Adeno-associated viral vector-induced overexpression of neuropeptide Y Y2 receptors in the hippocampus suppresses seizures

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Gene therapy using recombinant adeno-associated viral vectors overexpressing neuropeptide Y in the hippocampus exerts seizure-suppressant effects in rodent epilepsy models and is currently considered for clinical application in patients with intractable mesial temporal lobe epilepsy. Seizure suppression by neuropeptide Y in the hippocampus is predominantly mediated by Y2 receptors, which, together with neuropeptide Y, are upregulated after seizures as a compensatory mechanism. To explore whether such upregulation could prevent seizures, we overexpressed Y2 receptors in the hippocampus using recombinant adeno-associated viral vectors. In two temporal lobe epilepsy models, electrical kindling and kainate-induced seizures, vector-based transduction of Y2 receptor complementary DNA in the hippocampus of adult rats exerted seizure-suppressant effects. Simultaneous overexpression of Y2 and neuropeptide Y had a more pronounced seizure-suppressant effect. These results demonstrate that overexpression of Y2 receptors (alone or in combination with neuropeptide Y) could be an alternative strategy for epilepsy treatment.

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

Abbreviation: rAAV = recombinant adeno-associated viral

Introduction

Epilepsy affects ~0.4–1% of the world population and ~30% of all patients with epilepsy are resistant to currently available anti-epileptic drugs (Brodie and Dichter, 1996; Duncan et al., 2006). In temporal lobe epilepsy, the most common type of epilepsy in adults, approximately half of the patients are drug resistant, and ~50% of those suffer from mesial temporal lobe epilepsy with an epileptic focus in the hippocampus and/or amygdala (Yilmazer-Hanke et al., 2000). Since only few of these patients are candidates for surgical intervention in the form of temporal lobectomy (Wieser and Yasargil, 1982; Engel et al., 2003), the development of novel treatment strategies is highly warranted.
Recently, gene therapy has emerged as a promising alternative strategy for epilepsy treatment. Using viral vectors to transfer and overexpress genes of endogenous anti-epileptic agents, direct targeting of the epileptic focus and/or key seizure propagation pathways has become possible (Haberman et al., 2003; Richichi et al., 2004; Lin et al., 2006; McCown, 2006; Foti et al., 2007; Kanter-Schlifke et al., 2007a, b). Recombinant adeno-associated viral (rAAV) vectors are considered safe for human application (Warrington et al., 2006) and in recent clinical trials with rAAV in the central nervous system, evidence of both safety and efficacy has been obtained (Kapllt et al., 2007; Marks et al., 2008). Although clinical trials remain to be conducted in patients with epilepsy, rAAV vector-based neuropeptide Y gene therapy has been proposed as a feasible candidate for such trials (Löscher et al., 2008; Noé et al., 2008).

Neuropeptide Y is an endogenous neuropeptide with powerful anti-epileptic effects both in vivo and in vitro (Woldbye et al., 1996, 1997; Baraban et al., 1997; Vezzani et al., 1999; Woldbye and Kokaia, 2004; Xapelli et al., 2006). When overexpressed in the hippocampus or piriform cortex by rAAV vectors, transgene neuropeptide Y showed pronounced seizure-suppressant effects in both acute and chronic epilepsy models (Richichi et al., 2004; Lin et al., 2006; Foti et al., 2007; Noé et al., 2008, 2010; Sørensen et al., 2009). Neuropeptide Y exerts its effects in the brain by binding to the G-protein coupled receptors Y1, Y2 and Y5 (Berglund et al., 2003). In the hippocampus, Y2 receptors play a major role in mediating seizure-suppressant effects of neuropeptide Y (El Bahn et al., 2005) by presynaptically inhibiting glutamate release (Greber et al., 1994). In contrast, hippocampal Y1 receptors may facilitate seizure activity (Garibaldi et al., 1998; Benmaamar et al., 2003; Lin et al., 2006). Therefore, by acting at both Y2 and Y1 receptors, gene therapy based on transgene neuropeptide Y may potentially induce two opposing effects on seizures: (i) inhibition via Y2 and (ii) promotion via Y1. Y2 receptor binding is upregulated in human patients with temporal lobe epilepsy with hippocampal sclerosis (Furtinger et al., 2001) as well as in rodents after seizures (Röder et al., 1996; Gobbi et al., 1998). This Y2 receptor upregulation has been suggested to be a compensatory endogenous anticonvulsant mechanism (Vezzani and Sperk, 2004). To mimic this upregulation, we overexpressed transgene Y2 receptors in the hippocampus and tested whether this could exert a seizure-suppressant effect. Here we demonstrate for the first time that intrahippocampal infusion of the rAAV-Y2 receptor vector results in overexpression of functional Y2 receptors, leading to seizure-suppression in two temporal lobe epilepsy models: electrical kindling and kainate-induced seizures.

