Role of neurotrophins and neuropeptides in Genetic Absence Epilepsy Rats from Strasbourg (GAERS), a model for human generalized absence seizures

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Prof. Dr. B. Ernst (Fakultätsverantwortlicher)

Prof. Dr. U. Otten (Dissertationsleiter)

Prof. Dr. J. Lütschg (Korreferent)

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Prof. Dr. E. Parlow (Dekan)

Abstract

Several studies have shown that neurotrophins and neuropeptides contribute to epileptogenesis but their impact on idiopathic generalized epilepsies is not yet elucidated. Generalized absence seizures are a specific type of epilepsy occurring predominantly in children. Sudden onset and termination of typical bilaterally synchronous 3Hz spike-and-wave discharges on the electroencephalogram and a brief impairment of consciousness with interruption of ongoing activity are hallmarks of this disease. New classes of absence drugs designed to block the process of epileptogenesis are needed because known treatments are not effective in all patients and a broad spectrum of adverse reactions has been described. Drug screening is hindered because the molecular mechanisms underlying generalized absence seizures are still not completely clarified. Genetic Absence Epilepsy Rats from Strasbourg (GAERS) used in this study are a valid animal model that spontaneously displays many of the characteristics of human absence epilepsy.

The aim of this thesis was to define the potential role of neurotrophins and neuropeptides in generalized absence seizures with special regard to expression differences between GAERS and control animals, changes during maturation and region-specific expression alterations. Additionally, the consequences of their application on seizure initiation and termination were studied.

Brain-derived neurotrophic factor (BDNF) is ubiquitously expressed in brain and involved in several physiologic and pathologic processes including epilepsy. Glutamate release is enhanced, whereas inhibitory transmission is diminished by BDNF. Its signaling pathway is significantly impaired in adult GAERS after the onset of absence seizures due to reduced expression of BDNF receptors and transcription factors. Nevertheless, intracerebroventricular injection of BDNF significantly reduces the occurrence of spike-and-wave discharges in adult GAERS.

Neuropeptides are cofactors of the classical neurotransmitters and therefore important modulators of neuronal excitability. Expression of the anticonvulsant agent neuropeptide Y (NPY) is directly influenced by BDNF. The density of NPY-expressing cells is clearly increased in GAERS compared to control animals. Additionally, the onset of absence seizures in adult GAERS is associated with a drastic decrease of brain NPY content. Application of NPY and agonists to its receptors efficiently suppresses spike-and-wave discharges in adult GAERS. In contrast, absences are evoked in juvenile GAERS following treatment with specific NPY receptor antagonists.

In conclusion, this thesis demonstrates that BDNF as well as NPY exert potent anti-absence effects in adult GAERS. BDNF and NPY both represent accessible systems to intervene in brain excitability and thus provide new molecular targets for efficacious treatments against generalized absence epilepsy.

Zusammenfassung

Wie bereits in mehreren Studien gezeigt wurde, sind Neurotrophine und Neuropeptide in die Epileptogenese involviert, wobei ihre Wirkung bei idiopathischen generalisierten Epilepsien noch nicht geklärt ist. Generalisierte Absenzen sind eine spezifische Art von Epilepsie, die vor allem im Kindesalter auftritt. Besondere Merkmale sind das plötzliche Auftreten und Verschwinden von typischen Anfallsmustern im Elektroenzephalogramm mit einer Frequenz von 3Hz, die beide Gehirnhemisphären synchron betreffen, sowie eine kurze Bewusstseinsstörung, bei der laufende Aktivitäten unterbrochen werden. Die bisher bekannten Therapien sind nicht für alle Patienten wirksam und weisen ein breites Spektrum an unerwünschten Nebenwirkungen auf. Deshalb werden dringend neue Medikamente benötigt, welche die Entstehung der Epilepsie hemmen. Die Entwicklung von neuen Therapieansätzen wird jedoch dadurch erschwert, dass die molekularen Mechanismen, welche der Krankheit zu Grunde liegen, noch nicht vollständig bekannt sind.

Ziel dieser Arbeit war es, den möglichen Einfluss von Neurotrophinen und Neuropeptiden in der Entstehung von Absenzen aufzuklären. Dafür wurden GAERS-Ratten verwendet, die zahlreiche charakteristische Eigenschaften der menschlichen Absenzen aufweisen. Besondere Aufmerksamkeit wurde dabei auf Expressionsunterschiede zwischen GAERS und Kontrolltieren und Änderungen während der Entwicklung gelegt. Zusätzlich wurde die Auswirkung von Neurotrophinen und Neuropeptiden auf das Auftreten und Verschwinden der Anfälle untersucht.

Das Neurotrophin Brain-Derived Neurotrophic Factor (BDNF) ist im Gehirn weit verbreitet und spielt bei physiologischen wie auch pathologischen Prozessen so auch bei der Epilepsie eine wichtige Rolle. BDNF fördert die Ausschüttung von erregenden Neurotransmittersubstanzen wie Glutamat, während die hemmende Signalübermittlung abgeschwächt wird. Unsere Untersuchungen weisen darauf hin, dass die Signalkaskade von BDNF in adulten GAERS nach dem ersten Auftreten von Absenzen merklich gestört ist, da die Synthese von Rezeptoren und Transkriptionsfaktoren von BDNF reduziert ist. Funktionell bedeutsam ist, dass die intrazerebroventrikuläre Injektion von BDNF eine signifikante Verminderung der Absenzen bewirkt.

Neuropeptide sind Kofaktoren von klassischen Neurotransmittern und deshalb wichtige Modulatoren der neuronalen Erregbarkeit. Die Synthese des antikonvulsiven Neuropeptids NPY wird direkt durch BDNF reguliert. Die Dichte an NPY-exprimierenden Zellen ist in GAERS stark erhöht im Vergleich zu Kontrolltieren. Zusätzlich wird das erstmalige Auftreten von Absenzen von einem deutlichen Abfall der NPY Konzentration in verschiedenen Hirnregionen begleitet. Darüber hinaus unterdrückt die exogene Gabe von NPY und NPY-Rezeptor Agonisten das Auftreten von Absenzen in adulten GAERS. Antagonisten gegen NPY-Rezeptoren können hingegen Anfälle in jungen GAERS auslösen.

Zusammenfassend kann gesagt werden, dass BDNF wie auch NPY stark gegen Absenzen in adulten GAERS wirksam sind. Beide Substanzen bieten gut zugängliche Systeme, um die Erregbarkeit des Gehirns zu beeinflussen, und können deshalb als neue molekulare Angriffsorte für therapeutische Interventionen gegen Absenzen verwendet werden.

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1 Introduction

1.1 Epilepsy

Epilepsy is characterized by excessive and/or hypersynchronous, usually self-limited activity of neurons in the brain. Causes might be enhanced connectivity or excitatory transmission, a failure of inhibitory mechanisms or changes in intrinsic neuronal properties (Duncan, Sander et al. 2006). The International League Against Epilepsy (ILAE 2009) developed a standardized classification and terminology for epileptic seizures which distinguishes between focal and generalized seizures and status epilepticus (table 1). Focal seizures are initially activated in only a part of one cerebral hemisphere resulting in involuntary movement, unusual sensations or attention and behavioral changes. They can spread by recruitment of other brain areas. In contrast, in generalized forms of epilepsy the abnormal electrical activity encompasses both hemispheres of the brain leading to a complete loss of consciousness (Manning, Richards et al. 2003). Two major forms belong to generalized seizures: absence seizures (formerly known as petit mal) and tonic-clonic seizures (grand mal) which occur in two phases. In the first tonic phase muscles stiffen, the body grows rigid and the patient loses consciousness and falls. Afterwards, jerking and twitching of body extremities occur in the clonic phase. A second tonic phase may follow. Consciousness returns slowly and the patient is often confused and disoriented. Status epilepticus is a state of recurrent seizures during which consciousness does not return. It is a potentially life-threatening event which can lead to severe brain damage (according to ILAE, 1981; 1989; Engel 2001).

Table 1: Differentiation of epileptic seizure types with the focus on absence seizures *(adapted from ILAE 2009)*

Epileptic seizure type			
I	**Self-limited seizure types**		
	A	Focal seizures	
	B	Generalized seizures	
		i	Typical absence seizures
		ii	Atypical absence seizures
		iii	Myoclonic absence seizures
II	**Continuous seizure types**		
	A	Focal status epilepticus	
	B	Generalized status epilepticus	
		iv	Absence status epilepticus

1.1.1 Absence epilepsy

According to ILAE, absence seizures belong to idiopathic generalized epilepsies (Avoli, Rogawski et al. 2001). They can be divided in four subtypes (table 1): (i) typical absences, (ii) atypical absence seizures, (iii) myoclonic absence seizures and (iv) absence status epilepticus (Panayiotopoulos 1999b; ILAE 2009).
In table 1, the classification depends on the epileptic seizure type each of which represents a unique pathophysiological mechanism. It is a diagnostic entity with etiologic, therapeutic and prognostic implications.
Another possibility for classification is the epilepsy syndrome. It is a complex of different signs and symptoms that define a unique epilepsy condition involving more than just the seizure type (Engel 2001). Three epileptic syndromes belong to typical absences, namely childhood absence epilepsy, juvenile absence epilepsy and juvenile myoclonic epilepsy (table 2, Panayiotopoulos 1999a).

Table 2: Summary of the most important epilepsy syndromes mentioned in this thesis *(adapted from ILAE, Engel 2001)*

Epilepsy syndromes
▪ Typical absence seizures
o Childhood absence epilepsy
o Idiopathic generalized epilepsies with variable phenotypes
A Juvenile absence epilepsy
B Juvenile myoclonic epilepsy
▪ Myoclonic absence epilepsy
▪ Familial temporal lobe epilepsies
▪ Conditions with epileptic seizures that do not require a diagnosis of epilepsy
A Febrile seizures

1.1.1.1 Pathophysiology and epidemiology of absence seizures

Typical absence epilepsy consists of brief generalized epileptic seizures lasting up to 20 seconds with an abrupt onset and termination without a preceding aura (Panayiotopoulos 1999b). They are characterized by a sudden, brief impairment of consciousness and 3-4Hz bilaterally synchronous spike-and-wave discharges (SWDs) which can be recorded on the electroencephalogram (EEG, figure 1, according to ILAE 2009). An interhemispheric synchronization is required for the generalized nature which can be observed at seizure onset and throughout the whole discharge (Avoli, Rogawski et al. 2001). Patients interrupt ongoing activities, stare while their eyes are open, remain unresponsive and suddenly resume the pre-absence activity. Absence seizures occur very frequent, about ten to hundred times each day. As they occur mainly in children and are associated with a fixed, vacant stare, they are often mistaken for day-dreaming or remain unnoticed (Panayiotopoulos 1999b; Crunelli and Leresche 2002; Manning, Richards et al. 2003).

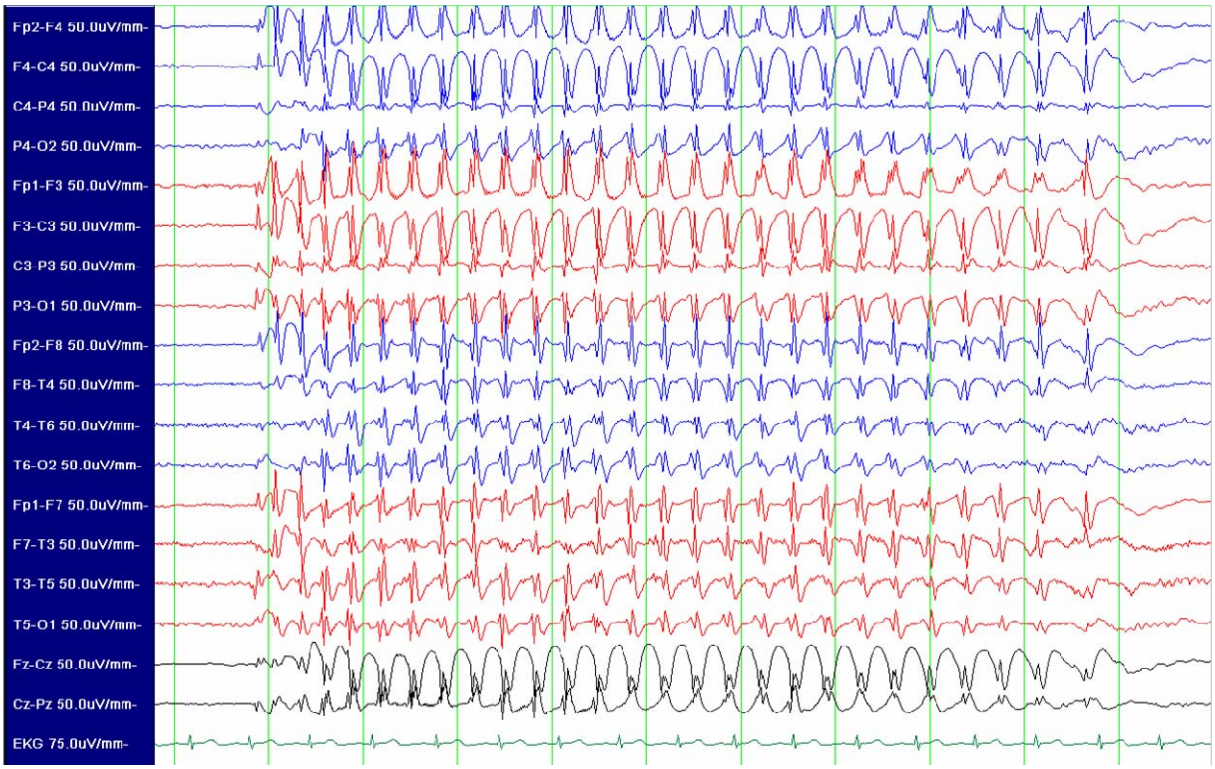

Figure 1: EEG of a child with typical absence seizures. *Typical 3Hz spike-and-wave discharges are recorded for 9 seconds. Absence epilepsy starts and ends abruptly. Green vertical lines mark each second on the time scale (kindly provided by Prof. J. Lütschg, UKBB, Basel).*

Absences are often associated with other symptoms such as mild clonic jerks, atonic components, tonic muscular contractions, automatisms (as e.g. lip smacking or licking, swallowing, fumbling with clothes, aimless walking), visual hallucinations and autonomic disturbances. Typical absences occur spontaneously but may be influenced by different factors such as anger, sorrow, fear, surprise, embarrassment, lack of interest, release of attention, meal- or school-time, awakening and metabolic factors as hypoglycemia (Loiseau 1992). For differential diagnosis, patients are asked to hyperventilate for three minutes while standing and count the breaths (for detection of impairment of consciousness) as absence seizures occur in more than 90% of the patients during hyperventilation (Panayiotopoulos 1999b).

Childhood absence epilepsy, also called pyknolepsy, occurs with an incidence of 1 in 1'000. Seizures start usually before the age of 10 years and peak at 5-6 years with higher prevalence in girls. In spite of the fact that pyknolepsy occurs very frequently during the day, it is the most innocuous type of generalized seizures because motor and autonomic manifestations are rare. The hallmark is a sudden onset and interruption of ongoing activities, often accompanied by a blank stare. An oligogenic or digenic model for inheritance is suggested. Patients are usually unaware of the seizure and respond well to medical treatment. Seizure remission occurs in more than 90% before the age of 12 years. Drug treatment can be withdrawn gradually after a seizure free time of 2-3 years (Snead 1995; Panayiotopoulos 1999a; Avoli, Rogawski et al. 2001; Manning, Richards et al. 2003). Factors for less favorable

prognosis are early or late onset (<4 or >9 years), initial drug resistance and photosensitivity. Persistence of seizures results usually in generalized tonic-clonic convulsions (Guerrini 2006).

Onset of **juvenile absence epilepsy** which is often accompanied by generalized tonic-clonic seizures and random myoclonic jerks occurs after the age of 10 years with a peak between 10 and 12 years. This type of seizures occurs with an incidence of 1 in 3'000 and is much more sporadic during the day than childhood absences. Girls and boys are equally affected. Frequency of SWDs can be faster (3.5-4.5Hz) than in childhood absences (Wolf 1993; Panayiotopoulos 1999a; Avoli, Rogawski et al. 2001).

Juvenile myoclonic epilepsy which starts in mid-teens is characterized by myoclonic jerks after awakening and generalized tonic-clonic seizures. Only one third of patients exhibit absences which are usually mild and without concurrent myoclonic jerks or automatisms. Both sexes are equally affected from juvenile myoclonic epilepsy which occurs in one out of 2'000 children (Panayiotopoulos 1999a; Avoli, Rogawski et al. 2001).

Atypical absences occur in the context of mainly severe symptomatic or cryptogenic epilepsies. Affected children have learning difficulties and suffer from frequent other types of seizures such as atonic, tonic and myoclonic seizures (Panayiotopoulos 1999a). SWDs are slower with a frequency between 1.5-2.5Hz and without precise beginning or ending. Additionally, eyelid clonia and automatisms may occur. The incidence of atypical absences varies from a few per day to nearly continuous. The lack of rhythmicity of the discharges is a remarkable feature of atypical absences which start in school age (Dulac 1999).

Patients suffering from **myoclonic absence seizures** have learning difficulties and a poor prognosis. Absence seizures are associated with bilateral rhythmic myoclonic jerks of severe intensity involving muscles of the shoulders, arms and legs. Facial muscles are less affected. The loss of consciousness may be complete or only partial. Seizure onset is around 7 years with a male preponderance. Hyperventilation, awakening and intermittent light can trigger a seizure (Tassinari, Rubboli et al. 1999; Panayiotopoulos 1999a).

Prolonged absence seizures lasting more than half an hour up to several days are called **absence status epilepticus.** It occurs in 10-30% of idiopathic generalized epilepsies with absences but not in the pure form of childhood absence epilepsy. Symptoms occur continuously or repetitively and last until the cessation of the status (Panayiotopoulos 1999b). Cardinal symptoms are clouding of consciousness, slow mind, withdrawal, slowness of responses and confusion often accompanied by eyelid, peri-oral or limb jerks. Verbal functioning is relatively well preserved and movement and coordination are intact. Absence status epilepticus is often not recognized or misdiagnosed (Agathonikou, Panayiotopoulos et al. 1998).

1.1.1.2 Molecular mechanisms underlying absence seizures

In contrast to generalized or focal convulsive seizures which are characterized by an excess of excitatory activity, absence epilepsy is related to a predominance of inhibition.

Seizure activity originates in the peri-oral region of the somatosensory cortex as shown for Wistar Albino Glaxo/Rijswijk (WAG/Rij) rats (Meeren, Pijn et al. 2002) and Genetic Absence Epilepsy Rats from Strasbourg (GAERS, Polack, Guillemain et al. 2007) and generalizes rapidly over the cortex (figure 2). Blockade of action potentials by tetrodotoxin in somatosensory cortex prevents the occurrence of local and distant SWDs (Polack, Mahon et al. 2009). The fact that injection of ethosuximide, a calcium channel blocking agent, into thalamic nuclei is not sufficient for a full anti-absence effect (Richards, Manning et al. 2003), whereas targeting the peri-oral region of the primary somatosensory cortex produces an immediate cessation of seizure activity (Manning, Richards et al. 2004) gives another indication for the focal origin of SWDs in the cortex. Secondly, oscillation is initiated within thalamocortical loops transforming the spike into spike-wave activity. In these first cycles of the seizure, the cortex drives the thalamus. Thereafter, rhythmic discharges are amplified and maintained by the interplay between cortex and thalamus alternatively leading and lagging in an unpredictable way (Meeren, van Luijtelaar et al. 2005). Neither cortex nor thalamus alone is able to sustain the discharges indicating that both structures are involved in the generation of SWDs (figure 2).

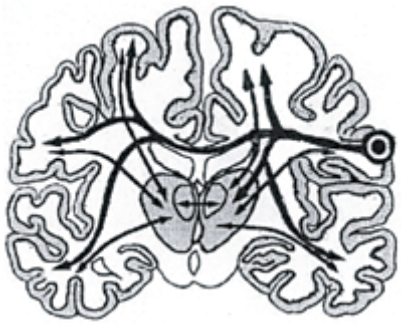

Figure 2: Cortical focus of absence epilepsy. *Seizures arise from a consistent focus within the peri-oral region of the somatosensory cortex (circle). Epileptic activity generalizes rapidly over the cortex and thereafter to the thalamocortical network. Then, thalamus and cortex drive each other and amplify and sustain the discharges (part of illustration from Meeren, van Luijtelaar et al. 2005).*

The thalamus modulates the flow of external information to the cortex by shifting between an oscillatory and tonic firing mode. Desynchronized EEG caused by tonic firing allows faithful signal transmission from the external environment to the cortex leading to an alert behavioral state. On the other hand, the threshold for excitatory postsynaptic potentials (EPSPs) is raised when thalamic neurons burst in an oscillatory, rhythmic firing mode leading to diminished signal transmission to the cortex and reduced consciousness (Snead 1995). Three neuronal classes exist in the thalamus: (i) Thalamocortical neurons projecting to the cortex are glutamatergic. (ii) Inhibitory neurons from the nucleus reticularis thalami (nRT) contain γ-amino-butyric

acid (GABA) and project back to thalamocortical neurons. (iii) Local GABAergic interneurons inhibit thalamic transmission (figure 3). GABAergic neurons of the nRT have the intrinsic ability to shift between the two firing modes and to impose their behavior on thalamocortical circuitry. Most parts of the thalamus are covered by the neuronal sheet of the nRT (Steriade 2001). Its neurons receive excitatory, glutamatergic input from thalamocortical as well as corticothalamic fibers. Additionally, nRT diffusely projects to virtually all dorsal thalamic territories (Steriade 2005). Therefore, nRT is uniquely situated to insure a wide synchronization of oscillations and to influence the flow of information between thalamus and cortex.

Generalized absence seizures represent a perturbation of the rhythmicity in favor of the oscillatory firing mode (Steriade and Contreras 1995) explaining the absence of incoming signals from the external world and the unconsciousness (Steriade 2001; Slaght, Leresche et al. 2002).

Firing of nRT units drastically changes with the appearance of SWDs in the EEG. Prolonged, high-frequency action potential bursts occur in the nRT at the same frequency as SWDs in the EEG as a response to cortical stimulation (Steriade 2001).

Glutamate-mediated EPSPs from the cortex activate neurons in the nRT which in turn release GABA. Activation of $GABA_B$ receptors leads to hyperpolarization which in turn converts low-threshold T-type Ca^{2+} channels in an activated state. Activation of these channels is the cellular event underlying the shift between oscillatory and firing mode (Slaght, Leresche et al. 2002). Hyperpolarization of the membrane potential in nRT to -75mV leads to spontaneous oscillations whereas depolarization to -55mV favors the tonic firing mode (Pape, Meuth et al. 2005). The following Ca^{2+}-mediated burst discharge facilitates the release of excitatory amino acids leading to EPSPs in the cortex. This process leads to another depolarization and the cycle repeats itself (Manning, Richards et al. 2003). Repolarization of thalamocortical neurons occurs by activation of voltage-gated K^+ channels (Feuerstein 2001). Spikes represent EPSPs, whereas slow waves are associated with inhibitory postsynaptic potentials (IPSPs, Giaretta, Avoli et al. 1987). In conclusion, the typical synchronized 3Hz SWDs in generalized absence seizures are generated by an overshooting inhibition of thalamic neurons (figure 3).

Sudden rhythmic membrane depolarizations temporally correlated with SWDs are also seen in cortico-striatal neurons. An abrupt membrane hyperpolarization in these neurons is correlated with the start of SWDs and its removal coincides with the end of SWDs (Panayiotopoulos 1999b).

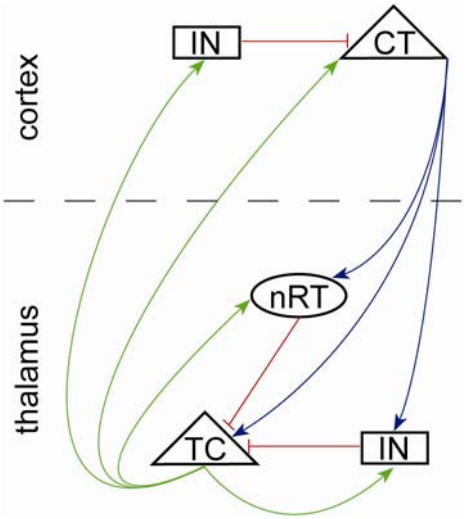

Figure 3: Thalamocortical network influencing absence seizures. *Descending corticothalamic projections (CT) activate neurons of the nucleus reticularis thalami (nRT), thalamic interneurons (IN) and thalamocortical (TC) neurons via excitatory glutamatergic transmission (blue). Thalamocortical neurons project back to cortical structures amplifying the excitation by activating other corticothalamic neurons. Additionally, inhibitory interneurons in thalamus and cortex as well as neurons of the nRT are activated (green). Projections form the nRT to other thalamic structures as well as interneurons block excessive excitation via inhibitory GABAergic transmission (red).*

1.1.1.3 Medical treatment of absence seizures

Generalized absence seizures are completely different from other type of seizures and therefore pharmacologically unique. The common antiepileptic drugs carbamazepine, vigabatrin and tiagabine are contradicted in absence seizures because they can induce seizures or even absence status epilepticus, whereas phenytoin, phenobarbitone and gabapentin are ineffective (Snead, Depaulis et al. 1999). First choice therapies of absence seizures are valproate (Convulex®, Orfiril®, Depakine®), ethosuximide (Petinimid®), mesuximide (Petinutin®) and benzodiazepines (for review see Feuerstein 2001; Manning, Richards et al. 2003). Ethosuximide blocks low-threshold calcium currents via direct action on T-type Ca^{2+} channels and decreases persistent Na^{+} and sustained Ca^{2+}-activated K^{+} currents (Broicher, Seidenbecher et al. 2007). Its direct application on the peri-oral regions of the primary somatosensory cortex exerts most efficacious inhibition of absence seizures (Manning, Richards et al. 2004). Intraperitoneal application of ethosuximide produces an immediate reduction of SWDs and a significant decrease in GABA levels in the primary motor cortex of GAERS (Terzioglu, Aypak et al. 2006). Mesuximide also blocks voltage-dependent T-type Ca^{2+} channels. Although any drug that facilitates GABA actions exacerbates absence seizures, valproate and benzodiazepines are commonly used for treatment. The mode of action of valproate on SWD suppression is not clear. Valproate increases GABA concentration by stimulating its synthesis and inhibiting its metabolism. The predominant action of valproate seems to involve inhibition of voltage-dependent Na^{+} and T-type Ca^{2+} channels (Broicher, Seidenbecher et al. 2007). Valproate additionally controls

myoclonic jerks and generalized tonic-clonic seizures. Benzodiazepines are believed to selectively affect nRT by enhancing GABA-mediated inhibitory neurotransmission via $GABA_A$ receptors and suppressing the spread of seizure activity. Another possibility to treat absences is lamotrigine (Lamictal®), a drug that inhibits Na^+ channels and reduces glutamate concentrations (Duncan, Sander et al. 2006).
Typical absences are normally treated with only one drug titrated up to the maximum tolerated dose. Polytherapy is required in only a small percentage of cases with typical absence epilepsy whereas therapy of atypical absences is more complex (Panayiotopoulos 1999b). Polytherapy increases the possibility of poor compliance, drug interactions, teratogenicity and long-term toxic effects (Duncan, Sander et al. 2006). Low doses of lamotrigine can be added to adequate doses of valproate in therapy-resistant patients. Other possibilities for the few remaining cases of treatment failure are acetazolamide (Diamox®) and benzodiazepines as clonazepam (Rivotril®). The latter is particularly effective in absences with myoclonic components (Dulac 1999). Atypical absences can also be treated with felbamate (Taloxa®), steroids and ketogenic diet (Panayiotopoulos 1999b). Intravenous diazepam (Valium®) or valproate or buccal application of midazolam (Dormicum®) are first choice treatments for absence status epilepticus (Panayiotopoulos 1999a).
Despite several treatment possibilities 10 to 20% of patients may not achieve control of seizures. Valproate is not desirable in women because of different side effects such as teratogenicity, weight gain and polycystic ovarian syndrome. Efficacy and adverse reactions have to be carefully balanced as treatment often last the whole life (Richards, Lemos et al. 1995). The need for new classes of absence drugs is urgent, because known treatments are not effective in all cases of absence epilepsy and a broad spectrum of side effects has been described (Koutroumanidis, Hennessy et al. 1999; Jeha, Morris et al. 2006).

