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Gene therapy for epilepsy: resculpturing synaptic transmission with neuropeptides

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Gene Therapy for Epilepsy:

Resculpturing Synaptic Transmission with Neuropeptides

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Gene Therapy for Epilepsy: Resculpturing Synaptic Transmission with Neuropeptides

Abstract   Intractable seizures and lack of effective antiepileptic drugs (AED) are severe and common conditions affecting many patients with epilepsy. Thus, there is an urgent need to develop new therapies in epilepsy. The search for novel treatments has identified several neuropeptide systems as potential targets for future therapeutic interventions. The neuropeptide Y (NPY) may represent one such target, as it plays a key role in controlling excitability in the hippocampus by suppressing glutamatergic transmission. If manipulated, the NPY system may be capable of restoring the imbalance between excitation and inhibition occurring in the epileptic brain. Indeed, emerging evidence has established a proof-of-principle for viral vector-mediated transfer of gene and expression of NPY in epileptogenic regions of the brain, providing effective suppression of both acute and chronic seizures in animal models of epilepsy. Therefore, NPY gene therapy strategies are currently under intense investigation and clinical trials are forthcoming. To implement an effective NPY gene therapy in patients, as well as to extend our general knowledge of how transgene NPY may act in the brain, the receptor subtypes mediating the antiepileptic action of NPY needs to be determined. Moreover, the mechanisms underlying the seizure-suppressant effects of transgene NPY are not well understood. Particularly, we need to know under which circumstances transgene NPY is released, and whether and how it acts on synaptic transmission within the area of viral vector transduction.

    In this thesis, evidences are provided showing that NPY is mediating its antiepileptic effect through activation of both Y2 and Y5 receptors in the hippocampus, and predominately via Y5 receptors in extra-hippocampal areas. Moreover, in rats, hippocampal NPY gene therapy generates long-lasting and neuronal-specific overexpression of transgene NPY. This is not associated with alterations in basal synaptic transmission, probably due to minor constitutive release of transgene NPY. However, as transgene NPY is preferentially released during high frequency neuronal activity, acting as a volume transmitter, it interferes with neuronal activity-dependent processes, reflected by suppressed long-lasting synaptic plasticity in hippocampal synapses and delayed, but not impaired, hippocampal-dependent learning capacity in naïve animals. This could be an unwanted adverse effect of transgene NPY, but since already existing impairment of long-term synaptic plasticity is not further exacerbated after electrical kindling-induced seizures, NPY gene therapy still remains a promising and novel antiepileptic treatment strategy.

Key words: epilepsy, gene therapy, hippocampus, neuropeptide Y (NPY), Y2 receptor, Y5 receptor, electrophysiology, synaptic transmission, synaptic plasticity, LTP, volume transmission, activity-dependent release, memory

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Gene Therapy for Epilepsy:

Resculpturing Synaptic Transmission with Neuropeptides

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Cover picture
A biocytin-labeled pyramidal neuron in the proximal subiculum.

Cover artwork by Bengt Mattsson and Andreas Toft Sørensen

To Clara and Anders

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SUMMARY

Epilepsy is a multifactoral and chronic neurological disorder affecting around 1% of the general population. It is characterized by an enduring predisposition to generate epileptic seizures, which are transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain. Current antiepileptic drug (AED) treatment is symptomatic and suppresses seizures without influencing the underlying disease to generate seizures. Despite continued progress in drug development, intractable seizures still affect around 30-40% of all patients with epilepsy, and particularly temporal lope epilepsy (TLE) remains amongst the most common and difficult types to treat. Thus, there is an urgent need to develop new therapies in epilepsy.

The search for novel treatments in epilepsy has identified several neuropeptide systems as potential targets for future therapeutic interventions. The neuropeptide Y (NPY) may represent one such target, as it plays a key role controlling excitability in the hippocampal formation. NPY suppresses glutamatergic transmission via various NPY receptors, which reduces Ca^{2+}-influx into presynaptic terminals of projection neurons. Therefore, if manipulated, the NPY system may be capable of restoring the imbalance between excitation and inhibition occurring in the epileptic brain. Indeed, emerging evidence has established a proof-of-principle for viral vector-mediated transfer of gene and expression of NPY in the hippocampal formation of the brain, providing effective suppression of both acute and chronic seizures in animal models of epilepsy. Therefore, NPY gene therapy strategies are currently under intense investigation and clinical trials are forthcoming. To implement an effective NPY gene therapy in patients, as well as to extend our general knowledge of how transgene NPY may act in the brain, the receptor subtypes mediating the antiepileptic action of NPY needs to be determined. Moreover, the mechanisms underlying the seizure-suppressant effects of transgene NPY are not well understood. Particularly, we need to know under which circumstances transgene NPY is released, and whether and how it acts on synaptic transmission within the area of viral vector transduction.

Using a receptor gene knockout (KO) strategy in mice, this thesis provides evidences that NPY is mediating its antiepileptic effect through activation of both Y2 and Y5 receptors in the hippocampal formation, and predominately via Y5 receptors in extra-hippocampal areas. In rats, viral vector-mediated NPY gene therapy in the hippocampus generates long-lasting and neuronal-specific overexpression of transgene NPY. In Schaffer collateral-CA1 synapses, such expression is not associated with alterations in basal synaptic transmission probably due to minor constitutive release of transgene NPY. However, as transgene NPY is preferentially released during high frequency neuronal activity, acting as a volume transmitter, it interferes with neuronal activity-dependent processes, reflected

by suppressed long-lasting activity-dependent synaptic plasticity in both Schaffer collateral-CA1 and CA1-subicular synapses, and delayed, but not impaired, hippocampal-dependent learning capacity in naïve animals. This could be an unwanted adverse effect of transgene NPY, but since already existing impairment of long-term synaptic plasticity is not further exacerbated after electrical kindling-induced seizures, NPY gene therapy still remains a promising and novel antiepileptic treatment strategy.

DANSK POPULÆRVIDENSKABLIG SAMMENFATNING

Epilepsi rammer ca. 1% af befolkningen og er en kronisk neurologisk lidelse, der viser sig ved pludselige, gentagne krampeanfald, som udløses af unormale elektriske impulser i større eller mindre dele af hjernen. Forstyrrelserne skyldes oftest, at balancen mellem de fremmende (eksitatorisk) og hæmmende (inhibitorisk) nerveimpulser forskydes, så nervetransmissionen bliver hyper-eksitatorisk. På trods af oftest god medicinsk behandling, oplever 30-40% af alle epilepsi patienter forsat tilbagevendende krampeanfald, og kun et fåtal af disse kan afhjælpes ved f.eks. et kirurgisk indgreb. Derfor er nye tiltag rettet mod at øge livskvaliteten samt erstatte en ofte mangeårig medicinsk behandling yderst ønskværdige. Dertil kræves dog, at vi fremskynder udviklingen af nye og mere effektive terapiformer, samt at vi bedre forstår de mekanismer som forårsager epilepsi.

Neuropeptide Y (NPY) er et naturlig forekommende peptid i hjernen, som har en kraftig hæmmende effekt på både udviklingen af epileptiske kramper og på selve krampeanfaldene. NPY virker fortrinsvis ved at dæmpe den eksitatoriske glutamaterge nervetransmission. Forskningsresultater på dyr har for nyligt vist, at øges NPY indholdet i hjernen vha. genterapi (en behandlingsform, hvor gener fungerer som lægemiddel), så kan både akutte og spontane kramper hæmmes. Disse positive resultater har bl.a. medført, at man nu igangsætter et klinisk NPY-baseret genterapi-forsøg på patienter med behandlingsresistent mesial temporallaps epilepsi (epilepsi associeret til tindingelappen).

I denne afhandling identificerer vi, fortrinsvis vha. elektrofysiologiske målinger, hvilke NPY receptorer i hjernen som er ansvarlige for den anti-epileptiske effekt. Til dette formål benytter vi mus, hvor det kodende gen for enten NPY Y2 receptoren, Y5 receptoren eller begge receptorer er inaktiveret (knockout). Vi undersøger også om den normale nervetransmission og dens plasticitet ændres når NPY mængden øges i hjernen på rotter vha. genterapi, og hvorvidt dette har indflydelse på hukommelsen. I sammenhæng med disse forsøg, har vi målt under hvilke omstændigheder NPY frigøres fra nerveenderne, samt undersøgt i hvilken udstrækning dette har indflydelse på den øvrige glutamaterge nervetransmission. Ønsket med disse forsøg er at belyse, hvordan NPY-baseret genterapi påvirker den generelle nervetransmission, samt at identificere nogle af de bivirkninger genterapi med NPY eventuelt måtte have.

ORIGINAL PAPERS AND MANUSCRIPTS

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

I.   Woldbye DPD, Nanobashvili A, Sørensen AT, Husum H, Bolwig TG, Sørensen G, Ernfors P, Kokaia M (2005) Differential suppression of seizures via Y2 and Y5 neuropeptide Y receptors.
Neurobiology of Disease 20:760-772.

II.   Sørensen AT, Kanter-Schlifke I, Carli M, Balducci C, Noé F, During MJ, Vezzani A, Kokaia M (2008) NPY gene transfer in the hippocampus attenuates synaptic plasticity and learning.
Hippocampus 18:564-574.

III.   Sørensen AT, Kanter-Schlifke I, Lin ED, During MJ, Kokaia M (2008) Activity-dependent volume-transmission by transgene NPY attenuates glutamate release and LTP in the subiculum.
Molecular and Cellular Neuroscience, in press

IV.   Sørensen AT, Nikitidou L, Ledri M, Lin ED, During MJ, Kanter-Schlifke I, Kokaia M (2008) Hippocampal NPY gene transfer attenuates seizures without affecting epilepsy- induced impairment of LTP.
Submitted manuscript

LIST OF ABBREVIATIONS

aCSF	artificial cerebrospinal fluid
AD	afterdischarge
AED	antiepileptic drug
BIIE0246	a selective NPY Y2 receptor antagonist
CA	cornu ammonis
CMV	cytomegalovirus
CNS	central nervous system
EEG	electroencephalogram
EPSC	excitatory postsynaptic current
fEPSP	field excitatory postsynaptic potential
GFP	green fluorescent protein
HFS	high frequency stimulation
IEI	inter-event interval
IR	immunoreactivity
ISI	inter-stimulus interval
KA	kainic acid
KO	knockout
LDCV	large dense core vesicle
LTP	long-term potentiation
mEPSC	miniature excitatory postsynaptic current
NPY	neuropeptide Y
NSE	neuron-specific enolase
PPF	paired-pulse facilitation
PSFV	presynaptic fibervolley
PTX	picrotoxin
rAAV	recombinant adeno-associated virus
TLE	temporal lobe epilepsy
TTX	tetrodotoxin
WT	wild type

INTRODUCTION

HIPPOCAMPAL INTERACTION WITH MEMORY FUNCTION

The hippocampal formation and its organization

The hippocampal formation is a group of structures located within the (medial) temporal lobe and comprises the dentate gyrus, the hippocampus (or hippocampus proper), which is subdivided into four fields (CA4, CA3, CA2 and CA1), and the subiculum. The hippocampus proper is also known as Cornu Ammonis (CA), and the principal cells in CA regions and subiculum are so-called pyramidal neurons (Amaral and Witter, 1989; Witter and Amaral, 2004). The hippocampal formation receives input from cortical association regions including visual, somotosensory, olfactory and auditory cortices, implying that the hippocampal formation is a central part of the sensory integration system (Lavenex and Amaral, 2000).

Sensory information from these various regions is conveyed to the entorhinal cortex, which then provides the granule cells of the dentate gyrus with its major input via the perforant pathway. Within the hippocampal formation the connectivity is largely unidirectional and dominated by excitatory pathways connecting each field. Dentate granule cells project via mossy fibers to CA3 pyramidal neurons, which then project to CA1 pyramidal neurons via Schaffer collateral fibers. CA1 pyramidal neurons connect with pyramidal neurons in the subiculum via the alveus, before subicular projections leave the hippocampal formation and reach back to the entorhinal cortex, completing the circuit. This particular arrangement was recognized by Andersen and colleagues in 1969 and referred to as a "feed-forward trisynaptic circuit" (Andersen et al., 1969).

Besides receiving input from the entorhinal cortex, the hippocampal formation also receives a wide variety of other extrinsic inputs from various nuclei consisting of cholinergic, noradrenergic, serotonergic and dopaminergic projections (Witter and Amaral, 2004). Intrinsic input to the hippocampal formation is mediated by GABAergic interneurons and various neuromodulating peptides, including neurotrophic factors and neuropeptides (Carnahan and Nawa, 1995). Both extrinsic and intrinsic inputs are thought to ensure proper function of the hippocampal formation, in which cognitive processing of time, space and relationship of environmental stimuli, and long-term memory appear to be the most important (Sweatt, 2004).

Memory and long-term synaptic plasticity

A variety of evidences have established the importance of the hippocampal formation and related temporal lobe areas to memory function, and damage to these areas in both humans and animals results in severe memory impairments (Rempel-Clower et al., 1996; Kaut and Bunsey, 2001; Bird et al., 2007). The under-

lying cellular mechanism for long-term memory in the hippocampal formation is uncertain, but repeatedly it has been suggested that synaptic modification must have a role in learning and memory processing (Malenka, 2003; Cooke and Bliss, 2006). The most attractive cellular candidate for memory is long-term potentiation (LTP), which is the persistent increase in synaptic strength (communication) between neurons following high frequency stimulation of their synapses (for comprehensive review see Lynch, 2004). It is important though to bear in mind that LTP is considered one of many forms of synaptic plasticity mechanisms that may contribute to learning and memory (Zucker and Regehr, 2002; Malenka and Bear, 2004).

NEUROPEPTIDE Y

NPY and its receptors

Neuropeptide Y (NPY) has been shown to be one of several neuromodulating peptides in the hippocampal formation involved in memory processing (Morley and Flood, 1990; McDonald et al., 1998; Tallent and Helen, 2007). NPY is a 36-amino acid peptide and one of the most abundant and widely distributed neuropeptides in the central nervous system (CNS) of mammals (de Quidt and Emson, 1986; Gray and Morley, 1986). It is also involved in a variety of other functions such as angiogenesis, neurogenesis, feeding behavior, blood pressure, circadian rhythms, anxiety and excitability (Wahlestedt and Reis, 1993; Balasubramaniam, 1997; Hökfelt et al., 1998; Ekstrand et al., 2003; Lin et al., 2004; Howell et al., 2005; Agasse et al., 2008; Stanic et al., 2008). In the CNS, NPY is contained within populations of GABAergic interneurons (Hendry et al., 1984), where it is expressed as an inactive precursor protein (prepro-NPY) in the cell soma. It is transported into axons and dendrites via large dense core vesicles (LDCV) (Thureson-Klein et al., 1986; Zhu et al., 1986; De Potter et al., 1988), where it is converted into its bioactive form, and released preferentially from neurons during high frequency activity (Hökfelt, 1991; Hökfelt et al., 2003).

NPY acts via five known receptor subtypes (Y1, Y2, Y4, Y5, Y6), all belonging to the G-protein coupled receptor superfamily (Michel et al., 1998). In the CNS, the Y1, Y2 and Y5 receptor subtypes are the most abundant, and are expressed in different areas including the hippocampal formation (Redrobe et al., 1999). How synaptic transmission is modulated after stimulation of these NPY receptors in hippocampus is uncertain, but the common functional response of the Y2 receptor appears to be inhibition of excitatory transmission. In CA3 and CA1, the Y2 receptor suppresses presynaptic glutamate release via inhibition of voltage-dependent Ca^{2+}-channels (Colmers et al., 1985; Haas et al., 1987; Colmers et al., 1988; Klapstein and Colmers, 1993; Qian et al., 1997). The Y5 receptor seems to have similar effect on synaptic transmission as the Y2 receptor, as shown in CA3 of mice (Guo et al.,
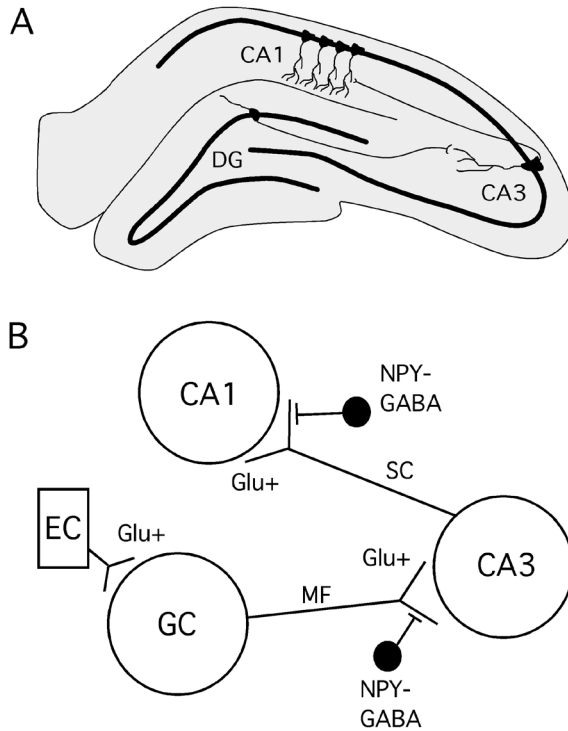
Figure 1. (A) Glutamatergic pathways connect the principal cells within the hippocampus. (B) The entorhinal cortex (EC) provides the granule cells (GC) in the dentate gyrus (DG) with its major input. Granule cells project via mossy fibers (MF) to CA3 pyramidal neurons, which then project to CA1 pyramidal neurons via Schaffer collaterals (SC). Endogenous NPY released from GABAergic interneurons may inhibit presynaptic glutamate release in MF-CA3 and SC-CA1 synapses. Modified from Baraban et al., 1997.

2002). The role of the Y1 receptor is more unclear, but it might reduce Ca^{2+}-influx into dentate granule cells (McQuiston et al., 1996) and function as an autoreceptor on NPY-containing hilar interneurons (Paredes et al., 2003) (Fig. 1).

NPY and seizures

In parallel with studies showing that NPY can inhibit glutamate release, it became clear that endogenous NPY plays a critical role in seizure control. Mice lacking the NPY gene (knockout; KO) experience spontaneous seizures and are more susceptible to chemically induced seizures as compared to wild-type (WT) control mice (Erickson et al., 1996; Baraban et al., 1997). Other studies have shown that

NPY decreases seizure susceptibility when overexpressed in transgenic animals (Vezzani et al., 2002) or when injected either intraventricularly (Woldbye et al., 1996; Woldbye et al., 1997) or into the hippocampus (Smialowska et al., 1996) in various animal models of epilepsy.

In addition, seizures induce prominent changes in NPY peptide levels, particularly evident in areas participating in the generation and propagation of seizures, as shown both in animal models of epilepsy (Vezzani and Sperk, 2004) and in patients with intractable temporal lobe epilepsy (TLE) (Mathern et al., 1995; Furtinger et al., 2001). In rodents, NPY expression is noticeably upregulated in NPY containing interneurons (Sperk et al., 1992; Gruber et al., 1994) even up to several months after status epilepticus (Gruber et al., 1994), and long-lasting ectopic expression of NPY is found in granule cell/mossy fibers (Marksteiner et al., 1990c; Marksteiner et al., 1990b; Marksteiner et al., 1990a; Vezzani et al., 1999b) and transiently in CA1 pyramidal cells (Gall et al., 1990; Sperk et al., 1992).

Together, these observations have lead to the concept that NPY might be an important endogenous inhibitor of seizure activity by controlling excitability (Vezzani et al., 1999a; DePrato Primeaux et al., 2000). Furthermore, it has lead to speculations whether NPY and its receptors may represent a potential clinical target to interfere with seizures and epileptogenesis in humans (Meurs et al., 2007; Noe et al., 2007).

Anticonconvulsive actions mediated by NPY receptors

At present, controversy remains regarding which NPY receptor subtypes are responsible for mediating the seizure-suppressant effect of NPY. Pharmacological studies in different *in vitro* and *in vivo* seizure models using various agonists and antagonists for NPY receptors have often generated conflicting results, suggesting either Y2 or Y5 subtypes are responsible for NPY action, and in fact many of the drugs used in these studies process an affinity for more than one receptor (Klapstein and Colmers, 1997; Woldbye et al., 1997; Reibel et al., 2001; Baraban, 2002; El Bahh et al., 2002; Nanobashvili et al., 2004; El Bahh et al., 2005). Some studies have even reported, that the Y1 receptor can mediate a proconvulsive effect in the hippocampus (Gariboldi et al., 1998; Benmaamar et al., 2003).

Apparently, another confounding factor that contributes to current disagreements and contradictory data is that seizure-induced changes in NPY expression are accompanied by modification in its receptor subtypes, which could be dependent on the type of epileptic insult. Most notably and consistently are hippocampal Y2 receptors levels found to be increased while Y1 receptors levels are decreased in various animal model of epilepsy (Gobbi et al., 1998; Kopp et al., 1999). Both up- and down-regulation in hippocampal Y5 receptors have been reported depending on the area where seizures are triggered, and these changes seem to be transient (Kopp et al., 1999; Bregola et al., 2000) (Fig. 2).
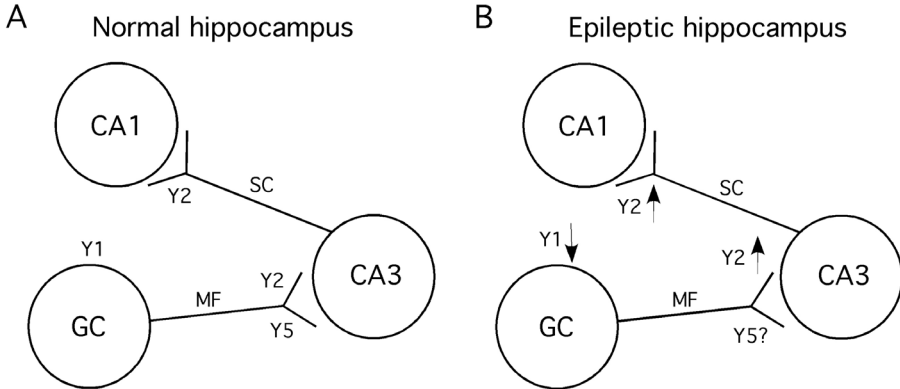
Figure 2. (A) In the normal hippocampus, Y1 receptors are most notably present close to granule cells (GC) in the dentate gyrus, particularly in the molecular layer. Y2 receptors are located presynatically on mossy fibers (MF) and on Schaffer collaterals (SC), where they may suppress glutamate release. Y5 receptors are located in CA3. (B) In the epileptic hippocampus, NPY peptide levels are increased in GABAergic interneurons and NPY is ectopically expressed in GC/MF. These changes are accompanied by down-regulation of the Y1 receptor, while the Y2 receptors are up-regulated in CA1 and CA3. Y5 receptor levels in CA3 remain unchanged, but see main text. Modified from Vezzani and Sperk, 2004.

Therefore, clarifying which NPY receptors are mediating the seizure-suppressant effect of NPY is important if pharmacological or other antiepileptic strategies directed against specific NPY receptors shall be optimized for future therapies in epilepsy.

EPILEPSY

Epilepsy and its current treatment

Affecting about 1% of the general population, epilepsy is one of the most common neurological disorders worldwide, and is associated with accumulating brain damage and neurological deficits. Epilepsy is not one condition, but a diverse family of disorders that can occur at all ages, and is characterized by the predisposition to generate epileptic seizures. The most recent definition, proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE), describes an epileptic seizure as *a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain.* Epilepsy is defined as *a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by the neurobiological, cognitive, psychological, and social consequences of this condition. The definition of epilepsy requires the occurrence of at least one epileptic seizure* (Fisher et al., 2005).

There are many distinct seizure types, several identifiable syndromes, but also many that are poorly classified, reflected by the fact that about 75% of all seizures are of no known etiology (Hauser et al., 1991; Duncan et al., 2006). Although current classification of epileptic seizures is continually evolving, an agreed classification of epilepsy recognizes two major categories, namely *partial* and *generalized* seizures (Seino, 2006). In humans, the most common type of epilepsy is temporal lobe epilepsy (TLE), which is characterized by complex partial seizures with secondary generalization. Initiation of TLE arises from a focal area (partial seizure) within limbic structures such as the hippocampal formation, amygdala, or temporal neocortex, and eventually generalizes by spreading throughout the brain (Engel, 2001).

Currently, the primary choice of treatment in TLE (and all other types of epilepsy) is antiepileptic drugs (AEDs). In general, these drugs enhance GABAergic inhibitory transmission, decrease excitatory transmission or modify sodium channel function, aiming to restore the imbalance in excitatory and inhibitory neurotransmitter systems by increasing net inhibition. No antiepileptic compounds aiming directly towards the NPY system (e.g. NPY ligands) have yet been tested in clinical trials in epilepsy patients (Meurs et al., 2007). Interestingly though, chronic valproic acid treatment, which is an AED primarily used for treating absence seizure but also effectively treats status epilepticus, significantly increases protein levels of NPY in the hippocampus and thalamus, and this mechanism has been proposed to underlie one of its antiepileptic actions (Brill et al., 2006).

In general, the main problem of AEDs is that they suppress the symptoms (seizures) rather than modifying the disease process (epileptogenesis), and when given systemically, they almost inevitably give rise to several side effects, such as nausea and general fatigue (Schachter, 2007). More importantly though, up to 40% of patients still develop pharmacoresistant epilepsy, particularly those patients with TLE (Engel, 2001; Duncan et al., 2006).

Advances in epilepsy treatment

New discoveries in AEDs have provided some advantages in terms of better tolerance, fewer drug interactions and simpler pharmacokinetics. Still, new classes of AEDs do not demonstrate superior efficacy compared to older drugs, and incidences of refractory patients are still unacceptably high, and remain a clinical problem (Perucca et al., 2007; Schachter, 2007). Therefore, novel treatment strategies are urgently needed, especially when the major determinant of good quality of life is total seizure remission. Certainly, surgical resection of an identifiable focus in TLE can have an excellent outcome, but this strategy is only appropriate for a minority of patients (Engel et al., 2003).

At the moment, several focal treatment strategies particularly suitable for TLE are under investigation, and they might be advantageous, if possible to target only epileptic regions involved in seizure generation and propagation. These strategies

include deep brain stimulation (Polkey, 2004; Su et al., 2008), neuronal stem cell grafting (Bjorklund and Lindvall, 2000; Lindvall and Kokaia, 2006), encapsulated cell biodelivery (Lindvall and Wahlberg, 2008), synthetic slow-release polymers (Boison et al., 1999) and gene therapy (McCown, 2004; Vezzani, 2007; Löscher et al., 2008).

GENE THERAPY

Gene therapy in epilepsy

Gene therapy in the brain is divided into two main approaches, namely *ex vivo* and *in vivo* gene therapy. These approaches both involve the transfer and expression of genes into the brain tissue to provide sustained levels of therapeutically active compounds in a localized manner. In *ex vivo* gene therapy this is achieved by transplanting genetically engineered cells in the target tissue, hereby serving as vehicles for expressing therapeutic genes. In *in vivo* gene therapy, viral vectors are used to directly transduce host brain neurons in order to express the desired gene product. Both approaches seem particularly suited when there is a defined seizure focus, where a single focal neuronal network (the seizure focus) drives seizure discharges across the brain. For patients with multiple foci, or without a clear focal onset, delivery and expression of therapeutic genes in key propagation pathways, might, if not completely prevent seizures, at least limit clinical severity (Detlev, 2007; Vezzani, 2007; Löscher et al., 2008). Additionally, focal gene therapy will allow restricted delivery, thereby minimizing the risk of widespread side effects, and could potentially improve intractable seizures by modulating endogenous antiepileptic mechanisms.

A handful of different viral vectors have been tested for *in vivo* gene therapy application in epilepsy, but currently, the two most promising vectors appear to be adeno-associated virus (AAV) and lentiviral vectors (McCown, 2004). Inserted into these vectors, several therapeutic genes have been tested in animal models of epilepsy, including: GAD65 (glutamic acid decarboxylase) (Epps et al., 2006), $GABA_A$ receptor subunit α-1 (Raol et al., 2006), glial cell line-derived neurotrophic factor (GDNF) (Kanter-Schlifke et al., 2007a), neuropeptide galanin (Haberman et al., 2003; Lin et al., 2003; Kanter-Schlifke et al., 2007b) and NPY (Richichi et al., 2004; Lin et al., 2006; Noe et al., 2008). The rationale of using these genes are several; i) restoring the imbalance between inhibition and excitation; ii) reinforcing ion channels; iii) providing neuroprotection; and iiii) supporting the function of endogenous neuromodulators.

NPY gene therapy in epilepsy

All of the above mentioned gene therapy strategies have been shown to suppress seizures, but presently the most promising gene therapy in epilepsy seems
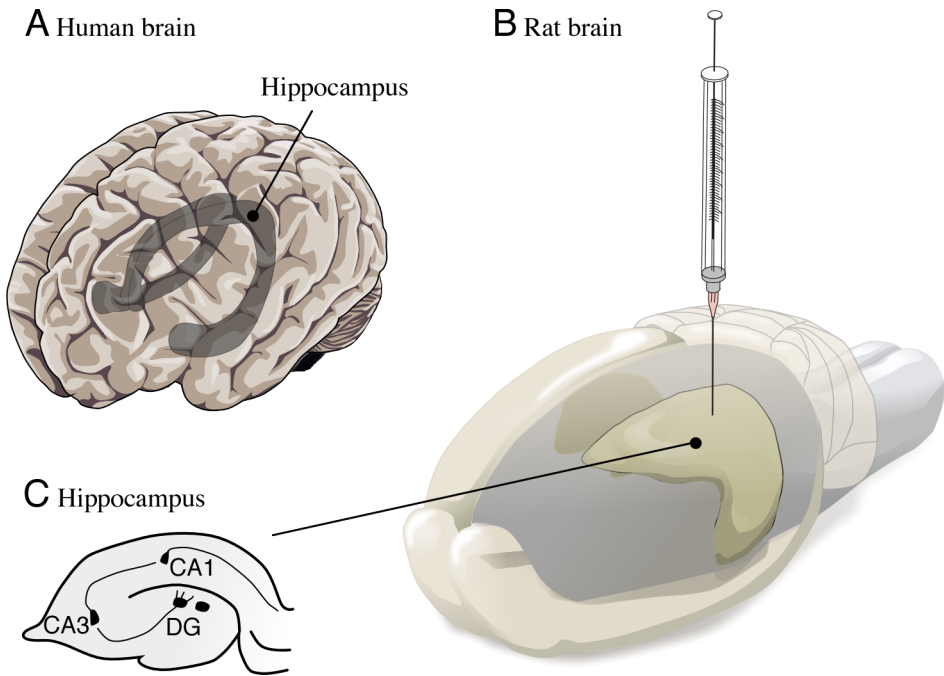
Figure 3. Location of the hippocampus in the human (A) and rat (B) brain. Schematic presentation of the trisynaptic hippocampal circuitry as seen in a transverse slice (C). In hippocampal NPY gene therapy, viral vector is injected directly into the hippocampal formation generating restricted and long-lasting expression of transgene NPY.

to be based on NPY. Annamaria Vezzani and her group in Milano were the first to show that using *in vivo* gene therapy to overexpress NPY in epileptogenic brain areas can have therapeutic effects on epilepsy (Fig. 3). In their first study, they demonstrated a wide-range of seizure suppressant effects by recombinant AAV (rAAV)-mediated overexpression of NPY (Richichi et al., 2004). Sustained overexpression of transgene NPY in the hippocampus of rats significantly suppressed acutely intrahippocampal kainate-acid (KA)-induced limbic seizures and intraventricular KA-induced status epilepticus. In addition, kindling acquisition was delayed and the electrical seizure threshold was increased, suggesting antiepileptogenic effects of transgene NPY (Richichi et al., 2004).

More recent studies have supported and extended these findings. Using a comparable rAAV vector construct, but with an additional insertion of a secretion signal sequence (fibronectin) promoting constitutive release, transduction of the NPY gene into the piriform cortex of rats was shown to attenuate acute intraperitoneal KA-induced limbic seizures. In addition, a similar vector, but with an insertion of the NPY13-36 C-terminal fragment of NPY (Y2 receptor preferring agonist) into the construct for preferential Y2 receptor activation, suppressed seizures comparable to the vector containing full length NPY cDNA (Foti et al., 2007). In another study, it was demonstrated that mice with hippocampal rAAV-mediated NPY overexpression experienced less severe systemic KA-induced seizures compared to controls, and by injecting the viral vector into Y1 and Y2 receptor KO mice, it was shown that the anticonvulsive effect was mediated by the Y2 and not Y1 receptors (Lin et al., 2006).

The more substantial proof of therapeutic efficacy of NPY gene therapy was most recently provided in a clinically relevant model of epilepsy (Noe et al., 2008). While the studies described above were carried out in animals expressing transgene NPY in the "normal brain" before induction of acute seizures, this study was conducted in epileptic rats experiencing spontaneous unprovoked seizures, mimicking human TLE. Noé and colleagues (2008) demonstrated, using a model of chronic epilepsy by electrically-induced status epilepticus, that in rats with rAAV-NPY transduced hippocampi, the progression and frequency of spontaneous seizures were significantly decreased as compared to controls.

Challenges of NPY gene therapy

It is clear that NPY gene therapy in epilepsy holds substantial therapeutic promises, but before moving from preclinical research to clinical application the side effects of NPY overexpression need to be evaluated, as well as addressing the long-term benefit-risks. This is particularly important as the studies discussed above were limited to a time period of weeks or a few months, and gene therapy in humans would be life-long.

Certainly, unpredictable side effects are possible, especially considering that the NPY system is involved in many different functions (described earlier). These concerns are partly overcome by using viral vectors providing highly localized transgene expression, and due to the fact that most of these brain functions are localized outside the hippocampal formation, which at present, seems to be the most feasible target for NPY gene therapy in epilepsy. However, as the hippocampal formation plays a significant role in the formation of new memories, and is important for storage and processing of spatial information (Eichenbaum et al., 1999; Sweatt, 2004), cognitive impairment could be a serious side effect of hippocampal NPY gene therapy. This concern is further justified by the fact that cognitive impairment is already a serious comorbidity of epilepsy.

Two distinct but somehow related mechanisms could potentially be unfavorable for learning and memory processing. First, NPY promotes precursor cell proliferation in the subgranular zone of the dentate gyrus (Howell et al., 2005; Howell et al., 2007). This could be a problem, as the formation of new neurons appears to be involved in hippocampal-dependent learning and memory processes (Shors et al., 2001; van Praag et al., 2002; Aimone et al., 2006). Second, increased levels of NPY could inhibit learning and memory by directly effecting synaptic transmission and plasticity within all regions of the hippocampal formation.

At present, our current knowledge of this latter issue is rather limited. Some data indicate that rats overexpressing NPY in the CA1 region of the hippocampus have impaired memory (Thorsell et al., 2000) and partial blockade of long-term synaptic plasticity in hippocampus, which is considered to be a synaptic correlate of hippocampal learning and memory (Martin and Morris, 2002; Lynch, 2004), has been shown after intraventricular infusion of NPY (Whittaker et al., 1999). Therefore, careful assessments of memory function, including aspect of synaptic transmission and plasticity, should be completed before NPY gene therapy is implemented for clinical trials in epilepsy patients.

AIMS OF THE THESIS

NPY gene therapy in epilepsy using viral vectors has evolved to a stage where clinical trials are a foreseeable future. The present work was conducted to strengthen the current knowledge and to further assess the clinical applicability of this strategy. The specific aims were:

1. To determine which NPY receptor subtypes are mediating the seizure suppressant effect of NPY in various brain structures (**paper I**).

2. To explore whether hippocampal NPY gene therapy influences neural transmission and synaptic plasticity in CA1 (**paper II**) and subiculum (**paper III**), and further determine whether transgene NPY overexpression affects hippocampal-dependent memory performance (**paper II**).

3. To examine the release mechanisms of transgene NPY in the hippocampal formation (**paper II, III**).

4. To determine the effect of transgene NPY on rapid kindling seizures, and further explore how transgene NPY is influencing synaptic plasticity in brain slices exposed to recurrent stimulation-induced seizure activity (**paper IV**).

RESULTS AND COMMENTS

ASSOCIATION OF NPY RECEPTORS WITH ANTIEPILEPTIC RESPONSE

The first part of the results section identifies possible NPY receptor subtypes involved in seizure suppression in various structures of the brain (paper I). This particular issue has been controversial, partially due to the lack of highly selective agonists and antagonists for various NPY receptor subtypes. To circumvent this problem, we used various NPY receptor KO mice with similar genetic background and explored the anti-epileptic action of NPY in *in vivo* and *in vitro* animal models of epilepsy.

Y2 and Y5 receptors suppress epileptiform activity in hippocampus *in vitro*

To study the seizure suppressant effect mediated by various NPY receptors situated in the hippocampus only, we used slices prepared from dissected hippocampi. In slices perfused with artificial cerebrospinal fluid (aCSF) with Mg^{2+} omitted, we recorded spontaneous $0\text{-}Mg^{2+}$-induced epileptiform bursts in the CA3 pyramidal layer. In WT mice, application of exogenous NPY decreased the frequency of epileptiform activity and this effect washed out after termination of NPY application. In slices from Y2-/- and Y5-/- mice, application of NPY had an anti-epileptiform effect comparable to that observed in slices from WT. However, some small differences were detected as compared to WT slices. In Y2-/- mice, the effect of NPY washed out faster, and in Y5-/- mice the peak inhibitory effect of NPY was lower. To exclude the involvement of the Y1 receptor (or other unidentified NPY receptors), NPY was also added to slices from Y2Y5-/- (double KO) mice. In this case, the anti-epileptiform effect of NPY was completely absent, and instead the epileptiform activity steadily progressed, indicating that these slices were far more excitable. This effect appeared to result from the absence of Y2 and Y5 receptors, rather than from a pro-epileptic effect of NPY (via Y1 receptors), as this observation was evident already during baseline recordings and throughout the entire washout period of NPY. Together, these data indicate that deletion of either Y2 or Y5 receptor had only a partial influence on the anti-epileptiform action of NPY, suggesting that both receptors could participate and mediate an antiepileptic response in the hippocampus.