Material and methods

Male Sprague Dawley rats (naïve rats and kindling experiment; B and K Sollentuna, Sweden) or male Wistar rats (kainate experiment; Taconic Europe, Denmark) weighing 250–280 g at the beginning of the experiments were housed in a 12 h light/dark cycle with ad libitum access to food and water. All experiments were performed according to the Swedish Animal Welfare Agency guidelines and approved by the local Ethical Committee for Experimental Animals.

Recombinant adeno-associated viral vector injections

The rAAV vectors derived from a mixture of serotypes 1 and 2 encoding the full-length cDNA for the mouse Y2 receptor (stock solutions: rAAV-Y2; 1.1 × 1012 genomic particles/ml) and empty vector (rAAV-empty; 1.2 × 1012 genomic particles/ml) were manufactured by GeneDetect (Auckland, New Zealand). The transgenes were subcloned into a rAAV expression cassette consisting of the rat neuron-specific enolase promoter, woodchuck post-transcriptional regulatory element and a bovine growth hormone polyA signal flanked by rAAV inverted terminal repeats (Richichi et al., 2004).

The injection method was described earlier (Kanter-Schlifke et al., 2007a, b; Sørensen et al., 2008b, 2009). Animals were anaesthetized by intraperitoneal injections of s-ketamine (80 mg/kg; Pfizer Inc., NY, USA) and xylazine (15 mg/kg; Sigma-Aldrich, St Louis, MO, USA) and placed into a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). A volume of 2 μl viral vector suspension was infused through a glass pipette (0.2 μl/min) bilaterally in the dorsal hippocampus (antero-posterior = −3.3 mm, medial-lateral = ±1.8 mm, dorsal-ventral = −2.6 mm) and in the ventral hippocampus (antero-posterior = −4.8 mm, medial-lateral = ±5.2 mm, dorsal-ventral = −6.4 and −3.8 mm; 1 μl at each location in the dorsal-ventral plane) (Paxinos and Watson, 2005). Some seizure-naïve rats only received unilateral injection. Reference points were bregma for the anterior-posterior axis, midline for the medial-lateral axis and dura for the dorsal-ventral axis with the toothbar at −3.3 mm. The pipette was left in place for an additional 3 min after injection to prevent backflow of viral particles through the injection track. Viral vector stock solutions were diluted 1:2 with sterile phosphate-buffered saline before use.

Recombinant adeno-associated viral-Y2-induced transgene overexpression

To confirm that the rAAV-Y2 vector was efficient at inducing transgene overexpression, we injected the vector uni- or bilaterally and studied hippocampal Y2 binding and functional Y2 neuropeptide Y receptor binding in seizure-naïve rats at 3 and/or 8 weeks after injection. We also performed Y2 in situ hybridization, binding and/or functional Y2 receptor binding in kindled and kainate-treated rats at 8 and 3 weeks after vector injection, respectively (see below).