1.1.2 Febrile seizures

Febrile seizures are the most common form of childhood seizures affecting 2-5% of all children and occurring in about 30% of patients with childhood absence epilepsy (Marini, Harkin et al. 2003). Children between 6 months and 5 years with a peak at 18-22 months are affected. Febrile seizures can be divided in simple and complex seizures. 75% of first febrile convulsions are simple with tonic-clonic seizures, duration less than 15 minutes and no recurrence within one day. Complex seizures are mainly focal with longer duration and a cluster of two or more convulsions during 24 hours. The earlier the age of onset, the greater is the risk of recurrence. Three features mainly underlie the genesis of febrile seizures: immature brain, fever and genetic predisposition. Increasing myelination, “dying back” of excessive neurons and augmentation of synaptic complexity occur in this time frame of brain maturation. Causes for fever may vary from different infections to noninfectious illnesses. A polygenetic inheritance is suggested for this disease. In a family study, febrile seizures seem to have an autosomal dominant mode of inheritance with 75% penetrance (Dube, Brunson et al. 2005). $GABA_A$ receptor γ2 subunit missense mutation (R43Q) was linked with childhood absence epilepsy and febrile seizures. Reduced cell surface expression of $GABA_A$ receptor results in increased neuronal excitability and epilepsy (Marini, Harkin et al. 2003). A family study showed that 64%

of all people with this mutation had febrile seizures, 21% childhood absence epilepsy and 15% a generalized epilepsy with febrile seizures plus phenotype (Doose 1998). Febrile seizures are usually brief and self-limited. A short febrile convulsion does not damage the brain. Prolonged convulsions are treated with intravenous or rectal anticonvulsants such as diazepam, midazolam or lorazepam. Rigorous use of antipyretic medication does not prevent febrile seizures. Although prophylactic daily therapy is not recommended, phenobarbital and valproate reduce the recurrence, but the potential side effects of drugs outweigh the benefits (for review see Arzimanoglou 2004). Long-lasting febrile convulsions (>1h) can lead to cognitive and motor developmental deficits and patients can later develop complex partial seizures and therapy-resistant epilepsies (Danober, Deransart et al. 1998).

1.1.3 Focal seizures

Focal seizures can be divided in complex and simple seizures depending on whether consciousness is affected or not. Seizures with a focal beginning can also secondarily generalize. Complex partial seizures usually arise from the limbic lobe including amygdala, hippocampus and less often from the temporal neocortex. Seizures are often accompanied by psychic, sensory and motor phenomena. The most frequent type of focal epilepsies with a high percentage of pharmacoresistant patients is temporal lobe epilepsy (TLE). Neuropathological properties as severe neurodegeneration in the hippocampus or related brain areas are characteristic for TLE which can be initiated by prolonged febrile convulsions in early life or by a status epilepticus. 40-50% of patients with TLE are not seizure free despite an adequate pharmacotherapy (Sperk 2006).

1.1.4 Animal models for focal and generalized tonic-clonic seizures

Animal models mimicking complex partial or generalized tonic-clonic seizures are frequently used to investigate epilepsy mechanisms (Sarkisian 2001).

1.1.4.1 Kindling model for brain plasticity

Kindling is a model to induce long-term plastic changes in brain excitability. Shortly, bipolar stimulating electrodes are implanted in amygdala or hippocampus. Then, daily electrical stimuli were applied for several days until electrical afterdischarges are observed which become progressively more complex and prolonged with each kindling stimulus. Spontaneous epileptic seizures in the absence of electrical stimuli occur after continued kindling for a few weeks (Fisher 1989). Kindled dentate gyrus has an increased efficiency to release glutamate (Rodi, Mazzuferi et al. 2003). The development of seizures can be divided into 5 different stages using the Racine scale: 1. mouth and facial movements; 2. head nodding; 3. forelimb clonus; 4. rearing; 5. falling. Full motor seizures with loss of postural control are referred to stage 5. Stages 4 and 5 are considered as models for secondarily generalized complex partial seizures (Racine 1972). Kindling leads to mossy fiber sprouting in the dentate gyrus and cell death in hilus, hippocampal cornu ammonis (CA) 3 and CA1

pyramidal neurons of hippocampus (Sarkisian 2001). This model is especially useful for investigations of changes that occur in brain over time. Seizures and the disposition of brain regions to develop them can be investigated (Fisher 1989).

1.1.4.2 Kainic acid-induced complex partial seizures

Kainic acid is a glutamate analog that can be systemically or intracerebrally injected into animals and rapidly produces acute seizures. In low doses, kainic acid induces complex motor activity and sometimes generalized tonic-clonic activity (Fisher 1989). Higher doses induce severe acute seizures with subsequent status epilepticus. After a quiescent period of several weeks spontaneous recurrent seizures develop. Kainic acid generates lesions similar to those observed in patients with temporal lobe sclerosis. It is able to produce selective lesions of cell bodies in the brain, especially in hippocampus including loss of GABAergic interneurons in the dentate hilus and pyramidal cell death within CA3 and CA1 (Sarkisian 2001), while axons are preserved (Fisher 1989). Disadvantages of this model are the difficult control of status epilepticus, the unpredictable spontaneous seizures and the extensive neural damage (Morimoto, Fahnestock et al. 2004).

1.1.4.3 Lithium-pilocarpine seizure model

Pilocarpine is a muscarinic cholinergic agonist which produces rapidly acute seizures after systemic or intracerebral injection. Large doses evoke acute seizures that are accompanied by status epilepticus followed by a quiescent period of several weeks (Sarkisian 2001). Neuronal damage develops during this silent period affecting hippocampus, cortex, amygdala as well as thalamus (Andre, Dube et al. 2007). In detail, loss of GABAergic interneurons in the dentate hilus and pyramidal cell death are observed. Afterwards, spontaneous recurrent seizures develop (Sarkisian 2001). Pretreatment with lithium increases the susceptibility of rats to pilocarpine-induced seizures (Turski, Ikonomidou et al. 1989).

1.1.4.4 Pentylenetetrazol-kindling

Parenteral application of pentylenetetrazol, a systemic convulsant, primarily produces myoclonic jerks which may lead to generalized tonic-clonic seizures. Pentylenetetrazol decreases the potency of GABA-mediated inhibition thus leading to seizure generation (Fisher 1989). Repeated injections produce a type of chemical kindling that resembles electrical kindling. Given systemically at very low doses, it elicits absence-like seizures (Sarkisian 2001).

1.1.4.5 Flurothyl model of epileptogenesis

Flurothyl is a chemical inhalant that causes myoclonic jerks followed by severe tonic-clonic convulsions (Sarkisian 2001). Application of the chemoconvulsant flurothyl on consecutive days each evoking a generalized seizure leads to a permanent and rapid reduction of seizure threshold and spreading of seizures in mice (Mhyre and Applegate 2003).

1.1.5 Drug-resistant epilepsies

The percentage of pharmacoresistant patients remains stable between 20 and 25% despite the development of new anticonvulsant drugs (Jeha, Morris et al. 2006). Multiple pathogenetic mechanisms such as differences in seizure onset and progression or different individual sensitivity may underlie refractoriness. 40% of drug-resistant childhood epilepsies are caused by malformations of the cerebral cortex (Guerrini 2006). Intractable epilepsies are often associated with hippocampal sclerosis. Temporal lobectomy is an established treatment for patients with refractory focal epilepsy (Crunelli and Leresche 2002), a cost-effective procedure which carries a chance of 60-70% to make the patient seizure-free accompanied by improved quality of life (Duncan, Sander et al. 2006). Another non-pharmacological option to treat severe forms of drug-resistant epilepsy in pediatric practice is a ketogenic diet consisting of high fat (80%), low protein (15%) and very low carbohydrate (5%) amounts. Increased formation of ketone bodies which are structurally similar to GABA seems to be the efficacious mechanism of the diet (Kossoff 2004).
Drug-intractable epilepsy is a major health problem, associated with morbidity and mortality and accounting for much of the economic burden of epilepsy (Regesta and Tanganelli 1999).

1.2 Genetic Absence Epilepsy Rats from Strasbourg (GAERS)

Studies of the pathophysiological mechanisms of absence epilepsy cannot be conducted in humans due to ethical reasons as mainly children and adolescents are affected and the disease has only moderate consequences. Therefore, most information derives from studies in animal models (Vergnes, Marescaux et al. 1982).

1.2.1 Characterization of GAERS

Coincidentally, it was observed that normal adult Wistar rats display spontaneous synchronous paroxysmal bursts consisting of SWDs in the EEG (Danober, Deransart et al. 1998). These rats had then been selectively inbred over 37 generations resulting in two different strains: all animals of the first group exhibit SWDs (GAERS) and the others were all seizure-free (non-epileptic controls (NEC)). No SWDs are observed in GAERS younger than 30 days. Then, SWDs gradually occur and at the age of 3 months 100% of GAERS are affected (Vergnes, Marescaux et al. 1986). At first, discharges are rare, irregular and brief with a low frequency (5-7Hz) called short irregular spike-and-wave discharges (SISWDs, figure 4A). Thereafter, number, duration and frequency increase progressively possibly as a consequence of neurochemical modifications occurring during brain development (Vergnes, Marescaux et al. 1982). A maximum of SWDs which persist until death is reached around 4-6 months. Typical SWDs feature a frequency of 7-11Hz, durations between 1-40s and spike amplitudes 2-6 times greater than background activity with an abrupt start and end on a normal EEG background (figure 4B). The average discharge lasts 6±3.4s with an incidence of 1.3±0.4/min. Concomitant phenomena are behavioral immobility, staring, rhythmic twitching of the vibrissae and facial muscles resulting in a gradual lowering of the head. A sudden rise of the head to its previous position marks the end of discharges which are preferentially observed during quite wakefulness. When discharges occur during active states, movement is suddenly interrupted and activity resumes as soon as the discharge stops. SWDs are immediately interrupted by unexpected sensory stimulation, although responsiveness to mild sensory stimuli is abolished (Crunelli and Leresche 2002; Rudolf, Bihoreau et al. 2004). Spontaneous activity, exploration, feeding, social interactions, learning and sexual behaviors are not impaired in GAERS as compared to NEC.
The transmission of seizures is polygenic and autosomal dominant as shown by Mendelian cross-breeding. A dominant gene is probably responsible for the epileptic phenotype and further genes modify duration and number of seizures (for review see Danober, Deransart et al. 1998; Rudolf, Bihoreau et al. 2004)

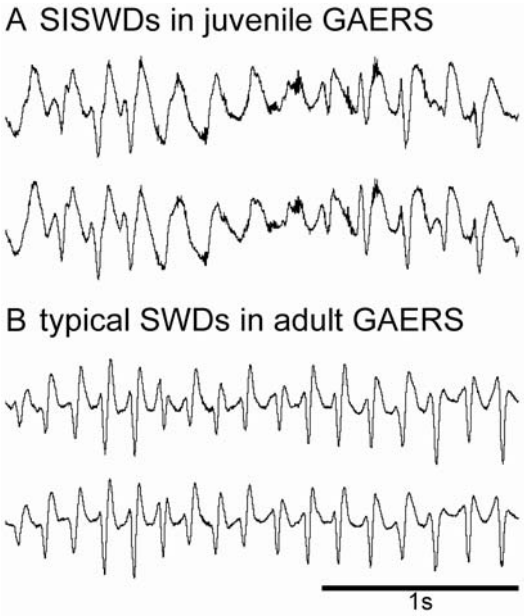

Figure 4: EEGs measured from juvenile and adult GAERS. *A, juvenile GAERS display short irregular spike-and-wave discharges (SISWDs) with frequencies between 5 and 7Hz and short duration. B, typical 7Hz spike-and-wave discharges (SWDs) recorded from adult GAERS.*

1.2.2 Prospects and constraints of this animal model

Convincing animal models for absence epilepsy should achieve the following criteria proposed by Snead et al. (1999):

1. Bilaterally synchronous SWDs associated with behavioral arrest with or without head drop and nystagmus
2. Reproducibility and predictability
3. Ability to standardize and quantify
4. Amelioration by ethosuximide, valproate and benzodiazepines
5. Exacerbation by GABAergic drugs ($GABA_A$ and $GABA_B$ agonists, GABA transaminase inhibitors)
6. Blockade by $GABA_B$ antagonists
7. SWDs originating from thalamus or cortex
8. Hippocampus is silent during seizure activity

Animals of the GAERS strain spontaneously display many of the characteristics of human absence seizures. (1) As described before, spontaneous synchronous SWDs occur bilaterally accompanied by behavioral immobility and lowering of the head (Rudolf, Bihoreau et al. 2004). (2) The epilepsy phenotype is polygenetically determined in an autosomal dominant inheritance (Micheletti, Vergnes et al. 1985; Manning, Richards et al. 2004) leading to a reproducible and predictable animal model. (3) EEG measurements allow quantification of seizure occurrence. (4) SWDs in GAERS are suppressed by ethosuximide, valproate and diazepam in a dose-dependent manner whereas carbamazepine and phenytoin are ineffective or aggravate seizures. Phenobarbital is only effective in small doses but loses its effect with higher doses (Danober, Deransart et al. 1998). (5) Additionally, SWDs are

aggravated by treatment with vigabatrin (GABA transaminase blocker), tiagabine (GABA-reuptake inhibitor), muscimol ($GABA_A$ agonist) and R-baclofen ($GABA_B$ agonist, Marescaux, Vergnes et al. 1992). (6) In contrast, $GABA_B$ antagonists suppress SWDs (Vergnes, Marescaux et al. 1990). (7) SWDs are predominantly located in a specific neuronal network involving cortical and thalamic areas whereas (8) no SWD was ever recorded from the hippocampus or any limbic structure (Polack, Guillemain et al. 2007). Discharges are initiated in deep layer somatosensory cortical neurons and spread within the thalamocortical network (Seidenbecher, Staak et al. 1998). During fully grown SWDs, burst discharges in the cortex significantly lag behind their thalamic counterparts which are responsible for the rhythmogenesis (Danober, Deransart et al. 1998). None of these two structures can sustain the SWDs alone (Manning, Richards et al. 2003).

All these investigations confirm that GAERS fulfill the requirements for a genetically based experimental model of human absence epilepsy. A great advantage of this genetic model is that seizures occur spontaneously and reflect therefore the underlying pathophysiology of human absence seizures (Loiseau 1992).

A major discrepancy between absence seizures in GAERS and humans is the age at onset. In humans, as mentioned above, childhood absence epilepsy develops before the age of 10 years and tends to disappear in puberty or adulthood (Depaulis and Van Luijtelaar 2005). Contrary in GAERS, SWDs start after full electrocortical maturation (around the 30^{th} postnatal day) and persist until death (Snead, Depaulis et al. 1999). This dichotomy is not surprising, since the maturation of human thalamocortical circuitry differs profoundly from those of rats (Seidenbecher, Staak et al. 1998). Another incongruity is the higher frequency of SWDs in GAERS as compared to human patients and the almost complete lack of polyspikes in SWDs (Danober, Deransart et al. 1998). SWD frequency seems to be species-dependent (Jobe, Mishra et al. 1991).

In conclusion, GAERS are helpful for further exploring the pathogenesis of and developing novel drugs for human absence epilepsy although this animal model does not completely mimic human SWDs (Nawa, Carnahan et al. 1995).

1.3 Neurotrophins

Neurotrophins are a family of secreted proteins that play a crucial role in the control of neuronal numbers and dendritic growth consisting of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3) and neurotrophin-4/5 (NT4/5, for review see Bibel and Barde 2000). After synthesis in the endoplasmatic reticulum, pro-neurotrophins (30-35kDa) are folded, sorted into the constitutive or regulated secretory pathway and transported to the appropriate subcellular compartment (Blumenfeld 2005). They are cleaved by metalloproteinases to mature proteins (12-13kDa) which often build noncovalently linked homodimers as active forms (for review see Bibel and Barde 2000). At least some neurotrophin subunits are able to form heterodimers (Huang and Reichardt 2001). Neurotrophins are secreted by both neuronal and non-neuronal cells (Morimoto, Fahnestock et al. 2004) in limited quantities resulting in a competition between innervating neurons. Only neurons with the appropriate synapses will survive (Tapia-Arancibia, Rage et al. 2004). Both, pro-neurotrophins and mature forms have biological activity (Kalb 2005) eliciting diverse but specific responses and regulating the structure and function of the nervous system throughout development and adulthood. Neurotrophins are involved in the correct morphologic, physiologic and chemical differentiation of neurons (Wirth, Patz et al. 2004), in cell fate decisions, axon growth, dendrite pruning, synaptic function and plasticity (Huang and Reichardt 2001). They also exert completely opposite actions such as proliferation or withdrawal from cell cycle, growth or shrinkage, survival or apoptosis (Dechant 2001). Rapid changes in synaptic activity seem to be mediated by activation of second messengers whereas differentiation occurs slowly through induction of new gene expression (Finkbeiner, Tavazoie et al. 1997).

1.3.1 Brain-derived neurotrophic factor (BDNF)

BDNF protein is expressed throughout the brain with highest concentration in hippocampus, followed by hypothalamus, septum, tectum, entorhinal cortex, frontal cortex, cerebellum, hindbrain, thalamus and striatum (Nawa, Carnahan et al. 1995; Conner, Lauterborn et al. 1997). BDNF distribution in soma, dendrites, fibers and nuclei suggests that BDNF enters the nucleus of neurons to directly influence transcription (Tapia-Arancibia, Rage et al. 2004). Translational and/or post-translational regulation of BDNF production or cellular transport of BDNF protein might influence relative regional disparities in protein and mRNA levels (Nawa, Carnahan et al. 1995).
BDNF is involved in several physiological and pathological processes. Various kinds of brain insults such as stress, ischemia and seizure activity alter BDNF expression in the central nervous system. Accordingly, changes in its expression contribute to different pathologies such as epilepsy, depression, eating disorders, Alzheimer's and Parkinson's disease (Tapia-Arancibia, Rage et al. 2004).

1.3.2 Neurotrophin receptors

Two different classes of transmembrane receptor proteins bind neurotrophins: the tropomyosin-related tyrosine kinase receptors (Trks) and the neurotrophin receptor p75 ($p75^{NTR}$). These receptors are able to interact allowing the transduction of very different signals following ligand binding.

Trks are a family of tyrosine kinase receptors consisting of TrkA which preferentially binds NGF, TrkB that prefers BDNF as well as NT4/5 and finally TrkC that binds NT3. However, NT3 is also able to interact with TrkA or TrkB and NT4/5 with TrkA (figure 5A). Trks have a size of about 140kD and express high affinity binding sites for neurotrophins (K_d~10^{-11}M). Binding of neurotrophins leads to receptor dimerization resulting in phosphorylation of specific tyrosine residues which act as docking sites for adapter proteins. Three main signaling cascades may be activated: (i) The Ras/Raf/extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway induces differentiation and neurite growth presumably via cAMP-response element binding protein (CREB) phosphorylation. (ii) Phosphoinositide 3-kinase (PI3K) activates protein kinase B or AKT kinases which in turn phosphorylate and therefore inactivate the proapoptotic protein Bcl-2 associated death promoter (BAD). (iii) The phospholipase Cγ (PLCγ)/inositol 1,4,5-triphosphate (IP_3)/Ca^{2+} pathway leads to neurotrophin release and synaptic plasticity. Activation of these pathways induces prevention of programmed cell death and neuronal differentiation. Neurons are also able to internalize neurotrophins in a receptor-dependent way and transport them retrogradely to the cell body. Trk receptors are expressed in a selective pattern leading to the exquisite neuronal specificity of neurotrophins (for reviews see Klesse and Parada 1999; Bibel and Barde 2000; Kaplan and Miller 2000). BDNF binding to TrkB is also able to evoke membrane depolarization through rapid activation of $Na_v1.9$ sodium channels. This mechanism does not require second messengers resulting in a rapid response without attenuation (Blum, Kafitz et al. 2002) and contributes to long-lasting potentiation of glutamatergic inputs (Scharfman 2005). Precursor forms of neurotrophins also bind to their specific Trk receptor and exert physiological activities (Fayard, Loeffler et al. 2005).

$p75^{NTR}$, a member of the tumor necrosis factor receptor superfamily, binds neurotrophins with low affinity (K_d~10^{-9}M) and mediates cell death independently from Trks. Pro-neurotrophins bind with higher affinity to $p75^{NTR}$ than the mature forms. This binding site is formed by a complex of $p75^{NTR}$ and sortilin (figure 5B, Kalb 2005). Signaling pathways of $p75^{NTR}$ are different with nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation preventing cell death or caspase activation mediating apoptosis. $p75^{NTR}$ expression is down-regulated during postnatal development but rapidly induced after nerve lesion or seizures (for review see Bibel and Barde 2000).

Ligands, receptors and their intracellular target proteins are linked by balanced biochemical interactions. Neurotrophin receptors form three different types of complexes: homodimers of Trk receptors, clusters of $p75^{NTR}$ receptors and a mixed complex containing both Trk and $p75^{NTR}$ (figure 5). TrkA, TrkB or TrkC receptor complexes may cross-talk to each other through $p75^{NTR}$ which provides a physical

link between them (Dechant 2001). The proapoptotic signals of $p75^{NTR}$ are largely suppressed by simultaneous activation of Trk receptors. $p75^{NTR}$ appears to refine the ligand-specificity of Trk receptors. It can even potentiate the activation of TrkA by subsaturating concentrations of NGF. Thus, $p75^{NTR}$ promotes elimination of neurons that are not exposed to appropriate levels of neurotrophins (Huang and Reichardt 2001).

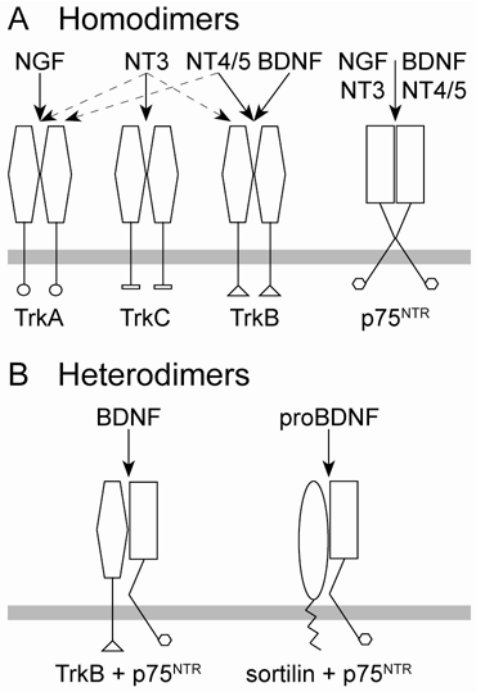

Figure 5: Interactions of neurotrophins with different receptor complexes. *A, NGF binds selectively to TrkA homodimers. NT3 has highest affinity to TrkC, but is also able to activate TrkA or TrkB homodimers. BDNF and NT4/5 preferentially bind to TrkB. All neurotrophins activate with low affinity a $p75^{NTR}$ receptor complex. B, Heterodimers with $p75^{NTR}$ increase affinity and selectivity of Trks for neurotrophin binding. Pro-forms of neurotrophins bind with high affinity to a heterodimer consisting of sortilin and $p75^{NTR}$.*

1.3.3 BDNF in epilepsy

Previous studies have shown that BDNF signaling is associated with epilepsy. BDNF appears to be both regulated by and a regulator of epileptogenesis.

BDNF mRNA or protein levels are significantly increased after epileptic stimuli as shown for limbic seizures induced by electrolytic lesions of the dentate gyrus (DG, Xapelli, Bernardino et al. 2008), non-convulsive doses of $GABA_B$ receptor antagonists (Heese, Otten et al. 2000), kainic acid (Zafra, Lindholm et al. 1992), pilocarpine (Kornblum, Sankar et al. 1997), flurothyl kindling (Mhyre and Applegate 2003) and after febrile seizures (Kim, Rhyu et al. 2001). After seizures, BDNF mRNA and protein are accumulated in dendrites of hippocampal neurons (Tongiorgi, Armellin et al. 2004).

Additionally, TrkB and TrkC, but not TrkA mRNA expression are concomitantly and transiently increased after focal and generalized seizures induced by hippocampal or

amygdala kindling (Bengzon, Kokaia et al. 1993). These changes are accompanied by a striking but transient induction of Trk phosphorylation in mossy fiber axons of dentate granule cells (Binder, Routbort et al. 1999). Expression of $p75^{NTR}$ is highly induced in neurons and microglia within the sclerotic hippocampus of pharmacoresistant patients with temporal lobe epilepsy (Ozbas-Gerceker, Gorter et al. 2004).

The impact of BDNF on epileptogenesis has been proven in several genetic modified mice models. Transgenic mice overexpressing BDNF are more susceptible to kainic acid-induced seizures and show hyperexcitability in hippocampal area CA3 and entorhinal cortex (Croll, Suri et al. 1999). In line with these results, transgenic mice overexpressing TrkB which leads to increased BDNF signaling experience more severe status epilepticus and have acute neuronal loss after kainic acid treatment. In contrast, the chronic increase in BDNF signaling does not alter the development of spontaneous seizures in transgenic mice. Changes in BDNF levels in the long run may be compensated by adjustments to the higher basal levels of TrkB signaling, whereas sudden changes such as occurring during status epilepticus cannot be antagonized (Lahteinen, Pitkanen et al. 2003).

Homozygous deletion of BDNF is lethal in knock-out mice, therefore heterozygotes showing a reduction of BDNF mRNA in cerebral cortex have been studied. Kindling development is markedly retarded in these mice reflecting dampening of the progression from focal to generalized seizures. In contrast, persistence of kindling is unaffected (Kokaia, Ernfors et al. 1995). Additionally, these mice have lower seizure susceptibility for limbic (6Hz), pentylenetetrazol- and kainic acid-induced seizures, but higher susceptibility to pilocarpine-induced seizures suggesting that lack of BDNF can confer resistance to some, but not all types of seizures (Barton and Shannon 2005). In line with these results, transgenic mice overexpressing truncated TrkB, a dominant negative receptor of BDNF, have less severe seizures with later onset and lower mortality after induction of status epilepticus by kainic acid (Lahteinen, Pitkanen et al. 2002).

The results obtained from genetic mice models have been corroborated by intracerebral injection studies. In the kindling paradigm, seizure scores are significantly higher in animals receiving multiple BDNF microinjections (Xu, Michalski et al. 2004). In contrast, chronic intrahippocampal infusion of BDNF delays the progression of hippocampal kindling, whereas infusion of BDNF antisense oligodeoxynucleotides aggravates seizures (Reibel, Larmet et al. 2000b). These discrepancies can be explained by investigating BDNF signaling pathways. Multiple bolus microinjections of BDNF do not affect TrkB, whereas continuous infusion of BDNF drastically decreases phosphorylation and expression of TrkB receptors thus leading to an attenuation of epileptogenic effects of BDNF (Xu, Michalski et al. 2004). Furthermore, continuous infusion of high BDNF concentrations may overcome the inhibition caused by TrkB receptor down-regulation and increases therefore excitability after pilocarpine injection and causes spontaneous seizures (Scharfman, Goodman et al. 2002).

Activation of Trk receptors and downstream-signaling play an important role in epileptogenesis. Perforant path kindling is significantly inhibited by blocking selectively TrkA, but not $p75^{NTR}$ receptors (Li, Saragovi et al. 2005). Only a modest impairment of limbic epileptogenesis is detected in BDNF knock-out mice, whereas no behavioral seizures occur in TrkB knock-out mice suggesting that compensatory

increases in NT3 expression may contribute to TrkB activation and epileptogenesis in BDNF knock-out mice (He, Kotloski et al. 2004).
In conclusion, these data support the hypothesis that BDNF predisposes the brain to epileptic seizures. Scharfman (2005) hypothesizes that increased levels of BDNF potentiate glutamatergic transmission leading to increased neuronal activity which in turn increases secondarily BDNF and TrkB levels. These events escalate by positive feedback and finally reach seizure threshold. Additionally, the seizure-induced increase of BDNF synthesis may constitute an intrinsic protective response counteracting cell death (Kokaia, Ernfors et al. 1995).

1.3.4 Mechanisms underlying the anticonvulsant effects of BDNF

BDNF induces a rapid and long-lasting enhancement of synaptic transmission at mature synapses (Kang and Schuman 1995) by increasing glutamate release via PLCγ/Ca^{2+} pathway (Toth 2005). Additionally, the regulated stimulation-evoked release of glutamate is increased in BDNF-treated cultured cortical neurons due to elevated expression of exocytosis-associated proteins and synaptic vesicles (Takei, Sasaoka et al. 1997).
In contrast, BDNF inhibits $GABA_A$-receptor mediated inhibitory post-synaptic currents in rat hippocampal slices facilitating neural excitation and generation of action potentials (Tanaka, Saito et al. 1997; Frerking, Malenka et al. 1998).
BDNF upregulation also mediates mossy fiber sprouting via activation of TrkB and Ca^{2+} release. Sprouting of mossy fibers generates a proepileptic recurrent circuit and is often seen in patients with TLE (Koyama, Yamada et al. 2004).

1.4 3',5'-cyclic adenosine monophosphate (cAMP)-response element binding protein (CREB)

Most of the mechanisms underlying neuronal reorganization are mediated via gene activation. The transcription factor CREB is ubiquitously expressed and a target for numerous signaling cascades that cause phosphorylation on the CREB activator site serine 133. This process causes a conformational change of the CREB molecule and allows pCREB to bind as dimer to certain DNA sequences called cAMP-response elements (CRE) in the promoters of many genes to modulate their transcription (Montminy, Gonzalez et al. 1990). CREB regulates the expression of different proteins as e.g. $GABA_B$ receptors (Steiger, Bandyopadhyay et al. 2004), the proto-oncogene c-fos, somatostatin, tyrosine hydroxylase, vasoactive intestinal peptide, pro-enkephalin and chorionic gonadotropin. Moreover, cellular morphology, intermediary metabolism and neuropeptide biosynthesis are affected (Montminy, Gonzalez et al. 1990). Thus, CREB plays an important role in neuronal connectivity and excitability leading finally to changes in the neuronal network (Chen, Chang et al. 2006). Within minutes after Ca^{2+} signals terminate, CREB is dephosphorylated and signaling pathways are stopped. CREB acts therefore as an on-off switch for gene expression (Chawla and Bading 2001).

