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Neuropeptides and neurotrophic factors in epilepsy: seizure suppressant actions of VEGF, NPY and galanin

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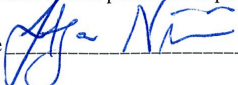Fakultetsopponent:
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Abstract

Epilepsy is a severe chronic neurological disorder, affecting about 1% of the population. The disease is manifested by spontaneous recurrent seizures, caused by hypersynchronized neuronal activity due to imbalance in the brain between the inhibition and excitation. Symptoms are treated with anti-epileptic drugs, but unfortunately, 30-40% of patients respond poorly to current treatment. Therefore, more efficient treatments with disease modifying or curing effects need to be developed. In the brain there are naturally occurring endogenous proteins affecting the survival and growth of brain cells, called neurotrophic factors. There are also neuropeptides, which are involved in signaling between brain cells. Both neurotrophic factors and neuropeptides have been shown to have an important role in suppressing epileptic activity. In this thesis, we focused on one neurotrophic factor and two neuropeptides that have demonstrated anti-epileptic properties; vascular endothelial growth factor (VEGF), neuropeptide Y (NPY), and galanin. To study these molecules, we have used different animal models of epilepsy and investigated the effect on epileptic seizures by enhancing the expression of these endogenously occurring proteins and/or their receptors in the brain of experimental animals. The levels of NPY, NPY receptors (Y2 or Y5), galanin and VEGF receptor 2 (Flk-1) were enhanced by using three different strategies. In the first study, we used genetically modified transgenic mice that increase the expression of VEGF receptor 2. In the second study, genetically modified cells were developed to release galanin. These cells were then placed into special capsules built of semipermeable membranes and subsequently implanted in the brain. The cells could thereby release galanin into the tissue through the membrane and in turn receive nutrients from the surrounding tissue. This approach has the advantage that, in case of adverse effects, the capsules filled with genetically modified cells easily can be removed from the brain. In the third and fourth studies we examined the effects of the combinatorial gene therapy of NPY and either Y2 or Y5 receptors on epileptic seizures by enhancing their expression with viral vectors. By enhancing the expression of the mentioned proteins and receptors in the brain, we have been able to reduce the number, duration and severity of epileptic seizures in animal models. Increased expression of VEGF receptor 2 (Flk-1) or increased extracellular levels of galanin by encapsulated cell biodelivery (ECB) inhibited focal epileptic seizures. The combinatorial treatment with NPY and either Y2 or Y5 receptors, on the other hand, also affected generalized seizures. All these approaches, particularly the viral vector-based treatment, have a potential to be developed into an alternative treatment strategies for epilepsy.

Key words: Epilepsy, Gene Therapy, Neuropeptide Y, Y2 receptor, Y5 receptor, Galanin, Encapsulated cell biodelivery, Vascular endothelial growth factor, Flk-1, Hippocampus

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Neuropeptides and neurotrophic factors in epilepsy:
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Cover artwork
The front cover is designed by my childhood friend and talented graphic designer Anna Cronquist

To my wonderful family
for your endless support and love

*An idea not coupled with action will never get
any bigger than the brain cell it occupied.*
Arnold H. Glasow

Index

Summary

Epilepsy is a severe chronic neurological disorder, affecting about 1% of the population. The disease is manifested by spontaneous recurrent seizures, caused by hypersynchronized neuronal activity due to imbalance in the brain between the inhibition and excitation. Symptoms are treated with anti-epileptic drugs, but unfortunately, 30-40% of patients respond poorly to current treatment. Therefore, more efficient treatments with disease modifying or curing effects need to be developed.

In the brain there are naturally occurring endogenous proteins affecting the survival and growth of brain cells, called neurotrophic factors. There are also neuropeptides, which are involved in signaling between brain cells. Both neurotrophic factors and neuropeptides have been shown to have an important role in suppressing epileptic activity. In this thesis, we focused on one neurotrophic factor and two neuropeptides that have demonstrated anti-epileptic properties; vascular endothelial growth factor (VEGF), neuropeptide Y (NPY), and galanin.

To study these molecules, we have used different animal models of epilepsy and investigated the effect on epileptic seizures by enhancing the expression of these endogenously occurring proteins and/or their receptors in the brain of experimental animals. The levels of NPY, NPY receptors (Y2 or Y5), galanin and VEGF receptor 2 (Flk-1) were enhanced by using three different strategies. In the first study, we used genetically modified transgenic mice that increase the expression of VEGF receptor 2. In the second study, genetically modified cells were developed to release galanin. These cells were then placed into special capsules built of semipermeable membranes and subsequently implanted in the brain. The cells could thereby release galanin into the tissue through the membrane and in turn receive nutrients from the surrounding tissue. This approach has the advantage that, in case of adverse effects, the capsules filled with genetically modified cells easily can be removed from the brain. In the third and fourth studies we examined the effects of the combinatorial gene therapy of NPY and either Y2 or Y5 receptors on epileptic seizures by enhancing their expression with viral vectors.

By enhancing the expression of the mentioned proteins and receptors in the brain, we have been able to reduce the number, duration and severity of epileptic

seizures in animal models. Increased expression of VEGF receptor 2 (Flk-1) or increased extracellular levels of galanin by encapsulated cell biodelivery (ECB) inhibited focal epileptic seizures. The combinatorial treatmentwith NPY and either Y2 or Y5 receptors, on the other hand, also affected generalized seizures. All these approaches, particularly the viral vector-based treatment, have a potential to be developed into an alternative treatment strategies for epilepsy.

Populärvetenskaplig sammanfattning

Epilepsi är en allvarlig kronisk neurologisk sjukdom, som drabbar ungefär 1% av befolkningen. Sjukdomen yttrar sig genom plötsliga återkommande krampanfall, som beror på en obalans i hjärnan mellan hämmande (inhibitoriska) och stimulerande (excitatoriska) nervimpulser. När de stimulerande impulserna tar överhand leder detta till överstimulering, också kallad för hyperexcitabilitet, som ger upphov till epileptiska kramper. Symptomen behandlas med väletablerade antiepileptiska läkemedel men mer än var tredje patient svarar inte som förväntat på behandlingen utan den epileptiska aktiviteten blir återkommande. För att öka livskvalitén hos epilepsipatienter genom att förebygga epileptiska kramper, samt att kunna ersätta en mångårig medicinsk behandling med svåra biverkningar krävs nya och mer effektiva behandlingsalternativ.

I hjärnan finns naturligt förekommande proteiner, ett exempel är neurotrofiska faktorer, som påverkar överlevnad och tillväxt av hjärnceller. Det finns också neuropeptider, som påverkar signalering mellan hjärnceller. Både neurotrofiska faktorer och neuropeptider har visat sig ha en hämmande roll vid epileptisk aktivitet i hjärnan. I den här avhandlingen har vi fokuserat på två neuropeptider och en neurotrofiska faktor som har uppvisat anti-epileptiska egenskaper, nämligen vaskulär endotelcellstillväxtfaktor (VEGF), neuropeptid Y (NPY) och galanin. För att studera dessa proteiner närmare, har vi använt oss av djurmodeller för epilepsi. Genom att förstärka uttrycket av dessa naturligt förekommande ämnen samt deras receptorer i hjärnan på försöksdjur har vi undersökt effekten på epileptiska kramper. Nivåerna har förstärkts genom tre olika strategier. I den första studien användes transgena möss, där en genetisk förändring påverkar uttrycket av VEGF receptor 2 (Flk-1). I den andra studien utvecklades genetiskt modifierade celler för att frisätta galanin som sedan placerades i speciella membran och implanterades i hjärnan i området där den epileptiska aktiviteten uppkommer. Cellerna kan på så sätt frisätta galanin genom det semipermeabla membranet samtidigt som de inkapslade cellerna kan ta upp näringsämnen från den omkringliggande vävnaden för överlevnad. De inkapslade cellerna integreras inte med den kroppsegna vävnaden, vilket möjliggör att de inkapslade cellerna kan avlägsnas vid eventuella biverkningar. I den tredje och fjärde studien undersöktes den hämmande effekten av epileptiska kramper genom att förstärka uttrycket av

både NPY och antingen Y2 eller Y5 receptorer. När NPY uttrycks i kombination med en av sina receptorer ökar effekten av behandlingen.

Genom att förstärka uttrycket av ovannämnda proteiner samt deras receptorer i hjärnan har vi kunnat reducera antalet, varaktigheten och svårighetsgraden av epileptiska kramper. Med ökat uttryck av VEGF receptor 2 (Flk-1) och med galanin har mildare epileptiska kramper kunnat hämmas. Den kombinatoriska behandlingen, med NPY och antingen Y2 eller Y5 receptorer påverkade även svårare generaliserade kramper, vilket gör den lämplig som en alternativ behandling för epilepsi.

Original papers and manuscripts

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. **Nikitidou L**, Kanter-Schlifke I, Dhondt J, Carmeliet P, Lambrechts D, Kokaia M. *VEGF receptor-2 (Flk-1) overexpression in mice counteracts focal epileptic seizures*, PLoS One. 2012;7(7):e40535.

II. **Nikitidou L**, Torp M, Fjord-Larsen L, Kusk P, Wahlberg LU, Kokaia M. *Encapsulated galanin-producing cells suppress focal epileptic seizures in the hippocampus*, Submitted.

III. Gøtzsche CR, **Nikitidou L**, Sørensen AT, Olesen MV, Sørensen G, Christiansen SH, Ängehagen M, Woldbye DP, Kokaia M. *Combined gene overexpression of neuropeptide Y and its receptor Y5 in the hippocampus suppresses seizures*, Neurobiol Dis. 2012 Jan;45(1):288-96.

IV. **Nikitidou L**, Gøtzsche CR, Christiansen SH, Woldbye DP, Kokaia M. *Neuropeptide-receptor combinatorial gene therapy for temporal lobe epilepsy in rats*, Manuscript.

Publications not included in the thesis

Ledri M, **Nikitidou L**, Erdelyi F, Szabo G, Kirik D, Deisseroth K, Kokaia M. *Altered profile of basket cell afferent synapses in hyper-excitable dentate gyrus revealed by optogenetic and two-pathway stimulations*, Eur J Neurosci. 2012 Jul;36(1):1971-83.

Olesen MV, Christiansen SH, Gøtzsche CR, **Nikitidou L**, Kokaia M, Woldbye DP. *Neuropeptide Y Y1 receptor hippocampal overexpression via viral vectors is associated with modest anxiolytic-like and proconvulsant effects in mice*, J Neurosci Res. 2012 Feb;90(2):498-507.

Woldbye DP, Ängehagen M, Gøtzsche CR, Elbrønd-Bek H, Sørensen AT, Christiansen SH, Olesen MV, **Nikitidou L**, Hansen TV, Kanter-Schlifke I, Kokaia M. *Adeno-associated viral vector-induced overexpression of neuropeptide Y Y2 receptors in the hippocampus suppresses seizures*, Brain. 2010 Sep;133(9):2778-88.

Sørensen AT, **Nikitidou L**, Ledri M, Lin EJ, During MJ, Kanter-Schlifke I, Kokaia M. *Hippocampal NPY gene transfer attenuates seizures without affecting epilepsy-induced impairment of LTP*, Exp Neurol. 2009 Feb;215(2):328-33.

List of abbreviations

AAV	Adeno-Associated Virus
aCSF	artificial Cerebro Spinal Fluid
AED	Anti-Epileptic Drug
AP	AnteroPosterior
ARPE	human Retinal Pigment Epithelial
BSA	Bovine Serum Albumin
CA	Cornu Ammonis
cDNA	complimentary DeoxyriboNucleic Acid
DAB	3,3-DiAminoBenzidine
DV	DorsoVentral
ECB	Encapsulated Cell Biodelivery
EEG	ElectroEncephaloGram
fEPSP	field Excitatory PostSynaptic Potential
Flk-1	Fetal Liver Kinase receptor 1
GABA	Gamma-AminoButyric Acid
GFAP	Glial Fibrillary Acidic Protein
GTP	Guanosine-5´-TriPhisphate
KA	Kainic Acid
ML	MedioLateral
MRI	Magnetic Resonance Imaging
NPY	Neuropeptide Y
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	ParaFormAldehyde
PPF	Paired-Pulse Facilitation
RNA	RiboNucleic Acid
RT	Room Temperature

rAAV	recombinant Adeno-Associated Virus
SE	Status Epilepticus
TLE	Temporal Lobe Epilepsy
VEGF	Vascular Endothelial Growth Factor

1 Introduction

1.1 Epilepsy

1.1.1 Definition of epilepsy

One of the first observations of epilepsy in humans was described by the Greek physician Hippocrates in 400 B.C. in his work, *"On the Sacred Disease"*. In the book, he stresses the fact that the disease has a natural cause and is not of divine nor sacred origin: *"It is thus with regard to the disease called Sacred: it appears to me to be nowise more divine nor more sacred than other diseases..."*. Despite this, even until the 1920's people with epilepsy were not allowed to get married or have children. It was thought that epilepsy was contagious, so to minimize the spread of the disease all epilepsy patients were isolated in mental hospitals. With time the scientific discoveries about the function of the brain allowed people with epilepsy to live a free life outside the mental hospitals.

Today, it is known that epilepsy is a serious chronic neurological disorder affecting about 1% of the general population. International League Against Epilepsy (ILAE) defines epilepsy as *"a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by the neurobiologic, cognitive, psychological, and social consequences of this condition"* (Fisher et al., 2005). Epilepsy is a family of disorders that can develop in all ages, but is most commonly diagnosed in children and people over the age of 65. Epilepsy can develop due to inherited genetic defects or after various insults to the brain, such as stroke, brain tumors, infections, head injuries and degenerative conditions (e.g. Alzheimer's disease). However, in most cases the cause of the disorder is unknown, also referred to as idiopathic epilepsy (Hauser et al., 1991;

Duncan et al., 2006). Regardless of the initial cause, epilepsy is characterized by appearance of epileptic seizures, defined as *"a transient occurrence of signs and/ or symptoms due to abnormal excessive or synchronous neuronal activity in the brain"* (Fisher et al., 2005). The epileptic seizures manifest in different ways in different people and depend on which part(s) of the brain that get affected. The symptoms can vary from lighter attention deficit, deafness and visual loss to periods of momentary loss of awareness and consciousness and severe generalized convulsions. Epileptic seizures are classified as partial seizures or generalized seizures (Seino, 2006). Partial seizures are limited to one brain area (the epileptic focus), but can spread to other areas of the brain, and transforms to generalized seizures that leads to loss of consciousness. If the seizures are lasting longer than 30 minutes without full recovery of consciousness, a life-threatening condition arises called status epilepticus (SE), which needs an immediate pharmacological treatment (Epilepsy Foundation of America, 1993).

In adults, the most common form of epilepsy is temporal lobe epilepsy (TLE) and manifests itself with initial partial seizures, which often develop into secondary generalization. In TLE the initiation of epileptic seizures is confined to a brain area in the temporal lobe, most often involving the hippocampus, amygdala or neocortex (Engel, 2001).

1.2 Organization of the hippocampal formation

The hippocampus is named after its shape resembling a seahorse. In medial temporal lobe epilepsy the epileptic focus is often localized to the hippocampus. In each side of the brain (in the different hemispheres) one hippocampus is located (Fig. 1). The hippocampus is a part of the limbic system and is an important structure in the brain involved in e.g. memory formation and navigation (Martin & Clark, 2007; Buzsaki & Moser, 2013).

The hippocampus is organized into three main subfields; the cornu ammonis (CA) 1, CA3 and the dentate gyrus. These three regions comprise the trisynaptic pathway, which involves three glutamatergic synapses (Fig. 1) (Andersen et al., 1969). First, afferents from the entorhinal cortex innervate the granule cell distal dendrites in the dentate gyrus via the perforant path. Then, the axons of the granule cells (the mossy fibers) project to proximal apical dendrites of CA3 pyramidal cells and subsequently the CA3 pyramidal cell axons (the Schaffer collaterals) innervate the proximal apical dendrites of CA1 pyramidal cells. The
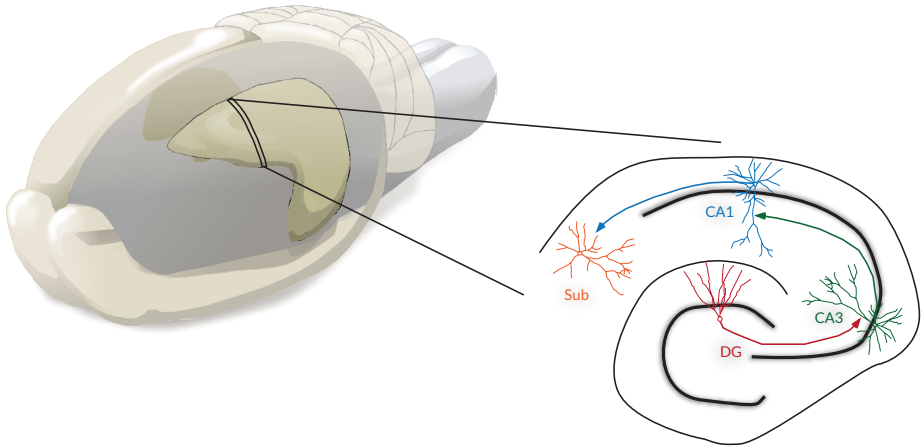
Figure 1 Location of the hippocampus in the rodent brain and a schematic of the organization of the rodent hippocampus. The trisynaptic pathway is illustrated. *DG* - Dentate Gyrus, *CA* - Cornus Ammonis, *Sub* - Subiculum

CA1 pyramidal cells in turn make contact with pyramidal cells in the subiculum and from there neurons connect to the entorhinal cortex, completing the pathway (Witter & Amaral, 2004). Moreover, the hippocampus receives different inputs from cholinergic, noradrenergic, serotonergic and dopaminergic projections (Cobb & Davies, 2005; Wisman et al., 2008).

The hippocampus is a plastic structure undergoing structural changes e.g. following epileptic seizures (Glass & Dragunow, 1995). In TLE patients, one common structural change is hippocampal sclerosis, which constitutes extensive cell loss and glial cell activation. Hippocampal sclerosis has been suggested to arise after prolonged seizure activity due to excitotoxicity in which neurons are killed and damaged because of excessive neurotransmitter levels (e.g. glutamate) (Fisher et al., 1998). On the other hand, cell death has also been proposed to be the cause of epileptic seizures (Kalviainen & Salmenpera, 2002; de Lanerolle & Lee, 2005). Another structural change in the hippocampus after prolonged seizure activity is synaptic reorganization. One example is mossy fiber sprouting, which is known to form new axon collaterals of granule cells in the dentate gyrus and create excitatory connections with nearby granule cells (Dudek & Shao, 2004). Mossy fiber sprouting is therefore considered to be pro-epileptogenic (Buckmaster et al., 2002; Nadler, 2003).

Taken together, the hippocampus seems to be a suitable structure for local

targeting of TLE treatment since inhibition of excessive neuronal activity generated in this brain area could suppress the epileptic seizures and the plastic structural changes.

1.3 Treatment of epilepsy

1.3.1 Current treatment strategies

The most common treatment strategy for epilepsy is the use of anti-epileptic drugs (AEDs). All AEDs are designed to decrease the severity, duration and frequency of the epileptic seizures by reducing the hyperexcitability in the brain and to recreate a balance in the neuronal network between excitation and inhibition (Avoli, 1983; Mody et al., 1992). These effects of AEDs can be achieved by increasing the inhibitory synaptic transmission via gamma-aminobutyric acid (GABA), decreasing excitatory glutamatergic transmission or reducing excitability by modulation of sodium channels generating action potentials. However, a major problem is that AEDs only control the symptoms (the epileptic seizures), while the progression of the disorder is not modified. More importantly, about 40 % of the patients and particularly those with TLE, respond poorly to the treatment and develop pharmacoresistance to AEDs. For a minority of these patients, surgical resection of the pre-determined seizure focus serves as the last treatment alternative (Engel et al., 2003). In addition, AEDs often give rise to several side effects due to the systemic administration. Therefore, the development of novel treatment strategies for epilepsy is highly needed.

To limit side effects and increase the efficacy, the treatment should ideally target only brain areas involved in the epileptic seizure generation and propagation. As previously described, in TLE the seizure focus is confined to one specific region (e.g. the hippocampus), which would be a favorable target for this type of treatment strategy.

1.3.2 Alternative treatment strategies

Currently, several treatment strategies to target specific regions involved in seizure generation and propagation are investigated for TLE. This type of treatment approaches includes gene therapy and encapsulated cell biodelivery (ECB) devices.

Gene therapy is divided into two categories; *ex vivo* and *in vivo*. For *ex vivo*
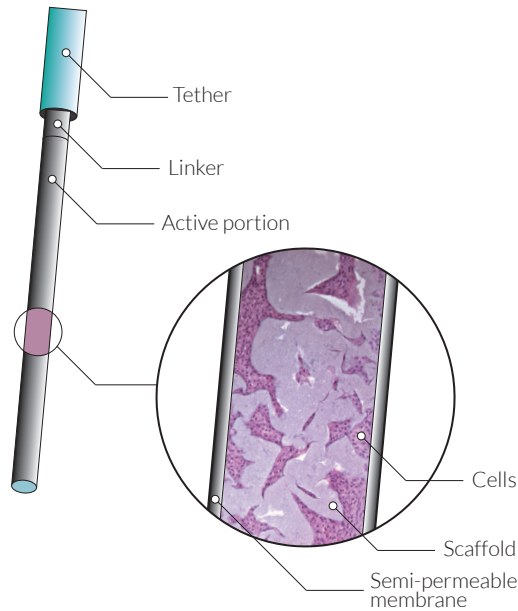
Figure 2 Illustration of the encapsulated cell biodelivery device showing the semi-permeable membrane filled with cells and held in place with scaffold foam.

gene therapy genetic material is inserted into cultured cells (*in vitro*), which are subsequently transplanted into the host tissue (*in vivo*) for sustainable therapeutic action. *In vivo* gene therapy, on the other hand, introduce the genetic material directly to the host brain cells. A common tool for the gene therapy approach is the viral vector, most common ones being lentiviral or adeno-associated viral (AAV) vectors. Lentiviral vectors have the ability to accommodate large transgene constructs (up to 10 kb), but the infection is more localized to a restricted brain area. AAVs on the other hand, can incorporate smaller transgene constructs (about 4.7 kb), but *in vivo* can spread more extensively to cover larger brain regions with a single viral vector injection. However, an important concern of this therapeutic strategy is an insufficient control of the transgene levels.

An alternative approach is *ex vivo* gene therapy, where the release of the therapeutic protein is more controlled, because of the possibility to titrate transgene expression *in vitro*. ECB devices consist of a semipermeable membrane filled with genetically modified cells that produce and release the therapeutic molecule (Fig. 2). Through the semipermeable membrane extracellular fluids can be exchanged between the surrounding host tissue and the encapsulated cells.

This enables a long-term viability of the encapsulated cells and a continuous local secretion of the therapeutic molecule into the host brain. Compared to AAVs, ECB devices have the advantage that if side effects appear the devices can be retracted, thereby eliminating the unwanted effects caused by the treatment.

The *ex vivo* and *in vivo* approaches are used to increase a therapeutic compound locally in the brain. Several therapeutic genes have been tested previously, often endogenous neuropeptides known to provide a neuroprotective effect or modulate the excitability in the brain (McCown, 2006; Kanter-Schlifke et al., 2007a; Paradiso et al., 2011).

1.4　Endogenous anti-epileptic agents

Neurotrophic factors are proteins that are important for growth, development and survival of neurons, one example of this type of protein being vascular endothelial growth factor (VEGF) (Sondell et al., 2000; Lambrechts et al., 2003; Lee et al., 2007). Neuropeptides, on the other hand, are important molecules involved in neuronal signaling. In the present work, we have investigated the seizure suppressant potential of VEGF, and two neuropeptides, neuropeptide Y (NPY) and galanin. VEGF, NPY and galanin have been suggested to decrease the excitability in the brain and thereby change the susceptibility for epileptic seizures (Gall et al., 1991).

1.4.1　Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is a homodimeric glycoprotein and, as the name indicates, it was first considered as an endothelial cell mitogen that promotes blood vessel formation. However, with time VEGF has been shown to have a neurotrophic function, being involved in growth and survival of neurons (Jin et al., 2000; Oosthuyse et al., 2001; Wick et al., 2002). VEGF exerts its effect mainly by binding to its tyrosine kinase receptors (VEGF receptor 1,2 and 3) on the cell membrane. When VEGF binds to its receptors, these dimerize and get activated through transphosphorylation (Petrova et al., 1999; Matsumoto & Mugishima, 2006).

In the hippocampus, VEGF has been shown to protect neuronal cells against glutamate excitotoxicity (Matsuzaki et al., 2001) and reduce neuronal loss associated to SE (Nicoletti et al., 2008). In previous *in vitro* studies, VEGF application on hippocampal slices from epileptic rats decreased epileptiform discharges

induced by bicuculline (McCloskey et al., 2005). In another study, addition of VEGF reduced ictal-like and interictal events in hippocampal rat slices, in the metabotropic glutamate receptor agonist AP4 (2-amino-4-phosphonobutyric acid) and low Mg^{2+} *in vitro* model (Cammalleri et al., 2011). The neurotrophic effect of VEGF is mainly mediated through VEGF receptor 2 signaling. VEGF receptor 2 is also referred to as kinase insert-domain receptor (KDR) in humans and fetal liver kinase 1 (Flk-1) in rodents. Whether inhibitory effect on seizures exerted by VEGF is also mediated via Flk-1 has not been investigated previously.

1.4.2 Galanin

Galanin is built up by a chain of 29 amino acids (30 amino acids in humans) and was first described in porcine intestines (Tatemoto et al., 1983). Since the first discovery, galanin has been isolated in the peripheral and central nervous system. Galanin has been reported to be involved in neurological disorders (Lundstrom et al., 2005), e.g. Alzheimer's disease (Counts et al., 2003), depression (Kuteeva et al., 2010) and epilepsy (Kokaia et al., 2001; Mazarati, 2004). Galanin often co-exists with neurotransmitters, including catecholamines, 5-hydroxytryptamine and GABA, and exerts its effect by modulating the release of these neurotransmitters.

In the rat hippocampus, galanin is located predominantly in noradrenergic fibers, originating from the locus coeruleus (Melander et al., 1986; Xu et al., 1998). Galanin acts through three G-protein-coupled receptors; galanin receptor 1, 2 and 3 (Iismaa & Shine, 1999; Branchek et al., 2000; Mitsukawa et al., 2008). However, only galanin receptor 1 and 2 are expressed in the rat hippocampus. Galanin receptor 1 has mainly been located to the ventral CA1 and CA3 hippocampal areas, while galanin receptor 2 is found in the dorsal and ventral dentate gyrus (Lu et al., 2005).

Infusion of galanin in the dentate gyrus prior and during self-sustained SE decreased the seizure duration or totally suppressed the seizures (Mazarati et al., 1998; Mazarati & Wasterlain, 2002). In the kindling model, transgenic mice overexpressing galanin or rats injected with AAV vector to overexpress galanin exhibited prolonged latent period to the convulsions and reduced susceptibility to generalized seizures (Schlifke et al., 2006; Kanter-Schlifke et al., 2007b). It has been suggested that galanin has the ability to modulate excitability by regulating voltage gated Ca^{2+}-channels and ATP-dependent K^{+}-channels (Palazzi et al., 1991; Zini et al., 1993; Kask et al., 1997). Therefore, the presynaptic effects of galanin lead to decreased glutamate release (Mazarati et al., 2000; Kokaia et al., 2001),

which could explain its inhibitory action on epileptic seizure activity.

1.4.3 Neuropeptide Y

NPY is a 36 amino acid peptide produced and released by neurons throughout the brain and by secretory cells in other parts of the body, such as the pancreas (Allen et al., 1983; Bennet et al., 1996). In the brain, NPY is involved in numerous physiological functions, including memory and anxiety, and pathologic conditions, such as epilepsy (Decressac & Barker, 2012).

In previous studies, NPY has been shown to decrease excitability in the hippocampus and to suppress epileptic activity. In NPY knock-out mice, where NPY is depleted, the susceptibility for developing epileptic seizures and the death rate is increased after kainic acid injection (Baraban et al., 1997). After seizures, the expression of NPY and its receptors are up-regulated both in animal models of TLE and in human tissue (Schwarzer et al., 1998; Vezzani et al., 1999; Furtinger et al., 2001). In addition, recurrent spontaneous seizures were suppressed by overexpression of NPY in the hippocampus using AAV vectors (Noe et al., 2008). Overexpressing NPY with AAV vectors have also been reported to have inhibitory effects on acute epileptic seizures, induced by kindling stimulations or by kainic acid (Richichi et al., 2004; Sorensen et al., 2009).

So far, five NPY receptor subtypes have been identified (Y1, Y2, Y4, Y5 and Y6), all being a part of the same G-protein-coupled receptor superfamily (Michel et al., 1998). Three NPY receptors (Y1, Y2 and Y5) are widely expressed in the central nervous system (Berglund et al., 2003). Both Y2 and Y5 receptors have been suggested to suppress epileptic seizures (Woldbye et al., 2005), while Y1 receptors are thought to promote epileptic seizures (Lin et al., 2006; Meurs et al., 2012; Olesen et al., 2012).

Epileptic seizures induced by kainic acid or kindling are inhibited by Y2 receptor overexpression, however by combining NPY with Y2 receptor the effect is superior to Y2 receptor alone (Woldbye et al., 2010).

1.5 Animal models of epilepsy

To study a human disease and to develop novel therapies is usually not possible directly in humans, in particularly investigations associated with invasive techniques (Engel, 1998). Therefore, a model for the disease is necessary. Some models can be performed *in vitro*, but the findings eventually need to

be investigated and confirmed in living animals, *in vivo.* Animal models allow us to reconstruct the symptoms and to investigate the underlying mechanisms associated with the disorder. Reconstructing human diseases allows us to understand and treat e.g. epilepsy.

The most common vertebrates used as research animals are mice and rats, with a fast reproduction, fairly low cost, easy handling and good availability. Moreover, with genetic manipulations transgenic animals can be generated to overexpress or knock-out a gene, thereby exploring the effect of the manipulated genes.

In the present work, we used well-established animal models of TLE in mice and rats. The work is focused on two different types of animal models of epilepsy: electrically and chemically induced seizures.

1.5.1 Electrical stimulation

To induce seizures electrically, an electrode is implanted into the brain, in our case the hippocampus (*papers I and II*). The electrical current intensity required to induce epileptic activity (seizure threshold) in the hippocampus is determined for each animal. After determination of the individual threshold, the animals are either stimulated at their threshold current once a day (kindling) or with 40 stimulations in one day, every five minutes (rapid kindling). At initial electrical stimulations focal epileptic seizures appear, while with repeated stimulations generalized seizures eventually develop.

Another electrically-induced animal model of epilepsy is SE, which is similar to the kindling model, but animals are instead stimulated with supra-threshold current every 10 minutes and interrupted with 1 minute EEG recording in between. After one hour of stimulation, self-sustained seizures arise and animals are further observed and EEG is recorded for additional 2 hours, during which seizures continue without any further stimulation. Finally the SE is terminated with injection of pentobarbital.

Kindling is an electrical-induced model to investigate epileptogenesis, the process underlying the development of epilepsy. In SE induced by electrical stimulation self-sustained epileptic seizures appear after the stimulus is turned off. After self-sustained seizures (SE) are terminated, spontaneous epileptic seizures could arise with a certain latent period (ranging from several days to weeks) which resembles post-insult epileptogenesis periods and could be studied experimentally.

1.5.2   Chemically induced seizures

Another approach to induce epileptic seizures is through specific chemical compounds, which can be injected either systemically (*paper III*) or locally in the brain (*paper IV*). The usage of chemical agents, such as kainic acid and pilocarpine, induces chronic epilepsy with recurrent spontaneous seizures. This animal model is often associated with neuronal damage, hippocampal sclerosis, axonal sprouting and synaptic reorganization, similar to what has been reported in epileptic patients (Davenport et al., 1990; Mello et al., 1993; Wenzel et al., 2000).

One chemical compound commonly used for induction of epileptic seizures is kainic acid (KA), an agonist of the kainate receptor that mimics the effect of glutamate. This model consists of different stages, starting with SE during the initial hours after the KA injection, followed by a latent period (lasting for several days to weeks) before recurrent spontaneous seizures appear, which gradually progress in severity and increase in frequency.

2 Aims of the thesis

The overall aim of this thesis was to explore the possible therapeutic effects and underlying mechanisms of increased signaling of neuropeptides and neurotrophic factors by *ex vivo* or *in vivo* gene overexpression, including their respective receptors, in diverse animal models of epilepsy.

The specific aims were:

- To investigate whether and how overexpression of VEGF receptor 2, Flk-1, affects electrical stimulation-induced seizures and their progression in the kindling model of epilepsy (*paper I*).

- To explore whether and how galanin-releasing encapsulated cell biodelivery devices influence electrical stimulation-induced seizures and their progression in the kindling model of epilepsy (*paper II*).

- To explore NPY and its receptor-based combinatorial gene therapy approach in acute (*paper III*) and chronic spontaneous epileptic seizures (*paper IV*).

3 Experimental procedures

3.1 Animals

All experimental procedures were approved by the local Ethical Committee for Experimental Animals and performed in agreement with the Swedish Animal Welfare Agency regulations. Animals were housed in standard cages at a 12 h light/dark cycle with *ad libitum* access to food and water (*papers I-III*) or *ad libitum* access to water and daily forage (15-20 g/day) (*paper IV*).

Male transgenic Thy1-Flk-1 mice expressing murine Flk-1 transgene in postnatal neurons under the Thy-1.2 promoter (Storkebaum et al., 2005) and FvB mice as control animals were used weighing 20 g at the beginning of the experiment (*paper I*). In *paper II*, male Sprague-Dawley (SD) rats (Charles River, Germany) weighing 200-230 g were used. For the studies with kainic acid-induced seizures, male Wistar rats (Charles River, Germany) weighing 250-350 g *(paper III)* or 200-230 g (*paper IV*) at the beginning of the experiments were used.

3.2 Viral vectors

Recombinant AAV (rAAV) vectors were produced by GeneDetect (Auckland, New Zealand) as previously described (During et al., 2003; Lin et al., 2003). AAVs of serotype 1/2 were used to produce viral vectors containing neuron-specific enolase (NSE) promoter coupled to human NPY, Y2 or Y5 gene constructs. As a control, an empty vector was used, that was carrying the same expression cassette but without the transgene. A woodchuck posttranscriptional regulatory element (WPRE) was inserted downstream to the transgenes to increase the gene

expression delivered by viral vectors. The final genomic titers (particles/ml) for the viral vectors were as follows: rAAV-NPY 1.0 x 10^{12} genomic particles/ml (*Paper III and IV*); rAAV-Y5 1.0 x 10^{12} genomic particles/ml (*Paper III*); rAAV-Y2 1.0 x 10^{12} genomic particles/ml (*Paper IV*); rAAV- Empty 1.0 x 10^{12} genomic particles/ml (*Paper III and IV*).

3.3 Electrode implantations

Animals were anesthetized by intraperitoneal injection of a mixture of ketamin (80 mg/kg) and xylazine (15 mg/kg) (*Paper I*) or by inhalation of isoflurane (*Papers II-IV*). Animals were placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA). For inducing epileptic seizures with electrical stimulations a bipolar stainless steel stimulation/recording electrode (PlasticsOne, Roanoke, VA) was implanted unilaterally in the hippocampus (*papers I and II*). The electrode was implanted using the following coordinates, in mice (*paper I*): Anteroposterior (AP) -2.9 mm, Mediolateral (ML) -3.0 mm, Dorsoventral (DV) -3.0 mm, and with the following coordinates in rats (*paper II*): AP -4.6 mm, ML -4.9 mm, DV -6.3 mm or AP -4.8 mm, ML -5.2 mm, DV -6.3 mm (reference points from bregma, midline, dura). A reference electrode was placed between the skull and the temporal muscle. Proximal electrode sockets were collected into a plastic pedestal (PlasticsOne, Roanoke, VA) and fixed on the skull with dental cement (Kemdent, Wiltshire, UK).

3.4 Implantation of ECB devices

At the same surgical session as the electrode implantation, ECB devices were implanted bilaterally (*paper II*). ECB devices were stored in an incubator (37ºC, 5% CO_2) until the time of implantation. In the first series of experiments, ECB devices were implanted in a vertical position, while in the second series of experiments in an angular position with 26º angle, to ensure better coverage of the hippocampus. Each ECB device was filled with 60 000 human retinal pigment epithelial cells (ARPE-19) either genetically modified to produce galanin or a parental cell line without the galanin gene modification. Two different active cell lines were tested, one with low release of galanin and one with high release of galanin, both developed by NsGene A/S. Moreover, empty devices were implanted as a control for the cell-filled ECB devices. ECB devices were 5 mm long for the vertical

placement or 7 mm long for the angular position (outer diameter: 725 μm; inner diameter: 525 μm). ECB devices were implanted with the following coordinates (reference points from bregma, midline, dura): Vertical placement: AP -4.8, ML ±4.1, DV -6.0; Angular placement: AP -5.3, ML ±2.7, DV -8.0.

3.5 Viral vector injection

Viral vectors were injected, 0.1 μl every 30 sec, using a glass capillary attached to a 5 μl Hamilton syringe. In *paper III*, 2 μl viral vector suspension was injected bilaterally in the dorsal hippocampus and another 2 μl in the ventral hippocampus, delivered at two different depths with 1 μl in each deposit. In *paper IV*, 2.5 μl viral vector suspension was infused unilaterally into the right dorsal hippocampus and additional 2.5 μl in the right ventral hippocampus. Viral vector injections were performed in the hippocampus with the following coordinates and sites (reference points from bregma, midline, dura): *Paper III*: AP -3.3 mm, ML ±1.8 mm, DV -2.6 mm and AP -4.8 mm, ML ±5.2 mm, DV -3.8 and -6.4 mm; *Paper IV*: AP -4.0 mm, ML -1.5 mm, DV -3.6 mm and AP -7.3 mm, ML -4.8 mm, DV -5.8 mm. After the viral vector injection, the glass capillary was left in place for 3 min (*paper III*) or 10 min (*paper IV*) to prevent backflow of viral particles.

3.6 Induction of epileptic seizures

3.6.1 Kindling

All animals were allowed to recover from surgery for one week before electrical stimulation was initiated. Electrical stimulations and EEG recordings were performed using a MacLab system (ADInstruments, Bella Vista, Australia).

In *paper I*, mice were stimulated according to the kindling protocol. The first days, the individual threshold was measured starting at a stimulation of 10 μA and increasing the current intensity with 10 μA steps (1 ms duration square wave pulse of 100 Hz for 1 sec). The animals were then stimulated at their individual threshold once a day, followed by a behavioral assessment of the seizure according to the Racine scale (Table 1) (Racine, 1972). Stimulations were ended after five stage 5 generalized seizures.

In *paper II*, the threshold was first measured (1 ms duration square wave pulse, 100 Hz for 1 sec) as described above. Subsequently animals were stimulated with

Table 1 The behaviour stages of the modified Racine scale.

Stage 1	Facial movements
Stage 2	Head nodding
Stage 3	Forelimb clonus
Stage 4	Rearing with forelimb clonus
Stage 5	Rearing and falling with forelimb clonus
Stage 6	Status epilepticus

40 stimulations every 5 min (1 ms duration square wave pulse, 10 Hz, 400 µA for 10 sec). After each stimulation the behavioral seizures was scored according the Racine scale (Racine, 1972).

3.6.2 Kainic acid

In *paper III*, 3-4 weeks after viral vector injection KA (10 mg/kg; Sigma, St. Louis, MO) was administered subcutaneous in the neck region in rats. The KA was diluted in sterile isotonic 0.9% saline and adjusted to pH 7.4. Animals were monitored for 2 hours and their seizures were scored according to the Racine scale (Racine, 1972) during that time.

In *paper IV*, intra-hippocampal KA injection was used to induce epileptic seizures. First, KA (Ascent scientific, Cambridge, UK) was dissolved in sterile isotonic 0.9% saline (0.4 µg/0.4 µl) and the pH was adjusted to 7.4. KA was injected with a glass capillary into the medial part of the right hippocampus with the following coordinates (reference points from bregma, midline, dura): AP -5.3 mm, ML -4.5 mm, DV -3.2 mm. The glass capillary was left in place for additional 5 min after injection.

3.7 Electrophysiology

For *in vitro* electrophysiological studies (*paper III*), the hippocampus were sliced from viral vector injected rats (4-5 weeks after viral vector injection) and non-injected control rats. Brains were immediately removed after decapitation and placed into ice-cold oxygenated (95% O_2 and 5% CO_2) sucrose-based solution (75 mM sucrose, 67 mM NaCl, 26 mM $NaHCO_3$, 25 mM glucose, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 0.5 mM $CaCl_2$, 7 mM $MgCl_2$; 308 mOsm and pH 7.4). Coronal slices of 300 µm thickness were cut on a Leica VT1200 vibratome using the

above solution. For the first hour, slices were kept in the oxygenated sucrose-based solution at 34ºC and thereafter slices were transferred to oxygenated artificial cerebrospinal fluid (aCSF) at room temperature (RT) (119 mM NaCl, 26 mM NaHCO$_3$, 25 mM glucose, 2.5 mM KCl, 1.25 mM NaH$_2$PO$_4$, 2.5 CaCl$_2$, 1,3 mM MgSO$_4$; 303 mOsm and pH 7.4). Individual slices were transferred to the recording chamber (at 32.5°C), and were constantly perfused with 2.5 ml/min oxygenated aCSF and 100 μM picrotoxin (Tocris Bioscience, Ellisville, MI) to block GABA-A receptors.

The electrophysiological measurements were performed in a submerged recording chamber as previously described (Sorensen et al., 2008). Field excitatory postsynaptic potentials (fEPSPs) were recorded in the Schaffer collaterals to CA1 area using a borosilicated glass pipette containing aCSF (3-4 MΩ resistance). The potentials were sampled at 10 kHz on a HEKA amplifier (HEKA Elektronik, Lambrecht, Germany). The glass pipette with aCSF was placed in the Schaffer collaterals which were stimulated by constant square-voltage pulses (0.1 μs), while the recording electrode was positioned in CA1 area (stratum radiatum). By stepwise increase of the stimulation strength (or current) the basal excitatory synaptic transmission, the "input-output" relationship for fEPSPs, was assessed in each slice.

Paired-pulse stimulations (25, 50, 100 and 200 ms interstimulus intervals; 0.05 Hz) were delivered to the same area to determine the short-term synaptic plasticity based on the paired-pulse facilitation (PPF) ratio. The PPF ratio was calculated as the initial slope of the second fEPSP divided by the initial slope of the first fEPSP.

The potential contribution of endogenous Y2 receptors were blocked with Y2 antagonist (BIIE0246; Tocris Bioscience, Ellisville, MI) that was first dissolved in 99 % ethanol and then diluted (1:10 000) in aCSF (0.3 μM final concentration). The slices were perfused with the Y2 antagonist at least 8 minutes before recordings were resumed.