Y2 receptor in situ hybridization

The in situ hybridization procedure was performed as described earlier, with minor modifications (Woldbye et al., 2005; Christensen et al., 2006). On the day of killing, the rats were decapitated and their brains were quickly removed, frozen on dry ice and cut into 15 μm coronal serial sections through the hippocampus using a cryostat. The sections were thaw-mounted onto Superfrost glass slides and stored at −80°C until further processing. The slides were defrosted for 10 min at room temperature and subsequently fixed in 4% paraformaldehyde for 5 min, rinsed briefly in phosphate-buffered saline and placed in phosphate-buffered saline for 5 min. The slides were then placed in 70% ethanol for 5 min and stored in 95% ethanol at 4°C until hybridization using the following synthetic anti-sense oligonucleotide DNA probe: Y2 mRNA: 5′-GAG-CAA-TGA-CTG-TCA-AAAG-TTA-TTG-TGG-ACA-CTT-GTA-CCG-CCA-GAC-CCA-G-3′ (Woldbye et al., 2005). The oligoprobe was labelled at the 3′-end with [35S]dATP (1250 Ci/mmol; NENG734H001MC; PerkinElmer, DK) using terminal deoxynucleotidyl transferase (Roche Diagnostics, Mannheim, Germany). The labelled probe was added to a specific activity of...
1–3 × 10⁵ cpm/100 μl to the hybridization buffer that contained 50% formamide (v/v). 4× saline sodium citrate (1× saline sodium citrate = 0.15 M NaCl, 0.015 M sodium citrate–2H₂O, pH 7.0), 10% dextran sulphate (w/v) and 10 mM dithiothreitol. After adding a volume of 120 μl hybridization mixture to each slide, the slides were covered with a parafilm and left at 42°C in humidity boxes overnight. At room temperature, the slides were subsequently briefly rinsed in 1× saline sodium citrate, washed for 30 min in 1× saline sodium citrate at 60°C, passed through a series of 1 min rinses in 1× saline sodium citrate, 0.1× saline sodium citrate, 70% ethanol and 95% ethanol. Finally, the slides were air-dried and exposed together with non-labelled 125I-albumin. The slides were then incubated at room temperature for 60 min in a binding buffer containing 0.1 nM [125I][Tyr36]monooi-odo-peptide YY (4000 Ci/mmol; porcine synthetic, #M259; Amershams Biosciences, DK) to which 10 nM Leu-Pro-Ala-neuropeptide Y (Y1/Y4/Y5 preferring agonist; rat/mouse synthetic, #H-3306, Bachem AG, Switzerland) was added to visualize Y2 binding or togeth-er with non-labelled 1 μM neuropeptide Y (human/rat synthetic, #H-5375, Bachem AG) to visualize non-specific binding. After a brief rinse, the slides were washed for 2× 30 min in binding buffer at room temperature and subsequently air-dried, before being exposed to 125I-sensitive Kodak Biomax MR films (Amersham Biosciences, DK) for 4–8 weeks and developed in Kodak GBX developer.

**Y2 receptor binding**

Visualization of Y2 receptor binding was performed as described earlier (Woldbye et al., 2005). The slides were defrosted at room temperature and preincubated for 20 min in a binding buffer (pH 7.4), containing 25 mM N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid) (HEPES), 2.5 mM CaCl₂, 0.5 g/l bacitracin and 0.5 g/l bovine serum albumin. The slides were then incubated at room temperature for 60 min in a binding buffer containing 0.1 nM [125I][Tyr36]monooi-odo-peptide YY (4000 Ci/mmol; porcine synthetic, #M259; Amershams Biosciences, DK) to which 10 nM Leu-Pro-Ala-neuropeptide Y (Y1/Y4/Y5 preferring agonist; rat/mouse synthetic, #H-3306, Bachem AG, Switzerland) was added to visualize Y2 binding or together with non-labelled 1 μM neuropeptide Y (human/rat synthetic, #H-5375, Bachem AG) to visualize non-specific binding. After a brief rinse, the slides were washed for 2× 30 min in binding buffer at room temperature and subsequently air-dried, before being exposed to 125I-sensitive Kodak Biomax MS films (Amersham Biosciences) for 4 days at −20°C with 125I-microscales (Amersham Biosciences). The films were developed in Kodak GBX developer.

**Y2 receptor functional binding**

The procedure was as described earlier (Christensen et al., 2006; Silva et al., 2007). Sections were defrosted and air-dried for 30 min at room temperature before being rehydrated in assay buffer A (50 mM Tris–HCl, 3 mM MgCl₂, 0.2 mM ethylene glycol tetraacetic acid (EGTA), 100 mM NaCl, pH 7.4) for 10 min at room temperature. Sections were pre-incubated in assay buffer B [assay buffer A + 0.2 mM dithiothreitol, 1 μM 1,3-di-propyl-8-cyclopentylxanthine (DPCPX; #C-101, Sigma-Aldrich, DK), 0.5% w/v bovine serum albumin and 2 mM guanosine-5’-diphosphate (GDP; #G7127, Sigma-Aldrich)] for 20 min at room temperature. The sections were subsequently incubated in assay buffer B [assay buffer A + 0.2 mM dithiothreitol, 1 μM 1,3-di-propyl-8-cyclopentylxanthine (DPCPX; #C-101, Sigma-Aldrich, DK), 0.5% w/v bovine serum albumin and 2 mM guanosine-5’-diphosphate (GDP; #G7127, Sigma-Aldrich)] for 20 min at room temperature. The sections were subsequently incubated in assay buffer B + 40 pM [35S]-GTP·S (1250 Ci/mmol; NE0309250UC; PerkinElmer, DK) for 1 h at 25°C with neuropeptide Y (rat synthetic, Schafer-N, Copenhagen, DK) at 10−6 M to which the Y1 receptor antagonist BIBP3226 (10−6 M; #E6320, Bachem AG, Switzerland) and the Y5 receptor antagonist L-152,804 (10−8 M; #1382, Tocris Cookson, UK) were added to visu-alize functional Y2 binding. To confirm the specificity of this binding, the Y2 receptor antagonist BIBL0246 (10−6 M; #1700, Tocris Cookson, UK) was used to add neuropeptide Y receptor ligands. Because rAAV-Y2 vector treatment increased basal binding, we also tested whether this increase was mediated by Y2 receptors by determining basal binding in the presence of BIBL0246 (10−8 M). Non-specific binding was determined by incubation in buffer B without neuropeptide Y receptor ligands + 40 pM [35S]-GTP·S + 10 μM non-labelled GTP·S (#89378; Sigma-Aldrich). Neuropeptide Y receptor antagonists were dissolved in dimethyl sulphoxide (DMSO), and DMSO was also added to other incubation buffers (0.1% as the final concentration). For blocking experiments, the pre-incubation buffer also contained neuropeptide Y antagonists.