1.4.1 Interaction between CREB and BDNF

CREB plays a major role in the realization of neurotrophin responses. Neurotrophins utilize at least two pathways for activating CREB by phosphorylation at serine 133. In a rapid signaling mechanism, activation of TrkB stimulates $PLC\gamma$ resulting in production of IP_3 which in turn releases intracellular Ca^{2+} leading to activation of calcium/calmodulin-dependent kinases (CaMK). The second slower signaling pathway includes activation of the Ras/ERK/ribosomal s6 kinase (RSK) cascade (figure 6). These different mechanisms enable BDNF to regulate specific neuronal functions and to broaden the number of genes responsive to BDNF (Finkbeiner, Tavazoie et al. 1997). BDNF-induced signaling pathways outlast the presence of BDNF due to the slow mechanism via Ras/ERK/RSK (Chawla and Bading 2001). Additionally, this pathway appears to be involved in BDNF-induced long-term memory formation (Alonso, Vianna et al. 2002).
BDNF does not only regulate transcription of genes via phosphorylation of CREB, it is also a CREB target gene by itself. Conti et al. (2002) showed in mice that CREB functions also as an upstream activator of BDNF expression.

1.4.2 Interaction between CREB and neuropeptide Y (NPY)

CREB is also involved in the signal transduction of NPY effects (Chance, Sheriff et al. 2000). Sheriff et al. (1998; 2002) showed that NPY mediates CREB phosphorylation and CRE binding via activation of Y1 and Y2 receptors leading to an increase in intracellular $[Ca^{2+}]$ and activation of protein kinase A (PKA) or CaMK (figure 6). Additionally, NPY expression itself is also regulated by CREB (Pandey 2003; Hsieh, Yang et al. 2007).

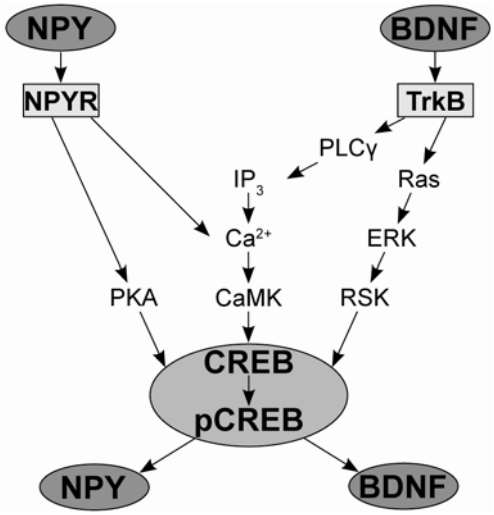

Figure 6: Interactions between BDNF, CREB and NPY. *BDNF binding to TrkB activates two signaling mechanisms: (i) Stimulation of phospholipase Cγ (PLCγ) produces inositol 1,4,5-triphosphate (IP_3) which in turn releases intracellular Ca^{2+}. Calcium/calmodulin-dependent kinases (CaMK) are activated by increased intracellular [Ca^{2+}]. (ii) The second signaling pathway involves phosphorylation of the proto-oncogene Ras, the extracellular signal-regulated kinases (ERK) and the ribosomal s6 kinase (RSK). Both pathways activate CREB and influence gene expression. Activated NPY-receptors (NPYR) lead to a direct phosphorylation of CREB via protein kinase A (PKA) or indirectly via elevation of intracellular [Ca^{2+}]. BDNF and NPY expression are both regulated themselves by the transcription factor CREB.*

1.4.3 CREB in epilepsy

The function of CREB in epileptogenesis is still not clarified. The number of pCREB-positive nuclei significantly decreases 24 hours and CREB mRNA expression 30 days after pentylenetetrazol-induced kindling in chronic epilepsy rats (Lou, Wang et al. 2007; Wang, Lou et al. 2008). Increases in seizure susceptibility to pentylenetetrazol of Sprague-Dawley rats by perinatal hypoxia are accompanied by decreases in CREB phosphorylation in midbrain, temporal cortex and hippocampus (Chen, Chang et al. 2006). Phosphorylation of CREB in hippocampal CA1 is also diminished 60 days after lithium-pilocarpine induced status epilepticus in Sprague-Dawley rats (Huang, Lai et al. 2003).
In contrast, increased CREB binding activity was demonstrated 2 hours after amygdala kindling in hippocampus (Kashihara, Sato et al. 2000).

1.5 Neuropeptides

Neuropeptides are a class of signaling molecules that often act as cofactors of classical transmitters including glutamate or GABA and are therefore important modulators of neuronal excitability (Wilson, Chung et al. 2005). They are synthesized as pro-forms in perikarya and wrapped into vesicles. Afterwards, they are transported to nerve endings, cleared by proteases and released by exocytosis. Neurons are not competent for neuropeptide reuptake, thus they have to continuously rebuild and transport them de novo (Sperk 2006).

1.5.1 Neuropeptide Y

NPY consists of 36 amino acids with tyrosine as its N-terminal, tyrosine amide as its C-terminal and a total of five tyrosine residues per molecule (figure 7). It is a member of the pancreatic polypeptide family having structural similarities with the peptide YY and the pancreatic polypeptide (Tatemoto, Carlquist et al. 1982). It is generated as pre-pro-NPY, then the signal peptide is removed in the endoplasmatic reticulum and the pro-form is cleaved by prohormone convertases. Functions of NPY include regulation of appetite, anxiety, sedation, epilepsy, circadian rhythms, memory processing, pain, drug addiction, blood pressure, rhinitis and endothelial cell dysfunctions (for review see Silva, Cavadas et al. 2002)

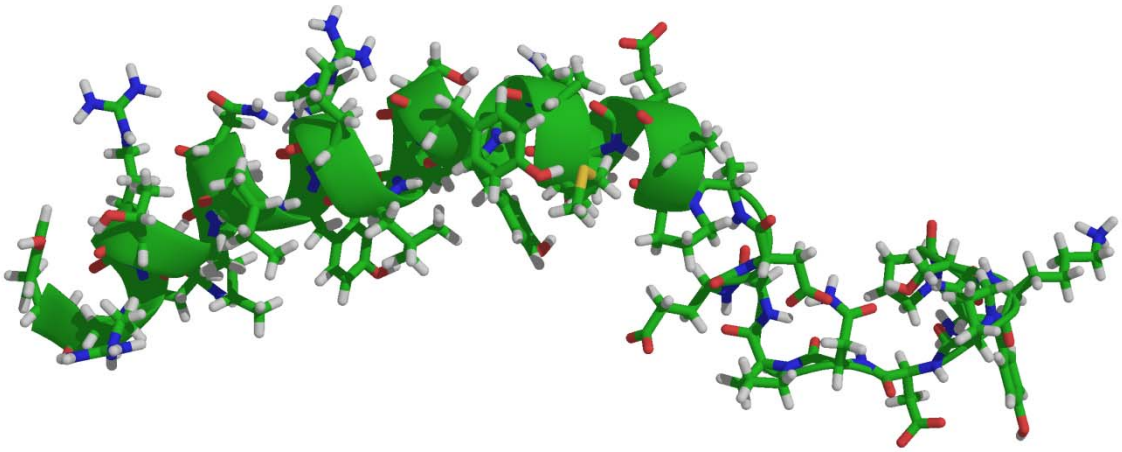

Figure 7: Structure of human neuropeptide Y. *NPY is a tyrosine rich protein consisting of 36 amino acids (from the protein data base according to the work of Monks, Karagianis et al. 1996).*

NPY is widely distributed throughout the central and peripheral nervous system and represents the most abundant neuropeptide in brain although the levels are low. It is co-localized in GABA-containing interneurons. Structures with highest levels of NPY mRNA are the hypothalamus, cerebral cortex and hilar region of the hippocampus. In addition, the majority of the perikarya in nRT expresses NPY mRNA (Nakagawa, Shiosaka et al. 1985), but not in the ventrobasal nucleus of the thalamus (Redrobe,

Dumont et al. 1999), whereas NPY-positive fibers are detected in several thalamic nuclei in rats (Walter, Mai et al. 1990). In human thalamus, NPY immunoreactivity is concentrated in the area of the midline nuclei, whereas immunoreactive fibers are found in various nuclei (Patrylo, van den Pol et al. 1999).
NPY is located within dense core vesicles (Pickel, Chan et al. 1995) that are believed to be released during high-frequency firing (Hokfelt 1991). Thus NPY may be released during epilepsy thereby limiting seizure severity (Brill, Lee et al. 2006).

1.5.2 NPY receptor subtypes

NPY signals through a family of G-protein-coupled receptors: Y1, Y2, Y3, Y4, Y5 and Y6; the latter is not functional in humans (Silva, Cavadas et al. 2002).
Colocalization of the receptor subtypes to the same region of a chromosome suggests that these subtypes have a common origin despite their structural differences (Ammar, Eadie et al. 1996). Distribution and expression of NPY receptor subtypes vary between species (Redrobe, Dumont et al. 1999).

Y1 receptor subtype is mainly expressed in blood vessels and the central nervous system, preferentially in frontoparietal cortex and thalamus (Dumont, Fournier et al. 1993; Silva, Cavadas et al. 2002). In hippocampus, moderate levels are found in the molecular layer of DG. High levels of Y1 receptor mRNA are restricted to the pyramidal cell layers of CA1, CA2 and CA3, whereas low levels were found in the granule cell layer of DG (Redrobe, Dumont et al. 1999).
The N-terminal part of NPY determines its affinity on the Y1 receptor. Vasoconstriction is the main effect mediated by this receptor subtype (Silva, Cavadas et al. 2002). Additionally, it promotes proliferation of neuronal precursor cells (Hansel, Eipper et al. 2001).
Postsynaptic Y1 receptors are proposed to reduce the excitability of neurons by activating G-protein-activated inwardly rectifying potassium (GIRK) channels in thalamic nuclei resulting in long lasting hyperpolarization (Sun, Akk et al. 2001; Brill, Kwakye et al. 2007). Furthermore, Y1 operates as autoreceptor regulating NPY release from interneurons (Baraban 2004).

Y2 receptors are expressed in the central and peripheral nervous system, intestine and certain blood vessels (Silva, Cavadas et al. 2002). In hippocampus, high expression levels of Y2 receptor mRNA are found throughout the pyramidal cell layer of each CA region, whereas expression is moderate in the granule cell layer and only very low in the molecular layer of DG (Redrobe, Dumont et al. 1999).
Activation of presynaptic Y2 receptors reduces intracellular [Ca^{2+}] (Qian, Colmers et al. 1997) and inhibits the K^{+}-stimulated and Ca^{2+}-dependent glutamate release in rat hippocampal slices (Greber, Schwarzer et al. 1994). In contrast, Y2 receptor mediated inhibition of Ca^{2+}-influx downregulates GABA release in thalamic nuclei (Sun, Akk et al. 2001).

Y3 receptor subtype is localized in the human adrenal medulla where it mediates NPY-induced secretion of catecholamines (Silva, Cavadas et al. 2002).

Y4 receptors are widely distributed throughout the whole body. Its expression in rat hippocampus is very limited and restricted to few specific neurons. Highest mRNA expression is detected in the pyramidal cell layer of CA3. Nevertheless, Y4 receptor mRNA is found in low levels in pyramidal neurons of all three CA regions and in granule cells of DG (Redrobe, Dumont et al. 1999).
Y4 receptor binds the pancreatic polypeptide with a higher affinity than NPY and seems therefore to be involved in the effects described for pancreatic polypeptide as inhibition of exocrine pancreatic secretion, induction of gall bladder relaxation and stimulation of luteinizing hormone secretion (Silva, Cavadas et al. 2002).

Y5 receptors are preferentially expressed in many peripheral organs and in hypothalamus where they stimulate appetite (Silva, Cavadas et al. 2002).
Y5 receptor mRNA is only expressed in regions where Y1 receptor mRNA is detected (Parker and Herzog 1999). In rat hippocampus, its mRNA is found in high levels throughout the pyramidal cell layers of all CA regions with peak levels in CA3. Y5 receptors are also expressed in significant amounts in the molecular layer of DG although levels are lower than the one of the Y1 receptor subtype (Rodi, Mazzuferi et al. 2003).

1.5.3 NPY in epilepsy

NPY expression undergoes drastic changes following epileptic seizures. Its protein levels decline immediately after seizure onset reflecting an enhanced release by seizure activity (Xapelli, Bernardino et al. 2008). This decrease occurs as neuropeptides do not undergo reuptake by neurons after their release. Thereafter, NPY is newly synthesized and NPY levels markedly increase. Changes in NPY expression following seizures occur only in brain regions that are crucial for the initiation and propagation of epileptic discharges (Vezzani, Sperk et al. 1999b) and represent an endogenous adaptive mechanism to counteract hyperexcitability (Vezzani and Sperk 2004).
Delayed and long-lasting increases in NPY expression are observed after severe kainic acid induced limbic seizures (Marksteiner, Sperk et al. 1989; Wilson, Chung et al. 2005; Brill, Lee et al. 2006), pentylenetetrazol-kindling (Marksteiner, Lassmann et al. 1990) and hyperthermic seizures (Kang and Macdonald 2004). Additionally, febrile seizures induce a release of endogenous NPY which in turn reduces the network excitability and increases threshold for further febrile seizures (Dube 2007). Elevated NPY immunoreactivity is also found in genetic animal models as Ihara's genetically epileptic rats which display spontaneous convulsions (Takahashi, Sadamatsu et al. 1997) and in Noda epileptic rats suffering from spontaneous generalized tonic-clonic seizures (Jinde, Masui et al. 2002).
Not only significant increases in NPY mRNA occur during kindling epileptogenesis, but also drastic cell- and region-specific changes in receptor expression. Rapid and transient decreases in Y1 mRNA are observed, whereas Y2 mRNA is elevated. Y5 mRNA expression is only transiently increased (Kopp, Nanobashvili et al. 1999). Similar changes occur in Noda epileptic rats (Jinde, Masui et al. 2002). In kainic acid-induced seizures, Y5 receptor protein is reduced accompanied by pyramidal and hilar cell loss (Vezzani, Moneta et al. 2000). During epileptogenesis, down-regulation of

Y1 receptors may favor the inhibitory effects of NPY mediated by Y2 receptors which are up-regulated.

NPY has also received considerable attention as an endogenous modulator of epileptic activity as corroborated by several genetic animal models. Rats overexpressing NPY show a significant reduction in the number and duration of seizures induced by kainic acid and an increase in electrical stimuli required to induce stage 5 seizures during kindling (Vezzani, Michalkiewicz et al. 2002). In accordance, adeno-associated virus vector mediated overexpression of NPY in rats reduces seizures induced by intrahippocampal kainic acid, delays seizure onset and impairs kindling epileptogenesis (Richichi, Lin et al. 2004). Additionally, selective down-regulation of Y1 receptors by infusion of antisense oligonucleotides significantly slows down hippocampal kindling development. In contrast, Y1 knock-out mice have a similar susceptibility to hippocampal kindling as control rats suggesting that compensatory mechanisms exist (Benmaamar, Pham-Le et al. 2003). Intrahippocampal administration of an Y1 receptor antagonist significantly reduces susceptibility to kainic acid, whereas an Y1 receptor agonist evokes wet dog shakes (Gariboldi, Conti et al. 1998). In line with these results, kainic-acid induced seizures progress uncontrollably finally leading to death in almost all NPY-deficient mice. Death can be prevented by intracerebroventricular (icv) infusion of NPY (Baraban, Hollopeter et al. 1997). Additionally, NPY-deficient mice are more susceptible to pilocarpine-induced seizures and kindling development (Shannon and Yang 2004).

In vitro and in vivo studies support the role of NPY as an endogenous anticonvulsant. Injection of NPY prior to kainic-acid significantly reduces total time spent in seizure and severity of seizures whereas latency to the first long lasting and grade 4/5 seizure is prolonged (Madsen, Woldbye et al. 1999). Chronic intrahippocampal infusion of NPY delays the progression of hippocampal kindling, whereas application of anti-NPY immunglobulins accelerates the development of generalized convulsive seizures (Reibel, Benmaamar et al. 2003). Additionally, NPY inhibits behavioral effects evoked by injection of the $GABA_A$-antagonist picrotoxin (Woldbye, Madsen et al. 1996). NPY and an Y2 receptor agonist reduce the frequency of spontaneous epileptiform discharges induced by picrotoxin in rat hippocampal slices suggesting that the antiepileptic effect is mediated by activation of Y2 receptors (Smialowska, Bijak et al. 1996). Icv applied NPY reduces the duration of afterdischarges following electrical stimulation in rat. Additionally, afterdischarge threshold is increased and accompanying wet dog shakes are blocked (Marksteiner and Sperk 1988). In epileptic humans, NPY is able to attenuate excitatory responses provoked by perforant path stimulation in DG and may be effective in reducing glutamate-mediated hyperactivity (Smialowska, Bijak et al. 1996). Recent data also implicate the involvement of NPY in the occurrence of absence seizures (Stroud, O'Brien et al. 2005; Morris, Gannan et al. 2007).

The neuroprotective effects of NPY highlight the possibility of developing novel therapeutic strategies for the treatment and management of intractable seizures.

1.5.4 Mechanisms underlying the antiepileptic effects of NPY

Exogenously applied NPY on neocortical slices of rats leads to a delayed and long-lasting potentiation of inhibitory post-synaptic currents and a depression of excitatory post-synaptic currents in pyramidal neurons by enhancing the GABA release (Frank, Ventimiglia et al. 1996) and suppressing depolarization-induced glutamate release (Richichi, Lin et al. 2004). NPY was shown to induce at least three different intracellular signaling events: (i) inhibition of adenylyl cyclase, (ii) variation of intracellular [Ca^{2+}] via IP_3 or direct activation or blocking of Ca^{2+} channels and (iii) activation or inhibition of K^+ channels (Silva, Cavadas et al. 2002).

The involvement of different NPY receptor subtypes in the antiepileptic effect is discussed controversially. Sun et al. (2003) propose that slow IPSPs in GABAergic nRT neurons are mediated by NPY binding to Y1 receptors leading to activation of GIRK channels causing a negative feedback for activity in the thalamic network and therefore suppression of epileptiform activity. In contrast, NPY released in the hippocampus following seizures exerts a proconvulsive action via Y1 receptors which are located on granule cells in the DG (Vezzani, Sperk et al. 1999b).

Y2 receptors are involved in the antiepileptic effect via reduction of glutamate outflow in synaptosomes of kindled rats (Colmers, Klapstein et al. 1991). NPY reversibly inhibits excitatory synaptic transmission from stratum radiatum to CA1 pyramidal cells via Y2 receptors (McQuiston, Petrozzino et al. 1996). During prolonged seizures, NPY is also spontaneously released from mossy fibers and subsequently by activating presynaptic Y2 receptors, glutamate release and granule cell epileptiform activity are reduced (Nadler, Tu et al. 2007).

Finally, other studies show that the Y5 receptor subtype also mediates anticonvulsant properties of NPY. In rat hippocampal slices, Y5 receptors play a major role in mediating NPY-exerted suppression of spontaneous bursting (Nanobashvili, Woldbye et al. 2004). Suppressive effects of NPY on kainic acid-induced seizures in rats are also mediated via Y5-like receptors (Woldbye, Larsen et al. 1997). Concomitantly, NPY has no effect on epileptiform activity recorded in hippocampal slices from Y5 receptor knock-out mice (Baraban 2002).

An extensive study that used in vitro and in vivo studies in rats and wild-type as well as knock-out mice confirms the involvement of Y2 receptors in the anticonvulsant effect of NPY, whereas involvement of Y5 receptors is not supported (El Bahh, Balosso et al. 2005).

In conclusion, Woldbye and Kokaia (2004) reviewed that Y2 and/or Y5 receptor agonists and Y1 receptor antagonists appear to inhibit seizures depending on the seizure model studied.

1.6 Interaction between BDNF and NPY

Icv administration of BDNF on newborn rats significantly increases NPY immunoreactivity and mRNA expression suggesting that BDNF induces NPY synthesis (Nawa, Pelleymounter et al. 1994). In line with this result, NPY-expression is long-lasting increased after chronic intrahippocampal infusion of BDNF in rats (Reibel, Larmet et al. 2000a; Scharfman, Goodman et al. 2002). Wirth et al. (2005) propose that BDNF is not able to induce NPY in the absence of activity. For induction of NPY, a continuous activation of TrkB by BDNF is required. The longer the BDNF exposure lasts, the longer the duration of TrkB phosphorylation and induction of the signaling pathway ERK that is involved in the induction of NPY (Barnea and Roberts 2001; Barnea, Roberts et al. 2004). Overexpression of NPY is long-lasting suggesting that BDNF can trigger long-term genomic effects and therefore contribute to neuroplasticity (Reibel, Vivien-Roels et al. 2000). BDNF and NPY play opposite roles in epilepsy. BDNF itself enhances hippocampal excitability, but it also induces NPY-expression which in turn blocks seizure propagation (Koyama and Ikegaya 2005). BDNF released by microglia and neurons can also affect neuroprotection against excitotoxicity which is mediated by NPY via Y1 and Y2 receptors (Xapelli, Bernardino et al. 2008). BDNF overexpression after AMPA (glutamate receptor agonist) exposure might be a response to excitotoxicity and reduces cell damage by inducing NPY production. Y2 receptors are thought to play an important role in this cross-talk between BDNF and NPY (Morris 1989).

2 Aim of this thesis

Epilepsy is currently among the most prevalent neurological disorders worldwide. The current treatment focuses exclusively on the prophylaxis or suppression of seizures and thus provides merely a symptomatic treatment without clear influence on the cause of the disease. There is an urgent need for new drugs that act at different molecular targets designed to block the process of epileptogenesis.

Goal of this thesis was to define the potential role of the neurotrophin/neuropeptide-system (including receptors and signaling pathways) in absence seizures. The following criteria are addressed in detail:

- To reveal expression differences in rats suffering from absence seizures, biochemical investigations are performed in various brain regions (e.g. cortex, thalamus and hippocampus) of adult GAERS as compared to NEC.

- To detect region- and neuronal subpopulation-specific expression patterns of neurotrophins and neuropeptides, brain tissue is investigated by immunohistochemical analyses.

- To expose differences that are linked to the occurrence of seizures, developmental studies are performed in juvenile, 3-4 weeks old GAERS which do not yet display SWDs. Early alterations in expression might have important consequences on brain development and therefore favor epileptogenesis.

- To verify the impact of expression differences on epileptogenesis, functional studies are performed. A dual approach is utilized to define the substances involved in possible anti-absence effects. In one series of experiments, effects on the initiation of SISWDs in juvenile GAERS are quantified following icv microinjection of drugs interfering with the neurotrophin/neuropeptide-system. In a further series of experiments, SWDs in adult GAERS are blocked by treatments inhibiting the neurotrophin/neuropeptide-system. Functional effects are monitored by recording SWDs using EEG measurements.

Results obtained should reveal new insights into the role of neurotrophins and neuropeptides in the causes of absence epilepsy. Both of them might represent accessible systems in specific brain regions to intervene in the excitability of neurons. Thus, novel therapeutic approaches aimed not only at the prevention or treatment of absence epilepsy, but also other neurological diseases with disturbed excitability in humans might emerge.

The results of my studies are summarized in the two manuscripts “Involvement of the brain-derived neurotrophic factor – tyrosine kinase receptor B – neuropeptide Y cascade in suppressing absence seizures in a genetic rat epilepsy model.” (red) and “On the protective role of neuropeptide Y against spike-and-wave discharges in a genetic rat model of absence epilepsy” (green) which are submitted for publication.

3 Results

3.1 Part I – Involvement of BDNF-TrkB-NPY in absence seizures

Involvement of the brain-derived neurotrophic factor – tyrosine kinase receptor B – neuropeptide Y cascade in suppressing absence seizures in a genetic rat epilepsy model.

Authors

Svenja Landweer[1,4], Ariane Zingg[1], Manuela Arzenti[1], Cordula Nitsch[2], Any Boehrer[3], Gabor Juhasz[4], Raymond Bernasconi[1] and Uwe Otten[1]

[1] Molecular Neurobiology, Department of Biomedicine, University of Basel, Pestalozzistrasse 20, CH-4056 Basel, Switzerland
[2] Functional Neuroanatomy, Department of Biomedicine, University of Basel, Pestalozzistrasse 20, CH-4056 Basel, Switzerland
[3] INSERM U596, Faculty of Medicine, 11 rue Humann, F-67085 Strasbourg Cedex, France
[4] Neurobiology Research Group, Hungarian Academy of Sciences, Eötvös Lorand University, Pazmany Peter setany 1/C, H-1117 Budapest, Hungary

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Abstract

Genetic Absence Epilepsy Rats from Strasbourg (GAERS) serve as a valid model for human generalized non-convulsive seizures according to their electroencephalographic, behavioural and pharmacological characteristics. Thus, exploring the pathogenesis of spike-and-wave discharges (SWDs) in GAERS may lead to a better understanding of the mechanisms underlying human absence epilepsy.
Here, we demonstrate that cortical and hippocampal brain-derived neurotrophic factor (BDNF) expression is elevated in adult GAERS as compared to age-matched non-epileptic controls (NEC). This is also the case in juvenile GAERS although they do not yet display SWDs. Tyrosine kinase receptor B (TrkB) mRNA expression is markedly decreased in adult GAERS and concomitantly, protein levels of phosphorylated cAMP response element binding protein (pCREB) are strongly reduced. Moreover, we found higher mRNA levels of neuropeptide Y (NPY) in cortex and hippocampus of both, adult and juvenile GAERS as compared to age-matched NEC although it has been demonstrated that NPY suppresses SWDs. In both brain areas, density of NPY expressing neurons is more pronounced in juvenile than in adult rats, suggesting that occurrence of absence seizures correlates with a significant age-dependent decrease of NPY expression. The majority of NPY-positive neurons also contain BDNF indicating a close correlation of expression. Intracerebroventricular injection of BDNF significantly reduces the occurrence of SWDs.
Taken together, it can be concluded that BDNF signalling is significantly altered in adult GAERS and plays an important role in the control of SWDs.

Introduction

Among the different types of epilepsy, the pathophysiology of human nonconvulsive absence seizures is poorly understood. This type of epilepsy is prevalent during childhood and potentially detrimental to the child's education, social integration and health. Studies of the pathophysiological mechanisms of human absence epilepsy cannot be conducted in human for ethical reasons (Vergnes *et al.*, 1982). Therefore, animal models are mandatory to understand this type of epilepsy and the mechanisms underlying the generation and control of spike-and-wave discharges (SWDs). The strain Genetic Absence Epilepsy Rats from Strasbourg (GAERS) appears to be a genetic model with clinical and pharmacological characteristics similar to those occurring in humans (Depaulis & Van Luijtelaar, 2005). In addition it is not pharmacologically induced and no neuronal loss has been described (Sabers *et al.*, 1996). The hallmark of absence seizures in GAERS is the appearance of bilaterally generalized SWDs in the electroencephalogram (EEG).
The goal of this study was to define the potential role of neurotrophins in generalized absence seizures with special regard to expression differences between GAERS and control animals as well as changes during maturation.
The role of brain-derived neurotrophic factor (BDNF) in epileptogenesis is controversial. Whereas Reibel et al. (2000b) observed that BDNF could inhibit the development of kindling-induced seizures, Scharfman et al. (2002) noted

spontaneous limbic seizures after intrahippocampal infusion of BDNF. The latter results were corroborated by conditional deletion experiments in the kindling model showing that tyrosine kinase receptor B (TrkB) deficiency in hippocampus blocks kindling (He *et al.*, 2004). TrkB is activated during epileptogenesis and it may be indispensable for the aggravating effect of BDNF (Binder *et al.*, 1999; He *et al.*, 2002). Controversial data were potentially reconciled by a study of Xu et al. (2004) showing that continuous infusion of BDNF downregulates TrkB receptors leading to an inhibitory effect on epileptogenesis, whereas bolus injection of BDNF does not alter TrkB expression and exerts epileptogenic effects.

BDNF has also been reported to regulate the expression of the transcription factor phosphorylated cAMP response element binding protein (pCREB, Wahlin *et al.*, 2000) and the potent endogenous antiepileptic transmitter neuropeptide Y (NPY, Nawa *et al.*, 1994; Reibel *et al.*, 2000a; Reibel *et al.*, 2003).

Juvenile GAERS younger than 30 days fail to show SWDs (Vergnes *et al.*, 1986). With increasing age the number of animals with SWDs gradually increases and reaches 100% at the age of 3 months. Comparing juvenile and adult GAERS and age-matched non-epileptic controls (NEC) allows to test whether alterations in the cerebral BDNF signalling cascade during brain development contribute to the occurrence of SWDs in GAERS by influencing NPY expression (Fig. 1). We hypothesize that either, BDNF binding on TrkB receptor results in phosphorylation of the receptor inducing the recruitment of a series of signalling proteins and finally leading to CREB phosphorylation, or BDNF directly phosphorylates CREB, known to act as transcription factor for NPY, or BDNF directly induces NPY expression.