3.8 Magnetic Resonance Imaging

In *paper IV*, magnetic resonance imaging (MRI) was performed to evaluate the damage caused by the intra-hippocampal KA injection. Animals were anesthetized with isoflurane and were placed in a special positioning system, where the head was stabilized. During the experiment, the breathing rate and

body temperature were controlled.

MRI was performed in a 9.4 T 400 MHz Agilent Technologies (Stockholm, Sweden) scanner with the 205/120 HD gradient coil. T_2-weighted images were obtained with the following parameters: Echo time 39.39 ms; Repetition time 4000 ms; five averages per cycle and flip angle of 180°. The field of view was 40.4 x 42 mm² on a 256 x 256 matrix. Totally 45 contiguous slices, with the thickness of 400 µm, were scanned for each animal.

Analysis of the scanned images was performed with the OsiriX software (Geneva, Switzerland). The lesion area was outlined in the obtained images in order for the software to calculate the lesion volume. Coordinates were then created based on the MRI images to target the remaining hippocampal tissue for viral vector treatment (Paxinos & Watson, 2006).

3.9 Transmitter implantation

About one year after induction of epileptic seizures with intrahippocampal KA (*paper IV*), animals were anesthetized with isoflurane and the skull was fixed into a stereotaxic frame (David Kopf Instruments, Tujunga, CA). One depth stainless steel electrode (Plastics One, Roanoke, VA), connected to one of the wires (signal input) of the F40-EET transmitter (Data Sciences International, St. Paul, Minnesota), was implanted into the hippocampus (left hemisphere) contralateral to KA injection with the following coordinates (reference points from bregma, midline, dura): AP -4.8 mm, ML +5.2 mm, V -6.3 mm. Another electrode was placed ipsilateral to the KA injection on dura mater, rostral to the coronal suture. For each of the electrodes a reference electrode was placed on the dura mater caudal to the lambdoid suture. All the electrodes were fixed to the skull with dental cement (Kemdent, Wiltshire, UK). The transmitter was subsequently placed in a subcutaneous pocket created from the incision by the skull.

3.10 Monitoring of spontaneous seizures

One week after transmitter implantation (*paper IV*), the transmitter was activated in each animal by a magnet and each cage with the animal was placed on top of a receiver. Every receiver obtains the generated information from a specific transmitter, via radio frequency (Fig. 3). Synchronous EEG and video recordings were collected from all animals for 2 consecutive weeks (24 hours/
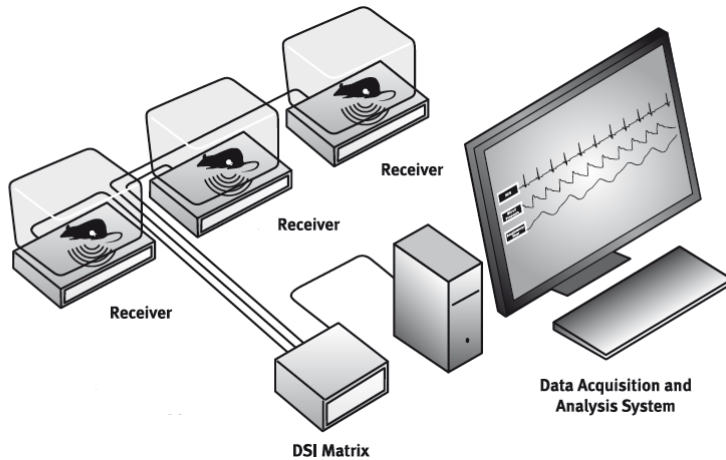
Figure 3 Overview of the different components for wireless EEG monitoring of spontaneous epileptic seizures. Modified illustration from Data Sciences International.

day). Another 2 week monitoring session was performed 4 weeks after viral vector injections. All EEG and video recordings were analyzed using the NeuroScore software (Data Sciences International, St. Paul, Minnesota, USA).

3.11 Termination of *in vivo* experiments

At the end of the experiments, the brains were collected for further histological analysis. For perfusions, animals were deeply anesthetized with pentobarbital (*paper I, II and IV*) and the transmitter was removed (*paper IV*). The animals were perfused transcardally with 0.9% NaCl, in *paper II* the ECB devices and brains were removed at this stage, while the rest of the brains were subsequently perfused with 4% paraformaldehyde (PFA). All brains were removed and post-fixed in 4% PFA (overnight in 4ºC), followed 20% sucrose in 0.1 M sodium phosphate buffered saline (PBS) (30% sucrose in *paper II*) overnight in 4ºC. Brains were cut on a microtome in 30 μm thick coronal sections and stored in cryoprotective solution (ethylene glycol and glycerol in PBS) in the freezer.

For following NPY receptor binding assays, *in situ* hybridization of NPY and Y5 receptor and some immunohistochemical stainings (VEGF, Flk-1, Glut1, Y5) animals were decapitated, brains were quickly removed and either post-fixed in 2% PFA (*paper I*) or instantly frozen on dry ice (*paper III and IV*). Coronal brain

sections were cut 10 μm (*paper I*) or 15 μm (*paper III and IV*) thick on a cryostat (Shandon Inc., Pittsburgh, PA).

3.12 Galanin ELISA

Galanin release from ECB devices (*paper II*) *in vitro* was determined one and three weeks (just prior to implantation) after encapsulation and immediately after removal from the brains at the end of the experiment. Galanin release from the ECB devices into the incubation medium (human endothelial serum-free medium) was sampled after 24 hours (37°C, 5% CO_2) and measured by galanin ELISA (Bachem, Bubendorf, Switzerland). Galanin ELISA was performed according to manufacturer's instructions. After explantation and galanin release determination, ECB devices were embedded in resin and cut in 5 μm tick sections that subsequently were stained with hematoxylin/eosin to evaluate cell survival.

3.13 Histological analysis

3.13.1 Immunohistochemistry

Free-floating slices were rinsed in KPBS, pre-incubated in blocking solution (5% normal serum and 0.25% Triton X-100 in KPBS) for 1 h and incubated with respective primary antibodies (Table 2) in blocking solution overnight in RT. The following day, slices were rinsed in KPBS and incubated with respective secondary antibody in blocking solution for 2 h in RT. Slices were finally rinsed in KPBS, mounted on coated glass slides and cover-slipped with DABCO (1,4-Diazabicyclo[2.2.2]octane solution).

For immunohistochemical stainings using 3,3-diaminobenzidine (DAB) reaction, slices were first quenched in 3% hydrogen peroxide and 10% methanol before pre-incubation in blocking solution and the incubation with primary antibody. After incubation with biotinylated secondary antibody (2 h in RT), slices were incubated with avidin-biotin conjugated horseradish peroxidase solution (Vector Laboratories, Peterborough, UK) for 1 h and finally visualized by 0.5 mg/ml DAB and 3% hydrogen peroxide.

Slices already mounted on glass slides, were first fixed in 4% PFA, rinsed in PBS and pre-incubated in blocking solution (5% normal serum, 1 % bovine serum albumin, 0.3% Triton X-100 in PBS). Slices were incubated with primary

Table 2 Primary antibodies used for immunohistochemical stainings.

Antigen	Raised in	Dilution	Provider
ED1	mouse	1:200	AbD Serotec
Flk-1	mouse	1:200	R&D Systems
GFAP	mouse	1:500	Sigma-Aldrich
Glut1	rabbit	1:20	Santa Cruz Biotechnology
IBA1	rabbit	1:1000	Wako
NPY	rabbit	1:10 000	Sigma-Aldrich
NeuN	rabbit	1:500	Millipore
VEGF	mouse	1:20	Santa Cruz Biotechnology
Y5	rabbit	1:300	Alomone Labs

antibody in blocking solution overnight at 4°C. Following day, the slices were washed in washing buffer (0.25% bovine serum albumin, 0.1% Triton X-100 in PBS). Slices were incubated in secondary antibody and finally rinsed a last time.

For validation of the electrode location (*paper I*) sections were stained with 0.5% cresyl violet (Sigma-Aldrich, Stockholm, Sweden). In *paper II*, the tissue damage and localization of the ECB devices were determined by hematoxylin/ eosin stainings.

In *paper I*, glial fibrillary acidic protein (GFAP) immunoreactive cells were quantified in Flk-1 overexpressing mice (n=3) and in control mice (n=3) in the hilus of the dentate gyrus. Three sections from each animal were quantified bilaterally in the dorsal hippocampus. Moreover, blood vessel area and density were assessed throughout the hippocampus of Flk-1 overexpressing mice (n=4) and control mice (n=4) based on the glucose transporter 1 (Glut1) immunohistochemical staining. Counting of Iba/ED1 inflammatory cells (*paper II*) were performed in in all layers of the cortex (1 mm^2) on three sections 180 µm apart in slices from animals implanted with vertical ECB devices.

3.13.2 Real-time PCR

Ribonucleic acid (RNA) was extracted (*paper I*) to measure the levels of Flk-1 and VEGF in the hippocampus of transgenic animals and controls. To extract RNA, hippocampi were dissected and homogenized in RLT lysis buffer (Qiagen, Venlo, Netherlands) using a FastPrep24 system (MP Biomedicals, Illkirch, France). RNA was extracted with DNase digestion (Qiagen, Venlo, Netherlands)

and was transcribed to complementary deoxyribonucleic acid (cDNA) by the QuantiTect reverse transcription kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. Gene expression was determined by the 7500 Fast Real-Time polymerase chain reaction (PCR) system (Applied Biosystems, Halle, Belgium) and cDNA was normalized to β-actin expression levels, with the following TaqMan gene expression assays: β-actin Mm00607939_s1; Flk1 Mm01222419_m1; VEGF Mm00437304_m1.

3.13.3 Binding autoradiography

Y5 receptor binding assay (*paper III*) was performed as previously described (Woldbye et al., 2005). Slices were defrosted at RT and preincubated for 20 min in binding buffer (pH 7.4), containing 25 mM N-(2-hydroxyethyl)-piperazine-N´-(2-ethanesulfonicacid) (HEPES), 2.5 mM $CaCl_2$, 0.5 g/l bacitracin, 0.5 g/l bovine serum albumin (BSA). Next, slices were incubated at RT for 60 min in binding buffer with the addition of 0.1 nM [Tyr^{36}]mono-iodo-PYY (Amersham Biosciences, Hørsholm, Denmark). Subsequently 100 nM Y1 receptor antagonist (BIBP3226; Bachem AG, Bubendorf, Switzerland) and Y2 receptor antagonist (BIIE0246; Tocris Bioscience, Bristol, UK) were added to the binding buffer, to visualize Y5 receptor binding.

After brief rinsing, the slices were washed twice for 30 min in binding buffer at RT and then air-dried. All slides were exposed to [^{125}I]-sensitive Kodak BioMax MS films (Amersham Biosciences, Hørsholm, Denmark) for 4 days at -20°C. Non-specific binding was performed by adding 1 μM unlabeled NPY, to displace the corresponding radioactive ligand binding. The films were developed in Kodak Processing Chemicals for Autoradiography Films and finally developed in Kodak GBX developer.

3.13.4 [^{35}S]GTPγS functional binding

The procedure was performed (paper III and IV) as previously described (Christensen et al., 2006; Agasse et al., 2008). All sections were defrosted for 30 min at RT before being rehydrated for 10 min at RT in assay buffer A (50 mM Tris-HCl, 3 mM MgCl, 0.2 mM ethylene glycol tetraacetic acid, 100 mM NaCl; pH 7.4). Sections were preincubated for 20 min at RT in buffer B (assay buffer A, 0.2 mM dithiothreitol, 1 μM 1,3-dipropyl-8-cyclopentylxanthine, 0.5% BSA, 2 mM guanosine-5´-diphosphate) and then incubated at 25°C for 2h in buffer B together with 40 pM [^{35}S]-guanosine-5´-triphosphate (GTP) γS (1250 Ci/mmol; PerkinElmer, Skovlunde, Denmark) and 1 μM NPY (Schafer-N, Copenhagen,

Denmark). To only visualize the Y5 functional binding, Y1 receptor antagonist (1 µM BIBP3226; Bachem AG, Bubendorf, Switzerland) and Y2 receptor antagonist (1 µM BIIE0246; Tocris Biosciene, Bristol, UK) were added to the preincubation and incubation buffers. For functional NPY receptor binding no receptor blockers were added. The basal binding was determined by incubation in buffer B with 40 pM [^{35}S]-GTPγS (1250 Ci/mmol) but without NPY. Non-specific binding was determined by incubation in buffer B (without NPY) with 40 pM [^{35}S]-GTPγS and 10 µM non-labeled GTPγS (Sigma-Aldrich, Copenhagen, Denmark). Incubation was terminated by washing twice for 5 min in ice-cold 50 mM Tris-HCl buffer (pH 7.4). Finally, sections were rinsed in ice-cold distilled H$_2$O, dried under a stream of cold air, before being exposed to [^{35}S]-sensitive Kodak BioMax MR films together with [^{14}C]-microscales (Amersham Biosciences, Hørsholm, Denmark) for 5 days and then being developed in Kodak Processing Chemicals for Autoradiography Films (Sigma-Aldrich, Copenhagen, Denmark). Autoradiographic image analysis and measurements were conducted using Scion Image software (National Institute of health, USA). Measurements were performed bilaterally, in 3-4 adjacent sections per animal, in 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).

3.13.5 In situ hybridization

The *in situ* hybridization was performed (*paper III*) as previously described (Woldbye et al., 2010). Slices were defrosted for 10 min in RT, fixed in 4% PFA for 5 min and briefly rinsed and left in PBS for 5 min. The slices were then transferred to 70% ethanol for 5 min and placed in 95% ethanol until hybridization. The following synthetic antisense oligonucleotide DNA probes were used for *in situ* hybridization: Y5 receptor mRNA: 5´-CGA GTC TGT TTT CTT TGT GGG ACA ATC CAC AGC TTA TAC TCC TGC-3´; prepro-NPY mRNA: 5´-GTC CTC TGC TGG CGC GTC CTC GCC CGG ATT GTC CGG CTT GGA GGG GTA-3´ (Mikkelsen & Woldbye, 2006). The oligoprobes were labeled with [α^{35}S] dATP (1250 Ci/mmol; PerkinElmer, Skovlunde, Denmark) at the 3´-end using terminal deoxynucleotidyl transferase (Roche Diagnostics, Mannheim, Germany). The labeled probes were added with a specific activity (1-3 x 10^5 counts per min/100 µl) to the hybridization buffer (50% formamide, 4 x saline sodium citrate (1 x saline sodium citrate: 0.15 M NaCl, 0.015 M sodium citrate-2H$_2$O; pH 7.0), 10% dextran sulfate, 10 mM dithiothreitol). The hybridization buffer was added

to slices (120 µl for each glass slide) and were then covered with parafilm and stored overnight at 42ºC in humidity boxes. The following day, slides were briefly rinsed at RT and washed for 30 min at 60ºC with 1 x saline sodium citrate. Finally, the slices were rinsed 1 min in each of the following solutions: 1 x saline sodium citrate, 0.1 x saline sodium citrate, 70% ethanol and 95% ethanol. The slices were air-dried before exposure to ^{14}C-microscales on ^{35}S-sensitive Kodak BioMax MR films (Amersham Biosciences, Hørsholm, Denmark) for 3-8 weeks. The films were finally developed in Kodak GBX developer.

3.14   Statistical methods

The statistical analysis of data was performed using Student's paired and unpaired *t*-test or one-way ANOVA, in *paper III* followed by Newman-Keuls post-hoc test. Electrophysiological data were analyzed with two-way ANOVA followed by Bonferroni post-hoc test. Differences between groups were considered significant at $p < 0.05$. Data are presented as mean ±SEM. The investigator analyzing and scoring the epileptic seizures, EEG traces and histological slices was unaware of the identity of individual animals.

4 Results and comments

4.1 Overexpression of Flk-1 in transgenic mice

4.1.1 Flk-1 overexpressing mice are more resistant to initiation of epileptic seizures

First we investigated the seizure suppressant potential of overexpressing Flk-1 (*paper I*). The overexpression of Flk-1 did not alter the number of stimulations needed to reach the fully kindled state (three stage 5 seizures). The threshold for induction of seizures, on the other hand, was more than twice as high for Flk-1 overexpressing animals as compared to controls. In addition, overexpression of Flk-1 decreased the afterdischarge duration of the focal seizures (stage 1 and 2), but the duration of the generalized seizures (stage 3-5) remained unaltered. This data indicates that overexpression of Flk-1 suppresses focal epileptic seizure activity.

4.1.2 Flk-1 overexpressing mice exhibit increased levels of VEGF

The overexpression of Flk-1 in the brain was verified with real-time PCR and immunohistochemistry. The Flk-1 mRNA levels in transgenic mice were increased with 5.58 fold in the hippocampus compared to control wild-type mice, as measured with real-time PCR. These results were further confirmed with Flk-1 immunohistochemistry, where distinct Flk-1 immunoreactivity was detected in neurons throughout the hippocampus in transgenic mice, while in control animals very few hippocampal neurons with Flk-1 immunoreactivity were identified.

To explore whether increased Flk-1 receptor expression led to any adaptive alterations of the ligand we estimated VEGF levels in the hippocampus.

Interestingly, the expression of VEGF was also increased as confirmed by real-time PCR. VEGF expression levels were 1.44 fold higher in the hippocampus of transgenic mice compared to control wild-type animals.

4.1.3   Blood vessel formation in the hippocampus of Flk-1 overexpressing mice

Further, we hypothesized that overexpression of Flk-1 could alter blood vessel formation via Flk-1 receptor activation, since it is known that VEGF mediates angiogenesis and vasculogenesis (Millauer et al., 1993; Carmeliet et al., 1996; Rosenstein et al., 1998; Yancopoulos et al., 2000). Both VEGF and Flk-1 were found to be up-regulated in Flk-1 overexpressing mice, therefore we wanted to explore if the blood vessel formation was altered. We quantified the area and the density of blood vessels based on Glut1 immunoreactivity, a protein highly expressed by endothelial cells in blood vessels. In both Flk-1 overexpressing animals and in control animals the blood vessel area was estimated to be around 2% of the hippocampus and the blood vessel density was not significantly different between the groups. Thus, overexpression of Flk-1 does not seem to induce alterations of the blood vessel area and density.

Moreover, in previous studies VEGF was found to increase astroglial proliferation (Krum et al., 2002; Thau-Zuchman et al., 2012), which can affect the epileptic seizures (Vessal et al., 2004). Therefore, we investigated if astrocytic proliferation occurred due to increased VEGF signaling in the Flk-1 overexpressing mice. However, the number of GFAP-positive cells in the hilus in transgenic animals was not different from control animals, indicating that gliosis was not involved in the effects seen on epileptic seizures.

4.2 Galanin-releasing encapsulated cell biodelivery devices in epilepsy

4.2.1   The duration of focal seizures is shortened with the high-releasing galanin cell line clone

First, we tested the levels of galanin release from different cell line clones by ELISA (*paper II*). Several cell lines were developed, but out of these two clones that secreted the highest and most consistent levels of galanin were selected. However, one of the cell line clones released superior levels of galanin than the

other, therefore we refer to them as a high-releasing clone and a low-releasing clone. The high-releasing clone released 30% more galanin compared to the low-releasing clone (12.6 versus 8.3 ng/ml/24 hours) at the time of implantation, as measured by ELISA. The afterdischarge threshold, determined at the initial phase of kindling stimulation, was not different between the experimental and control groups (empty ECB devices or ECB devices with non-genetically modified cells). However, the afterdischarge duration for focal seizures was decreased in animals implanted with high-releasing galanin ECB devices, regardless of the vertical or angular placement, as compared to control animals. On the other hand, the afterdischarge duration for generalized seizures was not affected by galanin-releasing ECB devices compared to control animals.

After removal of ECB devices, the galanin release was re-measured with ELISA by sampling the incubation medium 24 hours after explantation and were compared the release levels prior to implantation. The results show that the galanin levels from all explanted galanin-releasing ECB devices were reduced by 50% or more as compared to pre-implantation period. The galanin release levels are most likely related to the cell viability, therefore we investigated the cell survival in the explanted ECB devices by staining with hematoxylin/eosin. Only animals implanted with ECB devices that had viable cells and released galanin after explantation were included in the final analysis. Hematoxylin/eosin stainings were also performed in brain slices from animals implanted with ECB devices to confirm the position in the hippocampus.

Finally, we explored if the ECB devices releasing galanin could cause any inflammatory response, which could contribute to the observed seizure suppressant effects. The ECB devices filled with either galanin-producing cells or non-modified cells increased the number of Iba1 immunoreactive cells (Iba1 is expressed by activated microglia, charachteristic sign of inflammatory reaction) in the cortex compared to the animals implanted with empty devices. ED1 immunoreactivity, another marker of activated microglia during inflammatory response, was not different between the groups, neither was the double-labeling of Iba1/ED1. These results indicate that a relatively mild inflammatory reaction occurs in the cortex with the cell line containing ECB devices, while galanin *per se* did not induce any inflammatory response.

4.3 NPY gene therapy in epilepsy

4.3.1 Combinatorial gene therapy ameliorates epileptic seizures

Previous studies have shown an effect of rAAV gene transfer-based overexpression of NPY in the hippocampus on acute and spontaneous epileptic seizures (Richichi et al., 2004; Noe et al., 2008; Sorensen et al., 2009). This seizure suppressant effect has been suggested to be mediated by Y2 and Y5 receptors in the hippocampus (Woldbye et al., 2005). In the next study, we investigated if viral vector treatment with NPY in combination with Y2 or Y5 receptors in the hippocampus would have even stronger effect on epileptic seizures in an animal model resembling TLE in patients, the kainic acid model of epilepsy (*paper III and IV*). When overexpressing Y5 receptor alone no effect on epileptic seizures could be detected. However, when both NPY and Y5 were overexpressed at the same time epileptic seizures were inhibited. The combinatorial overexpression increased the latency to first motor seizure and SE. Moreover, it decreased the relative time spent in motor seizures and the severity of seizures during SE. These effects were always stronger than what was observed with NPY overexpression alone. Moreover, there was a reduction of generalized seizures with NPY/Y5 combination, while no such effect was detected with NPY viral vector alone.

Further on, we explored the combinatorial gene therapy treatment strategy with NPY and Y2 on spontaneous chronic seizures. About 10 months after induction of SE, the tissue damage in the hippocampus caused by the kainic acid and the following spontaneous seizures was determined by MRI. The MRI was performed to estimate coordinates for AAV vector injections and to correlate the extent of tissue damage with the number of spontaneous epileptic seizures. Most parts of the dorsal and ventro-caudal hippocampus were damaged, leaving the dorsal and ventral hippocampus intact and thereby enabling targeting of AAV vector injections in these areas. After MRI, animals were implanted with recording electrodes connected to a transmitter and were EEG and video recorded for 2 weeks. During these 2 weeks of recording, spontaneous epileptic seizures were observed in 54% of the animals. There was a high variability between animals in the total number of spontaneous seizures ranging between 2 and 94 during 2 weeks period. Focal and generalized seizures were distinguished by the video and EEG recordings. Epileptic EEG activity was recorded in the hippocampus but not in the cortex during focal seizures, while epileptic EEG activity was observed in both the cortex and the hippocampus during generalized seizures.

In the video recordings, generalized seizures were accompanied by clear whole body convulsions. We did not detect any correlation between the volume of the hippocampal damage and the number of seizures (either total, generalized or focal). After the video-EEG recording session, viral vectors were injected in the ipsilateral to kainic acid injected hippocampus and after 4 additional weeks animals were EEG and video recorded for a post-treatment 2 weeks session. The results show that animals injected with a combination of NPY and Y2 receptor viral vector exhibited shorter time spent in seizures compared to pre-treatment and to control animals injected with an empty vector. Moreover, the seizure frequency was increased in all 5 control animals after empty viral vector injections, while in 4 out of 5 NPY/Y2 viral vector injected animals the seizure frequency decreased.

4.3.2   Overexpression of NPY and its receptors

To quantify the amount and extent of transgene overexpression we first explored mRNA levels 3-4 weeks after rAAV-Y5 and rAAV-NPY of injected animals and observed increased mRNA levels of Y5 receptor and NPY in the hippocampus. The NPY and Y5 receptor mRNA was mainly localized in the principal layers of the hippocampus, such as the dentate granule cell layer and pyramidal cell layers of CA1 and CA3. Some expression was detected outside the principal layers, suggested to be labeling of interneurons.

Further on, we investigated if the NPY and Y5 mRNA levels were translated into protein expression by performing immunohistochemical stainings. Throughout the hippocampus dense NPY immunoreactivity was evident in NPY viral vector injected animals. Similarly, increased Y5 immunoreactivity was observed in the hippocampus in Y5 viral vector injected animals compared to non-treated control rats. In addition, we explored if the NPY binding to Y5 receptors also was elevated after injection of Y5 viral vector. Consistent with previous findings, with increased Y5 mRNA levels and immunoreactivity, binding of NPY to Y5 receptors was increased throughout the hippocampus compared to non-treated control rats. Increased expression of NPY was also detected with immunohistochemistry in the hippocampus after rAAV-NPY/Y2 vector injection. NPY immunoreactivity was mostly detected in fibers of hippocampal neurons.

Moreover, we wanted to investigate if NPY can activate the transgene Y5 receptors (*paper III*) or Y2 receptors (*paper IV*), which would suggest that they are functional. By performing [^{35}S]GTPγS functional binding assay we detected

a selective increase in [^{35}S]GTPγS radioactive signal indicating activation of Y5 (*paper III*) and Y2 receptors (*paper IV*) compared to control rats. Functional Y5 binding was increased in viral vector injected animals, both in rAAV-Y5 and rAAV-NPY/Y5 treated groups, after kainic acid compared to rAAV-Empty injected animals. We also observed an increase in basal functional binding in animals injected with the combination of rAAV-NPY/Y5 compared to rAAV-Y5 and rAAV-Empty injected animals. The specificity of the Y5 functional binding was verified by adding Y5 receptor antagonist, which blocked the [^{35}S]GTPγS signal. For Y2 receptor functional binding, we observed increased [^{35}S]GTPγS signal throughout the hippocampus, including the dorsal and ventral part, after injection with rAAV-NPY/Y2. In the latter case, other NPY receptors were not blocked (as was the case for Y5 functional binding in *paper III*), but the increased [^{35}S]GTPγS radioactive signal was most likely due to increased funtional binding of Y2 receptors. Despite the fact that the viral vector was injected unilaterally, increased [^{35}S]GTPγS signal was also detected in the contralateral hippocampus, mostly in the CA1 and CA3 areas.

4.3.3   Unaltered basal excitatory transmission after transgene overexpression

Previous studies suggested that transgene NPY can reduce presynaptic glutamate release from principal neurons in the hippocampus and thereby suppress epileptic seizures (Colmers et al., 1985; Vezzani et al., 1999). Therefore, effects of combinatorial NPY/Y5 gene therapy on excitatory synaptic transmission were investigated (*paper III*). Neither overexpression of rAAV-Y5 nor combined rAAV-NPY/Y5 altered basal excitatory transmission in Schaffer collateral-CA1 synapses, as determined by the relationship between the presynaptic fiber volley and the corresponding fEPSP amplitude. Next we applied paired-pulse stimulations in the same area as above with different interstimulus intervals to study possible alterations in glutamate release probability. Paired-pulse facilitation arises because the first stimulation increases concentration of presynaptic Ca^{2+}, which affects the release of neurotransmitter-containing synaptic vesicles by the second stimulation. Therefore, increased paired-pulse ratio (the difference in amplitude between the second and first fEPSP) indicates lower glutamate release probability. Paired-pulse facilitation was not significantly different in any treatment, suggesting that basal synaptic properties and glutamate release probability were unaltered by the transgenes.

5 General discussion

5.1 Seizure suppressant actions of VEGF through Flk-1 signaling

We have discovered that Flk-1 overexpression decreases the duration of focal seizures and transgenic mice become more resistant to initiation of epileptic seizures compared to control mice (*paper I*). The overall results from our study indicate that the seizure suppressant effect is mediated by the up-regulation of both Flk-1 and VEGF, thereby increasing the VEGF signaling in neurons.

An important factor that could affect the epileptic seizures after overexpression of Flk-1 is the formation of new blood vessels. Previous findings showed that VEGF increases angiogenesis and vasculogenesis in the brain, which are associated with changes in blood-brain barrier permeability, neuronal excitability and seizure susceptibility (Rigau et al., 2007; van Vliet et al., 2007; Ndode-Ekane et al., 2010). However, alterations in blood vessel formation were not observed in our study. Moreover, the number of GFAP immunoreactive cells in the hilus was not different in the Flk-1 transgenic mice compared to control mice, thereby suggesting that gliosis did not contribute to the seizure suppressant effect of Flk-1 overexpression. Most likely, the seizure suppressant actions observed in this study were mediated through modulation of excitatory synaptic transmission by increased VEGF signaling, as previously described in hippocampal slice preparations with VEGF application (McCloskey et al., 2005).

Despite the fact that VEGF can be protective against seizures, an excessive VEGF signaling can on the other hand lead to detrimental effects (Croll et al., 2004). For example, VEGF has the ability to trigger inflammatory cascades (Li et

al., 1995; Ryuto et al., 1996; Peltola et al., 2002). VEGF seems to have a complex role both in normal and pathological conditions and its action through Flk-1 signaling needs to be further investigated to get a better understanding of the cellular and molecular mechanisms of VEGF action.

Overexpression of Flk-1 modulates the excitability in the hippocampus through increased VEGF signaling. The VEGF up-regulation that was observed in the Flk-1 overexpressing mice presumably enhances the effect of Flk-1 overexpression on seizures, as is the case for a combinatorial therapy of NPY and Y2 or Y5 receptors (another part of the present thesis). It would be interesting to investigate if similar up-regulation of VEGF would occur when overexpressing Flk-1 by using the viral vector approach. In any case, targeting VEGF signaling through the Flk-1 receptor up-regulation could be considered as an alternative treatment strategy for epilepsy.

5.2 Seizure suppressant actions of galanin

Treatment with high-releasing galanin ECB devices reduced the duration of focal seizures, while the generalized seizures were unaffected (*paper II*). One explanation for this moderate effect could be that the galanin levels released by ECB devices were still not sufficient to reach a therapeutically effective dose.

Nevertheless, our results are in line with previous studies where galanin have been shown to inhibit epileptic seizures (Kokaia et al., 2001; Lin et al., 2003; Kanter-Schlifke et al., 2007b). One factor that can affect the seizure susceptibility is inflammation (Galic et al., 2008; Auvin et al., 2010). Therefore, we further hypothesized that despite the fact that the galanin-producing ARPE cells are behind the semipermeable membrane they might still be able to cause an inflammatory reaction, which could influence the duration of focal seizures. An increase in Iba1 immunoreactive cells in the cortex were detected in all cell-containing ECB devices, while the number of ED1 immunoreactive cells was not different between the groups. This indicates that there is a relatively mild inflammatory reaction caused by the encapsulated ARPE cells, irrespective of their galanin release. These data at the same time shows that an inflammatory reaction was not responsible for the effects on focal seizures exerted by high-releasing galanin ECB devices observed in this study.

5.3 Seizure suppressant actions of combination of NPY and NPY receptors

In *paper III*, we showed that simultaneous overexpression of NPY and Y5 receptor in the hippocampus resulted in seizure suppressant effect after systemic injection of kainic acid. However, when only Y5 receptor was overexpressed no effect was observed. In addition, the combinatorial treatment had a stronger effect compared to NPY overexpression alone, which is in line with previously described combinatorial effect of NPY and Y2 receptor overexpression in the hippocampus (Woldbye et al., 2010). However, in contrast to Y5, overexpression of Y2 receptor alone was sufficient to suppress epileptic seizures (Woldbye et al., 2010). One possible explanation for this diverse effect could be that a certain number of transgene Y5 receptors are not accessible to endogenous NPY, possibly because of uneven regional distribution of endogenous NPY release sites and transgene Y5 expression sites. This speculation seems however unlikely, since previous results with Y2 receptor overexpression using similar viral vector constructs demonstrated an effect on epileptic seizures (Woldbye et al., 2010). One alternative explanation could be that the endogenous expression patterns of the Y2 and Y5 receptors in the hippocampus are different (Gustafson et al., 1997; Durkin et al., 2000), which could influence the expression pattern of the transgene Y5 as well. Therefore, endogenous NPY may be less accessible for transgene Y5 receptors to activate them and inhibit seizure activity.

The transgene Y5 overexpression was verified by increased Y5 receptor mRNA, Y5 receptor immunoreactivity, Y5 receptor binding and functional Y5 binding. We observed slightly increased basal functional binding (without addition of NPY) in non-epileptic rAAV-Y5 treated rats. This indicates that endogenous NPY can under normal conditions activate at least some transgene Y5 receptors, as described previously (Woldbye et al., 2010). High basal binding was observed in rAAV-NPY/Y5 vector injected animals, suggesting that transgene Y5 receptors are activated during basal conditions and that transgene NPY is at least partially released from transduced cells. However, basal excitatory synaptic transmission and paired-pulse facilitation were unaltered, thereby arguing against the theory that transgene NPY is released during normal conditions.

In *paper IV*, we have detected a seizure suppressant effect of spontaneous epileptic activity after treatment with combination of NPY and Y2 receptor, in an animal model resembling human TLE. Spontaneous epileptic activity was

observed in 54% of the animals one year after induction of SE, which is in line with previous publications described at earlier timepoints (Bragin et al., 1999; Raedt et al., 2009). However, it is possible that the continuous monitoring period of 2 weeks is not sufficient to detect all seizure events, since spontaneous seizures often develop in clusters and therefore could have occurred outside the recording period. Despite this consideration, 2 weeks were sufficient to evaluate the positive therapeutic outcome of the combinatorial NPY and Y2 receptor treatment on spontaneous seizures. Animals injected with the rAAV-Empty vector exhibited increased frequency of seizures 4 weeks after viral vector injection. This indicates that more than one year after SE induced by KA, animals still experience progressive increase in seizure frequency. Another explanation could be that the rAAV-Empty vector exacerbates the excitability in the hippocampus, which could have led to increased seizure frequency. However, the rAAV-NPY/Y2 vector treatment prevented the progression in seizure frequency and prevented the progression of the disease. In this study, the gene therapy treatment was limited to the hemisphere where the seizure focus most likely was localized. Therefore, the selective targeting of the seizure focus seems to be sufficient to exert a seizure suppressant effect. This is a novel finding, since unilateral gene therapy approach with NPY has not been attempted previously. However, some overexpression of NPY was detected in the contralateral hippocampus as well, which could possibly contribute to the observed results. The overexpression by the rAAV-NPY/Y2 vector injection was verified by the increased functional NPY receptor binding and NPY immunoreactivity. The functional NPY receptor binding was increased probably due to the overexpression of Y2 receptors. The therapeutic outcome was variable between animals that may depend on the individual expression levels of NPY and Y2 receptors or due to uneven regional distribution of the overexpression in the hippocampus. This needs to be further investigated.

5.4 Clinical perspectives of ECB technology and gene therapy approaches

We have evaluated the effect of galanin-releasing ECB devices on epileptic seizures. The obtained results show that ECB devices could be an alternative treatment strategy for long-term delivery of proteins like neurotrophic factors or neuropeptides into the brain. There are several advantages with the ECB

technology. First, the ECB device can easily be removed in case of unwanted side effects or it can be replaced if needed. Another advantage is that the encapsulated cells do not have any direct interaction with the host tissue, thereby limiting the risk of an immune reaction. Moreover, there are no genetic manipulations of the host cells, thereby minimizing the risk of tumor formation and mutations. ECB devices have been tested in other neurological diseases, such as Alzheimer's disease, showing good safety, tolerability and positive functional outcomes (Eriksdotter-Jonhagen et al., 2012; Tornoe et al., 2012).

The other therapeutical approach evaluated here is the gene therapy strategy using viral vectors, such as rAAV vector. In the brain, overexpression with rAAV vectors has a long-lasting effect, therefore it is important to investigate if this long-term transgene up-regulation can give rise to any side effects. In addition, different side effects can appear depending on which region the viral vector carrying the transgene is injected into. Previous studies with rAAV-NPY vector treatment in the hypothalamus have shown increased body weight (Tiesjema et al., 2007) and Y5 receptor in the hypothalamus have been suggested to be involved in the body weight gain (Ishihara et al., 2006). However, after NPY viral vector treatments in the hippocampus such effects on body weight have not been observed (Richichi et al., 2004; Woldbye et al., 2010). Another potential side effect after rAAV-NPY vector treatment could be memory deficits as described in experimental animals (Sorensen et al., 2009). However, the memory deficits induced by AAV-NPY will probably not exacerbate the memory disturbances that already exist in epilepsy patients (Helmstaedter et al., 2003; Elger et al., 2004).

Gene therapy using rAAV-NPY has in various animal models of epilepsy shown promising results. A rAAV-NPY vector treatment is moving forward to human phase I clinical trials (Loscher et al., 2008). Similarly, AAV-mediated gene transfer in Parkinson patients with rAAV-GAD vector in the subthalamic nucleus has been proven to be a safe and well-tolerated treatment strategy, with no side effects for at least one year after treatment (Mandel & Burger, 2004; Kaplitt et al., 2007; LeWitt et al., 2011). Based on the findings presented here, the combinatorial therapy of NPY and Y2 or Y5 receptors, which mediate the seizure suppressant effect, could then be an additional more refined alternative for clinical application.

6 Concluding remarks

The work presented in this thesis increases our understanding of the seizure suppressant effects of VEGF, NPY and galanin in animal models of epilepsy. Increased levels of Flk-1 and galanin affected focal seizures, but no effect was detected on generalized seizures. On the other hand, the combinatorial gene therapy, NPY with its receptor Y2 or Y5, has more pronounced effect on generalized seizures. Therefore, based on the present studies the combinatorial treatment using viral vector based gene therapy could be developed into an alternative treatment strategy for epilepsy. However, since this type of treatment cannot be withdrawn once it is initiated, possible side effects need to be further explored.

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Paper I

PLoS one

VEGF Receptor-2 (Flk-1) Overexpression in Mice Counteracts Focal Epileptic Seizures

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Abstract

Vascular endothelial growth factor (VEGF) was first described as an angiogenic agent, but has recently also been shown to exert various neurotrophic and neuroprotective effects in the nervous system. These effects of VEGF are mainly mediated by its receptor, VEGFR-2, which is also referred to as the fetal liver kinase receptor 1 (Flk-1). VEGF is up-regulated in neurons and glial cells after epileptic seizures and counteracts seizure-induced neurodegeneration. *In vitro*, VEGF administration suppresses ictal and interictal epileptiform activity caused by AP4 and 0 Mg^{2+} via Flk-1 receptor. We therefore explored whether increased VEGF signaling through Flk-1 overexpression may regulate epileptogenesis and ictogenesis *in vivo*. To this extent, we used transgenic mice overexpressing Flk-1 postnatally in neurons. Intriguingly, Flk-1 overexpressing mice were characterized by an elevated threshold for seizure induction and a decreased duration of focal afterdischarges, indicating anti-ictal action. On the other hand, the kindling progression in these mice was similar to wild-type controls. No significant effects on blood vessels or glia cells, as assessed by Glut1 and GFAP immunohistochemistry, were detected. These results suggest that increased VEGF signaling via overexpression of Flk-1 receptors may directly affect seizure activity even without altering angiogenesis. Thus, Flk-1 could be considered as a novel target for developing future gene therapy strategies against ictal epileptic activity.

Introduction

Vascular endothelial growth factor (VEGF or VEGF-A) is a member of homodimeric glycoproteins and was initially shown to increase vascular permeability in tumor ascites fluid [1]. Since its discovery, VEGF has been found to have various roles in normal and pathologic conditions in the brain. For example, VEGF enhances neuronal proliferation [2,3,4,5,6,7], survival [8,9,10,11,12] and axonal outgrowth [13,14]. Both VEGF-A and VEGF-B, as well as their receptors, VEGFR-1, VEGFR-2 and neuropilin, are widely expressed in the brain with differential expression in distinct population of cells [15]. Immunohistological evaluation reveals their co-localization in all types of neural cells, including pyramidal neurons of the cortex and hippocampus, both in rodents after status epilepticus (SE), and human tissue resected from patients with focal cortical dysplasia-induced intractable epilepsy [15,16,17]. This suggests possible autocrine/paracrine mechanisms of action, and possible role in epileptogenesis and/or ictogenesis.

In the hippocampus, VEGF protects hippocampal neurons after hypoxia [10], glutamate excitotoxicity [18] and SE [16,17,19]. Following electroconvulsive seizures, the levels of VEGF mRNA are increased in brain areas susceptible to cell loss, such as the hippocampus [20]. In addition, VEGF protein is up-regulated 24 h after pilocarpine-induced seizures [17]. *In vitro*, hippocampal

slices from rats with recurrent spontaneous seizures show reduced bicuculline-induced epileptiform discharges after VEGF application [21]. Moreover, VEGF was shown to decrease both ictal and interictal activity induced by AP4 and 0 Mg^{2+} in rat hippocampal slices [22]. The neurotrophic and neuroprotective effects of VEGF are predominantly mediated by VEGF receptor-2, also called fetal liver kinase receptor 1 (Flk-1) [13,23,24], or kinase insert-domain containing receptor (KDR) in humans [25].

The mechanisms of action of VEGF through the Flk-1 receptor are still not completely understood. The pathway of signal transduction seems to be mediated by phosphatidylinositol 3'-kinase/Akt (PI3K/Akt), phospholipase C-gamma/protein kinase C (PLC-γ/PKC) and mitogen-activated protein kinase/extracellular signal-regulated protein kinase (MEK/ERK) pathways [26,27,28]. The neuroprotective effect is thought to be mediated by activation of the PI3K/Akt cascade, while the effects on axonal outgrowth and neuroproliferation are most likely dependent on the PKC- and ERK- dependent pathways.

The main objective of the present study was to investigate whether Flk-1 overexpression in transgenic mice, mimicking increased Flk-1 expression in the temporal lobe after epileptic seizures, would exert direct regulatory action on epileptogenesis and/or ictogenesis.

Methods

Ethics Statement

All experimental procedures were approved by the local Malmö/Lund Ethical Committee for Experimental Animals (Ethical permit number M87-06) and were performed according to the guidelines of the Swedish Animal Welfare Agency and in agreement with international guidelines.

Animals

Five male transgenic mice expressing murine Flk-1 transgene in postnatal neurons under the Thy-1.2 promoter (Thy1-Flk-1 OE) and 10 male FvB controls were used [29]. Only male animals were used to exclude effects of fluctuating female hormones on kindling epileptogenesis [30,31,32]. The animals weighing 20 g at the beginning of the experiment, had *ad libitum* access to food and water, and were housed at a 12 hour light/dark cycle.

Implantation of electrodes

Animals were anesthetized by intraperitoneal injection of ketamine (Ketalar, Pfizer; 80 mg/kg) and xylazine (Sigma-Aldrich, Stockholm, Sweden; 15 mg/kg). A bipolar stainless steel stimulation/recording electrode (Plastics One, Roanoke, VA) was stereotaxically (David Kopf Instruments, Tujunga, CA) implanted in the hippocampus at the following coordinates: AP -2.9, ML -3.0, DV -3.0 from bregma, midline and dura, respectively [33]. A reference electrode was placed in the temporal muscle. Proximal electrode sockets were inserted into a plastic pedestal (Plastics One, Roanoke, VA) and fixed on the skull with dental cement (Kemdent, Wiltshire, UK). The animals were allowed to recover for one week before electrical stimulation.