Incubation was terminated by 2× 5 min washing in ice-cold 50 mM Tris–HCl buffer (pH 7.4). Sections were dried and exposed to Kodak BioMax MR films together with 14C-microscales (Amersham Life Sciences) for 5 days at −20°C. Films were developed in Kodak GBX developer. Functional binding was quantified in the hippocampal den-tate gyrus, CA3 and CA1 of kindled and kainate-treated brains as described earlier (Christensen et al., 2006). Measurements were con ducted bilaterally in three to four adjacent sections per animal over the dorsal and ventral dentate gyrus (molecular layer), hippocampal CA3 (pyramidal layer and strata oriens, radiatum and lucidum) and CA1 (pyramidal layer and strata oriens and radiatum) at −4.68 mm to −5.64 mm in the coronal plane relative to bregma (Paxinos and Watson, 2005). Right- and left-side values were averaged per section and per animal.

**Electrical kindling**

Approximately 2 weeks after injections with rAAV-Y2 (n = 14) or rAAV-empty (n = 15) vectors, the rats were anaesthetized with 4-ketamine and xylazine. A bipolar stainless steel stimulating/recording electrode (Plastics One, Roanoke, VA, USA) was implanted stereotax-i-cally in the ventral hippocampus (anterior–posterior −4.8 mm, medial–lateral −5.2 mm, ventral −6.3 mm) and a recording electrode was placed in the piform cortex (anterior–posterior −2.3 mm, medial–lateral −6.0 mm, ventral −6.0 mm). A reference electrode was inserted into the temporal muscle and the electrodes were fixed to the skull with dental cement.

After 7–10 days of recovery (i.e. ~3 weeks after vector injection), the animals were subjected to rapid electrical hippocampal kindling. After connection to the recording system (MacLab/4e; AD Instruments, Milford, MA, USA), the electroencephalogram (EEG) of each animal was recorded continuously from the recording electrodes during kindling. First, the individual threshold was determined by delivering stimulations (1 s train consisting of 1 ms biphasic square wave pulses at 100 Hz) of increasing current in 10 μA steps (starting at 10 μA) until focal epileptiform afterdischarge activity of >5 s duration was detected by EEG recording. Subsequently, kindling was performed according to a previously described rapid kindling paradigm: 40 suprathreshold stimulations, 1 ms square-wave pulses of 400 μA intensity with 10 Hz intertrain frequency for 10 s every 5 min (Kopp et al., 1999; Sørensen et al., 2009). Behavioural seizures were scored accord-ing to Racine (1972): Grade 0 = arrest, wet dog shakes, normal behaviour; Grade 1 = facial twitches (nose, lips, eyes); Grade 2 = chewing, head nodding; Grade 3 = forelimb clonus; Grade 4 = rearing, falling on forelimbs; Grade 5 = imbalance and falling on side or back.