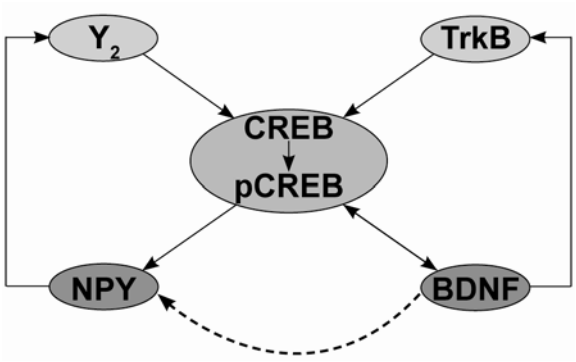

Figure 1. A summary of the signal cascade by which interaction between BDNF and NPY counteracts epileptogenesis. *BDNF binding to TrkB stimulates receptor phosphorylation, resulting in the recruitment of a series of signalling proteins to docking sites on the receptor, finally leading to CREB phosphorylation. This transcription factor in turn regulates the expression of NPY and BDNF. NPY via stimulation of NPY-receptors (probably Y2 receptors) is also competent to activate CREB by phosphorylation. Additionally, BDNF might phosphorylate CREB without binding to TrkB and/or directly induce NPY expression.*

Materials and methods

General chemicals used were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland) unless otherwise stated.

Animals
We performed our studies in juvenile rats at the age of 28-30 days and in adult rats older than 3 months. To avoid influence of cyclic changes in female sex hormones on BDNF and NPY expression (Scharfman & MacLusky, 2006), only male adult animals were used in these experiments. NEC and GAERS are from the original colony from Strasbourg and were kindly provided by Prof. C. Marescaux (University of Strasbourg, France). They were maintained on an ad libitum feeding schedule, housed on a 12h on/12h off light cycle and kept for at least one week on site before being used in experiments. All animal tests performed were in accordance with the guidelines of the Swiss Federal Veterinary Office.

Dissection of brain regions for biochemical analysis
The respiratory centre of animals was paralyzed through saturation with CO_2 (Carbagas, Basel, Switzerland). The brain was removed from the skull, dissected and brain regions (thalamus, hippocampus, and dorsolateral cortex, comprising mainly the sensorimotor cortex) were separated under a dissecting microscope (SZ-PT, Olympus, Tokyo, Japan), immediately frozen in liquid nitrogen and stored at -80°C until further use.

BDNF, proBDNF, $p75^{NTR}$ and pCREB protein determination by Western blot analysis
Individual samples were homogenized by sonication in 500µl, 300µl or 200µl RIPA-buffer (NaCl 150mM, Nonidet P-40 1%, Deoxycholate 0.5%, SDS 0.1%, Tris-HCl 50mM (pH 8.72), complete EDTA-free protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN, USA), $Na_4P_2O_7$ 20mM, Na_3VO_4 2mM, NaF 1mM (Merck, Darmstadt, Germany)). Afterwards, samples were purified by centrifugation for 1min at 16'000xg and for 20min at 50'000xg. Supernatants were further analyzed. Protein concentrations were determined using a Coomassie dye-based assay (BioRad, München, Germany). 25µg or 50µg of total protein content of homogenates were resolved on Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) by electrophoresis at 90V for 2h. Proteins were then transferred to Hybond® ECL® Nitrocellulose membranes (Amersham Pharmacia Biotech, Uppsala, Sweden) for 1h at 30V. For immunostaining, membranes were blocked at room temperature in 5% skim milk powder in phosphate buffered saline with 0.1% Tween-20 (Acros Organics, New Jersey, NJ, USA) for 30min. Incubation with primary antibodies was performed overnight at 4°C in 5% skim milk powder solution with the following dilutions: anti-BDNF 1:2'000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-proBDNF 1:2'000 (Alomone Labs, Jerusalem, Israel), anti-$p75^{NTR}$ 1:10'000 (kindly provided by Prof. Y.A. Barde, University of Basel, Switzerland), anti-pCREB 1:1'000 and anti-CREB 1:4'000 (both from Cell Signaling Technology, Beverly, MA, USA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 1:10'000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as housekeeping-protein for normalization of protein loading. After three 5min washes, blots were incubated for 1h at room temperature with the corresponding IgG-HRP-conjugated secondary

antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 5% skim milk powder solution. Signal intensities were determined using a chemiluminescence system (Roche Diagnostics, Indianapolis, IN, USA) and light emission was absorbed using Kodak Biomax XAR films. Images were captured in a MultiImage® Light Cabinet and quantified with FluorChem 8000 software (both from Alpha Innotech Corporation, Randburg, SA, USA). After subtraction of local background, pixel values of specific proteins were normalized to GAPDH values for each sample.

BDNF, TrkB and NPY mRNA determination by quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was prepared from rat dorsolateral cortex, hippocampus and thalamus using Trizol® Reagent (Invitrogen, Carlsbad, CA, USA) and purified by DNase treatment (Promega, Madison, WI, USA) according to the manufacturer's instructions. RNA quality was measured by means of agarose gel electrophoresis. First-strand cDNA synthesis was performed using a SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction with 2µg of total RNA, random hexamers, dNTPs and Superscript™ II reverse transcriptase. Reaction mixtures were subjected to PCR amplification using primers specific for BDNF, TrkB, NPY and β-actin (QuantiTect® Primer Assay (Qiagen, Hilden, Germany)) and Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) as fluorescent dye. PCR amplifications were performed in wells of the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the following conditions: 2min at 50°C, 10min at 95°C, followed by 40 cycles consisting of 15s at 95°C and 1min at 60°C. Melting curve analyses of PCR products were performed after each experiment to detect non-specific amplifications. Negative controls were transcribed both by omitting reverse transcriptase or RNA.

Quantification of RT-PCR results was performed using the comparative C_t method. Fluorescence signal intensities were plotted against the number of PCR cycles on a semi-logarithmic scale using the ABI Prism 7000 SDS software. A threshold cycle (C_t) was designated as the amplification cycle at which the first significant increase in fluorescence occurred. The C_t value of each sample was compared with that of β-actin as internal standard: $\Delta C_t = C_t(\text{target gene}) - C_t(\beta\text{-actin})$. In a second step, samples of GAERS were compared to those of age-matched NEC: $\Delta\Delta C_t = \Delta C_t(\text{GAERS}) - \Delta C_t(\text{NEC})$. The change of expression of the target gene in GAERS was calculated by the term $2^{-\Delta\Delta Ct}$.

BDNF, pCREB and NPY immunohistochemistry

Rats were deeply anesthetized with 0.5ml (juveniles) or 0.6ml (adults) Vetanarcol® (Veterinaria AG, Zürich, Switzerland; diluted 1:4 in NaCl 0.9%) and subsequently perfused through the ascending aorta with physiological saline followed by a phosphate buffered solution containing 0.2% glutaraldehyde and 4% paraformaldehyde for 10min and 4% paraformaldehyde in phosphate buffer for another 20min. 40µm thick sagittal sections were cut with a vibratome (Microtome HM 650V, Microme GmbH, Walldorf, Germany) through the whole brain. Free-floating sections were treated with 1% sodium borohydride in 0.05M tris buffered saline for 10min and 0.4% Triton X-100 in phosphate buffered saline for 90min at room temperature (both Merck, Darmstadt, Germany). Immunostaining was

performed using primary antibodies against BDNF (1:250, Santa Cruz Biotechnology, Santa Cruz, CA, USA), the phosphorylated form of CREB (1:100, Cell Signaling Technology, Beverly, MA, USA) and NPY (1:2'000, ImmunoStar Inc., Hudson, WI, USA) with the avidin-biotin-immunoperoxidase method. Primary antibodies were incubated in blocking solutions containing 5% normal goat serum (Vector Laboratories Inc., Burlingame, CA, USA) for 72h at room temperature. Incubation with goat biotinylated anti-rabbit IgG (H&L, 1:100; Antibodies Incorporated, Davis, CA, USA) took place for 2h and with ABC reagent (ABComplex/HRP, Dako Cytomation, Glostrup, Denmark; solutions A and B diluted 1:100 in phosphate buffered saline) for 90min at room temperature. Tissue-bound peroxidase was then developed using 0.03% H_2O_2 and 0.05% diaminobenzidine (DAB, Acros Organics, New Jersey, NJ, USA) as chromogen resulting in a brown (DAB in tris buffered saline, pH 7.4) or purple (DAB nickel in 0.1% $NiSO_4$ solution (Merck, Darmstadt, Germany), pH 8.6) staining, respectively. Sections were mounted on gelatinized slides, dried overnight at 37°C, completely dehydrated in 50% - 70% - 90% - 94% - 100% ethanol (Synopharm, Barsbüttel, Germany) solutions and cleared in xylol (Merck, Darmstadt, Germany). Finally, they were coverslipped with Eukitt® quick-hardening mounting media.
As a general rule all sections were processed with identical concentrations of primary and secondary antibody solutions, DAB and hydrogen peroxide. Chromogen reaction was initially standardized under a microscope to define the length of incubation resulting in optimal cell staining with minimal background.
Double stainings were performed consecutively since the primary antibodies were derived from the same species. We used first DAB nickel as chromogen and after blocking remaining peroxidase activity with 10% methanol and 0.3% H_2O_2 in 0.05M tris buffered saline for 10min, the whole procedure was repeated using another primary antibody and DAB without enhancement as chromogen.

Morphometry
NPY positive neurons were counted in three sections per animal, using similar laterality for all rats. The interface between two sections was 560µm. Brain sections were observed at 4fold magnification using a Nikon Eclipse E800 microscope (Nikon AG, Egg, Switzerland) interfaced with a ProgRes $C14^{plus}$ camera and pictures taken using the image capture software ProgRes Capture Pro 2.5 (Jenoptik, Jena, Germany). The three hippocampal subfields dentate gyrus (DG), hippocampal cornu ammonis regions 1 and 3 (CA1 and CA3, respectively) as well as the part of the frontoparietal cortex directly overlying the hippocampus were manually encircled and the areas calculated. The absolute number of NPY-immunopositive neurons was counted in each brain region under 20fold magnification. Cell densities and mean values were calculated for each animal.

Functional studies: Electroencephalogram (EEG) measurement after intracerebroventricular (icv) application of BDNF
Adult GAERS (n=3) were anesthetized with Narcotane® (0.8-1.0%; Leciva, Praha, Czech Republic) and placed in a stereotaxic frame (KOPF® Instruments, Tujunga, CA, USA). For cortical EEG recordings, four stainless steel screws (1.5mm diameter) were bilaterally placed on the frontal (AP: +2mm) and parietal cortex (AP: -4mm) according to the atlas of Paxinos and Watson (1997). Reference and ground

electrodes were placed over the cerebellum. A stainless steel icv cannula was implanted into the right lateral ventricle (AP: -1.0mm, L: -1.5mm) and lowered to a depth of 3.2mm below the dura. Electrodes and cannula were held in place with dental cement (SR Triplex® Cold Polymer, Ivoclar Vivadent® Technical, Schaan, Liechtenstein).
EEG was recorded in freely-moving animals 2 to 21 days after the operation. Electrical signals were amplified by a Grass 8-10B EEG machine (Grass Instrument CO., Quincy, MA, USA). The recording bandwidth was from 0.05 Hz to 10 kHz. Analogue to digital conversion was performed using a CED 1401µ type AD converter and data captured using the Spike2 software (Cambridge Electronic Design Ltd, Cambridge, United Kingdom) at 5 kHz conversion rate. After a 10min habituation, a baseline recording was acquired over 60min. 2µl of human recombinant BDNF (0.5µg/µl in 0.9% NaCl; Bachem AG, Bubendorf, Switzerland) or vehicle (0.9% NaCl) were then slowly (within 2min) injected into the lateral ventricle using a hand-held Hamilton syringe (Microliter™ Syringe, Hamilton, Bonaduz, Switzerland). A post-injection EEG was recorded for 120min. Subsequent treatments were performed at the same time point at following days. Additional control experiments were performed by only handling the animal.
SWDs were detected visually in the EEG before and after injection. Criteria for SWDs were high-amplitude asymmetric synchronized spikes and slow waves with an amplitude of at least 3 times higher than the EEG delta wave activity, a frequency of 7-11Hz and a duration longer than 1s (Dedeurwaerdere *et al.*, 2005). Begin and end of each SWD were manually marked and the duration was measured. All high voltage activities shorter than 1s were excluded. Percentage of total duration of seizures for each 30min interval was evaluated. The results were expressed as mean difference from the vehicle injection ± standard deviation (SD).
After performing the functional studies, rats were deeply anesthetized with urethane (1.4g/kg) and perfused intracardially with physiological saline and 4% paraformaldehyde. The cannula tract was visualized by means of the Gallyas silver staining method (Gallyas *et al.*, 1993).

Statistics
Results were subjected to statistical analysis using Student's t-test. Differences were considered to be statistically significant at p-values <0.05 (*) and highly significant at p-values <0.01 (**). Western blot and qRT-PCR experiments were performed with a total of 10 NEC and 10 GAERS and repeated 3 times. Immunohistochemical analyses were accomplished with 6 pairs of juvenile and 3 pairs of adult rats. The value for each brain region of control animals was set equal to 100% and appropriate values of GAERS were calculated relative to it. Functional studies were performed in 3 adult rats.

Results

BDNF mRNA and protein levels are increased in GAERS

In juvenile GAERS, BDNF mRNA expression is highly elevated in cortex (160%; p<0.01) and in hippocampus (212%; p<0.01; Fig. 2A) as compared to age-matched NEC, while in adult GAERS differences in BDNF mRNA expression are less pronounced displaying significant higher levels up to 140% (p<0.01) predominantly in cortex (Fig. 2B). Differences in BDNF mRNA expression are absent in hippocampus of adult animals (Fig. 2B) and in thalamus of both, juvenile and adult GAERS (Figs. 2A, 2B).

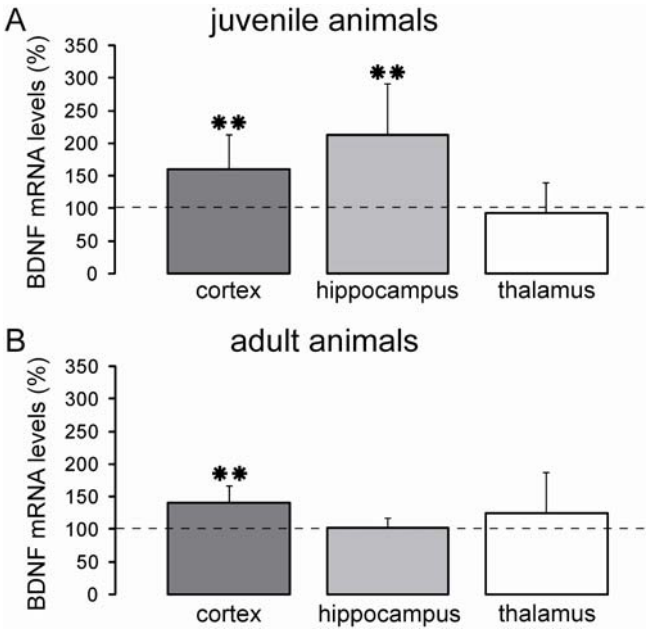

Figure 2. BDNF mRNA levels in cortex, hippocampus and thalamus of juvenile (A) and adult (B) GAERS as compared to age-matched NEC using qRT-PCR analyses. *Dashed line indicates level in the respective region of NEC set at 100%. Data show the mean ± SD. PCR runs were performed in quadruplicates and repeated three times per sample for 10 animals each (**p<0.01).*

Quite in contrast, cortical BDNF protein levels are significantly lower in juvenile GAERS (72%; p<0.01) as compared to NEC (Fig. 3A, left), whereas in hippocampus of juvenile GAERS significantly elevated BDNF levels are found (123%, p<0.01; Fig. 3A, right). In adult GAERS, increased BDNF levels are detected in both, cortex (121%; p<0.01; Fig. 3B, left) as well as hippocampus (119%; p<0.01; Fig. 3B, right) as compared to age-matched NEC. No differences in BDNF protein levels are observed in thalamus of juvenile or adult GAERS (data not shown).

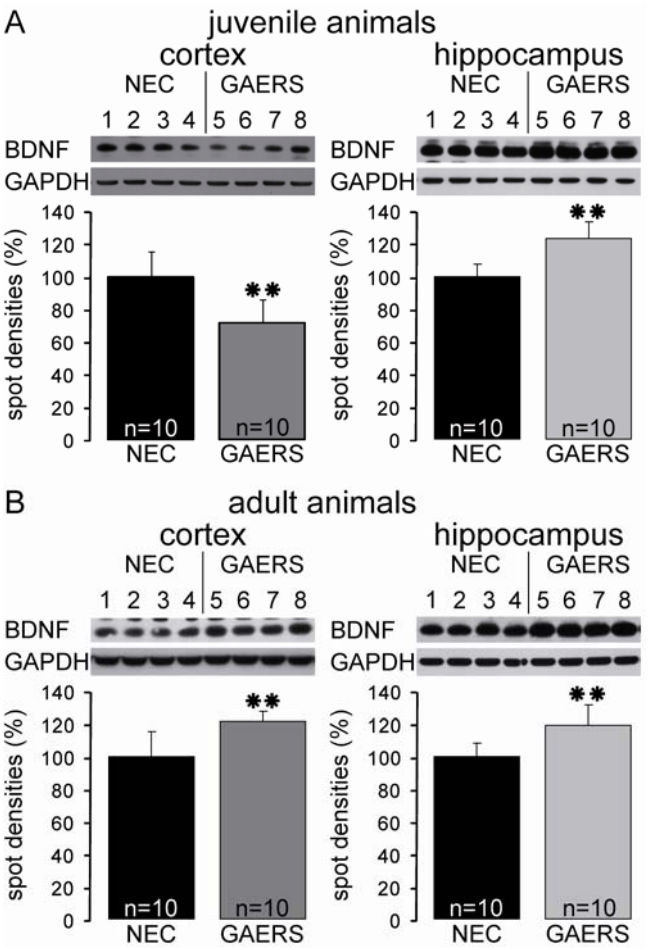

Figure 3. Western blot analysis of cortex (left) and hippocampus (right) homogenates derived from juvenile (A) and adult (B) NEC (1-4) and GAERS (5-8) immunoassayed with BDNF antibody. *Quantitative evaluation of BDNF levels is shown below. GAPDH immunoreactivity was used for normalization. Data are the mean ± SD of 10 animals per group. All assays were run in triplicates (**p<0.01 compared to age-matched NEC).*

The precursor form of BDNF (proBDNF) may itself have an independent biological activity. However, no changes in protein content of proBDNF are found in cortex, hippocampus and thalamus of both, juvenile and adult GAERS as compared to age-matched NEC (data not shown).

TrkB receptor mRNA is decreased in adult GAERS

Significant differences between juvenile and adult animals are found in BDNF's high affinity receptor TrkB. In adult GAERS TrkB mRNA expression is significantly reduced in both, cortex (83%; $p<0.01$) and hippocampus (69%; $p<0.01$), whereas no significant changes are found in thalamus (Fig. 4). However, in juvenile GAERS no significant differences in TrkB expression are detected in all three brain regions examined as compared to age-matched NEC (data not shown).

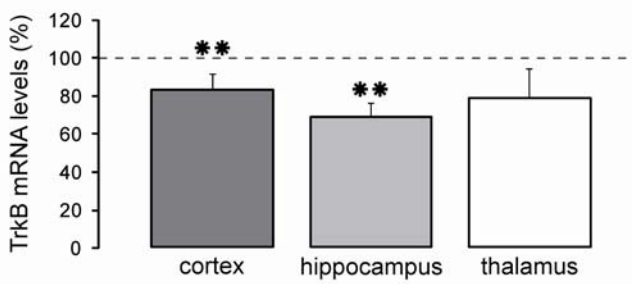

Figure 4. Expression levels of TrkB mRNA in cortex, hippocampus and thalamus of adult GAERS as compared to levels in age-matched NEC measured by qRT-PCR. *Dashed line indicates level in the respective region of NEC set at 100%. Data show the mean ± SD. PCR runs were performed in quadruplicates and repeated twice (n=10; **p<0.01).*

Western blot analyses of BDNF's low affinity receptor $p75^{NTR}$ reveal a significantly lower level only in cortex of juvenile GAERS (89%, $p<0.05$) as compared to age-matched NEC. Differences are neither observed in cortex of adult GAERS nor in hippocampus and thalamus of both, juvenile and adult GAERS as compared to NEC (data not shown).

Phosphorylation of CREB is reduced in adult GAERS

Several studies have shown that in neurons CREB phosphorylation follows TrkB activation by BDNF. Thus, it is of interest to investigate whether changes in CREB activation accompany alterations in receptor expression. CREB phosphorylation was examined using Western blot technique. In juvenile GAERS, no differences in the phosphorylation rate of CREB (pCREB) are found in cortex and hippocampus as compared to age-matched NEC, although total CREB expression is slightly elevated in cortex (111%; $p<0.05$) and hippocampus (131%; $p<0.05$; data not shown). No significant differences in CREB expression and phosphorylation are found in thalamus of juvenile GAERS as compared to age-matched NEC (data not shown).

In contrast to juvenile animals, CREB phosphorylation (pCREB) is markedly lower in adult GAERS. Phosphorylation rates are reduced to 49% in cortex, 39% in hippocampus and 68% in thalamus of adult GAERS as compared to age-matched NEC ($p<0.05$; Fig. 5). Expression of total CREB does not differ in all three examined brain regions between adult GAERS and NEC (data not shown).

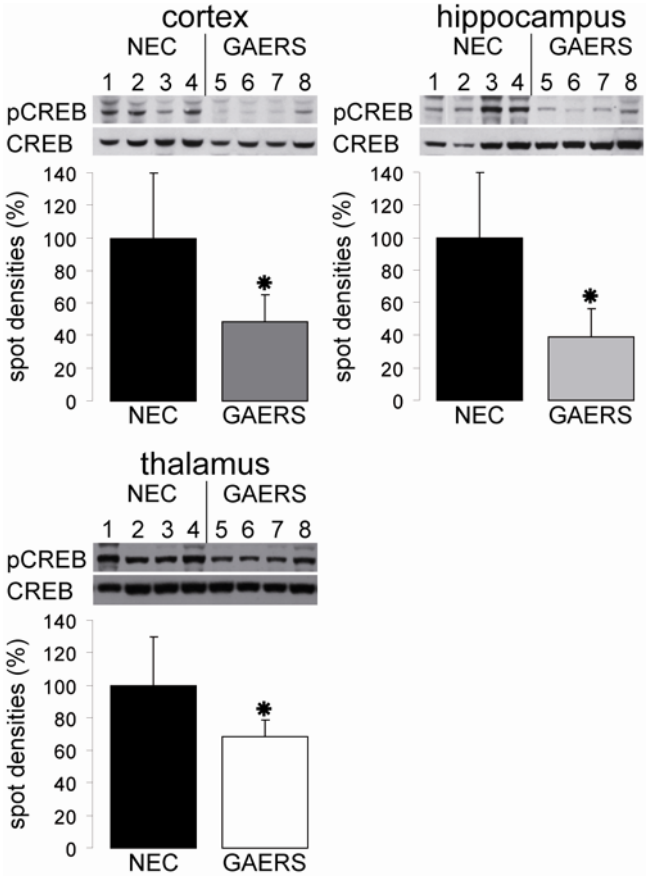

Figure 5. CREB phosphorylation rate in cortex, hippocampus and thalamus of adult GAERS analyzed by Western blot technique using pCREB (Ser133) antibody. *Quantitative evaluation of CREB phosphorylation is shown below. Total CREB protein expression was used for normalization. Data are the mean ± SD of 10 animals per group. All assays were run in triplicates (*p<0.05 compared to age-matched NEC).*

NPY mRNA is increased in cortex and hippocampus of GAERS

One of the consequences of the activation of the BDNF-TrkB system may be increased synthesis of neuropeptides including NPY. Therefore, expression of NPY was analyzed in various brain regions of GAERS. NPY mRNA expression is clearly higher in GAERS as compared to NEC (Fig. 6). In juvenile GAERS, NPY mRNA levels are elevated to 161% in cortex and 227% in hippocampus as compared to age-matched NEC (Fig. 6A; $p<0.01$). Similarly, in adult GAERS NPY mRNA expression is increased to 175% in cortex and 189% in hippocampus (Fig. 6B; $p<0.01$). No significant changes are found in thalamus of juvenile and adult animals.

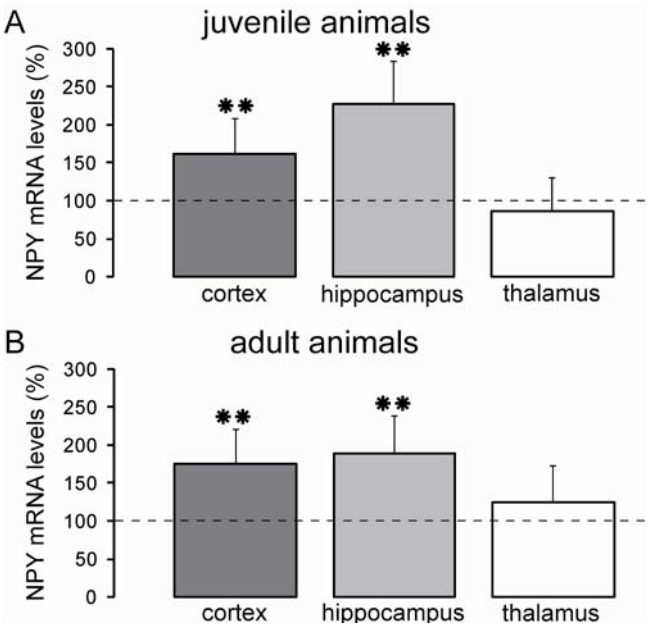

Figure 6. NPY mRNA expression in cortex, hippocampus and thalamus of juvenile (A) and adult (B) GAERS as compared to appropriate levels in age-matched NEC. *Dashed line indicates level in the respective region of NEC set at 100%. Data show the mean ± SD. PCR runs were performed in quadruplicates and repeated three times (n=10; **p<0.01).*

BDNF, pCREB and NPY immunohistochemistry

Morphological investigations were concentrated on frontoparietal cortex, hippocampus and thalamus in order to further verify the biochemical results.

Controversial data about the BDNF distribution in brain were published probably due to immunolabelling with different antibodies (Dugich-Djordjevic *et al.*, 1995; Conner *et al.*, 1997; Fusco *et al.*, 2003). BDNF distribution found in our experiments is in accordance with results of Fusco et al. (2003); it is localized throughout all cortical layers and in almost all subpopulations of hippocampal neurons (Fig. 7A). The distribution in cortex and hippocampus suggests a predominant localization in the cytoplasm of cell bodies and less in fibres or axon terminals. There is a tendency for less pronounced staining in adult rats. While no differences in perikaryal BDNF expression occur between NEC and GAERS, there is an increased staining of BDNF-positive nerve fibres in cortex and hippocampus of GAERS which is more common in juvenile than in adult animals (Figs. 7A1, 7A2, respectively). Stained nerve fibres occur in particular in hippocampal stratum lacunosum-moleculare of CA3.

Phosphorylation and therefore activation of CREB was investigated because this transcription factor might be involved in the NPY induction by BDNF. Staining for pCREB is exclusively found in the nuclei of nerve cells (Fig. 7B). pCREB staining in adult GAERS is fainter than in NEC. Differences are most pronounced in hippocampal CA1 and CA3 region, but are also detectable in DG and thalamus. Only in cortex, no clear differences are visible (Fig. 7B).

In frontoparietal cortex, NPY-immunopositive cells are distributed throughout all cortical layers. Density of NPY-positive cells is higher in cortex of GAERS as compared to age-matched NEC in juvenile as well as adult animals (Fig. 7C). In

hippocampus, density of NPY-positive cells is highest in DG and CA1, whereas fewer cells are detected in CA3. In DG, most of NPY-positive neurons are found in the hilus, occasionally at the inner margin of the granule cell layer.