Electrical kindling stimulation

On the first 3–5 days of kindling the individual threshold was measured by starting stimulations at 10 µA and increasing the current intensity in 10 µA steps (1 ms square wave pulse, 100 Hz) until a focal EEG epileptiform afterdischarge (AD) of at least 5 sec duration was observed. The threshold was determined once a day until ADs were evoked three times by the same minimal current intensity. Thereafter animals were stimulated at their individual threshold current once a day. Stimulation-induced behavioral seizures were scored according to the Racine scale [34]: Stage 0 – no behavioral changes; Stage 1 – facial twitches; Stage 2 – chewing and head nodding; Stage 3 – unilateral forelimb clonus; Stage 4 – rearing, body jerks, bilateral forelimb clonus; Stage 5 – imbalance. EEG was recorded on a MacLab system (ADInstruments, Bella Vista, Australia) 1 min before and 1 min after electrical stimulation. Animals were considered fully kindled when three stage 5 seizures were obtained.

Immunohistochemistry

Forty-eight hours after last stimulation-induced stage 5 seizure, animals were deeply anesthetized with pentobarbital and perfused through the ascending aorta with 0.9% NaCl followed by ice-cold 4% paraformaldehyde. Brains were post-fixed overnight at 4°C, cryoprotected in 20% sucrose (overnight at 4°C) and 30 µm coronal sections were cut on a microtome. The sections were stored in a cryoprotective solution at −20°C until use. For validation of electrode location sections were stained with 0.5% cresyl violet (Sigma-Aldrich, Stockholm, Sweden).

For glial fibrillary acidic protein (GFAP) immunostaining, slices were first rinsed in 0.02 M KPBS followed by preincubation with 5% normal goat serum (NGS) in 0.25% TKPBS and incubation with primary antibody mouse anti-GFAP (Sigma-Aldrich, Stock-

holm, Sweden; 1:500) overnight at room temperature (RT). The next day slices were rinsed with 0.02 M KPBS and incubated 2 hours with secondary antibody FITC-goat anti-mouse (Jackson Immunoresearch, Suffolk, UK; 1:400). Slices were rinsed once more and mounted on coated slides and coverslipped. The number of GFAP positive cells in the hilus was quantified by counting in Flk-1 OE mice (n = 3) and control mice (n = 3). From each animal three sections from the dorsal hippocampus were quantified bilaterally. The CA4 region was not included in the counting. Counting was conducted on Olympus BX61 microscope, while pictures were obtained with a Leica TCS SP2 confocal microscope.

Three different immunohistochemical stainings were performed with brain slices that did not undergo kindling stimulations; Flk-1 (WT n = 2; Flk-1 OE n = 2); VEGF (WT n = 2; Flk-1 OE n = 2) and glucose transporter 1, Glut1 (WT n = 4; Flk-1 OE n = 4). The brains were removed from the skull, fixed in 2% PFA and embedded in tissue-freezing medium (Tissue-Tek®, Sakura, Alphen aan den Rijn, Netherlands). The 10 µm coronal sections were incubated at RT overnight with primary antibodies directed against Glut1 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:20), VEGF (Santa Cruz Biotechnology, Santa Cruz, CA; 1:20) and Flk-1 (R&D Systems, Abingdon, UK; 1:200). Co-labeling with neuronal nuclei, NeuN (Millipore, Brussels, Belgium; 1:500) was carried out with VEGF and Flk-1 stainings. Subsequently, the sections were incubated with fluorescently-labeled secondary antibodies Alexa 488 or 568 (Molecular Probes, Eugene, Oregon; 1:200) for 2 h or with biotin-labeled IgG followed by amplification with a signal amplification system (Streptavidin-HRP-Fluorescein) for VEGF and Flk-1. Blood vessel area and density in the hippocampus were assessed using a Zeiss Axioplan microscope with KS300 image analysis software. VEGF and Flk-1 staining was visualized using a Zeiss LSM510 confocal microscope.

RNA extraction, cDNA preparation and real-time PCR

To extract RNA, hippocampi were dissected (WT n = 3; Flk-1 OE n = 4) and homogenized in RLT lysis buffer (Qiagen, Venlo, Netherlands) using a FastPrep24 system (MP Biomedicals, Illkirch, France). RNA was extracted with a DNase digestion (Qiagen, Venlo, Netherlands) and was transcribed to cDNA by the QuantiTect reverse transcription kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. Gene expression was assessed by the 7500 Fast Real-Time PCR system (Applied Biosystems, Halle, Belgium) and cDNA was normalized to β-actin expression levels, with the following TaqMan gene expression assays: β-actin Mm00607939_s1; Flk1 Mm01222419_m1; VEGF Mm00437304_m1.

Statistical analysis

Statistical analysis of data was performed using Student's unpaired *t*-test or one-way ANOVA. Differences between the group data were considered significant at $p < 0.05$. Data are presented as mean ±SEM. The investigator conducting the behavioral grading of animals, EEG analysis or histological and PCR analysis was unaware of the group identity of individual animals.

Results

Flk-1 OE increases threshold and shortens duration of focal seizures

The location of the implanted stimulation/recording electrodes was confirmed in hippocampal sections with standard cresyl violet staining. All electrode tips were placed within the expected same

area of the hippocampus in the two groups (Fig. 1C). Seizures arising during initial kindling stimulations were focal without any behavioral manifestations. Subsequent stimulations induced gradual development of generalized seizures with increasing severity of behavioral manifestations (Fig. 1A, one-way ANOVA and 1B). There was no significant difference between the Flk-1 OE and WT mice in the number of stimulations needed to reach each stage of kindling, including the fully kindled stage (three stage 5 seizures) that was reached after 21.6 ± 7.5 and 22.8 ± 4.6 days, respectively (Fig. 1B). Thus, overexpression of Flk-1 did not alter epileptogenesis *per se*. However, the threshold for seizure induction was more than twice as high in Flk-1 OE mice as compared to WT animals (WT 27.0 ± 2.9 µA; Flk-1 OE 60.0 ± 7.1 µA; $p<0.001$) (Fig. 2A). Also, the AD duration of focal stages was greatly shorter (less than half) in Flk-1 OE animals (Stage 0: WT 21.3 ± 2.8 sec, Flk-1 OE 10.5 ± 1.2 sec; Stage 1: WT 22.4 ± 0.7 sec, Flk-1 OE 11.4 ± 0.8 sec; Stage 2: WT 26.4 ± 1.5 sec, Flk-1 OE 10.6 ± 0.7 sec; $p<0.01$), but remained unaltered between the groups in more generalized stages (Stage 3–5: WT 41.7 ± 3.1 sec, Flk-1 OE 30.2 ± 5.4 sec; $p>0.05$) (Fig. 2B). Taken together, these data suggest that Flk-1 OE strongly suppresses focal seizure activity.

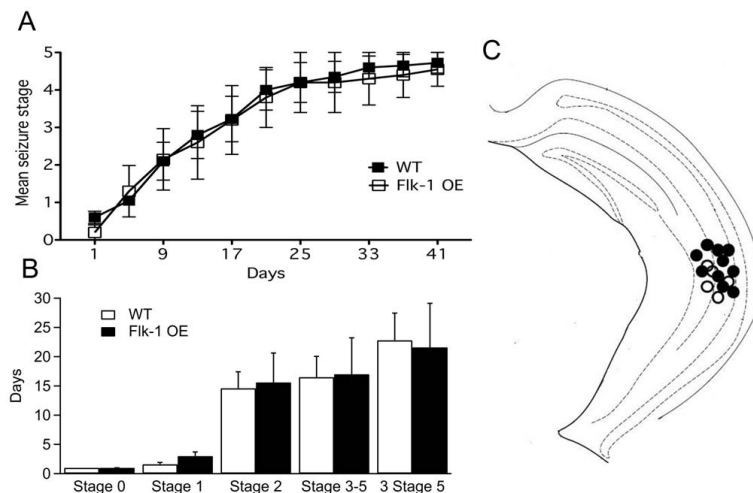### Flk-1 OE mice display elevated levels of Flk-1 and VEGF

The overexpression of Flk-1 in the transgenic mice was confirmed by real-time-PCR (Fig. 3A) and immunohistochemistry (Fig. 4). With immunostainings we just merely demonstrate expression pattern of the proteins, while using a more quantitative approach with real-time-PCR to estimate possible changes. The quantification of hippocampal mRNA showed a 5.58 fold increase ($p<0.01$) of Flk-1 in Flk-1 OE mice compared to WT (Fig. 3A). These quantitative results of real-time-PCR were supported by non-quantitative analysis of immunoreactivity, whereby the Flk-1 immunoreactivity was detected in numerous hippocampal neurons of Flk-1 OE mice, while WT mice displayed very few cells expressing Flk-1 (Fig. 4).

We hypothesized that Flk-1 overexpression could have led to compensatory changes and thereby decreased expression of its ligand, the VEGF. Therefore, we performed real-time-PCR (Fig. 3B) and immunohistochemistry (Fig. 5) to assess VEGF expression levels. Unexpectedly, VEGF mRNA expression was 1.44 fold higher in the hippocampus of Flk-1 OE mice compared to control mice ($p<0.001$) (Fig. 3B). Non-quantitative analysis of VEGF immunoreactivity showed VEGF positive neurons both in Flk-1 OE mice and in WT mice (Fig. 5). Taken together, our data suggest increased VEGF expression in the hippocampus of Flk-1 OE mice.

Unaltered blood vessel densities in Flk-1 OE mice in the hippocampus

It is well known that VEGF can increase the vasculogenesis and angiogenesis [35,36,37] through activation of Flk-1. Since both VEGF and Flk-1 were found to be up-regulated in Flk-1 OE mice, we asked whether Flk-1 OE mice exhibit altered quantity and/or morphology of blood vessels. We quantified the area and density of blood vessels positive for glucose transporter 1 (Glut1) in the hippocampus (Fig. 6A–D). The Glut1 immunohistochemistry was performed in animals that did not undergo kindling stimulation, since it is known that that the expression of Glut1 is regulated by epileptic seizures [38]. In both groups about 2% of the hippocampus were Glut1 positive (WT 2.1 ± 0.3%; Flk-1 OE 2.2 ± 0.2%) (Fig. 6C), and blood vessel density was not significantly different between the groups (WT 231.7 ± 3.6 vessels/µm^2; Flk-1 OE 252.5 ± 8.3 vessels/µm^2) (Fig. 6D). Thus, blood vessels did not seem to be altered in Flk-1 OE mice hippocampus.

Further, we explored whether increased VEGF signaling in Flk-1 OE mice could have led to gliogenesis [39]. To assess the number of glial cells, we performed a GFAP staining followed by quantification of GFAP-labeled cells in the hilus (Fig. 6E–G). The number of GFAP positive glial cells in the hilus of WT and Flk-1

Figure 1. Epileptogenesis is not alternated by overexpression of Flk-1. (A) Progression of kindling: mean stage during each kindling stimulation analyzed with one-way ANOVA. (B) Number of stimulations needed to reach different behavioral seizure stages. (C) Placement of electrodes for electrical stimulation in the hippocampus was verified by cresyl violet staining at the end of the experiments. Solid black dots for WT mice and black rings for Flk-1 OE mice.
doi:10.1371/journal.pone.0040535.g001

Figure 2. Flk-1 OE mice have higher threshold for seizure-induction and exhibit shorter afterdischarge durations of seizures. (A) Mean afterdischarge (AD) threshold during focal epileptiform activity of at least 5 sec duration measured one week after electrode implantation of WT and Flk-1 OE mice. (B) AD duration for different seizure stages during kindling stimulations. Values are presented as mean ±SEM, ** $p<0.01$ and *** $p<0.001$.
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OE mice was not significantly different (WT 91.8±5.0 cells per hilus; Flk-1 OE 77.4±7.1 cells per hilus) (Fig. 6G). Taken together, our data suggest unaltered glial number in the hilus in Flk-1 OE mice.

Discussion

Here, using a transgenic mouse line that overexpresses Flk-1 under the Thy-1 promoter, we show that increased levels of Flk-1 in the hippocampus suppress focal seizure susceptibility. In transgenic mice the threshold to generate ADs was significantly increased (twice as high) and the duration of ADs was markedly shortened (less than half) compared to WT animals.
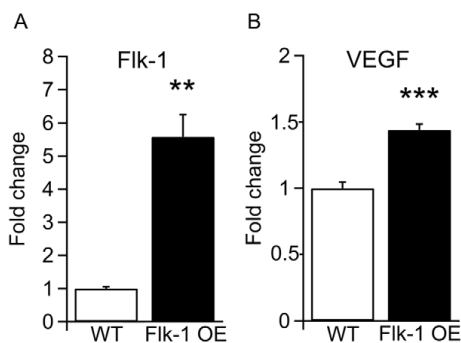
Epileptogenesis, however, was not altered by the overexpression of Flk-1. The Flk-1 OE mice and WT controls did not differ in the number of stimulations needed to reach different seizure stages or the fully kindled state (three stage 5 seizures). Taken together, these results indicate that overexpression of Flk-1 affects focal hippocampal seizure activity without having influence on kindling

Figure 3. Real-time-PCR shows high levels of Flk-1 and VEGF in the hippocampus of Flk-1 OE mice. (A) Flk-1 expression was 5.58 fold higher in Flk-1 OE mice. (B) VEGF levels in the hippocampus of animals with Flk-1 OE were 1.44 fold higher than WT mice. The cDNA was normalized to β-actin expression levels. Values are presented as mean ± SEM, ** $p<0.01$ and *** $p<0.001$.
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epileptogenesis or generalized seizure activity (i.e. seizures that have spread outside the focus, here being the hippocampus).

The Flk-1 OE mice exhibited increased expression VEGF, an endogenous ligand of Flk-1. The underlying molecular and cellular mechanism of such increase is currently unclear. A major limitation of traditional transgenic overexpression is the inability to control developmental compensatory or other alterations in signaling pathways. It would be highly warranted to address the question whether Flk-1 overexpression in adult stage (e.g. conditional overexpression, or viral vector-based approach) would also lead to up-regulation of VEGF. In any case, our data suggest that the observed effect on seizures is mediated by an overall increase of VEGF signaling through up-regulation of both Flk-1 and VEGF. Our immunoreactivity analysis indicates mostly neuronal expression of both Flk-1 and VEGF, though possibly in a variety of neuronal populations (see Figs. 4 and 5) In transgenic mice Flk-1 is overexpressed under the Thy-1 promoter, which has been shown to drive transgene expression (Flk-1) selectively in neurons. The identity of the Flk-1 positive and NeuN negative cells is not clear, but may represent a small population of non-neuronal cells due to some unspecificity of the used promoter. The cellular localization of up-regulated VEGF and Flk-1, and how it may influence its seizure modulating effects, remains to be studied in more details.

Our data are in line with previous observations showing that VEGF is able to suppress bicuculline-induced epileptiform discharges in slices from chronic epileptic rats [21], although it was largely unclear which VEGF receptor was responsible for these effects. Our data suggest that Flk-1 might play a major role in the seizure-suppressant effects of VEGF. As we observed a 1.44 fold increase in the expression of VEGF in Flk-1 OE mice, the anti-epileptic effects could be mediated by increased activation of conventional VEGF/Flk-1 signaling pathways described earlier. However, one cannot exclude that the seizure suppressant effect is partly mediated by other VEGF receptors, such as VEGFR-1 (Fms-related tyrosine kinase 1, Flt-1) or neuropilin [40]. It has been shown that VEGF can suppress glutamatergic synaptic transmission in all major synapses in the hippocampus [21,22]. It is likely that also Flk-1 overexpression exerts similar effect on glutamatergic transmission in the hippocampus, although it needs to be explored.

Recently, a novel concept has been put forward, suggesting that simultaneous overexpression of both the ligand and the receptor of

Figure 4. Immunoreactivity for Flk-1 in the hippocampal slices. Immunoreactivity for VEGF, NeuN and merged in the granule cell layer (GCL) and hilus (Hi) of the dentate gyrus. Flk-1 is barely detected in the hippocampus of wild-type (WT) mice, however the few cells that express Flk-1 are co-labeled with neuronal nuclei (NeuN) protein. In Flk-1 OE mice the Flk-1 immunoreactivity is mostly detected on the plasma membrane of the cells (indicated by the arrowheads). Scale bar is set to 20 μm.
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endogenous molecules may exert synergistic seizure-suppressant effect. This concept has been validated for endogenous seizure-suppressant molecules, such as e.g. neuropeptides and their receptors [41,42]. The finding that in Flk-1 OE mice also VEGF seems to be up-regulated, and potentially strengthens effects of Flk-1 OE, is in line with this conceptual framework.

Another potentially seizure-modulating aspect of Flk-1 overexpression could be the formation of new blood vessels (angiogenesis). It is well known that elevated VEGF signaling may lead to increased levels of vascularization in the brain [35,36,43], and increased angiogenesis and vascularization are associated with altered BBB function, neuronal excitability and seizure susceptibility [44,45,46]. Therefore, altered vascularization of the hippo-

campus in Flk-1 OE mice may have contributed to the observed changes in threshold and duration of ADs. However, we were not able to detect any significant alterations in vascularization of the hippocampus in Flk-1 OE mice, possibly due to some compensatory mechanisms. Similarly, no effect of Flk-1 OE has been shown on astrocytes as measured by GFAP immunolabeling, indicating that gliosis did not contribute to seizure-suppressant effects of Flk-1 overexpression. The most likely mechanism of action is modulation of synaptic transmission and plasticity by VEGF, as observed with acute VEGF application in hippocampal slice preparations [21]. However, this hypothesis needs to be tested in future studies.

Figure 5. Vascular endothelial growth factor (VEGF) is expressed by neurons in the hippocampus. Figure shows immunoreactivity of VEGF, NeuN and merged in the granule cell layer (GCL) and hilus (Hi) of the dentate gyrus. In both controls and Flk-1 OE mice, neurons in the hippocampus express VEGF. Scale bar is set to 20 μm.
doi:10.1371/journal.pone.0040535.g005

Figure 6. Glucose transporter 1 (Glut1) immunohistochemistry and quantification of blood vessels in the hippocampal slices. (A) Intensity and distribution of Glut-1 immunostaining is similar in the hippocampus of Flk-1 OE mice compared to (B) controls; Quantification of blood vessel area (C), as well as the blood vessel density (D) showed no significant differences ($p > 0.05$) between the groups. The number of glial fibrillary acidic protein (GFAP)-positive cells in wild-type mice (E) and Flk-1 OE mice (F) in the hilus of the dentate gyrus after kindling are unaltered (G). Values are presented as mean ±SEM. Scale bar is set to 30 µm.
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When considering the seizure modulating effects of VEGF signaling one has to keep in mind that excessive VEGF signaling may exert opposing effects: it could be protective after seizures, but also detrimental [16]. VEGF is for example capable of initiating inflammatory cascades in the brain [39], and could also cause BBB breakdown [47]. Interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α) are up-regulated after seizures, leading to subsequent up-regulation of VEGF [48,49]. On the other hand, VEGF, while triggering inflammatory cascades, has ability to protect brain cells from various insults. Selectively targeting overexpression of Flk-1 in neurons may avoid unwanted detrimental effects of increased VEGF signaling. Lastly, conventional overexpressing transgenic animals may have some undetected developmental alterations, which could be responsible for the effects observed here. Taken together, these considerations illustrate very complex role of VEGF in cellular and molecular events of normal and pathological conditions, and highlights the need for better understanding of the mechanisms involved in VEGF and its Flk-1 receptor signaling, which may help in developing novel therapeutic strategies for various diseases of the brain, including epilepsy.

Conclusion

In conclusion, the present study adds new insights on regulation of seizure activity by Flk-1. We have demonstrated that in Flk-1 OE mice the resistance to seizure induction is significantly increased and the duration of epileptic EEG activity decreased. Therefore, targeting Flk-1 receptors may represent a novel approach for optimizing regulation of VEGF signaling pathways in order to modulate excitability in the brain and counteract pathological activity such as in epileptic seizures.

Author Contributions

Conceived and designed the experiments: LN IKS DL MK. Performed the experiments: LN JD. Analyzed the data: LN JD. Contributed reagents/materials/analysis tools: PC DL. Wrote the paper: LN MK.

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Paper II

Encapsulated galanin-producing cells suppress focal epileptic seizures in the hippocampus

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Abstract

Galanin is a neuropeptide widely expressed throughout the central nervous system. In the hippocampus, galanin is mostly present in noradrenergic fibers arising from Locus Coeruleus, while its receptors GalR1-3 are expressed in various neurons. Galanin has been shown to exert strong inhibitory effect on seizures, most likely acting via decreasing glutamate release from excitatory synapses. Recent viral vector-based gene therapy approaches have demonstrated significant seizure-suppressant effects of galanin overexpression in a number of animal epilepsy models, suggesting its therapeutic potential.

Purpose - To explore whether a genetically modified galanin-producing human cell line could provide a seizure-suppressant effect, and test its possible translational prospect for clinical application, we implanted encapsulated cell biodelivery (ECB) devices into the hippocampus of rats exposed to a rapid kindling model of epilepsy.

Methods - Two clones from a genetically modified human cell line secreting different levels of galanin were tested. EEG recordings and stimulations were performed by stainless steel depth electrodes implanted into the hippocampus at the same surgical session as ECB devices. One week after the surgery, rapid kindling stimulations were initiated.

Key findings - ELISA measurements performed prior to ECB device implantation showed a release of galanin on average 8.3 ng/ml/24 h/device for the low-releasing clone, and 12.6 ng/ml/24 h/device for the high-releasing clone. Intrahippocampal high-releasing galanin-producing ECB devices moderately decreased stimulation-induced focal afterdischarge duration, while low-releasing ECB devices had no significant effect.

Significance - Our study shows that implantation of galanin-releasing ECB devices moderately suppress focal stimulation-induced recurrent seizures, and offers an alternative to gene therapy approaches in humans, with the advantage that the treatment could be terminated by removing the ECB devices from the brain. Thereby, this strategy provides a higher level of safety for future therapeutic applications, in which various anti-epileptic compounds produced and released by genetically modified human cell lines could be clinically evaluated.

Introduction

The neuropeptide galanin was first discovered in porcine intestine (Tatemoto et al., 1983), but later it has been found in various parts of the body, including the PNS and the CNS. Galanin has diverse physiological functions in the normal brain, but has also been implicated in pathophysiological conditions, for example depression (Lu et al., 2005a; Kuteeva et al., 2008), Alzheimer's disease (Steiner et al., 2001; Jhamandas et al., 2002; Ding et al., 2006; Counts et al., 2009) and epilepsy (Mazarati et al., 1998; Mazarati et al., 2000; Kokaia et al., 2001; Schlifke et al., 2006).

Galanin signaling occurs through G-protein coupled galanin receptor 1 (GalR1), 2 (GalR2) and 3 (GalR3) (Iismaa & Shine, 1999; Branchek et al., 2000; Mitsukawa et al., 2008). GalR1 and GalR2 are expressed within the hippocampus (Lu et al., 2005b). The mechanism of action of galanin through the galanin receptors is thought to be mediated through blockade of voltage gated Ca^{2+}-channels and/or activation of ATP-dependent K^+-channels (Palazzi et al., 1991; Zini et al., 1993; Kask et al., 1997).

Several studies suggest that galanin is involved in seizure regulation and can modulate epileptic activity in the brain. During the epileptic seizures, galanin is released and exerts a presynaptic inhibitory effect on the glutamatergic transmission (Zini et al., 1993; Mazarati et al., 2000; Kokaia et al., 2001). In galanin overexpressing transgenic mice or rats where galanin is overexpressed by gene transduction, prolonged latent period to the convulsions, and decreased susceptibility to generalized seizures have been observed in a kindling model of epilepsy (Schlifke et al., 2006; Kanter-Schlifke et al., 2007). Viral vector-based gene therapy approach has also demonstrated a powerful seizure-suppressant effect of transgene galanin in other animal models of epilepsy, such as chemical and electrical induced status epilepticus (Lin et al., 2003; Kanter-Schlifke et al., 2007).

Non-pathogenic viral vector-based gene delivery into the brain has been proven to be a safe procedure in phase

1-2 clinical trials, for e.g. Parkinson's disease (Mandel & Burger, 2004; Kaplitt et al., 2007; LeWitt et al., 2011). However, once the transgene of interest is expressed in the host brain cells, it is impossible to reverse the process and to terminate its action in case adverse effects arise. Encapsulated cell biodelivery (ECB) devices filled with genetically modified human cells to release gene products into the host tissue has an advantage of being a reversible treatment: the ECB devices can be removed from the brain with a relatively simple procedure and thereby terminate the exerted effect. The cells in the ECB devices can have long-term viability when implanted into the brain because the nutrients from the surrounding host tissue can penetrate the ECB device's semi-permeable membrane, and at the same time, the gene products can be released into the host tissue. The advantages with ECB devices as compared to direct genetic modification of the host cells by viral vector-based gene delivery, or direct cell transplantation, are that the encapsulated cells do not alter host cells or integrate into the host brain. Furthermore, the semi-permeable membrane isolates the cells in the ECB devices from immune reactions in the host brain. Thus, there is no need for immunosuppressant drugs. These considerations make it highly warranted from the translational perspective to explore possible therapeutic effects of compounds like galanin delivered into the brain by using ECB technology.

The main objective of the present study was to explore the therapeutic potential of intrahippocampally engrafted galanin-releasing ECB devices in an animal model of stimulation-induced recurrent epileptic seizures.

Experimental procedures

Ethics Statement
All experimental procedures were approved by the local Malmö / Lund Ethical Committee for Experimental Animals (Ethical permit number M187-09), were performed according to the guidelines of the Swedish Animal Welfare Agency and in agreement with international guidelines.

In vitro preparation and filling of ECB devices
ECB devices consisted of a semi-permeable polyethersulfone (PES) hollow fiber membrane filled with a polyvinyl alcohol (PVA) cylindrical matrix serving as support for the encapsulated cells. Devices were built and sterilized, before they were filled with low-passage human retinal pigment epithelial cell line (ARPE-19). The cells and the filled ECB devices were cultured in human endothelial serum-free media (HE-SFM) (Invitrogen, Stockholm, Sweden) in an incubator (37ºC, 5 % CO2). Several cell lines were generated by genetic modification to release galanin. Two cell clones that produced and released galanin were selected, one with a higher release (HR) and one with a lower release (LR) of galanin. The ECB devices were 5 mm long for the vertical placement in the hippocampus, and 7 mm long for the angular placement (see below). Both had an outer diameter of 725 μm and an inner diameter of 525 μm. Each ECB device was filled with 60 000 cells (optimized by pilot experiments). The ECB devices with non-modified ARPE-19 cells (not producing galanin) and empty ECB devices were used as controls. No differences in seizure control could be detected between groups treated with empty devices and ECB devices filled with control cells, thus the results from the rapid kindling from these two control groups were merged (data not shown). One and three weeks after encapsulation, galanin release from the ECB devices into the incubation medium was sampled after 24 hours and measured by a galanin ELISA (Bachem, Bubendorf, Switzerland).

Animals
Male Sprague-Dawley rats (Charles River, Germany) weighing 200-230g at the beginning of the experiment were used. The animals were housed individually at a 12 hour light / dark cycle with ad libitum access to food and water. All animals were weighted once a week throughout the experiment.

Implantation of ECB devices and electrode
Animals were anesthetized with isoflurane and fixed into a stereotaxic frame (David Kopf Instruments, Tujunga, CA). The ECB devices were implanted bilaterally in two different positions. One group of animals had the ECB devices implanted in a straight vertical position and the other group in a 26º angle, to ensure better coverage of the entire hippocampal axis. The following number of animals and groups were used: Vertical placement of ECB devices – Empty n=5, ARPE n=4, LR n=8, HR n=5; Angular placement of ECB devices – Empty n=12, ARPE n=12, LR n=10, HR n=7. The coordinates used for these two ECB device placements were as follows, reference points from bregma, midline and dura: AP -4.8, ML ±4.1, V -6.0 and AP -5.3, ML ±2.7, V -8.0, respectively. ECB devices were stored in an incubator at 37°C (5 % CO2) until implantation.

At the same surgical session a bipolar stainless steel stimulation / recording electrode (Plastics One, Roanoke, VA) was implanted into the hippocampus at the following coordinates: AP -4.6, ML -4.9, V -6.3 (vertically placed ECB devices) and AP -4.8, ML -5.2, V -6.3 (26º angular placed ECB devices) from bregma, midline and dura, respectively. A reference electrode was placed between the scull and the temporal muscle. Proximal electrode sockets were inserted into a plastic pedestal (Plastics One, Roanoke, VA) and fixed on the skull with dental cement (Kemdent, Wiltshire, UK). The animals were

allowed to recover for one week before rapid kindling electrical stimulations were started.
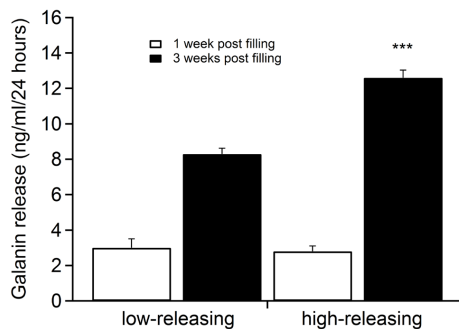
Rapid Kindling

One week after electrode and ECB device implantation, the individual current threshold for epileptiform afterdischarge (AD) induction was measured. The stimulation current started at 10 µA and increased by steps of 10 µA (1 ms square wave pulse, 100 Hz) until a focal EEG AD of at least 5 sec duration was elicited. Subsequently, induction of epileptic activity was initiated according to the rapid kindling protocol, 40 recurrent stimulations given every 5 min, consisting of trains of 10 sec duration, (1 ms bipolar square wave pulses at 10 Hz), with a current intensity of 400 µA.

The behavioral seizures during stimulation were scored according to the Racine scale (Racine, 1972): Stage 0 - no behavioral changes; Stage 1 - facial twitches; Stage 2 - chewing and head nodding; Stage 3 - unilateral forelimb clonus; Stage 4 - rearing, body jerks, bilateral forelimb clonus; Stage 5 - imbalance. EEG was recorded on a MacLab system (ADInstruments, Bella Vista, Australia) 1 min before and 1 min after electrical stimulation.

Explantation of ECB devices and perfusion

Four weeks after rapid kindling stimulation, the animals were deeply anesthetized with pentobarbital and were perfused transcardially with 0.9 % NaCl. The skull was opened and the ECB devices were removed and put into the pre-heated (37°C) medium (HE-SFM) and were stored in an incubator (37°C, 5 % CO2). Galanin levels in the incubation solution were measured after 24 hours by a galanin ELISA (Bachem, Bubendorf, Switzerland) to estimate galanin release from surviving encapsulated cells. Moreover, ECB devices were embedded in resin, cut (5 µm) and stained for hematoxylin/eosin to evaluate cell survival. The brains were taken out and fixed in 4

Figure 1. Galanin release from low-releasing and high-releasing ECB devices. Galanin release was measured using ELISA 1 week and 3 weeks after filling the ECB devices with ARPE-19 cell lines. Values are presented as mean ±SEM, *** p<0.001 compared between low-releasing and high-releasing clones.

% paraformaldehyde for 24 hours and then overnight in 30 % sucrose in 0.1 M sodium phosphate buffered saline. Brains were cut on a microtome in 30 µm thick slices and stored in a cryoprotective solution in the freezer until use.

Immunohistochemistry and other staining procedures for brain slices

To determine the extent of the damage and localization of the ECB devices hematoxylin/eosin stainings were performed on slices from all brains. To evaluate the inflammation, double immunohistochemical stainings of ionized calcium-binding adapter molecule 1, Iba1, together with ectodermal dysplasia 1, ED1, was performed. Slices were rinsed with 0.02 M KPBS and pre-incubated with 5 % normal goat serum and 5 % normal donkey serum in 0.25 % Triton-KPBS for one hour in room temperature (RT). The slices were then incubated with the sera, rabbit anti-Iba1 (Wako, Neuss, Germany; 1:1000) and mouse anti-ED1 (AbD Serotec, Puchheim, Germany; 1:200) overnight at room temperature (RT). Next day the slices were rinsed with 0.02 M KPBS, followed by incubation with secondary antibodies for Iba1 (FITC-goat anti-rabbit; Jackson Immunoresearch, Suffolk, UK; 1:400) and ED1 (Cy3-donkey anti-mouse; Jackson Immunoresearch, Suffolk, UK; 1:400) for two hours in RT. After two hours the slices were once again rinsed the same way as previously and were mounted on coated slides and coverslipped with DABCO (Sigma-Aldrich, Stockholm, Sweden).

Cell counting was performed in the motor cortex (1 mm2 including all layers of the cortex) on three serial (180 µm apart) Iba1/ED1 stained slices from each animal with vertically placed ECB devices. Four animals were counted bilaterally, but no difference in stained cell numbers was detected between the sides (data not shown), thus the rest of the animals were counted unilaterally, only ipsilateral to the electrode side. Cell counting was performed and the images for figures were acquired with Olympus BX61 fluorescence microscope.

Statistical analysis

Statistical analysis of data was performed using Student's unpaired t-test. Differences between groups were considered significant at p<0.05. Data are presented as mean ±SEM. The investigator conducting the behavioral grading of seizures in the animals, EEG and histological analysis was unaware of the group identity of individual animals.

Results

Implanted ECB devices with the HR galanin cell clone shortens duration of focal seizures

After filling ECB devices with galanin-producing cells,

3

Figure 2. High-releasing ECB devices with vertical placement attenuate focal epileptic seizures. (A) Afterdischarge (AD) duration at threshold stimulations was unchanged in animals implanted with galanin-producing ECB devices. (B) Average AD duration for kindling stages 1 and 2 was attenuated by high-releasing galanin-producing ECB devices. Values are presented as mean ±SEM, * p<0.05 and ** p<0.01 compared to control.
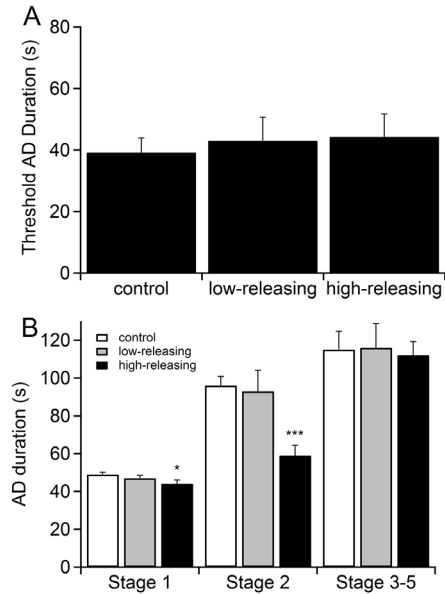
Figure 3. High-releasing ECB devices with angled placement attenuate focal epileptic seizures. (A) Afterdischarge (AD) duration at threshold stimulations was unchanged in animals implanted with galanin-producing ECB devices with different content. (B) Average AD for kindling stages 1 and 2 was attenuated by high-releasing galanin-producing ECB devices. Values are presented as mean ±SEM, * p<0.05 and *** p<0.001 compared to control.
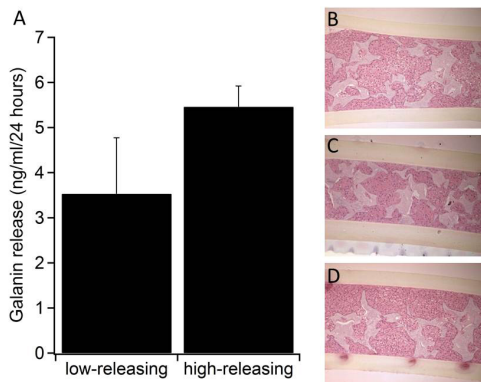
the release of galanin in the incubation culture media was measured (Fig. 1). One week after filling, the galanin release from the LR clone and the HR clone was similar, whereas three weeks after filling the HR galanin clone released about 30 % more galanin than the LR clone (HR 12.6±0.4 ng/ml/24h; LR 8.3±0.3 ng/ml/24h; p<0.05). During the initial phase of kindling stimulations, the seizures are usually focal, while subsequent stimulation-induced seizures spread and manifest as convulsions of increasing severity. The AD threshold for seizure induction was unaffected with vertically implanted and with angular ECB devices filled with galanin releasing cells (Vertical: control 48.9±8.1 µA; LR 52.5±10.1 µA; HR 50.0±6.3 µA; Angular: control 39.2±4.7 µA; LR 43.0±7.6 µA; HR 44.3±7.4 µA; p>0.05) (Fig. 2A and 3A). However, the AD duration of focal seizures was moderately decreased in animals with vertically implanted ECB devices releasing high levels of galanin compared to control (Stage 1: control 50.1±1.5 sec; LR, 46.0±1.4 sec; HR, 43.3±1.5 sec; Stage 2: control 83.3±6.0 sec; LR, 90.9±5.7 sec; HR, 61.9±5.5 sec), but was unaltered at the generalized stages (Stage 3-5: control 96.9±11.4 sec;

LR, 106.6±16.3 sec; HR, 90.5±14.0 sec; p>0.05) (Fig. 2B). The LR galanin clone showed a decreased AD duration during Stage 1, but not for any other seizure stages. Similarly, the angular placed ECB devices decreased AD duration for focal seizures in animals with only the HR clone (Stage 1: control 49.4±1.2 sec; LR 47.0±1.6 sec; HR 43.7±2.0 sec; Stage 2: control 95.9±4.8 sec; LR 93.3±11.1 sec; HR 59.4±5.4 sec) (Fig. 3B). There was no effect of LR ECB devices on AD duration at any seizure stage. The AD duration of generalized seizures remained unaltered (Stage 3-5: control 115.0±9.6 sec; LR 116.1±12.8 sec; HR 112.4±7.2 sec; p>0.05).

After retrieval of the ECB devices, galanin release levels were measured in the culture media once again. The galanin release was decreased for about 50 % or more (LR 3.5±0.5 ng/ml/24h; HR 5.5±1.2 ng/ml/24h; p>0.05) compared to the levels right before the implantation (Fig. 4A). No galanin release was detected from empty or parental ARPE-19 cell line filled ECB devices. Cell survival in the retrieved ECB devices was also verified with hematoxylin/eosin stainings of ECB device sections (Fig. 4B-D). Overall, almost all ECB devices

4

Figure 4. Galanin release after explantation of ECB devices. (A) Average galanin levels measured by ELISA in ECB devices after explantation. Hematoxylin-eosin staining of a section from an ECB device filled with (B) ARPE-19 control cells, (C) low-releasing galanin cells and (D) high-releasing galanin cells. Values are presented as mean ±SEM, p>0.05.

demonstrating galanin release also contained surviving cells. Only animals implanted with ECB devices that still released galanin after retrieval and those that had viable cells were included in the analysis.

Hematoxylin/eosin stainings were performed on sections from brains of all animals to confirm the position of the ECB devices in the hippocampus. The expected positions from two experimental animals are exemplified in schematic drawings on Fig. 5A and 5C. The examples of actual sections showing ECB device position in the hippocampus is shown on Fig. 5B and 5D. Scoring of the extent of the hippocampal damage exerted by the ECB device implantation did not reveal any correlation between the extent of damage and the severity of seizures (data not shown).

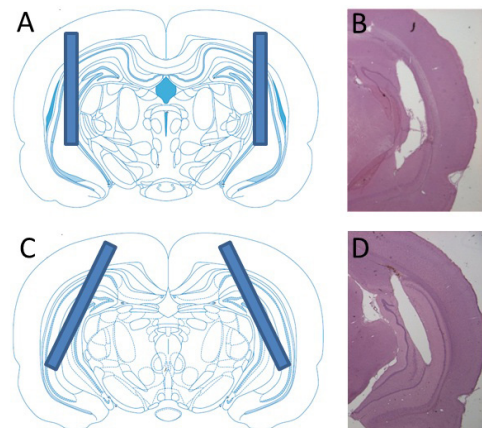### Inflammatory response in the motor cortex caused by encapsulated cells

Next we asked whether implantation of the ECB devices with galanin-producing cells could cause an inflammatory response, thereby contributing to the observed effects on seizures. However, the ECB devices, regardless whether they contained galanin-producing cells or those not modified, induced inflammatory reaction of the same magnitude as judged by estimating the numbers of activated microglia (number of Iba1-positive cells; Fig. 6A). The number of activated microglia was however less in the brain slices from animals implanted by empty ECB devices (empty 204.4±1.2 cells; ARPE 220.7±4.2 cells; LR 219.1±4.1 cells; HR 218.4±2.2 cells) (Fig. 6A). When counting ED1 immunoreactive cells in the cortex, no difference between the different groups could be detected

(empty 12.1±0.7 cells; ARPE 13.7±1.0 cells; LR, 11.3±0.7 cells; HR, 12.1±1.0 cells; p>0.05) (Fig. 6B). Similarly, double-labeled for Iba1 and ED1 immunoreactive cell numbers were not different in various groups (empty 6.1±0.4 cells; ARPE 6.8±0.8 cells; LR 5.7±0.5 cells; HR 5.7±0.4 cells; p>0.05) (Fig. 6C). Taken together, our data suggest increased number of activated microglia in the cortex from implanted cell-containing ECB devices.

Discussion

Here we demonstrate that implanted encapsulated cells releasing galanin decrease AD duration of focal seizures in a well-established model of epilepsy, rapid kindling. These data suggest that ECB devices could potentially be an alternative source for exogenous long-term galanin delivery to the brain, in particular to the hippocampus, to suppress focal epileptic seizures. The observed seizure-suppression was relatively moderate, shortening the focal seizures but having no significant effect on duration of generalized ones.

Overall, the observed effect of grafted galanin-releasing ECB devices is in line with previous publications, whereby galanin has been shown to exert an inhibitory effect on seizures (Kokaia et al., 2001; Lin et al., 2003; Kanter-Schlifke et al., 2007). The novel finding is that the ECB technology, which is a relatively safe treatment strategy compared to direct gene or cell therapy approaches, is a valid alternative, and may be considered for translational development towards clinical applications, e.g., in temporal lobe epilepsy patients. The advantage of the ECB

Figure 5. Predicted placement of ECB devices in the hippocampus. (A) Predicted placement of vertically implanted ECB devices. (B) An example of actual vertical placement of the ECB device in the hippocampus. (C) Predicted placement of the ECB devices implanted with a 26° angle. (D) An example of actual placement of the ECB device implanted with an angle.