Y5 receptors attenuate kainate acid-induced seizures *in vivo*

To further address the involvement of NPY receptors in seizure termination, we next explored the effect of exogenous NPY on *in vivo* seizures. Systemic injection of KA into Y2Y5-/- mice generated far more severe seizures as compared to WT mice, revealed by higher seizure grades and shorter latencies to first convulsion and loss of posture. In receptor KO mice, the effect of KA in Y5-/- was similar to Y2Y5-/- mice, whereas the effect in Y2-/- mice was similar to WT mice. There-

fore, systemically KA-induced seizures are likely suppressed mainly by Y5 receptors and to a minor extent by Y2 receptors. To further confirm this anticonvulsive effect mediated by Y5 receptors, we also tested the effect of Y5 specific antagonist, L-152,804 on systemic KA seizures in normal NMRI mice. This combined treatment had a clear proconvulsive effect as compared to control conditions revealed by shorter latencies to first convulsion and loss of posture, and by higher seizure grades. Similar results were observed when KA and the Y5 specific antagonist were injected in WT mice, although latency to loss of posture did not reach statistical significance (p = 0.06). In addition, Y2-/- and WT mice injected with KA and the Y5 receptor antagonist displayed a similar degree of seizures (with regard to any seizure parameter tested), thus supporting the significance of Y5 receptor as the predominate receptor subtype suppressing seizures *in vivo*.

Y5 receptors inhibit kindling-induced seizures in extra-hippocampal regions *in vivo*

In parallel to the KA experiments, we also used the kindling model of epilepsy, another widely used seizure model. Receiving electrical stimulations once daily in the left ventral hippocampal CA1/CA3 area, WT mice reached only stage 2 seizures within 55 days. During the same kindling period, the majority of Y5-/- mice developed stage 3 seizures and more than half of the mice were fully kindled, having displayed 5 grade 5 seizures. In the hippocampus, no differences between Y5-/- and WT mice were detected in seizure threshold level or mean afterdischarge (AD) duration. However, an electrode placed in the amygdala revealed longer mean duration of both primary and secondary and total AD duration in Y5-/- mice as compared to WT mice. Taken together, these two *in vivo* experiments suggest that endogenous and exogenous NPY primarily inhibits seizures via Y5 receptors, most likely in extra-hippocampal regions.

NPY receptor mRNA expression and receptor binding

To determine whether compensatory changes in gene expression or binding of Y1, Y2 and Y5 receptors could somehow account for the observed results using these mutant KO mice, we performed extensive *in situ* hybridization and radioactive ligand binding assays. Analysis and comparison of *in situ* hybridization signals of brain slices from mutant and corresponding WT mice did not reveal any changes in mRNA expression of Y1, Y2 and Y5 receptor in any KO strain (see result section in paper I for details about expression levels of various receptors in the hippocampal formation and neocortical regions).

The binding study revealed several alterations in Y1 binding. Generally, all mutant mice displayed significantly lower levels of Y1 binding than WT mice, and these changes appeared to be restricted to the hippocampal formation as no changes were observed in basolateral amygdala, piriform cortex or primary cortex. Specific Y2 receptor binding was similar between Y5-/- and WT mice in all

regions studied. Specific Y5 receptor binding was also observed in all examined regions, but the levels were in general lower in all regions as compared to both Y1 and Y2 binding. In comparison to WT mice, specific Y5 binding in Y2-/- mice was reduced in dorsal, but not ventral hippocampus.

NPY GENE THERAPY, SYNAPTIC TRANSMISSION AND PLASTICITY

The second part of the result section mainly focuses on changes in synaptic transmission and plasticity as a consequence of hippocampal NPY gene therapy (paper II, III and IV).

Viral vector-mediated transgene expression

To gain expression patterns of previous studies on NPY gene transfer in epilepsy, we used the rAAV vector with neuron-specific enolase (NSE) promoter (Richichi et al., 2004; Lin et al., 2006). This vector has been shown to selectively transduce neurons, generate long-lasting transgene expression and does not activate the host immune system (During et al., 2003; Burger et al., 2005).

In paper II, III and IV, injection of rAAV-NPY vector into the hippocampal formation induced expression patterns of transgene NPY showing low variability among animals and slices within individual experiments. A notable age-dependent variation in the extent of transgene expression was found in slices, evaluated approximately 3 to 9 weeks after viral injection. In paper II, where adult rats received a single viral injection, high levels of transgene NPY-immunoreactivity (IR) tended to be more restricted and confined around the injection, and subsequently recording site and surrounding areas of CA1 (i.e. stratum lacunosum-moleculare, radiatum, pyramidale and oriens). In paper III pup rats received a single viral injection at postnatal day 2 to 4. In this case, despite receiving half the volume of viral suspension as adult rats, high levels of transgene NPY-IR were found throughout the hippocampal formation, including all layers of dentate gyrus, CA3, CA1 and proximal subiculum.

In those experiments where adult rats received multiple injections (paper II and IV), high levels of transgene NPY-IR were found throughout the hippocampal formation. For both pups and adult rats maximal level of NPY expression was obtained within three weeks after rAAV-NPY injection and was stable for at least 3 months, well within the time frame used for electrophysiological recordings, kindling and behavioral testing. In the rare case that transgene NPY-IR was low or undetectable in the area of recording, slices/recordings were discarded from further analysis.

In general, the expression of transgene NPY was not restricted to any specific neuronal population in the hippocampus, and in transduced areas almost all projection neurons and interneurons were NPY-positive, although we cannot exclude some non-neuronal tropism. However, to better evaluate neuronal tro-

pisms, some neonatal and adult rats were injected with rAAV-GFP vector (paper II and III). Based on epifluorescent illumination of hippocampal slices, the serotype 1/2 rAAV vector with NSE promoter showed clear neuronal tropism, with GFP expression detected within the cell soma and fibers of transduced neurons. Spread of transgene, in this case GFP, was similar to transgene NPY as described above and was detected throughout the hippocampus including the subiculum.

Animals injected with rAAV-empty (control) vector displayed few scattered NPY-positive cells throughout the hippocampal formation as shown in young (paper III) and adult (II and IV) rats. These cells most probably resembled inhibitory GABAergic interneurons containing NPY. In young rats (paper III), a weak band of NPY-containing fibers was observed within the molecular layer of the dentate gyrus and the hilus. In agreement with previous observations, this NPY-containing band was not seen in adult rats (paper II and IV) as these NPY-positive fibers disappear during early adulthood (Moryś et al., 2002). Although episodes of seizures are associated with an acute and sometime long-lasting upregulation of endogenous NPY expression (Vezzani et al., 1999a), we were unable to detect any such differences in hippocampus between kindled and non-stimulated rats 4-6 weeks after rapid kindling (paper IV). In none of the examined brain slices (paper II, III and IV) was there any apparent brain damage caused by either viral injection or viral vectors consistent with previous observations (Richichi et al., 2004).

Transgene NPY suppresses kindling-induced seizures

To determine the effect of transgene NPY on neural transmission and plasticity in brain slices from epileptic, electrically kindled animals, we used the rapid kindling model of epilepsy previously employed by Richichi and colleagues (2004). Using this model (paper IV), we found that development of seizure severity over time and number of focal and generalized seizures did not differ between animals pre-injected with rAAV-NPY and rAAV-empty (control) vector. However, animals transduced with rAAV-NPY exhibited shorter AD duration at threshold stimuli intensity and showed shorter AD duration during both focal and generalized seizures. Overall, the cumulative AD duration during rapid kindling (all seizure grades) for each animal was reduced by approximately 50% in rAAV-NPY as compared to rAAV-empty treated animals. These data suggest that rapid kindling epileptogenesis was unaltered by rAAV- NPY treatment, but as duration of convulsions was suppressed once they were initiated, it could indicate that transgene NPY provides anticonvulsive rather than antiepileptogenic effects.

Basal synaptic transmission

To explore whether viral vector-mediated transgene NPY was influencing basal synaptic transmission in the hippocampus, assuming that some transgene NPY could be constitutively released from transduced neurons, we performed input-

output analysis of field excitatory postsynaptic potentials (fEPSPs) recorded in CA1 stratum radiatum. For this type of analysis, the amplitude of the presynaptic fibervolley (PSFV) was plotted against the values of the initial slope of the respective fEPSPs at various stimulation intensities. Theoretically, if transgene NPY was constantly inhibiting presynaptic glutamate release, this should suppress the magnitude of fEPSPs without affecting the PSFV. In slices derived from animals injected with rAAV-NPY and rAAV-empty vectors no differences were detected at any given stimulus intensity (paper II). Similar results were obtained in slices prepared from rapid kindled rats (paper IV). In this study, no differences were detected between rAAV-NPY and rAAV-empty treated kindled animals as well as compared to non-stimulated control rats. Overall, these results indicate that basal synaptic transmission in the hippocampus is unaffected by transgene NPY, irrespectively of the precondition of the animals.

Paired-pulse facilitation and short-term plasticity

Using a different, but more indirect, approach to detect constitutive release of transgene NPY, we examined the ratio of paired-pulse facilitation (PPF) of fEPSP in CA1 stratum radiatum (paper II and IV). Paired-pulse facilitation is considered a form of short-term synaptic plasticity and describes the ability of synapses to increase neurotransmitter release in response to the second of two closely spaced afferent stimulations, and depends on residual Ca^{2+} concentration in the presynaptic terminals (Zucker and Regehr, 2002; Nicoll and Schmitz, 2005). In the hippocampus, the amount of PPF is inversely related to the overall release probability (P_r) of the synapses (Dobrunz and Stevens, 1997). Since NPY has been shown to decrease the P_r of glutamate via modulation of Ca^{2+}-influx into afferent synaptic terminals in CA1 (Colmers et al., 1988; Qian et al., 1997), transgene NPY should theoretically, if released constantly, increase the PPF ratio. In slices from animals with intact hippocampus (paper II), we found that PPF was slightly (on average 6%), but significantly, higher in rAAV-NPY as compared to rAAV-empty treated slices at interstimulus intervals (ISIs) of 50, 100 and 200 ms. To confirm that this effect was dependent on transgene NPY, we applied the highly selective NPY Y2R antagonist, BIIE0246, to slices from rAAV-NPY injected rats and observed a small, but significant, reduction of PPF. A similar type of experiment was conducted in slices derived from animals exposed to rapid kindling (paper IV). Again, paired stimulation at different ISIs induced fEPSPs with pronounced PPF. No differences were detected at ISIs of 25, 50 and 100 ms between rAAV-NPY and RK-rAAV-empty treated slices. However, at ISI of 200 ms the PPF ratio was significantly higher in rAAV-NPY treated slices as compared to non-kindled control slices, but similar to RK-rAAV-empty treated slices. No differences at any ISI were detected between RK-rAAV-empty and non-kindled control slices. These results could indicate that transgene NPY might be constitutively released, but considering the relatively minor changes in PPF, the amount of transgene NPY tonically inhibit-

ing presynaptic glutamate release is very small, thus transgene NPY only causes relatively minor changes in short-term plasticity.

Minor constitutive release of transgene NPY

In paper III, we adopted another technique to explore whether transgene NPY was constitutively released. Blocking action potentials and $GABA_A$ receptors with TTX and PTX, respectively, we isolated miniature excitatory postsynaptic currents (mEPSCs) recorded from pyramidal neurons in proximal subiculum using whole-cell patch-clamp. In both rAAV-NPY and rAAV-empty treated slices, the cumulative probability of interevent intervals (IEIs) was unaffected by BIIE0246 application, suggesting insignificant amount of transgene NPY influencing spontaneous glutamate-mediated synaptic events and activation of non-NMDA receptor type of glutamate receptors. Interestingly, a direct comparison revealed significantly higher event frequencies of mEPSCs in rAAV-empty as compared to rAAV-NPY treated slices, independent of Y2 receptor activation, since BIIE0246 application did not increase the frequency of mEPSCs in rAAV-NPY (or rAAV-empty) treated slices. These results may indicate that transgene NPY is tonically suppressing spontaneous glutamate release via mechanisms not involving NPY Y2 receptors, or it may reflect a kind of homeostatic change in the neural network as a consequence of chronic inhibition mediated by transgene NPY.

Other results also supported the notion that there is no or a relatively small amount of constitutive release of transgene NPY. In paper III, the amplitudes of single evoked EPSCs recorded from subicular pyramidal cells in rAAV-NPY treated slices were not affected by BIIE0246 application. Moreover, the PPF ratio of the two first EPSCs (with ISI of 25 ms) evoked during high-frequency stimulation (HFS) was similar in rAAV-NPY and rAAV-empty treated animals both before and after BIIE0246 application (paper III), suggesting no alteration in short-term synaptic plasticity and low presence of transgene NPY in the extracellular spaces.

Based on the series of observations described above, it is most reasonable to assume that only a small fraction of transgene NPY is constitutively released. This release does not influence basal synaptic transmission in the hippocampus, but is associated with minor alterations in short-term synaptic plasticity.

High frequency activity-dependent release of transgene NPY

Due to the fact that neuropeptides in general are released during neuronal high frequency firing activity (Hökfelt, 1991) and that we could hardly detect any constitutive release of transgene NPY in the studies, we designed experimental setups to promote and detect the release of transgene NPY. In paper II, this was attempted by applying HFS trains to Schaffer collaterals (10 and 25 Hz, 50 stimuli each), while recording fEPSP in CA1 stratum radiatum. Normally, there is a progressive attenuation of fEPSP during the time course of HFS, most pronounced

at the later stage of HFS. In Schaffer collateral-CA1 synapses this gradual reduction in fEPSP magnitude is caused by a depletion of the readily releasable pool of glutamate (Dobrunz and Stevens, 1997). In rAAV-NPY treated slices, we saw that this progressive attenuation was significantly slower as compared to rAAV-empty treated slices. It is possible that this effect could be accounted to transgene NPY, if we presume that HFS-mediated transgene NPY release suppresses concomitant glutamate release, and thus reduces the rate of glutamate depletion. However, to get some more solid evidence of HFS-mediated release of transgene NPY, we recorded EPSCs from pyramidal cell situated in proximal subiculum using whole-cell patch-clamp technique (paper III). In this case, we again applied HFS trains, but now stimulating the alveus pathway, i.e. afferent glutamatergic fibers from CA1 pyramidal axons making synapses onto subicular pyramidal neurons. In rAAV-NPY treated slices, applying HFS trains (consisting of 10 stimuli applied at 40 Hz) evoked EPSCs with attenuating amplitudes, and this attenuation was substantially increased after application of BIIE0246. Such an effect was not observed in rAAV-empty treated slices. Quantification of these responses before and after BIIE0246 application by dividing the total integral of EPSCs (induced by all 10 stimuli) by the integral of the first EPSC evoked by the train, revealed a significant decrease of the ratio in rAAV-NPY, but not in rAAV-empty treated slices (where no changes were observed). One possible explanation for this effect could be that transgene NPY begins to be released only after the first few HFS pulses, leading to inhibition of glutamate release and preventing fast depletion of the releasable pool of glutamate vesicles as reflected by a relatively small depression of EPSCs. Once BIIE0246 was applied to the slices, the effect of transgene NPY on glutamate release was blocked, resulting in more glutamate release during the later phase of HFS, thus leading to more rapid depletion of the releasable pool of glutamate vesicles in presynapses, and therefore more pronounced EPSC depression. Therefore, the observations made by whole-cell recordings of CA1-subicular synapses and field recordings in Schaffer collateral-CA1 synapses are rather identical, all suggesting that transgene NPY can be released by high-frequency neuronal activity, where it decreases the rate of glutamate depletion.

Volume transmission

To further consolidate that transgene NPY was preferentially released during high frequency firing activity, we did alternating stimulation of two independent afferent pathways (in alveus), on either side of the recorded cell, impinging onto the same subicular pyramidal neuron (paper III). Using this experimental setup, we speculated that the release of transgene NPY by HFS in one pathway could reach neighboring non-stimulated excitatory synapses on the same cell and thereby suppress glutamate release. Applying HFS to one afferent pathway decreased EPSC amplitudes by approximately 33% induced by single test stimulation of the other afferent pathway in rAAV-NPY treated slices. This effect totally disappeared

after BIIE0246 application. In rAAV-empty treated slices, the amplitudes of paired EPSCs induced by test stimulation were of similar magnitude, and were neither influenced by HFS applied to the other pathway nor to BIIE0246 application itself. These data show unequivocally that HFS-induced synaptic activation leads to transgene NPY release, and demonstrate that transgene NPY can diffuse and inhibit neighboring non-stimulated (non-activated) glutamatergic synapses. It also emphasizes the ability of transgene NPY to reach receptors far from the release site, so-called volume transmission (Fuxe and Agnati, 1991), which also have been reported for endogenous NPY (Fuxe et al., 1990). In contrast, a single stimulation does not seem to release sufficient transgene NPY to diffuse and inhibit glutamate release, as indicated by unaltered amplitude of the first EPSC evoked by the HFS train before and after application of BIIE0246 in rAAV-NPY slices.

Attenuation of long-term synaptic plasticity by transgene NPY

Once we established that transgene NPY was preferentially released during high frequency neuronal activity, one central issue was to determine which functional effect this could potentially have on neuronal circuitries in the hippocampal formation. Obviously, this release-mechanism seems feasible in respect to seizure suppression, but since LTP, which is thought to be a synaptic correlate of learning and memory processes (Lynch, 2004; Malenka and Bear, 2004), is heavily dependent on repetitive activation of excitatory synapses, this could well be affected by transgene NPY. In paper II, we found that the magnitude of LTP in Schaffer collateral-CA1 synapses was approximately 50% lower in rAAV-NPY as compared to rAAV-empty treated slices, but still significantly elevated above its own baseline values. In addition, constant application of BIIE0246 to slices rescued LTP in rAAV-NPY treated slices to similar levels as recorded in rAAV-empty treated slices. These results strongly suggested involvement of Y2 receptor in inhibiting long-lasting synaptic plasticity in the hippocampus. In paper II, we also observed that the induction rate of LTP was similar between the groups. Similar results were found in proximal subiculum, studying CA1-subicular synapses using whole-cell patch clamp recordings of pyramidal cells (paper III). In these synapses, LTP was significantly attenuated in rAAV-NPY as compared to rAAV-empty treated slices. During the phase of post-tetanic potentiation (PTP; 0-5 min post tetanus) and during LTP (5-25 min post tetanus) the amplitudes of EPSCs were generally smaller in rAAV-NPY treated slices, but the LTP induction rate was similar.

To further address the action of transgene NPY on LTP, we tested several possible scenarios by applying exogenous NPY to slices from naïve (non-injected) rats at different time points (paper II). A test experiment showed that exogenous application of NPY reduced evoked fEPSP in CA1 by approximately 50%, and full washout was achieved after approximately 45-50 min. In cases where exogenous NPY was applied prior to tetanus stimulation, a brief phase of PTP was observed, but induction of LTP failed. These data indicated that increasing NPY levels by

exogenous application prior to tetanization blocks LTP induction in Schaffer collateral-CA1 synapses in naïve hippocampal slices. Since LTP induction was not blocked in rAAV-NPY treated slices, one could assume that extracellular transgene levels were low in these animals (in agreement with our other results), and due to the preferential release of transgene NPY during tetanic HFS-induced activation of Schaffer collaterals, this activity-dependent release of transgene NPY should compromise the level of LTP. To address this hypothesis, we applied exogenous NPY to naïve slices 20 min after LTP induction. A significant, but transient, decrease of fEPSP responses with approximately the same magnitude and time-course as in non-tetanized slices was observed. The fEPSP magnitude returned to the same potentiated levels as observed before NPY application 50-55 min after the peak of the NPY effect. These data indicate that the reduced magnitude of LTP in Schaffer collatral-CA1 synapses of rAAV-NPY treated animals could be a result of HFS-induced increase in released transgene NPY, which interferes with LTP induction, but not LTP maintenance.

Delayed but not impaired learning

To determine whether deficits in LTP caused by transgene NPY was associated with impairments of hippocampal-dependent learning processes, animals injected with rAAV-NPY and rAAV-empty vector were monitored for spatial discrimination and learning abilities (paper II) using a test sensitive to hippocampal manipulations (Morris et al., 1986; Carli et al., 2001). On the first day of acquisition, seven weeks after viral injections, the performance of both rAAV-NPY and rAAV-empty treated rats was at chance (chance level = 50% correct choices). Both groups improved their performance with training, but rAAV-empty treated rats learned faster, and during all seven days of training, rAAV-NPY treated rats made fewer correct choices as compared to controls. At the same time, both groups showed similar choice latency, and errors of omission were present only on the first day of training and were equal in both groups. These results suggest that hippocampal rAAV-NPY gene therapy delays spatial discrimination abilities, but does not prevent the animals from learning, and may raise some concerns about the clinical applicability of a rAAV-NPY gene-based therapy, as memory performance in epilepsy patients is often already compromised.

To further address this point (paper IV), we explored whether rAAV-NPY gene transfer in epileptic, electrically kindled animals, would further aggravate possible seizure-induced reduction of LTP in the hippocampus. In control animals, rapid kindling reduced the magnitude of LTP in CA1 region of the hippocampus. The rAAV-NPY vector injection into the hippocampus decreased seizure duration during rapid kindling, but did not have any further effect on rapid kindling-induced attenuation of LTP. Therefore, our data suggest that rAAV-NPY treatment does not prevent kindling-induced LTP impairment, but does not aggravate it either. In summary, NPY gene transfer may result in memory impairment in the

normal brain, but in the epileptic brain it appears to represent a relatively limited risk. Still, it needs to be tested in other animal models of epilepsy.

GENERAL DISCUSSION

ROLE OF Y2 AND Y5 RECEPTORS IN SEIZURE SUPPRESSION

Y2 and Y5 receptors in hippocampus

In hippocampal slices, we show that NPY provides anti-epileptiform action in Y2-/- and Y5-/- mice (with mixed BALB/c x 129/SvEv background), and generates no effect in Y2Y5-/- slices, suggesting that both Y2 and Y5 receptors can inhibit spontaneous 0-Mg^{2+}-induced bursting. The effect of NPY was less pronounced in single KO mice as compared to WT mice, indicating that activation of both receptors could act in an additive manner to suppress epileptiform activity in the hippocampus. Consistently, other studies have shown that application of Y2 and/or Y5 receptor agonists reduces 0-Mg^{2+}-induced bursting in hippocampal slices of both mice and rats (Klapstein and Colmers, 1997; Bijak, 1999; Marsh et al., 1999; Nanobashvili et al., 2004).

Contradictory to these results, inhibition of epileptiform bursting by NPY was shown to be mediated solely by Y2 receptors in hippocampal slices in two models of epileptiform activity *in vitro*, the 0-Mg^{2+} and the stimulus train-induced bursting (STIB) model (El Bahh et al., 2005). In slices from C57 x 129 WT mice, it was shown that NPY reduced the frequency of 0-Mg^{2+}-induced bursting, and this effect was totally blocked in the presence of a selective Y2 receptor antagonist (BIIE0246), but not by a selective Y5 receptor antagonist (Novartis 1). In addition, adding an Y5 receptor agonist had no measurable effect on 0-Mg^{2+}-induced bursting. In the STIB model using the same mice, both NPY and an Y2 preferring agonist, but not Y1 and Y5 preferring agonists, suppressed stimulation-induced ADs. Moreover, in slices from Y2-/- mice (mixed C57BL/6 x 129/svJ background), application of neither NPY nor Y1, Y2 and Y5 receptor-preferring agonists had any effect on STIB-induced epileptiform activity (El Bahh et al., 2005).

These results are in contrast to our and previous studies (Marsh et al., 1999; Baraban, 2002), which suggest that the Y5 receptor is important for NPY's action in the hippocampus. It is difficult to explain these conflicting results, but there could be several reasons (see below).

The genetic background of the strains used to generate NPY receptor KO mice seems to play an important role in determining the phenotypic characteristics of NPY's effect. The anti-epileptiform effect of NPY in 0-Mg^{2+}-induced bursting was totally absent in slices from Y5-/- mice in an inbred 129/sv background (Marsh et al., 1999; Baraban, 2002) as compared to the blunted peak effect of NPY in our Y5-/- strain on a mixed BALB/c x 129/SvEv background in our study. In addition, in slices from Y2-/- mice with a mixed C57BL/6 x 129/svJ background, a Y5 receptor-preferring agonist had no measurable effect in the STIB model (El Bahh et al., 2005). Since none of these studies were made on mice with similar genetic background, it is reasonable to believe that some of the conflicting results could be due to differences in strains.

Another pitfall of the KO strategy is the potential influence of compensatory changes in other genes and proteins. We found a down-regulation of Y1 receptor binding in the mutant strains, and based on the seizure permissive nature of Y1 receptor activation (Gariboldi et al., 1998; Benmaamar et al., 2003), it is possible that down-regulation of Y1 binding sites could compensate for the loss of one of the other NPY receptors (Y2 or Y5), which act in opposite direction. A similar reduction in Y1 receptor binding has been reported in different seizure models, and might reflect a compensatory mechanism to counteract increased excitability (Gobbi et al., 1998; Kopp et al., 1999; Husum et al., 2004).

In agreement with the El Bahh study (2005), we found prominent Y1 and Y2 receptor binding in DG and CA areas, respectively. However, we detected Y5 receptor binding in WT and Y2-/- mice, while El Bahh *et al*. (2005) could not provide any evidences for the presence of detectable Y5 receptors in mouse hippocampus. Generally, localization of Y5 mRNA signals suggests that this receptor should be abundantly expressed throughout the brain, including the hippocampus (Kopp et al., 1999; Parker and Herzog, 1999), in agreement with our observations. However, attempts to localize the Y5 receptor via autoradiograhic methods have demonstrated only little specific receptor binding in various brain structures including the hippocampus (Dumont et al., 1998a; Dumont et al., 1998b; Parker and Herzog, 1999; Grove et al., 2000), again consistent with our observations. In our study, Y5 receptor binding was visualized by radioactive labeled PYY (non-specific NPY agonist), which was added together with Y1 and Y2 receptor-preferring antagonists to displace these receptor sites. The value of specific Y5 receptor binding was then calculated by subtracting the non-specific binding (NPY) from total Y5 receptor binding. One criticism of this particular approach could be that we did not further displace the Y5 receptor signal by adding an Y5 receptor antagonist in order to verify that this signal corresponded with the background signal. Thus, we did not demonstrate that all NPY receptor sites could be occupied by adding all three antagonists, and therefore cannot completely exclude the possibility that receptors other than Y1, Y2 and Y5 could be present.

Interaction of NPY receptors (e.g. dimerization) could be another possible explanation for the conflicting data. Heterodimerization between Y1 and Y5 receptors has been described *in vitro*, and the Y5 receptor efficacy increases in the heterodimer configuration (Y1Y5) as compared to Y5 receptor alone (Gehlert et al., 2007). If such dimerization also occurs *in vivo*, it could potentially influence the functional response mediated by Y1 and Y5 receptors when using selective agonists and antagonists (and for instance KO animals). Whether interaction between Y2 receptors and other NPY receptors take place *in vivo* and *in vitro* is currently unknown, but there appears to exist some kind of physiological cross talk between Y1 and Y2 and also between Y2 and Y5 receptors, in which Y2 receptors play a predominant role (Silva et al., 2003).

Taken together, our *in vitro* study suggests that NPY can suppress epileptiform activity in the hippocampus via both Y2 and Y5 receptors, although any final conclusions must be taken with caution.

Y5 receptors in extra-hippocampal regions

In the KA model of seizures, we found that Y2-/- mice experienced seizures comparable to those of WT, whereas Y5-/- mice experienced more severe seizures as compared to both Y2-/- and WT mice. Thus, we found no indication of an additive effect of Y2 and Y5 receptors *in vivo*. Similar findings have been reported previously in the KA model of epilepsy: the pharmacological profile of centrally administered NPY analogues suggested the involvement of Y5 receptors (Woldbye et al., 1997; Reibel et al., 2001), while Y5-/- mice were more sensitive to systemic KA-induced seizures (Marsh et al., 1999). The reason for a lack of an additive effect of Y2 and Y5 receptors might be explained by the involvement of hippocampal versus extrahippocampal NPY receptors. This hypothesis is supported by our finding, showing that faster progression of hippocampal kindling in Y5-/- mice is associated with longer AD durations in amygdala but not in hippocampus. Supporting this notion, application of a selective Y5 receptor agonist inhibited the occurrence of generalized seizures during kindling without affecting focal AD in hippocampus (Benmaamar et al., 2005). The involvement of extrahippocampal Y5 receptors inhibiting generalized seizures also corresponds well with the more abundant expression of Y5 receptor found in amygdala and temporal cortex as compared to hippocampus (Dumont et al., 1998a; Parker and Herzog, 1999; Grove et al., 2000).

We also observed that systemic KA-induced seizures were aggravated by co-administration of a selective Y5 receptor antagonist, and further detected that the proconvulsive effect of the antagonist did not differ in Y2-/- and WT mice. These data further confirm that the Y2 receptor does not appear to play a major role in the regulation of systemically induced KA seizures. Instead, the Y2 receptors seem to play an important role in regulating focal hippocampal KA-induced seizures (El Bahh et al., 2005).

Interestingly, in a model of genetic generalized epilepsy, using genetic absence epilepsy rats of Strasbourg (GAERS), primarily involving seizures in thalamicocortical and to less extent limbic (hippocampus) circuitries, NPY suppressed generalized seizures predominately via Y2 receptors and to a lesser extent via Y5 receptors (Morris et al., 2007). This latter finding supports our conclusion that both Y2 and Y5 receptors are involved in the regulation of seizure activity by NPY and their differential contributions to the seizure-suppressant effect of NPY appears to be largely determined by the seizure model used. Our data suggest that the Y5 receptor is an important receptor for suppressing limbic seizures, predominately outside the hippocampal formation.

HIPPOCAMPAL NPY GENE THERAPY

Transduction pattern of rAAV-NPY vector

The level of transgene expression is dependent on a number of factors. The choice of rAAV serotype influences the cell-type specificity, the design of the expression cassette maintains control over the level and duration of transgene expression and the dose combined with the transduction efficiency of the serotype controls the spread (Burger et al., 2005).

In paper II, III and IV, we used the serotype 1/2 rAAV-NPY vector with NSE promoter, which previously has been shown to cause widespread, strong and long-lasting neuron-specific expression of transgene NPY in the hippocampal formation associated with prominent antiepileptic and antiepileptogenic effects in various animal models of epilepsy (Richichi et al., 2004; Lin et al., 2006). In paper IV, we extended these previous findings by showing that overexpression of transgene NPY in hippocampus also provides anticonvulsive effects during rapid kindling. Together, these studies clearly demonstrate that NPY gene transduction of the hippocampal formation using the serotype 1/2 rAAV vector with NSE promoter can effectively suppress seizure activity.

In paper III and IV, strong transgene expression of NPY was detected throughout the hippocampal formation, covering all cell layers of the DG, CA areas and proximale subiculum. In paper II, where adult rats received a single injection of the rAAV-NPY vector, transgene NPY expression was more restricted and confined towards the injection site in CA1 and subsequent recording site. In agreement with previous results (Richichi et al., 2004), transgene NPY expression reached maximal expression levels within 3 weeks after viral injection and remained stable for at least 3 months. In contrast, slices from animals injected with the rAAV-empty vector showed only few NPY-positive cells (paper II, III and IV). These cells were scattered throughout the hippocampal formation, most numerable in the hilus of the DG, and resembled NPY containing GABAergic interneurons.

Additional evaluation of transgene expression in slices not used for electrophysiology demonstrated that transgene NPY was retained within the borders of the hippocampal formation. Transgene NPY was restricted to neurons and fibers, and further evaluation of neuronal tropism by evaluating rAAV-GFP vector transduction pattern, revealed that transgene expression was restricted within cell soma and processes of virtually all neurons within the hippocampal formation including principal projection neurons and interneurons (paper II and III). Such confined expression of transgene NPY might be advantageous as it reduces the likelihood of possible side effects initiated from areas outside the hippocampal formation. For example, rAAV-mediated expression of transgene NPY in the rat hypothalamic paraventricular nuclei results in obesity (Tiesjema et al., 2007a; Tiesjema et al., 2007b).

Basal synaptic transmission and short-term synaptic plasticity

As the viral vector was constructed with the prepro-NPY cDNA sequence, an interesting question was whether transgene NPY is released via similar mechanisms as endogenous NPY. Normally, neuropeptides are preferentially released during high frequency neuronal activity (i.e. high firing rates) (Whim and Lloyd, 1989; Hökfelt, 1991; Vilim et al., 1996) and studies have shown that endogenous NPY can be released during seizure-like activity (Husum et al., 2002; Sun et al., 2003). Thus, if transgene and endogenuos NPY share similar release mechanisms, one should assume that only a small fraction of total transgene NPY content should be present in the extracellular spaces during normal conditions.

Input-output relationship of fEPSPs did not reveal any changes in basal synaptic transmission in Schaffer collateral-CA1 synapses in either naïve or kindled slices pretreated with rAAV-NPY as compared to control conditions (paper II and IV). This could reflect that transgene NPY does not provide any significant tonic inhibition on glutamate release and suggests that transgene NPY is either absent or present in low amounts extracellularly. This assumption is further supported by electron microscopy data indicating that transgene NPY is localized within LDCV of neurons (Noe et al., 2008), which is indicative of a similar transport- and release-mechanism as endogenous NPY.

In subicular pyramidal neurons though, we found that mEPSC frequencies were significantly lower in slices from rAAV-NPY treated animals as compared to control slices (paper III). This may actually suggest that transgene NPY, at least in CA1-subicular synapses, can also be constitutively released. Inability to reverse the decreased mEPSC frequency by BIIE0246 application could be explained by involvement of receptors other than Y2 (e.g. Y1 and/or Y5) in mediating transgene effect. However, the age of the animals do not support the involvement of Y5 receptors, as the inhibitory effect of Y5 receptors in proximal subiculum disappear within the first weeks after birth (Ho et al., 2000). Moreover, the NPY receptor subtype(s) that mediate effects of transgene NPY could vary between regions of the hippocampal formation. For example, in granule cells of epileptic animals, BIIE0246 application increases mEPSC frequency (Tu et al., 2005), suggesting Y2 receptor activation by de novo expressed and tonically released NPY from recurrent mossy fiber afferents to granule cells. In normal rats, which lack such NPY expression in mossy fibers, both BIIE0246 and NPY application have no effect on the frequency of mEPSCs recorded in granule cells (Tu et al., 2005). Similarly, NPY application does not alter the frequency of mEPSCs in CA3 pyramidal neurons of naïve rats (McQuiston and Colmers, 1996), despite high expression levels of Y2 receptor in this subregion (Redrobe et al., 1999).

Another reason why we were unable to reverse the decreased mEPSC frequency could be due to homeostatic plasticity. This type of plasticity refers to the capacity of neurons to regulate their own excitability relative to network activity, and is thought to be a compensatory adjustment that occurs over the timescale of

days (Turrigiano and Nelson, 2004). Compensatory changes in mEPSC frequencies have been described to occur after chronic excitation or inhibition (Turrigiano et al., 1998), so it is possible that changes could take place in the rAAV-NPY transduced hippocampal formation since activation of Y2 (and Y5) receptors decreases overall excitability (Vezzani et al., 1999a; Vezzani and Sperk, 2004). Therefore, alteration in mEPSC frequency could reflect chronic, rather than acute changes in the neural network, and for that reason acute application of the Y2 receptor antagonist did not reverse these changes. Moreover, we have no reason to believe that the Y2 receptor antagonist did not work in our experimental setup as alterations in synaptic transmission in the same synapses during high frequency neuronal activity could be blocked by BIIE0246, and long-lasting rAAV-NPY overexpression is not associated with a down-regulation of Y2 receptors in the hippocampal formation (Noe et al., 2008).

In combination with the input-output recordings of fEPSPs in Schaffer collateral-CA1 synapses, we also monitored changes in PPF in these synapses to address whether transgene NPY was altering short-term synaptic plasticity. Minor alterations were observed in rAAV-NPY treated slices from naïve animals (paper II), while short-term plasticity was almost unchanged in rAAV-NPY treated slices from kindled animals (paper IV). In paper II, rAAV-NPY treated slices displayed PPF ratios that were slightly higher as compared to control slices. This effect was sensitive to BIIE0246, which decreased the facilitation ratio. This suggests that short-term plasticity, at least in naïve slices, is slightly affected by transgene NPY. This could be a result of small amounts of transgene NPY being constitutively released from neurons, which does not interfere with basal synaptic transmission (as measured by fEPSPs).

Volume transmission – a beneficial antiepileptic mechanism
In the next series of experiments, we observed that during the time course of HFS of Schaffer collateral-CA1 synapses the progressive attenuation of fEPSPs was slower in rAAV-NPY treated slices as compared to control slices (paper II). In CA1-subicular synapses we observed a similar trend that HFS-induced EPSCs decayed faster in rAAV-NPY treated slices after application of Y2 receptor antagonist, but not in control slices (paper III). Thus, several results indicate that transgene NPY can be released during high frequency neuronal activity, whereby it suppresses concomitant glutamate release and decreases the rate of glutamate depletion in activated synapses.

The strongest evidence that transgene NPY is released during HFS was obtained from the two-pathway stimulation experiments, where HFS of one afferent pathway decreased the amplitude of EPSC elicited by a single pulse stimulation of another afferent pathway impinging onto the same subicular neuron (paper III). This effect was totally blocked by Y2 receptor antagonist application, suggesting that it was due to transgene NPY, and shows that transgene NPY may act as a volume transmitter.