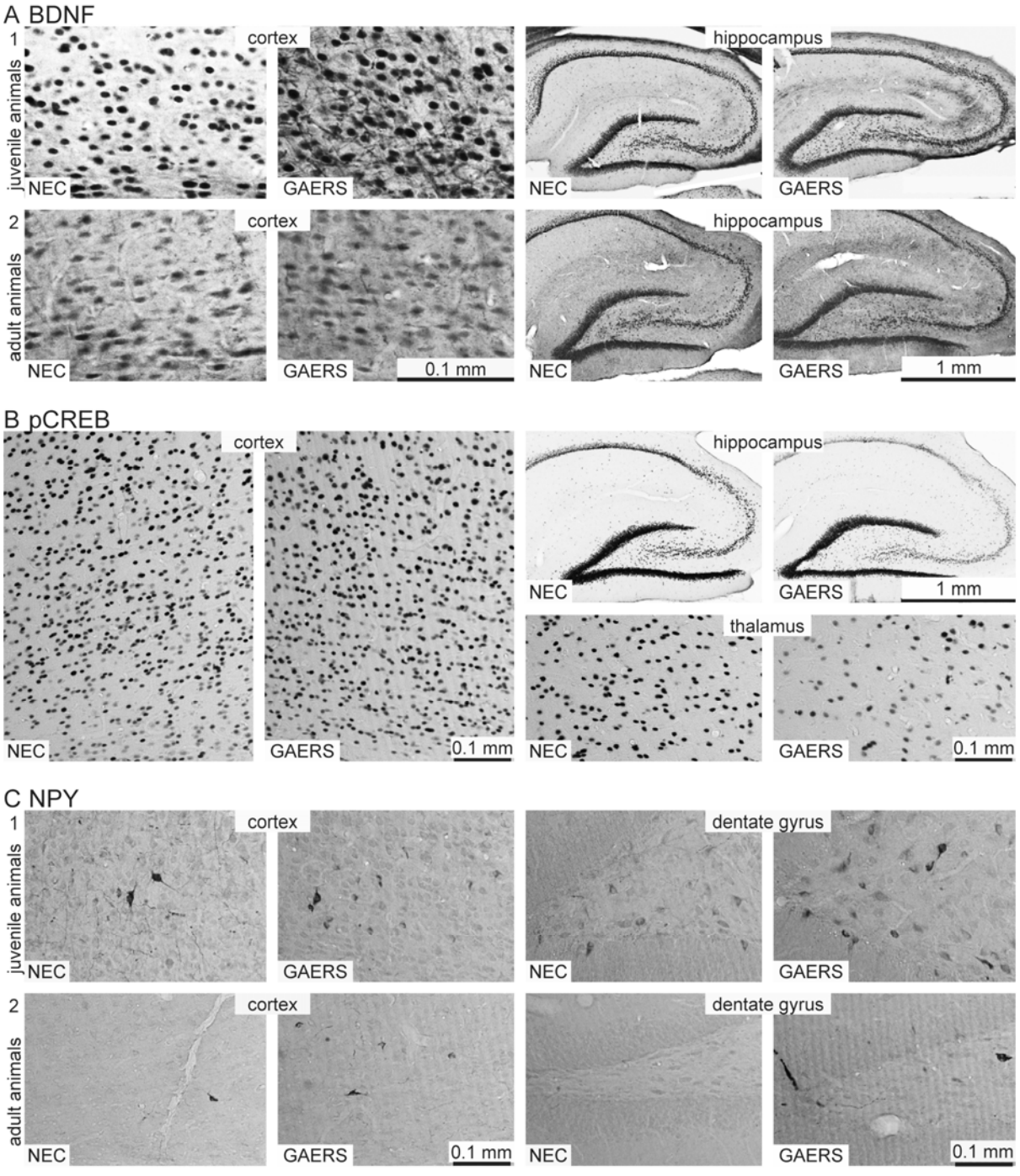

Figure 7. Visualization of BDNF, pCREB and NPY expression differences performed with immunohistochemistry. *(A) Comparison of BDNF expression in cortex and hippocampus of juvenile (1) and adult (2) NEC and GAERS. Differences in BDNF expression in cell bodies are barely detectable, whereas in both brain regions, more BDNF-immunostained nerve fibres are detected in juvenile and adult GAERS compared to age-matched NEC. (B) Phosphorylation of CREB in cortex, hippocampus and thalamus of adult NEC and GAERS. A reduction of pCREB*

immunoreactivity is detectable in all three hippocampal subregions and thalamus of adult GAERS as compared to NEC. (C) Immunohistochemistry of NPY-positive cells in cortex and dentate gyrus of juvenile (1) and adult (2) NEC and GAERS. The number of NPY-positive cells in cortex and DG of GAERS is markedly higher than in age-matched NEC. Note the distinct decline in NPY-immunoreactivity with maturation. Scale bar represents 0.1mm for cortex, thalamus and dentate gyrus or 1mm for hippocampus.

NPY-immunopositive neurons are more numerous in DG of juvenile GAERS than in age-matched NEC (Fig. 7C1). A smaller increase in expression levels is found in DG of adult GAERS (Fig. 7C2). Comparison of NPY-staining in juvenile and adult rats reveals a prominent reduction of NPY-immunoreactivity with maturation (Fig. 7C, Table 1). Density of NPY-immunoreactive neurons is markedly reduced in frontoparietal cortex and in all three hippocampal subregions examined of adult GAERS as compared to juvenile GAERS. The number of NPY-immunopositive cells in adult GAERS is only half of the levels measured in juvenile GAERS ($p<0.01$ for all brain regions; Table 1).

Table 1. Number of NPY-positive cell counts in frontoparietal cortex, DG, CA3 and CA1 of juvenile and adult GAERS expressed as cells/mm^2. *The data shown are the mean ± SD (n=6 for juvenile and n=3 for adult GAERS; **p< 0.01 compared to juvenile GAERS).*

	cortex	DG	CA3	CA1
juvenile	36.62 ± 6.16	68.20 ± 11.17	41.40 ± 10.97	61.32 ± 11.66
adult	15.67 ± 1.44**	14.35 ± 0.99**	15.40 ± 2.89**	20.98 ± 2.48**

To investigate whether BDNF, pCREB and NPY are expressed in the same neuron, colocalization studies were performed with DAB nickel for the first antigen resulting in a purple staining and with DAB without enhancement for the second antigen leading to a brown coloration (Fig. 8).
BDNF-positive neurons (brown) are very common throughout the whole cortical and hippocampal structures (see also Fig. 7A), while NPY-positive neurons (purple) are very rare. Notably, most of the NPY-expressing cells are also BDNF-immunopositive (Fig. 8A; arrowheads) in cortex and hippocampal CA1 and DG regions. Only a few NPY-positive cells do not express BDNF (Fig. 8A; arrows).
Three different colours are visible in double stainings for pCREB and BDNF (Fig. 8B). Brown cells express only BDNF (open arrowheads), purple cells are selective for pCREB (arrowheads) and a coexpression of pCREB and BDNF occurs in black cells (arrows). Less than half of all neurons are positive for both, pCREB and BDNF. Remaining neurons stain either only for pCREB or BDNF.
NPY-positive neurons (purple) in cortex and hippocampus of GAERS express generally pCREB (Fig. 8C; arrowheads). This colocalization is even more common than the coexpression of NPY and BDNF. Only very few NPY-expressing cells are not positive for pCREB (arrows). Taken together, the vast majority of NPY-containing neurons also express BDNF and pCREB.

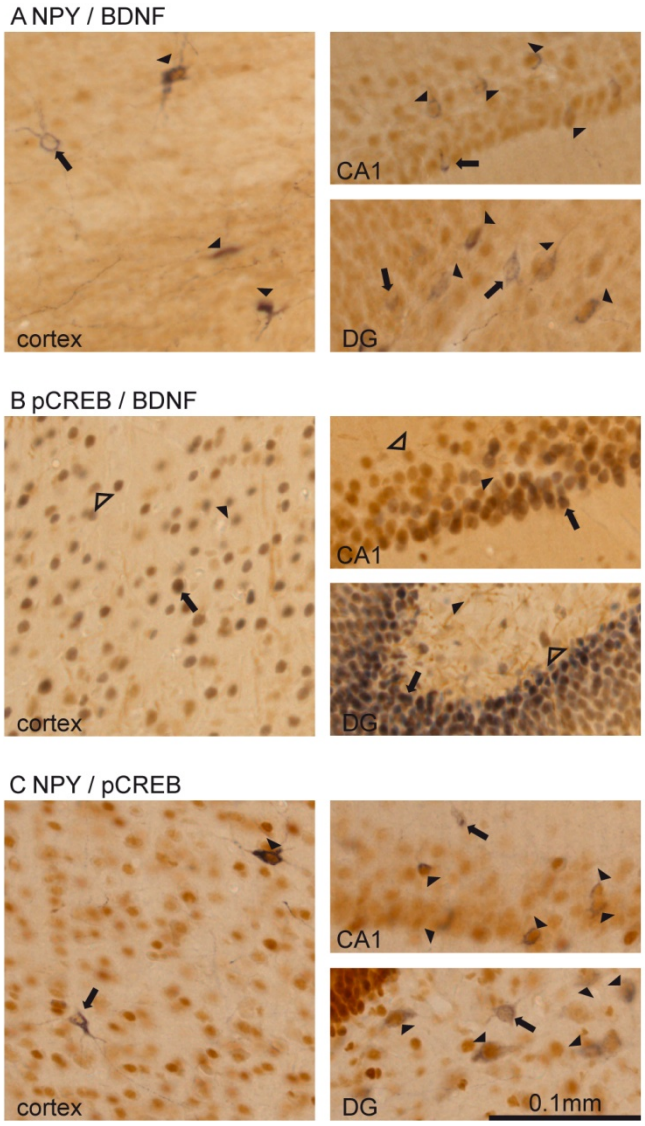

Figure 8. Colocalization of two proteins in cortex and hippocampal subregions of GAERS. *(A) In the majority of cases, NPY-positive neurons (purple colour) are also expressing BDNF (arrowheads) and only few neurons are present in CA1, DG and cortex which express NPY only (arrows). (B) Colocalization of pCREB and BDNF occurs in a small percentage of neurons (black colour; arrows). There are many neurons which express detectable levels of either only BDNF (brown colour; open arrowheads) or pCREB (purple colour; arrowheads). (3) NPY (purple colour) and pCREB (brown colour) are mostly colocalized in cortical or hippocampal neurons (arrowheads). There are only few neurons that express solely NPY (arrows). Black line indicates the distance of 0.1mm.*

Icv application of BDNF suppresses occurrence of SWDs

In order to verify whether changes in the BDNF-system are directly linked to the occurrence of SWDs, functional studies were performed by icv application of BDNF to adult GAERS. The typical morphology of SWDs – asymmetric spikes and slow waves with a frequency of 7Hz - is shown in Fig. 9 in a narrow (above) and broad (below) range. Icv injection of BDNF markedly reduces SWDs in adult GAERS. 60min after BDNF injection, the seizure-time is significantly reduced to 35% as compared to the seizure incidence after injection of the vehicle only. Occurrence of SWDs completely recovers within 120min after injection of BDNF (Fig. 9).

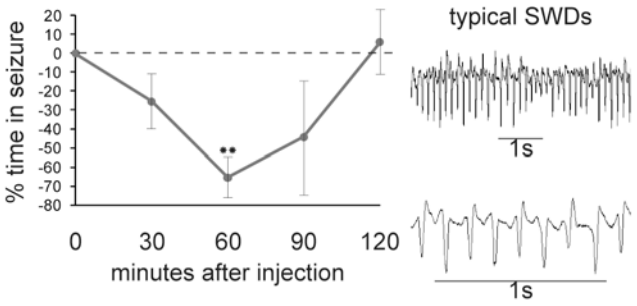

Figure 9. Time course showing the effect of icv injection of BDNF in adult GAERS. *Injection of BDNF into the lateral ventricle significantly decreases the percentage time spent in seizure 1 hour after application. Occurrence of SWDs completely recovers within 120min. Dashed line indicates percentage of time spent in seizures measured after injection of 0.9% NaCl. Data are the mean difference from the vehicle injection ± SD of experiments performed in 3 different adult GAERS (**p<0.01). The typical morphology of SWDs is shown in a narrow and broad range.*

Discussion

This study reveals marked differences in BDNF expression in GAERS as compared to NEC indicating that BDNF may contribute to the pathophysiology of absence epilepsy.

The BDNF system in GAERS

Our data show that the BDNF signalling-pathway is altered in GAERS and that enhanced neuronal activity in rats with generalized non-convulsive seizures (Wallengren *et al.*, 2005) is accompanied by increased BDNF expression. Juvenile GAERS express high BDNF levels, i.e. at an age at which SWDs are not yet present. BDNF-expression remains increased in adult GAERS. These characteristic high levels of the neurotrophin BDNF are found in cortex, but also in hippocampus, a brain structure that is primarily not involved in absence seizures. Thus, the BDNF system is altered in GAERS, but it may not be exclusively involved in the initiation of absence seizures.

Our results show discrepancies between BDNF protein and mRNA expression levels in the cortex of juvenile and in the hippocampus of adult GAERS. It should be stressed that there is no evidence for a tight correlation of BDNF protein

concentrations and mRNA levels in normal or epileptic brain tissues (Nawa *et al.*, 1995). Possible reasons for the discrepancy between BDNF mRNA and protein expression may be (1), that BDNF synthesis is regulated at a post-transcriptional level (Lipshitz & Smibert, 2000) and (2) that BDNF protein is transported from cell bodies of central and peripheral neurons to nerve endings and may share neurotransmitter-like functions in the brain (Altar *et al.*, 1997; Altar & DiStefano, 1998; Jacobsen & Mork, 2004).
Since it was not possible to demonstrate a direct correlation between changes in BDNF expression levels and the onset of SWDs, we extended our analyses to BDNF receptors. Adult GAERS display significant decreases in TrkB mRNA expression both in cortex and hippocampus, whereas no differences were found in juvenile rats. It is conceivable that long-time exposure to high levels of BDNF induces a down-regulation in TrkB expression or phosphorylation resulting in a loss of responsiveness to BDNF. This effect has been previously reported in cultured hippocampal and cortical neurons and in adult rat brain upon chronic exposure to BDNF (Sommerfeld *et al.*, 2000; Xu *et al.*, 2004). Such an adaptive mechanism requires time to establish and may not be fully operative in juvenile animals.

CREB and pCREB in GAERS
In addition to its role as a neurotrophic factor, BDNF has emerged as an important signalling molecule for the immature and adult nervous system. Recent studies characterizing intracellular signalling pathways have shown that BDNF induces activation of transcription factors, including CREB (Finkbeiner *et al.*, 1997; Blanquet *et al.*, 2003). CREB phosphorylation in turn regulates the expression of cAMP-inducible genes including those responsible for the synthesis of various neuropeptides, as e.g. NPY (Pandey, 2003; Wand, 2005). These molecular interactions are facilitated by the colocalization of these factors in identical neuron subpopulations as indicated by our immunohistochemical analysis. pCREB might be the molecular link between BDNF and NPY expression. Notably, most of the cortical and hippocampal neurons positive for NPY also express pCREB. The coexistence of BDNF and pCREB in the same neuron is predominant, but there are also neurons that either express detectable levels of only BDNF or only pCREB suggesting that also other transcription factors contribute to BDNF effects.
Interestingly, in adult GAERS the rate of CREB phosphorylation decreases dramatically as compared to NEC, whereas in juvenile GAERS CREB phosphorylation does not differ from that of age-matched NEC. The reduction of pCREB concentrations in adult GAERS coincides with the decrease of TrkB expression and the onset of SWDs, pointing to an important role of this transcription factor in the initiation and/or propagation of absence seizures. The precise relationship between pCREB and non-convulsive epileptic seizures, however, remains to be defined. It should be noted that pCREB initiates transcription of a variety of other proteins which may also be involved in seizure suppression.

NPY in GAERS
CREB modulates transcription of genes that encode NPY and its receptors (Chance *et al.*, 2000), supporting the notion that NPY expression is regulated by BDNF as well as by CREB. NPY functions as a potent endogenous antiepileptic compound in temporal lobe epilepsy (Vezzani *et al.*, 1999b) and experimental evidence indicates

that it is also implicated in absence seizures (Stroud *et al.*, 2005; Morris *et al.*, 2007). Our data show that NPY mRNA expression is significantly higher in cortex as well as in hippocampus of juvenile and adult GAERS as compared to age-matched NEC. Surprisingly, absence seizures occur in adult GAERS although brain concentrations of the endogenous anti-absence factor NPY are higher than in NEC but lower than in juvenile animals. It is conceivable that newborn GAERS suffer already from absence epilepsy, but that high levels of NPY prevent the occurrence of SWDs. The decrease in the number of NPY-expressing neurons in adulthood as shown by our immunohistochemical findings (many more NPY-positive cells in hippocampus and cortex of juvenile as compared to adult GAERS) would then cause a critical decline in the endogenous antiepileptic potential, resulting in the onset of SWDs.

In summary, we hypothesize that juvenile GAERS are protected against SWDs by high levels of NPY. The decrease in NPY expression with maturation allows the onset of SWDs. This might be influenced upstream by the chronic overexpression of BDNF in adult GAERS leading to a reduction in TrkB expression and CREB phosphorylation, which in turn results in the marked decrease in the density of NPY-positive cells during maturation.
Although BDNF expression is chronically elevated in adult GAERS, intracerebroventricular application of BDNF induces a marked reduction of SWDs. The time course appears slightly delayed to that observed after icv injection of NPY (Stroud *et al.*, 2005; Morris *et al.*, 2007) and strongly suggests that the anti-absence effects of BDNF are mediated at least in part via additional factors as, e.g., up-regulation of NPY expression. This assumption is corroborated by findings of Vezzani et al. (1999a) showing similar timing for increases in BDNF and NPY expression after acute seizures and Reibel et al. (2000a) reporting increases in NPY expression following BDNF-infusion. In addition, our current results indicate that the majority of NPY-positive neurons in the frontoparietal cortex and hippocampus contain BDNF. As reported before, the distribution of BDNF-immunoreactivity is in good accordance with that of NPY (Iritani *et al.*, 2000), but a direct colocalization has not yet been proven. Our results are supported by the fact that nitric oxide synthase expressing interneurons which are in the majority NPY-positive also contain BDNF (Fusco *et al.*, 2003). We assume that only BDNF neurons that simultaneously express NPY contribute to anticonvulsant effects.
Furthermore, we hypothesize that after bolus injection the BDNF-signalling cascade is distinct from the common mechanism via TrkB and CREB phosphorylation as discussed for chronic BDNF effects. Our results support the notion that suppression of SWDs after exogenous application of BDNF in adult GAERS is mediated by a direct induction of NPY (Fig. 1).

Absence seizures versus temporal lobe epilepsy
Epidemiological studies in patients have shown that temporal lobe epilepsy and non-convulsive generalized epilepsies rarely coexist (Koutroumanidis *et al.*, 1999; Sofue *et al.*, 2003; Nicolson *et al.*, 2004; Jeha *et al.*, 2006). This has also been observed in the GAERS model of absence epilepsy where induction of epileptogenesis by amygdala kindling or injection of kainic acid into the amygdaloid nucleus is impaired (Eskazan *et al.*, 2002; Gurbanova *et al.*, 2008). The resistance of GAERS against

limbic seizures may at least in part be explained by the decreased capacity of the BDNF-signalling cascade in adult rats.

In conclusion, this study shows that the BDNF-signalling cascade is dramatically impaired in adult GAERS leading to a marked decrease in the expression of the endogenous antiepileptic factor NPY. These dramatic changes come along with the onset of SWDs. Finally, exogenously applied BDNF markedly reduces the occurrence of SWDs in adult GAERS. We assume that this effect is at least partially mediated by NPY.

Acknowledgements

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3.2 Part II – Protective role of NPY against absence seizures

On the protective role of neuropeptide Y against spike-and-wave discharges in a genetic rat model of absence epilepsy

Authors

Svenja Landweer[1,3], Cordula Nitsch[2], Gabor Juhasz[3], Any Boehrer[4], Raymond Bernasconi[1] and Uwe Otten[1]

[1] Molecular Neurobiology, Department of Biomedicine, University of Basel, Pestalozzistrasse 20, CH-4056 Basel, Switzerland
[2] Functional Neuroanatomy, Department of Biomedicine, University of Basel, Pestalozzistrasse 20, CH-4056 Basel, Switzerland
[3] Neurobiology Research Group, Hungarian Academy of Sciences, Eötvös Lorand University, Pazmany Peter setany 1/C, H-1117 Budapest, Hungary
[4] INSERM U596, Faculty of Medicine, 11 rue Humann, F-67085 Strasbourg Cedex, France

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Abstract

Various studies have shown that neuropeptide Y (NPY) possesses anticonvulsant properties in models of temporal lobe epilepsy. In addition, NPY also suppresses spike-and-wave discharges in a genetic model of generalized non-convulsive epilepsy (GAERS).
Here we demonstrate that the density of NPY-expressing cells is increased in cortex and hippocampus of both, juvenile and adult GAERS as compared to age-matched non-epileptic controls. Additionally, NPY expression is more pronounced in juvenile than in adult GAERS, suggesting that occurrence of absence seizures correlates with a significant decrease in NPY synthesis. Moreover, the occurrence of spike-and-wave discharges in adult GAERS is impaired by intracerebroventricular application of NPY and a selective Y2 receptor agonist. In juvenile GAERS, onset of absences is facilitated by the treatment with specific NPY receptor antagonists.
These data strongly corroborate the involvement of NPY in the occurrence of absence seizures suggesting the NPY-system as a potential therapeutic target.

Introduction

Epilepsy remains a major health problem associated with recurrent spontaneous seizures and about 30% of the patients with epilepsy are refractory to drug therapy (Rang et al., 2007). The probability of intractability largely depends on the type of seizures with temporal lobe epilepsy (TLE) having the poorest prognosis of all seizure types in adults (Regesta and Tanganelli, 1999). A major obstacle in developing new strategies for treatment is that mechanisms of refractoriness are only poorly understood.
Recent studies have shown that the process of epileptogenesis, as induced by amygdala kindling (Eskazan et al., 2002) or intra-amygdaloid kainic acid injection (Gurbanova et al., 2008) is retarded in Genetic Absence Epilepsy Rats from Strasbourg (GAERS). These two studies demonstrate that GAERS, a well validated animal models for human absence epilepsy (Depaulis and Van Luijtelaar, 2005), are resistant to secondary generalization of focal limbic seizures. In this polygenic model (Rudolf et al., 2004) the duration of spike-and-wave discharges (SWDs) correlates negatively with kindling rates strongly suggesting an interplay between limbic and thalamo-cortical mechanisms (Onat et al., 2007). Kainic acid or kindling models in GAERS provide an opportunity for studying the mutual exclusivity of TLE and idiopathic generalized epilepsy as observed in the clinical situation (Jeha et al., 2006; Koutroumanidis et al., 1999; Nicolson et al., 2004; Sofue et al., 2003).
Many studies have implicated neuropeptide Y (NPY) in the control of limbic seizures. NPY immunoreactivity increases in epileptic animals (Nadler et al., 2007) and kainate or kindling induced seizures are reduced and seizure onset markedly delayed in rats overexpressing NPY (Richichi et al., 2004; Vezzani et al., 2002), whereas NPY-deficient mice are more susceptible to seizures (Baraban et al., 1997; Shannon and Yang, 2004). Moreover, exogenously applied NPY functions as potent antiepileptic compound in kindling and kainate models (Reibel et al., 2003; Woldbye et al., 1997). On the other hand, in absence seizures, too, NPY and its receptors play a central role (Morris et al., 2007; Stroud et al., 2005). Also, the role of NPY receptor subtypes

in seizures is controversial. Benmaamar et al. (2003) found that down-regulation of hippocampal Y1 receptors attenuates kindling, while Brill et al. (2007) reported that activation of Y1 suppresses thalamic oscillations. In contrast, stimulation of Y2 receptors is necessary and sufficient to control limbic seizures (El Bahh et al., 2005; Nadler et al., 2007; Vezzani and Sperk, 2004). Finally, Y5 receptor agonists may constitute a novel group of drugs in antiepileptic therapy (Baraban, 2002; Woldbye et al., 1997).
Goal of this study is to investigate the potential role of NPY and its receptors in generalized non-convulsive epilepsy using juvenile and adult GAERS with regard to the resistance to secondary generalization of focal limbic seizures. Juvenile GAERS express not yet typical SWDs. Short irregular spike-and-wave discharges (SISWDs) appear around postnatal day 30 and disclose various shapes with a typical frequency of 5-7Hz (Dedeurwaerdere et al., 2005). With increasing age the number of animals with typical SWDs gradually increases and reaches 100% at the age of 3 months (Vergnes et al., 1986). First, we determined the number of NPY-positive cells of juvenile and adult GAERS in several brain regions as compared to age-matched non-epileptic controls (NEC). We also measured protein levels of Y2 receptors and tested whether absence seizures induce hypertrophy of the hilar area. Finally in direct functional experiments, we studied the effects of intracerebroventricular (icv) injection of NPY receptor agonists and antagonists on SWDs.

Materials and Methods

General chemicals used were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland) unless otherwise stated.

Animals
NEC and GAERS originating from the Strasbourg breeding colony were used for this study. The GAERS strain was selected by inbreeding Wistar rats with spontaneously occurring electroencephalographic SWDs characteristic for absence seizures (Danober et al., 1998). Seizures start to appear within 1 month-old animals, increase with age and are present in 100% of the rats after 3 months. NEC were normal outbred Wistar rats without spontaneous absence seizures. The experiments were performed in juvenile rats aged 26-30 days and in adult rats older than 3 months. To avoid influence of cyclic changes in female sex hormones (Veliskova and Velisek, 2007), only male adult animals were used. Animals were maintained on an ad libitum feeding schedule, housed up to five animals in one cage and kept on a 12h on/12h off light cycle. They were kept for at least one week on site before being used in experiments. All animal tests performed were in accordance with the guidelines of the Swiss Federal Veterinary Office.

Quantification of NPY-immunopositive cells by means of immunohistochemistry
Rats were deeply anesthetized with 0.5 ml (juvenile) or 0.6 ml (adults) Vetanarcol® (Veterinaria AG, Zürich, Switzerland; diluted 1:4 in NaCl 0.9%) and subsequently perfused through the ascending aorta with physiological saline followed by a phosphate buffered solution containing 0.2% glutaraldehyde and 4% paraformaldehyde for 10min and 4% paraformaldehyde for another 20min. Serial

40µm thick sagittal sections were cut (Microtome HM 650V, Microme GmbH, Walldorf, Germany). Free-floating sections were treated with 1% sodium borohydride in 0.05M tris buffered saline for 10min and 0.4% Triton X-100 in phosphate buffered saline for 90min at room temperature (both Merck, Darmstadt, Germany). Immunostaining was performed using a primary antibody against NPY (1:2'000, ImmunoStar Inc., Hudson, WI) with the avidin-biotin-immunoperoxidase method. Primary antibody was incubated in a blocking solution containing 5% normal goat serum (Vector Laboratories Inc., Burlingame, CA) for 72 h at room temperature. Incubation with goat anti-rabbit IgG (H&L) Biotin (1:100; Antibodies Incorporated, Davis, CA) took place for 2 h and with ABC reagent (ABComplex/HRP, Dako Cytomation, Glostrup, Denmark; solutions A and B 1:100 diluted in phosphate buffered saline) for 90 min at room temperature. Tissue-bound peroxidase was then developed using 0.03% H_2O_2 and 0.05% diaminobenzidine (Acros Organics, New Jersey, NJ) as chromogen resulting in a brown staining. Chromogen reaction was initially standardized under a microscope to define the length of incubation resulting in optimal cell staining with minimal background. Sections were mounted on gelatinized slides, dried overnight at 37°C, completely dehydrated, cleared in xylol (Merck, Darmstadt, Germany) and finally coverslipped with Eukitt® quick-hardening mounting media.
NPY-positive neurons were counted in three sections per animal, using similar lateralities for all rats. The interface between two sections was 560µm. Brain sections were evaluated using a Nikon Eclipse E800 microscope (Nikon AG, Egg, Switzerland) interfaced with a ProgRes C14^{plus} camera and pictures taken using the image capture software ProgRes Capture Pro 2.5 (Jenoptik, Jena, Germany). The three hippocampal subfields dentate gyrus (DG), hippocampal cornu ammonis regions 1 and 3 (CA1 and CA3, respectively) as well as the part of the frontoparietal cortex directly overlying the hippocampus and striatum were manually encircled and areas calculated. The absolute number of NPY-immunopositive neurons was counted in each brain region. Cell densities and mean values were calculated for each animal.

Measurement of hilar area
Every 7th section per animal was stained with cresyl violet to obtain a general estimate of the status of tissue preservation. Dried brain sections mounted on glass slides were incubated at room temperature as follows: 10min in 0.4% acetic acid (Merck, Darmstadt, Germany), 10min in cresyl violet solution (0.2% cresyl violet acetate, 0.02% sodium acetate-3-hydrate, 0.2% acetic acid, pH 3.5), another 10min in 0.4% acetic acid and shortly rinsed with bidistilled water. Then, sections were dehydrated for 5 min in 50% and 70% ethanol (Synopharm, Barsbüttel, Germany), decolored for 20min in 4% acetic acid in ethanol, followed by 5min in pure ethanol and finally cleared in xylol.
Hilar area of each animal was measured in four different brain slices with similar laterality and mean values were calculated. The hilus is a part of DG and represents the area within the inner edge of granule cell layer and the lines connecting the tips of the two granule cell blades to the end of CA3 pyramidal cell layer.

Sectioning of different brain regions for biochemical analyses
The respiratory centre of animals was paralyzed through saturation with CO_2 (Carbagas, Basel, Switzerland). The brain was removed from the skull, dissected and brain regions (thalamus, hippocampus and dorsolateral cortex, comprising mainly the sensorimotor cortex) were separated under a dissecting microscope (SZ-PT, Olympus, Tokyo, Japan), immediately frozen in liquid nitrogen and stored at -80°C until further use.