Figure 6. Inflammatory response in the cortex after implantation of ECB devices. (A) Average number of Iba1 immunoreactive cells in the cortex of animals implanted with different types of ECB devices. (B) Average number of ED1 immunoreactive cells, a marker for activated microglia in the cortex of same animals as in (A). (C) Double-labeled Iba1 and ED1 immunoreactive cells in the same animals as in (A) and (B). Values are presented as mean ±SEM, ** $p<0.01$ and *** $p<0.001$ compared to empty.

technology from the point of patient safety is several-fold. First, the grafted cells are isolated from the host cells by a semi-permeable membrane, and therefore, it is possible to remove the whole graft in case of adverse effects or replace it if necessary; second, immune cells from the host cannot access the grafted cells, and therefore the risk of graft rejection is minimized; third, there is no genetic manipulation of host cells, diminishing the risk of unwanted mutations and carcinogenesis; fourth, there is no direct contact or interaction between the grafted and host cells, reducing the risk for transgene down-regulation or some other direct regulatory effects from the host. Despite these advantages, the ECB devices may not be a first choice when graft-host direct interaction and bi-directional integration is desirable, since ECB does not provide this possibility. This approach excludes that grafted cells would integrate and become part of the existing network, and thereby restricting release the product of interest in more regulated manner, just when it is needed. Such possible regulatory mechanism would allow for perhaps more physiological interaction of the graft and the host, preventing e.g. down-regulation of the receptors in the host cells due to permanent high levels of the ligands, as is the case for the ECB. Yet another limitation of the ECB technology may be a requirement of relatively high levels of the gene product released by the encapsulated cells to reach a therapeutically effective dose in the host brain tissue. The moderate effect of galanin-releasing ECB devices on seizures, observed in the present study, may be related to insufficient galanin levels provided by the implanted devices.

One possible confounding factor for seizure-suppressant effects observed in this study could be the inflammatory response to the galanin-producing ECB devices using ARPE cell line-containing devices. Inflammation has been shown to play a profound role in epileptogenesis and ictogenesis (Galic et al., 2008; Auvin et al., 2010). Therefore, possible inflammatory reaction of the host, despite the fact that these cells are behind the semi-permeable membrane (see above), could modulate seizures and contribute to the observed effects. Indeed, our data suggest that ECB devices containing ARPE cell lines, galanin-producing or not, elevate Iba1-positive cell counts in the cortical region of the grafted animals as compared to those implanted with empty devices. It should be noted that the ECB devices increased the number of Iba1 immunoreactive cells, but did not alter the number of ED1 immunoreactive cells. This would indicate a relatively mild level of inflammatory reaction caused by the ECB devices. However, the number of Iba1-positive microglia was similar in all groups with ECB devices containing ARPE cell lines, regardless whether they released low or high levels of galanin or no galanin at all. This would suggest that galanin-releasing ECB devices per se did not induce any additional inflammatory reaction (as judged by Iba1 immunostaining). Moreover, these data support the idea that galanin released from ECB devices was responsible for the observed seizure-suppressant effect but not the inflammatory reaction of the host. The moderate effect of galanin-releasing ECB devices could be related to several factors. One possibility is low levels of galanin released by implanted ECB

6

devices. In support of this notion, ELISA measurements of galanin release from explanted ECB devices (5 weeks after initial implantation) were less than half of what was measured prior to implantation. Such decrease in galanin release could be due to compromised survival of the encapsulated cells, which was apparent at least in some of the explanted ECB devices. Another reason for the moderate effect could be a glial scar formed around the devices. This would restrict diffusion of galanin into the host brain. Yet another factor potentially affecting the outcome of the present experiments could be related to down-regulation of galanin receptors induced by permanent increase of galanin levels around the ECB devices. These questions need to be addressed in future studies in more detail.

Conclusion

The ECB technology has previously been tested in other neurological diseases, such as Alzheimer's disease, demonstrating good safety, tolerability and positive functional outcomes (Eriksdotter-Jonhagen et al., 2012; Tornoe et al., 2012). Our data suggest that ECB devices could be a feasible strategy for delivering galanin or other seizure-suppressant agents locally into the focus of epileptic seizures. The various advantages of the ECB devices compared to other gene or gene product delivery techniques should be evaluated against the efficacy and functional outcomes. In our study, the observed seizure-suppressant effects by galanin-releasing ECB devices were moderate, and therefore may need further optimization before it can be considered for clinical application. Higher levels of galanin release over longer time periods seem to be necessary to achieve better outcomes in seizure control.

Acknowledgements

Disclosure statement

None of the authors have any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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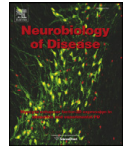
Paper III

Combined gene overexpression of neuropeptide Y and its receptor Y5 in the hippocampus suppresses seizures

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ABSTRACT

We recently demonstrated that recombinant adeno-associated viral vector-induced hippocampal overexpression of neuropeptide Y receptor, Y2, exerts a seizure-suppressant effect in kindling and kainate-induced models of epilepsy in rats. Interestingly, additional overexpression of neuropeptide Y in the hippocampus strengthened the seizure-suppressant effect of transgene Y2 receptors. Here we show for the first time that another neuropeptide Y receptor, Y5, can also be overexpressed in the hippocampus. However, unlike Y2 receptor overexpression, transgene Y5 receptors in the hippocampus had no effect on kainate-induced motor seizures in rats. However, combined overexpression of Y5 receptors and neuropeptide Y exerted prominent suppression of seizures. This seizure-suppressant effect of combination gene therapy with Y5 receptors and neuropeptide Y was significantly stronger as compared to neuropeptide Y overexpression alone. These results suggest that overexpression of Y5 receptors in combination with neuropeptide Y could be an alternative approach for more effective suppression of hippocampal seizures.

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Introduction

Epilepsy is a severe brain disorder, affecting approximately 1% of the world population, and is associated with reduced life expectancy and quality of life (Duncan et al., 2006; Jacoby and Baker, 2008). Despite significant efforts in development of novel antiepileptic drugs, 30–40% of patients respond insufficiently to current pharmacological treatments (Duncan et al., 2006). Particularly patients suffering from temporal lobe epilepsy (TLE) are often medically intractable, and although some of these may benefit from established surgical interventions (i.e., resection of epileptic focus, vagal nerve or deep brain stimulation), there is still need for development of new therapeutic strategies (Duncan et al., 2006; Thom et al., 2010).

Neuropeptide Y (NPY) is a 36-amino acid polypeptide transmitter abundantly expressed in the brain where it predominantly acts by binding to three G-protein coupled receptors (Y1, Y2, and Y5) (Berglund et al., 2003; de Quidt and Emson, 1986). NPY application exerts prominent seizure-suppressant effects in rodents in vitro and in vivo (Baraban et al., 1997; Klapstein and Colmers, 1997; Vezzani et al., 1999; Woldbye et al., 1996, 1997, 2005) and recombinant adeno-associated viral

(rAAV) vector-mediated overexpression of NPY also suppresses seizures in both acute and chronic models of epilepsy (Foti et al., 2007; Noe et al., 2008, 2010; Richichi et al., 2004; Sørensen et al., 2009). Consequently, rAAV-mediated NPY gene therapy has been proposed as an alternative treatment strategy for patients suffering from intractable TLE (McCown, 2010; Riban et al., 2009).

In the hippocampus, antiepileptic effects of NPY are mediated predominantly via binding to presynaptic Y2 receptors (El Bahh et al., 2005; Vezzani and Sperk, 2004) which subsequently inhibit glutamate release at excitatory synapses (Colmers et al., 1985; Greber et al., 1994). In addition, Y5 receptors have also been implicated in seizure-suppressant effects (Baraban, 2002; Benmaamar et al., 2005; Woldbye et al., 1997, 2005) while Y1 receptors may mediate opposite, seizure-promoting effects in the hippocampus (Benmaamar et al., 2003). Thus, once released, transgene NPY would activate both seizure-suppressant Y2/Y5 and seizure-promoting Y1 receptors at the same time. Indeed, we recently showed in two TLE models, kindling and kainate-induced seizures, that rAAV-mediated overexpression of Y2 receptors in the hippocampus exerts antiepileptic effect in rats and that, more importantly, combined overexpression of Y2 receptors and NPY had even broader seizure-suppressant action (Woldbye et al., 2010). To further capitalize on the novel concept of selective therapeutic overexpression of seizure-suppressant NPY receptors alone or in combination with NPY, we tested whether also transgene Y5 receptors would suppress kainate-induced seizures. Here we show that overexpression of functional Y5 receptors alone in the hippocampus has no detectable

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antiepileptic effect, but the combination of Y5 receptors with NPY exerts seizure-suppression, which is significantly stronger as compared to NPY alone.

Materials and methods

Animals

Adult male Wistar rats (Charles River, Germany) weighing 250–350 g at the beginning of the experiments were used. Animals were housed on 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.

Viral vector injections

The rAAV vector was a chimeric serotype, consisting of serotype 2 inverted terminal repeats (ITRs) and combined serotype 1 and 2 capsid proteins. The transgenes were subcloned into an expression cassette consisting of the rat neuron-specific enolase promoter, Woodchuck post-transcriptional regulatory element, and a bovine growth hormone polyA signal flanked by viral ITRs (Richichi et al., 2004). The rAAV vectors were manufactured by GeneDetect (Auckland, New Zealand) and encoded the full-length cDNA for the mouse Y5 receptor (accession number: AF049329; rAAV-Y5: stock solutions 1.0×10^{12} genomic particles/ml), human prepro-NPY (rAAV-NPY: stock solution 1.0×10^{12} genomic particles/ml), or empty cassette vector (rAAV-Empty; 1.0×10^{12} genomic particles/ml). Injections were performed as previously described (Kanter-Schlifke et al., 2007a, 2007b; Sørensen et al., 2008). Animals were anesthetized by inhalation of isoflurane (Baxter Medical AB, Sweden) and gently fixed in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). Before and after surgery, the local analgesic Marcain (AstraZeneca, Denmark) was subcutaneously administered around the wound. A volume of 2 μl viral vector phosphate-buffered saline (PBS) suspension was infused through a glass pipette (0.2 μl/min) bilaterally into the dorsal hippocampus (anterior–posterior (AP) −3.3 mm, medial–lateral (ML) ±1.8 mm, dorsal–ventral (DV) −2.6 mm) and the ventral hippocampus (AP −4.8 mm, ML ±5.2 mm, DV −6.4 and −3.8 mm; 1 μl at each location in the DV plane) (Paxinos and Watson, 2005). Reference points were bregma for the AP axis, midline for the ML axis, and dura for the DV axis. The pipette was left in place for an additional 3 min after injection to prevent backflow of viral particles through the injection track. The viral vector suspensions were mixed (1:1) from viral vector stock solutions diluted with sterile PBS or in a mixture of equal portions of the two in the combination gene therapy group (rAAV-Y5/NPY). Non-treated or rAAV-Empty treated rats were used as controls.

Transgene overexpression

To confirm the presence of rAAV vector mediated transgene overexpression we investigated hippocampal Y5 receptor and NPY gene expression, Y5 receptor-like and NPY-like immunoreactivity, Y5 receptor binding, and functional Y5 receptor binding.

Y5 receptor and NPY mRNA in situ hybridization

The procedure was performed as previously described (Woldbye et al., 2010) with only minor modifications. At the time of sacrifice, the rats were decapitated and their brains were quickly removed and frozen on dry ice. Subsequently, 15 μm thick coronal serial sections at the hippocampal level were cut on a cryostat and thaw-mounted on Superfrost glass slides, dried on a hotplate and stored at −80 °C. The slides were defrosted for 10 min at room temperature (R_T), subsequently fixed for 5 min in 4% PFA, and rinsed briefly and placed for 5 min in PBS. Then,

the slides were transferred to 70% ethanol for 5 min and stored in 95% ethanol at 4 °C until hybridization. Synthetic antisense oligonucleotide DNA probes were used for *in situ* hybridization: Y5 receptor mRNA: 5′-CGA GTC TGT TTT CTT TGT GGG ACA ATC CAC AGC TTA TAC TCC TGC-3′; prepro-NPY mRNA: 5′-GTC-CTC-TGC-TGG-CGC-GTC-CTC-GCC-CGG-ATT-GTC-CGG-CTT-GGA-GGG-GTA-3′ (Mikkelsen and Woldbye, 2006). The oligoprobes were labeled at the 3′-end with $[\alpha^{35}S]dATP$ (1250 Ci/mmol; #NEG734H001MC; PerkinElmer, Denmark) using terminal deoxynucleotidyl transferase (Roche Diagnostics, Mannheim, Germany). Labeled probes were added with a specific activity of $1–3 \times 10^5$ cpm/100 μl to the hybridization buffer containing 50% formamide (v/v), 4× saline sodium citrate (SSC; 1× SSC = 0.15 M NaCl, 0.015 M NaCitrate–2H$_2$O, pH 7.0), 10% dextran sulfate (w/v) and 10 mM dithiothreitol. After adding a volume of 120 μl hybridization mixture to each slide, they were covered with Parafilm and left overnight at 42 °C in humidity boxes. The slides were then briefly rinsed in 1× SSC at R_T, washed for 30 min in 1× SSC at 60 °C, passed through a series of 1 min rinses in 1× SSC, 0.1× SSC, 70% ethanol, and 95% ethanol at R_T, and finally air-dried. The slides where exposed together with ^{14}C-microscales on ^{35}S-sensitive Kodak BioMax MR films (Amersham Biosciences, Denmark) for 3–8 weeks and developed in Kodak GBX developer.

Y5 receptor immunohistochemistry

Sections were defrosted for 10 min at R_T and subsequently fixed for 30 min in 4% PFA, washed three times for 5 min in PBS, incubated in preincubation buffer (5% goat serum, 1% bovine serum albumin (BSA), 0.3% Triton X-100 in PBS) for 30 min, and placed in incubation buffer with rabbit anti-Y5 receptor antibody (1:300, Alomone Labs, Israel) overnight at 4 °C. The slides were then washed three times for 5 min in washing buffer (0.25% BSA, 0.1% Triton X-100 in PBS), left in incubation buffer with Alexa 568 goat anti-rabbit (1:200, Invitrogen, Denmark) for 1 h and washed three times for 5 min, once in washing buffer and twice in PBS. The sections were then mounted on glass slides using ProLongGold Antifade (Invitrogen).

NPY immunohistochemistry

Seizure-naïve rats injected 3 weeks previously with rAAV-NPY into the right hippocampus were deeply anesthetized with sodium pentobarbital and perfused through aorta with 0.9% NaCl followed by 4% PFA. The contralateral non-injected hemisphere served as control. Brains were post-fixed overnight at 4 °C, transferred to 20% sucrose, and stored overnight at 4 °C. Then the brains were cut into 30 μm thick slices on a microtome. Free-floating sections were rinsed in potassium phosphate buffer (KPBS) and incubated overnight at R_T with rabbit anti-NPY antibody (1:10,000; Sigma-Aldrich, St. Louis, MO, USA) in a solution of 5% goat serum, 0.25% Triton X-100 in KPBS. The slices were then rinsed and incubated with secondary biotinylated goat-anti rabbit antibody (1:200; Vector Laboratories, Burlingame, CA, USA), and staining was visualized by 3-3′-diaminobenzidine reaction.

Y5 receptor binding

Y5 receptor binding was performed as previously described (Woldbye et al., 2005). The slides were defrosted at R_T and subsequently preincubated for 20 min in binding buffer (pH 7.4), containing 25 mM N-(2-hydroxyethyl)-piperazine-N′-(2-ethanesulfonic acid) (HEPES), 2.5 mM CaCl$_2$, 0.5 g/l bacitracin, 0.5 g/l BSA. The slides were then incubated at R_T for 60 min in binding buffer containing 0.1 nM $[^{125}I][Tyr^{36}]$mono-iodo-PYY (4000 Ci/mmol; porcine synthetic, #IM259; Amersham Biosciences, Denmark) to which was added 100 nM BIBP3226 (Y1 receptor antagonist; #E3620, Bachem AG, Switzerland) + 100 nM BIIE0246 (Y2 receptor antagonist; #1700, Tocris Bioscience, UK) to visualize Y5 receptor binding. After a brief rinse, the slides were washed for 2 × 30 min in binding buffer at R_T

and subsequently air-dried, before being exposed to ^{125}I-sensitive Kodak BioMax MS films (Amersham Biosciences) for 4 days at −20 °C with ^{125}I-microscales (Amersham Biosciences). Non-specific binding was performed by adding of unlabeled NPY (1 μM) to displace [^{125}I]-PYY binding. The films were developed with Kodak GBX developer.

[^{35}S]GTPγS functional Y5 receptor binding

Functional binding was performed as previously described (Agasse et al., 2008; Christensen et al., 2006; Silva et al., 2007). Sections were defrosted for 30 min at R_T before being rehydrated for 10 min at R_T in assay buffer A (50 mM Tris–HCl, 3 mM MgCl, 0.2 mM ethylene glycol tetraacetic acid, 100 mM NaCl, pH 7.4). Sections were preincubated for 20 min at R_T in buffer B [assay buffer A, 0.2 mM dithiothreitol, 1 μM 1,3-dipropyl-8-cyclopentylxanthine (#C-101, PerkinElmer, Denmark), 0.5% w/v BSA, and 2 mM guanosine-5′-diphosphate (#G7127, Sigma-Aldrich, Denmark)] and then incubated at 25 °C for 1 h in buffer B together with 40 pM [^{35}S]GTPγS (1250 Ci/mmol; #NEG030H250UC; PerkinElmer, Denmark) and 1 μM NPY (rat synthetic, Schafer-N, Copenhagen, Denmark). For visualization of functional Y5 receptor binding, the Y1 receptor antagonist BIBP3226 (1 μM; #E3620, Bachem AG, Switzerland) and the Y2 receptor antagonist BIIE0246 (1 μM; #1700, Tocris Bioscience, UK) were added to both preincubation and incubation buffers. To block and confirm the specificity of functional Y5 receptor binding, the Y5 receptor antagonist L-152,804 (10 μM; #1382, Tocris Bioscience, UK) was added to both preincubation and incubation buffers together with Y1 and Y2 receptor antagonists at same concentrations as above. Basal binding was determined by incubation in buffer B with 40 pM [^{35}S]GTPγS (1250 Ci/mmol) but without NPY receptor ligands. Non-specific binding was determined by incubation in buffer B (without NPY receptor ligands) with 40 pM [^{35}S] GTPγS and 10 μM non-labeled GTPγS (#89378; Sigma-Aldrich, Denmark). Y1, Y2, and Y5 receptor antagonists were dissolved in dimethyl sulphoxide to a final concentration of 0.1%, and dimethyl sulphoxide was, likewise, added to the other incubation buffers (0.1% final concentration). Incubation was terminated by two times 5 min washing in ice-cold 50 mM Tris–HCl buffer (pH 7.4). Finally, sections were rinsed in ice-cold demineralized H_2O and dried under a stream of cold air. Sections were exposed to ^{35}S-sensitive Kodak BioMax MR films together with ^{14}C-microscales for 5 days and then developed in Kodak Processing Chemicals for Autoradiography Films (Sigma-Aldrich, Denmark). Computer-assisted autoradiographic image analysis was performed using Scion Image (National Institute of Health, USA). Measurements were conducted by a person blinded to the vector treatment of the animals bilaterally in 3–4 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.

Kainate-induced seizures

Three to 4 weeks after virus injections, rats treated with rAAV-Y5, rAAV-NPY, rAAV-Y5/NPY, or rAAV-Empty vectors were injected subcutaneously in the neck region with kainate (10 mg/kg; diluted in 0.9% isotonic saline; pH 7.4; Sigma #K2389; Sigma, St. Louis, MO, USA). Subsequently, the animals were placed in individual Plexiglas boxes (30×19×29 cm) and observed for motor seizures as previously described (Woldbye et al., 1997). Each rat was rated for 2 h by an observer unaware of the treatment condition, and latencies to first motor seizure and status epilepticus, percentage time spent in motor seizures as well as the number of seizures were determined. Motor seizures were defined as clonic movements involving fore-

and/or hindlimbs of at least 15 s duration, and status epilepticus was defined as continuous clonic motor seizure activity of at least 10 min duration. Moreover, a total seizure score was determined based on a modified rating scale of Racine (1972): grade 0 (no motor seizure occurrence), grade 1 (starring or facial movements), grade 2 (head nodding or isolated twitches), grade 3 (motor seizure with limb clonus), grade 4 (motor seizure with rearing), grade 5 (motor seizure with loss of posture), 6 (status epilepticus) and 7 (death). Three hours after the kainate injection, the experiment was terminated by decapitation whereupon the brains were quickly removed and processed as described above.

Electrophysiology

For electrophysiology, hippocampal slices were prepared from rats injected bilaterally in the hippocampus 4 to 5 weeks previously with rAAV-Y5 or rAAV-Y5/NPY as above. Age-matched non-injected rats served as controls. After decapitation, their brains were immediately immersed into oxygenated (95% O_2/5% CO_2) and ice-cold sucrose-based solution containing (in mM): 75 sucrose, 67 NaCl, 26 NaHCO₃, 25 glucose, 2.5 KCl, 0.5 CaCl₂, 7 MgCl₂; adjusted to 308 mOsm and 7.4 pH. Coronal slices of 300 μm thickness were prepared in the same solution using a Leica VT1200 vibratome. Slices were kept for 1 h at 34 °C in oxygenated sucrose-based solution, and then stored in oxygenated artificial cerebrospinal fluid (aCSF; containing in mM: 119 NaCl, 26 NaHCO₃, 25 glucose, 2.5 KCl, 1.25 NaH₂PO₄, 2.5 CaCl₂, 1.3 MgSO₄; 303 mOsm; 7.4 pH) held at room temperature until further use. In a submerged recording chamber, individual slices were constantly perfused at 2.5 ml/min with oxygenated aCSF at 32.5 °C. It is well established that NPY reduces excitatory glutamatergic transmission in the hippocampus (Colmers et al., 1985; Sørensen et al., 2008; Vezzani et al., 1999). Our recent finding shows that NPY may also affect GABAergic synaptic transmission onto basket cells of the dentate gyrus (Ledri et al., 2011). Therefore, picrotoxin (PTX; 100 μM; Tocris Bioscience, Ellisville, MI, USA) was added to the perfusion medium to analyze excitatory synaptic transmission in isolation.

The recordings were conducted as described elsewhere (Sørensen et al., 2008). Briefly, in Schaffer collateral–CA1 synapses, field excitatory postsynaptic potentials (fEPSPs) were recorded at 10 kHz with a borosilicated glass pipette containing aCSF (3–4 MΩ resistance) using HEKA amplifier and PATCHMASTER software (HEKA Elektronik, Lambrecht, Germany). The recording electrode was positioned in CA1 stratum radiatum, while orthodromic current stimulations of Schaffer collateral fibers were applied in the same subfield (~500 μm towards CA3) by a glass pipette containing aCSF. Basal excitatory synaptic transmission was determined in each slice by stepwise increasing the stimulation strength until maximal fEPSP was generated. The presynaptic fiber volley (mV) was plotted against the initial slope of the fEPSP (mV/ms) and an input–output (I–O) relationship was generated. For paired-pulse stimulations, submaximal stimulation intensities generating 40–50% of maximal fEPSP were used. These stimuli were applied at 0.05 Hz with 25, 50, 100, and 200 ms interstimulus intervals (ISI) to elicit paired-pulse fEPSPs. Short-term synaptic plasticity was determined by the paired-pulse facilitation (PPF) ratio, and calculated, based on the average of four consecutive stimuli, as the initial slope of fEPSP[2] divided by the initial slope of EPSP[1]. To block endogenous Y2 receptors, BIIE0246 (Tocris Bioscience) was initially dissolved in ethanol (99.5%) and then diluted 1:10,000 in aCSF to reach a final concentration of 0.3 μM. BIIE0246 was applied for at least 8 min before resuming the recordings. After recordings, slices fixed overnight in 4% PFA at 4 °C, rinsed in KPBS and stored in anti-freeze at −20 °C until further processing. Researchers were blinded with regard to vector treatment during acquisition and analysis of data.

Statistical analysis

Differences between groups were analyzed using one-way ANOVA followed by Newman–Keuls post-hoc test or two-tailed Student's *t*-test. Electrophysiological data were analyzed with two-way ANOVA followed by Bonferroni post-hoc test. Data are presented as mean ± SEM. $P < 0.05$ was considered statistically significant.

Results

Y5 receptor and NPY mRNA in situ hybridization

Three to 4 weeks after intrahippocampal injections of rAAV-Y5 or rAAV-NPY prominent increases in mRNA levels of Y5 receptor (Fig. 1A) or NPY (Fig. 1G), respectively, were observed in the hippocampal dentate gyrus and CA1–CA3 regions as compared to non-treated animals (Figs. 1B and H). Consistent with previous observations (Richichi et al., 2004; Woldbye et al., 2010), NPY and NPY receptor expression was mainly restricted to the principal layers of the hippocampus, i.e., the dentate granule layer and pyramidal layers of CA1–CA3 along with scattered dotted expression around these layers, most likely due to labeling of interneurons. In slices used for electrophysiology, overexpression of Y5 receptor and NPY was also confirmed using *in situ* hybridization (data not shown).
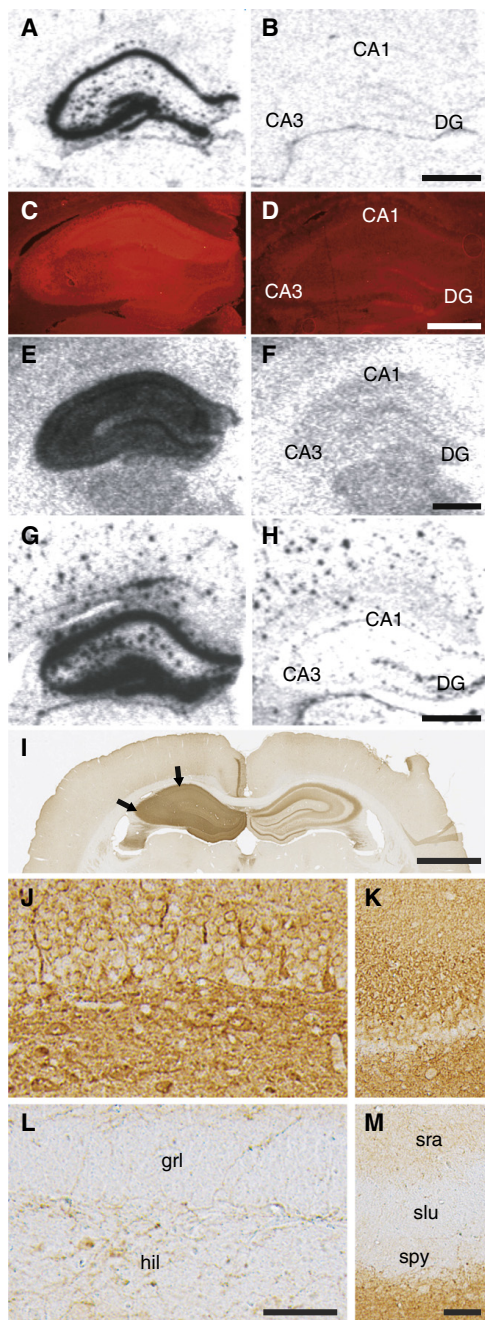
Y5 receptor and NPY immunohistochemistry

Next we explored whether rAAV-induced increased Y5 receptor mRNA levels were translated into protein using Y5 receptor immunohistochemistry. Indeed, dense Y5 receptor-like immunoreactivity (Fig. 1C) was evident throughout the hippocampus in slices obtained from rAAV-Y5 treated animals, while levels were low in slices obtained from non-treated rats (Fig. 1D). Y5 receptor overexpression in slices used for electrophysiology was also confirmed by Y5 receptor immunohistochemistry (data not shown).

In accordance with previous studies using a similar vector construct (Richichi et al., 2004; Sørensen et al., 2009), rAAV-NPY injections resulted in increased NPY-immunoreactivity throughout the ipsilateral hippocampus as compared to the contralateral non-treated side (Figs. 1I–M). A dense band of NPY-immunoreactivity in the contralateral inner molecular layer of the dentate gyrus most likely represents labeling of hippocampal commissural fibers since this was not observed in rAAV-Empty treated rats (Richichi et al., 2004; Sørensen et al., 2009).

Y5 receptor binding

We further explored whether also Y5 receptor binding ([^{125}I]-peptide YY + Y1 and Y2 receptor antagonist) was increased after

Fig. 1. Levels of Y5 and NPY mRNA, Y5 and NPY-immunoreactivity, and Y5 receptor binding in seizure-naive rats at 3–4 weeks after intrahippocampal injection of rAAV-Y5 or rAAV-NPY. (A) Increased Y5 mRNA labeling after bilateral rAAV-Y5 treatment as compared to (B) non-treated control. (C) Increased Y5-like immunoreactivity in an animal bilaterally injected with rAAV-Y5 as compared to (D) non-treated control. (E) Increased Y5 receptor binding (^{125}I-PYY + BIBP3226 + BIIE0246) after bilateral rAAV-Y5 treatment as compared to (F) non-treated control. (G) Increased NPY mRNA labeling after bilateral rAAV-Y5/NPY treatment as compared to (H) non-treated control. Scale bars = 1 mm. (I) Increased NPY-immunoreactivity (arrows) ipsilaterally as compared to the contralateral side after unilateral injection of rAAV-NPY vector in the dorsal hippocampus. Scale bar = 2 mm. High power magnifications show increased NPY-immunoreactivity ipsilaterally (J) in the grl and hil of the DG as well as (K) in the slu, sra, and spy of CA3 after unilateral injection of rAAV-NPY vector as compared to the corresponding contralateral side (L and M, respectively). Scale bars = 30 μm. NPY, neuropeptide Y; rAAV, recombinant adeno-associated virus; PYY, peptide YY; grl, granular layer; hil, hilus. DG, dentate gyrus; slu, stratum lucidum; sra, stratum radiatum; spy, pyramidal cell layer.

rAAV-Y5 vector injections. Consistent with Y5 receptor-like immuno-reactivity, Y5 binding was also elevated throughout the hippocampus as compared to non-treated rats (Figs. 1E and F).

[^{35}S]GTPγS functional Y5 receptor binding

Next we investigated whether binding of NPY could activate transgene Y5 receptors, thus suggesting that they were functional. We consequently conducted Y5 receptor [^{35}S]GTPγS functional binding assay (NPY + Y1 and Y2 receptor antagonists). Three to 4 weeks after injections of rAAV-Y5 we found prominent increases in functional Y5 receptor binding as compared to non-treated control animals (Figs. 2A and D). Basal [^{35}S]GTPγS binding (without addition of NPY) in rAAV-Y5 treated animals was also increased as compared to non-treated controls (Figs. 2B and E). Specificity of functional Y5 receptor binding was confirmed by addition of the Y5 receptor antagonist L-152,804 which blocked the [^{35}S]GTPγS signal (Figs. 2C and F). Functional Y5 binding was also strongly increased in kainate-treated animals after either rAAV-Y5 or rAAV-Y5/NPY vector injections as compared to rAAV-Empty treated animals (Figs. 2G–I). Interestingly, the corresponding basal functional binding in animals treated with the rAAV-Y5/NPY combination was remarkably high as compared to both rAAV-Y5 and rAAV-Empty treated animals (Figs. 2J–L).

Functional Y5 receptor binding in kainate-treated animals after viral vector injections was quantified in the hippocampal dentate gyrus, CA3, and CA1 (Fig. 2M). Both rAAV-Y5 and rAAV-Y5/NPY vector treatments resulted in significantly higher functional Y5 receptor binding in all measured regions (dentate gyrus: by 106% and 71%, CA3: by 342% and 384%, and CA1: by 331% and 333% for rAAV-Y5 and rAAV-Y5/NPY, respectively) as compared to rAAV-Empty. Basal functional binding levels in animals treated with the rAAV-Y5/NPY combination were significantly increased in all measured regions (dentate gyrus: by 67%, CA3: by 338%, and CA1: by 275%) as compared to rAAV-Empty (Fig. 2N). In fact, functional Y5 receptor binding in rAAV-Y5/NPY vector treated animals was only elevated modestly above corresponding basal functional binding in the CA3 (14.0%, $P = 0.023$, paired t-test), not in the dentate gyrus and CA1, indicating that transgene Y5 receptors were close to maximal functional activity under basal conditions.

Seizure-suppressant effects of transgene overexpression

Next we investigated if overexpression of Y5 receptor or the combination of Y5 receptor and NPY could affect kainate-induced seizures in rats 3 to 4 weeks after bilateral vector injections. In contrast to Y2 receptor overexpression in a previous study (Woldbye et al., 2010), rAAV-Y5 vector treatment alone did not induce seizure-suppressant effects on any of the seizure parameters analyzed in this study. However, when Y5 receptor overexpression was combined with NPY, there was a significant seizure-suppressant effect as revealed by increased latencies to first motor seizure and status epilepticus (Fig. 3A), as well as decreased relative time (in percent) spent in motor seizures (Fig. 3B) and decreased seizure scores (Fig. 3D). These effects on seizures were significantly stronger as compared to seizure-suppressant effects exerted after single rAAV-NPY treatment (Figs. 3A, B and D). In addition, the number of motor seizures was significantly reduced only after rAAV-Y5/NPY vector treatment but not after rAAV-NPY alone (Fig. 3C).

Since NPY and Y5 receptors are implicated in stimulating appetite (Ishihara et al., 2006; Tiesjema et al., 2007), we also studied body weight of the animals after the different vector treatments. We found no significant differences in body weight at the start of the experiment (data not shown; $P = 0.86$, one-way ANOVA) and no significant differences in weight gain 3 weeks after viral vector injections (rAAV-Y5 by 27.9 ± 2.2%, rAAV-NPY by 30.7 ± 3.7%, rAAV-Y5/NPY by 32.3 ± 2.7%, rAAV-Empty: 30.0 ± 2.3%; $P = 0.71$, one-way ANOVA).

Basal excitatory synaptic transmission after transgene overexpression
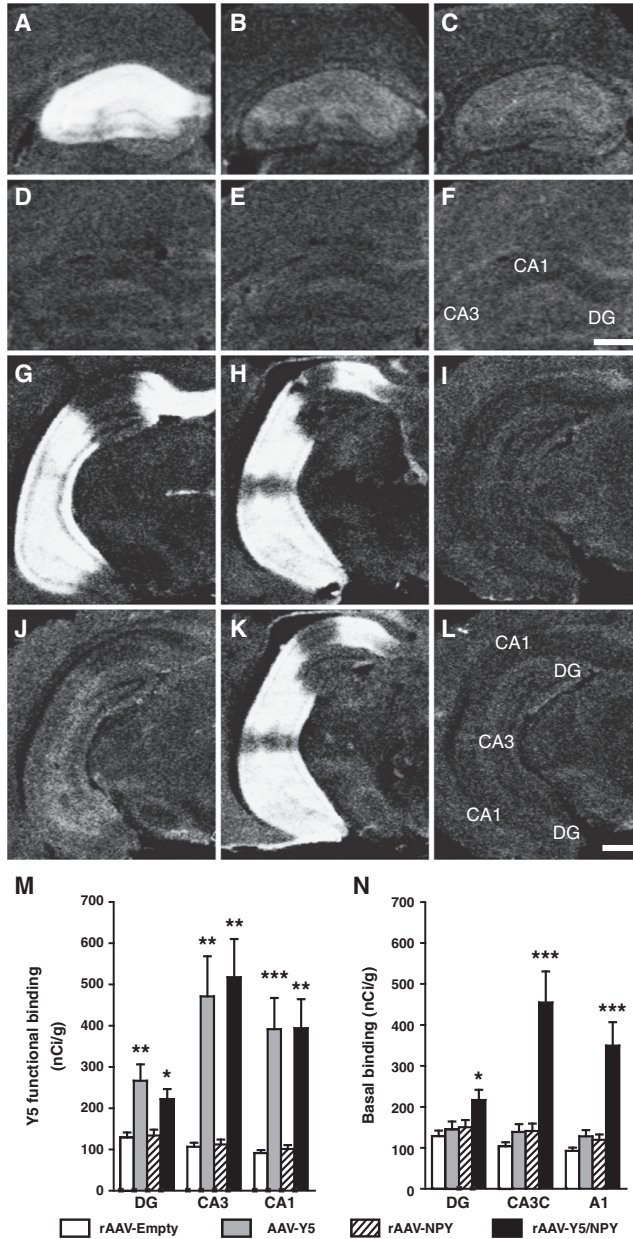
Since the seizure-suppressant effect of transgene NPY is most likely induced by suppression of presynaptic glutamate release from principal synapses in the hippocampus (Colmers et al., 1985; Vezzani et al., 1999), we asked whether combined Y5/NPY overexpression in the hippocampus would compromise normal excitatory synaptic transmission. We therefore recorded evoked field excitatory postsynaptic potentials (fEPSPs) in Schaffer collateral–CA1 synapses in slices from rAAV-Y5/NPY (n = 10 slices, 5 rats), rAAV-Y5 (n = 10 slices, 5 rats), and non-treated (n = 15 slices, 5 rats) animals. Throughout the experiments, GABA-A receptors were blocked by picrotoxin (PTX) application. Basal excitatory synaptic transmission was examined by determining the relationship between the presynaptic fiber volley (PSFV) and the corresponding fEPSPs amplitude (I–O relationship) at various stimulation strengths. This analysis revealed that neither combined rAAV-Y5/NPY nor rAAV-Y5 vector treatment affected the I–O relationship, indicating no alterations in basal excitatory synaptic transmission in Schaffer collateral–CA1 synapses (Fig. 4A). To further explore possible changes in these synapses, we applied paired-pulse stimulations at different interstimulus intervals (ISIs) to Schaffer collaterals and analyzed paired-pulse facilitation (PPF) of generated fEPSPs. The potential contribution of Y2 receptors was blocked by the adding of the Y2 receptor antagonist BIIE0246. No significant changes were detected in PPF between the three treatments (Fig. 4B).
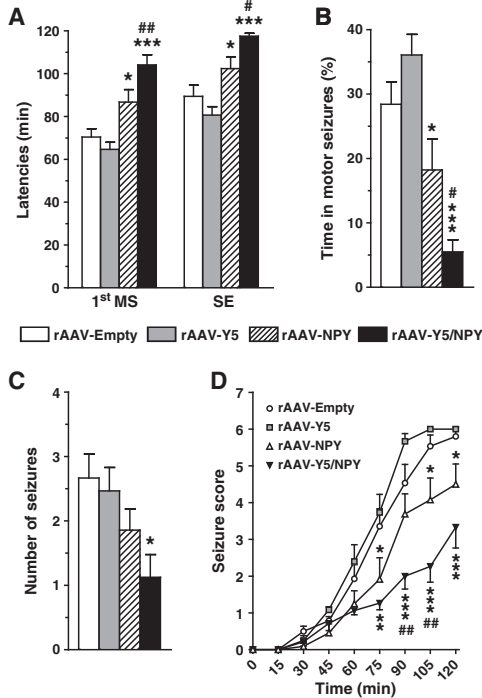
Discussion

In the present study, we show for the first time that rAAV-mediated overexpression of functional NPY Y5 receptors can be achieved in the rat hippocampus. However, overexpression of transgene Y5 receptors alone did not suppress kainate-induced seizures. Only when combined with NPY, Y5 receptor overexpression in the hippocampus resulted in pronounced seizure-suppressant effect. This effect was significantly stronger than that of NPY overexpression alone.

We previously achieved rAAV-based gene transfer for functional Y2 receptors in the hippocampus, and now demonstrate that this is also possible for Y5 receptors. Overexpression induced by rAAV-Y5 was documented by elevated Y5 receptor mRNA levels, Y5 receptor-like immunoreactivity, Y5 receptor binding, and functional Y5 receptor binding. The latter was blocked by the simultaneous addition of the Y5 receptor antagonist L-152,804, confirming the specificity of the functional Y5 receptor binding signal. We also observed slightly increased basal functional binding (without NPY added) in seizure-naive rAAV-Y5 treated animals. This may suggest that endogenous NPY can activate some of the transgene Y5 receptors under normal conditions, similar to what has been observed previously after hippocampal overexpression of Y2 receptors (Woldbye et al., 2010). When, in addition to Y5 receptors, NPY was overexpressed in the hippocampus, both seizure-naive and kainate-treated rats showed pronounced increases in basal functional binding as compared to both rAAV-Y5 and rAAV-Empty treated animals. Moreover, addition of exogenous NPY caused no further increase in functional Y5 receptor binding in the dentate gyrus and CA1 of rAAV-Y5/NPY treated rats as compared to basal functional binding (without NPY addition), and only resulted in a modest increase in the hippocampal CA3 region. This suggests that transgene Y5 receptors are activated under normal basal conditions, and indicates that transgene NPY is, at least partially, constitutively released from transduced neurons. In contrast, we previously observed that functional Y2 receptor binding was substantially increased above basal binding (100–170%) after addition of exogenous NPY (in the presence of Y1 and Y5 receptor antagonists) in rats treated with the rAAV-Y2/NPY vector combination in the hippocampus (data not shown). Likewise, rAAV-Y2/NPY vector treated rats showed

Fig. 2. Increased functional Y5 receptor [^{35}S]GTPγS binding at 3–4 weeks after bilateral intrahippocampal injection of rAAV-Y5 and rAAV-Y5/NPY (inverted colors). (A) Increased functional Y5 receptor binding (NPY + BIBP3226 + BIIE0246) in rAAV-Y5 injected seizure-naïve rat as compared to (D) non-treated rat. (B and E) Basal levels of functional binding (without adding NPY) in animals from A and D, respectively. (C and F) Blocking of functional Y5 binding with the Y5 antagonist L-152,804 in animals from A and D, respectively. Functional Y5 receptor binding displayed in inverted colors in kainate-treated animals after injection of (G) rAAV-Y5, (H) rAAV-Y5/NPY or (I) rAAV-Empty. (J–L) Corresponding basal levels of functional binding (without adding NPY) in rats from G–I. (M) Increased functional Y5 receptor binding and (N) corresponding basal binding measured in hippocampal DG, CA3 and CA1 of kainate-treated rats after rAAV-Y5, rAAV-NPY, or rAAV-Y5/NPY treatment as compared to rAAV-Empty (*$P<0.05$, **$P<0.01$, ***$P<0.001$ versus rAAV-Empty, Newman–Keuls post-hoc test following significant one-way-ANOVA). Data are mean ± SEM (n = 9–10). rAAV, recombinant adeno-associated virus; NPY, neuropeptide Y; DG, dentate gyrus.

Fig. 3. Bilateral intrahippocampal injections of rAAV-Y5 potentiate the seizure-suppressant effect of rAAV-NPY but fail to affect seizures on its own. (A) Latencies to 1st MS and SE were significantly increased and (B) percent time in motor seizures was significantly decreased after rAAV-Y5/NPY and rAAV-NPY treatment. rAAV-Y5/NPY effects were significantly stronger as compared to rAAV-NPY. No significant effects were detected after rAAV-Y5 treatment compared to rAAV-Empty controls. (C) Only the rAAV-Y5/NPY combination decreased the number of motor seizures. (D) The seizure score was significantly decreased after treatment with both rAAV-Y5/NPY and rAAV-NPY. The rAAV-Y5/NPY combination had a significantly stronger effect as compared to rAAV-NPY. Data are mean ± SEM (n = 13–16). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. rAAV-Empty; #$P < 0.05$, ##$P < 0.01$ vs. rAAV-NPY and rAAV-Y5, Newman–Keuls post-hoc test following significant one-way-ANOVA. rAAV, recombinant adeno-associated virus; NPY, neuropeptide Y; MS, motor seizure; SE, status epilepticus.