This particular release pattern of transgene NPY may have some importance for hippocampal NPY gene therapy in epilepsy. First, it shows that transgene NPY can suppress glutamate release via Y2 receptors, which are strong regulators of hippocampal excitability. Second, it demonstrates that NPY may preferentially be released during pathophysiological conditions characterized by increased neuronal high frequency firing, such as seizures, which will reduce the risk of side effects. Third, it shows that released transgene NPY can inhibit glutamate release even in distant synapses by volume transmission. Such volume transmission may allow transgene NPY to reach receptors far from the release site and influence large areas and may prevent involvement of synapses not yet recruited in epileptic activity, thereby limiting their involvement and contribution to seizures. These effects may significantly contribute to the antiepileptic effects of NPY gene therapy using rAAV-NPY vectors (Richichi et al., 2004; Lin et al., 2006; Noe et al., 2008). Noticeably, the EEG pattern and the behavioral seizures during rapid kindling observed in paper IV can also be largely explained by such release pattern of transgene NPY. We observed that the threshold for inducing focal epileptiform activity was unaltered in rAAV-NPY treated animals. This could indicate that transgene NPY is present in low amounts in the extracellular spaces, and thereby does not provide any tonic inhibition. However, once seizure activity was induced, it promoted the release of transgene NPY, which reduced the time spent in seizures by suppressing glutamate release.

Long-term synaptic plasticity

The release pattern of transgene NPY raises the possibility that not only seizures, but also normal physiological processes, such as long-term synaptic plasticity (i.e. LTP), which also depend on high frequency neuronal activity, can be affected by transgene NPY. Indeed, in both Schaffer collateral-CA1 synapses (paper II) and CA1-subicular synapses (paper III), we found that LTP was significantly reduced in rAAV-NPY treated slices as compared to control slices. This effect appeared to be dependent on transgene NPY acting via Y2 receptors, as application of the BIIE0246 prior to tetanization increased the magnitude of LTP to levels of control slices (paper II). Although, we did not apply BIIE0246 to control slices, it is unlikely that the effect of BIIE0246 can be attributed to blockade of signaling exerted by endogenous NPY, since in NPY KO mice (NPY-/- mice) neither paired-pulse, HFS-induced fEPSPs, nor input-out relationships were altered in CA1 (Baraban et al., 1997). Also, in naïve rat hippocampal slices perfused with BIIE0246, fEPSPs in CA1 remained unchanged (El Bahh et al., 2002), even though Y2 receptors are abundantly expressed in this area (Redrobe et al., 1999).

As we were able to rescue LTP by applying the Y2 receptors antagonist prior to tetanization, inhibition of voltage-gated Ca^{2+}-channels via Y2 receptor appears to be the most dominant mechanism by which NPY suppresses LTP in CA1 (and proximal subiculum). However, we cannot exclude partial involvement of both

Y1 and Y5 receptors, as these receptors in hippocampal synaptosomes, can inhibit presynaptic Ca^{2+}-influx and glutamate release similarly to Y2 receptors (Silva et al., 2001; Silva et al., 2003). Thus, the most straightforward explanation for impaired LTP in CA1 synapses is that transgene NPY inhibits presynaptic glutamate release onto dendrites on CA1 pyramidal neurons, which leads to fewer NMDA receptor-mediated AMPA receptor insertions into the postsynaptic membrane. This coincides with studies showing that NPY decreases presynaptic glutamate release by reducing Ca^{2+}-influx into axonal terminals of principal glutamatergic neurons (Colmers et al., 1988; Klapstein and Colmers, 1993; Qian et al., 1997). Similar mechanisms were also suggested by Whittaker *et al.* (1999), who showed that NPY injected intracerebroventricularly prior to LTP induction resulted in an inhibition of the induction and maintenance of perforant path LTP *in vivo*.

Likely, transgene NPY-mediated inhibition of presynaptic glutamate release may play a significant role during LTP induction, but the effect of transgene NPY could be more complex. This is reflected by the observations that the induction rate of LTP in both CA1 (paper II) and subiculum (paper III) did not differ between rAAV-NPY and rAAV-empty slices, and by the observation that exogenous NPY could totally block the induction of LTP and reduce already potentiated fEPSP magnitude when NPY was applied after tetanus (paper II). Therefore, it is possible that small constitutive release of transgene NPY may partially interfere with LTP induction, while additional, HFS-induced release of transgene NPY could cause a decrease in the magnitude of potentiated responses.

In proximal subiculum, the reasons for impaired LTP in CA1-subicular synapses are complicated by the fact that two different forms of LTP exist in these synapses. In the majority (60-75%) of subicular pyramidal neurons, the so-called bursting cells (Kokaia, 2000; Wozny et al., 2008), LTP induction seems to be independent of postsynaptic depolarization and postsynaptic calcium influx, whereas in so-called regular firing cells, LTP induction seems to be both NMDA receptor-dependent and postsynaptic depolarization-dependent (Wozny et al., 2008). Although, we did not distinguish between these subtypes of subicular pyramidal neurons (paper III), transgene NPY released by HFS may interfere with presynaptic calcium influx, resulting in weaker LTP in both types of cells.

Transgene NPY - good or bad for memory performance?

The results of the LTP experiments in naïve brain slices (paper II and III) raise some concerns about the clinical applicability of a rAAV-NPY gene-based therapy. Impaired LTP in the hippocampal formation could reflect a possible deficit in learning and memory function (Morris et al., 1987; Morris et al., 1990; Lynch, 2004), and therefore represent a potential side effect of such antiepileptic treatment.

In paper II, the two-platform spatial discrimination water maze test revealed a transient learning deficit in rAAV-NPY treated rats as compared to control animals. This learning deficit was mostly manifested at days 3 and 4. However, during the following days rAAV-NPY injected rats performed as control animals when assessed by the number of correct choices. These data indicate that rAAV-NPY treated animals could still acquire memory but had a delayed process of learning. To date, no learning and memory tests have been performed in mice overexpressing NPY (Thiele et al., 1998; Kaga et al., 2001), but transgene rats selectively overexpressing NPY in CA1 neurons had impaired ability in finding the hidden platform in Morris water maze during 4 days of trials (Thorsell et al., 2000). Since no further trials were performed in that study, it is unclear whether these animals would improve their performance on consecutive days of testing. However, similar rats had completely ameliorated their learning and memory deficit at one-year of age (Carvajal et al., 2004), and more recent studies failed to show any deficits in passive avoidance learning tests after NPY infusion into the CA3 region of rats (Ishida et al., 2007).

These data indicate that increased levels of NPY can lead to certain inhibitory effects on learning and memory. However, contradictory to these findings, intraventricular or direct injection of NPY into the hippocampus of mice has been shown to improve memory processing as demonstrated by enhanced memory retention for T-maze footshock avoidance test training (Flood et al., 1987; Flood et al., 1989). This effect was only evident when NPY was administered immediately after training, one week prior to testing. The reason for the discrepancy in comparison to this study is uncertain, but it is likely that the timing of NPY bioavailability (either release from the tissue, or applied exogenously) during the time-course of a learning process plays a central role by which NPY can modulate memory processing. In general, the effect of NPY on cognitive function seems to be more pronounced in the initial phase of the learning process but the animals do learn the task eventually.

Interestingly, in a most recent study, no learning deficit was observed in naïve rats injected with rAAV-NPY vector with cytomegalovirus (CMV)-chicken ß-actin (CBA) recombinant promoter (Noe et al., 2008) despite similar experimental conditions as were used in paper II. It is unclear why different promoters may differentially affect the functional outcome of NPY overexpression on learning and memory performance. One possibility is that different promoters (NSE versus CMV-CBA) provide different expression levels of transgene NPY in subpopulations of projection neurons and/or interneurons. This may also be the main reason for some conflicting results obtained in a gene therapy study using a rAAV vector containing a NMDA receptor 1 (NMDAR1) antisense subunit, where focal seizure sensitivity was either increased (with tetracycline-off regulatable promoter) or decreased (with CMV promoter) after transduction of the rat inferior collicular cortex (Haberman et al., 2002). As claimed by the authors in this study, the

contrary results were due to changes in promoter design that resulted in different transduction ratios between inhibitory interneurons and primary output neurons (Haberman et al., 2002).

Together, these studies demonstrate that different promoters might have different effects on possible side effects, and indicate that individual promoters may have different therapeutic profiles. Thus, detailed studies examining the links between various promoters, learning and memory function and antiepileptic effect are clearly required.

One main concern of using rAAV-NPY gene therapy in clinical applications is the risk that transgene NPY may impair cognitive function, which is usually already compromised in epilepsy patients (Helmstaedter et al., 2003; Elger et al., 2004). In surgically resected human hippocampal specimens from TLE patients, the ability to express LTP is markedly reduced. Similarly, in animals, isolated CA1 slice preparations exposed to repeated seizure-like activity can totally lose the ability to generate LTP (Hu et al., 2005), and several studies have demonstrated that both electrical and chemical kindling can severaly attenuate LTP *in vitro* (Leung and Wu, 2003; Schubert et al., 2005) and induce spatial memory deficits (Leung and Shen, 1991; Leung et al., 1994; Mortazavi et al., 2005).

Therefore, to mimic more closely the clinical situation, we also explored whether LTP was affected in the slices exposed to 40 rapid kindling stimulation-induced seizures (paper IV), which trigger a process of epileptogenesis and leads to permanent hyperexcitability in the hippocampus (Elmér et al., 1996). We observed that rapid kindling itself significantly impaired LTP in rAAV-empty treated slices, but to similar levels as in slices from rAAV-NPY treated slices. Thus, transgene NPY does not further attenuate LTP in slices exposed to repeated stimulation induced seizure activity, which might suggest that rAAV-NPY therapy in the epileptic brain may not exacerbate the magnitude of memory deficit already existing in epileptic patients. Still, the detailed mechanisms of why transgene NPY limit seizure duration without affecting LTP need to be further investigated, and testing of different promoters are required (as discussed above).

Neurogenesis and GABAergic transmission

Two mechanisms that were not addressed in the papers and manuscripts, but which are likely affected by transgene NPY in hippocampus are neurogenesis and GABAergic transmission. These mechanisms could be important determinates of successful gene therapy in epilepsy as they may either ameliorate or deteriorate the general condition after treatment. Therefore, these aspects will be briefly discussed.

Neurogenesis: It is hypothesized that the maintenance of hippocampal learning and memory function requires continuous addition of newly functional neurons into the granule cell layer circuitry (Shors et al., 2001; van Praag et al., 2002; Jessberger

and Kempermann, 2003; Aimone et al., 2006). However, during the chronic phase of TLE, neurogenesis substantially decreases in the dentate gyrus (Hattiangady et al., 2004; Kralic et al., 2005). Thus, it is possible that cognitive impairment in patients with a longer duration of intractable TLE could be at least partially linked to diminished dentate gyrus neurogenesis, although such cause-effect-relationship needs to be further evaluated (Hattiangady and Shetty, 2008).

Since NPY has been shown to strongly stimulate neuronal proliferation in the subgranular zone of the dentate gyrus via Y1 receptor mechanisms (Howell et al., 2005; Howell et al., 2007), augmentation of neurogenesis could also be a possible outcome of hippocampal NPY gene therapy. From this perspective, it is intriguing to think whether incorporations of additional new neurons might eventually influence the disease process and also memory performance.

If a major fraction of the newly born cells are incorporated inappropriately in the dentate hilus or molecular layer by aberrant migration, as observed in several animal models of epilepsy (Scharfman et al., 2000; Pierce et al., 2007), this could exacerbate the epileptic condition. This atypical migration pattern has been suggested to contribute to network hyperexcitablity and memory and learning disturbances (McCloskey et al., 2006; Parent, 2007; Scharfman and Gray, 2007). Alternatively, if the majority of newborn cells are incorporated in the granule cell layer, this could positively interfere with the disease process by reducing hyperexcitability. Jakubs and colleagues (2006) described that newborn cells situated into the granule cell layer of rats exposed to status epilepticus receive reduced excitatory drive and exhibits an enhanced inhibitory drive, suggesting that these cells may restrain seizure generation and perhaps even alleviate learning and memory impairments.

From these two points of views, the functional outcome of increased neurogenesis will likely be dependent on the behavior and connectivity of the newly born neurons (for review, see Hattiangady and Shetty, 2008), but studies need to establish a casual link between transgene NPY and increased neurogenesis.

GABAergic transmission: Outside the hippocampal formation in the arcuate nuclei, NPY has been found to decrease the firing rate and hyperpolarize GABAergic interneurons, whereby it presumably inhibits GABA release from these neurons (Acuna-Goycolea et al., 2005). In the hypothalamus, NPY inhibits GABA-mediated spontaneous inhibitory input onto hypocretin neurons (Fu et al., 2004), and both spontaneous and evoked GABAergic input onto reticular and ventrobasal nucleus neurons of the thalamus is reduced by NPY probably via inhibition of Ca^{2+}-influx from voltage-gated Ca^{2+}-channels (Sun et al., 2001b; Sun et al., 2001a). Thus, outside the hippocampal formation GABAergic transmission may be modified by NPY.

Currently, our knowledge of NPY's effect on GABAergic interneurons and transmission in the hippocampus is very limited (Sperk et al., 2007), and no stud-

ies have yet addressed the effect of transgene NPY on inhibitory networks. Electrophysiological recordings of hilar interneurons, presumably NPY-positive, have demonstrated the presence of a NPY-sensitive G-protein-coupled inward rectifying potassium current (GIRK) dependent on Y1 receptor activation (Paredes et al., 2003). This has led to speculations that the Y1 receptor may function as an autoreceptor on subpopulations of hilar interneurons that can be inhibited by NPY. Such inhibition of inhibitory interneurons could be one of several possible explanations for the proconvulsive action of Y1 receptor (Gariboldi et al., 1998; Benmaamar et al., 2003). Importantly, in relation to NPY gene therapy, these studies demonstrate that viral vectors not stimulating the Y1 receptor could be more efficient. Therefore, derivates of the NPY peptide, which have preferential affinity for the Y2 and/or Y5 receptors, should be tested. Such an attempt has already been successfully completed using the NPY13-36 C-terminal fragment of NPY inserted into a rAAV vector for preferential Y2 receptor activation (Foti et al., 2007), but more studies should specifically address the question whether viral vectors aimed for specific receptor activation are more beneficial in suppressing seizures.

Our own (unpublished) data indicate that exogenously applied NPY, acting via Y2 receptors, has an effect on both excitatory and inhibitory transmission onto a subpopulation of inhibitory interneurons in the dentate gyrus. Whether this dual action of NPY leads to net inhibition or excitation of these interneurons is uncertain, but it raises the possibility that transgene NPY may actually affect GABAergic interneurons in a way that might not be feasible when considering seizures suppression. Thus, studies evaluating GABAergic transmission and synaptic plasticity in rAAV-NPY gene therapy are required.

Clinical prospectives of NPY gene therapy in epilepsy

So far no clinical trials of in vivo gene therapy in epilepsy have been undertaken, but based on the promising results of rAAV-NPY gene therapy in animal models of epilepsy, a protocol for a human phase I clinical trial is subject to approval discussion by the Food and Drug Administration (FDA) (Löscher et al., 2008). A similar trial in Parkinson's disease patients using a rAAV-GAD vector has already been performed with a positive outcome, and shows that AAV-mediated gene transfer can be done safely in the human brain with no evidence of toxic effects or adverse events for at least 1 year after treatment (Kaplitt et al., 2007). This suggests that *in vivo* gene therapy in the human adult brain might be safe for various neurodegenerative diseases. Most likely, the first clinical trial in epilepsy using rAAV-NPY vector will be aimed towards patients with intractable TLE, which are already considered candidates for surgical dissection of the pathological tissue. Thus, viral delivery at stages prior to resective surgery will allow evaluation of treatment efficacy and potential side effects over a period before tissue resection, if still required.

CONCLUDING REMARKS

NPY is a neuropeptide involved in various and essential brain functions, most of them unexplored in details. Therefore, the therapeutic relevance of hippocampal NPY gene therapy and approach to human epilepsy will highly depend on possible unwanted side effects in animal models. Still, the evidences from animal seizure models are clear and point toward beneficial antiepileptic effects, and focal viral delivery seems to be well tolerated at least in the short term in patients. This thesis provides evidences that learning and memory deficits could be a potential side effect of hippocampal NPY gene therapy, although it does not appear detrimental for hippocampal function. For this reason, NPY gene therapy still represents a novel treatment strategy in epilepsy with hope of successful management of intractable seizures in the near future.

EXPERIMENTAL METHODS

Animals

All experimental procedures were approved by the local Ethical Committee for Experimental Animals, and followed guidelines in accordance with European Community Council Directive for the Care and Use of Laboratory Animals. Animals were kept in standard cages on a 12-h light/dark cycle with *ad libitum* access to laboratory food and water.

In paper I, NPY receptor deficient mice, Y2-/- and Y5-/-, were generated as previously described (Naveilhan et al., 1999; Naveilhan et al., 2001), and were maintained on a mixed genetic background (BALB/c x 129/SvEv, 50%), and generated by mating heterozygotes (i.e. Y2-/+ or Y5-/+) for the receptor mutation. Double receptor deficient mice, Y2Y5-/-, were generated by mating homozygotes (Y2-/- and Y5-/-) as separate breeding lines. WT control mice had similar genetic background as both single and double KO mice (for details see paper I).

In paper II and IV, adult Sprague Dawley (SD) rats, weighing 230-350 g at the beginning of the experiments, were used. These rats were purchased from general supplies. In paper III, neonatal SD rats derived from time-mated female SD rats were used. Pups underwent surgery on postnatal day 2-4, and were kept together with their mothers until weaning approximately three weeks after birth.

Viral vectors

The rAAV vectors used in paper II, III and IV, were designated as rAAV-NPY, rAAV-empty and rAAV-GFP according to their construct. In brief, the human pre-pro-NPY cDNA was subcloned into an expression cassette made of the rat NSE promoter, woodchuck post-translational regulatory element (WPRE) and a bovine growth hormone polyA (BGHpA) signal, which was flanked by AAV2 inverted terminal repeats (pAM/NSE-NPY-WPRE-BGHpA) (Fig. 4). A similar expression cassette without any transgene (pAM/NSE-empty-WPRE-BGHpA) served as the control (empty) vector, whereas an AAV expression cassette containing enhanced GFP (pAM/NSE-eGFP-WPRE-BGHpA) was used to evaluate viral transduction. Plasmids were cloned into the backbone of a chimeric AAV vector having a mix of rAAV serotype 1 and serotype 2 capsid helper plasmids and purified by heparin affinity columns, as the rAAV2 capsid proteins retained the heparin-binding domain (for details about construction and purification of vectors see During et al., 2003; Richichi et al., 2004). For all injections, the viral titer was 1.0×10^{13} genome copies per ml as determined by quantitative PCR of rAAV vector genomes, apart from the behavioral study in paper II, where the titer was 5.4×10^{13} genome copies per ml.
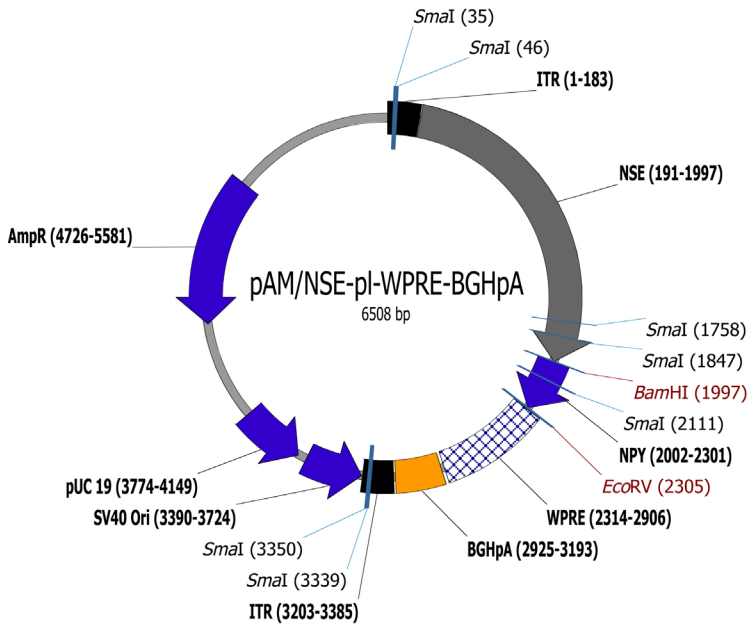
Figure 4. Viral construct of pAM/NSE-NPY-WPRE-BGHpA termed rAAV-NPY in paper II, III and IV. The construct of the rAAV-empty and rAAV-GFP vectors contained similar elements.

Injection of viral vectors

Before injections of viral vectors, adult rats (paper II and IV) were anesthetized with a ketamine (80 mg/kg) xylazine (15 mg/kg) mixture or Equithesin, whereas neonatal rats (paper III) were anesthetized using hypothermia. Rats were placed in a stereotaxic frame, and vector suspension (rAAV-NPY, rAAV-empty or rAAV-GFP) was injected through drill holes made in the skull using a thin glass micropipette attached to a 5 μl Hamilton syringe.

In paper II, rats used for electrophysiology received a unilateral injection of 1 μl vector suspension into the dorsal hippocampus (toothbar: -3.3; AP -3.3, ML +1.8, DV -2.4), whereas rats used for behavioral study received a bilateral injection (3 μl at each injection site) into ventral (toothbar: -2.5; AP: -6.0, ML: ±5.0, DV: -4.5) and dorsal (AP: -3.1, ML: ±2.0, DV: -3.2) hippocampus. In paper III, injection of 0.5 μl vector suspension was conducted bilaterally (AP -1.5, ML ± 1.9, DV -1.8) into the hippocampus. In paper IV, the vector suspension (1 μl at each site) was injected bilaterally at one site in dorsal (toothbar: -3.3; AP - 3.3, ML ± 1.8, V - 2.6) and at two sites in ventral (AP - 4.8, ML ± 5.2, V - 6.4 and - 3.8) hippocampus. Ref-

erence points for all coordinates (in mm) were calculated from bregma, midline and dura according to Paxinos and Watson (1996).

To minimize backflow of viral particles, the vector suspension was always injected at slow speed (0.1-0.2 μl per min) and the injection needle was left in place for several minutes. Animals were allowed to recover for at least one week before any other experimental procedures were undertaken, and electrophysiological experiments were not initiated until at least 3 weeks after viral delivery to ensure maximal expression of the transgene.

Slice preparation

Animals were killed by decapitation, and their brains were immediately removed and immersed into ice-cooled aCSF (paper I) or MaCSF (paper II, III, and IV) consisting of (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 26 $NaHCO_3$, 1.0 NaH_2PO_4, 11 glucose (pH 7.4, 296 mOsm); and 195 sucrose, 2.5 KCl, 0.5 $CaCl_2$, 7.0 $MgCl_2$, 28 $NaHCO_3$, 1.25 NaH_2PO_4, 7.0 glucose, 1.0 ascobate and 3.0 pyruvate (pH 7.4, 300 mOsm), respectively. Both solutions were constantly equilibrated with 95% O_2/ 5% CO_2. Please notice, that the concentration of sucrose is indicated as 225 mM in paper II, but should correctly be 195 mM. Since MaCSF solution significantly improves the quality of slices, thus making more viable cells for electrophysiology, this medium is now our preferable medium for dissection and cutting (but see Kuenzi et al., 2000). Following dissection of the hippocampal formation, slices were cut on a vibratome. For whole-cell patch-clamp and field recordings the desired thickness of slices was 210-250 μm and 350-450 μm, respectively. Slices were maintained for at least one hour in aCSF at room temperature before individual slices were transferred to the recording stage (Olympus BX51 microscope fitted with Luigs & Neumann equipment, Ratingen, Germany) perfused with gassed aCSF at 2 ml per min.

Electrophysiology

To investigate alterations in synaptic transmission and plasticity, two common electrophysiological techniques were used. In practice, field and whole-cell patch-clamp recordings can be a difficult task to perform, but for most the main difficulty is to understand how is works.

Principles of field recording: Extracellular signals generated from a single neuron are hardly measurable. However, the principal neurons in hippocampus are arranged so they both receive synaptic inputs in the same area and are oriented in the same direction. In this way, the extracellular signals do not cancel out, but rather add up to give a signal that can be measured by a field electrode placed in the extracellular spaces (Fig. 5A). This sum of signals is the field potential. When stimulating Schaffer collaterals (CA3 pyramidal axons) activating excitatory synapses impinging onto dendrites of CA1 pyramidal neurons in the hippocampus,
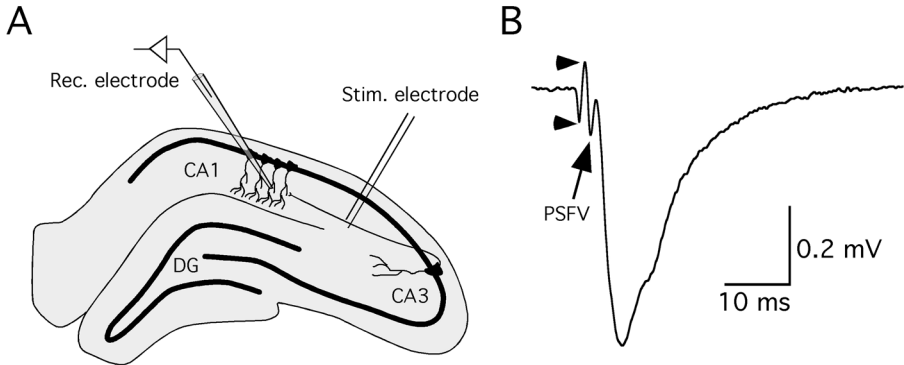
Figure 5. (A) Typical experimental setup for fEPSP recording in a hippocampal slice with extracellular recording electrode and stimulation electrode placed in CA1 stratum radiatum. (B) Field EPSP recorded in CA1 stratum radiatum. The first negative and positive deflection is a voltage artifact (two small arrows) caused by the stimulation electrode. The next negative voltage deflection is the presynatic fibervolley (PSFV) due to action potentials (generated in afferent CA3 axons) passing by the recording electrode. The PSFV is followed by a prominent fEPSP.

a current sink is generated in stratum radiatum. This signal is called the field excitatory postsynaptic potential (fEPSP), and is characterized by a negative voltage deflection (Fig. 5B). The reason for this is; during depolarization of the cell membrane, the flow of ions (primarily the sodium anion) flow into the cell, which is away from the recording electrode. Due to the random orientation of interneurons in the hippocampus, recording inhibitory field potentials is impractical.

Field recordings (Paper I, II and IV): Field EPSPs were recorded with recording pipettes filled with 3 M NaCl (paper I and II) and aCSF (paper IV) having pipette tip resistance of 0.3-1 MΩ. In paper I spontaneous epileptiform bursts were recorded from the CA3 pyramidal layer of the ventral hippocampus (for details see paper I). In paper II and IV, electrically evoked fEPSPs were generated by a bipolar, stainless steel electrode placed in stratum radiatum of CA1 and recorded by a recording electrode placed in the same subfield. The stimulation and recording electrode were separated by approximately 500 μm. To determine slices quality, the input-output relationship of stimulation-induced fEPSPs was examined by plotting the PSFV (mV) against the slope (mV/ms) of the resultant fEPSP at gradually increasing intensities. Slices generating fEPSPs with PSFV/EPSP ratio of more than 1:3, and/or those with maximal amplitudes of less than 1 mV were excluded from the experiments. Test stimuli generating stable submaximal fEPSPs (30-50% of maximal fEPSP) were used throughout the experiments.

Paired-pulse facilitation of fEPSPs were generated by paired stimulations using ISIs of 25, 50, 100 and 200 ms, and calculated as percentage change in initial slope (1 ms) of the second fEPSP as compared to the first (P2-P1/P2 x 100). This facilitation ratio was used to evaluate short-term synaptic plasticity. In paper II, HFS-induced fEPSPs were generated by stimulation trains applied to slices, consisting of 50 stimuli (at 10 or 25 Hz) each. To prevent induction of LTP in stimulated synapses, the specific N-methyl-D-aspartate (NMDA) antagonist, D(-)-2-Amino-5-phosphonopentanoic acid (D-AP5; 50 μM, Tocris) was applied together with aCSF, and to avoid interference with post-tetanic potentiation (PTP) each train was delivered at 5 min intervals.

For LTP experiments (paper II and IV), a 10-20 min fEPSP baseline was recorded (at 0.067 Hz and with 15% acceptable variability) before one HFS train (100 Hz, 1 sec; tetanic stimulation) was applied to slices. Field EPSP responses were recorded for another 60 min, and analyzed by measuring the initial slope (1 ms), normalized to average baseline values and plotted against time. In separated LTP experiments (paper II), the specific NPY Y2 receptor antagonist, (S)-N2-[[1-[2-[4-[(R,S) - 5,11-dihydro - 6(6h) - oxodibenz[b,e]azepin – 11 - yl] – 1 - piperazinyl] – 2 -oxoethyl]cyclopentyl]-acetyl]-N-[2-[1,2-dihydro-3,5 (4H)-dioxo-1,2-diphenyl-3H-1,2,4-triazol-4 yl] ethyl]-argininamide (BIIE0246; 0.3 μM, Tocris), pre-diluted in ethanol and dissolved into aCSF (1:10.000) was applied to slices. In additional LTP studies, exogenous NPY (1 μM; rat synthetic; Schafer-N, Copenhagen, Denmark) was dissolved in 10 ml aCSF and applied directly into the recording chamber. In these experiments, NPY was applied to slices either 10 min before or 20 min after HFS, and to slices not receiving HFS. LTP was considered significant if the mean response, as calculated 10-15 min after HFS, was increased by > 15% from baseline values.

Principles of whole-cell patch-clamp recording: This technique is more refining than field recordings and permits recordings of single neurons. The first step in whole-cell patch-clamp is to attach a recording glass pipette to the membrane of a desired cell. The small tip opening (about 1 μm) of the pipette is pressed against the cell membrane and small suction to the pipette assists forming a high-resistance (gigaohm) seal to the membrane. More suction is applied until the membrane inside the glass pipette ruptures while leaving the seal intact, thus providing access to the cytoplasm of the cell now with the pipette solution in direct contact with the interior of the cell (Fig. 6). With a silver electrode placed inside the pipette, electrical activity can be conducted to an amplifier. In voltage clamp, the voltage is kept constant while observing the current passing the cell membrane, whereas in current-clamp, the current is kept constant while observing the membrane potential changes. In this way, intrinsic membrane properties and synaptic transmission (generated by other neurons) can be measured.
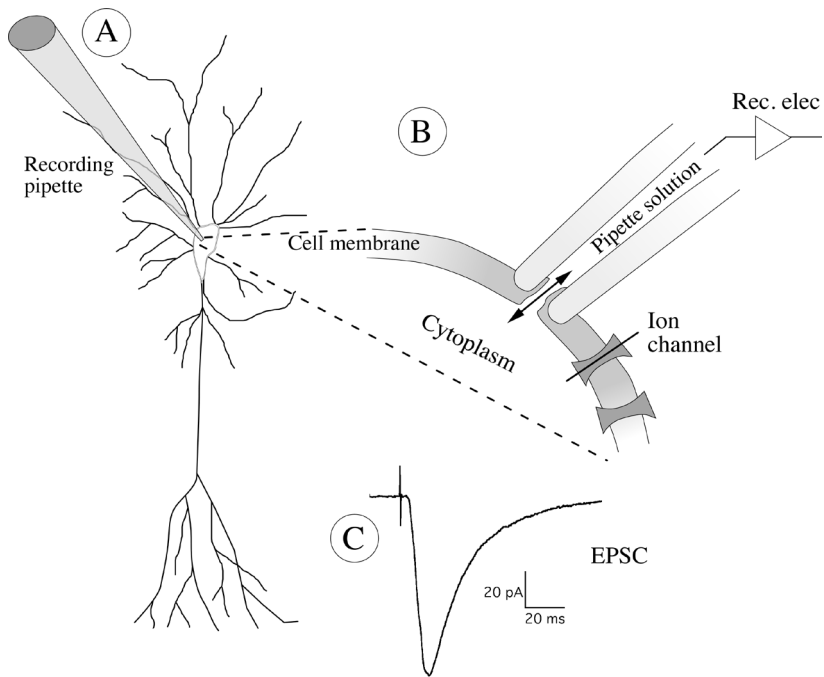
Figure 6. (A) The recording pipette in contact with the membrane of a pyramidal cell. (B) In the whole-cell patch-clamp configuration, the membrane under the pipette tip is ruptured while leaving the seal intact. The pipette solution is now in direct contact with cytoplasm of the cell. Charged ions crossing the cell membrane generate currents which can be measured by the recording electrode. (C) Typical excitatory postsynaptic current (EPSC) recorded by whole-cell patch-clamp.

Whole-cell patch-clamp recordings (Paper III): Whole-cell patch-clamp recordings of subicular pyramidal neurons localized in the proximal subiculum were completed at holding potential of -70 mV (via patch pipette). EPSCs were recorded with pipettes filled with (in mM): 117.5 K-gluconate, 17.5 CsCl, 8.0 NaCl, 10 CsOH-HEPES, 0.2 CsOH-EGTA, 2.0 MgATP, 0.3 Na_3GTP, and 5.0 QX-314 (pH 7.2; 295 mOsm; pipette tip resistance of 4 - 5 MΩ. Biocytin (0.5%) was freshly dissolved in the pipette solution immediately before use. Putative pyramidal neurons were first identified based on their characteristic triangle shaped cell soma and prospectively by immunohistochemistry of biocytin-labeling of recorded cell. Acceptable access resistance was set to 12-25 MΩ as measured by delivery of -5 mV voltage steps, and recordings during which series resistance varied more than 20% were excluded from analysis. For recording of evoked EPSCs, the intensity of the stimulation was always adjusted to give constant and submaximal currents. Miniature EPSCs were recorded with PTX (100 μM; Tocris) and TTX (1 μM; Toc-

ris) in aCSF to block $GABA_A$ receptor activation and action potential generation, respectively. After 3 min baseline recording, BIIE0246 (0.3 μM; Tocris) was applied to slices for 8 min, before mEPSCs were recorded for another 3 min. As a final step, NBQX (5 μM; Tocris) was added to aCSF to block non-NMDA receptors mediated mEPSC.

For LTP recordings, a single stimulation electrode was positioned in the alveus pathway close to CA1. Within 10 min after breaking into the whole-cell configuration, a single HFS train (100 Hz, 1 s) was applied to slices to induce LTP, which was considered significant if the mean amplitude of the responses (4 per min), calculated 10-15 min after HFS, was increased by > 15% from baseline values.

Two-pathway and HFS was accomplished by positioning two stimulation electrodes in the alveus pathway on either side of the recorded cell. In this setup, two independent pathways (termed P1 and P2) were stimulated to activate separated synapses on the same cell, as confirmed by paired stimulations of each pathway (P1-P1, or P2-P2) evoking EPSCs displaying PPF, while EPSCs remained unchanged when paired stimulations were delivered to alternate pathways (P1-P2 or P2-P1). First, one paired-pulse stimulation (with ISI of 850 ms) was delivered to P1 (the first stimulation is termed P1.1 and the next is termed P1.2). After 30 s, this paired-pulse stimulation by P1 was repeated, but with an intermediate HFS train (10 stimulations at 40 Hz) delivered to P2, 300 ms after P1.1 stimulation. This stimulation protocol was repeated 10 times with 1 min intervals. Following, BIIE0246 (0.3 μM; Tocris) was applied to slices for 10 min and alternate stimulations were resumed (10 stimulations each). Percent change [(P1.2-P1.1)/P1.1) x 100] of average EPSCs induced by P1 stimulation were compared between baseline and intermediate stimulations, both before and after BIIE0246 application. The experimental setup is illustrated in figure 3A in paper III. Additionally, the average synaptic response evoked by HFS of P2 was evaluated separately before and after BIIE0246 application by dividing the total area of all ten EPSCs in the train by the area of the first EPSC. In addition, the ratio of EPSC amplitudes between the two first evoked EPSCs during HFS was calculated to evaluate changes in PPF.

Data acquisition

Data from electrophysiology was acquired at 10 kHz and filtered at 2.9 kHz using HEKA amplifier and software (EPC 9 or 10 amplifier, PATCHMASTER, HEKA Elektronik, Lambrecht, Germany) and off-line analysis was performed using FITMASTER (HEKA Elektronik), Igor Pro (Wavemetrics, Lake Oswego, OR) or Mini-Analysis (Synaptosoft, Decatur, GA) software, whenever appropriated.

Epilepsy models

The animal models of epilepsy used in this thesis are briefly presented below, whereas the applied procedures and analysis can be found in the designated papers.

Kainate acid-induced seizures (paper I): Kainate acid (KA) is a neurotoxin and agonist for the KA subtypes of ionotropic glutamate receptors. Due to the highest density of KA in hippocampus, amygdala, perirhinal and entorhinal cortex, KA usually serves as a valuable model of partial seizures and secondary generalization from the limbic focus, reminiscent of TLE in humans. Systemically administration of KA at relative high dose, as used in paper I, induces severe motor seizures, but the insult strongly depends on the treatment protocol used (for further reading see Ben-Ari and Cossart, 2000; Leite et al., 2002).

Conventional kindling (paper I): Kindling is defined as a progressive increase in severity of both EEG discharges and seizure behavior. Initially, with electrodes stereotaxically implanted into the brain, constant and low-intensity electrical stimulation triggers initially focal low-frequency ADs with few or no behavioral responses. But with repeated stimulations, typically once daily, seizure activity spreads together with gradually increasing high-frequency ADs and convulsive behavior (Goddard, 1967; Goddard et al., 1969). In this sense, the kindling model of epilepsy is regarded as a highly suitable model of studying epileptogenesis by the ive development of focal partial seizures into complex partial seizures with secondary generalization.

Rapid kindling (paper IV): As the name says, this model uses shorter intervals between the kindling stimuli ranging from minutes to seconds and is usually completed within one day. Rapid kindling is characterized by progressive excitability during repeated stimulations (often using high-intensity electrical stimulation) as evident by increasing duration of EEG discharges and severity of behavioral seizures. The model is not as laborious as conventional kindling with stimulation once daily, but instead lack spatial resolution for studying kindling epileptogenesis, and the pathological hallmarks have yet not been systematically characterized (Lothman and Williamson, 1994; Elmér et al., 1996).