At 1 month after rapid kindling (i.e. 8 weeks after vector injection), the hippocampal afterdischarge threshold was determined again, and the rats received five rapid kindling stimulations with 5 min intervals, as described above (re-kindling). The following day, the animals were killed; their brains were rapidly removed, frozen on dry ice and stored at −80°C until further processing as described earlier. The location of kindling electrodes was checked and only animals with correctly placed electrodes were included.
Kainate-induced seizures

Three weeks after virus injections, rats treated with rAAV-Y2 (n = 7) and rAAV-empty (n = 7) vectors were injected subcutaneously with kainate (10 mg/kg; diluted in 0.9% isotonic saline; pH 7.4; Sigma-Aldrich) in the neck region and immediately placed in individual Plexiglas boxes (30 x 20 x 30 cm). The animals were observed for motor seizures as described earlier (Woldbye et al., 1997). Motor seizures were defined as clonic movements involving fore- and/or hind limbs of at least 15 s duration. Each rat was rated for 2 h by an observer unaware of the treatment condition, and latency to first motor seizure, percent time spent in motor seizures and status epilepticus were determined. Status epilepticus was defined as continuous clonic motor seizure activity of at least 10 min duration. The experiment was terminated 3 h after kainate administration with the decapitation of the animals. After killing, the brains were quickly removed and processed as described earlier.

Statistics

Most data were analysed using Student’s t-test or post hoc t-test after significant treatment effect in two-way ANOVA. In addition, the log-rank test was used for analysing mortality and number of stimulations to seizure grade since survival statistics are preferable for this type of data due to the so-called censoring (only some animals displayed mortality or the given seizure grade) (Altman, 1991). Extreme values defined as being more than three interquartile ranges below the 25th or above the 75th percentile (Statistical Package for the Social Sciences (SPSS) computer program) were excluded from statistical analysis. Data are presented as mean ± SEM if not stated otherwise. *P < 0.05 was considered statistically significant.

Results

Electrical kindling

Once we established in pilot experiments that overexpression of functional transgene Y2 receptors could be achieved in the hippocampus (data not shown), we asked whether and how this treatment affected recurrent seizures induced by rapid kindling in rats. The rAAV-Y2 vector treatment moderately but significantly decreased the average afterdischarge duration per kindling stimulation (Fig. 1A) and, more importantly, retarded kindling progression as revealed by reduced cumulative seizure grades (calculated as the mean seizure grade that animals experienced after a given number of kindling stimulations and maintenance thereafter until the next grade was reached; Fig. 1B). In addition, rAAV-Y2 vector treatment reduced the number of the severe Grade 4–5 seizures (Fig. 1C), and increased the number of stimulations necessary to reach Grade 3 or 4–5 seizures (Fig. 1D).

Next we asked whether overexpression of transgene Y2 receptors would also affect chronically increased excitability (maintenance of the kindled state) that has been shown to gradually develop 4 weeks after initial exposure of the animals to rapid kindling (Elmer et al., 1998). Animals were subjected to five re-kindling stimulations 4 weeks after the initial rapid kindling stimulations. During the first stimulation with threshold current inducing afterdischarges, the number of secondary after discharges (Fig. 2A) and their duration (data not shown) were found to be significantly decreased in the rAAV-Y2 group as compared to rAAV-empty treatment (Fig. 2A). Moreover, kindling progression during re-kindling was retarded as revealed by reduced cumulative seizure grades (Fig. 2B). In addition, rAAV-Y2 vector treatment reduced the total number of Grade 4–5 seizures (Fig. 2C) and increased the number of stimulations necessary to reach Grade 2–3 and Grade 4–5 seizures during re-kindling (Fig. 2D).

No alterations in afterdischarge durations induced by Y2 over expression in the hippocampus were observed in the piriform cortex, where an additional recording electrode was implanted, indicating focal effect of the transgene (Supplementary Fig. 1).

We then asked whether the transgene Y2 effect could be further enhanced by additional overexpression of transgene neuropeptide Y (i.e. rAAV-Y2/neuropeptide Y). To address this question, we injected a group of rats with a mixture of rAAV-Y2 and rAAV-neuropeptide Y (see ‘Methods’ section in
Supplementary material, and subjected these animals to rapid electrical kindling. When rAAV-Y2 treatment was combined with rAAV-neuropeptide Y, in addition to rAAV-Y2 effects described in the earlier section, some additional kindling-induced seizure parameters were also affected. Thus, the rAAV-Y2/neuropeptide Y combination modestly, but significantly, increased the threshold current necessary to induce afterdischarges (Fig. 3), and reduced the afterdischarge duration at threshold current intensity (Supplementary Fig. 2A) as compared to rAAV-empty or rAAV-Y2. In addition, rAAV-Y2/neuropeptide Y treatment significantly reduced the average afterdischarge duration of both mild (Grade 1–3) and severe (Grade 4–5) seizures compared to rAAV-empty or rAAV-Y2 (Supplementary Fig. 2B–D).