Fig. 4. Basal excitatory synaptic transmission and short-term synaptic plasticity are unchanged 4–5 weeks after bilateral intrahippocampal injection of rAAV-Y5/NPY and rAAV-Y5. (A) The relationship between the PSFV and the slope of the corresponding fEPSP was similar in slices from rAAV-Y5/NPY, rAAV-Y5, and non-treated animals, suggesting unaltered basal excitatory synaptic transmission. (B) The PPF ratio was found to be similar between the three groups at different ISIs, after blocking a potential contribution from Y2 receptors with the Y2 antagonist BIIE0246. Insert: representative fEPSPs recorded in a slice obtained from a rAAV-Y5/NPY treated animal showing PPF at ISI of 100 ms. Scale bar: 0.4 mV and 20 ms, respectively. Data are mean ± SEM (n = 10–15). rAAV, recombinant adeno-associated virus; NPY, neuropeptide Y; PSFV, presynaptic fiber volley; fEPSP, field excitatory postsynaptic potential; PPF, paired-pulse facilitation; ISI, interstimulus interval.

more or less similar modest elevated hippocampal levels of basal binding as compared to rAAV-Y2 treated animals (data not shown), while basal binding of rAAV-Y5/NPY vector treated rats of the present study was clearly above that of rAAV-Y5 single vector-injected animals. At present, it is not clear why transgene Y5 receptors appear to be more activated by transgene NPY (without exogenous NPY addition), and thereby differ from transgene Y2 receptors. Nevertheless, our data suggest that Y5 transgene receptors are somewhat more prone to be activated under basal conditions after rAAV-Y5/NPY injection.

The present study showed that rAAV-induced hippocampal NPY overexpression caused substantial suppressant effect on kainate-induced seizures. This finding is consistent with previous work from our and other groups (Noe et al., 2008; Richichi et al., 2004; Sørensen et al., 2009). In contrast, overexpression of functional Y5 receptors *per se* caused no significant changes in any measured seizure parameter as compared to control animals. Nonetheless, the combined overexpression of functional Y5 receptors and NPY exerted seizure-suppressant effects superior to the effects seen after single NPY

transgene overexpression. It remains to be explored why rAAV-Y5 treatment alone, as opposed to the rAAV-Y5/NPY vector combination, did not lead to seizure-suppressant effects. One may speculate that a proportion of transgene Y5 receptors after rAAV-Y5 vector treatment is not accessible to released endogenous NPY, perhaps due to divergent cellular or regional spatial distribution of endogenous NPY release sites and transgene Y5 receptor expression sites. This scenario, however, seems less likely, since we recently demonstrated that overexpression of the Y2 receptor alone was sufficient to exert prominent seizure-suppressant effects on rapid kindling and kainate-induced seizures (Woldbye et al., 2010). One has to bear in mind though that the endogenous Y2 receptor expression pattern in the hippocampus (Gustafson et al., 1997) is fundamentally different from that of Y5 receptors (Durkin et al., 2000), which may also influence the transgene Y5 receptor expression pattern. The special distribution of transgene NPY, on the other hand, may match closer the transgene Y5 receptor expression pattern, and thereby more readily activate them, leading to seizure-suppressant effects. In favor of this interpretation, we observed high basal functional binding in rAAV-Y5/NPY-treated animals. This finding would also imply that transgene NPY is released at basal conditions even during normal, low frequency, synaptic activity, possibly resembling an adaptive mechanism of hippocampal circuits to counteract seizures. Our findings of unaltered basal excitatory synaptic transmission and PPF in the CA1 area, however, argue against such interpretation.

In the present study, viral vectors were injected prior to the induction of acute seizures. Therefore, seizure-suppressant effect of Y5 and

NPY overexpression in the hippocampus can be taken as an initial proof-of-principle. To mimic more closely the clinical situation, similar treatment should be applied to animals with already established spontaneous, recurring chronic seizures. Nonetheless, the results obtained with the present design of the study give a good indication that such approach may also work in chronic seizure models. In support, rAAV-NPY treatment has been shown to exert a seizure-suppressant effect both in an acute (present design) and in a chronic rat TLE model, in which vector treatment was applied after the occurrence of spontaneous, recurrent seizures (Noe et al., 2008). In line with this notion, NPY ligands effectively decrease excitatory synaptic transmission in the hippocampal neurons from surgically resected human TLE of pharmacoresistant epilepsy patients (Colmers et al., 1997; Patrylo et al., 1999).

The present finding that combined overexpression of NPY and its Y5 receptor exerted superior effects as compared to single vector treatment lends further support to the concept of combination gene therapy with overexpression of endogenous ligands and their respective receptors (Woldbye et al., 2010). This is in line with our previous findings using a combination of NPY and Y2 receptor overexpression (Woldbye et al., 2010). Moreover, this concept can be extended to overexpression of different combinations of endogenous ligands to achieve seizure-suppressant effects, as was shown with fibroblast growth factor-2 and brain-derived neurotrophic factor (Paradiso et al., 2009). Thus, our study underscores the importance of targeting various endogenous therapeutic molecules and their receptors for combined gene therapy approaches in developing more effective and tailored future strategies for epilepsy treatment.

From the clinical perspective, it will be important to carefully explore possible adverse effects associated with long-term upregulation of NPY and its receptors. One possible concern is the effect of vector treatment on basal excitatory synaptic transmission and normal information processing, since NPY is known to suppress glutamate release in hippocampal excitatory synapses (Colmers et al., 1985; Greber et al., 1994). We found no evidence of altered basal excitatory synaptic transmission in rAAV-Y5 or combined rAAV-Y5/NPY vector treated animals, which is in line with our previous observations in rAAV-NPY treated rats (Sørensen et al., 2008, 2009). Previously, pronounced increases in body weight were observed after hypothalamic rAAV-NPY injections (Tiesjema et al., 2007), and Y5 receptors have been implicated in mediating feeding stimulatory effects of NPY in the hypothalamus (Ishihara et al., 2006). None of the three vector treatments in the present study (rAAV-Y5, rAAV-NPY, and rAAV-Y5/NPY) caused significant changes in body weight. A study from another group also did not observe changes in body weight after intrahippocampal injection of rAAV-NPY (Richichi et al., 2004) and neither did we in our previous study using rAAV-NPY, rAAV-Y2, and rAAV-Y2/NPY (Woldbye et al., 2010, unpublished observations), indicating that hippocampal overexpression of NPY transgenes does not lead to changes in body weight.

In conclusion, our data suggest that combined gene therapy targeting the Y5 receptor and NPY together could be a more optimal alternative approach for suppression of hippocampal seizures as compared to NPY alone, while Y5 receptor overexpression by itself appears to have limited antiepileptic potential.

Conflict of interest

The authors have no conflict of interest to disclose.

Acknowledgments

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Paper IV

Neuropeptide-receptor combinatorial gene therapy for temporal lobe epilepsy in rats

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Abstract

We have previously discovered that simultaneous overexpression of NPY and its Y2 receptor exerts stronger seizure-suppressant effect as compared to single gene expression strategy in electrical stimulation or systemically administered kainate-induced acute seizures in rats. Although this study proved a principle that NPY and Y2 receptor combination-based gene expression is an effective anticonvulsant, the evidence that such approach could be a relevant clinical strategy was still lacking. In the present study, rats were injected with kainate unilaterally into the hippocampus, to induce status epilepticus (SE) followed by recurrent spontaneous seizures. This model closely resembles human chronic mesial temporal lobe epilepsy (mTLE). To assess hippocampal degeneration and correctly target viral vector treatment, brain magnetic resonance imaging (MRI) was performed in all animals 10 months after kainate injection. After MRI, recurrent seizure frequency and their duration were assessed continuously for 2 weeks by wireless video-EEG monitoring system with electrodes implanted contralateral to the kainate-injected hippocampus and the ipsilateral cortex. The kainate-injected hippocampus (seizure focus) was then treated unilaterally with combination of NPY and Y2 receptor AAV vector injection, followed by another video-EEG monitoring session. We demonstrate, for the first time, that such clinically relevant unilateral combinatorial gene therapy approach effectively decreased frequency of spontaneous seizures (in 4 out of 5 epileptic animals). In contrast, seizure frequency was increased in all control animals treated with empty viral vectors. In addition, overall seizure duration was shorter in AAV-NPY/Y2 treated animals compared to controls. These data suggest that simultaneous overexpression of NPY and Y2 in the seizure focus area is a relevant approach and could be developed as an alternative treatment strategy for mTLE, which often is refractory to antiepileptic drugs (AEDs).

Introduction

Epilepsy is a severe neurological disease affecting 1 % of the population. Between 30-40 % of the epilepsy patients respond poorly to the anti-epileptic drugs (AEDs) currently available (Duncan et al., 2006). Therefore, the development of new treatment strategies is highly warranted. Gene therapy has recently emerged as such alternative treatment strategy for epilepsy. With recombinant adeno-associated viral (rAAV) vectors endogenous seizure-suppressant compounds can be overexpressed long-term in the epileptic focus (Richichi et al., 2004; McCown, 2006; Kanter-Schlifke et al., 2007; Noe et al., 2008). Such AAV-based gene therapy approach in CNS clinical trials has been demonstrated to be a safe procedure with positive therapeutic outcomes (Kaplitt et al., 2007; Marks et al., 2008).

Neuropeptide Y (NPY), a polypeptide widely expressed in the brain, has been shown to be involved in various processes in the brain controlling behavior, such as food intake (Dryden et al., 1995; Hanson & Dallman, 1995), stress (Zhou et al., 2008) and even seizure activity (Baraban et al., 1997; Woldbye et al., 1997). NPY overexpression by recombinant AAV (rAAV) vectors in the hippocampus

exerts a strong suppressant effect on stimulation-induced seizures in animal models of epilepsy (Richichi et al., 2004; Sorensen et al., 2009). Such seizure-suppressant effect of NPY overexpression has been shown even for spontaneous recurrent seizures (Noe et al., 2008). NPY exerts its action mainly through three G-protein coupled receptors: Y1, Y2 and Y5 (Berglund et al., 2003). rAAV-based overexpression of Y2 but not Y5 receptors inhibited kindling and kainate-induced epileptic seizures (Woldbye et al., 2010; Gotzsche et al., 2012). Combination of the ligand (NPY) and the receptors (Y2 or Y5) showed a superior effect compared to the overexpression of the ligand or receptor alone in acute stimulation-induced seizure models (Woldbye et al., 2010; Gotzsche et al., 2012). In contrast, overexpression of Y1 receptor seems to be proconvulsive in kainate-induced seizures in mice (Olesen et al., 2012). In line with this observation, Y1 receptor activation increases extracellular glutamate levels in the hippocampus (Meurs et al., 2012).

Here, in a preclinical study, we investigate whether combined overexpression of NPY and Y2 receptors are effective in controlling spontaneous seizures in a chronic rat model of epilepsy (> 1 year) induced by

intrahippocampal kainate injection. This model is highly relevant to human mesial temporal lobe epilepsy (mTLE) with hippocampal sclerosis. Epileptic animals were treated with a rAAV vector for overexpression of NPY and Y2 unilaterally in the hippocampal epileptic focus, i.e. ipsilateral to the kainate injection. Such therapeutic scheme is considered as clinically plausible scenario for future gene therapy in epilepsy.

Experimental procedures

Ethical statement

All experimental procedures were approved by the local Malmö/Lund Ethical Committee for Experimental Animals (Ethical permit number M190-09), and were performed according to the guidelines of the Swedish Animal Welfare Agency and in agreement with international guidelines.

Animals

Male Wistar rats (Charles River, Germany) weighing 200-230 g at the initiation of the experiments were used. The animals were housed individually at a 12 hour light/dark cycle and were given daily forage (15-20 g/day).

Induction of epileptic seizures

Animals were anesthetized with isoflurane mixture and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA). Kainic acid (Ascent scientific, Cambridge, UK) was dissolved in sterile PBS (0.4 µg/0.4 µl) and the pH was adjusted to 7.4. Kainic acid was injected through a glass capillary into the medial part of the right hippocampus with the following coordinates (reference points from bregma, midline, dura): AP -5.3 mm, ML -4.5 mm, V -3.2 mm. The glass capillary was left for additional 5 min after injection. All animals were weighed before surgery and once a week until sacrificed.

Magnetic resonance imaging

To assess the extent of the damage caused by the intrahippocampal kainic acid injection magnetic resonance imaging (MRI) was performed on all animals about 10 months after induction of epileptic activity.

For the MRI investigation all animals were anesthetized with an isoflurane mixture (4 % initially and 2 % for further maintenance) together with nitrous oxide and oxygen. Animals were placed in a special positioning system and the head was stabilized in the head holder. Throughout the experiment the breathing rate and body temperature was controlled.

MRI was performed in a 9.4 T 400 MHz Agilent Technologies (Stockholm, Sweden) with the 205/120 HD gradient coil. T2-weighted images were obtained with the following parameters: Echo time 39.39 ms; Repetition time 4000 ms; five averages per cycle and flip angle of 180°. Totally 45 contiguous slices were scanned for each animal, with the thickness of 400 µm. The field of view was 40.4 x 42 mm^2 on a 256 x 256 matrix. With the mentioned parameters each scan took approximately 11 min.

Analysis of data obtained from the MRI was performed with the OsiriX software (Geneva, Switzerland). The region of interest (ROI), in our case the lesion area, was outlined in each image. In T2-weighted images the damaged area was distinguished by being lighter than the rest of the brain tissue. The software could then based on the acquisition settings calculate the volume of the lesion. Based on the remaining hippocampus, coordinates were created to target the dorsal and ventral part of the hippocampus for gene therapy treatment.

Electrode and transmitter implantation

About one year after induction of epileptic seizures a F40-EET transmitter (Data Sciences International, St. Paul, Minnesota) was implanted for EEG monitoring to record spontaneous epileptic activity. Animals were anesthetized as described previously and the skull was fixed into a stereotaxic frame (David Kopf Instruments, Tujunga, CA). One depth stainless steel electrode (Plastics One, Roanoke, VA), connected to one of the electrodes of the transmitter, was implanted in the contralateral hippocampus (left hemisphere) with the following coordinates (reference points from bregma, midline, dura): AP -4.8 mm, ML +5.2 mm, V -6.3 mm. Another electrode was placed on dura mater rostral to the coronal suture for cortical EEG recordings in the cortex ipsilateral to the kainic acid injection. For each of the electrodes a reference electrode was placed on the dura mater caudal to the lambdoid suture. The transmitter was subsequently placed in a subcutaneous pocket created from the incision by the skull.

Monitoring of spontaneous seizures

One week after implantation, the transmitter was activated by a magnet and each cage was placed on top of a receiver. Hardware and software for monitoring spontaneous epileptic activity were produced by Data Sciences International (St. Paul, Minnesota, USA). Synchronous EEG and video data were collected with the ART software. The animals were monitored continuously for 2 consecutive weeks. Another monitoring session was conducted 4 weeks after virus injections. All the EEG and video analysis was conducted using the NeuroScore software (Data Sciences International, St. Paul, Minnesota, USA) by experimenter blind to the treatment of the particular animals.

Viral vector injection

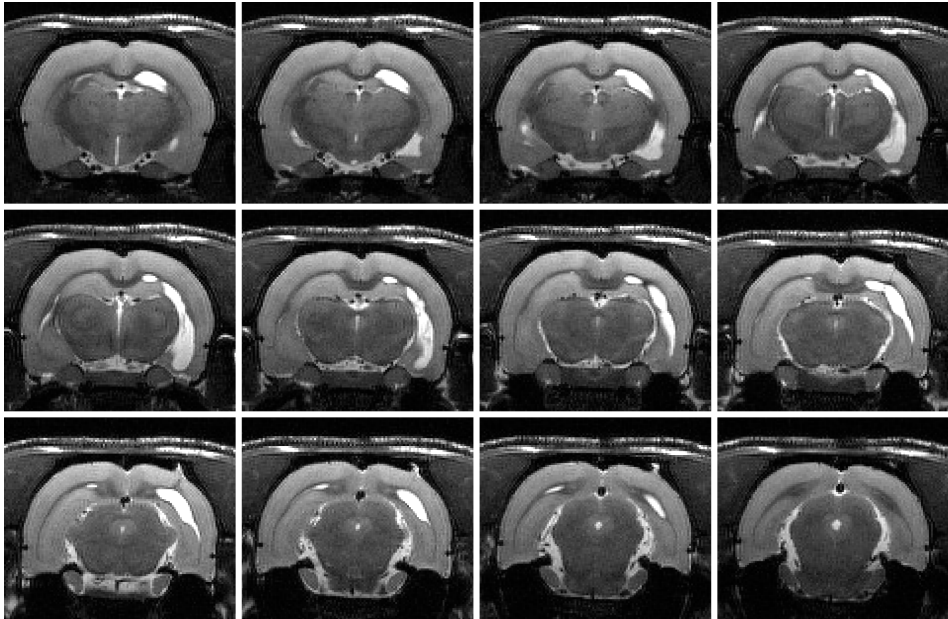Animals were anesthetized and fixed in the stereotaxic

Figure 1. Tissue damage caused by intrahippocampal kainic acid. A sequence of T2-weighted MR images showing the lesion in the right hippocampus.

frame as previously described. Adeno-associated viral vectors (GeneDetect, Auckland, New Zealand) with serotype 1/2 and expressed under the neuron specific enolase (NSE) promoter were used and had the following titers; rAAV-Empty 1.0×10^{12} genomic particles/ml, rAAV-NPY 1.0×10^{12} genomic particles/ml, rAAV-Y2 1.0×10^{12} genomic particles/ml. The rAAV-Empty vector was carrying the same expression cassette, but without the transgene and was used as a control. Viral vector injections were performed in the right hippocampus, which had previously been injected with kainic acid. Injections were performed using a glass capillary and the viral vector was injected in the dorsal and the ventral hippocampus with the following coordinates (reference points from bregma, midline, dura): Dorsal hippocampus: AP -4.0 mm, ML -1.5 mm, V -3.6 mm; Ventral hippocampus: AP -7.3 mm, ML -4.8 mm, V -5.8 mm. Animals were either injected with 2.5 µl Empty vector per site or a combination of NPY and Y2 (1 µl NPY and 1.5 µl Y2). The experimental groups were randomized by the GraphPad QuickCalcs software (La Jolla, CA). After injection the glass capillary was left in place for additional 10 min, before it was retracted.
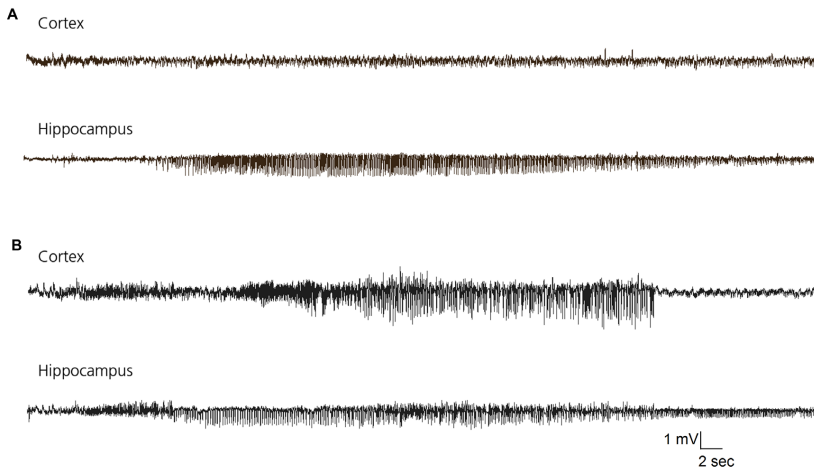
Perfusions and immunohistochemistry
At the end of the experiment all animals were deeply anesthetized with pentobarbital before the transmitter was removed and the animals were perfused transcardally with 0.9 % NaCl followed by 4 % paraformaldehyde. The brain was removed and post-fixed in 4 % paraformaldehyde for 24 hours and then overnight in 20 % sucrose in 0.1 M sodium phosphate buffered saline. The following day the brains were cut on a microtome in 30 µm thick sections and NPY immunohistochemistry was initiated. First, the slices were rinsed in 0.02 M KPBS, quenched with 3 % H_2O_2 and 10 % MeOH in 0.02 M KPBS for 10 min and then blocked in 5 % normal goat serum (NGS) in 0.25 % Triton X-100-KPBS for 1 h. The slices were then incubated with rabbit anti-NPY antibody (1:10 000; Sigma-Aldrich) in 5 % NGS overnight in room temperature and further on washed in 0.02 M KPBS before the incubation with goat anti-rabbit antibody (1:200, Vector Laboratories, Burlingame, CA) in 5 % NGS and 0.25 Triton X-100-KPBS for 2 h. The immunoreaction was amplified with an avidin-biotin complex kit (Vectastain ABC kit, Vector Laboratories) and finally visualized by application of 0.5 mg/ml 3,3´-diaminobenzidine (DAB) and 3 % H_2O_2. Bright-field images were obtained on an Olympus BX61 microscope.

Functional NPY receptor binding assay
Six additional animals were injected with intrahippocampal kainic acid (as above) and were used for functional NPY receptor binding. Three animals were

Figure 2. EEG patterns of spontaneous epileptic seizures. An example of a spontaneous focal seizure (A) and a spontaneous generalized seizure (B) recorded in the contralateral hippocampus and in the ipsilateral cortex, from the same animal.

injected with rAAV-NPY/Y2, while the rest were not viral vector treated. These animals were decapitated 4 weeks after viral vector injection and the brains were frozen in dry ice when removed. The brains were mounted on a cryostat (Shandon Inc., Pittsburgh, PA) using Cryo-Embed (Ax-Lab A/S, Vedbæk, Denmark) and were cut in 15 μm thick sections, thaw-mounted onto Superfrost Plus slides (VWR International, Herlev, Denmark) and were gently dried on a hotplate.

The sections were defrosted for 30 min in room temperature before being rehydrated in assay buffer (50 mM Tris-HCl, 3 mM MgCl, 0.2 mM ethylene glycol tetraacetic acid, 100 mM NaCl; pH 7.4) for 10 min at room temperature. Moreover, the sections were preincubated in buffer B (assay buffer A, 0.2 mM dithiothreitol, 1 μM 1,3-dipropyl-8-cyclopentylxanthine (#C-101, PerkinElmer, Skovlunde, Denmark), 0.5% w/v BSA, and 2 mM guanosine-5´-diphosphate (#G7127, Sigma-Aldrich, Brøndby, Denmark)) and 1 μM NPY (Schafer-N, Copenhagen, Denmark) for 20 min in room temperature. The basal binding was determined by incubation in buffer B with 40 pM [35S]-GTPγS (1250 Ci/mmol) but without NPY. Non-specific binding was determined by incubation in buffer B (without NPY) with 40 pM [35S]-GTPγS and 10 μM non-labeled GTPγS (#89378; Sigma-Aldrich, Brøndby, Denmark). Finally, the incubation was terminated by washing twice in ice-cold 50 mM Tris-HCl buffer (pH 7.4) for 5 min. All sections were rinsed in ice-cold distilled H_2O, dried under a stream of cold air, before being exposed to [35S]-sensitive Kodak BioMax
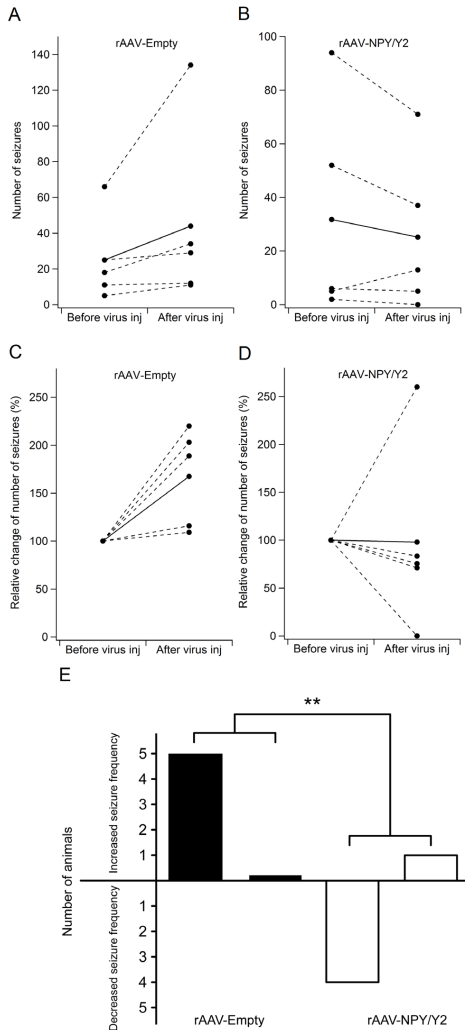
MR films together with [14C]-microscales for 5 days and then being developed in Kodak Processing Chemicals for Autoradiography Films (Sigma-Aldrich, Brøndby, Denmark).

Statistical analysis

Statistical analysis of data was performed using Student's t-test. Differences between groups (unpaired) and before and after treatment (paired) were considered significant at $p<0.05$. All data are presented as mean ±SEM. The investigator conducting the behavioral grading of the epileptic seizures in the animals, EEG and histological analysis was unaware of the group identity of individual animals.

Results

After the injection of intrahippocampal kainate, all animals developed status epilepticus (SE). About 10 months after induction of SE, MRI was performed to determine the extent of the tissue damage created by the kainic acid and subsequent spontaneous seizures (Fig. 1). The MRI was necessary to determine the exact coordinates for AAV injections, as well as to correlate extent of damage to severity of epilepsy in these animals. The damage to the kainate injected hippocampus was observed in all animals, and the volume of the damage was estimated to be 32.7 ± 4.7 mm^3 on average. Most of the damage was localized between the dorsal and ventro-caudal hippocampus, while dorsal and ventral hippocampi were spared. This allowed for targeting unilateral rAAV vector

4

Figure 3. Four out of five animals decrease in seizure frequency with rAAV-NPY/Y2 treatment. The number of seizures before and after viral vector injections of rAAV-Empty (A) and rAAV-NPY/Y2 (B) and the relative change of the number of seizures (C and D, respectively). The number of animals in each group that increased respectively decreased in number of seizures after treatment (E). ** p<0.01
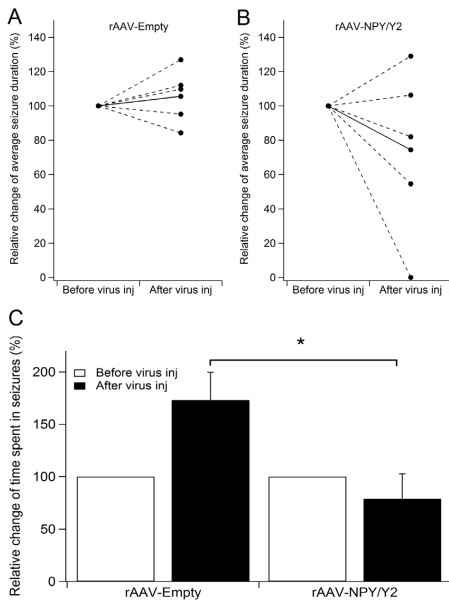
injections to the spared hippocampal areas ipsilateral to the kainate injection. Contralateral to the kainate injection hippocampi were spared in most of the cases without any significant damage observed on the MRI.

After MRI and hippocampal damage analysis in individual

animals, EEG recording electrodes were implanted in the hippocampus contralateral to the kainate injection and in the cortex ipsilateral to the kainate-injected hippocampus. After recovery from surgery, continuous video-EEG recordings were performed for 2 weeks on all animals. During the monitoring period, spontaneous seizures were detected in 13 out of 24 animals (54 %). Focal and generalized seizures were distinguished by the EEG and the video recording analysis. During focal seizures, epileptic EEG activity was detected in the hippocampus but not in the cortex (Fig. 2A), while during generalized seizures epileptic EEG activity was present both in hippocampal and cortical recordings (Fig 2B). Moreover, only generalized seizures were accompanied by clear whole-body convulsions observed on video recordings. The number of spontaneous seizures was highly variable between the animals during the 2 weeks of initial recording period, ranging between 2 and 94 of total number of detected seizures (Fig 3A and B). Average number of seizures was 28.4±10.0. Number of focal seizures on average was 5.2±4.6, while average number of generalized seizures was 23.2±9.2. No correlation was detected between the number of seizures (either total, generalized or focal) and the volume of the hippocampal damage (for total number of seizures $R^2=0.00008$, focal seizures $R^2=0.05230$ and generalized seizures $R^2=0.00840$).

Animals that exhibited spontaneous seizures were randomized in two groups: rAAV-Empty and a treatment group, which received rAAV vector carrying NPY and Y2 receptor transgenes. Only 5 animals from each group with recurrent spontaneous seizures survived to the end of the experiments and were therefore included in the final analysis.

Four weeks after rAAV vector injection, animals were monitored with the video-EEG system continuously for 2 weeks. All animals in the rAAV-Empty group exhibited increased number of seizures (Fig 3A, C and E), while four out of five animals in rAAV-NPY/Y2 group showed decreased number of seizures after the treatment (Fig 3B, D and E). One animal in rAAV-NPY/Y2 group became seizure free. The average number of all seizures (both generalized and focal) in rAAV-Empty group increased by 167.4±23.0 %, while it was largely unaltered in the rAAV-NPY/Y2 injected group (98.0±43.2 %, Fig 3C and D; p>0.05).

The average duration of individual seizures was not significantly changed after the treatment as compared to that of pre-treatment in both groups (rAAV-Empty 105.6±7.3 % of pre-treatment, rAAV-NPY/Y2 74.4±22.3 % of pre-treatment; p>0.05) (Fig 4A and B). However, in the rAAV-Empty group the overall time spent in seizures compared to pre-treatment was significantly longer compared to that in the rAAV-NPY/Y2 group

5

Figure 4. Duration of seizures before and after rAAV-Empty and rAAV-NPY/Y2 vector injection. The relative change of the average seizure duration in rAAV-Empty (A) and rAAV-NPY/Y2 (B) injected animals. The relative change in total time spent in seizures before and after treatment (D). Values are presented as mean ±SEM, * p<0.05.

(rAAV-Empty 173.2±26.4 %, rAAV-NPY/Y2 79.0±23.6 %; p<0.05) (Fig. 4C). Taken together, these data suggest that NPY/Y2 receptor rAAV treatment exerted inhibitory effect on spontaneous seizures.

NPY and Y2 receptor overexpression in rAAV vector injected animals

To confirm the overexpression of the NPY/Y2 after viral vector injections, NPY functional binding and NPY immunohistochemistry was performed. NPY functional binding was increased in the dorsal and ventral parts of the hippocampus (Fig. 5 A and C) as compared to basal functional binding (Fig. 5 B and D). NPY receptor functional binding was also detected in the contralateral hippocampus, mostly in the CA1 and CA3 areas (Fig. 5A). This suggests that rAAV-NPY/Y2 injection resulted in increased expression of Y2 receptor, which was also functionally active. Strong NPY immunoreactivity was detected in all parts of the hippocampus injected with rAAV-NPY/Y2 (Fig. 5E) as compared to the rAAV-Empty treated animals (Fig. 5F). NPY was mostly expressed in fibers rather than in the cell bodies of the hippocampal neurons (Fig. 5G and H). These data suggest that rAAV-NPY/Y2 viral vector injection resulted
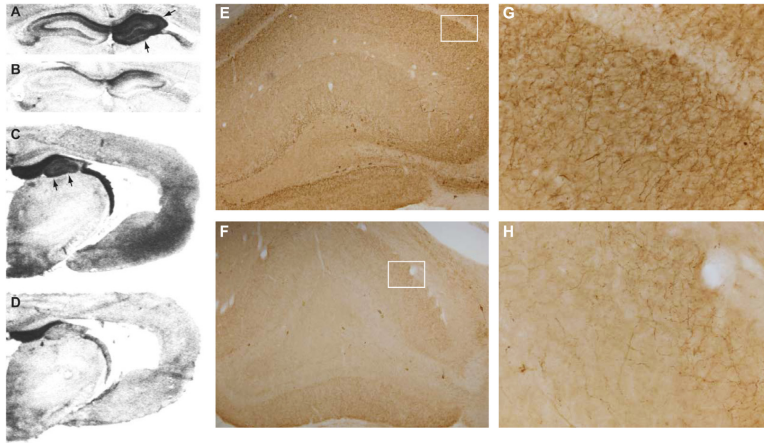
in increased expression of NPY in the viral vector treated hippocampus.

Discussion

The present study demonstrates for the first time that NPY/Y2 overexpression using viral vectors exerts seizure-inhibiting therapeutic effect on chronic spontaneous seizures occurring post-status epilepticus, in an animal model closely resembling human mTLE. This finding suggests that combinatorial NPY/Y2 gene therapy approach could be considered as a relevant approach and a possible alternative treatment strategy for clinical application, particularly for patients with mTLE, which often are pharmacoresistant and require surgical resection of the temporal lobe including hippocampus.

In our hands, spontaneous epileptic activity was detected in 54 % of kainate-injected animals, which is in line with previous observations (Bragin et al., 1999; Raedt et al., 2009). However, these data should be interpreted with certain caution, since continuous monitoring of 2 weeks may not be sufficient to detect seizures that could occur outside the recording period. Longer EEG monitoring periods are necessary to determine whether indeed some animals do not develop seizures at all. Also the number of seizures detected in different animals was variable in this model. Nevertheless, 2 weeks recording period and relatively low number of animals included in the analysis was sufficient to allow for assessment of the positive therapeutic outcome of the combinatorial NPY/Y2 gene therapy approach in these chronically epileptic animals.

In the rAAV-Empty group, animals exhibited increased number of seizures after viral vector injections. This may indicate that even one year after the initial insult (kainate-induced status epilepticus) there is a progressive increase in seizure frequency. Another explanation could be that the rAAV-Empty viral vector injection into the hippocampus may have exacerbated excitability of the seizure focus, and therefore may have led to increased seizure frequency in these animals. In any case, rAAV-NPY/Y2 vector treatment not only prevented the progressive increase in seizure frequency post-treatment, but also reversed it, as reflected by decreased seizure frequency and overall time spent in seizures.

In the present study, we deliberately chose to unilaterally deliver rAAV-NPY/Y2 treatment ipsilateral to the kainate-injected hippocampus, where most likely the seizure focus was induced and localized. To some extent, inhibitory effect of such approach on spontaneous seizures may have been unexpected in rats, since extensive inter-hemispheric connections between rat hippocampi allows for rapid spread of seizure activity from one hippocampus, where seizures arise, to the other (contralateral side) (Fernandes de Lima et al., 1990). Thus, the inhibition of

Figure 5. Functional NPY binding and NPY immunoreactivity after unilateral intrahippocampal injection of rAAV-NPY/Y2. Treatment with rAAV-NPY/Y2 vector causes prominent upregulation of functional Y2 receptors as revealed by an increase in NPY-stimulated [^{35}S]GTPγS functional binding. (A) and (C) show pronounced NPY-stimulated [^{35}S]GTPγS binding (arrows) 4 weeks after unilateral intrahippocampal rAAV-NPY/Y2 vector injection as compared to basal binding (B and D, respectively). NPY immunoreactivity in the hippocampus 6 weeks after rAAV-NPY/Y2 (E) and rAAV-Empty (F) injection. Boxed areas in (E) and (F) are shown magnified in (G) and (H).

spontaneous seizures by unilateral rAAV treatment could be taken to suggest that the majority of the seizures were indeed initiated in the kainate-injected hippocampus and that these seizures and their spread were inhibited by viral vector treatment. A contributing factor to limited spread of the seizures to the contralateral hemisphere could be a damage of interhemispheric connections caused by kainate. Regardless, the present results would imply that selective targeting the seizure focus by combinatorial NPY/Y2 rAAV gene therapy might be sufficient to exert therapeutic effects even on generalized chronic epileptic seizures. However, this statement needs to be taken with certain caution, since some spread of the transgenes to the contralateral hippocampus has been observed and may have contributed to the results.

We were not able to detect any positive correlation between the damage of the hippocampus and the frequency of the seizures, which could be counterintuitive. One would have expected higher seizure frequency in animals with more extensive hippocampal damage, since seizures are thought to cause hippocampal sclerosis (Kalviainen & Salmenpera, 2002). One explanation could be that spontaneous seizures occurring after kainate injection may exacerbate hippocampal sclerosis over time, causing degeneration of the seizure-generating hippocampal network and thereby stop the seizures. This could be one of the reasons as to why we were not able to detect seizures in some animals during the 2 week initial monitoring period. Speaking against this interpretation, the inverse correlation between the

hippocampal damage and seizure frequency was not observed either. Higher number of animals to increase the power of analysis may be needed to address this question in more detail.

Increased functional NPY receptor binding was found in animals from rAAV-NPY/Y2 group compared to basal functional NPY receptor binding. This supports the interpretation that the decreased seizure frequency in these animals was most likely due to transgene overexpression. The individual variations of the therapeutic outcomes may depend on the individual levels of NPY/Y2 overexpression, but could also be due to uneven regional distribution of the overexpression within the hippocampus in different animals.

In conclusion, this study suggests that combinatorial gene therapy is a valid alternative to single gene therapy approach, and even unilateral combinatorial treatment results in a positive therapeutic outcome. In this respect, clinical testing of this approach in pharmacoresistant mTLE patients assigned to surgical removal of the temporal lobe seems to be a viable concept, provided that the viral vector-transduced tissue can still be removed in case adverse effects of the gene therapy arise, serving as a safety aspect of the procedure. In general, combinatorial gene therapy overexpressing both the ligand and the receptor seems to offer better tuning of the desired therapy by strengthening the effect of the ligands via specific receptors that have been shown to mediate the therapeutic effect (Woldbye et al., 2010; Gotzsche et al., 2012).

Acknowledgements

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Paper I

PLoS one

VEGF Receptor-2 (Flk-1) Overexpression in Mice Counteracts Focal Epileptic Seizures

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Abstract

Vascular endothelial growth factor (VEGF) was first described as an angiogenic agent, but has recently also been shown to exert various neurotrophic and neuroprotective effects in the nervous system. These effects of VEGF are mainly mediated by its receptor, VEGFR-2, which is also referred to as the fetal liver kinase receptor 1 (Flk-1). VEGF is up-regulated in neurons and glial cells after epileptic seizures and counteracts seizure-induced neurodegeneration. *In vitro*, VEGF administration suppresses ictal and interictal epileptiform activity caused by AP4 and 0 Mg^{2+} via Flk-1 receptor. We therefore explored whether increased VEGF signaling through Flk-1 overexpression may regulate epileptogenesis and ictogenesis *in vivo*. To this extent, we used transgenic mice overexpressing Flk-1 postnatally in neurons. Intriguingly, Flk-1 overexpressing mice were characterized by an elevated threshold for seizure induction and a decreased duration of focal afterdischarges, indicating anti-ictal action. On the other hand, the kindling progression in these mice was similar to wild-type controls. No significant effects on blood vessels or glia cells, as assessed by Glut1 and GFAP immunohistochemistry, were detected. These results suggest that increased VEGF signaling via overexpression of Flk-1 receptors may directly affect seizure activity even without altering angiogenesis. Thus, Flk-1 could be considered as a novel target for developing future gene therapy strategies against ictal epileptic activity.

Introduction

Vascular endothelial growth factor (VEGF or VEGF-A) is a member of homodimeric glycoproteins and was initially shown to increase vascular permeability in tumor ascites fluid [1]. Since its discovery, VEGF has been found to have various roles in normal and pathologic conditions in the brain. For example, VEGF enhances neuronal proliferation [2,3,4,5,6,7], survival [8,9,10,11,12] and axonal outgrowth [13,14]. Both VEGF-A and VEGF-B, as well as their receptors, VEGFR-1, VEGFR-2 and neuropilin, are widely expressed in the brain with differential expression in distinct population of cells [15]. Immunohistological evaluation reveals their co-localization in all types of neural cells, including pyramidal neurons of the cortex and hippocampus, both in rodents after status epilepticus (SE), and human tissue resected from patients with focal cortical dysplasia-induced intractable epilepsy [15,16,17]. This suggests possible autocrine/paracrine mechanisms of action, and possible role in epileptogenesis and/or ictogenesis.

In the hippocampus, VEGF protects hippocampal neurons after hypoxia [10], glutamate excitotoxicity [18] and SE [16,17,19]. Following electroconvulsive seizures, the levels of VEGF mRNA are increased in brain areas susceptible to cell loss, such as the hippocampus [20]. In addition, VEGF protein is up-regulated 24 h after pilocarpine-induced seizures [17]. *In vitro*, hippocampal

slices from rats with recurrent spontaneous seizures show reduced bicuculline-induced epileptiform discharges after VEGF application [21]. Moreover, VEGF was shown to decrease both ictal and interictal activity induced by AP4 and 0 Mg^{2+} in rat hippocampal slices [22]. The neurotrophic and neuroprotective effects of VEGF are predominantly mediated by VEGF receptor-2, also called fetal liver kinase receptor 1 (Flk-1) [13,23,24], or kinase insert-domain containing receptor (KDR) in humans [25].

The mechanisms of action of VEGF through the Flk-1 receptor are still not completely understood. The pathway of signal transduction seems to be mediated by phosphatidylinositol 3'-kinase/Akt (PI3K/Akt), phospholipase C-gamma/protein kinase C (PLC-γ/PKC) and mitogen-activated protein kinase/extracellular signal-regulated protein kinase (MEK/ERK) pathways [26,27,28]. The neuroprotective effect is thought to be mediated by activation of the PI3K/Akt cascade, while the effects on axonal outgrowth and neuroproliferation are most likely dependent on the PKC- and ERK- dependent pathways.

The main objective of the present study was to investigate whether Flk-1 overexpression in transgenic mice, mimicking increased Flk-1 expression in the temporal lobe after epileptic seizures, would exert direct regulatory action on epileptogenesis and/or ictogenesis.

Methods

Ethics Statement

All experimental procedures were approved by the local Malmö/Lund Ethical Committee for Experimental Animals (Ethical permit number M87-06) and were performed according to the guidelines of the Swedish Animal Welfare Agency and in agreement with international guidelines.

Animals

Five male transgenic mice expressing murine Flk-1 transgene in postnatal neurons under the Thy-1.2 promoter (Thy1-Flk1 OE) and 10 male FvB controls were used [29]. Only male animals were used to exclude effects of fluctuating female hormones on kindling epileptogenesis [30,31,32]. The animals weighing 20 g at the beginning of the experiment, had *ad libitum* access to food and water, and were housed at a 12 hour light/dark cycle.

Implantation of electrodes

Animals were anesthetized by intraperitoneal injection of ketamine (Ketalar, Pfizer; 80 mg/kg) and xylazine (Sigma-Aldrich, Stockholm, Sweden; 15 mg/kg). A bipolar stainless steel stimulation/recording electrode (Plastics One, Roanoke, VA) was stereotaxically (David Kopf Instruments, Tujunga, CA) implanted in the hippocampus at the following coordinates: AP -2.9, ML -3.0, DV -3.0 from bregma, midline and dura, respectively [33]. A reference electrode was placed in the temporal muscle. Proximal electrode sockets were inserted into a plastic pedestal (Plastics One, Roanoke, VA) and fixed on the skull with dental cement (Kemdent, Wiltshire, UK). The animals were allowed to recover for one week before electrical stimulation.