$0-Mg^{2+}$ (paper I): The $0-Mg^{+2}$-model (or low Mg^{2+}) is an acute model of epilepsy used in hippocampal slices that is highly reproducible and generates spontaneous epileptiform activity. Normally, Mg^{2+} is blocking the NMDA receptor channel, but with removal of Mg^{2+} from aCSF, it allows NMDA receptors to respond directly and strongly to glutamatergic neurotransmission generating paroxysmal discharges (Quilichini et al., 2003).

Immunohistochemistry

An important step in paper II, III and IV was to evaluate and confirm viral expression of transgene NPY in brain slices derived from animals injected with rAAV-NPY and subsequently used for electrophysiology. After recordings, these slices were incubated in 4% paraformaldehyde overnight, rinsed and stored in

anti-freeze. Slices were rinsed, quenched (3% H_2O_2, 10% MeOH in KPBS) and incubated in a 1:5000 dilution of rabbit antiserum to rat NPY (Sigma-Aldrich, Sweden) in 5% normal goat serum in KPBS for either 24 hours at room temperature (paper II, III) or four days at 4°C (paper IV). The latter approach using a long incubation period significantly improves the final staining pattern of NPY in thick slices. Finally, slices were incubated in biotinylated secondary antibody (BA1000; 1:200; Vector Laboratories, Burlingame, CA), the reaction was amplified (Vectastain ABC KIT, Vector Laboratories) and visualized by 3-3'-diaminobenzidine (DAB). For improved quantification and illustration of viral expression and extent of transgene transduction, 30 μm brain sections from either whole brains (paper II, III) or brain slices used for electrophysiology (paper IV) were processed as described above, but instead using primary NPY antibody in a 1:1000 dilution for 24 hours. Native GFP expression was visualized in 30 μm brain sections by epifluorescent illumination (paper II, III).

Statistics

A wide range of statistical tests have been used for data analyses in paper I, II, III and IV. These tests were used whenever appropriate and included: paired and unpaired two-tailed Students t-test, two-way ANOVA followed by post-hoc least squares means t-tests or Bonferonni-Dunn post hoc test, repeated-measures ANOVA, Mann-Whitney test, X^2-test followed by Fisher's exact test, two-tailed Kolmogorov-Smirnov test and log rank test. Level of significance was $p < 0.05$ unless otherwise stated, and values are presented as mean ± SEM. Data collection and analysis were always conducted by investigators blind to group identities of experimental animals and pre-treatment conditions.

Additional methods

Details about work conducted by collaborators outside the lab are not further described here, but can be found in the original papers; *in situ* hybridization and binding study (paper I); behavioral hippocampal learning and memory test (paper II).

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this day isn't really made for patch-clamp. I believe, mainly because of you, **Katie Jakubs**, that all other days were different. I thank you for all your support and compassion, and for having a great mind and always being willing to solve problems whenever needed. The same goes for you, **Irene Kanter-Schlifke.** You have not only been my third and fourth hand, you have also been my second brain throughout the years. Thank you for outstanding support on almost all my projects, for being a nice traveling partner and for all, being the one you are.

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I don't know whether I'm boring, but when **Jan Tønnesen** arrived to the lab, I realized, that he perhaps better represents what it really means to be a Dane. You do not mind lying, enjoy drinking (so you sometimes need accommodation in the basement of A9) and have great humor. But above all, you are open minded, relaxed and at the same time my best sparring partner. These are some of your many qualities that I really appreciate.

In the EEG group, I would also like to thank **Mikael Ängehagen**, **Angelica Andersson** and **Natalia Avaliani**.

I can only admit, that bringing up the theme about Sweden and Denmark is solely due to my great roommates **Pär Thored** and **Robert Iosif**, although, the latter in my mind, is not really a Swede! Our never-ending conversations, discussions and enjoyment of all stupid things that exist between our countries (and on the internet!) have given me so much energy that traveling to Lund every day has been a pleasure. Strange enough, **Pär** now works in Denmark! I perhaps agree, that Sweden is better in sports than Denmark, but as long as they keep on wearing yellow shirts, it can never be as beautiful as if they were wearing red ones. Anyway, I thank you both for all our enjoyable moments, and I wish you good luck in the future. In addition, I should not forget my old roommate **Avto Nanobashvili**. When I arrived to the lab, you taught me everything, anytime. Thanks a lot.

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gic cells become to live in striatum has deeply inspired me. Most peculiarly, you are perhaps the person that most clearly shows signs of being a Swede and not a Dane. You hide some liquor bottles behind the curtains in your office for celebrating great achievements, but we never drink it! To my knowledge this is definitely not a Danish behavior. Nevertheless, because I really enjoy your friendly atmosphere blended beautifully with your scientific brilliance, I suggest we one day drink it together.

My work throughout the years at Restorative Neurology could not have been accomplished without a great team. Thanks to **Zaza**, **Henrik**, **Christine**, **Andreas A**, **Ursula**, **Nuño**, **Susan**, **Carlo**, **Katarina**, **Alexandre**, **Sara**, **Maurizio**, **Irakli**, **James**, **Camilla**, **Jan R**, **Emanuella**, **Ramiro** and especially **Therése Kallur** and **Monica Lundahl**. I would also like to thank all people in the Neurobiology Group, in particular **Josephine Hebsgaard**. I suppose, you are this typical Danish girl that makes most men feel uneasy, because when we observe you, we get the impression that we aren't as hard working as we should be. However, I'm impressed about you, especially your bright talent, and I enjoy being in your company.

At first glance, you would expect that the people at Molecular Psychiatry Unit (MPU) are crazy. The cover art on their former homepage had this great touch of black humor that we love so much in Denmark. I think that's why **Pär Thored** once didn't mind calling them the Mongoloid Parents United. Strikingly, this is the only group that is purely Swedish! Unfortunately, this brilliant art does not exist any more, and this brings me to another point. Humor no one understands is not as tolerated and joyful in Sweden as in Denmark. Anyway, thanks to all at MPU – **Linda**, **Malin**, **Karin**, **Johan**, **Joakim** and **Anders Tingström** – some of you are crazy, and I love it.

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Going back to December 2002, where this acknowledgement started, precisely a month before I went to Lund. Here I meet **Clara**, my beautiful girlfriend and later wife of my life. I can only say, that I have loved you ever since I saw you for the first time and will forever do. You make me happy and I really appreciate that you constantly remind me to talk about things not related to science, although I realize it can be tough. Thanks for your deep love, generosity and support throughout the years. **Anders**, not only are you a wonderful son with eyes of your mother, you are certainly also my infinite love that make me proud of being your dad. Kys kys to both of you…

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Differential suppression of seizures via Y2 and Y5 neuropeptide Y receptors

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Neuropeptide Y (NPY) prominently inhibits epileptic seizures in different animal models. The NPY receptors mediating this effect remain controversial partially due to lack of highly selective agonists and antagonists. To circumvent this problem, we used various NPY receptor knockout mice with the same genetic background and explored anti-epileptic action of NPY in vitro and in vivo. In Y2 (Y2−/−) and Y5 (Y5−/−) receptor knockouts, NPY partially inhibited 0 Mg^{2+}-induced epileptiform activity in hippocampal slices. In contrast, in double knockouts (Y2Y5−/−), NPY had no effect, suggesting that in the hippocampus in vitro both receptors mediate anti-epileptiform action of NPY in an additive manner. Systemic kainate induced more severe seizures in Y5−/− and Y2Y5−/−, but not in Y2−/− mice, as compared to wild-type mice. Moreover, kainate seizures were aggravated by administration of the Y5 antagonist L-152,804 in wild-type mice. In Y5−/− mice, hippocampal kindling progressed faster, and afterdischarge durations were longer in amygdala, but not in hippocampus, as compared to wild-type controls. Taken together, these data suggest that, in mice, both Y2 and Y5 receptors regulate hippocampal seizures in vitro, while activation of Y5 receptors in extra-hippocampal regions reduces generalized seizures in vivo.
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Introduction

Neuropeptide Y (NPY), a 36-amino-acid residue polypeptide, is widely distributed in the central nervous system, including the hippocampus (De Quidt and Emson, 1986; Köhler et al., 1986). NPY belongs to a family of peptides, also including peptide YY (PYY) and pancreatic polypeptide (PP), which exert biological effects via binding to G-protein-coupled receptors (Y1, Y2, Y4, Y5, y6), leading to reduced levels of cyclic AMP (Berglund et al., 2003). In the brain, NPY acts predominantly via binding to Y1, Y2, and Y5 receptors, which are present in many regions, including the hippocampus (Redrobe et al., 1999). There is increasing evidence that NPY plays an important role in regulation of epileptic seizures (Baraban, 1998; Vezzani et al., 1999; Woldbye and Kokaia, 2004). In different rodent models, seizures cause substantial increase in synthesis of NPY as well as changes in expression and binding of NPY receptors in hippocampus and other forebrain regions (Sperk et al., 1992; Mikkelsen et al., 1994; Kopp et al., 1999; Vezzani et al., 1999; Husum et al., 2000, 2004). In hippocampus, single seizures are associated with acute NPY release, while repeated seizures lead to increased basal levels of NPY (Husum et al., 2000, 2002). Seizure-induced increases in synthesis and release of NPY are generally considered to be a compensatory anti-epileptic response. Consistent with this view, transgenic rats overexpressing NPY in hippocampus display less severe kainate or hippocampal kindling seizures than wild-type (WT) controls (Vezzani et al., 2002). Conversely, NPY gene knockout mice develop more severe kainate or pentylenetetrazole seizures (Erickson et al., 1996; Baraban et al., 1997; DePrato Primeaux et al., 2000). Moreover, exogenous NPY application has inhibitory effect in several seizure models (Woldbye and Kokaia, 2004). In vitro, NPY inhibits epileptiform activity in hippocampal and cortical

slices induced by 0 Mg^{2+}, picrotoxin, or electrical stimulation (stimulus-induced bursting; STIB) (Smialowska et al., 1996; Klapstein and Colmers, 1997; Bijak, 1999, 2000; Marsh et al., 1999; El Bahh et al., 2002; Woldbye et al., 2002). In vivo, central administration of NPY suppresses seizures induced by kainate (Woldbye et al., 1997), pentylenetetrazole (Woldbye, 1998), and electrical hippocampal stimulation (Woldbye et al., 1996; Reibel et al., 2000, 2001, 2003; Klemp and Woldbye, 2001; Mazarati and Wasterlain, 2002).

At present, controversy remains regarding which NPY receptor subtypes are responsible for mediating seizure-suppressant effect of NPY. Pharmacological studies in different in vitro and in vivo seizure models using various existing agonists and antagonists for NPY receptors have often generated conflicting results, suggesting either Y2 or Y5 subtypes responsible for NPY action (Klapstein and Colmers, 1997; Woldbye et al., 1997; Bijak, 1999; Marsh et al., 1999; Vezzani et al., 2000; Reibel et al., 2001; El Bahh et al., 2002; Nanobashvili et al., 2004). One of the obstacles for resolving this issue has been a lack of highly selective agonists and antagonists for different NPY receptor subtypes. Diverse species, genetic backgrounds, and epileptic seizure models used in previous studies have also contributed to the existing controversy. To circumvent all these problems, we adopted a gene knockout strategy of loss-of-function for Y2 (Y2−/−), Y5 (Y5−/−), or both (Y2Y5−/−) NPY receptor subtypes in mice with the same genetic background and studied the seizure-suppressant effect of NPY in different in vitro and in vivo seizure models. The objectives of this study were (i) to determine which receptor subtypes mediate inhibitory effect of NPY on focal hippocampal and generalized seizures in mice and (ii) to explore whether compensatory changes in mRNA expression or binding sites for different NPY receptor subtypes occur in various receptor knockout strains, thus possibly altering the anti-epileptic effect of NPY.

Materials and methods

Animals

The Y2 or Y5 receptor genes were disrupted in mouse TC1 (129/SvEv) embryonic stem cells, and mice deficient in the Y2 receptor (Y2−/−), the Y5 receptor (Y5−/−), or both receptors (Y2Y5−/−) were generated as previously described (Naveilhan et al., 1999, 2001). The Y2 and Y5 receptor mutations were maintained on a mixed genetic background (BALB/c × 129/SvEv, 50%; B&K AB, Sweden). NPY receptor deficient and WT control mice were obtained either by mating heterozygotes for the NPY receptor mutations (Y2−/− and Y5−/−; littermates) or mating homozygotes as separate breeding lines for the NPY receptor mutations or WT from 1st generation offsprings (Y2Y5−/−; age-matched). Both male and female adult mice (16–36 g) were used, kept in standard cages on a 12-h light/dark cycle with access to laboratory food and tap water ad libitum. All experiments were performed according to the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the local Swedish Ethical Committee. Mice were genotyped by PCR (Fig. 1) using the primers as described previously for Y2−/− (Naveilhan et al., 1999) and Y5−/− (Naveilhan et al., 2001) mice, with 35 cycles at 92°C for 60 s, 50°C for 60 s, and 70°C for 90 s.
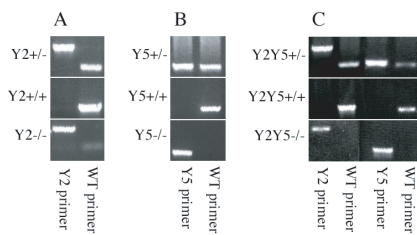

Fig. 1. PCR genotyping of knockout mice. White band demonstrates respective PCR-generated mutant DNA. (A) Y2+/− (heterozygous), Y2+/+ (WT), and Y2−/− (homozygous) knockout genotype. (B) Y5+/− (heterozygous), Y5+/+ (WT), and Y5−/− (homozygous) knockout genotype. (C) Y2Y5+/− (heterozygous for both receptors), Y2Y5+/+ (WT), and Y2Y5−/− (homozygous) double knockout genotype.

Effects of NPY on 0 Mg^{2+}-induced bursting in CA3 of hippocampal slices

Mice were anesthetized with halothane, decapitated, and their brains were rapidly removed. The hippocampus was dissected out, and transverse slices were cut (450 μm thick, Vibratome 1000 Plus, Vibratome Company, USA) in ice-cold artificial cerebrospinal fluid (aCSF) consisting of (in mM): 119 NaCl, 2.5 KCl, 1.3 MgSO$_4$, 2.5 CaCl$_2$, 26.2 NaHCO$_3$, 1 NaH$_2$PO$_4$, and 11 glucose, gassed with 95% O$_2$ and 5% CO$_2$, as previously described (Kokaia et al., 1998). After storage at room temperature for at least 1 h in a submerged chamber containing gassed aCSF, the slices were transferred to the submerged recording chamber, continuously perfused at a rate of 2 ml/min at 34°C with gassed aCSF as above but reduced calcium concentration (1.6 mM) and devoid of MgSO$_4$ (0 Mg-aCSF; Woldbye et al., 2002).

Spontaneous epileptiform bursts were recorded as extracellular field potentials from the CA3 pyramidal layer of the ventral hippocampus using a glass pipette containing a solution of 3 M NaCl (resistance 0.3–1 MΩ). Field potentials were amplified and filtered at 2.9 kHz, sampled at 10 kHz with an EPC-9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany), and stored on a G4 Power Macintosh computer. Traces were analyzed using Igor Pro software (WaveMetrics, Inc., version 4.0, Oregon, USA) with binomial smoothing algorithm (factor 30).

Following equilibration of hippocampal slices in the recording chamber for 40–60 min, when the frequency of arising spontaneous epileptiform discharges reached stable levels, NPY (1–4 μM; human/mouse synthetic, #H-6375, Bachem AG, Bubendorf, Switzerland) or free acid NPY (1 μM; human/mouse synthetic, #H-3322, Bachem AG) dissolved in 0 Mg-aCSF was introduced into the perfusion medium once for 10 min. After termination of peptide application, all slices were washed with 0 Mg-aCSF for at least 30 min. Standard (1 μM) as well as relatively high concentrations (2–4 μM) of NPY were tested to ensure that potential differences between genotypes were not caused by unspecific genetic changes affecting the concentration reached in the slices. Concentrations of a similar dose-range were used in a previous 0 Mg^{2+} study using Y5−/− mice (Marsh et al., 1999). No evidence of dose-dependency was observed in any of the genotypes regarding the magnitude of NPY's anti-epileptiform effect. The mean frequency of epileptiform discharges was determined at 2 min intervals, and the percentage change from

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individual baseline (last 10 min before NPY or free acid NPY application) was calculated for each slice (Woldbye et al., 2002). In addition, the peak effect of drug application was evaluated based on the maximal percentage change from individual baseline values during 1 min intervals (Nanobashvili et al., 2004).

Kainate seizures: NPY receptor knockout and WT mice

Y2−/− (n = 8), Y5−/− (n = 5), and Y2Y5−/− (n = 28) mice were injected subcutaneously (s.c.) with kainate dissolved in isotonic saline and adjusted to pH 7.4 (40 mg/kg; #2020, Ocean Produce International, Canada). Pilot studies showed it was necessary to use a 40 mg/kg dose of kainate in order to produce motor seizures in a high number of WT animals. Separate WT controls for Y2−/− (n = 6), Y5−/− (n = 8), and Y2Y5−/− (n = 24) mice were used. To exclude a possible effect of sex on response to kainate, we compared seizure parameters in male and female Y2Y5−/− mice with those of WT animals of corresponding sex (male: Y2Y5−/−, n = 17, WT, n = 8; female: Y2Y5−/−, n = 11, WT, n = 16). We did not observe sex differences in any seizure parameter tested (data not shown), and, therefore, the sexes of each genotype were pooled together for further analysis. The Y2Y5−/− mice were not different in weight from their age-matched controls (female mutants and WT [mean ± SEM]: 22.4 ± 1.2 g and 21.3 ± 1.1 g, respectively; male mutants and WT: 30.2 ± 1.4 g and 32.8 ± 2.1 g, respectively).

The animals were placed in individual boxes (10 × 10 × 10 cm) and were observed during 90 min for motor seizures defined as continuous forelimb clonic activity lasting for at least 5 s. Mice were decapitated immediately after the observation period. Seizure severity was scored according to a modified rating scale of Marsh et al. (1999): 0 = no seizure activity, 1 = staring or facial movements, 2 = head nodding or isolated twitches, 3 = motor seizure with forelimb clonus, 4 = motor seizure with rearing, 5 = motor seizure with loss of posture or status epilepticus (at least 10 min of continuous motor seizure activity), 6 = death. The seizure score and latencies to first motor seizure (when scored as at least 3) and motor seizure with loss of posture were determined for each genotype by an observer blinded to the identity of the mice.

Kainate seizures and L-152,804

Intraperitoneal (i.p.) injection of the selective non-peptide Y5 receptor antagonist L-152,804 (Kanatani et al., 2000) has previously been shown to modulate ethanol self-administration in mice at doses of 10 mg/kg or higher (Schroeder et al., 2003). Oral administration of L-152,804 (10 mg/kg) also inhibited the feeding stimulatory effect of centrally injected Y5 agonist bovine pancreatic polypeptide (bPP; Kanatani et al., 2000). Therefore, we aimed at doses around 10 mg/kg in the present study. Male NMRI mice (Taconic M&B, DK; 23–30 g) were injected i.p. with L-152,804 (#1382, Tocris Cookson Ltd., UK) at doses of 0.2, 1, 10, or 20 mg/kg (n = 7–8) suspended in vehicle containing 0.05% bovine serum albumin in 10 mM phosphate-buffered saline (PBS; 0.13 M NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4). A control group received only vehicle i.p. (n = 28). Five minutes later, all animals received an injection of kainate (30 mg/kg, s.c.) and were rated for seizures as described above. According to Kanatani et al. (2000), oral administration of L-152,804 at a dose of 10 mg/kg results in good brain bioavail-

ability after 2 h. Similarly, intracerebroventricular (i.c.v.) L-152,804 blunts the orexigenic effect of the Y5 agonist bPP during 2 h. Since i.p. injection of L-152,804 should result in brain bioavailability intermediary between oral and i.c.v. administration and since kainate seizures develop with increasing severity over the 90 min period, we chose an interval of 5 min between administration of L-152,804 and kainate. The 30 mg/kg dose of kainate was used because pilot studies showed it to be optimal in the NMRI strain, being the lowest dose causing motor seizures in a maximum number of animals. In a separate experiment, the effects of L-152,804 (10 mg/kg, i.p.) followed 5 min later by kainate (40 mg/kg, s.c.) were also tested in WT mice from our transgenic background strain (n = 12; BALB/c x 129/SvEv; age-matched controls for Y2Y5−/− mice; 14–28 g) and in Y2−/− mice (n = 5; 16–24 g). WT mice receiving vehicle for Y5 antagonist served as a control group (n = 10; 14–28 g).

Ventral hippocampal kindling

Male Y5−/− (n = 9) and age-matched WT control (n = 5) mice were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and mounted in a Kopf stereotaxic frame. A bipolar stainless steel electrode for stimulation and recording was implanted as previously described (Nanobashvili et al., 2000) in the left ventral hippocampal CA3/CA1 (coordinates: tooth bar at flat-skull position, 2.9 mm caudal to bregma, 3.0 mm lateral to midline, and 3.0 mm ventral to dura; Franklin and Paxinos, 1997). To monitor seizure spread to extra-hippocampal regions, a recording electrode was simultaneously implanted in the right amygdala (coordinates: tooth bar at flat-skull position, 1.5 mm caudal to bregma, 3.0 mm lateral to midline, and 4.0 mm ventral to dura; Kokaia et al., 1995). Following 7–10 days recovery, Y5−/− and WT mice received electrical stimulations at the afterdischarge threshold (1 ms bipolar square pulses of 100 Hz for 1 s) via the hippocampal electrode once daily. The threshold for eliciting focal epileptiform activity (afterdischarge) was determined on the first day of stimulation by increasing the current intensity with 10 μA steps, starting at 10 μA, until an afterdischarge lasting at least 5 s was elicited. Seizures were scored blindly according to a modified scale of Racine (1972): grade 0, no response; grade 1, facial twitches; grade 2, chewing and head nodding; grade 3, forelimb clonus; grade 4, rearing, full body jerks, and tail upholding; grade 5, rearing with loss of posture, hindlimb clonus, and vocalization. The mice were considered to be fully kindled when a total of 5 grade 5 seizures had been displayed. For each kindling stimulation, the seizure grade and duration of the primary (1AD) and secondary (2AD) afterdischarges for both hippocampus and amygdala were determined.

Brain sectioning for NPY receptor in situ hybridization and binding

The Y2−/− (n = 10), Y5−/− (n = 7), Y2Y5−/− (n = 15), and WT (n = 9) mice were decapitated. The brains were rapidly removed, frozen on dry ice, and stored at −80°C. Coronal serial sections (15 μm) were cut on a cryomicrotome at the level of the dorsal (−1.70 to −2.18 mm from bregma) and ventral (−2.92 to −3.28 mm from bregma) parts of hippocampus (Franklin and Paxinos, 1997). The sections were thaw-mounted onto Superfrost® glass slides, gently dried on a hotplate, and stored at −80°C until further processing for in situ hybridization or binding.

to compensate for potential risk of multi-significance. Data are presented as mean ± SEM.

Results

Effects of NPY on 0 Mg²⁺-induced bursting in CA3 of hippocampal slices

First, we established in WT mice that application of NPY caused a significant prolonged decrease in epileptiform burst frequency as compared to its own baseline, starting at 4 min and lasting until 16 min after termination of NPY application (Figs. 2A, C). The anti-epileptiform effect of NPY subsequently washed out.

In contrast, free acid NPY, which is considered biologically inactive (Wahlestedt and Reis, 1993), had no anti-epileptiform action (Fig. 2B; baseline: 0.26 ± 0.02 Hz; application: 0.27 ± 0.02 Hz; wash-out first 10 min: 0.26 ± 0.02 Hz). To further confirm the specificity of NPY's effect under our experimental conditions, the effects of NPY were compared directly to those of free acid NPY in WT mice. This comparison also revealed a significant anti-epileptiform effect of NPY which started already 2 min after the beginning of peptide application and was detectable as long as 14 min into the wash-out period (data not shown). These series of experiments provided the basic conditions for testing the effects of NPY in slices from mutant mice.

In hippocampal slices from Y2−/− mice, similar to slices from WT mice, NPY also had an anti-epileptiform effect of comparable
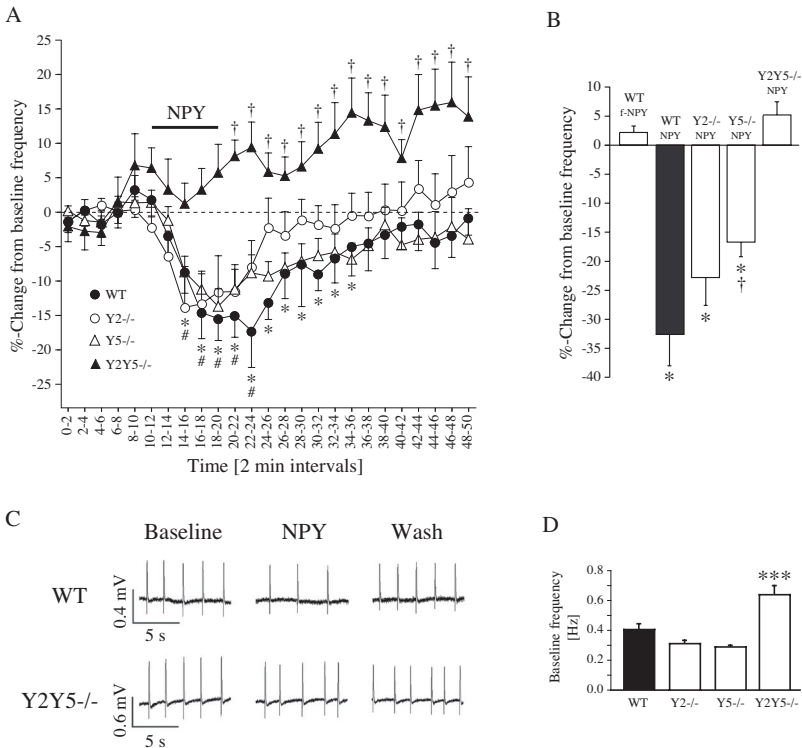

Fig. 2. The inhibitory action of NPY on 0 Mg²⁺-induced epileptiform activity in the CA3 region of hippocampal slices is completely absent in Y2Y5−/− mice. (A) Spontaneous epileptiform burst frequencies at 2 min intervals before, during, and after application of 1–4 μM NPY in WT ($n = 13$ slices sampled from six animals), Y2−/− ($n = 11$ slices from seven animals), Y5−/− ($n = 10$ slices from three animals), and Y2Y5−/− ($n = 8$ slices from four animals) mice calculated as percentage change from baseline values of individual slices. Statistics are based on frequency values. *$P < 0.05$ vs. baseline (WT and Y5−/− mice), #$P < 0.05$ vs. baseline (Y2−/− mice), †$P < 0.05$ vs. baseline (Y2Y5−/− mice), paired two-tailed t test. (B) The peak inhibitory effect of NPY in WT, Y2−/−, and Y5−/− mice or the mean percentage change from baseline during application of NPY in Y2Y5−/− mice or free acid NPY (f-NPY; $n = 8$) in WT mice in the CA3 of hippocampal slices with 0 Mg²⁺-induced spontaneous epileptiform bursting. *$P < 0.05$ vs. baseline, paired two-tailed t test; †$P < 0.05$ vs. WT NPY. (C) Traces showing spontaneous bursting during baseline, application of NPY, and wash-out periods from WT and Y2Y5−/− mice. Note that the anti-epileptiform effect of NPY is abolished in Y2Y5−/− mice. (D) Y2Y5−/− were hyperexcitable as compared to WT mice with significantly higher baseline frequencies (before application of NPY). ***$P < 0.001$ vs. WT, Bonferroni/Dunn post-hoc test following one-way ANOVA.

magnitude, starting at 4 min after application (Fig. 2A). However, the effect of NPY appeared to wash out faster in Y2−/− mice, and the spontaneous burst frequency remained significantly lower than baseline for only over the first 4 min of the wash-out period (Fig. 2A). This is consistent with our previous findings in rats under similar experimental conditions (Nanobashvili et al., 2004) and indicates that NPY could exert an anti-epileptiform effect via Y5 receptors. To confirm this hypothesis, we tested the effect of NPY on epileptiform bursting in slices from Y5−/− mice. Unexpectedly, NPY also suppressed epileptiform activity in these slices with a similar time course as in WT mice (Fig. 2A). However, the peak inhibitory effect of NPY was significantly lower in Y5−/− (−17%) as compared to WT (−33%) mice, with a similar tendency in Y2−/− mice (−23%; Fig. 2B). These data indicated that deletion of either the Y2 or Y5 receptor had only a partial influence on the anti-epileptiform action of NPY, suggesting that both receptors could mediate this effect.

To exclude the possible involvement of Y1, or some other, yet un-identified NPY receptor in the observed NPY effect, we added NPY to slices from Y2Y5−/− mice. Indeed, in this case, the anti-epileptiform effect of NPY was completely abolished (Figs. 2A–C). In addition, the hippocampal slices from Y2Y5−/− mice appeared to be more excitable compared to slices from WT mice. This was revealed by significantly higher baseline frequencies of epileptiform bursting in Y2Y5−/− mice (Fig. 2D). The frequencies further increased during NPY application and in the wash-out period (mean during entire wash-out: 11 ± 3%; Fig. 2A). The increase during application and wash-out appeared to result from the absence of anti-epileptiform effect of endogenous NPY at Y2 and Y5 receptors rather than a pro-epileptic effect of exogenously applied NPY. Thus, when 0 Mg-aCSF without NPY was applied to slices from Y2Y5−/− mice, a similar gradual increase was observed during the wash-out period (mean during entire wash-out: 13 ± 8%; *n* = 4 slices). In contrast, WT mice treated with inactive free acid NPY remained at baseline levels during wash-out (mean during entire wash-out: 2 ± 2%).

Kainate seizures

Once we established that both Y2 and Y5 receptors could mediate anti-epileptiform action of exogenous NPY in our in vitro model, we next asked whether this would also be the case for endogenous NPY in in vivo seizures. To address this question, we systemically injected kainate in Y2Y5−/− mice. This induced seizures, which were significantly more severe than in WT controls, as revealed by shorter latencies to the first convulsion and to the loss of posture, and by higher seizure grades (Fig. 3A). These results were in line with our in vitro data. However, to our surprise, Y5−/− mice were found to display more severe seizures similar to Y2Y5−/− mice (Fig. 3B), while kainate seizures in Y2−/− animals were comparable to those of WT littermates (Fig. 3C). Direct comparison of the groups revealed no significant differences in any seizure parameters tested between Y2Y5−/− and Y5−/−, or Y2−/− and WT mice, respectively. These data demonstrate that deletion of the Y2 receptor (as opposed to Y5) does not influence seizure severity following systemic kainate administration.

To further explore an anticonvulsant role for Y5 receptors, we tested the effect of the Y5 antagonist L-152,804 on systemic kainate seizures. In normal NMRI mice, L-152,804 dose-dependently aggravated kainate seizures with maximal effect at 10 mg/kg (Fig. 4). This proconvulsant effect was revealed by shorter latencies to
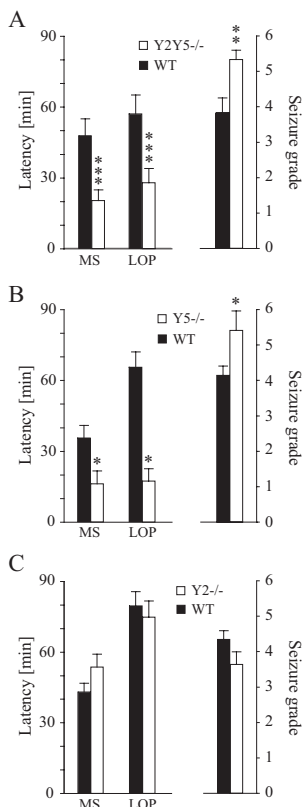

Fig. 3. Y2Y5−/− (A) and Y5−/− (B), but not Y2−/− (C), display more severe kainate seizures than WT controls as revealed by shorter latencies to first motor seizure (MS) and loss of posture (LOP), as well as higher seizure grades. *P < 0.05, **P < 0.01, ***P < 0.001 vs. WT mice, logrank test (latencies) or Mann–Whitney U test (seizure grades).

first convulsion (Fig. 4A) and loss of posture (Fig. 4B), as well as higher seizure grades (Fig. 4C). Likewise, in our WT control strain, L-152,804 at the 10 mg/kg dose caused more severe seizures than vehicle, as seen by significantly shorter latency to first convulsion and higher seizure grades (Fig. 4D). Latency to loss of posture also appeared to be shorter in L-152,804-treated WT mice, but this did not reach statistical significance (P = 0.06, Fig. 4D). There was no difference between the effect of 10 mg/kg L-152,804 in WT and Y2−/− mice with regard to any seizure parameter tested (P = 0.51–0.95, logrank test or Mann–Whitney U test, Fig. 4D).

Ventral hippocampal kindling

To further confirm our findings in the kainate model, we utilized another widely used seizure model, hippocampal
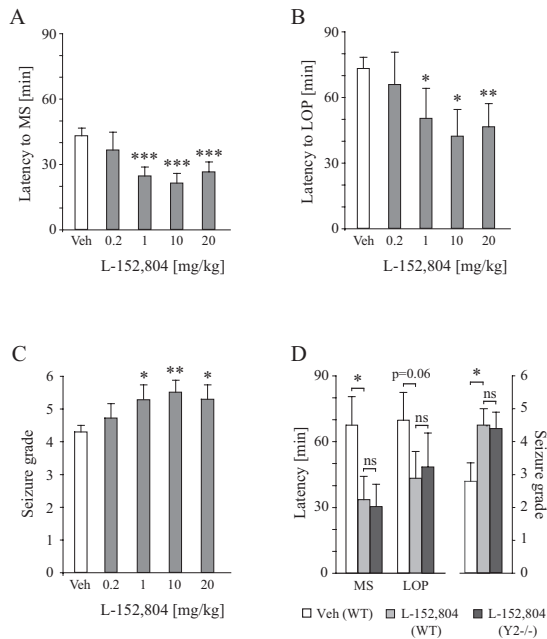
Fig. 4. The Y5 antagonist L-152,804 administered i.p. dose-dependently aggravates systemic kainate seizures in NMRI mice (A–C) as revealed by shorter latencies to first motor seizure (MS) and loss of posture (LOP), as well as higher seizure grades. Proconvulsant effects of 10 mg/kg L-152,804 are also observed in our WT mice, while there was no significant (ns) difference between the effects of L-152,804 in Y2−/− and in WT mice (D). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, logrank test (latencies) or Mann–Whitney U test (seizure grades).

kindling. WT mice of the particular strain used in this study displayed relatively slow kindling, all mice reaching only grade 2 seizures within 55 daily stimulations. This could be attributable to slower ventral hippocampal kindling or also to a relatively high resistance to seizures in the BALB/c strain (Frankel et al., 2001). This strain contributes to 50% of the breeding background for our WT and mutant mice. However, during the same kindling period, Y5−/− mice clearly exhibited faster epileptogenesis (Fig. 5A). Thus, within the 55 stimulations, 7 out of 9 Y5−/− mice ($P < 0.05$, Fisher's Exact Test) had developed grade 3 seizures, and 5 out of 9 Y5−/− mice were fully kindled, having displayed 5 grade 5 seizures. The area under curve (Fig. 5B) and mean seizure grade (WT: 1.3 ± 0.0, Y5−/−: 2.5 ± 0.1) of Y5−/− mice were significantly higher than that of WT mice. No differences between the two groups were found in afterdischarge threshold (Y5−/−: 38 ± 3 μA; WT: 40 ± 4 μA) or mean afterdischarge durations (Fig. 5D) focally in the hippocampus at the location of the stimulating electrode. In the amygdala, however, significantly longer mean durations of the primary, secondary, and total (primary + secondary) afterdischarges were observed in Y5−/− as compared to WT mice (Fig. 5C).

NPY receptor mRNA in situ hybridization

Next, we asked whether, in our mutant strains, compensatory changes in gene expression of Y1, Y2, or Y5 receptors could occur that might account for the observed results. Analysis of in situ hybridization of brain slices showed that, in all mutant and WT mice Y1 mRNA (Figs. 6A and 7), expression levels were above non-specific labeling (Fig. 6D) both in the dorsal and ventral hippocampal formation, including CA3, CA1, and dentate gyrus. Specific labeling was also found in neocortical regions, including primary motor cortex (Figs. 6A and 7), as well as piriform cortex and basolateral amygdala (Fig. 7). The highest levels of Y1 mRNA were observed in the dentate granule layer (Figs. 6A and 7). In WT and Y5−/− mice, specific Y2 mRNA labeling was observed in the same hippocampal regions as that of Y1 mRNA, but levels were lower in the dentate gyrus (Figs. 6B, E and 7). The levels of Y2 mRNA labeling were also lower than that of Y1 mRNA in the basolateral amygdala and piriform cortex (Fig. 7). Y2 mRNA labeling was very low in neocortical areas (Figs. 6B and 7). In WT and Y2−/− mice, specific Y5 mRNA labeling was found in hippocampal regions at even lower levels than that of Y2 mRNA
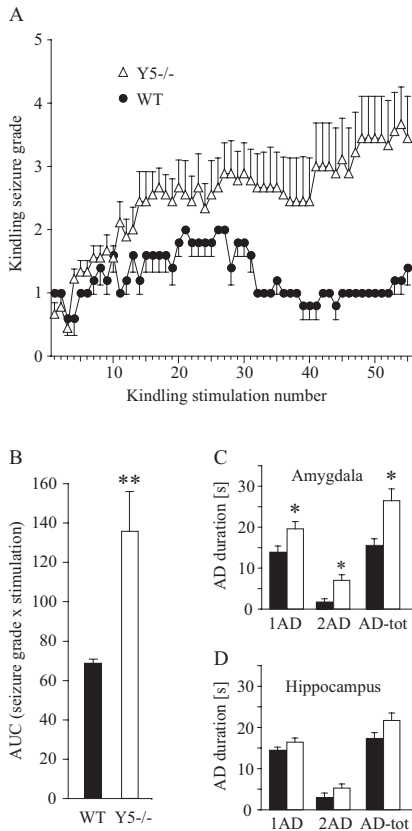
Fig. 5. Repeated daily electrical stimulation of ventral hippocampus induces more severe seizures during the kindling period in Y5−/− as compared to WT mice. (A) Mean seizure grades during kindling development. (B) Area under curve (AUC) is higher in Y5−/− as compared to WT mice. **$P <$ 0.01 vs. WT, Mann–Whitney U test. The Y5−/− mice (white columns) display longer mean durations of the primary (1AD), secondary (2AD), and total (AD-tot) afterdischarges in the amygdala (C) but not in ventral hippocampus (D). *$P <$ 0.05 vs. WT (black columns), two-tailed t test.