Kainate-induced seizures

To explore whether Y2 receptor overexpression was also effective at suppressing seizures in other models of epilepsy, we used a subcutaneous kainate-induced status epilepticus model in rats. In this model, rAAV-Y2 vector treatment also exerted a seizure-suppressant effect as compared to rAAV-empty (Fig. 4). Thus, the rAAV-Y2 vector significantly increased the latencies to first motor seizure and to status epilepticus (Fig. 4A), as well as reduced relative time (fraction of whole observation time expressed as percent) spent by the animals in motor seizures (Fig. 4B). In addition, rAAV-Y2 vector treatment significantly
Overexpression of Y2 receptors by the recombinant adeno-associated viral-Y2 vector

To confirm overexpression of Y2 receptors in experimental animals, we assessed transduction of the transgenes using in situ hybridization, Y2 receptor binding and Y2 receptor functional binding assays.

In situ hybridization

The rAAV-Y2 vector induced a prominent increase in mRNA levels for the Y2 receptor in the hippocampal dentate gyrus, CA3 and CA1 regions in kindled rats at 8 weeks after injection, as compared to rAAV-empty vector treated animals (Fig. 5A, B, M and N). Y2 mRNA expression was mainly restricted to the principal cell layers of the hippocampus, including the dentate granule cell layer and pyramidal layers of CA1–CA3, suggesting neuronal specificity as expected from the neuron-specific enolase promoter of the vector construct. A dotted-like hybridization signal was also observed in fibre layers of the dentate gyrus and CA1–CA3, most likely representing Y2 mRNA overexpression in transduced interneurons. Similar effects of the rAAV-Y2 vector were observed in seizure-naive rats (data not shown).

Y2 binding

We examined whether increased Y2 mRNA levels were translated into elevated Y2 receptor binding. Indeed, increased Y2 receptor binding ([35S]-peptide YY + Leu31, Pro34-neuropeptide Y) was observed in all parts of the hippocampus of kindled rats injected with rAAV-Y2 compared to rAAV-empty treated rats (Fig. 5C–F and O–Q). The distribution of Y2 binding in the hippocampus closely resembled that of Y2 mRNA, except that binding was not confined to principal cell layers, but distributed also to fibre layers, indicating that there was increased production and membrane insertion of transgene Y2 receptor proteins in these animals. Similar results were observed in seizure-naive rats (data not shown).

Functional Y2 binding

To confirm that rAAV-Y2 vector-induced gene transfer resulted in overexpression of functional Y2 receptors, [35S]-GTP[S] functional Y2 binding assay (neuropeptide Y + Y1 antagonist + Y5 antagonist) was conducted. We found that functional Y2 binding was strongly increased after injection of rAAV-Y2 as compared to the rAAV-empty vector (in kindled rats Figs 5G, H and 6A–D; in kainate-treated rats Fig. 7A–E). Addition of the Y2 receptor antagonist BIIE0246 blocked rAAV-Y2-induced functional Y2 binding (Figs 5K, L and O–Q), confirming that it was specific for Y2 receptors. Functional Y2 binding was quantified in the hippocampal dentate gyrus, CA3 and CA1 (in kindled rats Figs 6E; in kainate-treated rats Fig. 7F). Treatment with the rAAV-Y2 vectors resulted in significant increases in all measured regions (dentate gyrus: by 224 and 345%, CA3: by 165 and 235%, CA1: by 490 and 317% for kindled and kainate-treated rats, respectively) as compared to rAAV-empty.

We also observed that basal [35S]-GTP[S] binding (without addition of neuropeptide Y ligands) in animals treated with rAAV-Y2 was increased (in kindled rats Figs 5I, J and 6C, D, G, H; in kainate-treated rats Fig. 7B, E). Quantification of basal binding (in kindled and kainate-treated rats) as compared to rAAV-empty vector-treated rats showed significant increases after injection of the rAAV-Y2 vector in the CA3 (by 32 and 39%; kindled and kainate-treated rats, respectively) and CA1 (by 69 and 42%), but only reached significance in the dentate gyrus of kindled rats (by 33%) (Figs 6F and 7G). The rAAV-Y2 vector-induced increases in basal binding were blocked by the addition of BIIE0246 (Fig. 6H and I), indicating that, even under basal physiological conditions (i.e. during the absence of seizure-induced increase in endogenous neuropeptide Y release), transgene Y2 receptors mediate increased neuropeptide Y signalling. Similar increases in [35S]-GTP[S] functional Y2 binding and basal binding were observed in seizure-naive rats after rAAV-Y2 vector injection (data not shown).