Electrical kindling stimulation

On the first 3–5 days of kindling the individual threshold was measured by starting stimulations at 10 μA and increasing the current intensity in 10 μA steps (1 ms square wave pulse, 100 Hz) until a focal EEG epileptiform afterdischarge (AD) of at least 5 sec duration was observed. The threshold was determined once a day until ADs were evoked three times by the same minimal current intensity. Thereafter animals were stimulated at their individual threshold current once a day. Stimulation-induced behavioral seizures were scored according to the Racine scale [34]: Stage 0 – no behavioral changes; Stage 1 – facial twitches; Stage 2 – chewing and head nodding; Stage 3 – unilateral forelimb clonus; Stage 4 – rearing, body jerks, bilateral forelimb clonus; Stage 5 – imbalance. EEG was recorded on a MacLab system (ADInstruments, Bella Vista, Australia) 1 min before and 1 min after electrical stimulation. Animals were considered fully kindled when three stage 5 seizures were obtained.

Immunohistochemistry

Forty-eight hours after last stimulation-induced stage 5 seizure, animals were deeply anesthetized with pentobarbital and perfused through the ascending aorta with 0.9% NaCl followed by ice-cold 4% paraformaldehyde. Brains were post-fixed overnight at 4°C, cryoprotected in 20% sucrose (overnight at 4°C) and 30 μm coronal sections were cut on a microtome. The sections were stored in a cryoprotective solution at −20°C until use. For validation of electrode location sections were stained with 0.5% cresyl violet (Sigma-Aldrich, Stockholm, Sweden).

For glial fibrillary acidic protein (GFAP) immunostaining, slices were first rinsed in 0.02 M KPBS followed by preincubation with 5% normal goat serum (NGS) in 0.25% TKPBS and incubation with primary antibody mouse anti-GFAP (Sigma-Aldrich, Stock-

holm, Sweden; 1:500) overnight at room temperature (RT). The next day slices were rinsed with 0.02 M KPBS and incubated 2 hours with secondary antibody FITC-goat anti-mouse (Jackson Immunoresearch, Suffolk, UK; 1:400). Slices were rinsed once more and mounted on coated slides and coverslipped. The number of GFAP positive cells in the hilus was quantified by counting in Flk-1 OE mice (n = 3) and control mice (n = 3). From each animal three sections from the dorsal hippocampus were quantified bilaterally. The CA4 region was not included in the counting. Counting was conducted on Olympus BX61 microscope, while pictures were obtained with a Leica TCS SP2 confocal microscope.

Three different immunohistochemical stainings were performed with brain slices that did not undergo kindling stimulations; Flk-1 (WT n = 2; Flk-1 OE n = 2); VEGF (WT n = 2; Flk-1 OE n = 2) and glucose transporter 1, Glut1 (WT n = 4; Flk-1 OE n = 4). The brains were removed from the skull, fixed in 2% PFA and embedded in tissue-freezing medium (Tissue-Tek®, Sakura, Alphen aan den Rijn, Netherlands). The 10 μm coronal sections were incubated at RT overnight with primary antibodies directed against Glut1 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:20), VEGF (Santa Cruz Biotechnology, Santa Cruz, CA; 1:20) and Flk-1 (R&D Systems, Abingdon, UK; 1:200). Co-labeling with neuronal nuclei, NeuN (Millipore, Brussels, Belgium; 1:500) was carried out with VEGF and Flk-1 stainings. Subsequently, the sections were incubated with fluorescently-labeled secondary antibodies Alexa 488 or 568 (Molecular Probes, Eugene, Oregon; 1:200) for 2 h or with biotin-labeled IgG followed by amplification with a signal amplification system (Streptavidin-HRP-Fluorescein) for VEGF and Flk-1. Blood vessel area and density in the hippocampus were assessed using a Zeiss Axioplan microscope with KS300 image analysis software. VEGF and Flk-1 staining was visualized using a Zeiss LSM510 confocal microscope.

RNA extraction, cDNA preparation and real-time PCR

To extract RNA, hippocampi were dissected (WT n = 3; Flk-1 OE n = 4) and homogenized in RLT lysis buffer (Qiagen, Venlo, Netherlands) using a FastPrep24 system (MP Biomedicals, Illkirch, France). RNA was extracted with a DNase digestion (Qiagen, Venlo, Netherlands) and was transcribed to cDNA by the QuantiTect reverse transcription kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. Gene expression was assessed by the 7500 Fast Real-Time PCR system (Applied Biosystems, Halle, Belgium) and cDNA was normalized to β-actin expression levels, with the following TaqMan gene expression assays: β-actin Mm00607939_s1; Flk1 Mm01222419_m1; VEGF Mm00437304_m1.

Statistical analysis

Statistical analysis of data was performed using Student's unpaired *t*-test or one-way ANOVA. Differences between the group data were considered significant at $p < 0.05$. Data are presented as mean ±SEM. The investigator conducting the behavioral grading of animals, EEG analysis or histological and PCR analysis was unaware of the group identity of individual animals.

Results

Flk-1 OE increases threshold and shortens duration of focal seizures

The location of the implanted stimulation/recording electrodes was confirmed in hippocampal sections with standard cresyl violet staining. All electrode tips were placed within the expected same

area of the hippocampus in the two groups (Fig. 1C). Seizures arising during initial kindling stimulations were focal without any behavioral manifestations. Subsequent stimulations induced gradual development of generalized seizures with increasing severity of behavioral manifestations (Fig. 1A, one-way ANOVA and 1B). There was no significant difference between the Flk-1 OE and WT mice in the number of stimulations needed to reach each stage of kindling, including the fully kindled stage (three stage 5 seizures) that was reached after 21.6 ± 7.5 and 22.8 ± 4.6 days, respectively (Fig. 1B). Thus, overexpression of Flk-1 did not alter epileptogenesis *per se*. However, the threshold for seizure induction was more than twice as high in Flk-1 OE mice as compared to WT animals (WT 27.0 ± 2.9 μA; Flk-1 OE 60.0 ± 7.1 μA; $p < 0.001$) (Fig. 2A). Also, the AD duration of focal stages was greatly shorter (less than half) in Flk-1 OE animals (Stage 0: WT 21.3 ± 2.8 sec, Flk-1 OE 10.5 ± 1.2 sec; Stage 1: WT 22.4 ± 0.7 sec, Flk-1 OE 11.4 ± 0.8 sec; Stage 2: WT 26.4 ± 1.5 sec, Flk-1 OE 10.6 ± 0.7 sec; $p < 0.01$), but remained unaltered between the groups in more generalized stages (Stage 3–5: WT 41.7 ± 3.1 sec, Flk-1 OE 30.2 ± 5.4 sec; $p > 0.05$) (Fig. 2B). Taken together, these data suggest that Flk-1 OE strongly suppresses focal seizure activity.

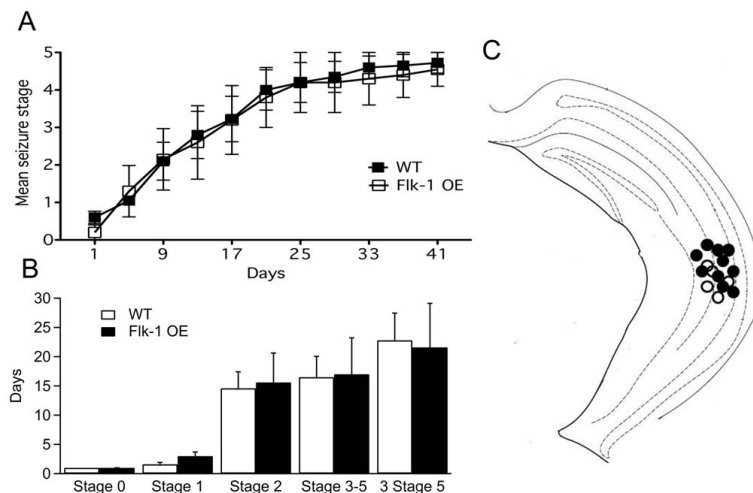### Flk-1 OE mice display elevated levels of Flk-1 and VEGF

The overexpression of Flk-1 in the transgenic mice was confirmed by real-time-PCR (Fig. 3A) and immunohistochemistry (Fig. 4). With immunostainings we just merely demonstrate expression pattern of the proteins, while using a more quantitative approach with real-time-PCR to estimate possible changes. The quantification of hippocampal mRNA showed a 5.58 fold increase ($p < 0.01$) of Flk-1 in Flk-1 OE mice compared to WT (Fig. 3A). These quantitative results of real-time-PCR were supported by non-quantitative analysis of immunoreactivity, whereby the Flk-1 immunoreactivity was detected in numerous hippocampal neurons of Flk-1 OE mice, while WT mice displayed very few cells expressing Flk-1 (Fig. 4).

We hypothesized that Flk-1 overexpression could have led to compensatory changes and thereby decreased expression of its ligand, the VEGF. Therefore, we performed real-time-PCR (Fig. 3B) and immunohistochemistry (Fig. 5) to assess VEGF expression levels. Unexpectedly, VEGF mRNA expression was 1.44 fold higher in the hippocampus of Flk-1 OE mice compared to control mice ($p < 0.001$) (Fig. 3B). Non-quantitative analysis of VEGF immunoreactivity showed VEGF positive neurons both in Flk-1 OE mice and in WT mice (Fig. 5). Taken together, our data suggest increased VEGF expression in the hippocampus of Flk-1 OE mice.
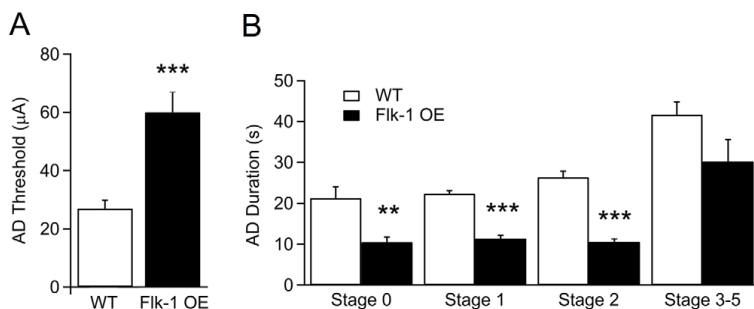
Unaltered blood vessel densities in Flk-1 OE mice in the hippocampus

It is well known that VEGF can increase the vasculogenesis and angiogenesis [35,36,37] through activation of Flk-1. Since both VEGF and Flk-1 were found to be up-regulated in Flk-1 OE mice, we asked whether Flk-1 OE mice exhibit altered quantity and/or morphology of blood vessels. We quantified the area and density of blood vessels positive for glucose transporter 1 (Glut1) in the hippocampus (Fig. 6A–D). The Glut1 immunohistochemistry was performed in animals that did not undergo kindling stimulation, since it is known that that the expression of Glut1 is regulated by epileptic seizures [38]. In both groups about 2% of the hippocampus were Glut1 positive (WT 2.1 ± 0.3%; Flk-1 OE 2.2 ± 0.2%) (Fig. 6C), and blood vessel density was not significantly different between the groups (WT 231.7 ± 3.6 vessels/μm^2; Flk-1 OE 252.5 ± 8.3 vessels/μm^2) (Fig. 6D). Thus, blood vessels did not seem to be altered in Flk-1 OE mice hippocampus.

Further, we explored whether increased VEGF signaling in Flk-1 OE mice could have led to gliogenesis [39]. To assess the number of glial cells, we performed a GFAP staining followed by quantification of GFAP-labeled cells in the hilus (Fig. 6E–G). The number of GFAP positive glial cells in the hilus of WT and Flk-1

Figure 1. Epileptogenesis is not alternated by overexpression of Flk-1. (A) Progression of kindling: mean stage during each kindling stimulation analyzed with one-way ANOVA. (B) Number of stimulations needed to reach different behavioral seizure stages. (C) Placement of electrodes for electrical stimulation in the hippocampus was verified by cresyl violet staining at the end of the experiments. Solid black dots for WT mice and black rings for Flk-1 OE mice.
doi:10.1371/journal.pone.0040535.g001

Figure 2. Flk-1 OE mice have higher threshold for seizure-induction and exhibit shorter afterdischarge durations of seizures. (A) Mean afterdischarge (AD) threshold during focal epileptiform activity of at least 5 sec duration measured one week after electrode implantation of WT and Flk-1 OE mice. (B) AD duration for different seizure stages during kindling stimulations. Values are presented as mean ±SEM, ** $p<0.01$ and *** $p<0.001$.
doi:10.1371/journal.pone.0040535.g002

OE mice was not significantly different (WT 91.8±5.0 cells per hilus; Flk-1 OE 77.4±7.1 cells per hilus) (Fig. 6G). Taken together, our data suggest unaltered glial number in the hilus in Flk-1 OE mice.

Discussion

Here, using a transgenic mouse line that overexpresses Flk-1 under the Thy-1 promoter, we show that increased levels of Flk-1 in the hippocampus suppress focal seizure susceptibility. In transgenic mice the threshold to generate ADs was significantly increased (twice as high) and the duration of ADs was markedly shortened (less than half) compared to WT animals.
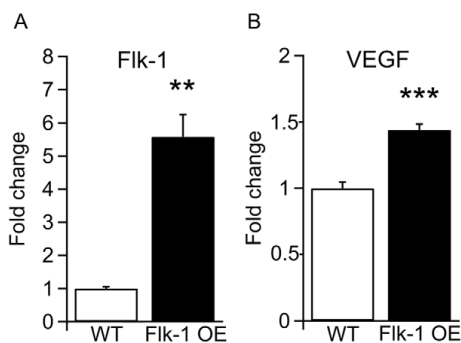
Epileptogenesis, however, was not altered by the overexpression of Flk-1. The Flk-1 OE mice and WT controls did not differ in the number of stimulations needed to reach different seizure stages or the fully kindled state (three stage 5 seizures). Taken together, these results indicate that overexpression of Flk-1 affects focal hippocampal seizure activity without having influence on kindling

Figure 3. Real-time-PCR shows high levels of Flk-1 and VEGF in the hippocampus of Flk-1 OE mice. (A) Flk-1 expression was 5.58 fold higher in Flk-1 OE mice. (B) VEGF levels in the hippocampus of animals with Flk-1 OE were 1.44 fold higher than WT mice. The cDNA was normalized to β-actin expression levels. Values are presented as mean ± SEM, ** $p<0.01$ and *** $p<0.001$.
doi:10.1371/journal.pone.0040535.g003

epileptogenesis or generalized seizure activity (i.e. seizures that have spread outside the focus, here being the hippocampus).

The Flk-1 OE mice exhibited increased expression VEGF, an endogenous ligand of Flk-1. The underlying molecular and cellular mechanism of such increase is currently unclear. A major limitation of traditional transgenic overexpression is the inability to control developmental compensatory or other alterations in signaling pathways. It would be highly warranted to address the question whether Flk-1 overexpression in adult stage (e.g. conditional overexpression, or viral vector-based approach) would also lead to up-regulation of VEGF. In any case, our data suggest that the observed effect on seizures is mediated by an overall increase of VEGF signaling through up-regulation of both Flk-1 and VEGF. Our immunoreactivity analysis indicates mostly neuronal expression of both Flk-1 and VEGF, though possibly in a variety of neuronal populations (see Figs. 4 and 5) In transgenic mice Flk-1 is overexpressed under the Thy-1 promoter, which has been shown to drive transgene expression (Flk-1) selectively in neurons. The identity of the Flk-1 positive and NeuN negative cells is not clear, but may represent a small population of non-neuronal cells due to some unspecificity of the used promoter. The cellular localization of up-regulated VEGF and Flk-1, and how it may influence its seizure modulating effects, remains to be studied in more details.

Our data are in line with previous observations showing that VEGF is able to suppress bicuculline-induced epileptiform discharges in slices from chronic epileptic rats [21], although it was largely unclear which VEGF receptor was responsible for these effects. Our data suggest that Flk-1 might play a major role in the seizure-suppressant effects of VEGF. As we observed a 1.44 fold increase in the expression of VEGF in Flk-1 OE mice, the anti-epileptic effects could be mediated by increased activation of conventional VEGF/Flk-1 signaling pathways described earlier. However, one cannot exclude that the seizure suppressant effect is partly mediated by other VEGF receptors, such as VEGFR-1 (Fms-related tyrosine kinase 1, Flt-1) or neuropilin [40]. It has been shown that VEGF can suppress glutamatergic synaptic transmission in all major synapses in the hippocampus [21,22]. It is likely that also Flk-1 overexpression exerts similar effect on glutamatergic transmission in the hippocampus, although it needs to be explored.

Recently, a novel concept has been put forward, suggesting that simultaneous overexpression of both the ligand and the receptor of

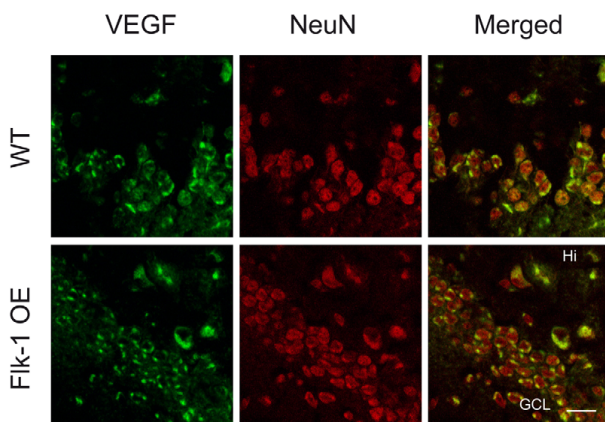**Figure 4. Immunoreactivity for Flk-1 in the hippocampal slices.** Immunoreactivity for VEGF, NeuN and merged in the granule cell layer (GCL) and hilus (Hi) of the dentate gyrus. Flk-1 is barely detected in the hippocampus of wild-type (WT) mice, however the few cells that express Flk-1 are co-labeled with neuronal nuclei (NeuN) protein. In Flk-1 OE mice the Flk-1 immunoreactivity is mostly detected on the plasma membrane of the cells (indicated by the arrowheads). Scale bar is set to 20 µm.
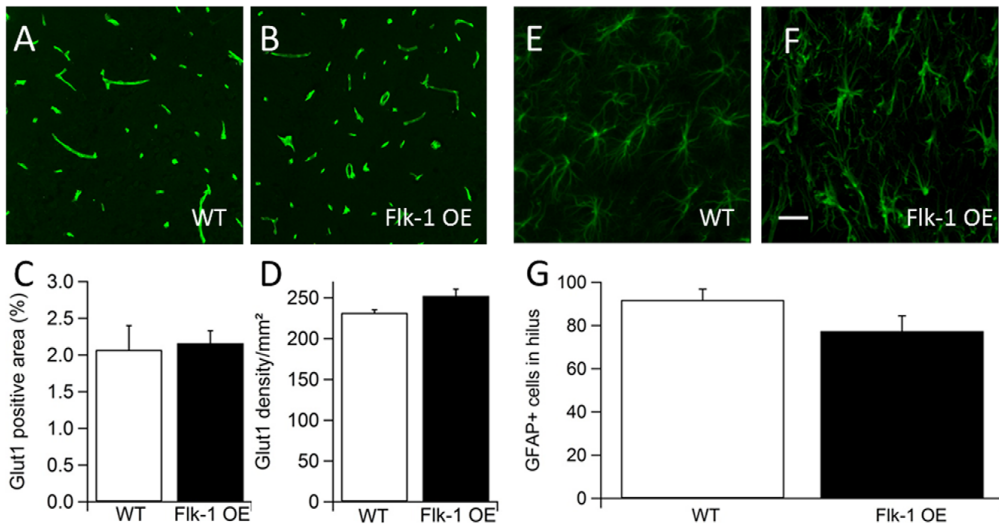doi:10.1371/journal.pone.0040535.g004

endogenous molecules may exert synergistic seizure-suppressant effect. This concept has been validated for endogenous seizure-suppressant molecules, such as e.g. neuropeptides and their receptors [41,42]. The finding that in Flk-1 OE mice also VEGF seems to be up-regulated, and potentially strengthens effects of Flk-1 OE, is in line with this conceptual framework.

Another potentially seizure-modulating aspect of Flk-1 overexpression could be the formation of new blood vessels (angiogenesis). It is well known that elevated VEGF signaling may lead to increased levels of vascularization in the brain [35,36,43], and increased angiogenesis and vascularization are associated with altered BBB function, neuronal excitability and seizure susceptibility [44,45,46]. Therefore, altered vascularization of the hippo-

campus in Flk-1 OE mice may have contributed to the observed changes in threshold and duration of ADs. However, we were not able to detect any significant alterations in vascularization of the hippocampus in Flk-1 OE mice, possibly due to some compensatory mechanisms. Similarly, no effect of Flk-1 OE has been shown on astrocytes as measured by GFAP immunolabeling, indicating that gliosis did not contribute to seizure-suppressant effects of Flk-1 overexpression. The most likely mechanism of action is modulation of synaptic transmission and plasticity by VEGF, as observed with acute VEGF application in hippocampal slice preparations [21]. However, this hypothesis needs to be tested in future studies.

Figure 5. Vascular endothelial growth factor (VEGF) is expressed by neurons in the hippocampus. Figure shows immunoreactivity of VEGF, NeuN and merged in the granule cell layer (GCL) and hilus (Hi) of the dentate gyrus. In both controls and Flk-1 OE mice, neurons in the hippocampus express VEGF. Scale bar is set to 20 µm.
doi:10.1371/journal.pone.0040535.g005

Figure 6. Glucose transporter 1 (Glut1) immunohistochemistry and quantification of blood vessels in the hippocampal slices. (A) Intensity and distribution of Glut-1 immunostaining is similar in the hippocampus of Flk-1 OE mice compared to (B) controls; Quantification of blood vessel area (C), as well as the blood vessel density (D) showed no significant differences ($p>0.05$) between the groups. The number of glial fibrillary acidic protein (GFAP)-positive cells in wild-type mice (E) and Flk-1 OE mice (F) in the hilus of the dentate gyrus after kindling are unaltered (G). Values are presented as mean ±SEM. Scale bar is set to 30 μm.
doi:10.1371/journal.pone.0040535.g006

When considering the seizure modulating effects of VEGF signaling one has to keep in mind that excessive VEGF signaling may exert opposing effects: it could be protective after seizures, but also detrimental [16]. VEGF is for example capable of initiating inflammatory cascades in the brain [39], and could also cause BBB breakdown [47]. Interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α) are up-regulated after seizures, leading to subsequent up-regulation of VEGF [48,49]. On the other hand, VEGF, while triggering inflammatory cascades, has ability to protect brain cells from various insults. Selectively targeting overexpression of Flk-1 in neurons may avoid unwanted detrimental effects of increased VEGF signaling. Lastly, conventional overexpressing transgenic animals may have some undetected developmental alterations, which could be responsible for the effects observed here. Taken together, these considerations illustrate very complex role of VEGF in cellular and molecular events of normal and pathological conditions, and highlights the need for better understanding of the mechanisms involved in VEGF and its Flk-1 receptor signaling, which may help in developing novel therapeutic strategies for various diseases of the brain, including epilepsy.

Conclusion

In conclusion, the present study adds new insights on regulation of seizure activity by Flk-1. We have demonstrated that in Flk-1 OE mice the resistance to seizure induction is significantly increased and the duration of epileptic EEG activity decreased. Therefore, targeting Flk-1 receptors may represent a novel approach for optimizing regulation of VEGF signaling pathways in order to modulate excitability in the brain and counteract pathological activity such as in epileptic seizures.

Author Contributions

Conceived and designed the experiments: LN IKS DL MK. Performed the experiments: LN JD. Analyzed the data: LN JD. Contributed reagents/materials/analysis tools: PC DL. Wrote the paper: LN MK.

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Paper II

Encapsulated galanin-producing cells suppress focal epileptic seizures in the hippocampus

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Abstract

Galanin is a neuropeptide widely expressed throughout the central nervous system. In the hippocampus, galanin is mostly present in noradrenergic fibers arising from Locus Coeruleus, while its receptors GalR1-3 are expressed in various neurons. Galanin has been shown to exert strong inhibitory effect on seizures, most likely acting via decreasing glutamate release from excitatory synapses. Recent viral vector-based gene therapy approaches have demonstrated significant seizure-suppressant effects of galanin overexpression in a number of animal epilepsy models, suggesting its therapeutic potential.

Purpose - To explore whether a genetically modified galanin-producing human cell line could provide a seizure-suppressant effect, and test its possible translational prospect for clinical application, we implanted encapsulated cell biodelivery (ECB) devices into the hippocampus of rats exposed to a rapid kindling model of epilepsy.

Methods - Two clones from a genetically modified human cell line secreting different levels of galanin were tested. EEG recordings and stimulations were performed by stainless steel depth electrodes implanted into the hippocampus at the same surgical session as ECB devices. One week after the surgery, rapid kindling stimulations were initiated.

Key findings - ELISA measurements performed prior to ECB device implantation showed a release of galanin on average 8.3 ng/ml/24 h/device for the low-releasing clone, and 12.6 ng/ml/24 h/device for the high-releasing clone. Intrahippocampal high-releasing galanin-producing ECB devices moderately decreased stimulation-induced focal afterdischarge duration, while low-releasing ECB devices had no significant effect.

Significance - Our study shows that implantation of galanin-releasing ECB devices moderately suppress focal stimulation-induced recurrent seizures, and offers an alternative to gene therapy approaches in humans, with the advantage that the treatment could be terminated by removing the ECB devices from the brain. Thereby, this strategy provides a higher level of safety for future therapeutic applications, in which various anti-epileptic compounds produced and released by genetically modified human cell lines could be clinically evaluated.

Introduction

The neuropeptide galanin was first discovered in porcine intestine (Tatemoto et al., 1983), but later it has been found in various parts of the body, including the PNS and the CNS. Galanin has diverse physiological functions in the normal brain, but has also been implicated in pathophysiological conditions, for example depression (Lu et al., 2005a; Kuteeva et al., 2008), Alzheimer's disease (Steiner et al., 2001; Jhamandas et al., 2002; Ding et al., 2006; Counts et al., 2009) and epilepsy (Mazarati et al., 1998; Mazarati et al., 2000; Kokaia et al., 2001; Schlifke et al., 2006).

Galanin signaling occurs through G-protein coupled galanin receptor 1 (GalR1), 2 (GalR2) and 3 (GalR3) (Iismaa & Shine, 1999; Branchek et al., 2000; Mitsukawa et al., 2008). GalR1 and GalR2 are expressed within the hippocampus (Lu et al., 2005b). The mechanism of action of galanin through the galanin receptors is thought to be mediated through blockade of voltage gated Ca^{2+}-channels and/or activation of ATP-dependent K^+-channels (Palazzi et al., 1991; Zini et al., 1993; Kask et al., 1997).

Several studies suggest that galanin is involved in seizure regulation and can modulate epileptic activity in the brain. During the epileptic seizures, galanin is released and exerts a presynaptic inhibitory effect on the glutamatergic transmission (Zini et al., 1993; Mazarati et al., 2000; Kokaia et al., 2001). In galanin overexpressing transgenic mice or rats where galanin is overexpressed by gene transduction, prolonged latent period to the convulsions, and decreased susceptibility to generalized seizures have been observed in a kindling model of epilepsy (Schlifke et al., 2006; Kanter-Schlifke et al., 2007). Viral vector-based gene therapy approach has also demonstrated a powerful seizure-suppressant effect of transgene galanin in other animal models of epilepsy, such as chemical and electrical induced status epilepticus (Lin et al., 2003; Kanter-Schlifke et al., 2007).

Non-pathogenic viral vector-based gene delivery into the brain has been proven to be a safe procedure in phase

1-2 clinical trials, for e.g. Parkinson's disease (Mandel & Burger, 2004; Kaplitt et al., 2007; LeWitt et al., 2011). However, once the transgene of interest is expressed in the host brain cells, it is impossible to reverse the process and to terminate its action in case adverse effects arise. Encapsulated cell biodelivery (ECB) devices filled with genetically modified human cells to release gene products into the host tissue has an advantage of being a reversible treatment: the ECB devices can be removed from the brain with a relatively simple procedure and thereby terminate the exerted effect. The cells in the ECB devices can have long-term viability when implanted into the brain because the nutrients from the surrounding host tissue can penetrate the ECB device's semi-permeable membrane, and at the same time, the gene products can be released into the host tissue. The advantages with ECB devices as compared to direct genetic modification of the host cells by viral vector-based gene delivery, or direct cell transplantation, are that the encapsulated cells do not alter host cells or integrate into the host brain. Furthermore, the semi-permeable membrane isolates the cells in the ECB devices from immune reactions in the host brain. Thus, there is no need for immunosuppressant drugs. These considerations make it highly warranted from the translational perspective to explore possible therapeutic effects of compounds like galanin delivered into the brain by using ECB technology.

The main objective of the present study was to explore the therapeutic potential of intrahippocampally engrafted galanin-releasing ECB devices in an animal model of stimulation-induced recurrent epileptic seizures.

Experimental procedures

Ethics Statement

All experimental procedures were approved by the local Malmö / Lund Ethical Committee for Experimental Animals (Ethical permit number M187-09), were performed according to the guidelines of the Swedish Animal Welfare Agency and in agreement with international guidelines.

In vitro preparation and filling of ECB devices

ECB devices consisted of a semi-permeable polyethersulfone (PES) hollow fiber membrane filled with a polyvinyl alcohol (PVA) cylindrical matrix serving as support for the encapsulated cells. Devices were built and sterilized, before they were filled with low-passage human retinal pigment epithelial cell line (ARPE-19). The cells and the filled ECB devices were cultured in human endothelial serum-free media (HE-SFM) (Invitrogen, Stockholm, Sweden) in an incubator (37ºC, 5 % CO2). Several cell lines were generated by genetic modification to release galanin. Two cell clones that produced and

released galanin were selected, one with a higher release (HR) and one with a lower release (LR) of galanin. The ECB devices were 5 mm long for the vertical placement in the hippocampus, and 7 mm long for the angular placement (see below). Both had an outer diameter of 725 μm and an inner diameter of 525 μm. Each ECB device was filled with 60 000 cells (optimized by pilot experiments). The ECB devices with non-modified ARPE-19 cells (not producing galanin) and empty ECB devices were used as controls. No differences in seizure control could be detected between groups treated with empty devices and ECB devices filled with control cells, thus the results from the rapid kindling from these two control groups were merged (data not shown). One and three weeks after encapsulation, galanin release from the ECB devices into the incubation medium was sampled after 24 hours and measured by a galanin ELISA (Bachem, Bubendorf, Switzerland).

Animals

Male Sprague-Dawley rats (Charles River, Germany) weighing 200-230g at the beginning of the experiment were used. The animals were housed individually at a 12 hour light / dark cycle with ad libitum access to food and water. All animals were weighted once a week throughout the experiment.

Implantation of ECB devices and electrode

Animals were anesthetized with isoflurane and fixed into a stereotaxic frame (David Kopf Instruments, Tujunga, CA). The ECB devices were implanted bilaterally in two different positions. One group of animals had the ECB devices implanted in a straight vertical position and the other group in a 26º angle, to ensure better coverage of the entire hippocampal axis. The following number of animals and groups were used: Vertical placement of ECB devices – Empty n=5, ARPE n=4, LR n=8, HR n=5; Angular placement of ECB devices – Empty n=12, ARPE n=12, LR n=10, HR n=7. The coordinates used for these two ECB device placements were as follows, reference points from bregma, midline and dura: AP -4.8, ML ±4.1, V -6.0 and AP -5.3, ML ±2.7, V -8.0, respectively. ECB devices were stored in an incubator at 37°C (5 % CO2) until implantation.

At the same surgical session a bipolar stainless steel stimulation / recording electrode (Plastics One, Roanoke, VA) was implanted into the hippocampus at the following coordinates: AP -4.6, ML -4.9, V -6.3 (vertically placed ECB devices) and AP -4.8, ML -5.2, V -6.3 (26º angular placed ECB devices) from bregma, midline and dura, respectively. A reference electrode was placed between the scull and the temporal muscle. Proximal electrode sockets were inserted into a plastic pedestal (Plastics One, Roanoke, VA) and fixed on the skull with dental cement (Kemdent, Wiltshire, UK). The animals were

allowed to recover for one week before rapid kindling electrical stimulations were started.
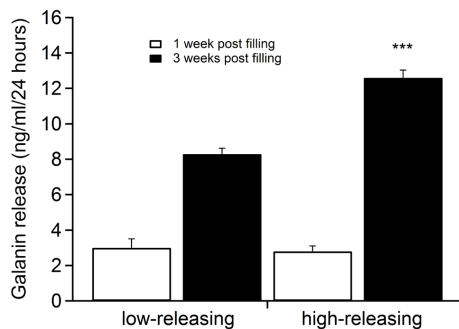
Rapid Kindling

One week after electrode and ECB device implantation, the individual current threshold for epileptiform afterdischarge (AD) induction was measured. The stimulation current started at 10 µA and increased by steps of 10 µA (1 ms square wave pulse, 100 Hz) until a focal EEG AD of at least 5 sec duration was elicited. Subsequently, induction of epileptic activity was initiated according to the rapid kindling protocol, 40 recurrent stimulations given every 5 min, consisting of trains of 10 sec duration, (1 ms bipolar square wave pulses at 10 Hz), with a current intensity of 400 µA.

The behavioral seizures during stimulation were scored according to the Racine scale (Racine, 1972): Stage 0 - no behavioral changes; Stage 1 - facial twitches; Stage 2 - chewing and head nodding; Stage 3 - unilateral forelimb clonus; Stage 4 - rearing, body jerks, bilateral forelimb clonus; Stage 5 - imbalance. EEG was recorded on a MacLab system (ADInstruments, Bella Vista, Australia) 1 min before and 1 min after electrical stimulation.

Explantation of ECB devices and perfusion

Four weeks after rapid kindling stimulation, the animals were deeply anesthetized with pentobarbital and were perfused transcardially with 0.9 % NaCl. The skull was opened and the ECB devices were removed and put into the pre-heated (37°C) medium (HE-SFM) and were stored in an incubator (37°C, 5 % CO_2). Galanin levels in the incubation solution were measured after 24 hours by a galanin ELISA (Bachem, Bubendorf, Switzerland) to estimate galanin release from surviving encapsulated cells. Moreover, ECB devices were embedded in resin, cut (5 µm) and stained for hematoxylin/eosin to evaluate cell survival. The brains were taken out and fixed in 4

Figure 1. Galanin release from low-releasing and high-releasing ECB devices. Galanin release was measured using ELISA 1 week and 3 weeks after filling the ECB devices with ARPE-19 cell lines. Values are presented as mean ±SEM, *** p<0.001 compared between low-releasing and high-releasing clones.

% paraformaldehyde for 24 hours and then overnight in 30 % sucrose in 0.1 M sodium phosphate buffered saline. Brains were cut on a microtome in 30 µm thick slices and stored in a cryoprotective solution in the freezer until use.

Immunohistochemistry and other staining procedures for brain slices

To determine the extent of the damage and localization of the ECB devices hematoxylin/eosin stainings were performed on slices from all brains. To evaluate the inflammation, double immunohistochemical stainings of ionized calcium-binding adapter molecule 1, Iba1, together with ectodermal dysplasia 1, ED1, was performed. Slices were rinsed with 0.02 M KPBS and pre-incubated with 5 % normal goat serum and 5 % normal donkey serum in 0.25 % Triton-KPBS for one hour in room temperature (RT). The slices were then incubated with the sera, rabbit anti-Iba1 (Wako, Neuss, Germany; 1:1000) and mouse anti-ED1 (AbD Serotec, Puchheim, Germany; 1:200) overnight at room temperature (RT). Next day the slices were rinsed with 0.02 M KPBS, followed by incubation with secondary antibodies for Iba1 (FITC-goat anti-rabbit; Jackson Immunoresearch, Suffolk, UK; 1:400) and ED1 (Cy3-donkey anti-mouse; Jackson Immunoresearch, Suffolk, UK; 1:400) for two hours in RT. After two hours the slices were once again rinsed the same way as previously and were mounted on coated slides and coverslipped with DABCO (Sigma-Aldrich, Stockholm, Sweden).
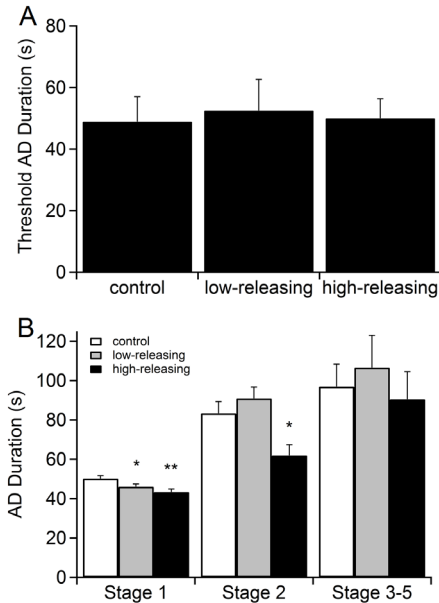
Cell counting was performed in the motor cortex (1 mm2 including all layers of the cortex) on three serial (180 µm apart) Iba1/ED1 stained slices from each animal with vertically placed ECB devices. Four animals were counted bilaterally, but no difference in stained cell numbers was detected between the sides (data not shown), thus the rest of the animals were counted unilaterally, only ipsilateral to the electrode side. Cell counting was performed and the images for figures were acquired with Olympus BX61 fluorescence microscope.

Statistical analysis

Statistical analysis of data was performed using Student's unpaired t-test. Differences between groups were considered significant at p<0.05. Data are presented as mean ±SEM. The investigator conducting the behavioral grading of seizures in the animals, EEG and histological analysis was unaware of the group identity of individual animals.
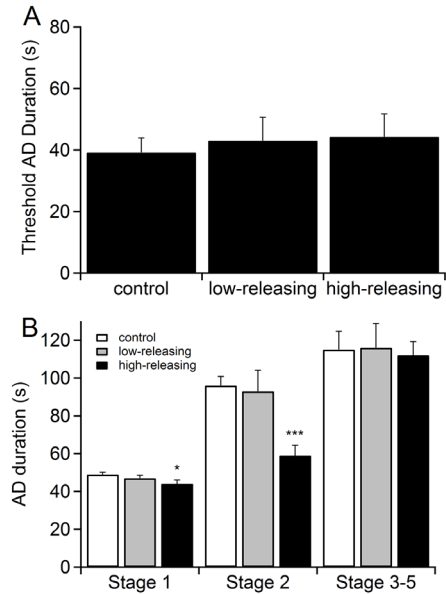
Results

Implanted ECB devices with the HR galanin cell clone shortens duration of focal seizures

After filling ECB devices with galanin-producing cells,

Figure 2. High-releasing ECB devices with vertical placement attenuate focal epileptic seizures. (A) Afterdischarge (AD) duration at threshold stimulations was unchanged in animals implanted with galanin-producing ECB devices. (B) Average AD duration for kindling stages 1 and 2 was attenuated by high-releasing galanin-producing ECB devices. Values are presented as mean ±SEM, * p<0.05 and ** p<0.01 compared to control.

Figure 3. High-releasing ECB devices with angled placement attenuate focal epileptic seizures. (A) Afterdischarge (AD) duration at threshold stimulations was unchanged in animals implanted with galanin-producing ECB devices with different content. (B) Average AD for kindling stages 1 and 2 was attenuated by high-releasing galanin-producing ECB devices. Values are presented as mean ±SEM, * p<0.05 and *** p<0.001 compared to control.
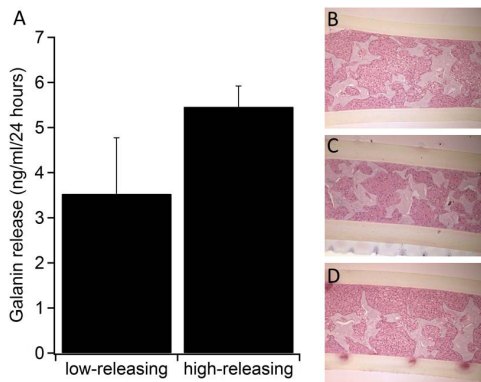
the release of galanin in the incubation culture media was measured (Fig. 1). One week after filling, the galanin release from the LR clone and the HR clone was similar, whereas three weeks after filling the HR galanin clone released about 30 % more galanin than the LR clone (HR 12.6±0.4 ng/ml/24h; LR 8.3±0.3 ng/ml/24h; p<0.05). During the initial phase of kindling stimulations, the seizures are usually focal, while subsequent stimulation-induced seizures spread and manifest as convulsions of increasing severity. The AD threshold for seizure induction was unaffected with vertically implanted and with angular ECB devices filled with galanin releasing cells (Vertical: control 48.9±8.1 µA; LR 52.5±10.1 µA; HR 50.0±6.3 µA; Angular: control 39.2±4.7 µA; LR 43.0±7.6 µA; HR 44.3±7.4 µA; p>0.05) (Fig. 2A and 3A). However, the AD duration of focal seizures was moderately decreased in animals with vertically implanted ECB devices releasing high levels of galanin compared to control (Stage 1: control 50.1±1.5 sec; LR, 46.0±1.4 sec; HR, 43.3±1.5 sec; Stage 2: control 83.3±6.0 sec; LR, 90.9±5.7 sec; HR, 61.9±5.5 sec), but was unaltered at the generalized stages (Stage 3-5: control 96.9±11.4 sec;

LR, 106.6±16.3 sec; HR, 90.5±14.0 sec; p>0.05) (Fig. 2B). The LR galanin clone showed a decreased AD duration during Stage 1, but not for any other seizure stages. Similarly, the angular placed ECB devices decreased AD duration for focal seizures in animals with only the HR clone (Stage 1: control 49.4±1.2 sec; LR 47.0±1.6 sec; HR 43.7±2.0 sec; Stage 2: control 95.9±4.8 sec; LR 93.3±11.1 sec; HR 59.4±5.4 sec) (Fig. 3B). There was no effect of LR ECB devices on AD duration at any seizure stage. The AD duration of generalized seizures remained unaltered (Stage 3-5: control 115.0±9.6 sec; LR 116.1±12.8 sec; HR 112.4±7.2 sec; p>0.05).

After retrieval of the ECB devices, galanin release levels were measured in the culture media once again. The galanin release was decreased for about 50 % or more (LR 3.5±0.5 ng/ml/24h; HR 5.5±1.2 ng/ml/24h; p>0.05) compared to the levels right before the implantation (Fig. 4A). No galanin release was detected from empty or parental ARPE-19 cell line filled ECB devices. Cell survival in the retrieved ECB devices was also verified with hematoxylin/eosin stainings of ECB device sections (Fig. 4B-D). Overall, almost all ECB devices

Figure 4. Galanin release after explantation of ECB devices. (A) Average galanin levels measured by ELISA in ECB devices after explantation. Hematoxylin-eosin staining of a section from an ECB device filled with (B) ARPE-19 control cells, (C) low-releasing galanin cells and (D) high-releasing galanin cells. Values are presented as mean ±SEM, p>0.05.

demonstrating galanin release also contained surviving cells. Only animals implanted with ECB devices that still released galanin after retrieval and those that had viable cells were included in the analysis.

Hematoxylin/eosin stainings were performed on sections from brains of all animals to confirm the position of the ECB devices in the hippocampus. The expected positions from two experimental animals are exemplified in schematic drawings on Fig. 5A and 5C. The examples of actual sections showing ECB device position in the hippocampus is shown on Fig. 5B and 5D. Scoring of the extent of the hippocampal damage exerted by the ECB device implantation did not reveal any correlation between the extent of damage and the severity of seizures (data not shown).