(Figs. 6C, F and 7). Outside the hippocampus, low levels of Y5 mRNA expression were also detected in cortical regions and basolateral amygdala (Figs. 6C, F and 7). Comparison between the mutant and corresponding WT mice did not reveal any significant changes in mRNA expression in any knockout strain (Figs. 6G–I and 7). Thus, our data showed no evidence of compensatory changes in NPY receptor expression in the mutant mice strains. To exclude the possibility that absence of changes in mRNA expression was due to a methodological problem, we hybridized brain slices from kainate-treated WT mice in the same hybridization session as the rest of the slices from mutant non-treated

mice. As expected, substantial changes in Y1, Y2, and Y5 mRNA levels in kainate-treated mice were found (data not shown), consistent with a previous study from our group (Kopp et al., 1999).

NPY receptor binding

To explore whether compensatory changes in the receptors might have occurred at the post-transcriptional level (e.g., faster degradation/internalization of the receptor proteins), we used radioactive ligand binding assays. First, we demonstrated that total ^{125}I-PYY binding was almost completely abolished by adding "cold" NPY (non-specific binding), confirming that the used method visualizes actual binding to NPY receptors (Figs. 8A, B, I, J).

Specific Y1 binding

In all 4 genotypes, specific Y1 binding was observed in dorsal and ventral hippocampal CA3, CA1, and dentate gyrus regions (Figs. 8C, D and 9), as well as in cortical areas (Figs. 8D and 9) and basolateral amygdala (Fig. 9). The highest levels were found in the dorsal dentate gyrus (Figs. 8C and 9). Y1 binding comparisons between the different mutant mice using two-way ANOVA revealed a significant effect of genotype ($P < 0.0001$) and region ($P < 0.0001$) with no interaction ($P < 0.29$) in hippocampal regions. Further post-hoc least squares means t tests showed that all 3 mutant mice displayed significantly lower
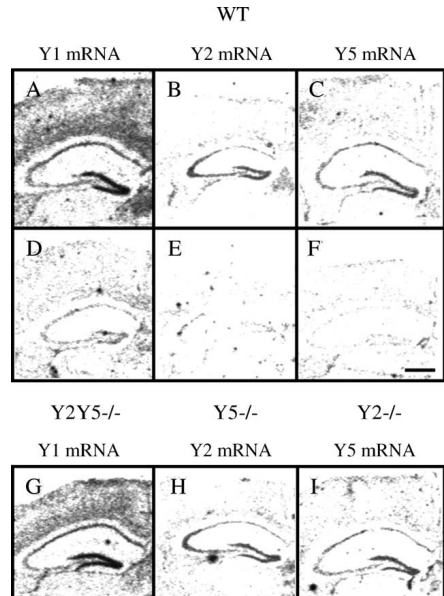

Fig. 6. In situ hybridization autoradiograms showing Y1, Y2, and Y5 mRNA expression (A–C) with corresponding non-specific (D–F) labeling (competitive controls) in the dorsal hippocampus of WT mice. No changes were detected in any mutant mouse strains (G–I). Scale bar = 0.5 mm.
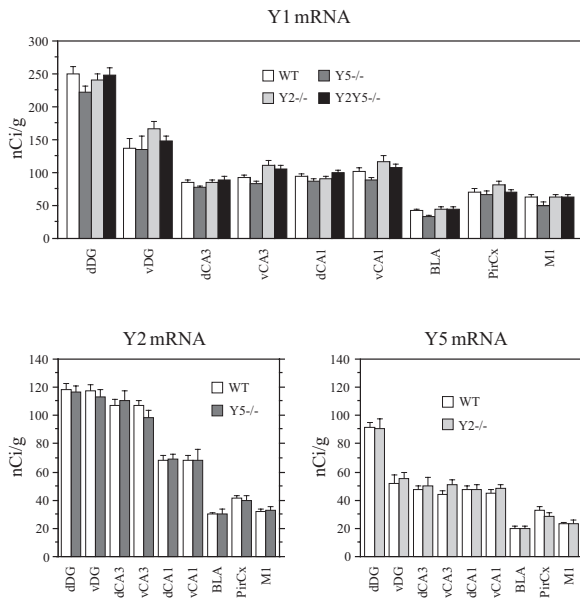
Fig. 7. Y1, Y2, and Y5 mRNA receptor expression in dorsal (d) and ventral (v) hippocampal areas as well as the basolateral amygdala (BLA), piriform cortex (PirCx), and primary motor cortex (M1) of Y2−/−, Y5−/−, Y2Y5−/−, and WT mice. Data are actual values without subtraction of competitive control values. No significant differences between the mutant strains and WT mice were detected in any region.

overall levels of Y1 binding than WT mice ($P < 0.01$). Y1 down-regulation was most pronounced in Y2Y5−/− mice, reaching statistically significant levels in all examined hippocampal regions, amounting to 15% in the dorsal dentate gyrus and about 60–85% in the remaining hippocampus (Figs. 8K and 9). Y2−/− mice displayed significantly lower specific Y1 binding in both dorsal and ventral CA3 and CA1 (Figs. 8L and 9), whereas Y1 binding was only significantly decreased in dorsal CA3 and CA1 of Y5−/− mice as compared to WT mice (Fig. 9). No significant changes in Y1 binding were found in any of the knockout strains in the basolateral amygdala, piriform cortex, or primary motor cortex (Fig. 9).

Specific Y2 binding

In both WT and Y5−/− mice, specific Y2 binding was found in the same hippocampal regions as that of Y1 binding, with levels being lower in the dorsal dentate gyrus and higher in dorsal CA3 and CA1 (Figs. 7E, F and 8). Specific Y2 binding was also found in the basolateral amygdala and piriform cortex (Fig. 9). There were no significant differences in Y2 binding between the Y5−/− and WT mice (Fig. 9).

Specific Y5 binding

In WT mice, specific Y5 binding was found in CA3, CA1, and dentate gyrus regions, but levels were lower in all regions as compared to Y1 and Y2 binding (Figs. 8G, H and 9). Specific Y5 binding was also found in the basolateral amygdala and piriform

cortex (Fig. 9). Two-way ANOVA revealed a significant effect of genotype ($P < 0.0001$) and region ($P < 0.0001$) with interaction ($P < 0.001$) in hippocampal regions. Further analysis showed that Y5 binding was significantly reduced in dorsal, but not ventral, hippocampal regions of Y2−/− as compared to WT mice (Fig. 9). No significant changes were found outside the hippocampus (Fig. 9).

Discussion

Using a gene knockout strategy, we show for the first time that in mice: (i) in in vitro hippocampal slice preparations, suppression of 0 Mg^{2+}-induced epileptiform bursting by exogenously applied NPY can be mediated by both Y2 and Y5 receptor subtypes, possibly in an additive manner; and (ii) endogenous NPY in systemic seizure models exerts its inhibitory effect predominantly via Y5 receptor activation, most likely in extra-hippocampal regions. This is the first study examining Y2−/− and Y2Y5−/− mice in seizures.

In vitro epileptiform activity

Our data showing anti-epileptiform action of NPY both in Y2−/− and Y5−/− mice, in combination with absence of NPY effect in Y2Y5−/− mice, suggest that both Y2 and Y5 receptors can mediate this action of NPY in the 0 Mg^{2+} model. The less pronounced effect of NPY in the single receptor knockout strains is
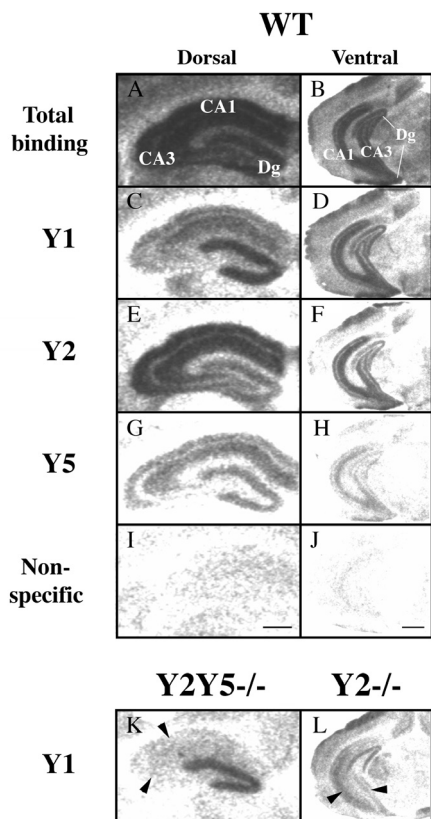
WT

Dorsal　　　　　**Ventral**

Fig. 8. Autoradiograms showing total NPY receptor Y1, Y2, Y5, and non-specific binding in the dorsal and ventral hippocampus of WT mice (A–J). Y1 binding is reduced in all hippocampal regions of Y2Y5−/− mice, particularly in CA1 and CA3 (arrows on K). Y2−/− mice also display reduced Y1 binding in CA1 and CA3 (arrows on L). Left scale bar = 0.5 mm, right scale bar = 1 mm.

consistent with the hypothesis that activation of these two NPY receptor subtypes act in an additive manner to suppress epileptiform activity in the hippocampus. In line with these observations, agonists for both Y2 and/or Y5 receptors reduce 0 Mg^{2+}-induced spontaneous epileptiform bursting in hippocampus of mice and rats (Klapstein and Colmers, 1997; Bijak, 1999; Marsh et al., 1999; Nanobashvili et al., 2004), and the Y5 antagonist CGP71683A blunts the anti-epileptiform effect of NPY (Nanobashvili et al., 2004). Moreover, ligands with efficacy at both Y2 and Y5 receptors (NPY, PYY_{3-36}) appear to have higher peak effects than agonists with preference for Y2 (Ahx_{5-24}-NPY) or Y5 (Leu^{31}, Pro^{34}-NPY, [cPP_{1-7},NPY_{19-23},Ala^{31},Aib^{32},Gln^{34}]hPP) alone (Klapstein and Colmers, 1997; Nanobashvili et al., 2004). As a potential anti-epileptic mechanism, NPY appears to inhibit

glutamate release via modulation of Ca^{2+} influx by binding to both Y2 and Y5 presynaptic receptors in the hippocampus (Colmers et al., 1991; Greber et al., 1994; Guo et al., 2002; Rodi et al., 2003; Silva et al., 2003). Consistent with the present finding of an additive effect between Y2 and Y5 receptors on seizure activity, recent data show an interaction between Y2 and Y5 receptors in modulation of glutamate release and calcium currents in hippocampal synaptosomal preparations (Silva et al., 2003).

Genetic background of the strains used to generate NPY receptor knockout mice seems to play an important role in determining the phenotypic characteristics of the NPY effect. Thus, the anti-epileptiform effect of NPY was totally absent in slices from Y5−/− mice on an inbred 129/Sv background (Baraban, 2002; Marsh et al., 1999 and personal communication) as compared to the blunted peak effect of NPY in our Y5−/− strain on a mixed BALB/c × 129/SvEv background. One of the pitfalls of the knockout strategy is the potential influence on the normal development of the brain and possible compensatory changes in other genes and proteins. Indeed, we found decreased Y1 receptor binding in our mutant mice. Considering the proposed seizure permissive nature of Y1 receptor activation (Gariboldi et al., 1998; Vezzani et al., 1999; Benmaamar et al., 2003), one could speculate that down-regulation of binding-sites for the Y1 receptor could compensate for the loss of one of the other NPY receptors (e.g., Y2 or Y5), which act in opposite direction. However, this down-regulation was apparently not sufficient to reverse excitability of the slices when both Y2 and Y5 receptors were absent, as revealed by higher basal frequency of epileptiform discharges in slices from Y2Y5−/− mice as compared to WT, Y2−/−, and Y5−/− mice.

In contrast to the 0 Mg^{2+} model, there is evidence for inhibition of ictal discharges in the STIB model exclusively via Y2 receptors in the hippocampus of rats (El Bahh et al., 2002). It would be interesting to test the STIB model in NPY receptor knockout mice to determine whether there are rat vs. mouse species differences or whether it is merely a question of the seizure model used.

In vivo seizure models

Our data show that, in the kainate seizure model, absence of the Y5 receptor in Y5−/− mice results in more severe seizures as compared to WT mice. Similar findings were also reported in previous work with the kainate model in Y5−/− mice from an inbred 129/Sv background (different from our mice) (Marsh et al., 1999). In contrast to the 0 Mg^{2+} model, we found no indication of an additive action of Y2 and Y5 receptors in vivo. The reason for these differences might be explained by involvement of hippocampal versus extra-hippocampal NPY receptors. Thus, in the 0 Mg^{2+} model, NPY acts via activation of local hippocampal NPY receptors, whereas, in the kainate model, extra-hippocampal NPY receptors are also likely to play a role. This hypothesis is further substantiated by our finding that faster progression of hippocampal kindling in Y5−/− mice was associated with longer afterdischarge durations in amygdala but not in hippocampus. In addition, there were no compensatory changes in expression or binding of other NPY receptors outside the hippocampal formation in Y5−/− mice that would account for observed effects.

A role for Y5 receptors in regulating systemic seizures was further supported in our study by the novel finding that the
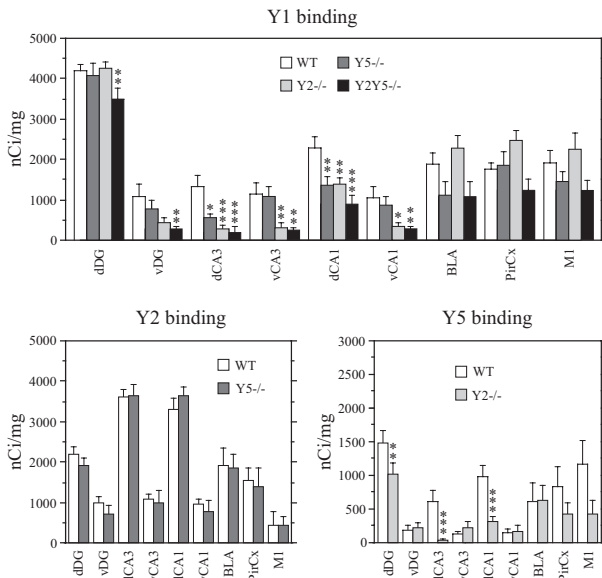
Fig. 9. Specific Y1, Y2, and Y5 receptor binding in dorsal and ventral hippocampus of Y2−/−, Y5−/−, Y2Y5−/−, and WT mice. *$P < 0.02$, **$P < 0.01$, ***$P < 0.001$ vs. WT, least squares means post-hoc t tests following two-way ANOVA (genotype and region as factors). Abbreviations as in Fig. 7.

selective Y5 antagonist L-152,804 aggravated systemic kainate seizures in WT mice as well as in mice with a completely different genetic background (NMRI mouse strain). The proconvulsant effect of L-152,804 did not differ in Y2−/− and WT mice, further confirming that the absence of Y2 receptors does not appear to play a role in regulation of systemic kainate seizures. Consistent with our findings in Y5−/− mice, it was recently shown in rats that the selective Y5 antagonist GW438014A accelerates rapid ventral hippocampal kindling while the selective Y5 agonist Ala[31],Aib[32]-NPY inhibits the development of generalized seizures during kindling without affecting the duration of CA3 afterdischarges (Benmaamar et al., 2005).

Compensatory changes in mutant mice

Distribution of NPY receptor expression and binding in our study was consistent with previous observations by us and others in rodents, showing the presence of Y1, Y2, and Y5 receptors throughout the hippocampal formation and in extra-hippocampal areas (Dumont et al., 1998; Naveilhan et al., 1998; Kopp et al., 1999; Redrobe et al., 1999; Gackenheimer et al., 2001; Trivedi et al., 2001; Guo et al., 2002; Wolak et al., 2003; Husum et al., 2004). In our study, a consistent finding was that all mutant mice had a robust decrease in Y1 receptor binding in hippocampal regions. In situ hybridization revealed no significant changes in Y1 mRNA in any of the mutant strains, indicating that Y1 down-regulation occurs post-transcriptionally. Y1 receptor internalization (Gicquiaux et al., 2002) might account for these

results, and, as mentioned above, could be a compensatory mechanism to counteract increased excitability in the brain due to lack of Y2, Y5, or both receptor subtypes. Consistent with this interpretation, reductions in Y1 mRNA expression and/or binding have been reported in different seizure models (Kofler et al., 1997; Gobbi et al., 1998; Kopp et al., 1999; Husum et al., 2004).

Surprisingly, Y5 binding in Y2−/− mice was reduced in the dorsal hippocampus, but not in the ventral hippocampus, where our in vitro recording electrode was placed in the 0 Mg^{2+} model. Similar to Y1, Y5 down-regulation did not result from a reduction in mRNA expression. Previous studies have shown that seizures cause prolonged reduction in Y5 binding (Bregola et al., 2000), though Y5 mRNA expression is acutely increased (Kopp et al., 1999). Decreased Y5 binding after seizures would be expected to further promote epileptogenesis and might be involved in seizure-induced hyperexcitability occurring in the hippocampus. Conversely, increased Y5 binding in hippocampus during kindling was recently suggested as the anti-epileptic mechanism of the drug levetiracetam (Husum et al., 2004). Further studies will be required to clarify the possible implications of the demonstrated decreased binding of Y5 receptor in the dorsal hippocampus.

Conclusion

Use of loss-of-function gene knockout strategy in mice with the same genetic background allowed us to show that both Y2 and Y5

receptors are involved in regulation of seizure activity by NPY. Their differential contribution to the seizure-suppressant effect of NPY appears to be largely determined by the seizure model used. The present data also suggest that Y5 is an important receptor subtype mediating anti-epileptic effect of NPY, predominantly outside the hippocampal formation.

Acknowledgments

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II

NPY Gene Transfer in the Hippocampus Attenuates Synaptic Plasticity and Learning

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ABSTRACT: Recombinant adeno-associated viral (rAAV) vector-induced neuropeptide Y (NPY) overexpression in the hippocampus exerts powerful antiepileptic and antiepileptogenic effects in rats. Such gene therapy approach could be a valuable alternative for developing new antiepileptic treatment strategies. Future clinical progress, however, requires more detailed evaluation of possible side effects of this treatment. Until now it has been unknown whether rAAV vector-based NPY overexpression in the hippocampus alters normal synaptic transmission and plasticity, which could disturb learning and memory processing. Here we show, by electrophysiological recordings in CA1 of the hippocampal formation of rats, that hippocampal NPY gene transfer into the intact brain does not affect basal synaptic transmission, but slightly alters short-term synaptic plasticity, most likely via NPY Y2 receptor-mediated mechanisms. In addition, transgene NPY seems to be released during high frequency neuronal activity, leading to decreased glutamate release in excitatory synapses. Importantly, memory consolidation appears to be affected by the treatment. We found that long-term potentiation (LTP) in the CA1 area is partially impaired and animals have a slower rate of hippocampal-based spatial discrimination learning. These data provide the first evidence that rAAV-based gene therapy using NPY exerts relative limited effect on synaptic plasticity and learning in the hippocampus, and therefore this approach could be considered as a viable alternative for epilepsy treatment. © 2008 Wiley-Liss, Inc.

KEY WORDS: neuropeptide Y; gene therapy; memory; LTP; synaptic transmission

INTRODUCTION

Neuropeptide Y (NPY), a 36 amino acid residue-long polypeptide, and some of its G-protein coupled receptors are widely distributed throughout the brain (de Quidt and Emson, 1986; Michel et al., 1998; Redrobe et al., 1999), and have been implicated in various functions, such as regulation of blood pressure, circadian rhythms, feeding behav-ior, anxiety, memory, and cognition (Wahlestedt and Reis, 1993; Pedrazzini et al., 2003; Tschenett et al., 2003; Greco and Carli, 2006). Accumulating evidence indicates that NPY can modulate excitatory (Haas et al., 1987; Klapstein and Colmers, 1993) and inhibitory (Chen and van den Pol, 1996; Bacci et al., 2002) synaptic transmission in the brain and it has been proposed as one of the candidate molecules (Mody, 2005) involved in the regulation of homeostatic plasticity of central neuronal circuitries (Turrigiano, 1999). In the hippocampus, NPY inhibits excitatory synaptic transmission by decreasing glutamate release via reducing Ca^{2+} influx into the presynaptic terminals (Colmers et al., 1988; Colmers et al., 1991; Qian et al., 1997). This effect of NPY on excitatory synaptic transmission is probably responsible, at least partly, for its well-documented antiepileptic and antiepileptogenic properties. NPY exerts inhibitory action on seizures when overexpressed in transgenic animals (Vezzani et al., 2002) or injected either intraventricularly (Woldbye et al., 1996, 1997) or into the hippocampus (Smialowska et al., 1996) in various seizure models. Moreover, NPY KO mice are more susceptible to seizures (Baraban et al., 1997). These observations have led to the concept that NPY could be an important endogenous inhibitor of seizure activity (Vezzani et al., 1999). Therefore, using gene-therapy to overexpress NPY in epileptogenic brain areas could be a potential treatment strategy for epilepsy. Indeed, recent data show that recombinant adeno-associated viral (rAAV) vector-based transduction of the NPY gene into the piriform cortex can attenuate limbic seizures (Foti et al., 2007), whereas transduction of the hippocampus can have both anticonvulsant and antiepileptogenic effects (Richichi et al., 2004). The mechanisms by which transgene overexpression of NPY suppresses seizures are not known, although pharmacological experiments and seizure susceptibility studies in NPY receptor knock-out mice point to a prominent role of Y2 receptors (Y2R) decreasing glutamate release in the hippocampus (for review see Vezzani and Sperk, 2004). Most importantly, it is not clear whether increased NPY levels could affect other brain functions, such as hippocampal learning and memory. This is of particular significance if this kind of approach shall be developed for clinical application in epilepsy patients. Our current knowledge of

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whether increased levels of NPY inhibit learning and memory is rather limited. Some data indicate that rats overexpressing NPY in the CA1 region of the hippocampus have impaired memory (Thorsell et al., 2000), but this deficit is completely restored when the animals become older (Carvajal et al., 2004). Various studies in rats reported either some attenuation (Cleary et al., 1994) or no effect (Ishida et al., 2007) on learning and memory exerted by intraventricular infusion of NPY.

The objective of this study was to determine in naïve rats under which circumstances transgene NPY is released from the transduced hippocampal neurons, and whether rAAV-NPY vector-based gene transfer into the hippocampus could provide an alternate strategy for epilepsy treatment considering the potential side-effects of NPY on (i) alterations in excitatory synaptic transmission and plasticity, and on (ii) hippocampal learning and memory.

MATERIALS AND METHODS

Animals

All animals were housed in a 12-h light/dark cycle with ad libitum access to food and water. Viral vectors were injected into the hippocampus of Sprague Dawley rats ($n = 39$; B&K, Denmark or Charles River, Italy; 250–350 g; males). In some control experiments, nontreated naïve rats were used ($n = 19$; 4–7 weeks old; B&K, Denmark; females and males). All experimental procedures were conducted in accordance to the guidelines of European Community for the Care and Use of Laboratory Animals and were approved by the local Ethical Committee.

Vector Production

The rAAV vector was produced as previously described (During et al., 2003; Richichi et al., 2004). Briefly, plasmids were constructed by subcloning human prepro-NPY cDNA into an expression cassette consisting of the rat neuron-specific enolase (NSE) promoter, the woodchuck post-translational regulatory element, and a bovine growth hormone poly(A) signal using standard cloning procedures. Plasmids were then cloned into the backbone of the chimeric serotype 1/2 rAAV vector, having both a rAAV1 and rAAV2 capside helper plasmid in a 1:1 ratio. Vectors with or without insert were designated rAAV-NPY and rAAV-empty, respectively. The latter served as a control. To evaluate viral transduction, the same vector-construct containing GFP was used (designated as rAAV-GFP). The viral titer was 1.0×10^{13} particles per ml for all vectors (and 5.2×10^{12} per ml for the behavioral study) as determined by quantitative PCR of rAAV vector genomes.

Vector Injections

Rats used for electrophysiological experiments received a single rAAV vector injection unilaterally (right hemisphere) in the CA1 region of the dorsal hippocampus under ketamine-xylazine (80 mg/kg ketamine, 15 mg/kg xylazine) anesthesia. A KOPF stereotaxic frame was used with the following coordinates (in mm): anteriorposterier (AP) -3.3 from bregma; mediolateral (ML) $+1.8$ from midline; dorsoventral (DV) -2.4 from dura mater (toothbar -3.3). A total volume of 1 μl vector suspension (rAAV-NPY or rAAV-empty) was injected during 5 min, and the pipette was left in place for an additional 3 min.

For the behavioral study, rats were injected bilaterally under Equithesin anesthesia with rAAV-NPY or rAAV-empty vector in the ventral (in mm; AP: -6.0 from bregma; ML: ± 5.0 from midline; DV: -4.5 below dura mater; toothbar -2.5) and dorsal (AP: -3.1; ML: ± 2.0; DV: -3.2 below dura; toothbar: -2.5) hippocampus. Three μl vector suspensions were infused into each brain site together with 0.5 μl of heparin at the flow rate of 0.1 μl per min, to enhance viral distribution inside the brain parenchyma, and the needle was left in place for additional 3 min.

Electrophysiology

Three to seven weeks after vector injection, individual rats were randomly collected and killed by decapitation, their brains were quickly removed, and the hippocampus was dissected in cold modified artificial cerebrospinal fluid (aCSF) solution (mM; 225 sucrose, 2.5 KCl, 0.5 CaCl$_2$, 7.0 MgCl$_2$, 28 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 7.0 glucose, 1.0 ascobate, and 3.0 pyruvate; adjusted to pH 7.4; osmolarity 300 mOsm) equilibrated with 95% O$_2$/5% CO$_2$. In the same solution, transverse hippocampal slices (400 μm) were cut on a vibratome (Vibratome 3000, Ted Pella, Redding, CA) at 4°C, and then placed in a holding chamber containing aCSF (mM; 119 NaCl, 2.5 KCl, 1.3 MgSO$_4$, 2.5 CaCl$_2$, 26 NaHCO$_3$, 1.0 NaH$_2$PO$_4$, and 11 glucose; pH 7.4; 296 mOsm) oxygenated at room temperature (RT). After 1 h of resting, slices were transferred to a submerged recording chamber (perfused with oxygenated aCSF; RT; 2 ml per min).

Field EPSP Recordings in CA1

All recordings were conducted in the CA1 region of the hippocampus. Schaffer collaterals were stimulated by constant square-voltage pulses (0.1 μs) through a bipolar stainless steel electrode placed in the stratum radiatum layer of area CA1. Field excitatory postsynaptic potentials (fEPSPs) were recorded from the same layer with a recording pipette filled with 3 M NaCl (with pipette resistance 0.5–1 MΩ). Only slices capable of generating fEPSP amplitudes of more than 1 mV were included in the study. Test stimuli inducing 30–50% of the maximal fEPSP responses were used for baseline and tetanus stimulation throughout all experiments.

Paired-Pulse Stimulation

Short-term plasticity of fEPSPs was assessed by paired-pulse (PP) stimulations at different interstimulus intervals (ISI; 25,

50, 100, 200 ms; 0.033 Hz). Paired-pulse facilitation (PPF) of fEPSPs was calculated in individual slices (average of 6 recordings) as percentage change in the initial slope of the second fEPSP as compared to the first (P2−P1/P1*100). In an additional experiment, using PP stimulations as described earlier, the specific NPY Y2R antagonist, (S)-N2-[[1-[2-[4-[(R,S)-5,11-dihydro-6(6h)-oxodibenz[b,e]azepin-11-yl]-1-piperazinyl]-2-oxoethyl]cyclopentyl]-acetyl]-N-[2-[1,2-dihydro-3,5(4H)-dioxo-1,2-diphenyl-3H-1,2,4-triazol-4 yl]ethyl]-argininamide (BIIE0246; 0.3 μM), prediluted in ethanol and dissolved into aCSF (1:10,000) (El Bahh et al., 2002), was applied to slices from rAAV-NPY-injected rats.

High-Frequency Stimulation

To examine the effect of transgene NPY expression on excitatory neurotransmission during repetitive activation, high-frequency stimulation (HFS) trains of varying frequencies (10 and 25 Hz; 50 stimulations each) were used. Stimulation at each frequency was repeated three times and the responses were averaged. To prevent long-term potentiation (LTP) induction in stimulated synapses, the specific N-methyl-D-aspartate (NMDA) antagonist, D(−)-2-Amino-5-phosphonopentanoic acid (D-AP5; 50 μM) was applied together with aCSF. To avoid interference from post-tetanic potentiation (PTP) each train was delivered at 5 min intervals. The absence of LTP induction was verified by unchanged fEPSPs throughout the experiment (data not shown).

Long-Term Potentiation

LTP of excitatory postsynaptic responses was induced by tetanic stimulation consisting of one train of stimuli (1 μs, 100 Hz, during 1 s) using the test stimulus intensity. The magnitude of LTP was calculated in individual slices by normalizing the initial slope of fEPSPs to average baseline values (10–20 min stable fEPSP evoked by 0.067 Hz stimulations), expressed as percentage change. LTP was considered significant if the mean response, as calculated 10–15 min after tetanic stimulation, was increased by >15% from baseline values. In a separate LTP experiment, we applied BIIE0246 (0.3 μM) continuously to slices obtained from rAAV-NPY-njected rats, starting 20 min before baseline recordings.

In a series of additional experiments, using similar stimulating-recording parameters as described earlier, the effect of exogenously applied NPY on LTP was investigated in hippocampal slices from naïve rats. Immediately before use, exogenous NPY (1 μM; rat synthetic) was dissolved in 10 ml aCSF and applied directly into the recording chamber at 2 ml per min. In these experiments, NPY was applied to the slices either 10 min before or 20 min after tetanization. NPY was also applied to naïve slices without tetanization. All drugs used for electrophysiology were purchased from Tocris Cookson (Bristol, UK), except NPY, which was custom synthesized by Schafer-N (Copenhagen, Denmark).

Hippocampus

Data Acquisition

Data was sampled at 10 kHz and filtered at 2.9 kHz using an EPC10 amplifier (HEKA Elektronik, Lambrecht, Germany) connected to a G4 Macintosh computer equipped with PATCHMASTER software (HEKA Elektronik). Off-line analyses were performed using FITMASTER (HEKA Elektronik) and IgorPro (Wavemetrics, Lake Oswego, OR). Experimenters performing data acquisition and analysis for group comparisons were unaware of the identity of the experimental animals.

Hippocampal Learning and Memory Test

Spatial discrimination abilities of rAAV-empty (i.e., controls) and rAAV-NPY-injected rats were assessed 7 weeks after vector injection by a test sensitive to hippocampal manipulations (Morris et al., 1986; Carli et al., 2001). A circular "swimming pool" (150 cm × 50 cm), filled to a depth of 0.29 m with water at 26°C ± 1°C, was used. The pool was placed in the middle of a large room and was surrounded by various visual cues. Two gray visible square (11 cm × 11 cm) platforms protruding 1.2–2.0 cm above the water were used. One platform was rigid and provided support and the other sank when rats tried to climb onto it. Rats were trained to swim to the rigid escape platform while avoiding the floating one. For all rats, the fixed escape platform (correct) was always in the same place at the center of one of the eight sectors. The floating platform (incorrect) was positioned over successive trials in a quasi-random sequence of eight locations around the pool. The rats were trained with 10 trials a day for 7 days. A trial began with the rat being placed in the pool and ended when the rat chose one of the two platforms. In the case of the rigid one it was allowed to sit on the platform for 15 s. Intertrial intervals were ~2–4 min so each rat's daily testing lasted ~30 min. A correct trial was one in which the rat climbed onto the rigid platform without touching the floating platform with its forepaws or snout. If the rat did not choose to escape onto either platform (correct or incorrect) within 60 s it was taken to the rigid platform and left on it for 15 s. We measured (1) the first choice in each trial (correct/ incorrect), (2) the latency to escape (s), and (3) the number of omissions.

Immunohistochemistry

For histological analysis of transgene NPY expression and viral transduction, hippocampal tissue from additional 11 rats injected by viral vectors was examined 3 weeks after rAAV-NPY, rAAV-empty, or rAAV-GFP treatment. These animals were not used for electrophysiological analysis, and they were perfused through the ascending aorta with 0.9% NaCl and 4% paraformaldehyde (pH 7.4), and their brains were post fixed overnight at 4°C. Brains were cut on a microtome, slices (30 μm) were collected, and stored in antifreeze solution. Slices used for electrophysiology (400 μm) were fixed in 4% paraformaldehyde overnight, rinsed, and stored in antifreeze solution. All slices were rinsed, quenched (3% H_2O_2, 10% MeOH in KPBS) for 10 min, and incubated overnight with rabbit

anti-NPY antibody (1:1,000 for 30 μm slices; 1:5,000 for 400 μm slices; Sigma). Slices were then incubated with secondary antibody (BA 1000; 1:200; Vector Laboratories, Burlingame, CA) for 2 h, and with ABC solution for 1 h (Vectastain ABC Kit, Vector Laboratories), and subsequently visualized by 3-3′-diaminobenzidine reaction. Epifluorescence illumination was used to visualize native GFP fluorescence (Olympus BX61). All images were digitally acquired. In all examined brain slices, no brain damage caused by either surgery or viral vectors was observed in accordance with previously published evidence (Richichi et al., 2004).

Statistics

Statistical significance was set at $P < 0.05$ and all data are presented as mean ± standard error of mean (SEM) A two-tailed Student's t-test was used for statistical analysis between groups, and paired t-test for differences within groups (i.e., before and after drug treatment). For behavioral learning and memory testing, repeated measure analysis of variance (ANOVA) with factors treatment and days was used. In addition, the better than chance performance was calculated using a binomial distribution and one-tailed t-test. The χ^2-test followed by Fisher's exact test was performed to evaluate differences in LTP induction rate between groups.
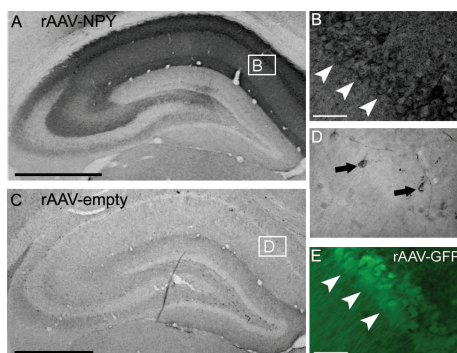
RESULTS

Transgene NPY Expression

Injection of rAAV-NPY vector into the hippocampus of rats resulted in an extensive and widespread overexpression of transgene NPY as confirmed by NPY immunoreactivity. In all slices from rats injected with rAAV-NPY vector and used for electrophysiology 3–7 weeks later, high levels of transgene NPY-immunoreactivity were detected at the recording site and the surrounding areas (Figs. 1A,B). Maximal levels of NPY expression were obtained within 3 weeks after the rAAV-NPY injection, and persisted constant and stable for at least 3 months (data not shown, but see Richichi et al., 2004). The NPY expression was mostly confined to the injection (and subsequent recording) site, causing a preferential and strong expression within the cell layers of CA1, including stratum oriens, pyramidale, radiatum, and lacunosum-moleculare. Expression of NPY was not restricted to any specific neuronal population in the hippocampus, and in transduced areas almost all projection neurons and interneurons were NPY-positive. Transgene NPY expression extended ∼1.9 mm along the medio-lateral axis of the hippocampus within the CA1 area, limited by dorsal hippocampal fissure. In the ventral direction from the injection site, transgene NPY expression in CA1 cell layers extended for ∼4.0 mm. Injections of the rAAV-GFP vector into rats confirmed that neurons within the hippocampus were effectively transduced with the virus (Fig. 1E). However, based on the
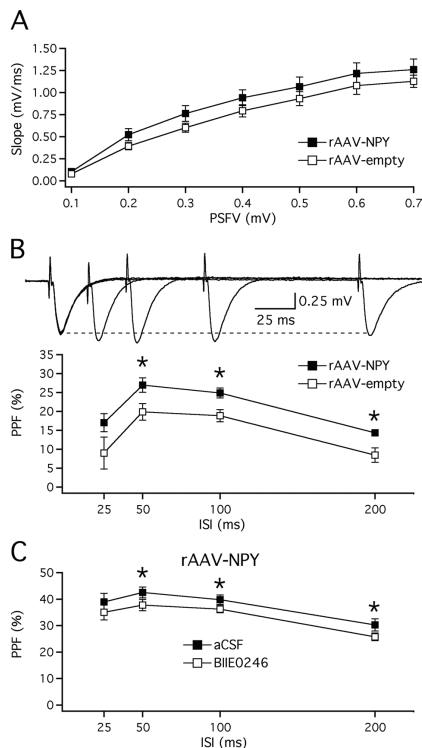
epifluorescent visualization of native GFP in slices, we cannot exclude that some neurons were not transduced with the viral vector. In control animals, injected with the rAAV-empty vector, only scattered, presumably endogenous NPY-positive interneurons were detected (Figs. 1C,D). Control recordings were conducted in CA1 area of rAAV-empty vector injected animals. Adeno-associated viral vectors do not seem to induce any significant immunogenicity in the brain (During, 1997) and, therefore, rAAV-empty viral vector injection most likely had negligible effect on normal hippocampal function. In accordance with previous study by Richichi et al. (2004) transgene NPY expression in rats used for the behavioral study was extensive throughout the entire hippocampus (data not shown).