Y2 receptor protein determination by Western blot analysis
Individual samples were homogenized by sonication in 500µl, 300µl or 200µl RIPA-buffer (NaCl 150mM, Nonidet P-40 1%, deoxycholate 0.5%, SDS 0.1%, Tris-HCl 50mM (pH 8.72), complete EDTA-free protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN), $Na_4P_2O_7$ 20mM, Na_3VO_4 2mM, NaF 1mM (Merck, Darmstadt, Germany)). Afterwards, samples were purified by centrifugation at 16'000xg for 1min and at 50'000xg for 20min. Protein concentrations in supernatants were determined using a Coomassie dye-based assay (BioRad, München, Germany). Homogenates containing 50µg of total protein were resolved on 10% Bis-Tris gels with MOPS running buffer (both from Invitrogen, Carlsbad, CA) by electrophoresis at 90V for 2h. Proteins were then transferred to Hybond™-P PVDF membranes (Amersham Pharmacia Biotech, Uppsala, Sweden) for 1h at 30V. For immunostaining, membranes were blocked at room temperature for 30min in 5% skim milk powder in phosphate buffered saline with 0.1% Tween-20 (Acros Organics, New Jersey, NJ). Incubation with NPY2R (L-17) primary antibody (Santa Cruz Biotechnolgy, Santa Cruz, CA) was performed overnight at 4°C diluted 1:2'000 in 5% skim milk powder solution. Anti-glyceraldehyd-3-phosphate dehydrogenase (GAPDH, 1:10'000, Santa Cruz Biotechnology, Santa Cruz, CA) was used as housekeeping-protein for normalization of protein loading. After three 5min washes, blots were incubated for 1h at room temperature with bovine anti-goat IgG-HRP-conjugated secondary antibodies diluted 1:2'000 or goat anti-mouse IgG-HRP 1:10'000 (both from Santa Cruz Biotechnology, Santa Cruz, CA) in 5% skim milk powder solution for NPY2R and GAPDH, respectively. Signal intensities were determined using a chemiluminescence system (Roche Diagnostics, Indianapolis, IN) and light emission was absorbed using Kodak Biomax XAR films. Images were captured in a MultiImage™ Light Cabinet and quantified with FluorChem 8000 software (both from Alpha Innotech Corporation, Randburg, SA). After subtraction of local background, pixel values of specific proteins were normalized to GAPDH values for each sample.

Functional studies: Electroencephalogram (EEG) measurement after intracerebroventricular (icv) application of NPY, NPY receptor agonists and antagonists
Adult (n=4) and juvenile GAERS (n=6) were anesthetized by inhaling Narcotane® (0.8-1.0%; Leciva, Praha, Czech Republic) and placed in a stereotaxic frame (KOPF® Instruments, Tujunga, CA). For cortical EEG recordings, four stainless steel screws (1.5mm and 0.8mm diameter for adult and juvenile animals, respectively) were bilaterally placed on the frontal (AP: +2mm) and parietal cortex (AP: -4mm) according to the atlas of Paxinos and Watson (1997). Reference and ground electrodes were placed over the cerebellum. A stainless steel icv cannula was implanted into the right lateral ventricle (AP: -1.0mm, L: -1.5mm, depth: -3.2mm for adult animals and AP: -

0.9mm, L: -0.6mm; depth -3.5mm for juvenile animals). Electrodes and cannula were held in place by dental cement (SR Triplex® Cold Polymer, Ivoclar Vivadent® Technical, Schaan, Liechtenstein).
EEG was recorded in freely-moving animals 2 to 21 days after the operation. Electrical signals were amplified by a Grass 8-10B EEG machine (Grass Instrument CO., Quincy, MA). The recording bandwidth was from 0.05Hz to 10kHz. Analogue to digital conversion was performed using a CED 1401µ type AD converter and data captured using the Spike2 software (Cambridge Electronic Design Ltd, Cambridge, United Kingdom) at 5kHz conversion rate. After 10min habituation, a baseline recording was acquired over 60min. 2µl of various drugs were then slowly (within 2min) injected into the lateral ventricle using a hand-held Hamilton syringe (Microliter™ Syringe, Hamilton, Bonaduz, Switzerland). A post-injection EEG was recorded for 120min. Subsequent treatments were performed at the same time point at following days.
SWDs and SISWDs were detected visually in the EEG before and after injection. Criteria for SWDs in adult GAERS were high-amplitude asymmetric synchronized spikes and slow waves with an amplitude of at least 3 times higher than the EEG delta wave activity, a frequency of 7-11Hz and a duration longer than 1s. SISWDs observed in juvenile animals disclosed variable discharges with spike-and-wave aspects. Characteristics are lower amplitude, frequency of 5-7Hz and duration longer than 0.5s (Dedeurwaerdere et al., 2005). Start and end of each discharge were manually marked and the duration was measured as described (Stroud et al., 2005). Percentage of total duration spent in seizure for each 30min interval was evaluated. The results were expressed as mean difference from the vehicle injection.
After performing the functional studies, rats were deeply anesthetized with urethane (1.4g/kg) and perfused intracardially with physiological saline and 4% paraformaldehyde in phosphate buffer. The cannula tract was visualized by means of Gallyas silver staining method (Gallyas et al., 1993).

Drugs injected for functional studies
In one set of experiments, adult GAERS received 2µl icv injections of NPY (Neuropeptide Y (human, rat) trifluoroacetate salt, Bachem AG, Bubendorf, Switzerland; 1.5mM in 0.9% NaCl), Y2 receptor agonist (Acetyl-(Leu^{28,31})-Neuropeptide Y (24-36), Bachem AG, Bubendorf, Switzerland; 1.5mM in 0.9% NaCl) and Y2 receptor antagonist (BIIE0246 formate, Tocris Bioscience, Ellisville, MO; 10mM in 40% methanol in 0.9% NaCl).
In a second series of injections, juvenile GAERS (aged 26-50 days) received 2µl of Y1 receptor antagonist (BIBP3226, Bachem AG, Bubendorf, Switzerland; 10mM in 20% dimethylsulfoxide (DMSO) in 0.9% NaCl), Y2 receptor antagonist (BIIE0246) and Y5 receptor antagonist (CGP71683 hydrochloride, Tocris Bioscience, Ellisville, MO; 10mM in 20% DMSO in 0.9% NaCl).
After dissolving, drugs were stored in aliquots at -20°C until use. Doses of drugs were chosen on the basis of previous studies showing that antagonists provide a complete receptor block (Morris et al., 2007; Polidori et al., 2000).
Injections of 0.9% NaCl, 40% methanol (Reanal, Budapest, Hungary) in 0.9% NaCl, 20% DMSO in 0.9% NaCl and artificial cerebrospinal fluid (ACSF, Szent Rokus Korhaz es Intezmenyei, Budapest, Hungary) as well as handling without injection were used as controls.

Statistics
Results were subjected to statistical analysis using Student's t-test. Differences were considered to be statistically significant at p-values <0.05 (*) and highly significant at p-values <0.01 (**). Results are expressed as mean values ± standard deviation (SD). Immunohistochemical analyses were accomplished with 6 pairs of juvenile and 3 pairs of adult rats. Western blot experiments were performed with a total of 10 juvenile and adult NEC and GAERS and repeated 3 times. Functional studies were performed in 4 adult and 6 juvenile GAERS.
Differences in the number of NPY-positive cells per mm^2 between the four groups (juvenile, adult, NEC, GAERS) were investigated using univariate two-way ANOVA. Kolmogorov-Smirnov- and Shapiro-Wilk-tests were utilized to check normal distribution of mean values. Normal distribution of error variances was tested using Levene-test.

Results

To analyze the role of NPY in the onset and maintenance of SWDs, expression of NPY is investigated in different brain regions of juvenile and adult GAERS as well as NEC by means of immunohistochemistry.

NPY expression in different brain regions
Immunohistochemical investigations of NPY expression were concentrated on different brain regions such as the three hippocampal subregions DG, CA1 and CA3 as well as on frontoparietal cortex, thalamus and striatum.
Generally, only few NPY-immunopositive neurons are found in the investigated brain areas. Perikarya are stained whereas only few dendrites or axons of the multipolar or bipolar neurons are visible. NPY is preferentially expressed in GABAergic interneurons with long smooth dendrites. The highest number of NPY-positive perikarya is found in basal forebrain (Fig. 2G) showing also the longest dendrites. In contrast, only NPY-positive nerve terminals but no perikarya are observed in hypothalamus (Fig. 2H). In cortex, NPY-immunopositive cells are distributed throughout all cortical layers (Fig. 1B, 2B). In hippocampus, density of NPY-positive cells is highest in DG and CA1 in GAERS, whereas fewer cells are detectable in CA3. In DG, most of the NPY-positive neurons are found in the hilus or occasionally at the inner margin of the granule cell layer (Fig. 1C, 2C). NPY-immunoreactivity is low in the vicinity of the pyramidal cell layer in CA3 (Fig. 1D, 2D), whereas in CA1 most of the NPY-positive neurons are scattered around this layer (Fig. 1E, 2E). In thalamus, only the nucleus reticularis thalami (nRT) shows immunoreactivity for NPY in some of its GABAergic neurons (Fig. 1G). Intensity of staining is very weak making it hard to distinguish NPY-immunopositive cells properly from the background. Quantification of NPY-positive cells in this brain area is therefore not feasible. NPY-positive axons projecting from nRT to the thalamus proper are not traceable with this staining method. No other thalamic nuclei are found to contain NPY-positive perikarya (Fig. 1H). NPY-immunopositive cells are distributed throughout the whole striatum showing clear dendrites traversing the bundles of white matter (Fig. 1F, 2F).

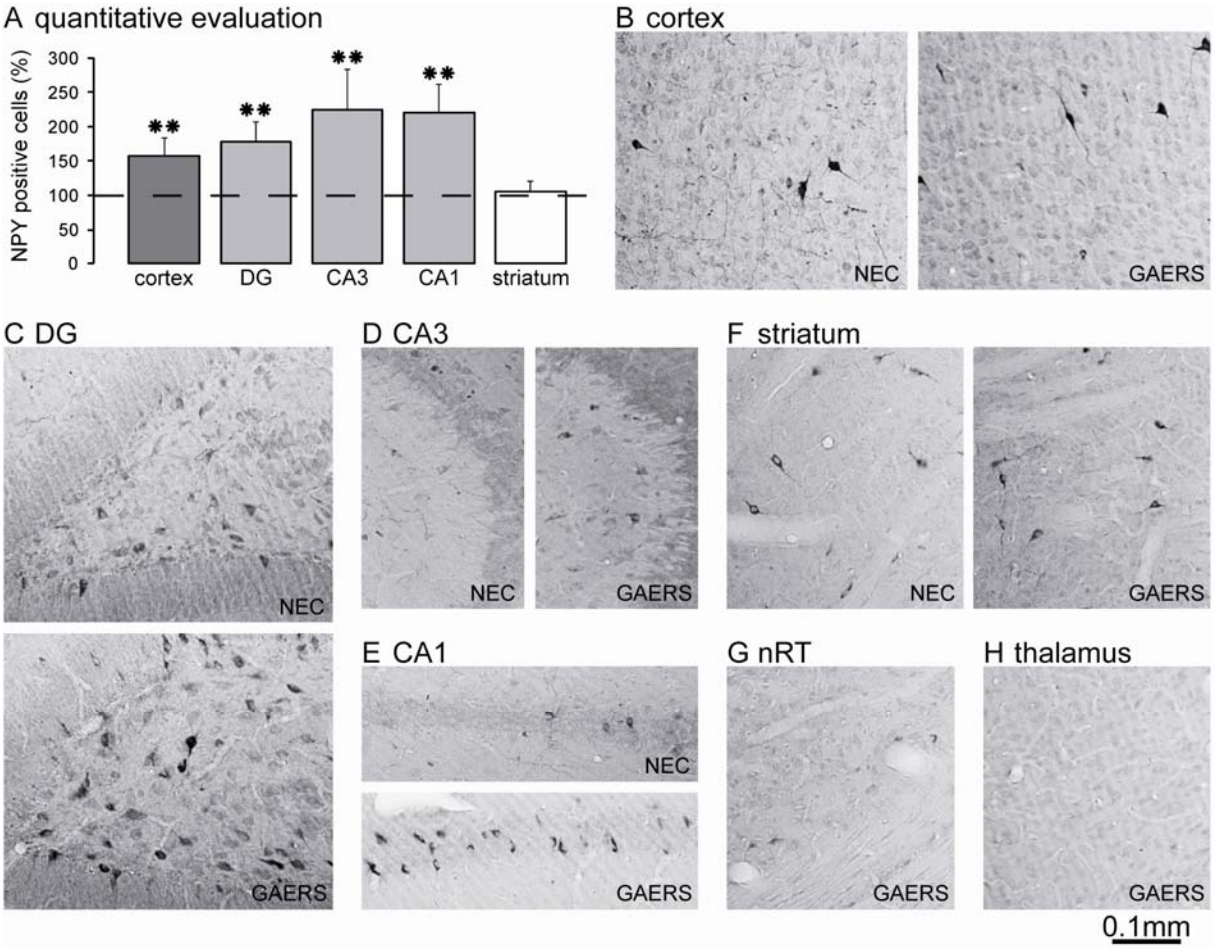


Fig. 1. Immunohistochemical analysis of NPY-positive cells in juvenile GAERS as compared to age-matched NEC. *Number of NPY-positive cells (A) in the brain regions cortex (B), DG (C), CA3 (D) and CA1 (E) is highly elevated in juvenile GAERS as compared to the appropriate levels in age-matched NEC (**p<0.01). No differences are detected in striatum (F). In thalamus, NPY-positive cells are only weakly visible in nRT (G), whereas no other thalamic nuclei were found to contain NPY-positive perikarya (H). Dashed line in A indicates number of NPY-positive cells in the respective region of NEC set at 100%.*

Density of NPY-positive cells is elevated in the three hippocampal subregions and in cortex of juvenile and adult GAERS

To quantify expression differences between GAERS and NEC, the density of NPY-positive cells was determined by morphometry. Mean value for each brain region of NEC is set equal to 100% and appropriate values of GAERS are calculated relative to it. NPY-immunopositive neurons are highly elevated in the three hippocampal subregions DG (178%, $p<0.01$), CA3 (224%, $p<0.01$) and CA1 (220%, $p<0.01$) as well as in cortex (158%, $p<0.01$) of juvenile GAERS as compared to age-matched NEC. In contrast, no differences in expression levels between GAERS versus NEC are found in striatum (Fig.1A).

In all investigated brain regions, NPY-immunoreactivity is much lower in adult animals than in juvenile ones. Nevertheless, levels in adult GAERS are significantly higher than in age-matched NEC. Higher numbers in hippocampal subregions are

not as pronounced as in juvenile animals but still exhibit increases up to 138% in DG ($p<0.05$), 141% in CA3 ($p>0.05$) and 164% in CA1 ($p<0.05$). In cortex, the increase in NPY-immunopositive cells (182%, $p<0.01$) is even more pronounced than in juvenile animals. No differences are observed in striatum (Fig. 2A). Absolute cell densities of NPY-positive neurons (cells/mm^2) are shown in table 1 (mean ± SD).

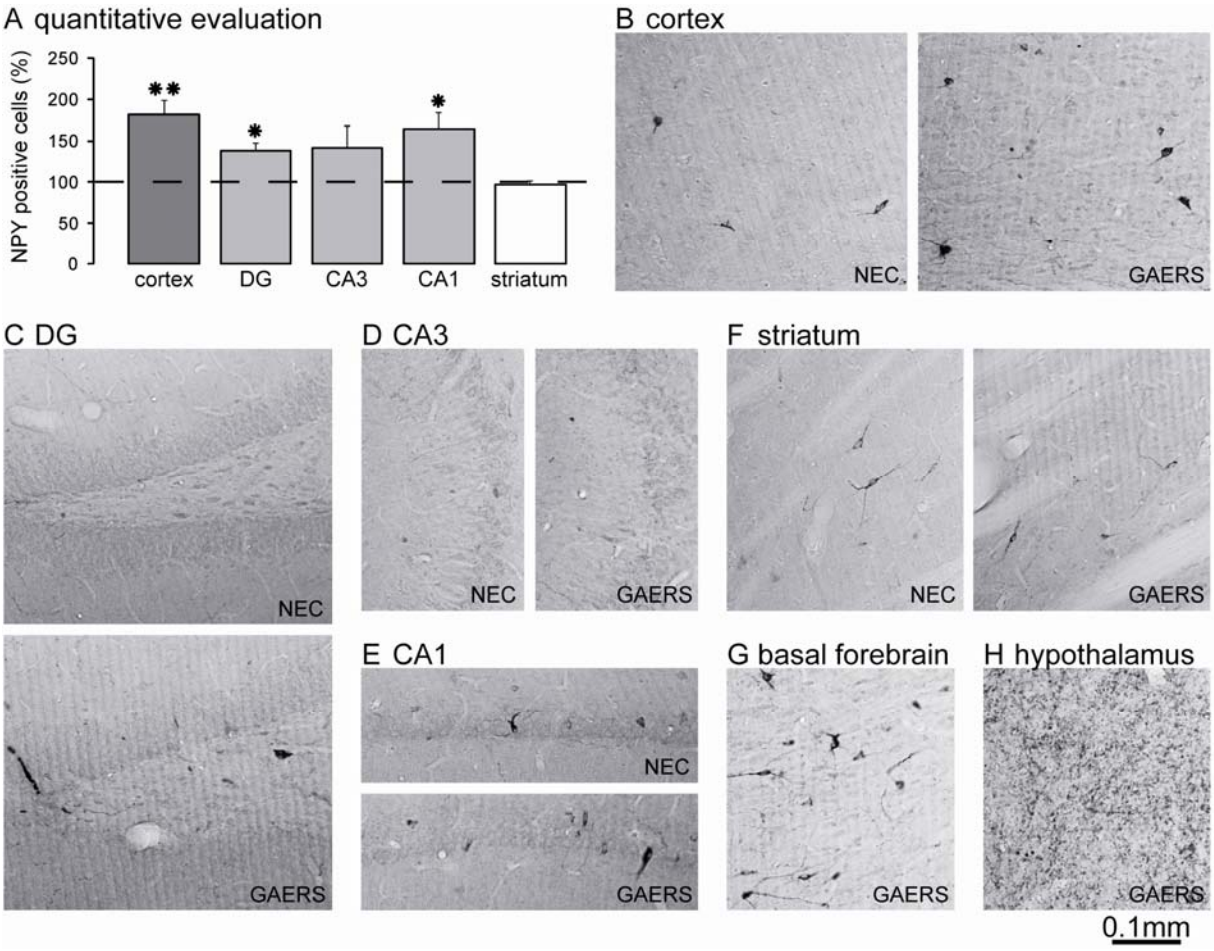


Fig. 2. Immunohistochemical analysis of NPY-positive cells in adult GAERS as compared to age-matched NEC. *Number of NPY-positive cells (A) is strongly increased in cortex (B; **p<0.01) and somewhat less in DG (C) and CA1 (E; *p<0.05 for both) of adult GAERS compared to the appropriate levels in age-matched NEC. The slight increase in NPY-immunoreactivity in CA3 (D) is not significantly distinct from NEC. No significant differences were found in striatum (F). Highest density of NPY-positive perikarya is present in basal forebrain (G), whereas density of NPY-positive nerve terminals is high in hypothalamus (H). Dashed line in A indicates number of NPY-positive cells in the respective region of NEC set to 100%.*

Table 1. Density of NPY-positive cells in individual brain regions. *Number of NPY-positive cell counts in cortex, DG, CA3, CA1 and striatum of juvenile and adult NEC and GAERS expressed as cells/mm^2. Shown are mean ± SD (*p<0.05; **p<0.01 compared to age-matched NEC; ‡p<0.01 compared to corresponding juvenile animals).*

		cortex	DG	CA3	CA1	striatum
juvenile	NEC	23.24 ± 2.41	38.30 ± 6.53	18.51 ± 2.44	27.87 ± 4.89	30.02 ± 2.17
	GAERS	36.62 ± 6.16 **	68.20 ± 11.17 **	41.40 ± 10.97 **	61.32 ± 11.66 **	31.67 ± 4.68
adult	NEC	8.60 ± 0.71 ‡	10.42 ± 1.96 ‡	10.91 ± 0.94 ‡	12.78 ± 2.80 ‡	20.09 ± 0.18 ‡
	GAERS	15.67 ± 1.44 **‡	14.35 ± 0.99 *‡	15.40 ± 2.89 ‡	20.98 ± 2.48 *‡	19.47 ± 0.93 ‡

A prominent reduction of NPY-immunoreactivity occurs with maturation and onset of SWDs

As juvenile animals show more frequent staining for NPY, cell densities are compared between juvenile and adult GAERS revealing a dramatic decrease in NPY expression with maturation. Mean density of NPY-positive cells is set equal to 100% for each brain region of juvenile animals. In adult GAERS, NPY-immunopositive cells are reduced to 43% in cortex, 21% in DG, 37% in CA3, 34% in CA1 and 61% in striatum as compared to juvenile GAERS ($p<0.01$ for all brain regions; Fig. 3A). A significant decrease of NPY-immunoreactivity occurs in all examined brain regions with differences only in intensity. Similar data are found by comparing adult with juvenile NEC. The number of NPY-positive cells in adult NEC is decreased to 37% in cortex, 27% in DG, 59% in CA3, 46% in CA1 and 67% in striatum as compared to juvenile NEC ($p<0.01$ for all brain regions; Fig. 3B). Thus, the decrease with maturation only slightly differs between NEC versus GAERS.

Comparison of changes occurring in the area of various brain regions and in the absolute number of NPY-positive cells reveals that decreases in cell densities with maturation are not only due to enlargement of brain area but rather to a drastic loss of NPY-expressing cells (table 2).

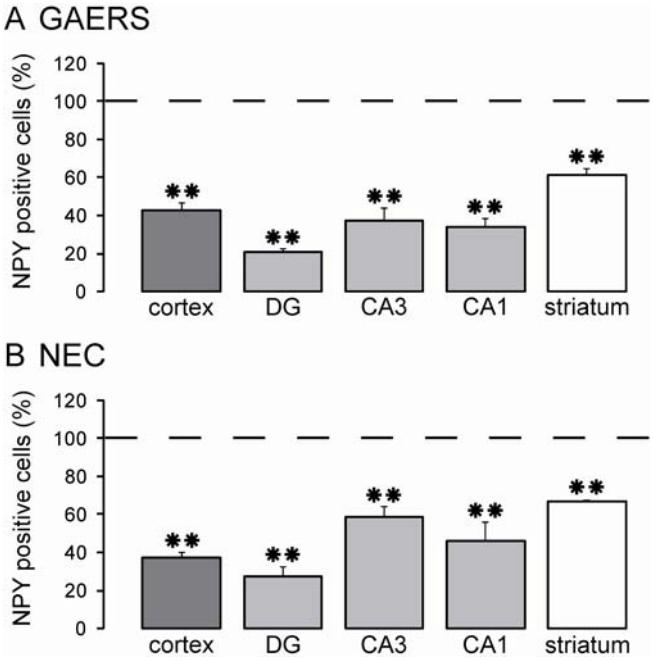
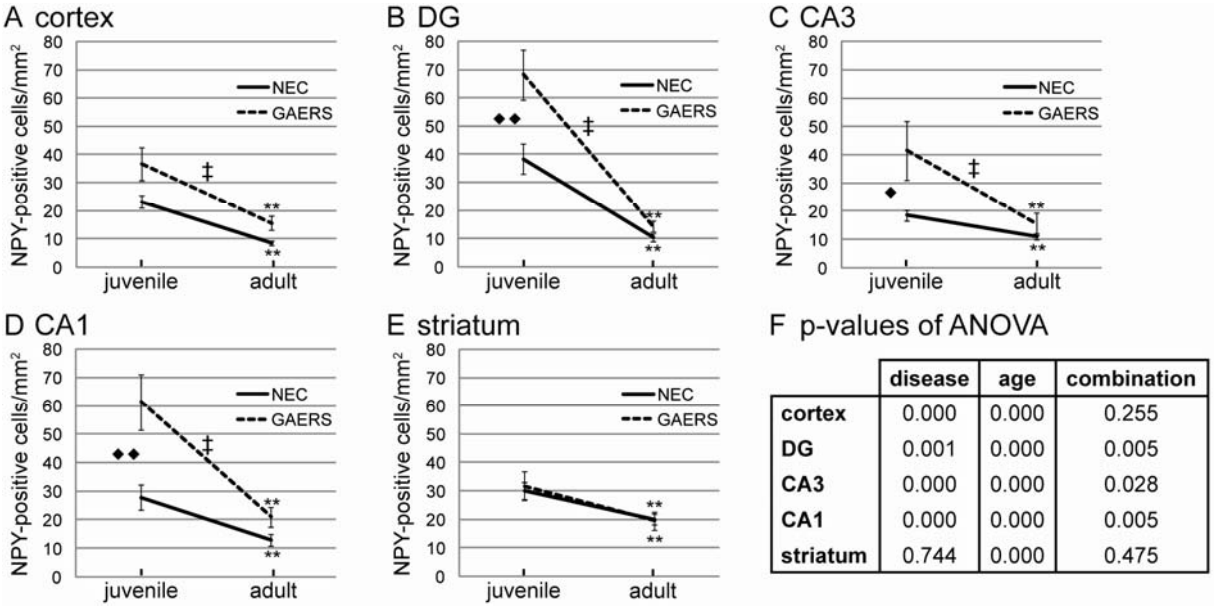


Fig. 3. Decline in the number of NPY-positive cells with maturation. *A, adult GAERS express markedly fewer NPY-positive neurons in cortex, DG, CA3, CA1 and striatum as compared to the corresponding levels counted in juvenile GAERS, which was set to 100% for each brain region (dashed line). B, similar results were found in all examined brain regions of adult NEC compared to the appropriate levels in juvenile NEC (**p<0.01).*

Table 2. Changes in area size, number of NPY-positive cells and cell density of various brain regions. *Four different analyses were performed: (1) juvenile GAERS compared to juvenile NEC, (2) adult GAERS compared to adult NEC, (3) adult GAERS compared to juvenile GAERS and (4) adult NEC versus (vs.) juvenile NEC. Results are expressed as percentages. Reference values were set equal to 100% (*p<0.05; **p<0.01). Note that not total cortices were measured but only that part of the frontoparietal cortex directly overlying the dorsal hippocampus, i.e. variations in this parameter indicate differences in the thickness of the cortex.*

		cortex	DG	CA3	CA1	striatum
juvenile GAERS vs. juvenile NEC	area [mm²]	99%	116% *	101%	99%	100%
	# of cells	156% **	204% **	219% **	218% **	104%
	density [mm⁻²]	158% **	178% **	224% **	220% **	106%
adult GAERS vs. adult NEC	area [mm²]	91%	129% **	99%	118%	106%
	# of cells	166% *	179% **	141%	193% *	102%
	density [mm⁻²]	182% **	138% *	141%	164% *	97%
adult GAERS vs. juvenile GAERS	area [mm²]	115%	166% **	140% *	147% **	113%
	# of cells	50% **	36% **	54% **	51% **	70% *
	density [mm⁻²]	43% **	21% **	37% **	34% **	61% **
adult NEC vs. juvenile NEC	area [mm²]	123% *	149% **	143% **	124% **	106%
	# of cells	47% **	41% **	84%	58% **	71% *
	density [mm⁻²]	37% **	27% **	59% **	46% **	67% **

Two-way univariate ANOVA reveals that the number of NPY-positive cells in cortex depends on age (juvenile vs. adult, $p<0.01$) and disease (NEC vs. GAERS, $p<0.01$) but that the decrease of NPY-immunoreactivity with maturation is independent of the disease state of the animal (Fig. 4A and F). In contrast, in all three hippocampal subregions the number of NPY-immunopositive cells is highly regulated by disease state and age ($p<0.01$) and additionally, the decline in NPY-immunoreactivity is greater in GAERS as compared to NEC ($p<0.01$ for DG and CA1, $p<0.05$ for CA3; Fig. 4B-D, F). In striatum, changes in the number of NPY-positive cells occur only with age ($p<0.01$), whereas the disease state of the animal does not reveal any influence (Fig. 4E-F).



	disease	age	combination
cortex	0.000	0.000	0.255
DG	0.001	0.000	0.005
CA3	0.000	0.000	0.028
CA1	0.000	0.000	0.005
striatum	0.744	0.000	0.475

Fig. 4. ANOVA evaluation of NPY-positive cells in juvenile and adult NEC and GAERS. *ANOVA for repeated measures revealed a significant effect for age (**p<0.01) on the density of NPY-positive cells in all examined brain regions (A-E). Differences in NPY-positive cells due to the disease state were found in cortex, DG, CA1 and CA3 (A-D, ‡p<0.01 compared to NEC) with exception of striatum (E). The interaction between age and disease state influences NPY-immunoreactivity in DG and CA1 (◆◆p<0.01; B and D) as well as in CA3 (◆p<0.05; C). F, calculated p-values for disease, age and the interaction between these two factors in all examined brain regions.*

Hilar area is enlarged in GAERS

To investigate more precisely differences observed in the area of DG between GAERS and NEC, we performed a quantitative measurement of the size of the hilus. The hilar area is defined by the inner edge of the granule cell layer and the lines connecting them with the beginning of the pyramidal cell layer of Ammon‘s horn (Fig. 5A).