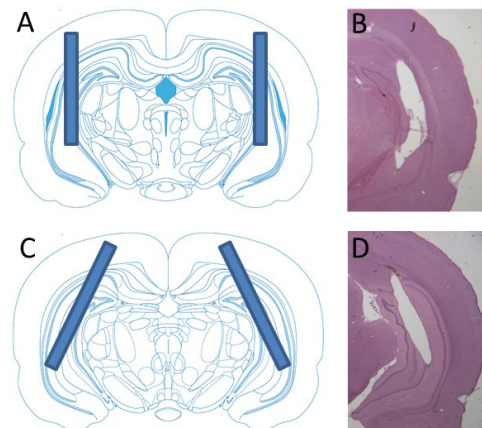### Inflammatory response in the motor cortex caused by encapsulated cells

Next we asked whether implantation of the ECB devices with galanin-producing cells could cause an inflammatory response, thereby contributing to the observed effects on seizures. However, the ECB devices, regardless whether they contained galanin-producing cells or those not modified, induced inflammatory reaction of the same magnitude as judged by estimating the numbers of activated microglia (number of Iba1-positive cells; Fig. 6A). The number of activated microglia was however less in the brain slices from animals implanted by empty ECB devices (empty 204.4±1.2 cells; ARPE 220.7±4.2 cells; LR 219.1±4.1 cells; HR 218.4±2.2 cells) (Fig. 6A). When counting ED1 immunoreactive cells in the cortex, no difference between the different groups could be detected

(empty 12.1±0.7 cells; ARPE 13.7±1.0 cells; LR, 11.3±0.7 cells; HR, 12.1±1.0 cells; p>0.05) (Fig. 6B). Similarly, double-labeled for Iba1 and ED1 immunoreactive cell numbers were not different in various groups (empty 6.1±0.4 cells; ARPE 6.8±0.8 cells; LR 5.7±0.5 cells; HR 5.7±0.4 cells; p>0.05) (Fig. 6C). Taken together, our data suggest increased number of activated microglia in the cortex from implanted cell-containing ECB devices.
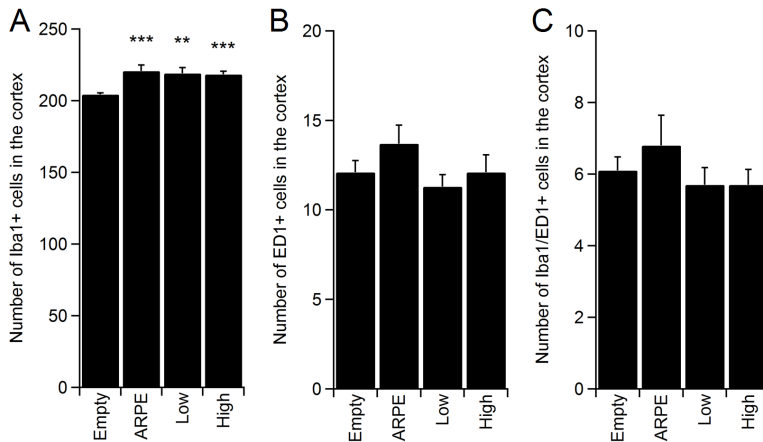
Discussion

Here we demonstrate that implanted encapsulated cells releasing galanin decrease AD duration of focal seizures in a well-established model of epilepsy, rapid kindling. These data suggest that ECB devices could potentially be an alternative source for exogenous long-term galanin delivery to the brain, in particular to the hippocampus, to suppress focal epileptic seizures. The observed seizure-suppression was relatively moderate, shortening the focal seizures but having no significant effect on duration of generalized ones.

Overall, the observed effect of grafted galanin-releasing ECB devices is in line with previous publications, whereby galanin has been shown to exert an inhibitory effect on seizures (Kokaia et al., 2001; Lin et al., 2003; Kanter-Schlifke et al., 2007). The novel finding is that the ECB technology, which is a relatively safe treatment strategy compared to direct gene or cell therapy approaches, is a valid alternative, and may be considered for translational development towards clinical applications, e.g., in temporal lobe epilepsy patients. The advantage of the ECB

Figure 5. Predicted placement of ECB devices in the hippocampus. (A) Predicted placement of vertically implanted ECB devices. (B) An example of actual vertical placement of the ECB device in the hippocampus. (C) Predicted placement of the ECB devices implanted with a 26º angle. (D) An example of actual placement of the ECB device implanted with an angle.

Figure 6. Inflammatory response in the cortex after implantation of ECB devices. (A) Average number of Iba1 immunoreactive cells in the cortex of animals implanted with different types of ECB devices. (B) Average number of ED1 immunoreactive cells, a marker for activated microglia in the cortex of same animals as in (A). (C) Double-labeled Iba1 and ED1 immunoreactive cells in the same animals as in (A) and (B). Values are presented as mean ±SEM, ** $p < 0.01$ and *** $p < 0.001$ compared to empty.

technology from the point of patient safety is several-fold. First, the grafted cells are isolated from the host cells by a semi-permeable membrane, and therefore, it is possible to remove the whole graft in case of adverse effects or replace it if necessary; second, immune cells from the host cannot access the grafted cells, and therefore the risk of graft rejection is minimized; third, there is no genetic manipulation of host cells, diminishing the risk of unwanted mutations and carcinogenesis; fourth, there is no direct contact or interaction between the grafted and host cells, reducing the risk for transgene down-regulation or some other direct regulatory effects from the host. Despite these advantages, the ECB devices may not be a first choice when graft-host direct interaction and bi-directional integration is desirable, since ECB does not provide this possibility. This approach excludes that grafted cells would integrate and become part of the existing network, and thereby restricting release the product of interest in more regulated manner, just when it is needed. Such possible regulatory mechanism would allow for perhaps more physiological interaction of the graft and the host, preventing e.g. down-regulation of the receptors in the host cells due to permanent high levels of the ligands, as is the case for the ECB. Yet another limitation of the ECB technology may be a requirement of relatively high levels of the gene product released by the encapsulated cells to reach a therapeutically effective dose in the host brain tissue. The moderate effect of galanin-releasing ECB devices on seizures, observed in the present study, may be related to insufficient galanin levels provided by the implanted devices.

One possible confounding factor for seizure-suppressant effects observed in this study could be the inflammatory response to the galanin-producing ECB devices using ARPE cell line-containing devices. Inflammation has been shown to play a profound role in epileptogenesis and ictogenesis (Galic et al., 2008; Auvin et al., 2010). Therefore, possible inflammatory reaction of the host, despite the fact that these cells are behind the semi-permeable membrane (see above), could modulate seizures and contribute to the observed effects. Indeed, our data suggest that ECB devices containing ARPE cell lines, galanin-producing or not, elevate Iba1-positive cell counts in the cortical region of the grafted animals as compared to those implanted with empty devices. It should be noted that the ECB devices increased the number of Iba1 immunoreactive cells, but did not alter the number of ED1 immunoreactive cells. This would indicate a relatively mild level of inflammatory reaction caused by the ECB devices. However, the number of Iba1-positive microglia was similar in all groups with ECB devices containing ARPE cell lines, regardless whether they released low or high levels of galanin or no galanin at all. This would suggest that galanin-releasing ECB devices per se did not induce any additional inflammatory reaction (as judged by Iba1 immunostaining). Moreover, these data support the idea that galanin released from ECB devices was responsible for the observed seizure-suppressant effect but not the inflammatory reaction of the host. The moderate effect of galanin-releasing ECB devices could be related to several factors. One possibility is low levels of galanin released by implanted ECB

devices. In support of this notion, ELISA measurements of galanin release from explanted ECB devices (5 weeks after initial implantation) were less than half of what was measured prior to implantation. Such decrease in galanin release could be due to compromised survival of the encapsulated cells, which was apparent at least in some of the explanted ECB devices. Another reason for the moderate effect could be a glial scar formed around the devices. This would restrict diffusion of galanin into the host brain. Yet another factor potentially affecting the outcome of the present experiments could be related to down-regulation of galanin receptors induced by permanent increase of galanin levels around the ECB devices. These questions need to be addressed in future studies in more detail.

Conclusion

The ECB technology has previously been tested in other neurological diseases, such as Alzheimer's disease, demonstrating good safety, tolerability and positive functional outcomes (Eriksdotter-Jonhagen et al., 2012; Tornoe et al., 2012). Our data suggest that ECB devices could be a feasible strategy for delivering galanin or other seizure-suppressant agents locally into the focus of epileptic seizures. The various advantages of the ECB devices compared to other gene or gene product delivery techniques should be evaluated against the efficacy and functional outcomes. In our study, the observed seizure-suppressant effects by galanin-releasing ECB devices were moderate, and therefore may need further optimization before it can be considered for clinical application. Higher levels of galanin release over longer time periods seem to be necessary to achieve better outcomes in seizure control.

Acknowledgements

Disclosure statement

None of the authors have any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.
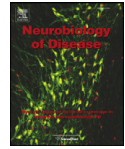
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Paper III

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Combined gene overexpression of neuropeptide Y and its receptor Y5 in the hippocampus suppresses seizures

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ABSTRACT

We recently demonstrated that recombinant adeno-associated viral vector-induced hippocampal overexpression of neuropeptide Y receptor, Y2, exerts a seizure-suppressant effect in kindling and kainate-induced models of epilepsy in rats. Interestingly, additional overexpression of neuropeptide Y in the hippocampus strengthened the seizure-suppressant effect of transgene Y2 receptors. Here we show for the first time that another neuropeptide Y receptor, Y5, can also be overexpressed in the hippocampus. However, unlike Y2 receptor overexpression, transgene Y5 receptors in the hippocampus had no effect on kainate-induced motor seizures in rats. However, combined overexpression of Y5 receptors and neuropeptide Y exerted prominent suppression of seizures. This seizure-suppressant effect of combination gene therapy with Y5 receptors and neuropeptide Y was significantly stronger as compared to neuropeptide Y overexpression alone. These results suggest that overexpression of Y5 receptors in combination with neuropeptide Y could be an alternative approach for more effective suppression of hippocampal seizures.

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Introduction

Epilepsy is a severe brain disorder, affecting approximately 1% of the world population, and is associated with reduced life expectancy and quality of life (Duncan et al., 2006; Jacoby and Baker, 2008). Despite significant efforts in development of novel antiepileptic drugs, 30–40% of patients respond insufficiently to current pharmacological treatments (Duncan et al., 2006). Particularly patients suffering from temporal lobe epilepsy (TLE) are often medically intractable, and although some of these may benefit from established surgical interventions (i.e., resection of epileptic focus, vagal nerve or deep brain stimulation), there is still need for development of new therapeutic strategies (Duncan et al., 2006; Thom et al., 2010).

Neuropeptide Y (NPY) is a 36-amino acid polypeptide transmitter abundantly expressed in the brain where it predominantly acts by binding to three G-protein coupled receptors (Y1, Y2, and Y5) (Berglund et al., 2003; de Quidt and Emson, 1986). NPY application exerts prominent seizure-suppressant effects in rodents *in vitro* and *in vivo* (Baraban et al., 1997; Klapstein and Colmers, 1997; Vezzani et al., 1999; Woldbye et al., 1996, 1997, 2005) and recombinant adeno-associated viral

(rAAV) vector-mediated overexpression of NPY also suppresses seizures in both acute and chronic models of epilepsy (Foti et al., 2007; Noe et al., 2008, 2010; Richichi et al., 2004; Sørensen et al., 2009). Consequently, rAAV-mediated NPY gene therapy has been proposed as an alternative treatment strategy for patients suffering from intractable TLE (McCown, 2010; Riban et al., 2009).

In the hippocampus, antiepileptic effects of NPY are mediated predominantly via binding to presynaptic Y2 receptors (El Bahh et al., 2005; Vezzani and Sperk, 2004) which subsequently inhibit glutamate release at excitatory synapses (Colmers et al., 1985; Greber et al., 1994). In addition, Y5 receptors have also been implicated in seizure-suppressant effects (Baraban, 2002; Benmaamar et al., 2005; Woldbye et al., 1997, 2005) while Y1 receptors may mediate opposite, seizure-promoting effects in the hippocampus (Benmaamar et al., 2003). Thus, once released, transgene NPY would activate both seizure-suppressant Y2/Y5 and seizure-promoting Y1 receptors at the same time. Indeed, we recently showed in two TLE models, kindling and kainate-induced seizures, that rAAV-mediated overexpression of Y2 receptors in the hippocampus exerts antiepileptic effect in rats and that, more importantly, combined overexpression of Y2 receptors and NPY had even broader seizure-suppressant action (Woldbye et al., 2010). To further capitalize on the novel concept of selective therapeutic overexpression of seizure-suppressant NPY receptors alone or in combination with NPY, we tested whether also transgene Y5 receptors would suppress kainate-induced seizures. Here we show that overexpression of functional Y5 receptors alone in the hippocampus has no detectable

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antiepileptic effect, but the combination of Y5 receptors with NPY exerts seizure-suppression, which is significantly stronger as compared to NPY alone.

Materials and methods

Animals

Adult male Wistar rats (Charles River, Germany) weighing 250–350 g at the beginning of the experiments were used. Animals were housed on 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.

Viral vector injections

The rAAV vector was a chimeric serotype, consisting of serotype 2 inverted terminal repeats (ITRs) and combined serotype 1 and 2 capsid proteins. The transgenes were subcloned into an expression cassette consisting of the rat neuron-specific enolase promoter, Woodchuck post-transcriptional regulatory element, and a bovine growth hormone polyA signal flanked by viral ITRs (Richichi et al., 2004). The rAAV vectors were manufactured by GeneDetect (Auckland, New Zealand) and encoded the full-length cDNA for the mouse Y5 receptor (accession number: AF049329; rAAV-Y5: stock solutions 1.0×10^{12} genomic particles/ml), human prepro-NPY (rAAV-NPY: stock solution 1.0×10^{12} genomic particles/ml), or empty cassette vector (rAAV-Empty; 1.0×10^{12} genomic particles/ml). Injections were performed as previously described (Kanter-Schlifke et al., 2007a, 2007b; Sørensen et al., 2008). Animals were anesthetized by inhalation of isoflurane (Baxter Medical AB, Sweden) and gently fixed in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). Before and after surgery, the local analgesic Marcain (AstraZeneca, Denmark) was subcutaneously administered around the wound. A volume of 2 μl viral vector phosphate-buffered saline (PBS) suspension was infused through a glass pipette (0.2 μl/min) bilaterally into the dorsal hippocampus (anterior–posterior (AP) −3.3 mm, medial–lateral (ML) ±1.8 mm, dorsal–ventral (DV) −2.6 mm) and the ventral hippocampus (AP −4.8 mm, ML ±5.2 mm, DV −6.4 and −3.8 mm; 1 μl at each location in the DV plane) (Paxinos and Watson, 2005). Reference points were bregma for the AP axis, midline for the ML axis, and dura for the DV axis. The pipette was left in place for an additional 3 min after injection to prevent backflow of viral particles through the injection track. The viral vector suspensions were mixed (1:1) from viral vector stock solutions diluted with sterile PBS or in a mixture of equal portions of the two in the combination gene therapy group (rAAV-Y5/NPY). Non-treated or rAAV-Empty treated rats were used as controls.

Transgene overexpression

To confirm the presence of rAAV vector mediated transgene overexpression we investigated hippocampal Y5 receptor and NPY gene expression, Y5 receptor-like and NPY-like immunoreactivity, Y5 receptor binding, and functional Y5 receptor binding.

Y5 receptor and NPY mRNA in situ hybridization

The procedure was performed as previously described (Woldbye et al., 2010) with only minor modifications. At the time of sacrifice, the rats were decapitated and their brains were quickly removed and frozen on dry ice. Subsequently, 15 μm thick coronal serial sections at the hippocampal level were cut on a cryostat and thaw-mounted on Superfrost glass slides, dried on a hotplate and stored at −80 °C. The slides were defrosted for 10 min at room temperature (R_T), subsequently fixed for 5 min in 4% PFA, and rinsed briefly and placed for 5 min in PBS. Then,

the slides were transferred to 70% ethanol for 5 min and stored in 95% ethanol at 4 °C until hybridization. Synthetic antisense oligonucleotide DNA probes were used for *in situ* hybridization: Y5 receptor mRNA: 5′-CGA GTC TGT TTT CTT TGT GGG ACA ATC CAC AGC TTA TAC TCC TGC-3′; prepro-NPY mRNA: 5′-GTC-CTC-TGC-TGG-CGC-GTC-CTC-GCC-CGG-ATT-GTC-CGG-CTT-GGA-GGG-GTA-3′ (Mikkelsen and Woldbye, 2006). The oligoprobes were labeled at the 3′-end with [α^{35}S]dATP (1250 Ci/mmol; #NEG734H001MC; PerkinElmer, Denmark) using terminal deoxynucleotidyl transferase (Roche Diagnostics, Mannheim, Germany). Labeled probes were added with a specific activity of $1–3 \times 10^{5}$ cpm/100 μl to the hybridization buffer containing 50% formamide (v/v), 4×saline sodium citrate (SSC; 1× SSC = 0.15 M NaCl, 0.015 M NaCitrate–2H$_2$O, pH 7.0), 10% dextran sulfate (w/v) and 10 mM dithiothreitol. After adding a volume of 120 μl hybridization mixture to each slide, they were covered with Parafilm and left overnight at 42 °C in humidity boxes. The slides were then briefly rinsed in 1× SSC at R_T, washed for 30 min in 1× SSC at 60 °C, passed through a series of 1 min rinses in 1× SSC, 0.1× SSC, 70% ethanol, and 95% ethanol at R_T, and finally air-dried. The slides where exposed together with ^{14}C-microscales on ^{35}S-sensitive Kodak BioMax MR films (Amersham Biosciences, Denmark) for 3–8 weeks and developed in Kodak GBX developer.

Y5 receptor immunohistochemistry

Sections were defrosted for 10 min at R_T and subsequently fixed for 30 min in 4% PFA, washed three times for 5 min in PBS, incubated in preincubation buffer (5% goat serum, 1% bovine serum albumin (BSA), 0.3% Triton X-100 in PBS) for 30 min, and placed in incubation buffer with rabbit anti-Y5 receptor antibody (1:300, Alomone Labs, Israel) overnight at 4 °C. The slides were then washed three times for 5 min in washing buffer (0.25% BSA, 0.1% Triton X-100 in PBS), left in incubation buffer with Alexa 568 goat anti-rabbit (1:200, Invitrogen, Denmark) for 1 h and washed three times for 5 min, once in washing buffer and twice in PBS. The sections were then mounted on glass slides using ProLongGold Antifade (Invitrogen).

NPY immunohistochemistry

Seizure-naïve rats injected 3 weeks previously with rAAV-NPY into the right hippocampus were deeply anesthetized with sodium pentobarbital and perfused through aorta with 0.9% NaCl followed by 4% PFA. The contralateral non-injected hemisphere served as control. Brains were post-fixed overnight at 4 °C, transferred to 20% sucrose, and stored overnight at 4 °C. Then the brains were cut into 30 μm thick slices on a microtome. Free-floating sections were rinsed in potassium phosphate buffer (KPBS) and incubated overnight at R_T with rabbit anti-NPY antibody (1:10,000; Sigma-Aldrich, St. Louis, MO, USA) in a solution of 5% goat serum, 0.25% Triton X-100 in KPBS. The slices were then rinsed and incubated with secondary biotinylated goat-anti rabbit antibody (1:200; Vector Laboratories, Burlingame, CA, USA), and staining was visualized by 3-3′-diaminobenzidine reaction.

Y5 receptor binding

Y5 receptor binding was performed as previously described (Woldbye et al., 2005). The slides were defrosted at R_T and subsequently preincubated for 20 min in binding buffer (pH 7.4), containing 25 mM N-(2-hydroxyethyl)-piperazine-N′-(2-ethanesulfonic acid) (HEPES), 2.5 mM CaCl$_2$, 0.5 g/l bacitracin, 0.5 g/l BSA. The slides were then incubated at R_T for 60 min in binding buffer containing 0.1 nM [^{125}I][Tyr^{36}]mono-iodo-PYY (4000 Ci/mmol; porcine synthetic, #IM259; Amersham Biosciences, Denmark) to which was added 100 nM BIBP3226 (Y1 receptor antagonist; #E3620, Bachem AG, Switzerland) + 100 nM BIIE0246 (Y2 receptor antagonist; #1700, Tocris Bioscience, UK) to visualize Y5 receptor binding. After a brief rinse, the slides were washed for 2×30 min in binding buffer at R_T

and subsequently air-dried, before being exposed to ^{125}I-sensitive Kodak BioMax MS films (Amersham Biosciences) for 4 days at −20 °C with ^{125}I-microscales (Amersham Biosciences). Non-specific binding was performed by adding of unlabeled NPY (1 μM) to displace [^{125}I]-PYY binding. The films were developed with Kodak GBX developer.

[^{35}S]GTPγS functional Y5 receptor binding

Functional binding was performed as previously described (Agasse et al., 2008; Christensen et al., 2006; Silva et al., 2007). Sections were defrosted for 30 min at R_T before being rehydrated for 10 min at R_T in assay buffer A (50 mM Tris–HCl, 3 mM MgCl, 0.2 mM ethylene glycol tetraacetic acid, 100 mM NaCl, pH 7.4). Sections were preincubated for 20 min at R_T in buffer B [assay buffer A, 0.2 mM dithiothreitol, 1 μM 1,3-dipropyl-8-cyclopentylxanthine (#C-101, PerkinElmer, Denmark), 0.5% w/v BSA, and 2 mM guanosine-5′-diphosphate (#G7127, Sigma-Aldrich, Denmark)] and then incubated at 25 °C for 1 h in buffer B together with 40 pM [^{35}S]GTPγS (1250 Ci/mmol; #NEG030H250UC; PerkinElmer, Denmark) and 1 μM NPY (rat synthetic, Schafer-N, Copenhagen, Denmark). For visualization of functional Y5 receptor binding, the Y1 receptor antagonist BIBP3226 (1 μM; #E3620, Bachem AG, Switzerland) and the Y2 receptor antagonist BIIE0246 (1 μM; #1700, Tocris Bioscience, UK) were added to both preincubation and incubation buffers. To block and confirm the specificity of functional Y5 receptor binding, the Y5 receptor antagonist L-152,804 (10 μM; #1382, Tocris Bioscience, UK) was added to both preincubation and incubation buffers together with Y1 and Y2 receptor antagonists at same concentrations as above. Basal binding was determined by incubation in buffer B with 40 pM [^{35}S]GTPγS (1250 Ci/mmol) but without NPY receptor ligands. Non-specific binding was determined by incubation in buffer B (without NPY receptor ligands) with 40 pM [^{35}S] GTPγS and 10 μM non-labeled GTPγS (#89378; Sigma-Aldrich, Denmark). Y1, Y2, and Y5 receptor antagonists were dissolved in dimethyl sulphoxide to a final concentration of 0.1%, and dimethyl sulphoxide was, likewise, added to the other incubation buffers (0.1% final concentration). Incubation was terminated by two times 5 min washing in ice-cold 50 mM Tris–HCl buffer (pH 7.4). Finally, sections were rinsed in ice-cold demineralized H_2O and dried under a stream of cold air. Sections were exposed to ^{35}S-sensitive Kodak BioMax MR films together with ^{14}C-microscales for 5 days and then developed in Kodak Processing Chemicals for Autoradiography Films (Sigma-Aldrich, Denmark). Computer-assisted autoradiographic image analysis was performed using Scion Image (National Institute of Health, USA). Measurements were conducted by a person blinded to the vector treatment of the animals bilaterally in 3–4 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.

Kainate-induced seizures

Three to 4 weeks after virus injections, rats treated with rAAV-Y5, rAAV-NPY, rAAV-Y5/NPY, or rAAV-Empty vectors were injected subcutaneously in the neck region with kainate (10 mg/kg; diluted in 0.9% isotonic saline; pH 7.4; Sigma #K2389; Sigma, St. Louis, MO, USA). Subsequently, the animals were placed in individual Plexiglas boxes (30×19×29 cm) and observed for motor seizures as previously described (Woldbye et al., 1997). Each rat was rated for 2 h by an observer unaware of the treatment condition, and latencies to first motor seizure and status epilepticus, percentage time spent in motor seizures as well as the number of seizures were determined. Motor seizures were defined as clonic movements involving fore-

and/or hindlimbs of at least 15 s duration, and status epilepticus was defined as continuous clonic motor seizure activity of at least 10 min duration. Moreover, a total seizure score was determined based on a modified rating scale of Racine (1972): grade 0 (no motor seizure occurrence), grade 1 (starring or facial movements), grade 2 (head nodding or isolated twitches), grade 3 (motor seizure with limb clonus), grade 4 (motor seizure with rearing), grade 5 (motor seizure with loss of posture), 6 (status epilepticus) and 7 (death). Three hours after the kainate injection, the experiment was terminated by decapitation whereupon the brains were quickly removed and processed as described above.

Electrophysiology

For electrophysiology, hippocampal slices were prepared from rats injected bilaterally in the hippocampus 4 to 5 weeks previously with rAAV-Y5 or rAAV-Y5/NPY as above. Age-matched non-injected rats served as controls. After decapitation, their brains were immediately immersed into oxygenated (95% O_2/5% CO_2) and ice-cold sucrose-based solution containing (in mM): 75 sucrose, 67 NaCl, 26 NaHCO$_3$, 25 glucose, 2.5 KCl, 1.25 NaH$_2$PO$_4$, 0.5 CaCl$_2$, 7 MgCl$_2$; adjusted to 308 mOsm and 7.4 pH. Coronal slices of 300 μm thickness were prepared in the same solution using a Leica VT1200 vibratome. Slices were kept for 1 h at 34 °C in oxygenated sucrose-based solution, and then stored in oxygenated artificial cerebrospinal fluid (aCSF; containing in mM: 119 NaCl, 26 NaHCO$_3$, 25 glucose, 2.5 KCl, 1.25 NaH$_2$PO$_4$, 2.5 CaCl$_2$, 1.3 MgSO$_4$; 303 mOsm; 7.4 pH) held at room temperature until further use. In a submerged recording chamber, individual slices were constantly perfused at 2.5 ml/min with oxygenated aCSF at 32.5 °C. It is well established that NPY reduces excitatory glutamatergic transmission in the hippocampus (Colmers et al., 1985; Sørensen et al., 2008; Vezzani et al., 1999). Our recent finding shows that NPY may also affect GABAergic synaptic transmission onto basket cells of the dentate gyrus (Ledri et al., 2011). Therefore, picrotoxin (PTX; 100 μM; Tocris Bioscience, Ellisville, MI, USA) was added to the perfusion medium to analyze excitatory synaptic transmission in isolation.

The recordings were conducted as described elsewhere (Sørensen et al., 2008). Briefly, in Schaffer collateral–CA1 synapses, field excitatory postsynaptic potentials (fEPSPs) were recorded at 10 kHz with a borosilicated glass pipette containing aCSF (3–4 MΩ resistance) using HEKA amplifier and PATCHMASTER software (HEKA Elektronik, Lambrecht, Germany). The recording electrode was positioned in CA1 stratum radiatum, while orthodromic current stimulations of Schaffer collateral fibers were applied in the same subfield (~500 μm towards CA3) by a glass pipette containing aCSF. Basal excitatory synaptic transmission was determined in each slice by stepwise increasing the stimulation strength until maximal fEPSP was generated. The presynaptic fiber volley (mV) was plotted against the initial slope of the fEPSP (mV/ms) and an input–output (I–O) relationship was generated. For paired-pulse stimulations, submaximal stimulation intensities generating 40–50% of maximal fEPSP were used. These stimuli were applied at 0.05 Hz with 25, 50, 100, and 200 ms interstimulus intervals (ISI) to elicit paired-pulse fEPSPs. Short-term synaptic plasticity was determined by the paired-pulse facilitation (PPF) ratio, and calculated, based on the average of four consecutive stimuli, as the initial slope of fEPSP[2] divided by the initial slope of EPSP[1]. To block endogenous Y2 receptors, BIIE0246 (Tocris Bioscience) was initially dissolved in ethanol (99.5%) and then diluted 1:10,000 in aCSF to reach a final concentration of 0.3 μM. BIIE0246 was applied for at least 8 min before resuming the recordings. After recordings, slices fixed overnight in 4% PFA at 4 °C, rinsed in KPBS and stored in anti-freeze at −20 °C until further processing. Researchers were blinded with regard to vector treatment during acquisition and analysis of data.

Statistical analysis

Differences between groups were analyzed using one-way ANOVA followed by Newman–Keuls post-hoc test or two-tailed Student's *t*-test. Electrophysiological data were analyzed with two-way ANOVA followed by Bonferroni post-hoc test. Data are presented as mean ± SEM. *P*<0.05 was considered statistically significant.

Results

Y5 receptor and NPY mRNA in situ hybridization

Three to 4 weeks after intrahippocampal injections of rAAV-Y5 or rAAV-NPY prominent increases in mRNA levels of Y5 receptor (Fig. 1A) or NPY (Fig. 1G), respectively, were observed in the hippocampal dentate gyrus and CA1–CA3 regions as compared to non-treated animals (Figs. 1B and H). Consistent with previous observations (Richichi et al., 2004; Woldbye et al., 2010), NPY and NPY receptor expression was mainly restricted to the principal layers of the hippocampus, i.e., the dentate granule layer and pyramidal layers of CA1–CA3 along with scattered dotted expression around these layers, most likely due to labeling of interneurons. In slices used for electrophysiology, overexpression of Y5 receptor and NPY was also confirmed using *in situ* hybridization (data not shown).
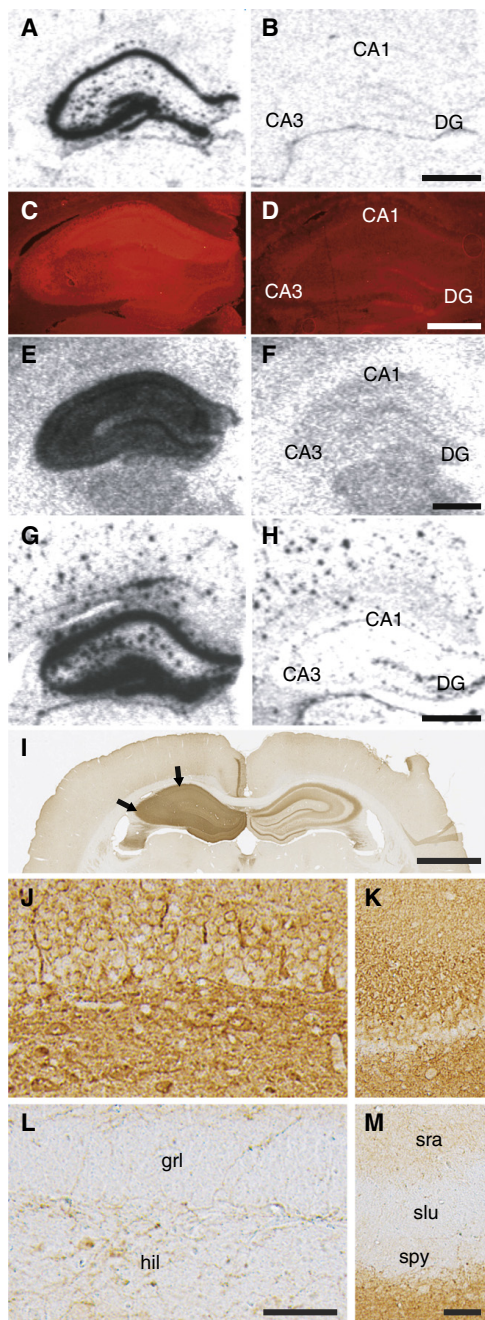
Y5 receptor and NPY immunohistochemistry

Next we explored whether rAAV-induced increased Y5 receptor mRNA levels were translated into protein using Y5 receptor immuno-histochemistry. Indeed, dense Y5 receptor-like immunoreactivity (Fig. 1C) was evident throughout the hippocampus in slices obtained from rAAV-Y5 treated animals, while levels were low in slices obtained from non-treated rats (Fig. 1D). Y5 receptor overexpression in slices used for electrophysiology was also confirmed by Y5 receptor immunohistochemistry (data not shown).

In accordance with previous studies using a similar vector construct (Richichi et al., 2004; Sørensen et al., 2009), rAAV-NPY injections resulted in increased NPY-immunoreactivity throughout the ipsilateral hippocampus as compared to the contralateral non-treated side (Figs. 1I–M). A dense band of NPY-immunoreactivity in the contralateral inner molecular layer of the dentate gyrus most likely represents labeling of hippocampal commissural fibers since this was not observed in rAAV-Empty treated rats (Richichi et al., 2004; Sørensen et al., 2009).

Y5 receptor binding

We further explored whether also Y5 receptor binding ([^{125}I]-peptide YY + Y1 and Y2 receptor antagonist) was increased after

Fig. 1. Levels of Y5 and NPY mRNA, Y5 and NPY-immunoreactivity, and Y5 receptor binding in seizure-naive rats at 3–4 weeks after intrahippocampal injection of rAAV-Y5 or rAAV-NPY. (A) Increased Y5 mRNA labeling after bilateral rAAV-Y5 treatment as compared to (B) non-treated control. (C) Increased Y5-like immunoreactivity in an animal bilaterally injected with rAAV-Y5 as compared to (D) non-treated control. (E) Increased Y5 receptor binding (^{125}I-PYY + BIBP3226 + BIIE0246) after bilateral rAAV-Y5 treatment as compared to (F) non-treated control. (G) Increased NPY mRNA labeling after bilateral rAAV-Y5/NPY treatment as compared to (H) non-treated control. Scale bars = 1 mm. (I) Increased NPY-immunoreactivity (arrows) ipsilaterally as compared to the contralateral side after unilateral injection of rAAV-NPY vector in the dorsal hippocampus. Scale bar = 2 mm. High power magnifications show increased NPY-immunoreactivity ipsilaterally (J) in the grl and hil of the DG as well as (K) in the slu, sra, and spy of CA3 after unilateral injection of rAAV-NPY vector as compared to the corresponding contralateral side (L and M, respectively). Scale bars = 30 μm. NPY, neuropeptide Y; rAAV, recombinant adeno-associated virus; PYY, peptide YY; grl, granular layer; hil, hilus. DG, dentate gyrus; slu, stratum lucidum; sra, stratum radiatum; spy, pyramidal cell layer.

rAAV-Y5 vector injections. Consistent with Y5 receptor-like immuno-reactivity, Y5 binding was also elevated throughout the hippocampus as compared to non-treated rats (Figs. 1E and F).

[^{35}S]GTPγS functional Y5 receptor binding

Next we investigated whether binding of NPY could activate transgene Y5 receptors, thus suggesting that they were functional. We consequently conducted Y5 receptor [^{35}S]GTPγS functional binding assay (NPY + Y1 and Y2 receptor antagonists). Three to 4 weeks after injections of rAAV-Y5 we found prominent increases in functional Y5 receptor binding as compared to non-treated control animals (Figs. 2A and D). Basal [^{35}S]GTPγS binding (without addition of NPY) in rAAV-Y5 treated animals was also increased as compared to non-treated controls (Figs. 2B and E). Specificity of functional Y5 receptor binding was confirmed by addition of the Y5 receptor antagonist L-152,804 which blocked the [^{35}S]GTPγS signal (Figs. 2C and F). Functional Y5 binding was also strongly increased in kainate-treated animals after either rAAV-Y5 or rAAV-Y5/NPY vector injections as compared to rAAV-Empty treated animals (Figs. 2G–I). Interestingly, the corresponding basal functional binding in animals treated with the rAAV-Y5/NPY combination was remarkably high as compared to both rAAV-Y5 and rAAV-Empty treated animals (Figs. 2J–L).

Functional Y5 receptor binding in kainate-treated animals after viral vector injections was quantified in the hippocampal dentate gyrus, CA3, and CA1 (Fig. 2M). Both rAAV-Y5 and rAAV-Y5/NPY vector treatments resulted in significantly higher functional Y5 receptor binding in all measured regions (dentate gyrus: by 106% and 71%, CA3: by 342% and 384%, and CA1: by 331% and 333% for rAAV-Y5 and rAAV-Y5/NPY, respectively) as compared to rAAV-Empty. Basal functional binding levels in animals treated with the rAAV-Y5/NPY combination were significantly increased in all measured regions (dentate gyrus: by 67%, CA3: by 338%, and CA1: by 275%) as compared to rAAV-Empty (Fig. 2N). In fact, functional Y5 receptor binding in rAAV-Y5/NPY vector treated animals was only elevated modestly above corresponding basal functional binding in the CA3 (14.0%, $P = 0.023$, paired t-test), not in the dentate gyrus and CA1, indicating that transgene Y5 receptors were close to maximal functional activity under basal conditions.

Seizure-suppressant effects of transgene overexpression

Next we investigated if overexpression of Y5 receptor or the combination of Y5 receptor and NPY could affect kainate-induced seizures in rats 3 to 4 weeks after bilateral vector injections. In contrast to Y2 receptor overexpression in a previous study (Woldbye et al., 2010), rAAV-Y5 vector treatment alone did not induce seizure-suppressant effects on any of the seizure parameters analyzed in this study. However, when Y5 receptor overexpression was combined with NPY, there was a significant seizure-suppressant effect as revealed by increased latencies to first motor seizure and status epilepticus (Fig. 3A), as well as decreased relative time (in percent) spent in motor seizures (Fig. 3B) and decreased seizure scores (Fig. 3D). These effects on seizures were significantly stronger as compared to seizure-suppressant effects exerted after single rAAV-NPY treatment (Figs. 3A, B and D). In addition, the number of motor seizures was significantly reduced only after rAAV-Y5/NPY vector treatment but not after rAAV-NPY alone (Fig. 3C).

Since NPY and Y5 receptors are implicated in stimulating appetite (Ishihara et al., 2006; Tiesjema et al., 2007), we also studied body weight of the animals after the different vector treatments. We found no significant differences in body weight at the start of the experiment (data not shown; $P = 0.86$, one-way ANOVA) and no significant differences in weight gain 3 weeks after viral vector injections (rAAV-Y5 by 27.9 ± 2.2%, rAAV-NPY by 30.7 ± 3.7%, rAAV-Y5/NPY by 32.3 ± 2.7%, rAAV-Empty: 30.0 ± 2.3%; $P = 0.71$, one-way ANOVA).
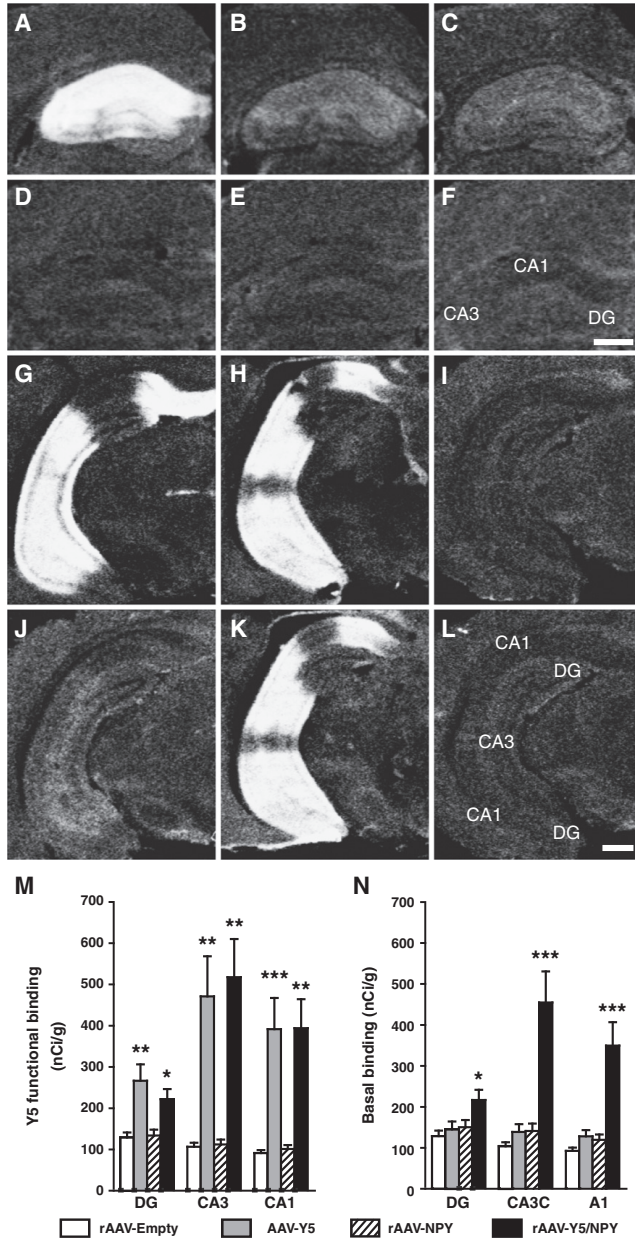
Basal excitatory synaptic transmission after transgene overexpression

Since the seizure-suppressant effect of transgene NPY is most likely induced by suppression of presynaptic glutamate release from principal synapses in the hippocampus (Colmers et al., 1985; Vezzani et al., 1999), we asked whether combined Y5/NPY overexpression in the hippocampus would compromise normal excitatory synaptic transmission. We therefore recorded evoked field excitatory postsynaptic potentials (fEPSPs) in Schaffer collateral–CA1 synapses in slices from rAAV-Y5/NPY (n = 10 slices, 5 rats), rAAV-Y5 (n = 10 slices, 5 rats), and non-treated (n = 15 slices, 5 rats) animals. Throughout the experiments, GABA-A receptors were blocked by picrotoxin (PTX) application. Basal excitatory synaptic transmission was examined by determining the relationship between the presynaptic fiber volley (PSFV) and the corresponding fEPSPs amplitude (I–O relationship) at various stimulation strengths. This analysis revealed that neither combined rAAV-Y5/NPY nor rAAV-Y5 vector treatment affected the I–O relationship, indicating no alterations in basal excitatory synaptic transmission in Schaffer collateral–CA1 synapses (Fig. 4A). To further explore possible changes in these synapses, we applied paired-pulse stimulations at different interstimulus intervals (ISIs) to Schaffer collaterals and analyzed paired-pulse facilitation (PPF) of generated fEPSPs. The potential contribution of Y2 receptors was blocked by the adding of the Y2 receptor antagonist BIIE0246. No significant changes were detected in PPF between the three treatments (Fig. 4B).
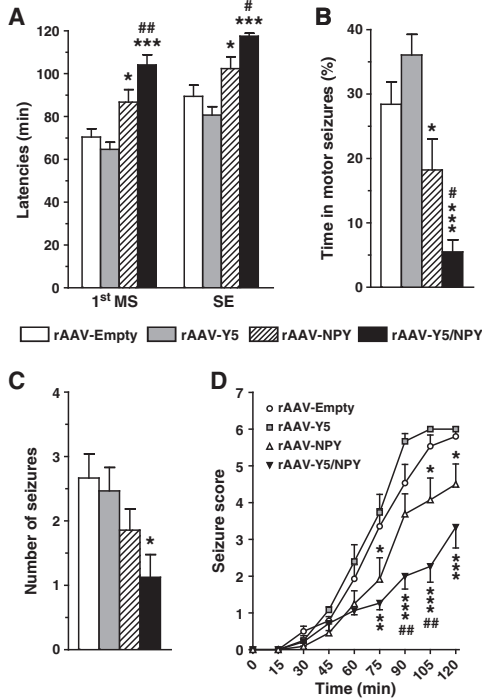
Discussion

In the present study, we show for the first time that rAAV-mediated overexpression of functional NPY Y5 receptors can be achieved in the rat hippocampus. However, overexpression of transgene Y5 receptors did not suppress kainate-induced seizures. Only when combined with NPY, Y5 receptor overexpression in the hippocampus resulted in pronounced seizure-suppressant effect. This effect was significantly stronger than that of NPY overexpression alone.