Short-Term Synaptic Plasticity in CA1 of rAAV-NPY-Treated Animals

First we explored possible alterations of synaptic transmission and plasticity in the hippocampus. To address this question we assessed "input–output" relationship for fEPSPs, a measure of basal synaptic transmission, at the Schaffer collateral-CA1 synapses in hippocampal slices of rAAV-NPY and rAAV-empty

FIGURE 1. **High and stable overexpression of transgene NPY after injection of viral vector encoding NPY into the hippocampus of rats.** (A) Immunohistochemical staining of a rat brain slice (as used for electrophysiology), as seen 3 weeks after rAAV-NPY vector injection, showing high levels of transgene NPY expression mainly within the CA1 region (injection and recording site) of the hippocampus. (B) Cells within the pyramidal layer in the CA1 region positively stained for NPY (as indicated by white arrow heads). (C) Slice from a control rat, injected with the rAAV-empty vector, having weak NPY immunoreactivity in all regions of the hippocampus with few dispersed NPY-positive interneurons. (D) The same slices as in (C) with scattered NPY-positive cell bodies, presumably NPY containing interneurons (shown by black arrows). (E) Viral transduction by rAAV-GFP vector, showing GFP expression mostly confined to pyramidal cell bodies and processes. Boxed areas on left images are magnified and shown on the right, and rAAV-mediated GFP expression is shown from the same region as boxed areas. Scale bars = 1 mm in A, C; 50 μm in B, D, E. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

FIGURE 2.   **Short-term plasticity, but not basal synaptic transmission, is altered by ectopic NPY overexpression. (A) The presynaptic fiber volley (PSFV) is plotted against the slope of the corresponding fEPSPs in rAAV-NPY (n = 18 slices from 3 animals) and rAAV-empty (n = 20 slices from 3 animals) injected rats. (B) Paired-pulse facilitation (PPF) at different ISI in rAAV-NPY (n = 22 slices from 4 animals) and rAAV-empty (n = 24 slices from 4 animals) injected adult rats (*P < 0.05, t-test). (Inset) Representative superimposed traces of PPF recorded at ISI of 25, 50, 100, and 200 ms from the same slice (obtained from a rAAV-NPY-injected rat). (C) Average PPF in slices from rAAV-NPY-injected rats before and after application of NPY Y2 receptor antagonist, BIIE0246 (*P < 0.05, paired t-test, n = 18 slices from 8 animals).**

injected rats (Fig. 2A). Resulting input–output relationship curves generated by plotting the amplitude of the presynaptic fibervolley (PSFV) against the values of the initial slope of corresponding fEPSPs were not different between rAAV-NPY (n = 18 slices from 3 animals) and rAAV-empty (n = 20 slices from 3 animals) injected rats at any given stimulus intensity (P > 0.05, t-test), suggesting that overall basal synaptic transmission was unaffected by transgene NPY.

Paired stimulations in the same area with different ISI induced PPF of fEPSPs. PPF was slightly (on average 6%) but significantly higher in rAAV-NPY-injected rats (n = 22 slices from 4 animals) at ISI of 50, 100, and 200 ms as compared to rAAV-empty injected animals (n = 24 slices from 4 animals) (P < 0.05, t-test; Fig. 2B). Application of the selective NPY Y2R antagonist, BIIE0246 (0.3 µM), to slices from rAAV-NPY-injected rats (n = 18 slices from 8 animals) slightly reduced the enhanced PPF (P < 0.05, paired t-test; Fig. 2C).

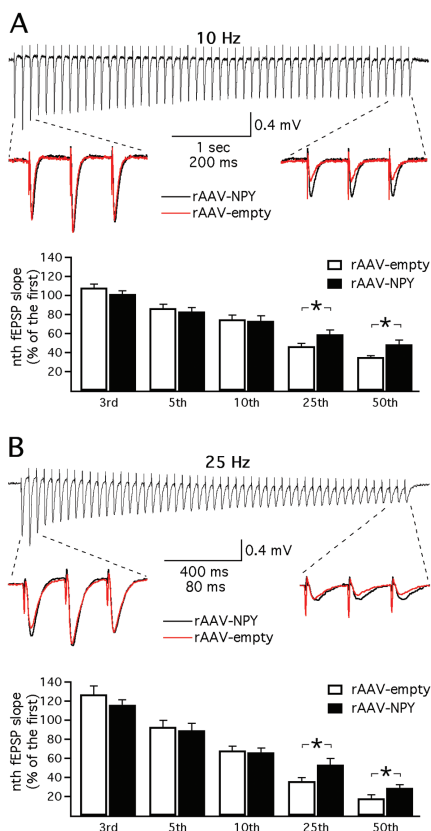### Transgene NPY is Released During HFS

Since endogenous neuropeptides are thought to be released during high frequency synaptic activity, we next explored whether transgene NPY could also be released during high frequency activity of the synapses. We stimulated Schaffer collaterals with 10 and 25 Hz (50 pulses) while recording fEPSPs in stratum radiatum (Figs. 3A,B). By comparing initial slopes of the first stimulation-evoked fEPSP with those of consecutive fEPSPs (3rd, 5th, 10th, 25th, and 50th) during the course of HFS, we found that in control slices (n = 6 slices from 3 animals) the fEPSPs were progressively attenuated at the later phase of HFS. This attenuation of fEPSPs is most likely due to the depletion of the readily releasable pool of glutamate in these synapses (Dobrunz and Stevens, 1997). In rAAV-NPY-treated animals, the attenuation of fEPSPs (at the 25th and 50th stimulations) was on average less pronounced (P < 0.05, t-test) (n = 12 slices from 4 animals; Figs. 3A,B) as compared to control slices. This could be interpreted as lower rate of glutamate depletion due to its lower release caused by HFS-induced transgene NPY release at the later phase of stimulation train.

LTP is Reduced in CA1 of rAAV-NPY-Treated Animals

We next analyzed whether LTP, a synaptic correlate of learning and memory in the hippocampus, was altered in rAAV-NPY-treated rats. Directly after tetanus, PTP of fEPSPs, another form of presynaptic plasticity, was evoked, lasting for a few minutes. The PTP was generally lower in rAAV-NPY injected compared to rAAV-empty injected rats. PTP was followed by LTP of fEPSPs, lasting for more than 50 min (Fig. 4A). The average magnitude of LTP was significantly reduced by about 50% in rAAV-NPY-injected rats (n = 20 slices from 10 animals) as compared to rAAV-empty injected animals (n = 20 slices from 12 animals) (∼140% in rAAV-empty and 120% in rAAV-NPY-treated animals, measured at 20–30 min after tetanus, as compared to respective baseline fEPSPs). Despite the reduced magnitude of LTP in rAAV-NPY-treated animals, the average fEPSP amplitude at 20–30 min after tetanus was still significantly higher compared to baseline values (Fig. 4A).

Although there was a clear difference in LTP magnitude between the two groups, the rate of LTP induction was not significantly different (estimated as at least 15% increase of fEPSP initial slope from the corresponding baseline; 75%, n = 20, and 45%, n = 20, in rAAV-empty and rAAV-NPY-injected

FIGURE 3. **Transgene NPY overexpression reduces synaptic depression in Schaffer-collateral CA1 synapses upon long-lasting stimulation at 10 and 25 Hz. (A) Representative fEPSP recorded during HFS at 10 Hz for 5 s from a rAAV-NPY-injected rat. Magnified traces highlight the first and last three fEPSP responses from the above trace, including a representative superimposed trace (red) recorded at similar time points from a rAAV-empty injected rat. The bars show percentage changes in initial slope of the first fEPSP to successive responses recorded at different time points (3rd, 5th, 10th, 25th, and 50th stimulation) from rAAV-empty (*n* = 6 slices from 3 animals) and rAAV-NPY (*n* = 12 slices from 4 animals) injected rats (*P < 0.05, *t*-test). (B) Representative fEPSPs during HFS at 25 Hz obtained from a rAAV-NPY-injected rat. Magnified traces show the above trace together with a superimposed representative trace (red) recorded from a rAAV-empty injected rat at similar time points. Bars are aligned as in A (*P < 0.05, *t*-test). Upper time scale applies to top traces, whereas lower time scale applies to magnified traces. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

rats, respectively; $P = 0.11$, χ^2-test followed by Fisher's exact test). To exclude the possibility that the observed reduction in LTP magnitude was dependent on a relatively lower (although nonsignificant) rate of LTP induction in rAAV-NPY-treated animals, we compared exclusively those recordings where LTP was successfully induced in both groups. This analysis revealed a significantly reduced LTP magnitude in rAAV-NPY-injected rats as compared to rAAV-empty controls ($\sim$130 and 160% at 20–30 min after tetanus, respectively; $P < 0.05$, t-test).

To confirm that the reduced magnitude of LTP was due to transgene NPY, we applied BIIE0246 (0.3 μM) to slices from rAAV-NPY-injected rats ($n = 11$ slices from 7 animals). This treatment completely restored LTP in Schaffer collateral-CA1 synapses of rAAV-NPY-treated animals to levels and with time-course as observed in rAAV-empty slices (Figs. 4A,B). The magnitude of LTP was also significantly higher as compared to rAAV-NPY slices perfused with aCSF only ($P < 0.05$, t-test; Fig. 4B). These results suggest that the LTP reduction was due to transgene NPY, acting via NPY Y2 receptors.

Exogenous Application of NPY Inhibits LTP

To further confirm that LTP reduction was due to NPY, a gain-of-function approach to mimic the effect of transgene NPY was adopted by applying NPY exogenously to naïve slices from rats. Application of 1 μM NPY for 5 min without tetanic stimulation significantly reduced the magnitude of evoked fEPSPs in CA1 for 45–50 min (Fig. 5A). Tetanus applied to the Schaffer collaterals 10 min after the start of NPY infusion evoked brief PTP, but failed to induce LTP. The fEPSP continued to decline, reaching the attenuated level comparable to that in nontetanized slices (Fig. 5A). These data suggest that increasing NPY levels by exogenously applied NPY prior to tetanization blocks LTP induction in naïve hippocampal slices. Since LTP induction was not blocked in rAAV-NPY-treated animals (although there was a trend toward lower induction rate in the CA1), one could hypothesize that extracellular transgene NPY levels (i.e., constitutively released NPY prior to tetanization) are low in these animals. It is possible though that the compromised LTP magnitude in rAAV-NPY-treated slices is due to additional release of transgene NPY during tetanic HFS-induced activation of Schaffer collaterals. To address this hypothesis, exogenous NPY was applied 20 min after LTP induction in naïve slices. A significant but transient decrease of fEPSP initial slopes with approximately the same magnitude and time-course as in nontetanized slices was observed. The fEPSP magnitude returned to the same potentiated levels as observed before NPY application 50–55 min after the peak of the NPY effect (Fig. 5B). These data indicate that the reduced magnitude LTP in rAAV-NPY-treated animals could be a result of HFS-induced increase in released transgene NPY.

Spatial Discrimination Learning is Delayed in rAAV-NPY-Treated Animals

Since LTP is thought to be a synaptic correlation of learning and memory, and LTP was partially compromised in rAAV-
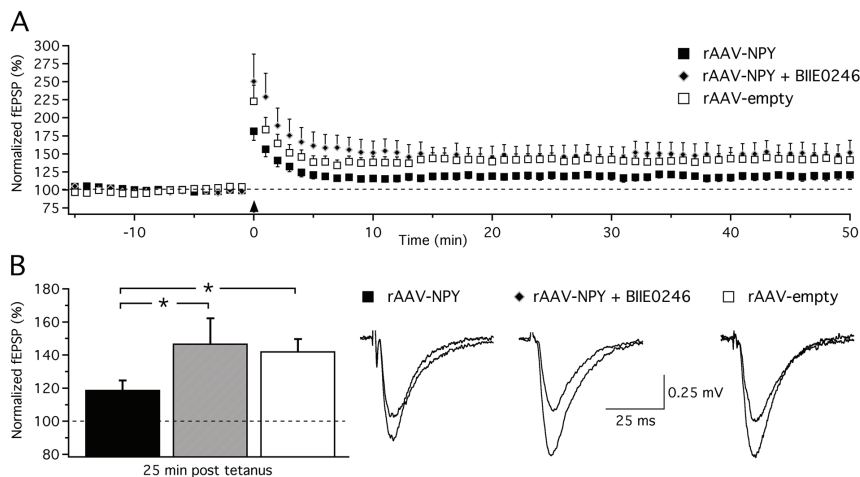
FIGURE 4.   Transgene NPY attenuates the magnitude of LTP in Schaffer collateral-CA1 synapses. (A) Pre- and post-tetanus fEPSP magnitudes are normalized to mean baseline values and plotted against time from rAAV-empty (n = 20 slices from 12 animals) and rAAV-NPY (n = 20 slices from 10 animals) injected rats during aCSF perfusion together with traces from rAAV-NPY (n = 11 slices from 7 animals) injected animals perfused with aCSF containing BIIE0246. During aCSF application, responses after tetanus between rAAV-empty and rAAV-NPY-treated animals are significantly different at all time points from 7 until 50 min post-tetanus (P < 0.05, t-test). Responses between rAAV-NPY slices perfused with aCSF alone or together with BIIE0246 are significantly different from 6 min post-tetanus and onward (P < 0.05, t-test). No differences are detected at any time-points between rAAV-NPY slices perfused with BIIE0246 and rAAV-empty slices. All three traces are significantly different at all time points when compared with their own baseline (P < 0.05, t-test). Bottom arrow indicates time-point of tetanic stimulation. (B) Bars show normalized fEPSP magnitudes 25 min after tetanization recorded in slices from rAAV-empty injected animals (white bar) and in slices from rAAV-NPY-injected animals during aCSF perfusion (black bar) and during BIIE0246 application (gray bar) (*P < 0.05, t-test). Superimposed traces show representative fEPSPs recorded 10 min before and 25 min after tetanus stimulation from a rAAV-NPY-injected rat during aCSF (black square) and BIIE0246 (gray square) application, and with traces from a rAAV-empty injected rat (white square).

NPY-treated animals, we next explored whether this impairment was paralleled by alterations in hippocampus-associated learning processes in rAAV-NPY-treated animals. For this purpose, we used a spatial discrimination-learning test. This experiment showed that on the first day of acquisition, the performance of both rAAV-empty (n = 5 animals) and rAAV-NPY (n = 5 animals) injected rats was at chance (chance level = 50% correct choices; Fig. 6A). With training, both groups improved their performance, but from Day 4 and onward the proportion of correct choices made by control rats (rAAV-empty) was significantly different from chance (P < 0.05), whereas rAAV-NPY-injected rats reached a performance of about 80% of correct choices only after 6 days of training. In agreement with this description the repeated measure ANOVA indicated that rAAV-empty and rAAV-NPY-treated rats learned the task at different rates [treatment $F(1,48) = 6.9$, $P = 0.03$; days × treatment: $F(6,48) = 2.1$, $P = 0.06$) (Fig. 6A)]. In addition, during all 7 days of training, rAAV-NPY-injected rats made fewer correct choices compared to controls (P < 0.05, t-test; Fig. 6B). At the same time, both groups showed similar

choice latency, and errors of omissions were present only on the first day of training and were equal in both groups (data not shown).

DISCUSSION

Here, we demonstrate for the first time that rAAV-based gene transfer of NPY in the hippocampal formation of naïve animals decreases hippocampal activity-dependent plasticity (LTP) in excitatory synapses and delays hippocampal learning. Moreover, we show that release of ectopic transgene NPY occurs from high frequency-activated excitatory synapses. These data provide the first experimental evidence that gene transfer using neuropeptides not only mitigates seizure activity, as has been shown previously, but also alters hippocampal synaptic plasticity and memory. However, despite the fact that LTP in Schaffer collatteral-CA1 synapses of the hippocampal formation was attenuated, it was still maintained. These alterations in
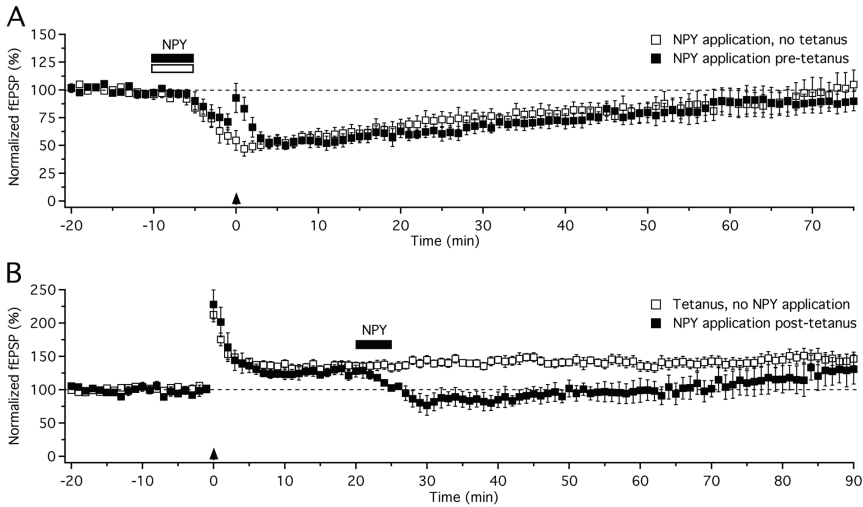
FIGURE 5. **Exogenously applied NPY inhibits LTP in Schaffer collateral-CA1 synapses. (A) Application of NPY (1 µM) for 5 min without tetanus stimulation (white squares, n = 5 naïve slices from 3 animals) significantly reduces evoked fEPSPs ($P < 0.001$, t-test, peak effect of 53% ± 7%, 11 min after NPY application) and the effect is detectable 47 min into the washout period. Application of NPY 10 min before tetanus stimulation (black squares, n = 5 naïve slices from 4 animals) significantly inhibits LTP ($P < 0.001$, t-test). PTP is brief and the following post-tetanus fEPSP responses are significantly attenuated for 52 min into the washout period (peak effect of 50% ± 4%, 12 min after NPY application). Field EPSP slopes are normalized to the first 10 min of baseline and plotted against time. Arrow and bars indicate the time point for tetanic stimulation and NPY application, respectively. (B) Alignment is made between slices perfused with NPY 20 min post-tetanus (black squares, n = 6 naïve slices from 5 animals) and slices receiving tetanus stimulation but no NPY application (white squares, n = 8 naïve slices from 6 animals). In both situations, tetanus stimulation induces LTP, but application of NPY 20 min post tetanus significantly attenuates the evoked fEPSP responses, having a peak effect 6 min after termination of NPY application ($P < 0.05$, t-test, 48% ± 14% reduction compared to values obtained immediately before NPY application). Full recovery of fEPSPs is obtained within 29 min of the washout period, and by the end of the recording period the mean responses are elevated ~29% above average baseline values. Pre- and post-tetanus fEPSP slopes are normalized to baseline, and plotted against time. Arrow and filled bar indicate time-points of tetanic stimulation and NPY application, respectively.**

synaptic plasticity were associated with delayed memory consolidation, although animals were still able to acquire new knowledge.

Transgene NPY Expression and Release

The pattern of transgene NPY expression in the present study closely resembles the previous study described in detail by Richichi et al. (2004), where the same chimeric serotype 1/2 rAAV vector was used. This recombinant AAV almost exclusively transduces neurons, including granule cells, hilar, and pyramidal neurons (Burger et al., 2004; Richichi et al., 2004). Normally, endogenous neuropeptides are stored in and released from large dense-core vesicles (LDCVs) (Thureson-Klein et al., 1986; Zhu et al., 1986; De Potter et al., 1988) and compared to classical neurotransmitters, higher frequencies of neuronal stimulation are in general required for neuropeptide release (Hokfelt, 1991). Whether ectopically expressed transgene NPY also is accumulated in and is released from LDCVs is not
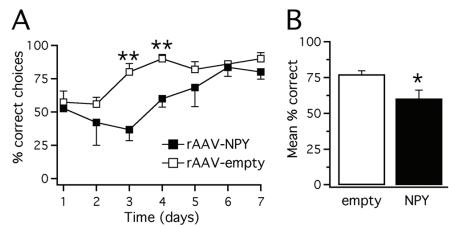
FIGURE 6. **Spatial learning rate is reduced by transgene NPY overexpression. (A) Percentage of correct choices made by rAAV-NPY (n = 5 animals) and rAAV-empty (n = 5 animals) injected rats during acquisition training (**$P < 0.01$, t-test). The repeated measure ANOVA revealed a significant effect of treatment (treatment $F(1,48)$ = 6.9, $P = 0.03$; days × treatment: $F(6,48)$ = 2.1, $P = 0.06$). (B) Overall mean of correct choices made during the 7 days of training sessions ($P < 0.05$, t-test).**

known, although, using electron microscopy, we have observed NPY immunoreactivity associated with LDCVs of rAAV-NPY-injected hippocampi (unpublished data). However, since the viral vector was constructed with the prepro-NPY cDNA sequence, it is conceivable that the transgene NPY trafficking occurs through normal peptide trafficking pathway. Supporting this assumption, studies have shown that if the rAAV vector encodes for NPY (or galanin) in combination with the fibronectin secretory signal sequence (FIB), which promotes constitutive release, immunohistochemical identification of transgene neuropeptide is hardly possible (Haberman et al., 2003; Foti et al., 2007). We easily detected transgene NPY immunoreactivity expression within all animals injected with rAAV-NPY, and our electrophysiological recordings also suggest that the dominating mode of transgene NPY release is dependent on high frequency synaptic activity. Thus, we found that in CA1, depression of consecutive fEPSPs was less pronounced at the later stages of HFS of Schaffer collaterals in rAAV-NPY-treated animals. These results suggest that high frequency activity-dependent release of transgene NPY during the initial phase of the stimulation train causes an overall decrease of glutamate release, resulting in slower depletion of its releasable pool during the last part of the stimulation train. This in turn leads to less pronounced depression of the responses in rAAV-NPY-treated animals compared to controls during HFS.

On the basis of our data, however, we cannot exclude that some small amount of the transgene NPY could be constitutively released from neurons: if the observed alteration of PPF had been due to transgene NPY released by the first pulse of paired stimulations, changes in the opposite direction (i.e., decreased second fEPSP, resulting in decreased PPF) would have been expected. The observed increase in PPF is inconsistent with this interpretation. Released NPY most likely acts via NPY Y2R activation, since application of BIIE0246 in rAAV-NPY-treated animals decreased PPF in Schaffer collateral-CA1 synapses. This notion is supported by relatively sparse expression of receptors (i.e., Y1 and Y5) other than Y2R subtype in the CA1 area (Redrobe et al., 1999). Moreover, only weak specific Y5R agonist-mediated inhibition of excitatory synaptic responses has been observed in CA1 area and subiculum in young animals (Ho et al., 2000). This effect of Y5R agonists completely disappeared at adulthood, while Y2R agonists still maintained their strong inhibitory action (Ho et al., 2000).

One could speculate that transgene NPY may downregulate Y2R expression, thus limiting NPY-induced activation of this receptor subtype. This scenario, though, seem less likely due to several considerations. We have observed that viral-mediated long-lasting overexpression of NPY in the hippocampus decreases only NPY-Y1R binding, while NPY-Y2R binding is unaltered (unpublished observations). In agreement, transgenic NPY overexpressing rats showed increased levels of prepro-NPY mRNA and NPY peptide in hippocampus, accompanied by decreased NPY-Y1R binding, but no alterations in NPY-Y2R binding has been observed (Thorsell et al., 2000). Similarly, in Chinese hamster ovary cells, expression of NPY Y1R, but not NPY Y2R, was downregulated by exposure to receptor-selective peptide agonists (Parker et al., 2001). Moreover, our previous studies show that recurrent epileptic seizures enhance the expression of NPY in the hippocampus without down-regulating Y2R (Vezzani et al., 1999; Vezzani and Sperk, 2004). Taken together, these finding support the notion that transgene NPY overexpression does not downregulate the Y2R, and could act via activation of this receptor.

Long-Term Potentiation

Activity-dependent long-lasting increase in synaptic strength, i.e., LTP, in the hippocampus is considered to be a synaptic correlate of hippocampal learning and memory (Stevens, 1996; Malenka and Nicoll, 1999). We found that the magnitude of LTP in CA1 of rAAV-NPY-treated animals is decreased by about 50%. This effect appears to be due to release of transgene NPY, since the selective antagonist of NPY Y2R, BIIE0246, rescued LTP in the CA1 area of rAAV-NPY-injected rats. This effect of BIIE0246 cannot be attributed to blockade of signaling exerted by endogenous NPY, since in mice lacking the NPY gene (NPY$^{-/-}$ mice) neither PP, nor HFS-induced field EPSPs, as well as input–out relationships were altered in CA1 as compared to wild-type littermates (Baraban et al., 1997). Also, in naïve rat hippocampal slices perfused with BIIE0246, field EPSPs remained unchanged (El Bahh et al., 2002) despite the fact that the Y2R subtype is the most abundant expressed NPY receptor in this area (Redrobe et al., 1999). The mechanism of transgene NPY effect on LTP seems to be complex. We observed a trend, though nonsignificant ($P = 0.11$), toward lower LTP induction rates in NPY overexpressing animals, paralleled by a significantly decreased LTP magnitude. Exogenously applied NPY to the naïve slices showed blockade of LTP induction by administration of NPY prior to tetanus, and reduction of already potentiated fEPSP magnitude when NPY was applied after tetanus. One could propose that small constitutive release of transgene NPY may partially interfere with LTP induction, while additional, tetanus-induced release of transgene NPY could cause a decrease in the magnitude of potentiated responses. Both effects can be explained by inhibition of glutamate release onto pyramidal cells caused by transgene NPY, reducing Ca^{2+} influx into presynaptic nerve terminals (Colmers et al., 1988; Qian et al., 1997). In line with our observations, partial blockade of LTP in the rat dentate gyrus (DG) has been shown after intraventricular infusion of NPY in vivo (Whittaker et al., 1999). Our data suggest that LTP, although partially compromised, is preserved in the hippocampus of rats with transgene NPY expression.

Learning and Memory

In the present study, the two-platform spatial discrimination water maze test revealed a transient learning deficit in rAAV-NPY-treated rats as compared to control animals. The learning deficit was mostly manifested at Days 3 and 4. However, during the following days rAAV-NPY-injected rats performed as control animals as assessed by the number of correct choices.

These data indicate that rAAV-NPY-treated animals could still acquire memory but had a delayed process of learning. Somewhat similar to our study, it has been shown that transgene rats selectively overexpressing NPY in CA1 neurons had impaired ability in finding the hidden platform in Morris water maze during 4 days of trials (Thorsell et al., 2000). Since no further trials were performed in that study, it is not clear whether these animals would improve their performance on consecutive days of testing. However, these rats had completely ameliorated their learning and memory deficit at 1-yr of age (Carvajal et al., 2004). To date, no learning and memory tests have been performed in mice overexpressing NPY (Thiele et al., 1998; Kaga et al., 2001). Increasing NPY levels by intraventricular infusion of NPY attenuates conditional discrimination accuracy in rats in a dose-dependent manner (Cleary et al., 1994). However, more recent studies failed to show any deficits in passive avoidance learning tests after NPY infusion (Ishida et al., 2007). These data indicate that increased levels of NPY either by viral vector injection in normal rats or by generating transgenic rats, as well as by intraventricular NPY infusions, leads to certain inhibitory effects on learning and memory. Somehow contradictory to these findings, intraventricular or direct injection of NPY into the hippocampus of mice has been shown to improve memory processing as demonstrated by enhanced memory retention for T-maze footshock avoidance test training (Flood et al., 1987, 1989). This effect was only evident when NPY was administered immediately after training, 1 week prior to testing. The reason for the discrepancy in comparison to this study is uncertain, but it is likely that the timing of NPY bioavailability (either release from the tissue, or applied exogenously) during the time-course of a learning process plays a central role by which NPY can modulate memory processing. In general, the effect of NPY on cognitive function seems to be more pronounced in the initial phase of the learning process but the animals do learn the task eventually.

CONCLUSION

The present study describes changes in synaptic transmission and plasticity and related alterations in learning and memory caused by transgene NPY ectopically overexpressed in the hippocampus of rats. The changes in synaptic transmission and plasticity are mostly due to high frequency, activity-dependent release of transgene NPY. Our data also provide evidence that NPY gene transfer only partly inhibits cognitive function of the hippocampus, delaying the process of learning but not preventing memory acquisition in rats. The impact of these effects on clinical applicability of NPY gene transfer in epilepsy will depend on how effectively transgene NPY suppresses seizures as compared to other pharmacological or surgical treatments, both of which have also certain negative impact on cognitive function (Motamedi and Meador, 2004; Hamberger and Drake, 2006). In drug resistant patients, the possible benefits of NPY gene transfer on seizures would most likely outweigh the

partially potentially cognitive function. Moreover, it still remains to be explored whether rAAV-NPY affects synaptic plasticity and cognitive performance in chronic epileptic animals mimicking more closely the clinical situation, where these functions are already compromised by the neuropathology and the recurrent seizures.

Acknowledgment

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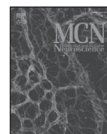
III

Activity-dependent volume transmission by transgene NPY attenuates glutamate release and LTP in the subiculum

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ABSTRACT

Neuropeptide Y (NPY) gene transduction of the brain using viral vectors in epileptogenic regions can effectively suppress seizures in animals, and is being considered as a promising alternative treatment strategy for epilepsy. Therefore, it is fundamental to understand the detailed mechanisms governing the release and action of transgene NPY in neuronal circuitries. Using whole-cell recordings from subicular neurons, we show that in animals transduced by recombinant adeno-associated viral (rAAV) vector carrying the NPY gene, transgene NPY is released during high-frequency activation of CA1-subicular synapses. Released transgene NPY attenuates excitatory synaptic transmission not only in activated, but also in neighboring, non-activated synapses. Such broad action of transgene NPY may prevent recruitment of excitatory synapses in epileptic activity and could play a key role in limiting the spread and generalization of seizures.

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Introduction

One of the most prominent novel strategies to interfere with neurological disease processes is considered to be a gene therapy approach based on viral vectors to transfer genes of interest into the brain. In this regard, NPY gene transduction of brain tissue has attracted particular interest due to its potential to regulate and perhaps ameliorate neurological conditions, in particular epileptic disorders (McCown, 2004; Noe' et al., 2007). It has been demonstrated that delivery of recombinant adeno-associated viral (rAAV) vector carrying the NPY gene into the rat hippocampus or piriform cortex can effectively suppress acutely intracerebroventricular and intrahippocampal (Richichi et al., 2004), as well as intra-peritoneal (Foti et al., 2007) kainic acid-induced limbic seizures, respectively. More recently, hippocampal rAAV–NPY vector-based gene therapy was shown to suppress the frequency of spontaneous seizures in chronically epileptic rats (Noè et al., 2008). The mechanisms underlying such seizure-suppressant effects of transgene NPY are not well understood. Particularly, under which circumstances transgene NPY is released, and whether and how it acts on synaptic transmission within the area of viral vector transduction is not known. These questions are of fundamental importance not only for the implementation of such

gene therapy approach in clinical trials with patients, but also for our general understanding of how transgene neuropeptides may act in the brain.

In the normal brain, exogenously applied NPY has been shown to modulate inhibitory (Bacci et al., 2002) and excitatory synaptic transmission (Haas et al., 1987) in cortical and hippocampal regions, respectively. The most thoroughly described action of NPY in the central nervous system is its pronounced inhibitory effects on excitatory synaptic transmission in the hippocampal formation (Colmers and Bleakman, 1994). NPY has been shown to decrease glutamate release (Colmers et al., 1988; Klapstein and Colmers, 1993) by inhibiting presynaptic, voltage-dependent Ca^{2+}-channels in glutamatergic terminals in CA1 (Qian et al., 1997).

In the present study, we explore under which circumstances transgene NPY is released from afferent synapses impinging onto subicular pyramidal neurons, and investigate how it modulates excitatory synaptic transmission and plasticity in these synapses. The subicular pyramidal neurons serve as main relay stations for outgoing efferents of CA1 pyramidal cells (O'Mara et al., 2001), and therefore may control spreading of seizure activity from the hippocampal formation. Moreover, some clinical and experimental evidence suggests that temporal lobe epileptic (TLE) activity could be even initiated in the subiculum (Stafstrom, 2003, 2005). In contrast to the CA1 and CA3 areas of the hippocampus, there is relatively little neuronal loss and insignificant reactive gliosis in the subiculum of patients with TLE (Fisher et al., 1998; Dawodu and Thom, 2005).

Therefore, the preserved subiculum in TLE could offer an attractive target area for gene transfer in order to suppress spreading of epileptic activity from the hippocampal formation.

Results

Transgene NPY expression

Viral transduction and transgene expression in brain slices, prepared three weeks after rAAV–NPY vector administration, was examined by immunohistochemistry. In these animals, high levels of transgene NPY immunoreactivity were detected throughout the hippocampal formation including CA1, CA3, dentate gyrus, dentate hilus and proximal subiculum (Fig. 1A). Dense transgene NPY expression was particularly observed within the inner molecular layer and hilus of the dentate gyrus, and also within the mossy fiber terminals in CA3 and pyramidal cell layer of CA1. Maximal expression of transgene NPY was observed within two weeks after rAAV–NPY vector injection, and it remained stable for at least three months (data not shown). Transgene NPY expression was selective to neurons,
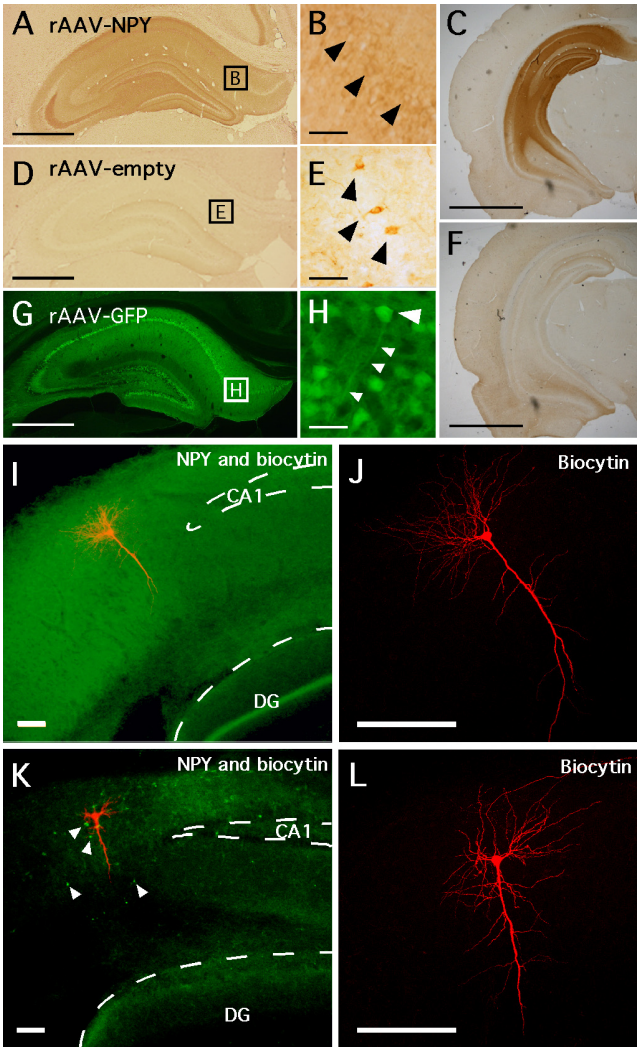
particularly to projection neurons, in agreement with previously demonstrated neuronal tropism of the rAAV serotype 1/2 vector used in this study (Richichi et al., 2004). In the proximal subiculum, which is the area bordering CA1, where the pyramidal neurons are less densely packed than in CA1, transgene expression of NPY was observed in all pyramidal neurons (Fig. 1B). Transgene NPY expression extended along the medio-lateral and dorso-ventral axis, covering most of the hippocampal formation, and was generally retained within the borders of the hippocampal fissure (Fig. 1C). Occasionally, transgene NPY expression was observed in neocortical areas, probably due to back-flow of the viral particles through the needle track following surgery. However, in all examined brain slices there were no apparent brain damage caused by either surgery or viral vectors, again consistent with previous observations (Richichi et al., 2004).

In contrast to slices obtained from rAAV–NPY injected animals, slices from time-matched control animals injected with rAAV-empty vector showed only few NPY immunoreactive cells scattered throughout the hippocampal formation, including subiculum. These cells most probably resemble GABAergic inhibitory interneurons containing endogenous NPY (Figs. 1D, E and F). In these control animals, a weak band of NPY-containing fibers was observed within the molecular layer of the dentate gyrus and within the hilus, as described previously in young rats (Moryś et al., 2002). Additionally, administration of rAAV–GFP vector clearly confirmed neuronal tropism of the rAAV serotype 1/2 vector, and spread of the transgene, in this case of GFP, throughout the hippocampus and subiculum (Fig. 1G). The GFP expression, as seen by epifluorescence microscope, was detected in both cell soma and fibers of the transduced neurons (Fig. 1H).

Slices prepared on the vibratome and used for whole-cell recordings were subsequently processed for immunohistochemistry to confirm both biocytin-labeling of recorded pyramidal neurons, as well as transgene NPY immunoreactivity. In these slices, all recorded cells had typical shape of subicular pyramidal neurons, were situated in proximal subiculum (i.e. the area bordering the CA1), and were surrounded by transgene NPY immunoreactivity in rAAV–NPY (biocytin-labeled pyramidal neurons in Figs. 1I and J), but not in rAAV-empty treated animals (Figs. 1K and L). These data suggested that rAAV–NPY vector injections successfully transduced neurons in the whole hippocampal formation of rats as assessed by NPY immunoreactivity.