Hilar area gradually increases with maturation of rat brain. Mean hilar area in juvenile NEC is 0.232mm^2 enlarging up to 0.277mm^2 in adult NEC (119%, $p<0.05$, compared to juvenile NEC). GAERS exhibit larger hilar areas in both ages: 0.295mm^2 in juvenile animals and 0.412mm^2 in adults (139%, $p<0.01$, compared to juvenile GAERS) resulting in an increase of 127% and 149%, respectively compared to age-matched NEC (Fig. 5B and C, $p<0.01$).

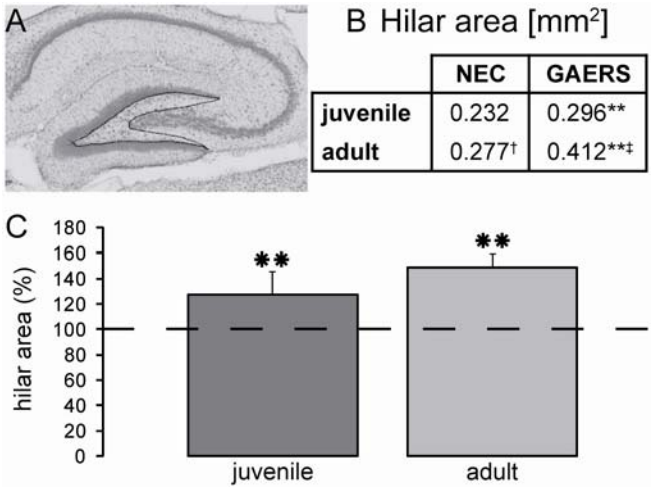


Fig. 5. Size of the hilar area is increased in juvenile and adult GAERS. *A, hilar area is defined by the area within the inner edge of the granule cell layer and the lines connecting the tips of the two granule cell blades to the end of CA3 pyramidal cell layer. B, mean of the measured hilar area in mm^2 of juvenile and adult NEC and GAERS (**p<0.01 compared to age-matched NEC; †p<0.05 compared to juvenile NEC; ‡p<0.01 compared to juvenile GAERS). C, hilar area of juvenile and adult GAERS compared to reference areas of age-matched NEC, which was set at 100% (dashed line; **p<0.01 compared to age-matched NEC).*

Y2 receptor protein levels are increased in adult GAERS

Y2 receptor is suggested as the NPY receptor subtype involved in the process of absence seizures (Morris et al., 2007). Its protein levels were measured by means of Western blot analysis to estimate whether the signaling pathway of NPY is also altered in GAERS. Slight increases of Y2 receptor protein levels are detected in adult GAERS, whereas no differences are found in all brain regions of juvenile GAERS compared to age-matched NEC. Cortical Y2 receptor levels are elevated to 138% ($p>0.05$; Fig. 6A) and hippocampal levels to 128% ($p<0.05$; Fig. 6B) in adult GAERS. No changes in expression levels are detected in thalamus.

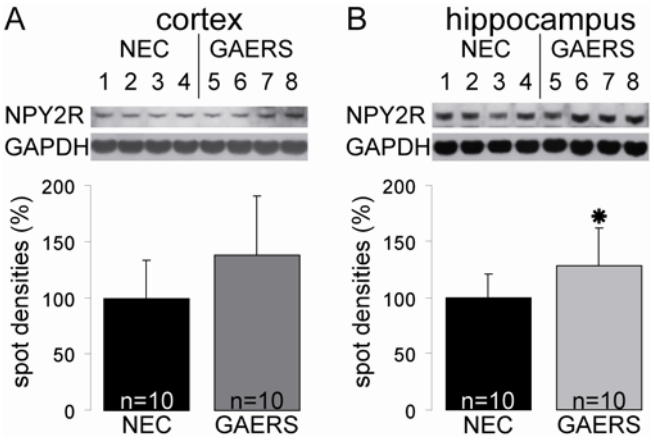


Fig. 6. Content of Y2 receptor protein is slightly elevated in adult GAERS. *A small but not significant increase of the Y2 receptor protein was found in cortex of adult GAERS (A; p>0.05), whereas the elevation in hippocampus reached significance (B; *p<0.05).*

Functional studies

In order to verify whether changes in the NPY-system are directly linked to the occurrence of SWDs, functional studies were performed both in juvenile and adult GAERS. Occurrence of SWDs and onset of SISWDs are directly modified by icv application of NPY receptor agonists and antagonists.

SISWDs in juvenile GAERS are less pronounced and more variable than the typical SWDs in adult GAERS

All adult GAERS examined exhibit typical and well distinguishable SWDs with high amplitude and a frequency of 7Hz (Fig. 7A). Discharges observed in juvenile GAERS (around 1 month) do not yet possess all the characteristics. SISWDs are more variable with smaller amplitude, shorter duration and a frequency of 5Hz. Nevertheless, the spike-and-wave morphology is clearly detectable (Fig. 7B). Two juvenile GAERS (aged 36 and 50 days, respectively) displayed SISWDs before the injection of the first drug and are therefore omitted for the analysis.

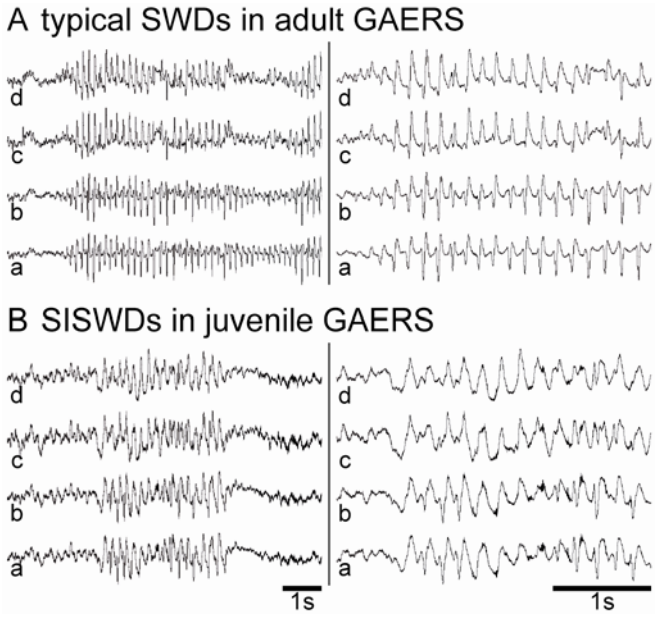


Fig. 7. Typical EEG discharges in GAERS. *A, SWDs observed in adult GAERS with high amplitude and a frequency of 7Hz shown in a narrow (left) and broad range (right). B, SISWDs seen in juvenile GAERS are variable with a shorter duration and smaller amplitude than typical SWDs displaying a frequency of 5Hz. Electrode locations: a, left frontal; b, right frontal; c, left posterior; d, right posterior*

NPY and a selective Y2 receptor agonist suppress SWDs in adult GAERS

To verify the inhibitory role of NPY on the occurrence of SWDs, adult GAERS are injected with NPY (3nmol, icv). This treatment markedly reduces the occurrence of SWDs in adult GAERS. 30min after injection, percentage time spent in seizure is reduced to 63% and further lowered to 44% ($p<0.05$) within 60min compared to seizure incidence after injection of 0.9% NaCl. The occurrence of SWDs completely recovers within 90min after NPY injection (Fig. 8).

To check the hypothesis of Morris et al. (2007) that the suppressing effect of NPY is primarily mediated through Y2 receptors, adult GAERS were treated with 3nmol of a selective Y2 receptor agonist. A clear reduction of SWDs to about 35% of seizure incidence following 0.9% NaCl treatment occurs between 30 and 90 minutes after injection. A further decrease to 10% is observed 2 hours after treatment ($p<0.05$). Seizure incidence completely recovers within 24 hours after mimicking NPY effects on the Y2 receptor (Fig. 8).

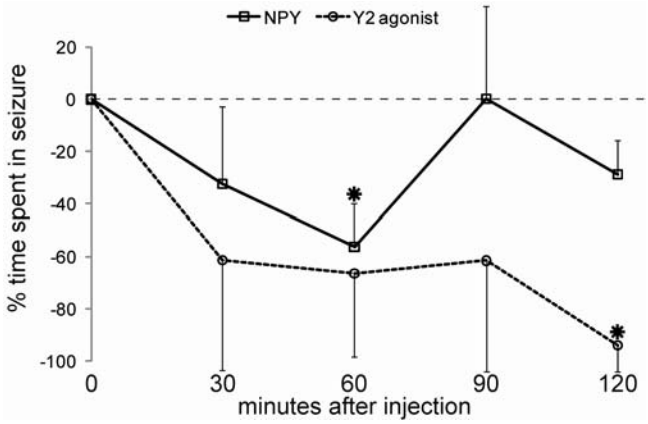


Fig. 8. NPY and a specific Y2 receptor agonist (Acetyl-(Leu^{28,31})-Neuropeptide Y (24-36)) reduce the occurrence of SWDs in adult GAERS. *The percentage time spent in seizure is impaired to 63% and 44% 30 and 60 minutes after NPY injection, respectively (continuous line, p<0.05). Treatment with the Y2 receptor agonist reduces SWDs to about 35% between 30 and 90 minutes and further to 10% 2 hours after application of the drug (dashed line, *p<0.05). Seizure occurrence completely recovers within 24 hours.*

Unexpectedly, injection of the Y2 receptor agonist evokes epileptogenic effects in the hippocampus. EEGs from 3 out of 4 adult GAERS display typical focal epilepsies with an initial flattening of the amplitude followed by an increase in amplitude of spikes with a decrease in frequency over time. Occurrence of focal seizures is first observed in electrodes ipsilateral to the injection site followed by a rapid spreading to the contralateral hemisphere. Morphology of focal seizures differs between animals, probably due to differences in the diffusion of the drug from the ventricle to the hippocampus. Two animals show epileptic discharges which are typical for stage 1 or 2 seizures after hippocampal kindling (Fig. 9A). One adult GAERS displays status epilepticus as seen after pilocarpine- or kainate-injection (Fig. 9B). Focal epilepsy concomitantly occurs with absence seizures (Fig. 9C). No overt motor convulsions were observed, but animals showed a very aggressive behavior.

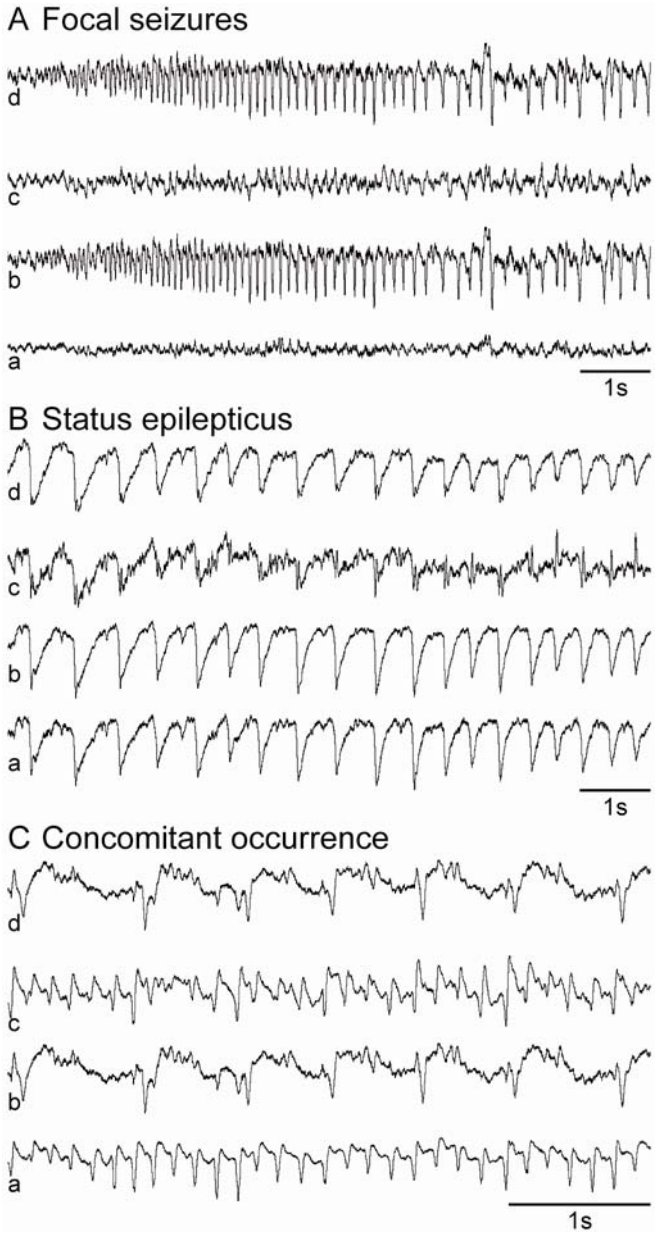


Fig. 9. Epileptogenic properties of the specific Y2 receptor agonist (Acetyl-(Leu^{28,31})-Neuropeptide Y (24-36)). *A, focal seizures which secondarily spread are observed on the ipsilateral site (b,d) of two animals after selective activation of Y2 receptors. Initial flattening of amplitude and decrease of spike frequency over time are two hallmarks of focal epilepsy. B, in one animal, icv application of the Y2 receptor agonist evokes status epilepticus with a spike frequency of 2-3Hz and duration longer than 1 hour. C, rarely, focal and absence seizures are concomitantly observed. Absence seizures occur on the hemisphere contralateral to the injection site (a,c) and focal seizures on the ipsilateral site (b,d). Electrode locations: a, left frontal; b, right frontal; c, left posterior; d, right posterior*

Y2 receptor antagonist is not able to increase the occurrence of SWDs in adult GAERS

To verify whether suppression of remaining NPY effects results in a further increase of SWD incidence, adult GAERS are injected with 20nmol BIIE0246, an Y2 receptor antagonist. However, this treatment does not change seizure incidence as compared to application of its solvent (40% methanol in 0.9% NaCl; data not shown).

Y1 receptor antagonist facilitates the onset of SISWDs in juvenile GAERS

To elaborate the protective effect of NPY on the occurrence of SWDs in juvenile GAERS, these animals were treated with three different NPY receptor antagonists, their solvents and controls. At the start of EEG measurement, SISWDs are not yet distinguishable in 4 out of 6 juvenile GAERS (aged 25–33 days). In 3 out of these 4 juvenile GAERS, first clear SISWDs (Fig. 7B) are observed following the injection of 20nmol of the Y1 receptor antagonist BIBP3226 at the age of 30, 31 and 39 days. The onset of SISWDs occurs 24 to 96 hours after injection of the Y1 receptor antagonist without any other treatment in between. The fourth animal shows its first SISWDs at the age of 33 days, 24 hours after injection of the Y5 receptor antagonist (CGP71683) and 120 hours after injection of the Y1 receptor antagonist.

Y2 receptor antagonist increases the occurrence of SISWDs in juvenile GAERS

Occurrence of SISWDs was investigated 24 hours after injection of the antagonist as compared to baseline incidence before treatment. As shown in figure 10, no differences in the SISWD incidence are observed after handling of animals without injection, application of ACSF, 40% methanol in 0.9% NaCl and 20% DMSO in 0.9% NaCl. Furthermore, application of the Y5 receptor antagonist (CGP71683, 20nmol) does not interfere with the occurrence of SISWDs, whereas injection of the Y1 receptor antagonist (BIBP3226, 20nmol) shows a tendency to aggravate seizures. Although the mean value of time spent in seizure is increased to 223% as compared to baseline, this augmentation does not reach statistical significance due to broad interindividual variability. Inhibition of Y2 receptors with 20nmol BIIE0246 results in a significant increase of time spent in seizure to 171% as compared to baseline values ($p<0.01$; Fig. 10). Additionally, the incidence of SISWDs after injection of the Y2 receptor antagonist is also elevated compared to application of its solvent (40% methanol in 0.9% NaCl, $p<0.05$).

Histological examination of brain sections revealed that all cannulae implanted in adult GAERS and half of the ones implanted in juvenile GAERS enter the ventricle. In the remaining 3 juvenile GAERS, cannulae tip was placed in the corpus callosum right above the ventricle. As no clear differences are observed between juvenile GAERS, all animals were included in our analyses.

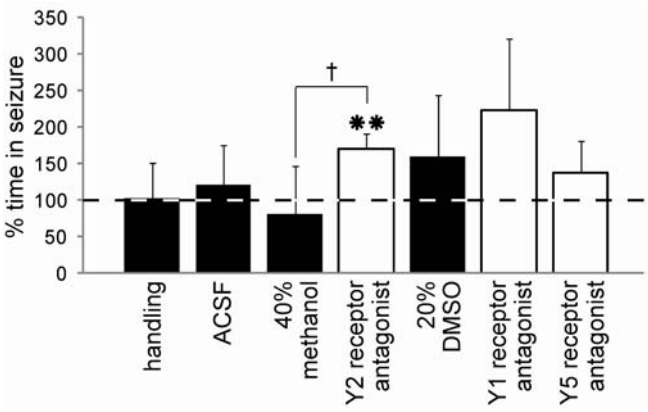


Fig. 10. Effects of different NPY receptor antagonists on the occurrence of SISWDs 24 hours after treatment. *Percentage time in seizure is influenced by the icv application of control substances (black bars) or various NPY receptor antagonists (white bars) compared to baseline values set at 100% (dashed line). Injection of an Y2 receptor antagonist (BIIE0246 formate) increases the percentage time in seizure compared to baseline (**p<0.01) as well as compared to its solvent (40% methanol in 0.9% NaCl; $^{\dagger}p<0.05$; n=4).*

Discussion

The objective of this study was to investigate the role of NPY and its receptors in an animal model for absence epilepsy with regard to the resistance to secondary generalization of focal limbic seizures. We show that the density of NPY-positive neurons is higher in GAERS and undergoes a prominent reduction with maturation. Moreover, SWDs in adult GAERS are reduced by icv application of NPY and a selective Y2 receptor agonist, while in juvenile GAERS, onset of SISWDs is facilitated by treatment with NPY receptor antagonists.

Density of NPY-positive cells in GAERS

Our data show a robust higher density of NPY-positive cells in cortex and hippocampus of juvenile as well as adult GAERS compared to age-matched NEC and support our previous results showing an increase in NPY mRNA expression in GAERS (Landweer et al., unpublished results). An increase of NPY expression was found in hippocampus of another genetic rat model for generalized epilepsy, the spontaneously epileptic rats (Sadamatsu et al., 1995). The increase of NPY synthesis is already observed in juvenile GAERS indicating that this effect is not the consequence of SWDs but rather the result of a genetic alteration in GAERS. Rats overexpressing NPY show a reduction in seizure frequency and a remarkable decrease in the progression of seizures in different animal models of TLE including a rat model of chronic epilepsy (Noe et al., 2008; Vezzani et al., 2002). The anti-epileptic effects of NPY are also observed in GAERS, although elevation of NPY expression is relatively moderate as compared to transgenic rats overexpressing NPY. Distinct differences in NPY expression occur also in hippocampus of GAERS, in a brain structure primarily not involved in absence seizures. Similarly, in stargazer mutant mice an increase of NPY-immunoreactivity occurs only in DG and not in neocortex and nRT, two regions involved in spike-wave synchronization (Chafetz et

al., 1995). In contrast, no differences were observed in striatum, a brain region that may contribute to an endogenous mechanism controlling the maintenance and duration of SWDs (Slaght et al., 2004). Our study reveals a marked decrease of NPY-positive cells during maturation. The decrease in density of hippocampal NPY neurons is more pronounced in GAERS than in NEC. The fact that this reduction coincides with the appearance of absence seizures is compatible with an anti-absence effect of NPY. We assume that newborn GAERS suffer already from absence epilepsy, but high levels of NPY prevent the occurrence of discharges. The age-dependent decrease in NPY expression allows then the onset of SWDs. On the other hand, the blockage of kindling development in adult GAERS is not compatible with decreased NPY-levels unless the difference of NPY expression between mature GAERS and NEC is still sufficient to protect GAERS from kindling generalization. This interpretation is supported by results of Onat et al. (2007) indicating that the rate of amygdala kindling correlates negatively with the intensities of SWDs.
In thalamus, NPY immunoreactivity is low suggesting that thalamic NPY is primarily not involved in the initiation of SWDs. Only nRT expresses NPY in its GABAergic neurons (Morris, 1989). In contrast, numerous NPY-positive neurons are found in the cortex and their number decreases dramatically with age. Expression of typical absence seizures is known to arise from an altered interplay between cortex and thalamus although the roles played by the components of this circuitry are still unclear. Electrophysiological data have identified a specific site of seizure generation within the primary somatosensory cortex in GAERS (Polack et al., 2007). Then, SWDs rapidly propagate to other cortical areas and related thalamic nuclei.

Increased size of dentate hilus in animals with absence seizures
Absence seizures cause a robust increase in the size of the hilar region of the hippocampal dentate gyrus. This increase is not the consequence of SWDs per se, since larger hilar area is already observed in juvenile GAERS before the appearance of SWDs. Increments were not only observed between NEC and GAERS but also among immature and adult rats suggesting that hilar hypertrophy is both disease- and age-dependent. This is the first evidence that generalized non-convulsive seizures elicit changes in the hilar area. The increase is of the same order of magnitude as observed after recurrent seizure activity induced during kindling (Adams et al., 1998). This observation underscores the strong link between generalized non-convulsive seizures originating in the corticothalamic loop and focal limbic seizures in the hippocampus.
Mechanisms underlying hilar enlargement are not clear. One possibility is reactive gliosis. Dutuit et al. (2000) showed that the increased neuronal activity in GAERS induces specific alterations in the expression of glial fibrillary acidic protein, which is considered as marker of reactive gliosis, in the cortex and thalamus of these rats. However, no change was observed in whole hippocampus and the hilus was not specifically investigated. Gliosis has been associated with neuronal loss in focal epilepsy and with the sclerosis observed in the foci. However, in GAERS no neuronal loss and no decrease in glutamate decarboxylase, which indicates a loss of GABA interneurons, were observed in the frontoparietal cortex and thalamus (Sabers et al., 1996; Spreafico et al., 1993).
In models of TLE, dentate granule cell excitability is regulated by hilar inhibitory interneurons (Buckmaster and Schwartzkroin, 1995; Ratzliff et al., 2004). Under

physiological conditions, NPY is expressed in these interneurons with a subpopulation especially vulnerable to seizure-induced injury (de Lanerolle et al., 1989). Loss of inhibitory interneurons contributes to increased dentate granule cell excitability (Martin and Sloviter, 2001) and may disrupt the ability of DG to filter incoming seizure activity (Heinemann et al., 1992). When the activity propagates into hippocampus, it may lead to neuronal injury and intractable seizures (Engel et al., 1997). A comparable mechanism has not yet been shown in the case of generalized absence seizures.

Y2 receptor protein in GAERS

Despite convergent evidence that NPY operates anti-epileptic, identifying specific receptor subtypes underlying this action has proven controversial. Y2 receptors are the primary mediators of the anti-epileptic activity in rats (Klapstein and Colmers, 1997), but evidence for a role of Y5 has emerged from mouse studies (Marsh et al., 1999). Based on the results of Morris et al. (2007) we concentrated our analysis on Y2 receptors. Interestingly, its expression is slightly elevated after the onset of SWDs only in adult GAERS, suggesting that changes in Y2 receptor protein may be a consequence of absence seizures. In line with our results, delayed hippocampal kindling leads to a transient increase of NPY mRNA in limbic structures and Y2 receptor mRNA in dentate granule cell layer, entorhinal cortex and amygdala leading to suppression of hyperexcitability (Kopp et al., 1999).

Pharmacological manipulation of SWDs in adult GAERS

Occurrence of SWDs in adult GAERS is drastically influenced by activation of NPY receptors. Icv application of NPY and a selective Y2 receptor agonist significantly diminishes SWDs with a maximum 60 minutes or 2 hours after injection, respectively. In our hands, time courses are slightly delayed compared to previous studies showing maximal effects between 15 and 45 minutes after treatment (Morris et al., 2007; Stroud et al., 2005).

Not NPY infusion itself, but selective activation of its Y2 receptors evokes focal seizures in GAERS. Mechanisms of focal and absence epilepsy do not interfere with each other as they occur concomitantly, even though the coincidence rarely exists. Motor effects of focal seizures are not observed, but treated animals show very aggressive behaviors. Therefore, the focus for epileptic discharges probably lies within the hippocampus and not the cortex. Selective targeting of NPY receptor subtypes seems to be more critical in interference with absence seizures than with the neuropeptide itself. Facilitating effects of NPY on limbic seizures have already been described in hippocampus, but Y1 receptors have been proposed to mediate this action (Vezzani et al., 1999b). Application of a high-affinity $GABA_B$ receptor antagonist to adult GAERS evokes similar phenomena. Intraperitoneal application of low doses reduces SWDs, whereas intracerebral injection induces focal seizures (Vergnes et al., 1997).

Treatment of adult GAERS with a selective Y2 receptor antagonist does not increase seizure incidence. These data indicate that the protective effect of endogenous NPY on SWD generation is completely abolished in adult GAERS or that other NPY receptor subtypes are involved in the anti-absence effect.

Interference with SISWDs in juvenile GAERS

Onset and incidence of SISWDs in juvenile GAERS can be changed by application of NPY receptor antagonists. First clear SISWDs are detectable after treatment with the Y1 receptor antagonist, whereas blocking of Y2 receptors resulted in elevated seizure incidence. Y5 receptor subtypes do not seem to be involved in the generation of absence seizures. Occurrence of SISWDs in juvenile GAERS is very susceptible to the environment. During long-term EEG recording incidence of SISWDs decreased due to diminished vigilance. The effect of treatment was delayed and therefore analysis was performed with data collected 24 hours after injection. Our data give first evidence that absence seizures can be evoked in juvenile GAERS by blocking NPY receptors. Sun et al. (2002) speculate that Y1 receptor activation leads to suppression of nRT excitability and therefore cessation of absence epilepsy.

Taken together, high NPY expression protects juvenile GAERS from absence seizures. Onset of SISWDs in juvenile GAERS can be elicited by NPY receptor antagonists. NPY expression levels decline with maturation allowing the onset of SWDs in adult GAERS. Suppression of absence seizures is achieved by NPY treatment. Specific receptor subtypes are involved in the anti-absence effect of NPY.
One of the mechanisms whereby GAERS are resistant to secondary generalization of focal limbic seizures may be related to the significant enhancement of NPY expression in GAERS compared to NEC. Absence seizure-induced overexpression of NPY may constitute an endogenous mechanism that controls hippocampal epileptogenesis. Data from the epileptic human hippocampus showing that NPY inhibits perforant path-evoked responses (Patrylo et al., 1999) reinforce the future strategy for treating epilepsy by specifically interacting with the brain NPY-system.

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4 Discussion

The aim of this doctoral thesis is to elucidate the mechanisms underlying absence seizures. This work reveals marked differences in neurotrophin and neuropeptide expression between GAERS and NEC as well as between juvenile and adult GAERS indicating that both systems may contribute to the pathophysiology of absence seizures (table 3).

Table 3: Summary of the main findings of this thesis. *Differences in protein or mRNA levels are investigated in cortex, hippocampus and thalamus of juvenile and adult GAERS as compared to age-matched NEC. Additionally, effects of icv application of various drugs on SWDs are tested. ↑: increase, ↓: decrease, ↔: no significant change, empty fields: not done*

		cortex		hippocampus		thalamus		effects on SWDs
		protein	mRNA	protein DG/CA1/CA3	mRNA	protein	mRNA	
juvenile GAERS	BDNF	↓	↑	↑	↑	↔	↔	
	proBDNF	↔		↔		↔		
	TrkB		↔		↔		↔	
	$p75^{NTR}$	↓		↔		↔		
	pCREB/CREB	↔		↔		↔		
	CREB	↑		↑		↔		
	NPY	↑	↑	↑ / ↑ / ↑	↑		↔	
	Y2-receptor	↔		↔		↔		
	Y1-antagonist							↑
	Y2-antagonist							↑
	Y5-antagonist							↔
adult GAERS	BDNF	↑	↑	↑	↔	↔	↔	↓
	proBDNF	↔		↔		↔		
	TrkB		↓		↓		↔	
	$p75^{NTR}$	↔		↔		↔		
	pCREB/CREB	↓		↓		↓		
	CREB	↔		↔		↔		
	NPY	↑	↑	↑ / ↑ / ↔	↑		↔	↓
	Y2-receptor	↔		↑		↔		
	Y2-agonist							↓
	Y2-antagonist							↔

In conclusion, although many aspects are still unknown, experimental evidence clearly demonstrates that:

- The hilar area of the hippocampal dentate gyrus is markedly enlarged in juvenile as well as adult GAERS as compared to age-matched NEC.
- Expression of the convulsant neurotrophin BDNF is altered in GAERS before and after the onset of absence seizures.
- As TrkB expression and CREB phosphorylation are markedly decreased in adult GAERS, we may assume that BDNF signaling is impaired in these animals.