We previously achieved rAAV-based gene transfer for functional Y2 receptors in the hippocampus, and now demonstrate that this is also possible for Y5 receptors. Overexpression induced by rAAV-Y5 was documented by elevated Y5 receptor mRNA levels, Y5 receptor-like immunoreactivity, Y5 receptor binding, and functional Y5 receptor binding. The latter was blocked by the simultaneous addition of the Y5 receptor antagonist L-152,804, confirming the specificity of the functional Y5 receptor binding signal. We also observed slightly increased basal functional binding (without NPY added) in seizure-naive rAAV-Y5 treated animals. This may suggest that endogenous NPY can activate some of the transgene Y5 receptors under normal conditions, similar to what has been observed previously after hippocampal overexpression of Y2 receptors (Woldbye et al., 2010). When, in addition to Y5 receptors, NPY was overexpressed in the hippocampus, both seizure-naive and kainate-treated rats showed pronounced increases in basal functional binding as compared to both rAAV-Y5 and rAAV-Empty treated animals. Moreover, addition of exogenous NPY caused no further increase in functional Y5 receptor binding in the dentate gyrus and CA1 of rAAV-Y5/NPY treated rats as compared to basal functional binding (without NPY addition), and only resulted in a modest increase in the hippocampal CA3 region. This suggests that transgene Y5 receptors are activated under normal basal conditions, and indicates that transgene NPY is, at least partially, constitutively released from transduced neurons. In contrast, we previously observed that functional Y2 receptor binding was substantially increased above basal binding (100–170%) after addition of exogenous NPY (in the presence of Y1 and Y5 receptor antagonists) in rats treated with the rAAV-Y2/NPY vector combination in the hippocampus (data not shown). Likewise, rAAV-Y2/NPY vector treated rats showed
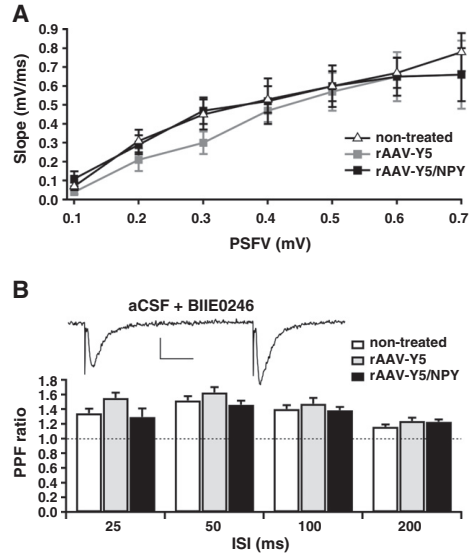
Fig. 2. Increased functional Y5 receptor [^{35}S]GTPγS binding at 3–4 weeks after bilateral intrahippocampal injection of rAAV-Y5 and rAAV-Y5/NPY (inverted colors). (A) Increased functional Y5 receptor binding (NPY + BIBP3226 + BIIE0246) in rAAV-Y5 injected seizure-naïve rat as compared to (D) non-treated rat. (B and E) Basal levels of functional binding (without adding NPY) in animals from A and D, respectively. (C and F) Blocking of functional Y5 binding with the Y5 antagonist L-152,804 in animals from A and D, respectively. Functional Y5 receptor binding displayed in inverted colors in kainate-treated animals after injection of (G) rAAV-Y5, (H) rAAV-Y5/NPY or (I) rAAV-Empty. (J–L) Corresponding basal levels of functional binding (without adding NPY) in rats from G–I. (M) Increased functional Y5 receptor binding and (N) corresponding basal binding measured in hippocampal DG, CA3 and CA1 of kainate-treated rats after rAAV-Y5, rAAV-NPY, or rAAV-Y5/NPY treatment as compared to rAAV-Empty (*P<0.05, **P<0.01, ***P<0.001 versus rAAV-Empty, Newman–Keuls post-hoc test following significant one-way-ANOVA). Data are mean ± SEM (n=9–10). rAAV, recombinant adeno-associated virus; NPY, neuropeptide Y; DG, dentate gyrus.

Fig. 3. Bilateral intrahippocampal injections of rAAV-Y5 potentiate the seizure-suppressant effect of rAAV-NPY but fail to affect seizures on its own. (A) Latencies to 1st MS and SE were significantly increased and (B) percent time in motor seizures was significantly decreased after rAAV-Y5/NPY and rAAV-NPY treatment. rAAV-Y5/NPY effects were significantly stronger as compared to rAAV-NPY. No significant effects were detected after rAAV-Y5 treatment compared to rAAV-Empty controls. (C) Only the rAAV-Y5/NPY combination decreased the number of motor seizures. (D) The seizure score was significantly decreased after treatment with both rAAV-Y5/NPY and rAAV-NPY. The rAAV-Y5/NPY combination had a significantly stronger effect as compared to rAAV-NPY. Data are mean ± SEM (n = 13–16). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. rAAV-Empty; #$P < 0.05$, ##$P < 0.01$ vs. rAAV-NPY and rAAV-Y5, Newman–Keuls post-hoc test following significant one-way-ANOVA. rAAV, recombinant adeno-associated virus; NPY, neuropeptide Y; MS, motor seizure; SE, status epilepticus.

Fig. 4. Basal excitatory synaptic transmission and short-term synaptic plasticity are unchanged 4–5 weeks after bilateral intrahippocampal injection of rAAV-Y5/NPY and rAAV-Y5. (A) The relationship between the PSFV and the slope of the corresponding fEPSP was similar in slices from rAAV-Y5/NPY, rAAV-Y5, and non-treated animals, suggesting unaltered basal excitatory synaptic transmission. (B) The PPF ratio was found to be similar between the three groups at different ISIs, after blocking a potential contribution from Y2 receptors with the Y2 antagonist BIIE0246. Insert: representative fEPSPs recorded in a slice obtained from a rAAV-Y5/NPY treated animal showing PPF at ISI of 100 ms. Scale bar: 0.4 mV and 20 ms, respectively. Data are mean ± SEM (n = 10–15). rAAV, recombinant adeno-associated virus; NPY, neuropeptide Y; PSFV, presynaptic fiber volley; fEPSP, field excitatory postsynaptic potential; PPF, paired-pulse facilitation; ISI, interstimulus interval.

more or less similar modest elevated hippocampal levels of basal binding as compared to rAAV-Y2 treated animals (data not shown), while basal binding of rAAV-Y5/NPY vector treated rats of the present study was clearly above that of rAAV-Y5 single vector-injected animals. At present, it is not clear why transgene Y5 receptors appear to be more activated by transgene NPY (without exogenous NPY addition), and thereby differ from transgene Y2 receptors. Nevertheless, our data suggest that Y5 transgene receptors are somewhat more prone to be activated under basal conditions after rAAV-Y5/NPY injection.

The present study showed that rAAV-induced hippocampal NPY overexpression caused substantial suppressant effect on kainate-induced seizures. This finding is consistent with previous work from our and other groups (Noe et al., 2008; Richichi et al., 2004; Sørensen et al., 2009). In contrast, overexpression of functional Y5 receptors *per se* caused no significant changes in any measured seizure parameter as compared to control animals. Nonetheless, the combined overexpression of functional Y5 receptors and NPY exerted seizure-suppressant effects superior to the effects seen after single NPY

transgene overexpression. It remains to be explored why rAAV-Y5 treatment alone, as opposed to the rAAV-Y5/NPY vector combination, did not lead to seizure-suppressant effects. One may speculate that a proportion of transgene Y5 receptors after rAAV-Y5 vector treatment is not accessible to released endogenous NPY, perhaps due to divergent cellular or regional spatial distribution of endogenous NPY release sites and transgene Y5 receptor expression sites. This scenario, however, seems less likely, since we recently demonstrated that overexpression of the Y2 receptor alone was sufficient to exert prominent seizure-suppressant effects on rapid kindling and kainate-induced seizures (Woldbye et al., 2010). One has to bear in mind though that the endogenous Y2 receptor expression pattern in the hippocampus (Gustafson et al., 1997) is fundamentally different from that of Y5 receptors (Durkin et al., 2000), which may also influence the transgene Y5 receptor expression pattern. The special distribution of transgene NPY, on the other hand, may match closer the transgene Y5 receptor expression pattern, and thereby more readily activate them, leading to seizure-suppressant effects. In favor of this interpretation, we observed high basal functional binding in rAAV-Y5/NPY-treated animals. This finding would also imply that transgene NPY is released at basal conditions even during normal, low frequency, synaptic activity, possibly resembling an adaptive mechanism of hippocampal circuits to counteract seizures. Our findings of unaltered basal excitatory synaptic transmission and PPF in the CA1 area, however, argue against such interpretation.

In the present study, viral vectors were injected prior to the induction of acute seizures. Therefore, seizure-suppressant effect of Y5 and

NPY overexpression in the hippocampus can be taken as an initial proof-of-principle. To mimic more closely the clinical situation, similar treatment should be applied to animals with already established spontaneous, recurring chronic seizures. Nonetheless, the results obtained with the present design of the study give a good indication that such approach may also work in chronic seizure models. In support, rAAV-NPY treatment has been shown to exert a seizure-suppressant effect both in an acute (present design) and in a chronic rat TLE model, in which vector treatment was applied after the occurrence of spontaneous, recurrent seizures (Noe et al., 2008). In line with this notion, NPY ligands effectively decrease excitatory synaptic transmission in the hippocampal neurons from surgically resected human TLE of pharmacoresistant epilepsy patients (Colmers et al., 1997; Patrylo et al., 1999).

The present finding that combined overexpression of NPY and its Y5 receptor exerted superior effects as compared to single vector treatment lends further support to the concept of combination gene therapy with overexpression of endogenous ligands and their respective receptors (Woldbye et al., 2010). This is in line with our previous findings using a combination of NPY and Y2 receptor overexpression (Woldbye et al., 2010). Moreover, this concept can be extended to overexpression of different combinations of endogenous ligands to achieve seizure-suppressant effects, as was shown with fibroblast growth factor-2 and brain-derived neurotrophic factor (Paradiso et al., 2009). Thus, our study underscores the importance of targeting various endogenous therapeutic molecules and their receptors for combined gene therapy approaches in developing more effective and tailored future strategies for epilepsy treatment.

From the clinical perspective, it will be important to carefully explore possible adverse effects associated with long-term upregulation of NPY and its receptors. One possible concern is the effect of vector treatment on basal excitatory synaptic transmission and normal information processing, since NPY is known to suppress glutamate release in hippocampal excitatory synapses (Colmers et al., 1985; Greber et al., 1994). We found no evidence of altered basal excitatory synaptic transmission in rAAV-Y5 or combined rAAV-Y5/NPY vector treated animals, which is in line with our previous observations in rAAV-NPY treated rats (Sørensen et al., 2008, 2009). Previously, pronounced increases in body weight were observed after hypothalamic rAAV-NPY injections (Tiesjema et al., 2007), and Y5 receptors have been implicated in mediating feeding stimulatory effects of NPY in the hypothalamus (Ishihara et al., 2006). None of the three vector treatments in the present study (rAAV-Y5, rAAV-NPY, and rAAV-Y5/NPY) caused significant changes in body weight. A study from another group also did not observe changes in body weight after intrahippocampal injection of rAAV-NPY (Richichi et al., 2004) and neither did we in our previous study using rAAV-NPY, rAAV-Y2, and rAAV-Y2/NPY (Woldbye et al., 2010, unpublished observations), indicating that hippocampal overexpression of NPY transgenes does not lead to changes in body weight.

In conclusion, our data suggest that combined gene therapy targeting the Y5 receptor and NPY together could be a more optimal alternative approach for suppression of hippocampal seizures as compared to NPY alone, while Y5 receptor overexpression by itself appears to have limited antiepileptic potential.

Conflict of interest

The authors have no conflict of interest to disclose.

Acknowledgments

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Paper IV

Neuropeptide-receptor combinatorial gene therapy for temporal lobe epilepsy in rats

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Abstract

We have previously discovered that simultaneous overexpression of NPY and its Y2 receptor exerts stronger seizure-suppressant effect as compared to single gene expression strategy in electrical stimulation or systemically administered kainate-induced acute seizures in rats. Although this study proved a principle that NPY and Y2 receptor combination-based gene expression is an effective anticonvulsant, the evidence that such approach could be a relevant clinical strategy was still lacking. In the present study, rats were injected with kainate unilaterally into the hippocampus, to induce status epilepticus (SE) followed by recurrent spontaneous seizures. This model closely resembles human chronic mesial temporal lobe epilepsy (mTLE). To assess hippocampal degeneration and correctly target viral vector treatment, brain magnetic resonance imaging (MRI) was performed in all animals 10 months after kainate injection. After MRI, recurrent seizure frequency and their duration were assessed continuously for 2 weeks by wireless video-EEG monitoring system with electrodes implanted contralateral to the kainate-injected hippocampus and the ipsilateral cortex. The kainate-injected hippocampus (seizure focus) was then treated unilaterally with combination of NPY and Y2 receptor AAV vector injection, followed by another video-EEG monitoring session. We demonstrate, for the first time, that such clinically relevant unilateral combinatorial gene therapy approach effectively decreased frequency of spontaneous seizures (in 4 out of 5 epileptic animals). In contrast, seizure frequency was increased in all control animals treated with empty viral vectors. In addition, overall seizure duration was shorter in AAV-NPY/Y2 treated animals compared to controls.
These data suggest that simultaneous overexpression of NPY and Y2 in the seizure focus area is a relevant approach and could be developed as an alternative treatment strategy for mTLE, which often is refractory to antiepileptic drugs (AEDs).

Introduction

Epilepsy is a severe neurological disease affecting 1 % of the population. Between 30-40 % of the epilepsy patients respond poorly to the anti-epileptic drugs (AEDs) currently available (Duncan et al., 2006). Therefore, the development of new treatment strategies is highly warranted. Gene therapy has recently emerged as such alternative treatment strategy for epilepsy. With recombinant adeno-associated viral (rAAV) vectors endogenous seizure-suppressant compounds can be overexpressed long-term in the epileptic focus (Richichi et al., 2004; McCown, 2006; Kanter-Schlifke et al., 2007; Noe et al., 2008). Such AAV-based gene therapy approach in CNS clinical trials has been demonstrated to be a safe procedure with positive therapeutic outcomes (Kaplitt et al., 2007; Marks et al., 2008).

Neuropeptide Y (NPY), a polypeptide widely expressed in the brain, has been shown to be involved in various processes in the brain controlling behavior, such as food intake (Dryden et al., 1995; Hanson & Dallman, 1995), stress (Zhou et al., 2008) and even seizure activity (Baraban et al., 1997; Woldbye et al., 1997). NPY overexpression by recombinant AAV (rAAV) vectors in the hippocampus exerts a strong suppressant effect on stimulation-induced seizures in animal models of epilepsy (Richichi et al., 2004; Sorensen et al., 2009). Such seizure-suppressant effect of NPY overexpression has been shown even for spontaneous recurrent seizures (Noe et al., 2008). NPY exerts its action mainly through three G-protein coupled receptors: Y1, Y2 and Y5 (Berglund et al., 2003). rAAV-based overexpression of Y2 but not Y5 receptors inhibited kindling and kainate-induced epileptic seizures (Woldbye et al., 2010; Gotzsche et al., 2012). Combination of the ligand (NPY) and the receptors (Y2 or Y5) showed a superior effect compared to the overexpression of the ligand or receptor alone in acute stimulation-induced seizure models (Woldbye et al., 2010; Gotzsche et al., 2012). In contrast, overexpression of Y1 receptor seems to be proconvulsive in kainate-induced seizures in mice (Olesen et al., 2012). In line with this observation, Y1 receptor activation increases extracellular glutamate levels in the hippocampus (Meurs et al., 2012).

Here, in a preclinical study, we investigate whether combined overexpression of NPY and Y2 receptors are effective in controlling spontaneous seizures in a chronic rat model of epilepsy (> 1 year) induced by

intrahippocampal kainate injection. This model is highly relevant to human mesial temporal lobe epilepsy (mTLE) with hippocampal sclerosis. Epileptic animals were treated with a rAAV vector for overexpression of NPY and Y2 unilaterally in the hippocampal epileptic focus, i.e. ipsilateral to the kainate injection. Such therapeutic scheme is considered as clinically plausible scenario for future gene therapy in epilepsy.

Experimental procedures

Ethical statement
All experimental procedures were approved by the local Malmö/Lund Ethical Committee for Experimental Animals (Ethical permit number M190-09), and were performed according to the guidelines of the Swedish Animal Welfare Agency and in agreement with international guidelines.

Animals
Male Wistar rats (Charles River, Germany) weighing 200-230 g at the initiation of the experiments were used. The animals were housed individually at a 12 hour light/dark cycle and were given daily forage (15-20 g/day).

Induction of epileptic seizures
Animals were anesthetized with isoflurane mixture and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA). Kainic acid (Ascent scientific, Cambridge, UK) was dissolved in sterile PBS (0.4 µg/0.4 µl) and the pH was adjusted to 7.4. Kainic acid was injected through a glass capillary into the medial part of the right hippocampus with the following coordinates (reference points from bregma, midline, dura): AP -5.3 mm, ML -4.5 mm, V -3.2 mm. The glass capillary was left for additional 5 min after injection. All animals were weighed before surgery and once a week until sacrificed.

Magnetic resonance imaging
To assess the extent of the damage caused by the intrahippocampal kainic acid injection magnetic resonance imaging (MRI) was performed on all animals about 10 months after induction of epileptic activity.
For the MRI investigation all animals were anesthetized with an isoflurane mixture (4 % initially and 2 % for further maintenance) together with nitrous oxide and oxygen. Animals were placed in a special positioning system and the head was stabilized in the head holder. Throughout the experiment the breathing rate and body temperature was controlled.
MRI was performed in a 9.4 T 400 MHz Agilent Technologies (Stockholm, Sweden) with the 205/120 HD gradient coil. T2-weighted images were obtained with the following parameters: Echo time 39.39 ms; Repetition

time 4000 ms; five averages per cycle and flip angle of 180°. Totally 45 contiguous slices were scanned for each animal, with the thickness of 400 µm. The field of view was 40.4 x 42 mm^2 on a 256 x 256 matrix. With the mentioned parameters each scan took approximately 11 min.
Analysis of data obtained from the MRI was performed with the OsiriX software (Geneva, Switzerland). The region of interest (ROI), in our case the lesion area, was outlined in each image. In T2-weighted images the damaged area was distinguished by being lighter than the rest of the brain tissue. The software could then based on the acquisition settings calculate the volume of the lesion. Based on the remaining hippocampus, coordinates were created to target the dorsal and ventral part of the hippocampus for gene therapy treatment.

Electrode and transmitter implantation
About one year after induction of epileptic seizures a F40-EET transmitter (Data Sciences International, St. Paul, Minnesota) was implanted for EEG monitoring to record spontaneous epileptic activity. Animals were anesthetized as described previously and the skull was fixed into a stereotaxic frame (David Kopf Instruments, Tujunga, CA). One depth stainless steel electrode (Plastics One, Roanoke, VA), connected to one of the electrodes of the transmitter, was implanted in the contralateral hippocampus (left hemisphere) with the following coordinates (reference points from bregma, midline, dura): AP -4.8 mm, ML +5.2 mm, V -6.3 mm. Another electrode was placed on dura mater rostral to the coronal suture for cortical EEG recordings in the cortex ipsilateral to the kainic acid injection. For each of the electrodes a reference electrode was placed on the dura mater caudal to the lambdoid suture. The transmitter was subsequently placed in a subcutaneous pocket created from the incision by the skull.

Monitoring of spontaneous seizures
One week after implantation, the transmitter was activated by a magnet and each cage was placed on top of a receiver. Hardware and software for monitoring spontaneous epileptic activity were produced by Data Sciences International (St. Paul, Minnesota, USA). Synchronous EEG and video data were collected with the ART software. The animals were monitored continuously for 2 consecutive weeks. Another monitoring session was conducted 4 weeks after virus injections. All the EEG and video analysis was conducted using the NeuroScore software (Data Sciences International, St. Paul, Minnesota, USA) by experimenter blind to the treatment of the particular animals.

Viral vector injection
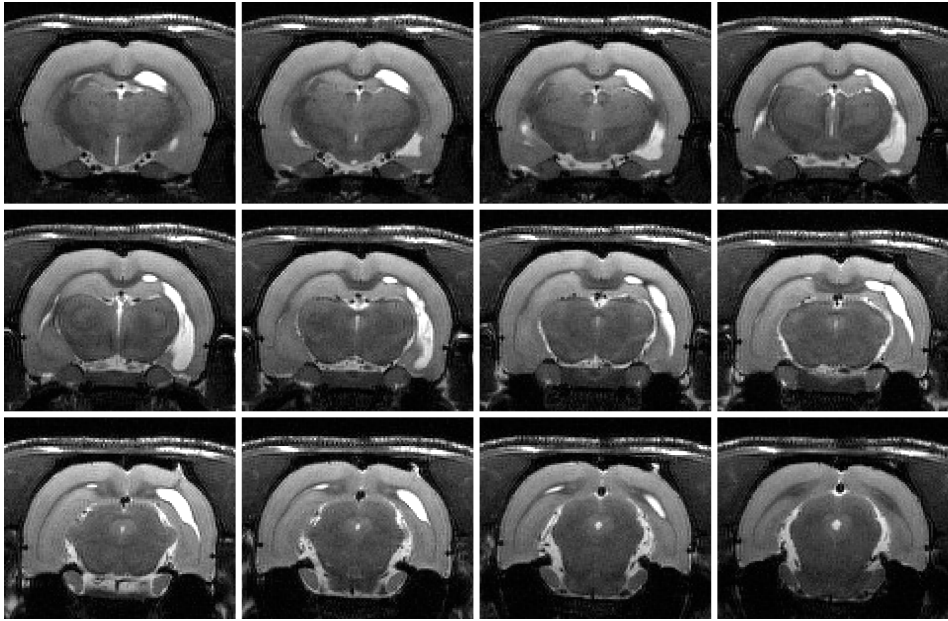Animals were anesthetized and fixed in the stereotaxic

Figure 1. Tissue damage caused by intrahippocampal kainic acid. A sequence of T2-weighted MR images showing the lesion in the right hippocampus.

frame as previously described. Adeno-associated viral vectors (GeneDetect, Auckland, New Zealand) with serotype 1/2 and expressed under the neuron specific enolase (NSE) promoter were used and had the following titers; rAAV-Empty 1.0×10^{12} genomic particles/ml, rAAV-NPY 1.0×10^{12} genomic particles/ml, rAAV-Y2 1.0×10^{12} genomic particles/ml. The rAAV-Empty vector was carrying the same expression cassette, but without the transgene and was used as a control. Viral vector injections were performed in the right hippocampus, which had previously been injected with kainic acid. Injections were performed using a glass capillary and the viral vector was injected in the dorsal and the ventral hippocampus with the following coordinates (reference points from bregma, midline, dura): Dorsal hippocampus: AP -4.0 mm, ML -1.5 mm, V -3.6 mm; Ventral hippocampus: AP -7.3 mm, ML -4.8 mm, V -5.8 mm. Animals were either injected with 2.5 µl Empty vector per site or a combination of NPY and Y2 (1 µl NPY and 1.5 µl Y2). The experimental groups were randomized by the GraphPad QuickCalcs software (La Jolla, CA). After injection the glass capillary was left in place for additional 10 min, before it was retracted.
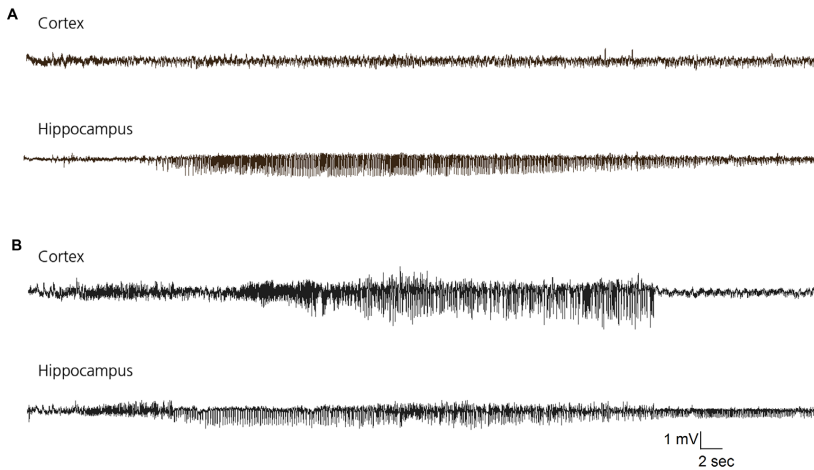
Perfusions and immunohistochemistry
At the end of the experiment all animals were deeply anesthetized with pentobarbital before the transmitter was removed and the animals were perfused transcardally with 0.9 % NaCl followed by 4 % paraformaldehyde. The brain was removed and post-fixed in 4 % paraformaldehyde for 24 hours and then overnight in 20 % sucrose in 0.1 M sodium phosphate buffered saline. The following day the brains were cut on a microtome in 30 µm thick sections and NPY immunohistochemistry was initiated. First, the slices were rinsed in 0.02 M KPBS, quenched with 3 % H_2O_2 and 10 % MeOH in 0.02 M KPBS for 10 min and then blocked in 5 % normal goat serum (NGS) in 0.25 % Triton X-100-KPBS for 1 h. The slices were then incubated with rabbit anti-NPY antibody (1:10 000; Sigma-Aldrich) in 5 % NGS overnight in room temperature and further on washed in 0.02 M KPBS before the incubation with goat anti-rabbit antibody (1:200, Vector Laboratories, Burlingame, CA) in 5 % NGS and 0.25 Triton X-100-KPBS for 2 h. The immunoreaction was amplified with an avidin-biotin complex kit (Vectastain ABC kit, Vector Laboratories) and finally visualized by application of 0.5 mg/ml 3,3´-diaminobenzidine (DAB) and 3 % H_2O_2. Bright-field images were obtained on an Olympus BX61 microscope.

Functional NPY receptor binding assay
Six additional animals were injected with intrahippocampal kainic acid (as above) and were used for functional NPY receptor binding. Three animals were

Figure 2. EEG patterns of spontaneous epileptic seizures. An example of a spontaneous focal seizure (A) and a spontaneous generalized seizure (B) recorded in the contralateral hippocampus and in the ipsilateral cortex, from the same animal.

injected with rAAV-NPY/Y2, while the rest were not viral vector treated. These animals were decapitated 4 weeks after viral vector injection and the brains were frozen in dry ice when removed. The brains were mounted on a cryostat (Shandon Inc., Pittsburgh, PA) using Cryo-Embed (Ax-Lab A/S, Vedbæk, Denmark) and were cut in 15 µm thick sections, thaw-mounted onto Superfrost Plus slides (VWR International, Herlev, Denmark) and were gently dried on a hotplate.

The sections were defrosted for 30 min in room temperature before being rehydrated in assay buffer (50 mM Tris-HCl, 3 mM MgCl, 0.2 mM ethylene glycol tetraacetic acid, 100 mM NaCl; pH 7.4) for 10 min at room temperature. Moreover, the sections were preincubated in buffer B (assay buffer A, 0.2 mM dithiothreitol, 1 µM 1,3-dipropyl-8-cyclopentylxanthine (#C-101, PerkinElmer, Skovlunde, Denmark), 0.5% w/v BSA, and 2 mM guanosine-5´-diphosphate (#G7127, Sigma-Aldrich, Brøndby, Denmark)) and 1 µM NPY (Schafer-N, Copenhagen, Denmark) for 20 min in room temperature. The basal binding was determined by incubation in buffer B with 40 pM [35S]-GTPγS (1250 Ci/mmol) but without NPY. Non-specific binding was determined by incubation in buffer B (without NPY) with 40 pM [35S]-GTPγS and 10 µM non-labeled GTPγS (#89378; Sigma-Aldrich, Brøndby, Denmark). Finally, the incubation was terminated by washing twice in ice-cold 50 mM Tris-HCl buffer (pH 7.4) for 5 min. All sections were rinsed in ice-cold distilled H_2O, dried under a stream of c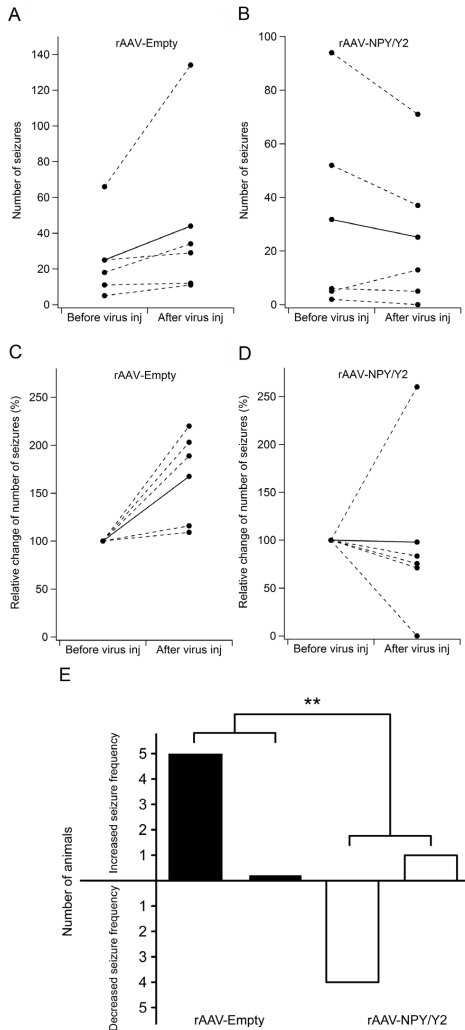old air, before being exposed to [35S]-sensitive Kodak BioMax MR films together with [14C]-microscales for 5 days and then being developed in Kodak Processing Chemicals for Autoradiography Films (Sigma-Aldrich, Brøndby, Denmark).

Statistical analysis

Statistical analysis of data was performed using Student's t-test. Differences between groups (unpaired) and before and after treatment (paired) were considered significant at $p<0.05$. All data are presented as mean ±SEM. The investigator conducting the behavioral grading of the epileptic seizures in the animals, EEG and histological analysis was unaware of the group identity of individual animals.

Results

After the injection of intrahippocampal kainate, all animals developed status epilepticus (SE). About 10 months after induction of SE, MRI was performed to determine the extent of the tissue damage created by the kainic acid and subsequent spontaneous seizures (Fig. 1). The MRI was necessary to determine the exact coordinates for AAV injections, as well as to correlate extent of damage to severity of epilepsy in these animals. The damage to the kainate injected hippocampus was observed in all animals, and the volume of the damage was estimated to be 32.7±4.7 mm^3 on average. Most of the damage was localized between the dorsal and ventro-caudal hippocampus, while dorsal and ventral hippocampi were spared. This allowed for targeting unilateral rAAV vector

4

Figure 3. Four out of five animals decrease in seizure frequency with rAAV-NPY/Y2 treatment. The number of seizures before and after viral vector injections of rAAV-Empty (A) and rAAV-NPY/Y2 (B) and the relative change of the number of seizures (C and D, respectively). The number of animals in each group that increased respectively decreased in number of seizures after treatment (E). ** p<0.01
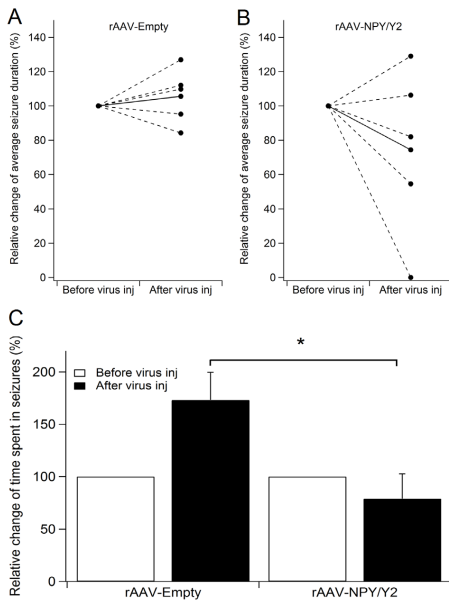
injections to the spared hippocampal areas ipsilateral to the kainate injection. Contralateral to the kainate injection hippocampi were spared in most of the cases without any significant damage observed on the MRI.

After MRI and hippocampal damage analysis in individual

animals, EEG recording electrodes were implanted in the hippocampus contralateral to the kainate injection and in the cortex ipsilateral to the kainate-injected hippocampus. After recovery from surgery, continuous video-EEG recordings were performed for 2 weeks on all animals. During the monitoring period, spontaneous seizures were detected in 13 out of 24 animals (54 %). Focal and generalized seizures were distinguished by the EEG and the video recording analysis. During focal seizures, epileptic EEG activity was detected in the hippocampus but not in the cortex (Fig. 2A), while during generalized seizures epileptic EEG activity was present both in hippocampal and cortical recordings (Fig 2B). Moreover, only generalized seizures were accompanied by clear whole-body convulsions observed on video recordings. The number of spontaneous seizures was highly variable between the animals during the 2 weeks of initial recording period, ranging between 2 and 94 of total number of detected seizures (Fig 3A and B). Average number of seizures was 28.4±10.0. Number of focal seizures on average was 5.2±4.6, while average number of generalized seizures was 23.2±9.2. No correlation was detected between the number of seizures (either total, generalized or focal) and the volume of the hippocampal damage (for total number of seizures $R2=0.00008$, focal seizures $R2=0.05230$ and generalized seizures $R2=0.00840$).

Animals that exhibited spontaneous seizures were randomized in two groups: rAAV-Empty and a treatment group, which received rAAV vector carrying NPY and Y2 receptor transgenes. Only 5 animals from each group with recurrent spontaneous seizures survived to the end of the experiments and were therefore included in the final analysis.

Four weeks after rAAV vector injection, animals were monitored with the video-EEG system continuously for 2 weeks. All animals in the rAAV-Empty group exhibited increased number of seizures (Fig 3A, C and E), while four out of five animals in rAAV-NPY/Y2 group showed decreased number of seizures after the treatment (Fig 3B, D and E). One animal in rAAV-NPY/Y2 group became seizure free. The average number of all seizures (both generalized and focal) in rAAV-Empty group increased by 167.4±23.0 %, while it was largely unaltered in the rAAV-NPY/Y2 injected group (98.0±43.2 %, Fig 3C and D; p>0.05).

The average duration of individual seizures was not significantly changed after the treatment as compared to that of pre-treatment in both groups (rAAV-Empty 105.6±7.3 % of pre-treatment, rAAV-NPY/Y2 74.4±22.3 % of pre-treatment; p>0.05) (Fig 4A and B). However, in the rAAV-Empty group the overall time spent in seizures compared to pre-treatment was significantly longer compared to that in the rAAV-NPY/Y2 group

Figure 4. Duration of seizures before and after rAAV-Empty and rAAV-NPY/Y2 vector injection. The relative change of the average seizure duration in rAAV-Empty (A) and rAAV-NPY/Y2 (B) injected animals. The relative change in total time spent in seizures before and after treatment (D). Values are presented as mean ±SEM, * p<0.05.

(rAAV-Empty 173.2±26.4 %, rAAV-NPY/Y2 79.0±23.6 %; p<0.05) (Fig. 4C). Taken together, these data suggest that NPY/Y2 receptor rAAV treatment exerted inhibitory effect on spontaneous seizures.

NPY and Y2 receptor overexpression in rAAV vector injected animals

To confirm the overexpression of the NPY/Y2 after viral vector injections, NPY functional binding and NPY immunohistochemistry was performed. NPY functional binding was increased in the dorsal and ventral parts of the hippocampus (Fig. 5 A and C) as compared to basal functional binding (Fig. 5 B and D). NPY receptor functional binding was also detected in the contralateral hippocampus, mostly in the CA1 and CA3 areas (Fig. 5A). This suggests that rAAV-NPY/Y2 injection resulted in increased expression of Y2 receptor, which was also functionally active. Strong NPY immunoreactivity was detected in all parts of the hippocampus injected with rAAV-NPY/Y2 (Fig. 5E) as compared to the rAAV-Empty treated animals (Fig. 5F). NPY was mostly expressed in fibers rather than in the cell bodies of the hippocampal neurons (Fig. 5G and H). These data suggest that rAAV-NPY/Y2 viral vector injection resulted in increased expression of NPY in the viral vector treated hippocampus.
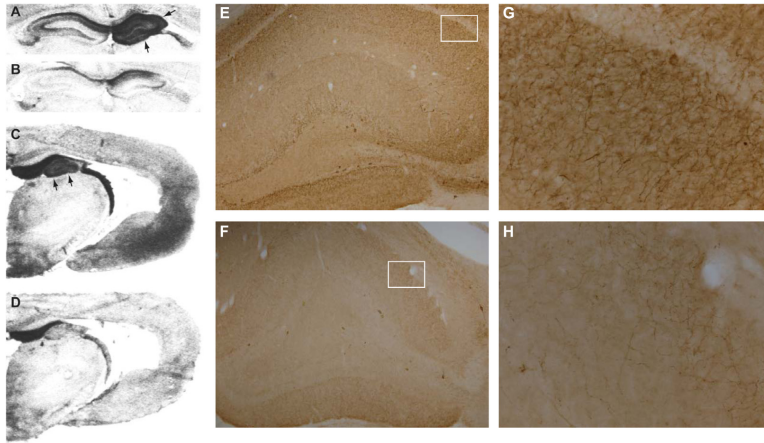
Discussion

The present study demonstrates for the first time that NPY/Y2 overexpression using viral vectors exerts seizure-inhibiting therapeutic effect on chronic spontaneous seizures occurring post-status epilepticus, in an animal model closely resembling human mTLE. This finding suggests that combinatorial NPY/Y2 gene therapy approach could be considered as a relevant approach and a possible alternative treatment strategy for clinical application, particularly for patients with mTLE, which often are pharmacoresistant and require surgical resection of the temporal lobe including hippocampus.

In our hands, spontaneous epileptic activity was detected in 54 % of kainate-injected animals, which is in line with previous observations (Bragin et al., 1999; Raedt et al., 2009). However, these data should be interpreted with certain caution, since continuous monitoring of 2 weeks may not be sufficient to detect seizures that could occur outside the recording period. Longer EEG monitoring periods are necessary to determine whether indeed some animals do not develop seizures at all. Also the number of seizures detected in different animals was variable in this model. Nevertheless, 2 weeks recording period and relatively low number of animals included in the analysis was sufficient to allow for assessment of the positive therapeutic outcome of the combinatorial NPY/Y2 gene therapy approach in these chronically epileptic animals.

In the rAAV-Empty group, animals exhibited increased number of seizures after viral vector injections. This may indicate that even one year after the initial insult (kainate-induced status epilepticus) there is a progressive increase in seizure frequency. Another explanation could be that the rAAV-Empty viral vector injection into the hippocampus may have exacerbated excitability of the seizure focus, and therefore may have led to increased seizure frequency in these animals. In any case, rAAV-NPY/Y2 vector treatment not only prevented the progressive increase in seizure frequency post-treatment, but also reversed it, as reflected by decreased seizure frequency and overall time spent in seizures.

In the present study, we deliberately chose to unilaterally deliver rAAV-NPY/Y2 treatment ipsilateral to the kainate-injected hippocampus, where most likely the seizure focus was induced and localized. To some extent, inhibitory effect of such approach on spontaneous seizures may have been unexpected in rats, since extensive inter-hemispheric connections between rat hippocampi allows for rapid spread of seizure activity from one hippocampus, where seizures arise, to the other (contralateral side) (Fernandes de Lima et al., 1990). Thus, the inhibition of

Figure 5. Functional NPY binding and NPY immunoreactivity after unilateral intrahippocampal injection of rAAV-NPY/Y2. Treatment with rAAV-NPY/Y2 vector causes prominent upregulation of functional Y2 receptors as revealed by an increase in NPY-stimulated [^{35}S]GTPγS functional binding. (A) and (C) show pronounced NPY-stimulated [^{35}S]GTPγS binding (arrows) 4 weeks after unilateral intrahippocampal rAAV-NPY/Y2 vector injection as compared to basal binding (B and D, respectively). NPY immunoreactivity in the hippocampus 6 weeks after rAAV-NPY/Y2 (E) and rAAV-Empty (F) injection. Boxed areas in (E) and (F) are shown magnified in (G) and (H).

spontaneous seizures by unilateral rAAV treatment could be taken to suggest that the majority of the seizures were indeed initiated in the kainate-injected hippocampus and that these seizures and their spread were inhibited by viral vector treatment. A contributing factor to limited spread of the seizures to the contralateral hemisphere could be a damage of interhemispheric connections caused by kainate. Regardless, the present results would imply that selective targeting the seizure focus by combinatorial NPY/Y2 rAAV gene therapy might be sufficient to exert therapeutic effects even on generalized chronic epileptic seizures. However, this statement needs to be taken with certain caution, since some spread of the transgenes to the contralateral hippocampus has been observed and may have contributed to the results.

We were not able to detect any positive correlation between the damage of the hippocampus and the frequency of the seizures, which could be counterintuitive. One would have expected higher seizure frequency in animals with more extensive hippocampal damage, since seizures are thought to cause hippocampal sclerosis (Kalviainen & Salmenpera, 2002). One explanation could be that spontaneous seizures occurring after kainate injection may exacerbate hippocampal sclerosis over time, causing degeneration of the seizure-generating hippocampal network and thereby stop the seizures. This could be one of the reasons as to why we were not able to detect seizures in some animals during the 2 week initial monitoring period. Speaking against this interpretation, the inverse correlation between the

hippocampal damage and seizure frequency was not observed either. Higher number of animals to increase the power of analysis may be needed to address this question in more detail.

Increased functional NPY receptor binding was found in animals from rAAV-NPY/Y2 group compared to basal functional NPY receptor binding. This supports the interpretation that the decreased seizure frequency in these animals was most likely due to transgene overexpression. The individual variations of the therapeutic outcomes may depend on the individual levels of NPY/Y2 overexpression, but could also be due to uneven regional distribution of the overexpression within the hippocampus in different animals.

In conclusion, this study suggests that combinatorial gene therapy is a valid alternative to single gene therapy approach, and even unilateral combinatorial treatment results in a positive therapeutic outcome. In this respect, clinical testing of this approach in pharmacoresistant mTLE patients assigned to surgical removal of the temporal lobe seems to be a viable concept, provided that the viral vector-transduced tissue can still be removed in case adverse effects of the gene therapy arise, serving as a safety aspect of the procedure. In general, combinatorial gene therapy overexpressing both the ligand and the receptor seems to offer better tuning of the desired therapy by strengthening the effect of the ligands via specific receptors that have been shown to mediate the therapeutic effect (Woldbye et al., 2010; Gotzsche et al., 2012).

7

Acknowledgements

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