High-frequency stimulation-dependent release of transgene NPY

Since endogenous neuropeptides are though to be released primarily during repetitive activation of presynaptic terminals (Hokfelt, 1991), we first asked whether transgene NPY is also released during high-frequency activity. To address this question, we applied high-frequency stimulation (HFS) trains to alveus, i.e., afferent glutamatergic fibers (CA1 pyramidal axons) to the subicular pyramidal neurons, while recording EPSCs from these cells. In rAAV–NPY treated animals (n=13 cells from 5 animals), the HFS trains of 10 pulses with 40 Hz frequency evoked EPSCs with attenuating amplitudes (Fig. 2A, aCSF trace). Application of BIIE0246 substantially increased the rate of EPSC attenuation (Fig. 2A, BIIE0246 trace), but did not affect the
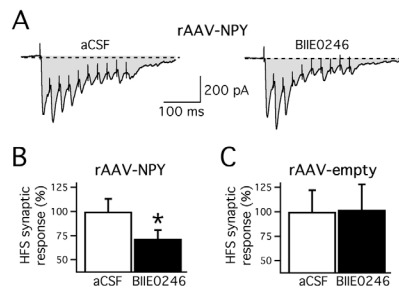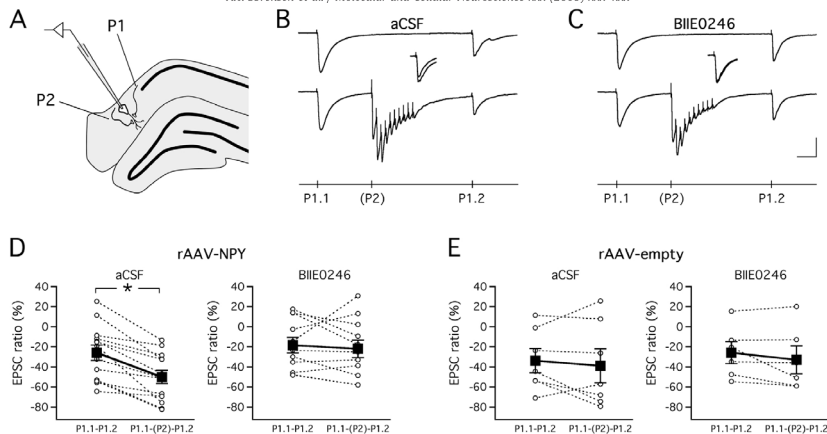

Fig. 2. HFS-induced excitatory synaptic transmission in CA1-subicular cell synapses of rAAV–NPY and rAAV-empty vector-treated animals. (A) Representative traces of HFS-induced EPSCs (10 stimulations at 40 Hz) before and after BIIE0246 application in a slice from a rAAV–NPY injected rat. (B) The average ratio of normalized synaptic responses in percent (integral of the total EPSC response during 350 ms divided by the integral of the first EPSC during 25 ms) recorded from rAAV–NPY injected rats (n=13 cells from 5 animals) before and after application of Y2 receptor antagonist, BIIE0246, during HFS of the alveus pathway (*p<0.01, paired t-test). (C) The average ratio of normalized synaptic responses in percent recorded from rAAV-empty injected rats (n=6 cells from 4 animals) before and after BIIE0246 application.

amplitude of the first EPSC of the response (p>0.05, paired t-test, rAAV–NPY: 265 pA±52 during aCSF, 239 pA±69 during BIIE0246). Moreover, quantification of these responses by dividing the total integral of the EPSCs (induced by all 10 stimulations) by the integral of the first EPSC of the train, revealed a significant decrease of this ratio after BIIE0246 application (p<0.01, paired t-test, Fig. 2B). In slices from rAAV-empty treated animals (n=6 cells from 4 animals), BIIE0246 application had no effect on EPSC attenuation rate, nor on the first evoked EPSC in the train (p>0.05, paired t-test, 183 pA±73 during aCSF, 129 pA±42 during BIIE0246), and therefore, the average integral ratio of synaptic responses was not changed (Fig. 2C). These data indicate, that transgene NPY could be released during high-frequency activation of the alveus pathway, significantly altering high-frequency responses in CA1-subicular synapses. Examination of the two first evoked EPSCs during HFS (separated by 2 ms), indicated that paired-pulse facilitation (PPF) was similar in both groups and was unaffected by BIIE0246 application (data not shown). These data could be taken to suggest that the first pulse of the stimulation train was either not releasing transgene NPY or the amount of transgene NPY released by a single stimulation was not sufficient to alter the second EPSC in the train, leaving this type of presynaptic short-term plasticity unaltered (Zucker and Regehr, 2002).

Released transgene NPY affects neighboring excitatory synapses

To further confirm that HFS evokes transgene NPY release, we used alternating stimulation of two independent afferent pathways impinging onto the same subicular pyramidal neuron (see Fig. 3A). We tested whether NPY released by HFS of one pool of excitatory

Fig. 1. Transgene expression after injection of rAAV–NPY and rAAV–GPF vector into the rat hippocampal formation. (A, B) Slice from the dorsal hippocampal formation showing immunoreactivity (IR) for NPY three weeks after injection of rAAV–NPY vector. Note high levels of transgene NPY expression throughout all hippocampal regions including the subiculum (inset B). In the proximal subiculum, pyramidal neurons were positive for NPY IR (indicated by arrow heads). (C) Extensive transgene NPY IR throughout the hippocampal formation. Note that NPY expression is enclosed by the surrounding fissure. (D) Slice from animal injected with rAAV-empty vector showing weak endogenous NPY IR in all regions of the hippocampal formation with dispersed NPY-positive cell bodies and fibers. (E) Scattered NPY-positive cell bodies in proximal subiculum, presumably resembling NPY-containing interneurons (shown by arrows). (F) NPY IR as seen in a coronal section of a rat injected with rAAV-empty vector. (G) GFP autofluorescence detected three weeks after rAAV–GFP vector administration, revealing widespread and efficient viral transduction throughout the hippocampal formation. (H) GFP expression mostly confined to pyramidal cell bodies (large arrow) and processes (small arrows). (I) Slice from a rAAV–NPY injected rat used for whole-cell patch-clamp recordings. Green color (NPY, FITC) shows NPY IR and red color shows biocytin-labeled recorded cell (CyTM3) in proximal subiculum. Dotted lines indicate the border of the CA1 pyramidal cell layer adjacent to the recorded cell with the dentate gyrus (DG) area situated below. (J) The same cell, as shown in image I, is visualized by confocal images with superimposed z-planes, showing characteristic shape of cell soma and dendrites of a subicular pyramidal neuron. (K) In slices from rAAV-empty injected rats, NPY IR (NPY, green, FITC) seen as green dots (marked by arrows), denoting the presence of endogenous NPY-containing interneurons within the proximal subiculum. (L) The same biocytin-labeled cell (red, CyTM3, arrow) as in image K shown by confocal imaging. Boxed areas on left images (A, D, G) are magnified and shown on right images (B, E, H). Scale bars=1 mm in A, D, G; 50 μm in B, E; 3 mm in C, F; 25 μm in H; 100 μm in I, J, K, L.

Fig. 3. Transgene NPY released from HFS-activated excitatory synapses inhibits glutamate release in neighboring non-activated synapses. (A) The stimulation electrodes are placed in the alveus on either side of the recorded cell in order to stimulate two separate afferent fibers from CA1 neurons (P1 and P2) impinging onto the same subicular cell. (B) Representative traces showing EPSCs induced by only paired stimulation (separated by 850 ms) of the P1 pathway (top trace, P1.1–P1.2), or P1 pathway with intermediate train stimulation of P2 pathway (lower trace, P1.1–(P2)–P1.2) in a slice from rAAV–NPY injected rat. Bottom line shows time points of stimulation for P1 and P2. Note the reduction of EPSC amplitude evoked by P1.2 stimulation after HFS of P2 (inset). (C) Similar traces as shown in B, but after BIIE0246 application. Note that EPSCs evoked by P1.2 stimulations are of the same amplitude. Scale bar is 100 ms and 100 pA, and applies for both B and C. (D) EPSC amplitude ratios (P1.2–P1.1)/P1.1) of paired EPSCs are plotted for each cell before and after BIIE0246 application in rAAV–NPY injected animals. Doted lines and circles show individual EPSC ratio changes. Thick line and closed squares show the mean±SEM values (*p<0.05, paired *t*-test). (E) Similar as in D for rAAV–empty injected rats.

synapses (P2, Fig. 3A) would be strong enough to diffuse and modulate excitatory transmission in a separate, neighboring pool of excitatory synapses on the same cells (P1, Fig. 3A). Indeed, we found that HFS applied to one afferent pathway decreased EPSC amplitudes induced by single test-stimulation of the other afferent pathway in rAAV–NPY slices (Fig. 3B). Normalized ratio (calculated as (P1.2−P1.1)/P1.1) of single EPSCs, induced by stimulation of the test afferent pathway, was significantly decreased (on average by 33%) when intermittent HFS (between P1.1 and P1.2) was applied to a separate afferent pathway (Fig. 3D, aCSF; n=13 cells from 5 animals; p<0.05, paired *t*-test). We confirmed that this effect was indeed caused by released NPY acting via Y2 receptor by applying BIIE0246 to slices, which completely restored the test EPSC amplitudes to control levels (Figs. 3C and D, BIIE0246; n=10 cells from 4 animals). The EPSC ratios were not altered by HFS in slices from rAAV–empty treated control animals during normal conditions (Fig. 3E; p>0.05, n=7 cells from 5 animals) or after application of BIIE0246 itself (Fig. 3E; n=6 cells from 5 animals). Taken together, these data show unequivocally that HFS-induced synaptic activation leads to transgene NPY release. In contrast, a single stimulation of the P1 pathway does not seem to release sufficient transgene NPY to diffuse and inhibit glutamate release, since the amplitude of the first EPSC evoked by the HFS train (P2) remained unchanged after application of BIIE0246 in rAAV–NPY slices (data not shown).

Attenuation of long-term potentiation in CA1-subicular synapses

Next we asked whether HFS-induced transgene NPY release had any functional effect on CA1-subicular cell synapses. In particular, we explored possible alterations in long-term activity-dependent plasticity (long-term potentiation − LTP) in these synapses. LTP in CA1-subicular synapses was induced by tetanic stimulation (at 100 Hz) of CA1 fibers in the alveus and stimulation-induced EPSCs were recorded by whole-cell patch-clamp technique from subicular neurons. We assumed that tetanic stimulation of CA1 fibers used for LTP induction would concomitantly release transgene NPY and could affect the potentiated presynapses (as shown by previous series of experiments;

see above). Directly after tetanus, post-tetanic potentiation (PTP) was evoked, lasting for a few minutes (at most for 5 min; Fig. 4). This PTP seemed to be lower (although reaching statistically significant levels only at 3rd and 4th min after tetanus; *t*-test, p=0.03 and 0.02, respectively) in rAAV–NPY as compared to rAAV–empty injected rats. Since PTP is considered as another form of presynaptic short-term plasticity (Zucker and Regehr, 2002), one could propose that transgene NPY released by tetanic stimulation decreased glutamate release from CA1-subicular cell synapses in rAAV–NPY treated animals. Long-term potentiation, estimated as at least 15% increase in EPSC amplitude at 15 min after tetanus, was significantly lower in rAAV–NPY treated
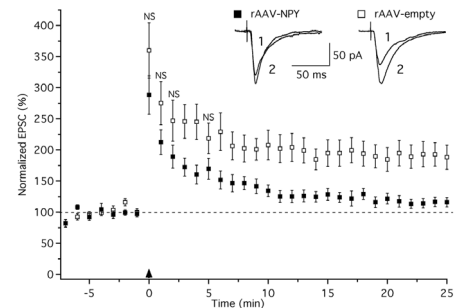
Fig. 4. Reduced LTP in CA1-subicular synapses of rAAV–NPY treated animals. Pre-and post-tetanus EPSC amplitudes are normalized to baseline values and plotted against time. Time point for tetanic stimulation is shown by arrow. Post-tetanic potentiation (PTP: max. 5 min after tetanus) during the 3rd and 4th min is significantly reduced (p<0.05, *t*-test) in rAAV–NPY (n=13 cells from 9 animals) as compared to rAAV–empty (n=11 cells from 8 animals) injected rats, while non-significant (NS) at other time points. From 6 min and onwards, LTP is significantly lower in rAAV–NPY as compared to rAAV–empty injected rats (p<0.05, *t*-test). Inset: Superimposed representative EPSCs acquired 5 min before (1) and 10 min after (2) tetanic stimulation from a rAAV–NPY and a rAAV-empty injected rat.

animals (on average 15 min after tetanus, $126 \pm 10\%$, $n = 13$ cells from 9 animals for rAAV–NPY; $199 \pm 20\%$, $n = 11$ cells from 8 animals for rAAV-empty; $p < 0.05$, t-test; Fig. 4). Despite these clear differences in magnitude and time-course of LTP between the two groups, the LTP induction rate was similar (rAAV–NPY, 68%, $n = 19$; rAAV-empty, 69%, $n = 16$; χ^2 test followed by Fisher's exact test) as calculated if the mean response was increased by >15% from baseline values 15 min after tetanic stimulation. These data suggest, that HFS of the alveus pathway releases transgene NPY, which suppresses glutamate release in CA1-subicular synapses as indicated by less PTP, and consequently weaker LTP in these synapses.

Miniature EPSCs

To explore whether transgene NPY could also be released during normal conditions without high-frequency stimulation, possibly tonically suppressing glutamate release from the presynaptic terminals, we studied isolated miniature excitatory postsynaptic currents (mEPSCs) (i.e. when action potentials are blocked by TTX and $GABA_A$ receptors by PTX) in subicular neurons using whole-cell recordings (Fig. 5A). After application of the specific Y2 receptor antagonist, BIIE0246, the mean frequency of mEPSCs was unchanged in both rAAV–NPY ($n = 7$ cells from 4 animals) and rAAV-empty-injected animals ($n = 5$ cells from 3 animals) (Fig. 5B). Further analysis of mEPSCs using cumulative probability plots followed by Kolmogorov–Smirnov (K–S) test confirmed these observations, resulting in statistically non-significant alterations of mEPSCs interevent intervals in rAAV–NPY (Fig. 5C) and rAAV-empty (Fig. 5D) injected animals induced by BIIE0246. Both amplitude and kinetic properties (e.g., rise and decay time constants) of mEPSCs remained unchanged in both groups after BIIE0246 application (data not shown). Addition of NBQX (AMPA receptor antagonist) to the perfusion medium completely abolished the mEPSCs (data not shown), confirming that the synaptic events were generated by activation of non-NMDA type of glutamate receptors.

Direct comparison of the mean values revealed that mEPSC frequency was similar between rAAV–NPY and rAAV-empty vector-treated animals both before and after BIIE0246 application ($p > 0.05$, t-test; Fig. 5B). However, as confirmed by cumulative probability plot analysis, the interevent intervals of mEPSCs were significant higher in rAAV–NPY injected animals both before and after BIIE0246 application ($p < 0.001$, K–S test; Figs. 5E and F), suggesting lower tonic excitatory input.

Discussion

We show here, for the first time, that rAAV–NPY vector-derived transgene NPY in the hippocampal formation is predominantly released during high-frequency neuronal activity. This finding suggests that transgene NPY exerts its action on synaptic transmission preferentially during elevated firing rate and/or synchronization of neuronal activity, which could occur during increased excitability and epileptic seizures in the hippocampal formation (Avoli et al., 2002; Cavazos and Cross, 2006). We also demonstrate, for the first time, that transgene NPY released from one set of activated excitatory synapses can diffuse and reach non-activated neighboring excitatory synapses on the same cell, decreasing glutamate release even in these synapses. Such action could prevent recruitment of the new excitatory synapses and perhaps even cells in the activity occurring during epileptic seizures, and may significantly contribute to the antiepileptogenic and antiepileptic effects of gene therapy using rAAV–NPY vectors (Richichi et al., 2004; Lin et al., 2006; Foti et al., 2007; Noè et al., 2008).

Transgene expression

In the proximal subiculum, NPY is normally expressed in a subset of GABAergic interneurons having multipolar and few dendritic

processes, which are mainly distributed subjacent to the alveus pathway (de Quidt and Emson, 1986). Similar expression pattern was also found in animals injected with rAAV-empty vector, most likely representing endogenous NPY expression. On the other hand, in rats with rAAV–NPY vector transduced hippocampi, massive transgene NPY immunoreactivity was detected in almost all neurons throughout the hippocampal formation. Similar expression pattern of transgene
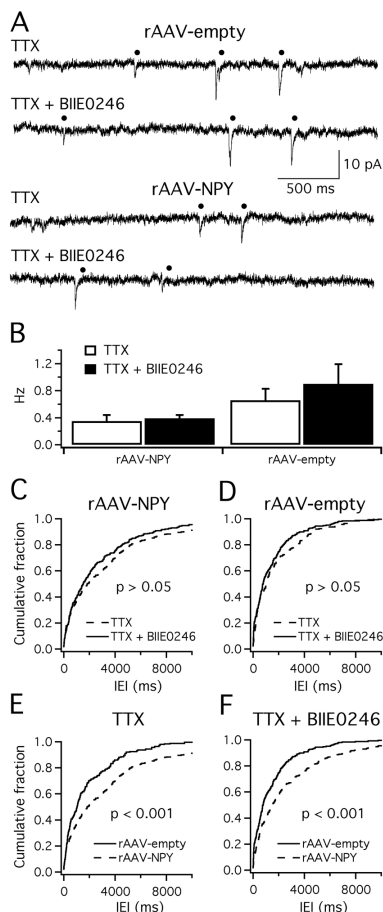
Fig. 5. Decreased frequency of mEPSCs recorded in subicular neurons of rAAV–NPY vector-treated animals. (A) Traces of representative mEPSCs recorded from subicular neurons in rAAV-empty and rAAV–NPY injected rats before and after BIIE0246 application (with TTX present in the perfusion medium). Synaptic events (mEPSC) are marked by dots and scale applies for all traces. (B) Mean frequency of mEPSCs recorded in rAAV-empty ($n = 5$ cells in 3 animals) and rAAV–NPY ($n = 7$ cells in 4 animals) treated animals before and after exposure to BIIE0246 ($p > 0.05$, t-test). (C, D) Cumulative probability plots of interevent intervals (IEIs) recorded before and after BIIE0246 application in rAAV-empty and rAAV–NPY injected animals ($p > 0.05$, K–S test). (E, F) Cumulative probability curves showing significantly higher IEIs in rAAV–NPY as compared to rAAV-empty injected animals both before and after BIIE0246 application ($p < 0.001$, K–S test).

NPY was also observed in a previous study using the same viral vector (Richichi et al., 2004). Predominantly neuronal tropism and long-term transgene expression of this viral vector was achieved by using the chimeric serotype 1/2, carrying the neuron-specific enolase (NSE) promoter (for review, see Burger et al., 2005). Based on the expression pattern of GFP autofluorescence in rAAV–GFP injected rats, we also confirmed that gene transduction was mostly restricted to neurons, although some non-neuronal transduction could not be completely excluded.

Release of transgene NPY

Several lines of evidence from our current study strongly suggest that transgene NPY is preferentially released during HFS. In subicular neurons of rAAV–NPY treated animals, HFS-induced depression of consecutive EPSCs was more pronounced after BIIE0246 application, suggesting acute blockade of released transgene NPY effect. This stronger HFS-induced depression after BIIE0246 application was mostly due to changes in the later phase of HFS. One possible explanation of such effect is that transgene NPY begins to be released only after the first few HFS pulses, leading to inhibition of glutamate release and preventing fast depletion of the releasable pool of glutamate vesicles as reflected by relatively small depression of EPSCs. Once BIIE0246 was applied to the slices, transgene NPY effect on glutamate release was blocked, resulting in more glutamate release during the later phase of HFS, thus leading to more rapid depletion of the releasable pool of glutamate vesicles in presynapses, and therefore more pronounced EPSC depression.

The more substantiated proof that transgene NPY is released during HFS was obtained from the two-pathway stimulation experiments, where HFS of one afferent pathway decreased the amplitude of EPSC elicited by a single pulse stimulation of another afferent pathway impinging onto the same subicular neuron. This effect was blocked by Y2 receptor antagonist BIIE0246, suggesting that it was due to transgene NPY. These data strongly support the idea that transgene NPY is indeed released during HFS. In addition, these data also indicate that HFS-activated axon terminals release such a large amount of transgene NPY that it is sufficient to diffuse and decrease glutamate release even in synapses that are not activated by the HFS. In contrast, activation of presynaptic fibers by single pulse stimulation does not seem to release enough transgene NPY to alter EPSCs in neighboring synapses (e.g., on Fig. 3, BIIE0246 application does not alter amplitude of the first EPSC evoked by HFS of P2, although P1 is stimulated by a single pulse (P1.1) 300 ms earlier). These findings imply that; (i) during epileptic seizures, transgene NPY could be released due to the high-frequency neuronal activation, counteracting the excessive glutamate release, and subsequently inhibiting seizure activity; and (ii) such release of transgene NPY could decrease glutamate release even in those synapses that are not yet recruited in high-frequency or epileptic activity, limiting their involvement and contribution to seizures. In line with our conclusion, previous publications indicate that neuropeptides are predominantly released by high-frequency stimulation (Whim and Lloyd, 1989; Hokfelt, 1991; Vilim et al., 1996). Since seizures induce de novo expression of NPY mRNA in hippocampal neurons, transgene NPY expression cannot be considered as completely ectopic for most of the principal cells in the hippocampal formation (Marksteiner et al., 1990; Sperk et al., 1992). Therefore, one could presume that neuropeptide release machinery, including large dense core vesicles, is present or at least can be easily re-expressed by these cells when NPY is present. Electron microscopy data indicate that rAAV–NPY vector transduced neurons do contain transgene NPY in large dense core vesicles (Noè et al., 2008), and may thus be released by this route.

Although, based on the results discussed above, it is tempting to propose that transgene NPY would be released exclusively by HFS, and not during relatively low frequency neuronal activity, and, therefore, would not interfere with normal brain function, some of our data may suggest opposite. For example, subicular neurons received lower tonic excitatory input, as judged by decreased mEPSC frequency in rAAV–NPY vector-treated animals. This may suggest that some limited amount of transgene NPY is released even without any action potential activity. Inability to reverse the decreased mEPSC frequency in subicular neurons of rAAV–NPY treated animals by BIIE0246 could be explained by involvement of other than Y2 NPY receptors (e.g. Y1 and/or Y5) in mediating transgene NPY effect. In support, application of a specific Y5 receptor agonist reduced EPSC amplitude recorded in pyramidal neurons of the proximal subiculum (Ho et al., 2000). However, this effect was relatively small compared to Y2 receptor agonist, and it totally disappeared in rats older than 22 days of age (Ho et al., 2000), which is about 12 days younger than the mean age of animals used in the present study. Moreover, the NPY receptor subtype that mediates effects of overexpressed NPY could vary between the regions of the hippocampal formation. For example, opposite to our findings in subicular neurons of rAAV–NPY injected rats, in granule cells of epileptic animals, BIIE0246 application increases mEPSC frequency (Tu et al., 2005), suggesting Y2 receptor activation by de novo expressed and tonically released NPY from recurrent mossy fiber afferents to granule cells even in the absence of action potential activity. In normal rats, which lack such NPY expression in mossy fibers, both BIIE0246 and NPY application had no effect on the frequency of mEPSCs recorded in granule cells (Tu et al., 2005). Similarly, NPY application did not alter the frequency of mEPSCs in CA3 pyramidal neurons of naïve rats, despite high expression levels of Y2 receptor in this subregion (McQuiston and Colmers, 1996). Alternatively, a strong generic transgene expression may lead to alleged partial failure in synthesis of other proteins (Beck et al., 2000). In our case, transgene NPY could have caused such synthesis failure for endogenous protein(s) someway involved in transmitter release machinery, as reflected by decreased mEPSC frequency.

Long-term potentiation

High-frequency activity in the hippocampal formation does not only occur exclusively during epileptic seizures. Long-term potentiation (LTP), which is considered as a synaptic correlate for learning and memory, also relies on repetitive activation of excitatory synapses and was compromised in CA1-subicular cell synapses of rAAV–NPY treated animals. These data suggest that transgene NPY is indeed released during HFS for LTP induction, and most likely attenuates LTP magnitude by simply inhibiting glutamate release (Klapstein and Colmers, 1993; Qian et al., 1997) from the potentiated synaptic terminals. This notion is supported by our data showing unaltered LTP induction rate in rAAV–NPY vector-treated animals with concomitant decrease of EPSCs during PTP immediately after HFS, as well as throughout the whole recording period during LTP maintenance phase. Such interpretation of our results requires an assumption that transgene NPY released by LTP-inducing HFS remains in the extracellular space throughout the whole LTP observation period, thereby continuously affecting glutamate release from the presynaptic terminals. In support, the effect during washout of exogenously applied NPY on synaptic transmission in our (Woldbye et al., 2005; Sørensen et al., 2008) and other previous studies (Colmers et al., 1988; Klapstein and Colmers, 1997) have been shown to take about 30 min or even longer. An alternative explanation could be that transgene NPY released by HFS may decrease glutamate release from the presynapses already during LTP induction phase (i.e., already during HFS), resulting in less pronounced depolarization of the postsynaptic neurons, and therefore less pronounced LTP. This could very well be the case at least in the minority of subicular neurons, so-called regular spiking neurons, in which LTP induction, at least in rats, seem to be NMDA receptor-dependent and postsynaptic depolarization-

dependent (Wozny et al., 2008). However, the majority (65–75%) of the subicular pyramidal neurons are so-called bursting cells (Kokaia, 2000; Wozny et al., 2008) in which LTP induction seems to be independent of postsynaptic depolarization and postsynaptic calcium influx in the middle portion of rat subiculum (Wozny et al., 2008). In mice, irrespective of the cell-type, LTP in proximal subiculum has been shown to be independent of postsynaptic depolarization and NMDA receptor activation (Kokaia, 2000). In bursting cells, both in mice and rats, LTP seems to be expressed presynaptically as evidenced by decreased PPF of EPSCs (Kokaia, 2000; Wozny et al., 2008). In this case, transgene NPY released by LTP-inducing HFS may interfere with presynaptic calcium influx, again resulting in weaker LTP. Smaller PTP after HFS observed in rAAV–NPY treated animals would also be consistent with the latter interpretation.

Conclusions

Our data suggest that transgene NPY is released in high-frequency activity-dependent manner in the subiculum and acts as a volume transmitter, affecting nearby populations of both activated and non-activated excitatory synapses. Such volume transmission exerted by released transgene NPY would strengthen the compensatory effect of de novo expressed endogenous NPY after insults in the hippocampus, and provides us with more detailed insight and understanding of the mechanisms by which rAAV–NPY vectors protect against epileptogenesis and seizure activity.

The results of our LTP experiments raise some concerns about the clinical applicability of a rAAV–NPY gene-based therapy, since an attenuated LTP in the subiculum could reflect a possible deficit in learning and memory function (Morris et al., 1987; Morris et al., 1990; Lynch, 2004), and therefore represent a potential side-effect of such antiepileptic treatment. However, our previous study suggests, that such deficit in learning and memory is most likely transient, and rAAV–NPY treated animals are able to eventually learn hippocampal-dependent tasks, although with certain delay (Sørensen et al., 2008). Moreover, in most recent study, no learning deficit was observed in rats injected with rAAV–NPY vector carrying the cytomegalovirus (CMV)-chicken β-actin (CBA) recombinant promoter (Noè et al., 2008). Further studies are needed to specifically address the question whether and how a selective NPY vector promoter may differentially affect the functional outcome of NPY overexpression on learning and memory. It remains, though, to be determined whether LTP or cognitive functions are affected by rAAV–NPY treatment in epileptic animals, which already exhibit some disturbances in learning and memory.

Experimental methods

Vector production

The expression cassettes of the plasmids and the chimeric AAV vector were produced as described previously (During et al., 2003; Richichi et al., 2004). In brief, human prepro-NPY cDNA was subcloned into an expression cassette made of the rat neuron-specific enolase (NSE) promoter, woodchuck post-translational regulatory element (WPRE) and a bovine growth hormone polyA (BGHpA) signal, which was flanked by AAV2 inverted terminal repeats (pAM/NSE–NPY–WPRE–BGHpA). A similar expression cassette without the transgene (pAM/NSE–empty–WPRE–BGHpA) was used as control. An AAV expression cassette containing enhanced GFP (pAM/NSE–eGFP–WPRE–BGHpA) was used to evaluate viral transduction. Plasmids were then cloned into the backbone of a chimeric AAV vector having mix of rAAV serotype 1 and serotype 2 capsid helper plasmids and purified by heparin affinity columns, as the rAAV2 capsid proteins retained the heparin-binding domain. The viral titer was 1.0×10^{13} genome copies per ml for all vectors as determined by quantitative

PCR of rAAV vector genomes. Vectors were designated as rAAV–NPY, rAAV–empty and rAAV–GPF according to their construct.

Animals and vector administration

Neonatal Sprague Dawley rats ($n = 64$; B and K, Denmark; females and males), derived from time-pregnant rats, were used as recipients for hippocampal injections. Rats were kept in a 12-h light/dark cycle with ad libitum access to food and water. All experimental procedures were conducted following the guidelines of European Community for the Care and Use of Laboratory Animals and were approved by the local Ethical Committee. To facilitate patch-clamp experiments, which are significantly easier to perform in relatively young animals, postnatal day 2–4 (P2–4) rats were used for rAAV–NPY vector injections. Transgene NPY expression after P2–4 viral injections resembles that of adult injected animals. Rats were anesthetized by hypothermia and mounted on a pup tray attached to a KOPF stereotaxic frame. Injections of rAAV–NPY, rAAV–empty or rAAV–GFP vectors were conducted bilaterally into the hippocampal formation using a thin glass micropipette attached to a 5 µl Hamilton syringe at the following coordinates (in mm): anteroposterior (AP)-1.5 from bregma; medio-lateral (ML) ±1.9 from midline; dorso-ventral (DV)-1.8 from dura mater. At each injection site, 0.5 µl vector suspension was injected during 3 min and the pipettes were left in place for an additional 3 min.

Brain slices

Three to seven weeks after viral vector injections, individual rats (average age 34±2 days) were randomly selected and killed by decapitation. Their brains were rapidly removed, and placed in ice-cold cutting solution containing (mM); 195 sucrose, 2.5 KCl, 0.5 CaCl$_2$, 7.0 MgCl$_2$, 28 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 7.0 glucose, 1.0 ascobate and 3.0 pyruvate (adjusted to pH 7.4, osmolarity of 300 mOsm, equilibrated with 95% O$_2$/5% CO$_2$) and the hippocampal formation was dissected. Transverse slices from the dorsal hippocampal formation were cut (210–250 µm; Vibratome 3000, Ted Pella, Inc., Redding, CA) at 4 °C using the same solution, and placed in a holding chamber filled with artificial cerebrospinal fluid (aCSF) (mM); 119 NaCl, 2.5 KCl, 1.3 MgSO$_4$, 2.5 CaCl$_2$, 26 NaHCO$_3$, 1.0 NaH$_2$PO$_4$, and 11 glucose; pH 7.4; 296 mOsm) oxygenated and held at room temperature (RT). Slices were rested for >1 h before transferred to a submerged recording chamber fitted to a Olympus microscope (BX50WI) equipped with infrared differential interference contrast (IR-DIC) video microscopy. Slices were continuously perfused at 2 ml/min with oxygenated aCSF at RT.

Whole-cell patch-clamp in subiculum

Electrophysiological recordings of proximal subicular pyramidal neurons were accomplished 21–51 days after the viral injections (i.e. postnatal day 23–55). Excitatory postsynaptic currents (EPSCs) were recorded at a holding potential of −70 mV via patch-pipettes filled with (in mM): 117.5 K-gluconate, 17.5 CsCl, 8.0 NaCl, 10 CsOH–HEPES, 0.2 CsOH–EGTA, 2.0 MgATP, 0.3 Na$_3$GTP, and 5.0 QX-314 (pH 7.2; 295 mOsm; pipette tip resistance of 4–5 MΩ). Pyramidal neurons were first identified based on their characteristic triangle shaped cell soma and prospectively by immunohistochemistry of biocytin-labeling of recorded cell. Acceptable access resistance, as measured by delivery of −5 mV voltage step, was set to 12–25 MΩ, and acceptable variability during recordings was less than 20%.

Miniature EPSC (mEPSC)

In the presence of picrotoxin (PTX; 100 µM; Tocris Cookson) and tetrodotoxin (TTX; 1 µM; Tocris Cookson) blocking GABA$_A$ receptor-mediated and transient Na$^+$ currents, respectively, mEPSC were recorded for 3 min. To block NPY-mediated effects, the specific NPY

Y2 receptor antagonist, (S)-N2-[[1-[2-[4-[(R,S)-5,11-dihydro-6(6h)-oxodibenz[b,e]azepin-11-yl]-1-piperazinyl]-2-oxoethyl]cyclopentyl]-acetyl]-N-[2-[1,2-dihydro-3,5 (4H)-dioxo-1,2-diphenyl-3H-1,2,4-tria-zol-4 yl] ethyl]-argininamide (BIIE0246; 0.3 μM; Tocris Cookson), pre-diluted in ethanol and dissolved into aCSF (1:10.000; (El Bahh et al., 2002), was applied to slices for 8 min before mEPSCs were recorded for another 3 min. In some slices, as a final step, 3-dihy-droxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX; 5 μM; Tocris Cookson) was applied to the perfusion medium to block α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid (AMPA) receptor-mediated EPSCs.

Two-pathway and high-frequency stimulation (HFS)

Bipolar stimulation electrodes were used to evoke EPSCs in recorded cells. Connected to constant voltage stimulators (DS2A, Digitimer Ltd, Hertfordshire, England), the intensity of the stimulations was adjusted to give stable and submaximal synaptic currents. Simultaneous use of stimulation electrodes was accomplished by positioning two electrodes, separated by approximately 400–500 μm, in the alveus pathway on either side of the recorded cell. In this setup, two pathways (termed P1 and P2) were stimulated to activate separate sets of synapses on the same cell (see Fig. 3A). Paired stimulations of each pathway (P1–P1, or P2–P2) induced EPSCs that displayed PPF, while EPSCs remained unchanged when paired stimulations were delivered to alternate pathways (P1–P2 or P2–P1), confirming independence and no overlap between these two afferent inputs (data not shown). Using this configuration, paired-pulse stimulation (with ISI of 850 ms) was delivered to P1 (first stimulation is termed P1.1 and the next is termed P1.2). After 30 s, paired-pulse stimulation was repeated, with an intermediate HFS train (10 stimulations at 40 Hz) delivered to P2, 300 ms after the first P1 stimulation. This stimulation protocol was repeated 10 times with 1 min intervals. Following, BIIE0246 (0.3 μM) was applied to slices for 10 min and alternate stimulations were resumed (10 stimulations each). Percent change [(P1.2–P1.1)/P1.1)×100%] of average EPSCs induced by P1 stimulation were compared with baseline and intermediate stimulations, both before and after BIIE0246 application. In the same experiment, the average synaptic response evoked by HFS of P2 was evaluated after BIIE0246 application by dividing the total area of all ten EPSCs in the train by the area of the first EPSC, and PPF was calculated as the ratio (EPSC amplitudes) between the two first evoked EPSCs during HFS.

Long-term potentiation (LTP)

A single stimulation electrode was positioned in the alveus pathway close to the CA1 region and the stimulation intensity was adjusted before baseline recording to give submaximal and constant synaptic currents. Within 10 min after breaking into whole-cell configuration, tetanic stimulation (single train, 1 ms, 100 Hz, during 1 s) was used to induce LTP. The magnitude of LTP was calculated in individual slices by normalizing the amplitude of EPSCs to average baseline values (minimum 5 min stable EPSCs evoked at 0.067 Hz). LTP was considered significant if the mean response, as calculated 10–15 min after tetanic stimulation, was increased by >15% from baseline values. Some control recordings were also conducted in animals injected with rAAV–GFP. The synaptic responses in these animals appeared normal, and since no difference was observed in LTP magnitude between rAAV–GFP and rAAV–empty injected animals, these data (not shown) were pooled for further analysis (together referred as rAAV-empty injected).

Data acquisition and statistics

All recordings were sampled at 10 kHz and filtered at 2.9 kHz using HEKA amplifier and software (EPC10 and PATCHMASTER, HEKA Elektronik, Lambrecht, Germany). Off-line analyses were performed using FITMASTER (HEKA Elektronik) and IgorPro (Wavemetrics, Lake Oswego, OR) software. Data acquisition and analysis were performed by experimenters unaware of the group identity of the animals. Statistical significance was set at $p < 0.05$ and all data are presented as mean±S.E.M. A two-tailed Student's t-test was used for statistical analysis between groups, and paired t-test for differences within groups (i.e. before and after drug treatment). The χ^2-test followed by Fisher's exact test was performed to evaluate differences in LTP induction rate between groups. Additionally, a two-tailed Kolmo-gorov–Smirnov (K–S) test was used to determine cumulative probability distribution differences of mEPSC (interevent interval, amplitude and kinetics) between and within groups. During recordings of mEPSC, access resistance was monitored before and after each recording, and continuously during drug infusion. For analysis, the first 50 events recorded before and after drug application, as automatically detected by MiniAnalysis Software (Synaptosoft, Decatur, GA), were selected using a three times RMS detection level (3.80 pA±0.25, $n = 12$) calculated by the software in individual recordings.

Immunohistochemistry

In conjunction to the electrophysiological recordings, additional 6 rats injected with rAAV–NPY, rAAV-empty or rAAV–GFP vector at P2–4 were examined separately to determine resultant vector-mediated NPY overexpression and viral transduction three weeks after injection. These animals were perfused through the ascending aorta with 0.9% NaCl and 4% PFA (pH 7.4), and their brains were post fixed overnight at 4 °C. Brains were cut on a microtome, and slices (30 μm) were collected and stored in anti-freeze solution. For NPY staining, slices were rinsed, quenched (3% H_2O_2, 10% MeOH in KPBS) for 10 min and incubated overnight with rabbit anti-NPY antibody (1:1000; Sigma). Slices were then incubated with secondary antibody (BA 1000; 1:200; Vector Laboratories, Burlingame, CA) for 2 h, and with ABC solution for 1 h (Vectastain ABC Kit, Vector Laboratories). Visualization of staining was done by DAB-reaction.