In rare cases, increased Y2 functional binding was also observed dorsally outside the hippocampus along the vector injection track.
in the adjoining neocortex, most likely caused by viral particles escaping from the injection needle during its insertion into or withdrawal from the brain tissue (data not shown). In some cases, extrahippocampal Y2 functional binding of varying intensity was seen ventrolaterally (Fig. 6A) in the adjoining lateral entorhinal cortex, caudal piriform cortex, amygdalo-hippocampal area, amygdalo–piriform transition area and/or posteromedial/lateral cortical amygdaloid nuclei (Paxinos and Watson, 2005).

Similar to the rAAV-Y2 vector treatment alone, intrahippocampal injection of the rAAV-Y2/neuropeptide Y combination strongly increased Y2 (but also neuropeptide Y) mRNA expression, Y2 binding and Y2 functional binding both in seizure-naı̈ve (Supplementary Fig. 3) and kindled rats. In the latter, quantification showed increased Y2 functional binding in rAAV-Y2/neuropeptide Y-treated rats by 224% in dentate gyrus, 85% in CA3 and 370% in CA1 and increased basal functional binding in dentate gyrus by 74%, in CA3 by 40% and in CA1 by 118%.

Discussion

Here we show for the first time that overexpression of functional Y2 receptors can be achieved in the hippocampal formation using
rAAV vectors. Moreover, we demonstrate that this hippocampal Y2 overexpression exerts a pronounced seizure-suppressant effect. Intrahippocampal injection of the rAAV-Y2 vector resulted in a prominent increase in Y2 mRNA expression, Y2 receptor binding and, most importantly, in functional Y2 receptor binding. The bulk of vector-induced transgene overexpression was confined to the hippocampus, although some transgene was also detected in adjoining regions (e.g. piriform and entorhinal cortices). Therefore, it

![Image](https://example.com/image.png)

**Figure 6** Functional Y2 receptor [³⁵S]GTPγS-binding 8 weeks after bilateral intrahippocampal injection of rAAV-Y2 or rAAV-empty in kindled rats. (A) Increased functional Y2 binding after injection of rAAV-Y2 or (B) rAAV-empty vector at the level of quantification. (C and D) Basal binding of rats from A and B, respectively. (E) Increased functional Y2 receptor [³⁵S]GTPγS-binding and (F) corresponding basal binding quantified in hippocampal dentate gyrus (DG), CA3 and CA1 after injection of rAAV-Y2 as compared to rAAV-empty (*P < 0.05, ***P < 0.001, Student's t-test following significant treatment effect in two-way ANOVA). Data are mean ± SEM (n = 13). (G) Pseudocoloured autoradiograms showing hippocampal basal [³⁵S]GTPγS binding (i.e. without the addition of neuropeptide Y ligands) after rAAV-empty or (H) rAAV-Y2 vector injection. Colours: blue, green, yellow and red indicate progressively increasing binding levels. Note that basal binding is increased in rAAV-Y2 vector-treated animals, and this increase is blocked with BIIE0246 (I), indicating that functional signalling via transgene Y2 receptors is increased under basal conditions. Scale bars = 1 mm (A–D) and 2 mm (G–I).
could not be completely ruled out that the latter may have contributed to the observed seizure-suppressant effects of transgene Y2. Increase in Y2 functional binding induced by the rAAV-Y2 vector was seen at both 3 and 8 weeks after viral vector injection and appeared to be of similar magnitude in kindled, kainate-treated and seizure-naive rats, suggesting long-lasting transduction, which was not affected by seizures. The vector-induced increase in functional Y2 binding was blocked by the Y2 antagonist BIIE0246, confirming that the functional response was Y2 receptor-mediated. Basal hippocampal functional binding in the [35S]GTPγS binding assay (i.e. without addition of exogenous neuropeptide Y) was also increased by the rAAV-Y2 vector treatment. This increase in basal binding was blocked by the BIIE0246 application, suggesting activation of some transgene Y2 receptors by endogenous neuropeptide Y present in the slide preparations during the functional assay. rAAV-Y2 vector-induced increased basal binding indicates that there could be elevated signalling via Y2 receptors in rAAV-Y2-treated hippocampi under normal conditions, and not exclusively during high-frequency-induced massive release of neuropeptide Y (e.g. during seizures) (Sørensen et al., 2008b) which, in the functional assay, is mimicked by exogenously added neuropeptide Y. It seems plausible that increased basal signalling via transgene Y2 receptors contributes to seizure-suppressant effects of the rAAV-Y2 vector.

