the hippocampus after seizure induction in rats (Vezzani et al., 1999; Vezzani and Sperk, 2004). As a reduction in seizure susceptibility has been demonstrated in rodents with increased hippocampal NPY (Colmers and El Bahh, 2003; Richichi et al., 2004), seizure-induced NPY upregulation has been considered as an endogenous protective mechanism even though it is not

Table 1

<table>
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<tr>
<th>Rat</th>
<th>Group</th>
<th>Subpopulation</th>
<th>Seizure frequency before injection</th>
<th>Seizure frequency after injection</th>
<th>Seizure Progression Index</th>
<th>NPY immunoreactivity score</th>
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<td>1.19</td>
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<td>0.71</td>
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<td>0.21</td>
<td>0.58</td>
<td>66.14</td>
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sufficient to prevent the recurrence of seizures. The introduction of extra copies of the transgene using the rAAV vector maintains sufficient peptide in the epileptic brain with recurrent seizures that would otherwise exhaust NPY in neuronal storage vesicles resulting in reduced NPY release (Bellmann et al., 1991).

Electron microscopic analysis of hippocampal tissue of rAAV-NPY injected rats not exposed to seizures (see Supplementary Fig. 1) showed that NPY immunoreactivity was found in both type 1 (presumably glutamatergic) and type 2 (presumably GABAergic) synapses. This observation is consistent with the immunohistochemical localization of the peptide in granule cell mossy fibres and in hilar interneurons. HPLC analysis showed that NPY encoded by the transfected neuronal cells is eluted with synthetic rat NPY which indicates that transduced and endogenous NPY do not differ (see Supplementary Fig. 2). Importantly, NPY is released to a significantly larger extent from hippocampal slices of rAAV-NPY injected rats as compared to Empty-vector injected rats when slices are stimulated using high KCl plus Ca²⁺ trigger (see Supplementary Fig. 2), thus indicating that the transduced peptide massively enters the vesicular release pool.

The mechanism(s) by which NPY reduces spontaneous seizures and arrests their progression involves the powerful inhibitory action of the peptide on pre-synaptic glutamate release via activation of NPY-Y2 receptors on glutamatergic terminals (Klapstein and Colmers, 1993; Vezzani et al., 1994). NPY-Y2 receptors are crucially involved in the anticonvulsant effects of this peptide as assessed in acute seizure models (El Bahh et al., 2005). This inhibitory action may indeed be amplified in rodent and human chronic epileptic hippocampi where the density of NPY-Y2 receptors is increased on mossy fibre terminals (Roder et al., 1996; Vezzani et al., 2000; Furtinger et al., 2001). Importantly, the chronic rAAV-mediated NPY over-expression did not reduce the level of NPY-Y2 receptors in the hippocampus in accordance with previous evidence showing Y2 receptors upregulation in the hippocampus associated with strong NPY immunoreactivity (Roder et al., 1996; Vezzani et al., 2000; Vezzani and Sperk, 2004). In contrast, NPY-Y1 receptor levels were significantly reduced (see Supplementary Fig. 3) in agreement with the ability of these receptors to internalize following the binding of agonists (Pheng et al., 2003). This phenomenon may favour the anti-convulsant actions of NPY since Y1 receptors can mediate excitatory effects in the hippocampus (Gariboldi et al., 1998).

Our findings, therefore, indicate that the transgene-derived NPY is transported to nerve terminals and released in large amount following neuronal depolarization. Thus, NPY encoded by our vector construct does not require any secretory signal sequence to be released, in contrast to the construct adopted for galanin over-expression by Haberman et al. (2003).

NPY is involved in various functions including the regulation of blood pressure, circadian rhythms, feeding behaviour, anxiety, memory processing and cognition.

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**Fig. 4** Increased expression of NPY in the hippocampus is associated prevention of progression in seizure frequency. (A) Locations of vector injections in the left (LHC) and right (RHC) hippocampus (septal and temporal injections): ‘+’ indicates optimal injection in the hippocampus resulting in prominent NPY expression; ‘±’ partially successful injection very rostrally in the septal hippocampus or laterally in the temporal hippocampus resulting in modest NPY expression; ‘−’ misplacement of injection or clogging of the injection cannula. rAAV-NPY group (yellow squares) had a higher expression of NPY in the hippocampus than rAAV-Empty group (blue triangles). All rats with at least two successful vector injections in the rAAV-NPY group had increased expression of NPY as compared to the rAAV-Empty group. Progression index: ratio of spontaneous seizures after vector injection as compared to respective baseline (cut-off of progression was set to >1.5-fold increase as compared to the baseline, arrow and red line). The green line at 1 indicates no changes in seizure frequency, values to its left indicate reduction in seizure frequency. Note that some of the rats in the rAAV-Empty group had up to 2-fold increase in seizure frequency whereas the highest increase found in the rAAV-NPY group was 4.5-fold. (B) Relation between the immunoreactive score in each rat and its seizure progression index. A significant inverse correlation was found ($y = -17.70x + 45.96; r^2 = 0.25; P < 0.05$) between these two parameters.
Danger et al., 1990; Hokfelt et al., 1998) raising the possibility of side-effects consequent to peptide over-expression. However, many of these functions are mediated by areas other than the hippocampus. The highly localized rAAV-vector-mediated over-expression of NPY (Richichi et al., 2004) makes unlikely that physiological processes mediated by extra-hippocampal brain regions are affected. Our experiments in normal rats injected with the rAAV-NPY vector, showed no significant alterations in learning and memory performance as compared to animals injected with Empty-vector (see Supplementary Fig. S4; Noe et al., 2007).

However, the effects of NPY over-expression on cognitive behaviour could be different in epileptic rats due to the multiple pathological changes and the known cognitive and memory deficits in these animals. Therefore, there is a need for future studies of potential effects of NPY transgene expression on learning and memory in chronic epileptic conditions.

In conclusion, our study shows that intra-hippocampal injection of rAAV-NPY in a rat model of chronic epilepsy results in long-lasting peptide over-expression in neurons, decreases spontaneous seizure frequency and arrests their progression. Our study provides first proof-of-principle evidence that transfer of a gene expressing an endogenous anticonvulsant peptide in chronic epileptic tissue may offer a novel therapeutic intervention, alternative to surgery, in patients with drug-refractory epilepsy.

**Supplementary material**

Supplementary material is available at *Brain* online.

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**References**