Slices used for electrophysiology (210–250 μm) were all post fixed in 4% PFA overnight, rinsed, and stored in anti-freeze solution. For NPY-biocytin visualization, slices were incubated with rabbit anti-NPY (1:2000) overnight at RT, followed by a 2 h incubation with the secondary antibodies CyTM3-Streptavidine (1:200) and FITC donkey anti-rabbit (1:200) (both Jackson ImmunoResearch). Epifluorescence illumination was used to visualize native GFP fluorescence (in 30 μm slices) or NPY-FITC together with biocytin-CyTM3 signaling (in 210–250 μm slices) using an upright Olympus BX61 microscope. Confocal laser-scanning microscopy (Leica TCS) was used for high magnification images of single biocytin-labeled cells. All images were digitally acquired.

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IV

Hippocampal NPY gene transfer attenuates seizures without affecting epilepsy-induced impairment of LTP

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Running title:
NPY reduce rapid kindling but not LTP

Keywords (8):
NPY; gene therapy; epilepsy; rapid kindling; synaptic transmission; synaptic plasticity; LTP; hippocampus.

ABSTRACT

Recently, hippocampal neuropeptide Y (NPY) gene therapy has been shown to effectively suppress both acute and chronic seizures in animal model of epilepsy, thus representing a promising novel antiepileptic treatment strategy, particularly for patients with intractable mesial temporal lobe (TLE) epilepsy. However, our previous studies show that recombinant adeno-associated viral (rAAV)-NPY treatment in naïve rats attenuates LTP and transiently impairs hippocampal learning process, indicating that negative effect on memory function could be a potential side effect of NPY gene therapy.

Here we report how rAAV vector-mediated overexpression of NPY in the hippocampus affects rapid kindling, and subsequently explore how synaptic plasticity and transmission is affected by kindling and NPY overexpression by field recordings in CA1 stratum radiatum of brain slices. In animals injected with rAAV-NPY, we show that rapid kindling-induced hippocampal seizures in vivo are effectively suppressed as compared to rAAV-empty (control) injected rats. Six to nine weeks later, basal synaptic transmission and short-term plasticity are unchanged after rapid kindling, while LTP is significantly attenuated in vitro. Importantly, transgene NPY overexpression has no effect on short-term synaptic plasticity, and does not further compromise LTP in kindled animals. These data suggest that epileptic seizure-induced impairment of memory function in the hippocampus may not be further affected by rAAV-NPY treatment, and may be considered less critical for clinical application in epilepsy patients already experiencing memory disturbances.

Introduction

Neuropeptide Y (NPY) is an abundantly expressed brain peptide involved in diverse functions in the brain such as food intake, anxiety, blood pressure and memory (Pedrazzini et al., 2003; Lin et al., 2004). Strong antiepileptic effects of NPY have also been reported in numerous studies, proposing a critical role of endogenous NPY in seizure regulation by controlling neuronal excitability (Vezzani et al., 1999; De-Prato Primeaux et al., 2000). Mice lacking the NPY gene are more disposed to seizures (Erickson et al., 1996; Baraban et al., 1997), whereas rats overexpressing NPY show decreased seizure susceptibility and epileptogenesis (Vezzani et al., 2002). At a cellular level, NPY is normally expressed in a subset of GABAergic interneurons and is preferentially released at high frequency neuronal firing (Thureson-Klein et al., 1986; Kits and Mansvelder, 2000). When released, NPY can modulate inhibitory GABA-mediated (Sun et al., 2003) and excitatory glutamate-mediated (Haas et al., 1987) synaptic transmission. In the hippocampus, NPY has pro-

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found inhibitory effect on presynaptic glutamate release and can reduce the magnitude of evoked excitatory responses (Colmers et al., 1985), probably by reducing Ca^{2+}-influx in axonal terminals of principal glutamatergic neurons (Colmers et al., 1988). This mechanism probably also underlies the inhibitory effect of NPY on the generation of long-term potentiation (LTP) in hippocampus (Whittaker et al., 1999), which is a form of activity-dependent synaptic plasticity probably underlying learning and memory (Lynch, 2004).

Recently, a novel gene therapy strategy based on the recombinant adeno-associated viral (rAAV) vector carrying the NPY gene has been developed to treat particularly intractable temporal lobe epilepsy (TLE) seizures (Noe et al., 2007). Strong seizure-suppressant effects have been demonstrated in both acute and chronic epilepsy models in animals with rAAV-mediated hippocampal NPY overexpression (Richichi et al., 2004; Lin et al., 2006; Noè et al., 2008). One main concern of using this treatment in clinical applications is a potential side effect that may exacerbate cognitive function, which is usually already compromised in epilepsy patients. Previously, we have shown that naïve rats with rAAV-mediated transgene NPY overexpression in the hippocampus have a transient delay of hippocampal-based learning which is paralleled by an attenuation of LTP in CA1 area (Sørensen et al., 2008b). However, until now it is unknown to what extent LTP is affected by transgene NPY in animals that have already experienced epileptogenesis. Therefore, in the present study we determined alterations in synaptic transmission and plasticity in slices from animals injected with rAAV-NPY and subjected to 40 rapid kindling stimulation-induced seizures, which trigger a process of epileptogenesis and leads to permanent hyperexcitability in the hippocampus (Elmér et al., 1996).

Methods and materials

Animals

A total of 34 adult male Sprague Dawley rats (250 grams; Charles River, Germany) were used. Animals were housed in individual cages at 22°C under a 12-hours light/dark cycle with free access to food and water. Experimental procedures were approved by the local Ethical Committee for Experimental Animals, and followed guidelines in accordance of European Community Council Directive for the Care and Use of Laboratory Animals.

Viral vector injection

The rAAV vectors were produced as described elsewhere (During et al., 2003; Richichi et al., 2004). Briefly, a plasmid containing the human prepro-NPY cDNA was subcloned into an expression cassette made of the rat neuron-specific enolase (NSE) promoter, woodchuck post-translational regulatory element (WPRE) and a bovine growth hormone polyA (BGHpA) signal, flanked by AAV2 inverted terminal repeats (pAM/NSE-NPY-WPRE-BGHpA). This cassette was cloned into the backbone of the chimeric AAV vector (mix of rAAV serotype 1 and serotype 2 capsid helper plasmid) and purified. An empty control vector carrying no transgene (pAM/NSE-empty-WPRE-BGHpA) was produced as above. For injection of viral vectors, animals were anesthetized by intra-peritoneal injection of ketamine (80 mg/kg) and xylazine (15 mg/kg) mixture, and placed into a KOPF stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). Through drill holes made in the skull, viral vector solutions were injected bilaterally at one site in the dorsal (AP -3.3, ML ±1.8, V -2.6) and at two sites in the ventral (AP -4.8, ML ±5.2, V -6.4 and -3.8) hippocampus (in mm). Reference points for all coordinates were bregma, midline and dura, tooth bar at -3.3 mm (Paxinos and Watson, 1996). At each site, 1 μl vector suspension (with genomic titers of $1x10^{13}$

for rAAV-NPY and rAAV-empty) was injected during 5 min (0.2 μl per min) and the pipette was left in place for additional 3 min to minimize backflow through the injection track when retracting the pipette.

Electrode implantation

Two weeks following viral vector injection, animals were anesthetized as described above. A bipolar stainless-steel stimulating/recording electrode (PlasticsOne, Roanoke, VA, USA) was implanted stereotactically into the left ventral hippocampus at the following coordinates (in mm): AP -4.8, ML -5.2, V -6.3 and tooth bar at -3.3. This electrode and a reference electrode (inserted into the cheek muscle) were fixed in a pedestale and onto the skull with dental cement. Animals were allowed to recover for one week before undergoing electrical stimulation.

Electrical rapid kindling stimulations

The threshold for inducing focal epileptiform activity of more than 5 s duration was determined in each animal by applying stepwise stimulations (10 μA steps, 1 s, 100 Hz) at increasing current intensity, starting at 10 μA. Focal epileptiform activity (afterdischarge, AD) was detected by electroencephalographic (EEG) recording. During rapid kindling stimulation, consisting of 40 suprathreshold stimulation trains (10 s, 1 ms square wave pulses at 50 Hz, 400 μA intensity) separated by 5 min interval between stimulations, EEG activity was continuously recorded on a MacLab system (ADInstruments, Bella Vista, Australia) for 200 min except during stimulations. Behavioral seizures were scored according to the scale of 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. Rats injected with rAAV-NPY or rAAV-empty, and used for rapid kindling (RK) were designated as RK-rAAV-NPY

(n = 12) and RK-rAAV-empty (n = 9) injected animals, respectively.

Electrophysiology

For electrophysiology, rats used for rapid kindling (RK-rAAV-NPY and RK-rAAV-empty injected animals) and 13 time-matched control rats not exposed to rapid kindling (8 rats injected with rAAV-empty vector and with no electrode implantation, and 5 naïve rats) were briefly sedated with isoflurane before decapitation. Their brains were quickly placed into ice-cold sucrose solution (in mM; 195 sucrose, 2.5 KCl, 0.5 $CaCl_2$, 7.0 $MgCl_2$, 28 $NaHCO_3$, 1.25 NaH_2PO_4, 7.0 glucose, 1.0 ascobate and 3.0 pyruvate; adjusted to pH 7.4; osmolarity 300 mOsm; oxygenated with 95% O_2/ 5% CO_2) and 350 μm transverse slices were prepared from the right hippocampus (i.e. contra-lateral to the stimulation/EEG electrode) using a vibratome (Vibratome 3000, Ted Pella, Inc., Redding, CA) containing the same solution. Slices were maintained for > 1 hour in artificial cerebrospinal fluid (aCSF) (mM; 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 26 $NaHCO_3$, 1.0 NaH_2PO_4, and 11 glucose; pH 7.4; 296 mOsm; oxygenated, RT) before transferred to the recording chamber, which was constantly perfused (2 ml per min) with oxygenated aCSF. In stratum radiatum of CA1, a bipolar stimulation electrode and a pipette filled with aCSF (tip resistance of 0.8-1.2 MΩ) were placed, separated by approximately 500 μm. Evoked field exitatory postsynaptic potentials (fEPSPs) at increasing intensities were recorded and used for input-output analysis by plotting the presynaptic fiber volley (PSFV; mV) against the slope (mV/ms) of the corresponding fEPSPs. Slices generating fEPSPs with PSFV/EPSP ratio of more than 1:3, and/or those with maximal fEPSP amplitudes of less than 1 mV were excluded from the analysis. Stable submaximal baseline fEPSPs (30-40% of maximal fEPSP) were continuously monitored for 5-10 min (at 0.05 Hz) before paired-pulse stimulations were delivered at interstimulus in-

tervals (ISI) of 25, 50, 100 and 200 ms. For LTP, a 15 min fEPSP baseline was recorded (at 0.067 Hz and with 15% acceptable variability) before high-frequency stimulation (HFS, 100 Hz, 1 s) was applied. Field EPSPs were recorded for another 60 min, and analyzed by measuring the initial slope (1 ms interval), normalized to average baseline values and plotted against time (average of four fEPSPs per min). Data was acquired at 10 kHz and filtered at 2.9 kHz using HEKA amplifier and software (EPC 10, PATCHMASTER, HEKA Elektronik, Lambrecht, Germany) and off-line analysis was performed using FIT-MASTER (HEKA Elektronik) and Igor Pro (Wavemetrics, Lake Oswego, OR) software.

Immunohistochemistry

All brain slices were processed for visualization of NPY immunoreactivity. Following recording, slices were post-fixed overnight in 4% paraformaldehyde (4°C), rinsed in KPBS, quenched (3% H_2O_2, 10% MeOH in KPBS) and incubated in a 1:5000 dilution of rabbit antiserum to rat NPY (Sigma-Aldrich, Sweden) in 5% normal goat serum in KPBS for four days at 4°C. Finally slices were incubated in biotinylated secondary antibody (BA1000; 1:200; Vector Laboratories, Burlingame, CA). The reaction was amplified (Vectastain ABC KIT, Vector Laboratories) and visualized by 3-3'-diaminobenzidine (DAB). For improved image illustrations, some microtome slices (30 μm) were prepared from the slices (350 μm) cut on the Vibratome. These slices were processed as above, but incubated in primary antibody in a 1:1000 dilution for 24 hours. All images were digitally acquired and no damage caused by either virus or surgery was observed in any slices.

Statistical analysis

The level of significance was p < 0.05 and data are presented as mean ± SEM. Kindling data was analyzed using two-tailed Student's t-test. Input-output and PPF (calculated as the mean of five consecutive fEPSPs) data was analyzed by ANOVA followed by Bonferroni-Dunn post-hoc test. For LTP analysis, repeated-measures ANOVA and Mann-Whitney test was used. Differences in rate of LTP induction between groups was evaluated by χ^2-test followed by Fisher's exact test, and determined in individual slices as an increase by > 15% from baseline values, calculated 20-24 min after HFS. Investigators conducting behavioral grading of seizures, EEG analysis, and electrophysiological recordings were blind to group identities of experimental animals and pre-treatment conditions. For electrophysiology, animals were randomly selected for experiments on a day-to-day basis. Since no differences in input-output, PPF and LTP were detected in slices prepared from naïve rats and non-stimulated rats injected with rAAV-empty vector, these animals were pooled in one group and are together referred as control slices.

Results

Endogenous and transgene NPY expression

Expression of endogenous and transgene NPY was determined in all slices by immunohistochemistry. Consistent with previous observations (Richichi et al., 2004; Lin et al., 2006; Noè et al., 2008; Sørensen et al., 2008b), injection of rAAV-NPY vector into the hippocampus gave rise to long-lasting and strong expression of transgene NPY throughout the hippocampus (Figs. 1A, B, C). RK-rAAV-NPY treated animals had a uniform expression pattern of transgene NPY immunoreactivity as revealed in hippocampal slices used for electrophysiology 7-9 weeks post viral vector injection. Transgene NPY was observed within the cell layers of CA1 and CA3, including stratum pyramidale, moleculare, radiatum and oriens, as well in the granule cell layer, molecular layer and hilus of the dentate gyrus (Figs. 1A, B, C). Transgene NPY was predominantly confined to neuronal cell bodies and fibers throughout the hippocampus. In slices from RK-rAAV-empty treated animals,
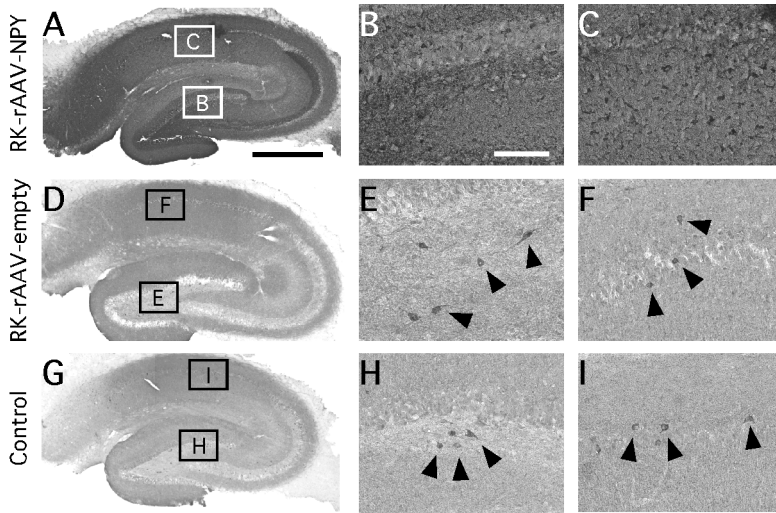
Figure 1. Expression of endogenous and transgene NPY in the hippocampus. (A, B, C) Representative slices from a RK-rAAV-NPY treated animal showing high levels of transgene NPY immunoreactivity throughout the whole hippocampus with particular dense transgene NPY expression in hilus of the dentate gyrus and in mossy fiber terminals in stratum oriens of CA3. (D, E, F) NPY immunoreactivity in a slice from a RK-rAAV-empty treated animal showing scattered NPY positive cells and fibers throughout the hippocampus (indicated by arrows in hilus of dentate gyrus), most likely resembling NPY containing interneurons. (G, H, I) Endogenous immunoreactivity of NPY is confined to interneurons clearly identified in the hilus of the dentate gyrus (shown by arrows), but is detectable throughout hippocampus. Boxes in left images are magnified and shown on right. Scale bar in A is 1 mm and applies for D and G. Scale in B is 100 μm and applies for C, E, F, H, and I.

scattered NPY immunoreactivity was observed throughout hippocampus, particularly within the hilus of the dentate gyrus, most likely representing NPY-positive interneurons (Figs. 1D, E, F). In slices from naïve control animals, we found similar expression pattern and intensity of NPY immunoreactivity (Figs. 1G, H, I) as described for RK-rAAV-empty treated slices (see above), suggesting that rapid kindling, at least 4-6 weeks post kindling, did not alter endogenous NPY expression.

Transgene NPY provides anticonvulsive effects during rapid kindling

Applying stepwise stimulations at increasing intensities until reaching focal epileptiform activity of more than 5 s duration did not reveal any difference in seizure threshold between RK-

rAAV-NPY (n = 12) and RK-rAAV-empty (n = 9) treated animals (Fig. 2A). Similarly, during the 40 supra-threshold stimulations with 5 min intervals during 3 hours and 20 min, the number of stimuli needed to reach seizure stage 1-5 was similar for both groups (Fig. 2B). However, the mean AD duration recorded using stimuli at threshold intensity strength was significantly shorter ($p < 0.01$, t-test) in RK-rAAV-NPY (25.1 ± 6.0 s) as compared to RK-rAAV-empty (72.3 ± 12.8 s) treated animals (Fig. 2C). Also, during the rapid kindling procedure, the mean AD duration at seizure stages 1-4, was significantly shorter in RK-rAAV-NPY as compared to RK-rAAV-empty treated animals (stage 1: 29.4 ± 1.5 s vs. 62.3 ± 3.8 s, $p < 0.001$; stage 2: 39.5 ± 3.1 s vs. 91.7 ± 9.6 s, $p < 0.001$; stage 3: 37.3 ± 8.3 s vs. 84.9 ± 10.8 s, $p < 0.01$; stage 4: 37.7 ± 3.0 s vs. 53.2 ± 5.4 s, $p < 0.05$; Fig. 2C). Finally,
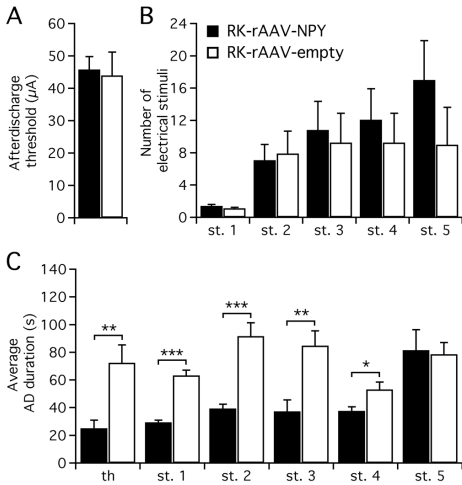
Figure 2. Transgene NPY decreases seizure duration in rapid kindling. (A) Mean afterdischarge (AD) threshold (μA) inducing focal epileptiform activity of more than 5 s duration. (B) Mean number of suprathreshold stimuli needed to reach seizure stage 1-5 during 40 rapid kindling stimulations. (C) Mean AD duration at threshold stimuli intensity (th), and during seizure stage 1-5 using at suprathreshold stimulation intensity. In all figures, black bars represent RK-rAAV-NPY (n = 12) and white bars represent RK-rAAV-empty (n = 9) treated animals, respectively. Values are mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, t-test.

the average duration of total ADs in each animal during kindling was reduced by ~50 % in RK-rAAV-NPY (23 ± 2 min) as compared to RK-rAAV-empty (44 ± 6 min) treated animals (p < 0.001, t-test).

*Basal synaptic transmission and short-term synaptic plasticity*

Four to six weeks after animals were subjected to rapid kindling, vibratome slices from the contralateral hippocampus (site with no stimulation/EEG electrode) were prepared. Input-output, paired-pulse and HFS-induced fEPSP in CA1 were analyzed to determine possible alterations in synaptic transmission and

plasticity. Serving as a control, recordings in slices from time-matched animals not subjected to rapid kindling were studied.

Evoking fEPSPs using stimulations with stepwise increasing intensities, thereby establishing an input-output relationship between the amount of activated afferent axons (estimated by amplitude of PSFV) and the corresponding magnitude of postsynaptic response (estimated by steepness of initial slope of the fEPSP), revealed that basal synaptic transmission was unaltered between RK-rAAV-NPY (n = 25 slices, 9 animals), RK-rAAV-empty (n = 23 slices, 11 animals) and control (n = 22 slices, 8 animals) slices (Fig. 3A). Paired stimulations at different interstimulus intervals (IEIs) induced fEPSPs with pronounced PPF (Fig. 3B). No differences in PPF were detected at IEIs of 25, 50 and 100 ms between the groups (RK-rAAV-NPY, n = 20 slices, 7 animals; RK-rAAV-empty, n = 21 slices, 9 animals; control, n = 22 slices, 8 animals). However, at IEI of 200 ms, PPF was significantly higher in RK-rAAV-NPY treated slices (138 ± 3 %) as compared to control slices (126 ± 4 %) (p < 0.05, ANOVA followed by Bonferroni-Dunn post hoc test), but similar to RK-rAAV-empty treated slices. No differences were detected between RK-rAAV-empty and control slices (Fig. 3B).

Long-term potentiation

In control slices, stable HFS-induced LTP was recorded in Schaffer collateral-CA1 cell synapses, with a 60 ± 4 % increase of fEPSP initial slope (calculated 20-24 min post HFS) as compared to baseline values (n = 10 slices, 8 animals), lasting for at least 60 min (Fig. 3C). At similar time point, the fEPSP initial slope in RK-rAAV-empty treated slices (n = 11 slices, 9 animals) was only increased by 43 ± 3 % (significantly lower as compared to controls, repeated measures ANOVA (p < 0.05) and Mann-Whitney test (p < 0.001)) and was similar to that in RK-rAAV-NPY treated slices (39 ± 3 %, n = 11 slices, 7 animals, repeated measures ANOVA
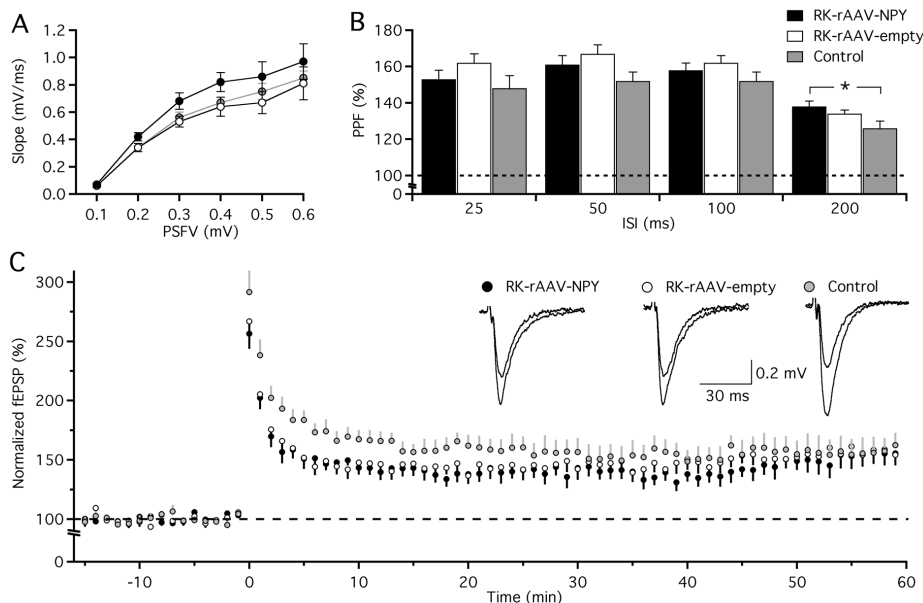
Figure 3. Alterations in synaptic transmission and plasticity in CA1 after rapid kindling in rAAV-NPY-treated and control animals. (A) The presynaptic fiber volley (PSFV) is plotted against the initial slope (mV/ms) of the corresponding fEPSP for RK-rAAV-NPY (n = 25 slices, 9 animals), RK-rAAV-empty (n = 23 slices, 11 animals) and control (n = 22 slices, 8 animals) animals (p > 0.05, ANOVA with Bonferroni-Dunn test). Each trace is labeled as shown in C. (B) Mean evoked fEPSP showing pronounced PPF at different ISI (at 25, 50, 100 and 200 ms) in RK-rAAV-NPY (n = 20 slices, 7 animals), RK-rAAV-empty (n = 21 slices, 9 animals) and control (n = 22 slices, 8 animals) animals. *p < 0.05, ANOVA followed by Bonferroni-Dunn test. (C) HFS-induced LTP of fEPSPs expressed as initial slope normalized to average baseline values and plotted against time (average of four fEPSP per min) for RK-rAAV-NPY (n = 11 slices, 7 animals), RK-rAAV-empty (n = 11 slices, 9 animals) and control (n = 10 slices, 7 animals) animals. HFS-induced LTP is significantly attenuated in RK-rAAV-NPY and RK-rAAV-empty treated slices as compared to control slices, revealed by repeated measures ANOVA (p < 0.05) and Mann-Whitney test (p < 0.001). Representative fEPSPs (average of four traces) acquired during baseline and 20 min post HFS are shown for each group. Data in A, B and C are shown as mean ± SEM.

(p < 0.05) and Mann-Whitney test (p < 0.001) (Fig. 3C). At the same time, the induction rate of LTP (i.e. at least 15% increase from baseline values) was similar between groups (73% in RK-rAAV-NPY, 68% in RK-rAAV-empty, 71% in control slices; χ^2-test followed by Fisher's exact test).

Discussion

The present study demonstrates that hippocampal rAAV vector-mediated NPY overexpression significantly reduces seizure duration during rapid kindling stimulations. We also demonstrate for the first time that after rapid kindling, LTP in hippocampus is attenuated, and that transgene NPY has no further detrimental effect on LTP. This is in contrast with our previous studies in naïve animals, where LTP was compromised by transgene NPY.

Transgene NPY expression and seizure suppression

In slices transduced with rAAV-NPY, immunohistochemistry revealed increased NPY levels mainly restricted to neurons and fibers throughout the hippocampus. This pattern of transgene NPY expression is in line with previous observations showing that injection of serotype 1/2 rAAV-NPY vector causes widespread expression of transgene NPY in the hippocampus (Richichi et al., 2004; Noè et al., 2008). In rats with such overexpression of transgene NPY, acute intrahippocampal and intraventricular kainic acid-induced seizures were significantly attenuated revealed by delayed onset and time spent in EEG seizures (Richichi et al., 2004), and after electrical induced status epilepticus, the progression and frequency of subsequent spontaneous seizures were decreased in a rat model of chronic epilepsy (Noè et al., 2008), indicating that transgene NPY has strong anticonvulsive and antiepileptic effects. In addition, in the rapid kindling model of epilepsy, Richichi and colleagues (2004) observed a significant delay in kindling acquisition at stage 3-5 and a significant increase in AD threshold, suggesting that transgene NPY may also effectively suppress epileptogenesis. In the present study, the threshold for inducing focal epileptiform activity and kindling development (at any seizure stage) was similar between RK-rAAV-NPY and RK-rAAV-empty treated animals. However, the duration of ADs was dramatically decreased by transgene NPY at threshold stimulation intensity and during seizure stage 1-4 (almost by 50 %). Therefore, our results also provide evidence that transgene NPY exerts anticonvulsive effects in the rapid kindling model.

Influence on synaptic transmission and short-term synaptic plasticity.

In vivo recordings reveal that stimulation-induced EPSPs in CA1 of kindled animals can be enhanced as long as up till 28 days after the last kindling stimulation (Leung and Shen, 1991), but remain unchanged when assessed by *in vitro* recordings in brain slices. In vitro, basal synaptic transmission of kindled animals remains unaltered both shortly after the last kindling stimulation (< 24 hours) (Leung and Wu, 2003) and at later time points (> 3 weeks) (Zhao and Leung, 1991; Leung et al., 1994). Similarly, our input-output analysis did not reveal any changes in basal synaptic transmission in slices from control rapid kindled animals, and was not influenced by injection of rAAV-NPY vector. Paired-pulse facilitation was also unchanged between the groups, except at ISI of 200 ms, where the rate of facilitation was slightly, but significantly, higher in RK-rAAV-NPY as compared to control slices. Thus, it appears that short-term plasticity is mostly unaffected by rapid kindling and transgene NPY. In other models of epilepsy, e.g., after traditional electrical kindling (Zhao and Leung, 1991; Leung and Wu, 2003) and during the latent period following status epilepticus (El-Hassar et al., 2007), an increase in the PPF has been observed *in vitro*. Interestingly, even after less severe seizures during conventional kindling, such as after partial kindling, i.e., when animals do not experience any generalized seizures, PPF was persistently increased 6-8 weeks after the last stimulation (Leung et al., 1994). Moreover, partial kindling induced similar synaptic changes in the contralateral to the stimulation hippocampal CA1 site (Leung et al., 1994). Taken together, these data indicate that these two models of epilepsy differ in how excitatory synaptic transmission and short-term synaptic plasticity are affected, and therefore may differ in mechanistic aspects of epileptogenesis and development of increased excitability in the hippocampus. The fact that transgene NPY had no effect on PPF indicates that probably very limited amount of transgene NPY is released during low frequency stimulations. This is in line with our previous studies, where we showed that transgene NPY is released predominantly during high frequency stimulation (Sørensen et al., 2008b; Sørensen et al., 2008a).

Attenuation of long-term potentiation

The ability to express LTP is markedly reduced in the surgically resected human hippocampal specimens from TLE patients (Beck et al., 2000) and often these patients have complains for impaired memory function (Helmstaedter et al., 2003; Elger et al., 2004), supporting the idea that LTP may be a synaptic correlate of memory (Lynch, 2004). Similarly, in animals, isolated CA1 slice preparation exposed to repeated seizure-like activity can totally loose the ability to generate LTP (Hu et al., 2005), and several reports have demonstrated that both electrical and chemical kindling can severely attenuate LTP *in vitro* (Leung and Wu, 2003; Schubert et al., 2005) and induce spatial memory deficits (Leung and Shen, 1991; Leung et al., 1994; Mortazavi et al., 2005). In the present study, we now also show for the first time that rapid kindling significantly reduces LTP. In addition, our data show, also for the first time, that in slices from RK-rAAV-NPY injected animals, LTP is impaired to a similar magnitude as slices from RK-rAAV-empty injected animals, both compared to control slices from non-kindled animals. This is an important finding bearing in mind that rAAV-NPY gene therapy is being considered for clinical application in epilepsy.

Concluding remarks

In contrast to our previous findings, where LTP was strongly attenuated in CA1 of hippocampal slices in naïve rats (Sørensen et al., 2008b), our present data suggest that rAAV-NPY therapy in epileptic brain does not seem to exacerbate the magnitude of memory deficit already existing in epileptic patients. The detailed mechanisms of why transgene NPY limit seizure duration without affecting LTP need to be further investigated. Nevertheless, it is clear that there may be multiple different mechanisms for how NPY regulates these two processes in normal versus epileptic hippocampus. For example, apart from inhibiting glutamate release from the excitatory synapses (Haas et al., 1987; Colmers et al., 1988), NPY has an effect on the excitatory and inhibitory transmission onto a subpopulation of inhibitory interneurons in the dentate gyrus (our unpublished data).

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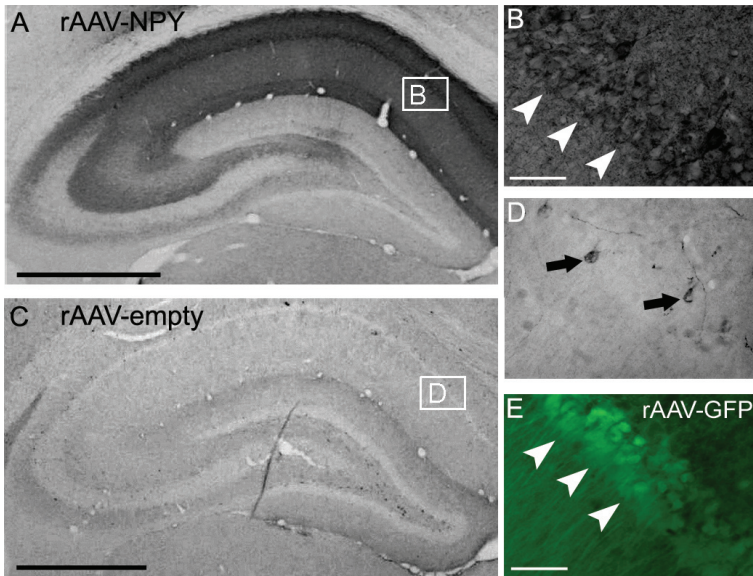
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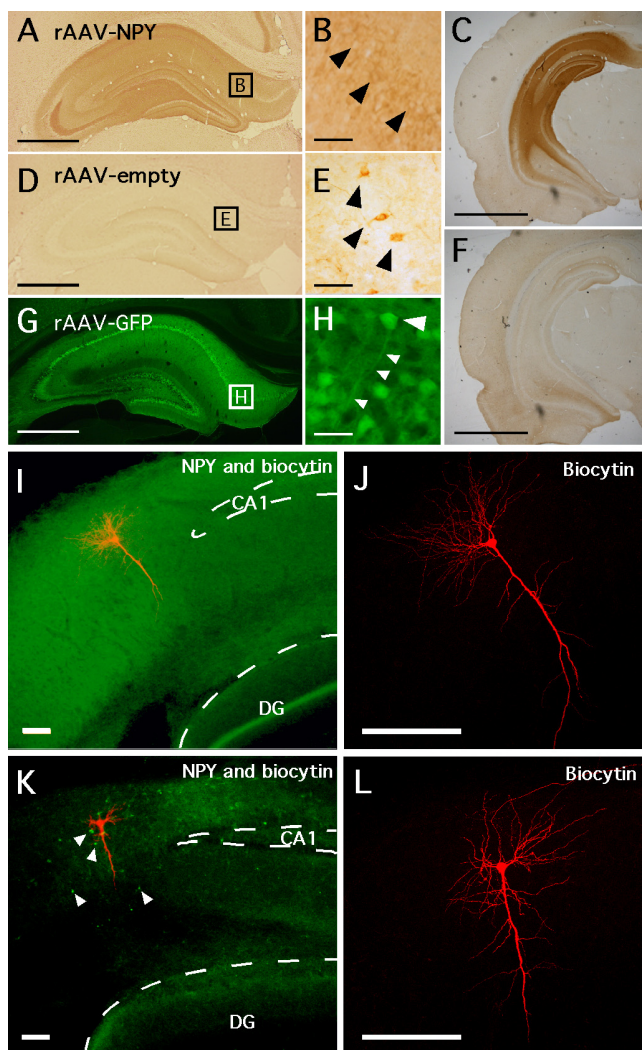
Color plates

FIGURE 1. High and stable overexpression of transgene NPY after injection of viral vector encoding NPY into the hippocampus of rats. (A) Immunohistochemical staining of a rat brain slice (as used for electrophysiology), as seen 3 weeks after rAAV-NPY vector injection, showing high levels of transgene NPY expression mainly within the CA1 region (injection and recording site) of the hippocampus. (B) Cells within the pyramidal layer in the CA1 region positively stained for NPY (as indicated by white arrow heads). (C) Slice from a control rat, injected with the rAAV-empty vector, having weak NPY immunoreactivity in all regions of the hippocampus with few dispersed NPY-positive interneurons. (D) The same slices as in (C) with scattered NPY-positive cell bodies, presumably NPY containing interneurons (shown by black arrows). (E) Viral transduction by rAAV-GFP vector, showing GFP expression mostly confined to pyramidal cell bodies and processes. Boxed areas on left images are magnified and shown on the right, and rAAV-mediated GFP expression is shown from the same region as boxed areas. Scale bars = 1 mm in A, C; 50 µm in B, D, E. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 1. Transgene expression after injection of rAAV–NPY and rAAV–GPF vector into the rat hippocampal formation. (A, B) Slice from the dorsal hippocampal formation showing immunoreactivity (IR) for NPY three weeks after injection of rAAV–NPY vector. Note high levels of transgene NPY expression throughout all hippocampal regions including the subiculum (inset B). In the proximal subiculum, pyramidal neurons were positive for NPY IR (indicated by arrow heads). (C) Extensive transgene NPY IR throughout the hippocampal formation. Note that NPY expression is enclosed by the surrounding fissure. (D) Slice from animal injected with rAAV-empty vector showing weak endogenous NPY IR in all regions of the hippocampal formation with dispersed NPY-positive interneurons and fibers. (E) Scattered NPY-positive cell bodies in proximal subiculum, presumably resembling NPY-containing interneurons (shown by arrows). (F) NPY IR as seen in a coronal section of a rat injected with rAAV-empty vector. (G) GFP autofluorescence detected three weeks after rAAV–GFP vector administration, revealing widespread and efficient viral transduction throughout the hippocampal formation. (H) GFP expression mostly confined to pyramidal cell bodies (large arrow) and processes (small arrows). (I) Slice from a rAAV–NPY injected rat used for whole-cell patch-clamp recordings. Green color (NPY, FITC) shows NPY IR and red color shows biocytin-labeled recorded cell (CyTM3) in proximal subiculum. (J) The same cell, as shown in image I, is visualized by confocal images with superimposed z-planes, showing characteristic shape of cell soma and dendrites of a subicular pyramidal neuron. (K) In slices from rAAV-empty injected rats, NPY IR (NPY, green, FITC) seen as green dots (marked by arrows), denoting the presence of endogenous NPY-containing interneurons within the proximal subiculum. (L) The same biocytin-labeled cell (red, CyTM3, arrow) as in image K shown by confocal imaging. Boxed areas on left images (A, D, G) are magnified and shown on right images (B, E, H). Scale bars = 1 mm in A, D, G; 50 µm in B, E; 3 mm in C, F; 25 µm in H; 100 µm in I, J, K, L.