Potential side effects of overexpressing the Y2 transgene remain to be determined, particularly on hippocampal-related learning and memory, in which Y2 receptor activation has been shown to play a role (Redrobe et al., 2004). Our previous results suggest that increased Y2 receptor signalling induced by transgene neuropeptide Y could lead to transient deficits in learning and memory (Sørensen et al., 2008a). However, we have also shown that already compromised cognitive function in epileptic animals does not appear to be further exacerbated by transgene neuropeptide Y (Sørensen et al., 2009).

Previous studies show that adeno-associated viral vector-based transduction of the neuropeptide Y gene exerts a long-lasting seizure-suppressant effect in the hippocampus (Richichi et al., 2004; Noé et al., 2008, 2010). Transgene neuropeptide Y, apart from binding to Y2 receptors, which mediates seizure-suppressant effects, can also bind to Y1 receptors that seem to have seizure-promoting properties (Vezzani et al., 1999). However, binding of transgene neuropeptide Y to Y1 receptors may be less of a problem in patients with temporal lobe epilepsy, in which the level of Y1 receptors has been shown to be lower (Furtinger et al., 2001). Moreover, rAAV-neuropeptide Y vector treatment by itself may decrease Y1 levels in the hippocampus as has been shown in rats (Noé et al., 2008). On the other hand, in patients with temporal lobe epilepsy without hippocampal sclerosis, a decrease in dentate Y1 binding is not observed (Furtinger et al., 2001). Furthermore, even activation of remaining decreased levels of Y1 receptors by transgene neuropeptide Y may be enough to promote seizures to some extent. If this is the case, transgene neuropeptide Y may counteract (by acting on Y2 receptors) and promote (by acting on Y1 receptors) seizures at the same time.
time. Overexpression of Y2 receptors alone or in combination with neuropeptide Y may thus favour neuropeptide Y signalling through seizure-suppressant Y2 receptors. Our present data support this idea, demonstrating that Y2 overexpression in the hippocampus exerts seizure-suppressant effects in two different epilepsy models. This is also consistent with the known anti-epileptic role of endogenous Y2 receptors (El Bahh et al., 2005) and indicates that gene therapy using Y2 receptor overexpression could be an alternative approach to neuropeptide Y (Richichi et al., 2004; Noé et al., 2008, 2010).

This study demonstrates seizure-suppressant effects of viral vectors applied prior to seizure induction that may not be a relevant scenario for the human epilepsy condition with already established and often progressive spontaneous, recurrent seizures. However, the previously reported effect of rAAV-neuropeptide Y on suppressing spontaneous recurrent seizures (Noé et al., 2008) provides some favourable prognosis for rAAV-Y2 treatment to be effective against spontaneous seizures as well. Our present results with re-kindling provide further indirect evidence for long-lasting maintenance of the seizure-suppressant effect by transgene Y2 receptors in chronic epileptic conditions. Yet another aspect that requires critical consideration is the upregulated Y2 receptor binding reported in patients with temporal lobe epilepsy with hippocampal sclerosis (Furtinger et al., 2001). This may render gene therapy-based overexpression of Y2 receptors in human patients unnecessary since endogenous Y2 receptors are already upregulated. Arguing in favour of rAAV-Y2 gene therapy, in patients with temporal lobe epilepsy without hippocampal sclerosis, such upregulation of endogenous Y2 receptors was not detected (Furtinger et al., 2001). Moreover, even in sclerotic temporal lobe epilepsy patients, Y2 hippocampal binding was estimated to increase by only 43–48% as opposed to 165–490% observed for functional Y2 binding in rAAV-Y2 treated animals in the present study. Taken together, it seems reasonable to expect that vector-induced overexpression of functional Y2 receptors may be beneficial for spontaneous seizure control in animal models and possibly in patients with temporal lobe epilepsy.

Our present study also demonstrates that simultaneous injection of rAAV-Y2 and rAAV-neuropeptide Y into the hippocampus does not have a simple additive effect as expected, but rather differentially strengthens seizure-suppressant action of rAAV-Y2, as revealed by higher afterdischarge threshold and shorter afterdischarge durations during both mild (Grade 1–3) and severe (Grade 4–5) generalized seizures. In conclusion, the present study suggests for the first time that rAAV-vector-induced overexpression of Y2 receptors (alone or in combination with neuropeptide Y) is a viable alternative gene therapy approach for epilepsy and underscores the potential of receptor gene transduction as a possible target for novel treatment strategies in brain diseases.

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Supplementary material

Supplementary material is available at Brain online.

References