- BDNF, CREB and NPY are expressed in identical neuronal subpopulations facilitating molecular interactions.
- Levels of the endogenous anticonvulsant compound NPY are highly increased in juvenile as well as adult GAERS as compared to age-matched NEC.
- A marked decrease in the density of NPY-positive cells occurs during maturation and coincides with the onset of absence seizures.
- Occurrence of SWDs in adult GAERS is reduced by icv application of NPY as well as BDNF.
- In juvenile GAERS, SISWDs are evoked or increased by treatment with antagonists against Y1- and Y2-receptors, but not with an antagonist against Y5-receptors.

4.1 BDNF is able to switch between pro- and anti-convulsant effects

Increased levels of the neurotrophin BDNF potentiate glutamatergic transmission and increase neuronal activity in brain finally leading to seizures (Kang and Schuman 1995; Toth 2005). However, epileptic patients do not constantly suffer from seizures. Three down-stream signaling effects of BDNF may cause the cessation of epileptic activity: (1) TrkB expression is diminished following chronic exposure to BDNF leading to an attenuation of BDNF action. (2) BDNF induces the expression of the anticonvulsant protein NPY. (3) Continuous exposure to BDNF significantly increases GABA-current amplitude in epileptic brain via PLCγ and protein kinase C pathway finally inducing an increase in inhibitory activity (Palma, Torchia et al. 2005).
The BDNF system is markedly altered in GAERS, but it may not be directly involved in the initiation of absence seizures. In adult GAERS, BDNF signaling is impaired due to marked decreases in TrkB expression and CREB phosphorylation. Therefore, adjustment to chronic neurotrophic input results in attenuation of signal transmission leading to a loss of responsiveness to BDNF. In line with our results, chronic exposure to BDNF markedly reduces protein and mRNA levels of TrkB and ligand-induced tyrosine phosphorylation in embryonic (Knusel, Gao et al. 1997) as well as adult neurons (Frank, Wiegand et al. 1997). This down-regulation might present a protective mechanism against potentially damaging levels of growth factors (Frank, Ventimiglia et al. 1996). The decline of TrkB expression recovers rapidly after termination of trophic factor administration. Nevertheless, functional desensitization to BDNF does not obligatory occur despite a clear reduction in TrkB protein (Frank, Wiegand et al. 1997). The decrease in TrkB protein may be explained by receptor-mediated internalization of the ligand or by clearance of the ligand-receptor complex by retrograde axonal transport (Frank, Wiegand et al. 1997). A specific amino acid sequence in the intracellular part of TrkB is competent for this process (Sommerfeld, Schweigreiter et al. 2000).
The temporal coincidence of decreases in BDNF responsiveness with the onset of SWDs suggests an important role for the TrkB/CREB signaling pathway in the initiation and/or propagation of absence seizures.

Despite a significant reduction in TrkB signaling and CREB phosphorylation, NPY protein and mRNA levels are still elevated in adult GAERS compared to age-matched NEC. This fact implicates that NPY expression is not solely regulated via TrkB/CREB signaling pathway. We assume that besides this conventional track, BDNF is also able to directly induce NPY expression.
A clue is given by the fact that bolus injection of BDNF drastically depletes the occurrence of SWDs in adult GAERS despite diminished TrkB and CREB signaling. We assume that the anti-absence effect of BDNF is at least in part mediated via its direct effect on NPY expression and that only BDNF neurons that simultaneously express NPY contribute to the anti-absence effect. A possible way to demonstrate this assumption is to test whether coapplication of NPY receptor antagonists abrogates the anti-absence effect of BDNF.
In juvenile GAERS, expression of both, BDNF and NPY, is already increased as compared to age-matched NEC although no epileptic activity is yet present at this age. Nevertheless, an underlying imbalance between excitation and inhibition may already be present leading to changes in protein expression and making the immature brain to a dynamically changing structure (for review see Holmes 1997). For example, immature hippocampal neurons respond to $GABA_A$-receptor activation by a transient Ca^{2+} influx, which increases BDNF mRNA expression (Berninger, Marty et al. 1995). Elevated BDNF levels in turn may induce hippocampal NPY synthesis.

4.2 NPY is a potent endogenous anti-absence factor acting via Y1 and Y2 receptors

NPY protein and mRNA concentrations are significantly increased in cortex and hippocampus of juvenile as well as adult GAERS as compared to age-matched NEC. Accordingly, NPY-immunoreactivity is markedly increased in adult spontaneously epileptic rats with absence-like seizures at an age at which animals already display seizures (Sadamatsu, Kanai et al. 1995). Additionally, in stargazer mutant mice, another model for non-convulsive SWDs, NPY expression is elevated solely after the onset of SWDs. Interestingly, aberrant expression is found in dentate granule cells which are normally devoid of NPY. The abnormal synaptic activity pattern during SWDs seems to be sufficient to induce ectopic NPY expression. But in sharp contrast to GAERS, SWDs are also detectable in hippocampus in this mice model (Chafetz, Nahm et al. 1995).
Therefore, GAERS seem to be the only known animal model with generalized absence seizures in which immature animals already display changes in NPY expression although seizures are not yet present. Taken together, these data implicate that increases in NPY contents in adult animals are a consequence of non-convulsive seizures intended to counteract epileptic activity. The seizure-induced overexpression of BDNF and NPY may be a first stage of an endogenous mechanism that controls epileptogenesis. Nevertheless, the epileptic process can overcome the endogenous protection and seizures may develop (Reibel, Larmet et al. 2000a). In line with this hypothesis, NPY is released from nRT neurons during electrical stimulation-induced oscillatory thalamocortical activity in non-epileptic rat brain slices. NPY release autoregulates the termination of absence seizures by

activation of Y1 receptors leading to an enduring suppression of nRT excitability (Sun, Bandrowski et al. 2002). Increased synthesis of NPY in juvenile GAERS is not a consequence of SWDs but rather a genetic alteration underlying absence seizures.
An outstanding finding of this thesis is the drastic loss of NPY immunoreactivity occurring during maturation. A similar decrease has been observed previously. In the early post-natal cortex, about 3% of all neurons are NPY-positive. The number of peptide containing neurons then drastically decreases in the second post-natal months to 0.5-1% which is not due to neuronal death but merely to a cessation of NPY-production. Neurons are still able to produce NPY on demand, as e.g. after application of BDNF (Wirth, Patz et al. 2004). Additionally, decreases in NPY-immunopositive neurons as well as in BDNF concentration and pCREB-immunoreactivity occur age-dependently in the hippocampus of Fischer 344 rats (Hattiangady, Rao et al. 2005). Age-related changes in NPY expression levels are depending on strain and sex of the animals investigated.
The decline in NPY content occurs in both, NEC and GAERS, but is significantly more pronounced in hippocampus of GAERS. Decrease of BDNF signaling cannot cause this radical changes in NPY expression as also NEC are affected although TrkB expression and CREB phosphorylation remain unchanged in these rats. Normal maturation seems to generate these alterations. The drastic diminishment in NPY expression in GAERS and therewith the critical decline in the endogenous antiepileptic potential allows the onset of SWDs. It is conceivable that newborn GAERS suffer already from absence epilepsy but that extremely high levels of the anticonvulsant neuropeptide NPY prevent the occurrence of SWDs.
Further evidence that NPY also acts as potent anti-absence agent originates from functional studies. Icv application of NPY reduces the occurrence of SWDs in adult GAERS without affecting the frequency of the remaining discharges. Therefore, Stroud et al. (2005) suggest that NPY does not inhibit nRT, the pacemaker of SWDs, but interrupts thalamocortical oscillations via its action on the ventrobasal thalamus or cortex. The authors hypothesize that sole activation of the Y2 receptor is sufficient for the anti-absence effect of NPY (Morris, Gannan et al. 2007). Our results corroborate this hypothesis. Treatment with an agonist specific for Y2 receptors mimics the NPY effect. The occurrence of SWDs is already markedly reduced 30 minutes after application and remains suppressed for the whole 2 hours recorded. Unfortunately, this specific agonist exerts epileptogenic effects in the hippocampus probably due to diffusion of the drug into this brain region. Support for the additional involvement of Y1 receptors derives from functional studies in juvenile GAERS. Application of an Y1 receptor antagonist leads to the first-time appearance of SISWDs, whereas Y2 receptor antagonists increase the incidence of the discharges. Selective blocking of Y5 receptors had no obvious effects on absence seizures suggesting that this subtype is not involved in the generalization of absence seizures. All these treatments were well tolerated by the experimental animals with no adverse behavioral changes. The immature SISWD pattern consists of slow wave train with a fundamental frequency significantly lower than in adult animals as comparable with the results obtained by Carcak et al. (2008). Development and propagation of seizures as well as their EEG features differ considerably between immature and mature brain (Holmes 1997).

4.3 Mechanisms of focal and generalized absence seizures partially exclude each other

Pharmacological differences between generalized and focal epilepsy exist both in humans and in experimental animals. Two classes of anticonvulsant drugs are established, those effective against absence attacks and those used to treat other epileptic seizures (Giaretta, Avoli et al. 1987). In humans, coexistence of focal epilepsy, such as TLE, and idiopathic generalized epilepsy in the same patient is very rare with an incidence of about 0.5-1% (Koutroumanidis, Hennessy et al. 1999; Nicolson, Chadwick et al. 2004). Febrile seizures and a positive family history are frequently observed in patients suffering from both seizure types. The reason for this rarity is poorly understood. Prognosis can be good if the disease is treated with an appropriate broad-spectrum antiepileptic drug (Regesta and Tanganelli 1999).
Similar observations are made in animal models for generalized absence seizures. Adult GAERS fail to progress beyond stage 2 seizures in the amygdala kindling paradigm and afterdischarge duration is markedly shorter than in NEC (Eskazan, Onat et al. 2002). During amygdala kindling, thalamic activity in GAERS may generate the resistance to secondary generalization of limbic seizures. In WAG/Rij, another model for typical absence seizures, rats can be divided into three groups according to their susceptibility to amygdala kindling: kindling-resistant, slow-kindled and rapid-kindled rats. Basal SWD durations are highest in GAERS, followed by kindling-resistant WAG/Rij rats and are significantly different from the durations measured in kindled WAG/Rij rats (Onat, Aker et al. 2007). Therefore, the intensity of SWDs correlates negatively with the susceptibility to amygdala kindling (Aker, Yananli et al. 2006). In accordance with these results, the first convulsive seizure as well as the first spontaneous seizure after intra-amygdaloid injection of kainic acid is significantly delayed in GAERS as compared to NEC (Gurbanova, Aker et al. 2008). Vergnes et al. (2000) tested the susceptibility of GAERS to kainic acid. First seizures after low doses of kainic acid are less severe in GAERS with longer latency, shorter duration and smaller quantity. In line with these results, status epilepticus occurs more frequently in NEC than in GAERS after high doses of kainic acid.
A maturation study in GAERS reveals that kindling rate in GAERS is slower than in NEC for all ages investigated. However, 20 days old GAERS are kindling-prone and the resistance against kindling starts at 30 days of age, when first SWDs appear, and increases with age. As 20 days old GAERS are already partially protected against kindling although no SWDs are present at this age, no causal link exists between SWDs themselves and the kindling resistance (Carcak, Aker et al. 2008). The inhibitory mechanisms underlying generalized absence seizures seem to have antagonistic effects on the kindling process. We hypothesize that the protection against limbic seizures in GAERS is at least partly due to increased expression of the endogenous antiepileptic peptide NPY in this animal strain.

A similar protection against generalized tonic-clonic seizures is observed in immature rats. In contrast to adult rats, it is not possible to induce status epilepticus by application of kainic-acid or pilocarpine in pups (Albala, Moshe et al. 1984; Cavalheiro, Silva et al. 1987). Additionally, repetitive status epilepticus episodes in juvenile rats induce electrographic and behavioral epileptic features but not TLE (dos

Santos, Arida et al. 2000). One possible explanation might be that the higher NPY concentrations observed in immature rats exert protective effects against limbic seizure as suggested for absence epilepsy.

Limbic structures are not involved in the occurrence of SWDs, although they are seizure-prone and participate in the generalization of several seizure models. Nonetheless, the rare coexistence of focal and generalized seizures provides evidence for an interplay between corticothalamic and limbic seizures. Vulnerability to seizure activity in any part of the neuronal network seems to be influenced by activity everywhere else. Brain regions beyond the thalamocortical network are participating in some aspects of absence seizures. The following results confirm this hypothesis. Measurement of local cerebral blood flow at early stages of amygdala kindling reveals that more brain regions are activated in GAERS as compared to NEC. In addition to somatosensory cortex and thalamus, numerous limbic regions are activated (Carcak, Ferrandon et al. 2009). Local cerebral metabolic rates of glucose stay elevated in adult GAERS even after suppressing SWDs by ethosuximide treatment (Nehlig, Vergnes et al. 1993). Although no SWDs are recordable in limbic structures, hippocampal circuits become synchronized as seizures occur within the thalamocortical loop. When inhibition in the hippocampus is diminished, thalamocortical SWDs spread into this brain region leading to atypical absence seizures (Velazquez, Huo et al. 2007). Further evidence for a thalamo-limbic interaction is the observation that simultaneous stimulation of nRT with hippocampal kindling counteracts the generalization of seizures. The nucleus reuniens is the only thalamic structure with direct projections to the hippocampus. nRT stimulation may influence spreading of discharges by inhibiting the neurons of this nucleus (Nanobashvili, Chachua et al. 2003).
In conclusion, these studies implicate a critical role for limbic structures in generalized absence seizures, possibly in preventing the spread of hyperactivity to the whole brain.

Information from cerebral cortex, thalamic nuclei, amygdala and hippocampus are processed to the striatum and transferred to basal ganglia which are involved in the control of seizure activity. Propagation and/or the generation of different types of seizure activity in animals can be controlled by the substantia nigra pars reticulata which contains GABAergic neurons. In GAERS, rhythmic discharges of cortico-striatal neurons contribute to the propagation of oscillatory cortical activity to basal ganglia (Slaght, Paz et al. 2002). Maturation studies reveal that local cerebral metabolic rates for glucose are already increased in 21 days old GAERS in four limbic structures (entorhinal and piriform cortices, hippocampus and basolateral amygdala) where no SWDs are recorded in mature GAERS as well as in structures of the nigral inhibitory system (substantia nigra pars reticulata, superior colliculus and globus pallidus) (Nehlig, Vergnes et al. 1998a; Nehlig, Vergnes et al. 1998b). Genetic mutations appear to be evident first in regions that could participate actively in the control of epileptogenesis. The cerebral metabolic activity is already increased when the anatomical substrate for absence seizures may still be too immature to generate SWDs. Genetic mutations seem to be responsible for these metabolic increases which may represent an inhibitory, seizure suppressing mechanism necessary to maintain the brain in the interictal state.

Based on a case report, Dematteis et al. (2003) speculate that increased activity in striatum may facilitate seizure arrest or limit seizure recurrence by inhibiting neurons of the substantia nigra pars reticulata. The involvement of the substantia nigra in the control of seizures has been proved by several lesions, biochemical and pharmacological studies. Bilateral activation of GABAergic transmission within the substantia nigra suppresses seizures in very different animal models of generalized seizures including the GAERS model or seizures with secondary generalization. Therefore, the striato-nigro-tectal pathway seems to play a critical role in seizure suppression (for review see Depaulis, Vergnes et al. 1994).

4.4 Memory performance in children and rats suffering from absence seizures

Discussing changes in expression or activation of BDNF and CREB inevitably leads to the question how learning and memory are affected.
General intelligence of children with typical absence seizures is within the normal range. Nevertheless, specific problems with attention, visual memory and fine-motor fluency are observed. Latter is improved with adequate anti-absence treatment (Siren, Kylliainen et al. 2007). Deficits in attention, verbal learning and word fluency persist despite well-controlled seizures (Henkin, Sadeh et al. 2005). Reading abilities of children with generalized absence seizures appear almost 2 years behind expectations based on school grade level. Some of the learning difficulties may be related to absences during school time and possible detrimental side effects of anticonvulsive medication (Vanasse, Beland et al. 2005).
In contrast, it has been shown that adult GAERS learn faster than NEC, though awareness is impaired during absences. This effect is not diminished by the application of $GABA_B$ receptor antagonists, although they completely suppress absence seizures (Getova, Bowery et al. 1997). Thus, the occurrence of SWDs is not directly associated with the superior performance of GAERS. Elevation of BDNF expression in GAERS might lead to this enhanced capacity. It has already been shown that the improved formation of spatial memory following exposure to an enriched environment is associated with an induction of BDNF mRNA expression in the pyramidal cells of hippocampal CA1 (Falkenberg, Mohammed et al. 1992). Elevated CA1 BDNF mRNA expression is also clearly associated with higher memory performance of senescent rats (Schaaf, Workel et al. 2001). A cause for better learning might be that BDNF is involved in the maintenance of long-term potentiation, possibly via activation of CREB (Alonso, Bekinschtein et al. 2005) or increased expression of CREB mRNA (Vaynman, Ying et al. 2003). Protein kinase A and CREB are activated in hippocampus during the process of spatial memory formation in rats (Mizuno, Yamada et al. 2002). Furthermore, BDNF is able to concurrently increase its mRNA levels and TrkB in the mechanism of synaptic plasticity (Vaynman, Ying et al. 2003). Learning training causes a time-dependent activation of the transcription factor CREB and simultaneous expression of BDNF gene in different regions of the forebrain (Ulloor and Datta 2005). BDNF signaling via TrkB/PLCγ/CaMK/CREB has been implicated in hippocampal long-term potentiation, acting largely independent from the Ras/ERK/RSK signaling pathway (Minichiello, Calella et al. 2002). Further support for the involvement of the BDNF signaling

cascade is the observation that learning and memory impairments observed during senescence are accompanied by considerable declines in BDNF, pCREB and NPY levels (Hattiangady, Rao et al. 2005). Moreover, reduced BDNF concentrations are found in hippocampus of patients with Alzheimer's disease (Hock, Heese et al. 2000) as well as a decline in NPY-immunoreactive cells (Chan-Palay, Lang et al. 1986).
In conclusion, elevated BDNF concentrations might at least in part contribute to the superior performance in adult GAERS.

4.5 Final conclusion

Animal models for absence seizures offer a unique opportunity for understanding the pathophysiology of epileptogenesis in animals and possibly, by extrapolation, in humans. Nevertheless, models show not necessarily the identical pathophysiology as human epilepsy, although the EEG and behavioral picture of a model looks similar to a clinical seizure type.
Notwithstanding our work clearly demonstrates the involvement of BDNF and NPY in the pathophysiology of absence seizures in GAERS, their role in human absence epilepsy has to be established in clinical studies.

5 Outlook

Epilepsy remains a major health problem associated with recurrent spontaneous seizures. The drug-resistant epilepsies and partially severe adverse reactions are two reasons for the need of new potent antiepileptic drugs designed to block the process of epileptogenesis.
For several reasons the choice of anticonvulsant drugs is especially difficult in women: (1) Reproductive endocrine disorders, e.g. irregular menstrual cycles, weight gain and hirsutism, have been associated with antiepileptic drugs, especially valproate. (2) Clearance of oral contraceptive pills is increased by hepatic enzyme-inducing antiepileptic drugs as phenytoin, carbamazepine, phenobarbital, topiramate and oxcarbazepine. (3) Oral contraceptive pills and pregnancy enhance the clearance of many antiepileptic drugs. (4) No antiepileptic drug can be considered as absolutely safe during pregnancy (French and Pedley 2008).
Valproate is currently the first-line treatment for generalized seizures. Weight gain and teratogenicity are two problematic adverse reactions associated with this drug. Valproate is a major antiepileptic drug with a broad spectrum of activity. It is effective against absence seizures as well as focal and generalized tonic-clonic seizures. The mechanism of action is still not completely clarified. An interesting observation is that chronic valproate treatment specifically increases NPY expression in the nRT (Brill, Lee et al. 2006).

The results of this thesis suggest the use of BDNF and NPY in the treatment of generalized absence seizures.
Anti-absence therapy with BDNF seems to be difficult because of its ambivalent actions in epileptogenesis. On one hand, BDNF is a key mediator of the epileptogenic process favoring the development and progression of epilepsy due to its excitatory properties. On the other hand, the neurotrophic actions of BDNF may prevent neuronal cell damage during seizures and induction of NPY synthesis counteracts epileptogenesis. Therefore, a therapeutic use of BDNF presumably will be associated with considerable challenges.
Implementation of NPY in the treatment of absence epilepsy appears to be more promising. First successes are achieved with NPY gene therapy against chronic spontaneous seizures in rats. Intra-hippocampal injection of a recombinant adeno-associated viral vector expressing the human NPY gene fourteen weeks after status epilepticus significantly reduces spontaneous seizure frequency and symptomatic progression of the disease (Noe, Pool et al. 2008). A major challenge will be the development of galenic forms which are non-invasively applied and tolerated by patients. An important point to keep in mind is the penetration into the brain by crossing the blood-brain-barrier of possible drug candidates. Therefore, low molecular weight non-peptidergic substances are preferred to NPY-alikes. They should be able to cross the blood-brain barrier and stimulate endogenous NPY synthesis and release or directly activate or block selectively NPY receptor subtypes.
The results of this thesis suggest that primarily Y2 and Y1 receptor subtypes are involved in the anti-absence effect of NPY. Drugs activating selectively these two receptor subtypes may be efficacious and well-tolerated new therapies for generalized epilepsies, for which current treatment options are limited. The adverse

reaction of weight gain will probably not be a problem, as mean food intake is significantly increased by NPY action via Y5 receptor (Morris, Gannan et al. 2007), whereas Y2 receptor subtypes are not involved in the orexigenic effects of NPY (Silva, Cavadas et al. 2002).

Drugs mimicking NPY represent powerful treatments not only against absence epilepsy (this study and Stroud, O'Brien et al. 2005; Morris, Gannan et al. 2007), but also against various other epileptic syndromes (Marksteiner and Sperk 1988; Smialowska, Bijak et al. 1996; Madsen, Woldbye et al. 1999; Reibel, Benmaamar et al. 2003). The wide range of therapeutic use is similar to valproate giving these drugs tremendous potential. Once the therapy is well-tolerated and without severe adverse reactions, it will be a valid alternative to valproate treatment.

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7 Appendix

7.1 Abbreviations

ACSF	artificial cerebrospinal fluid
BAD	Bcl-2 associated death promoter
BDNF	brain-derived neurotrophic factor
CA	hippocampal cornu ammonis
CaMK	calcium/calmodulin-dependent kinase
cAMP	3',5'-cyclic adenosine monophosphate
CRE	3',5'-cyclic adenosine monophosphate response element
CREB	3',5'-cyclic adenosine monophosphate response element binding protein
CT	corticothalamic
C_t	threshold cycle
DAB	diaminobenzidine
DG	dentate gyrus
DMSO	dimethylsulfoxide
EEG	electroencephalogram
EPSPs	excitatory postsynaptic potentials
ERK	extracellular signal-regulated kinase
GABA	γ-amino-butyric acid
GAERS	Genetic Absence Epilepsy Rats from Strasbourg
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GIRK	G-protein-activated inwardly rectifying potassium
icv	intracerebroventricular
ILAE	International League Against Epilepsy
IN	interneuron
IP_3	inositol 1,4,5-triphosphate
IPSPs	inhibitory postsynaptic potentials
MAPK	mitogen-activated protein kinase
NEC	non-epileptic controls
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	nerve growth factor
NPY	neuropeptide Y
NPYR	neuropeptide Y receptor
nRT	nucleus reticularis thalami
NT3	neurotrophin-3
NT4/5	neurotrophin-4/5
$p75^{NTR}$	neurotrophin receptor p75
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PLCγ	phospholipase Cγ
qRT-PCR	quantitative real-time polymerase chain reaction
RSK	ribosomal s6 kinase
SD	standard deviation
SISWDs	short irregular spike-and-wave discharges

SWDs	spike-and-wave discharges
TC	thalamocortical
TLE	temporal lobe epilepsy
Trk	tropomyosin-related tyrosine kinase receptor
WAG/Rij	Wistar Albino Glaxo/Rijswijk

7.2 Poster presentations

During the time of my doctoral thesis I had the opportunity to present my work to a broad audience of experts in neurobiology during a European and American congress. Abstract and poster for the respective conventions are given below.

7.2.1 6th FENS Forum of European Neuroscience

Geneva, Switzerland, July 12-16, 2008

The occurrence of spike-and-wave discharges in young Genetic Absence Epilepsy Rats from Strasbourg (GAERS) coincides with a decrease of cortical neuropeptide Y levels.

Landweer S[1], Otten U[1], Kunz D[1], Nitsch C[2], Boehrer A[3] and Bernasconi R[1]

[1] Institute of Physiology, University of Basel
[2] Institute of Anatomy, University of Basel
[3] INSERM U596 Strasbourg

Absence seizures are associated with the abrupt occurrence of bilaterally synchronous 3/s spike-and-wave discharges (SWDs) in the EEG over wide cortical areas. They arise from an aberration of the interplay between the cortex and thalamus. Non-linear association analysis has identified a specific site of seizure generation within the peri-oral region of the primary somatosensory cortex (Meeren et al. J. Neurosci. 2002, 22, 1480). Molecular and structural mechanisms involved in absence epilepsy have not yet been identified, while a genetic component for its aetiology has been suggested. We used juvenile and adult GAERS to study the role of neuropeptide Y (NPY) in absence seizures. Determination of mRNA levels and quantification of NPY-immunoreactive neurons show higher NPY levels in sensorimotor cortex of GAERS as compared to age-matched non-epileptic control (NEC) rats. There are much more NPY-positive cells in CA1 and dentate gyrus regions of GAERS as compared to NEC. In both, cortex and hippocampus, NPY expression is more pronounced in juvenile than in adult rats. In contrast, there is no difference in expression levels between GAERS versus NEC in striatum and basal forebrain. Our results show that occurrence of SWDs correlates with a significant decrease of NPY expression suggesting that NPY prevents SWDs.

The occurrence of spike-and-wave discharges in adult Genetic Absence Epilepsy Rats from Strasbourg (GAERS) coincides with a decrease of cortical neuropeptide Y levels.

Landweer S[1], Otten U[1], Kunz D[1], Nitsch C[2], Boehrer A[3] and Bernasconi R[1]

[1] Institute of Physiology, University of Basel; [2] Institute of Anatomy, University of Basel, [3] INSERM U596 Strasbourg

150.18

Introduction

Absence seizures are associated with the abrupt occurrence of bilaterally synchronous 3 Hz spike-and-wave discharges (SWD) in the EEG over wide cortical areas. They arise from an aberration of the interplay between the cortex and thalamus. Non-linear association analysis has identified a specific site of generation within the peri-oral region of the primary somatosensory cortex (Meeren et al., 2002, Journal of Neuroscience, 22, 1480-1495). Molecular and structural mechanisms involved in absence epilepsy have yet not been identified, while a genetic component for its aetiology has been suggested.

Using juvenile and adult male GAERS we investigated the role of neuropeptide Y (NPY) in generalized non-convulsive epilepsy. Juvenile GAERS which are younger than 30 days fail to show SWD. With increasing age the number of GAERS with SWD gradually increases and reaches 100% at the age of 3 months (Danober et al., 1998, Progress in Neurobiology, 55, 27-57).

Goal of this study was to demonstrate the impact of NPY on the occurrence of SWD.

Methods

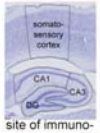
Experiments were performed in juvenile (<30 days) and adult (>3 months) GAERS and age-matched non-epileptic control animals (NEC).

Quantitative immunohistochemical analysis with stereological counting of NPY immunopositive neurons was carried out in the three hippocampal subregions (dentate gyrus (DG), CA1 and CA3), somatosensory cortex and striatum of juvenile and adult GAERS and NEC.

Stainings were performed using the avidin-biotin-immunoperoxidase method with diaminobenzidine as chromogen.

NPY mRNA expression was investigated in homogenates of total hippocampus, somatosensory cortex and thalamus using real-time polymerase chain reaction with SYBR green as fluorescent dye. Beta-actin was used as internal standard. Quantification of results was performed using the comparative C_t method.

Results were subjected to statistical analysis using the Student's t-test. Differences were considered to be statistically significant at p-values <0.05 (*) or highly significant at p-values <0.01 (**).

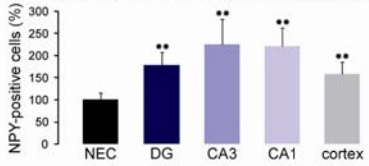


site of immuno-histochemical analyis

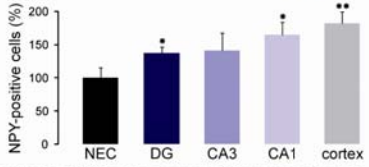
Results

1. More NPY-positive cells are visible in the hippocampal subregions DG, CA1 and CA3 and the somatosensory cortex of juvenile as well as adult GAERS as compared to age-matched NEC.

A Quantification of NPY-positive neurons in juvenile animals

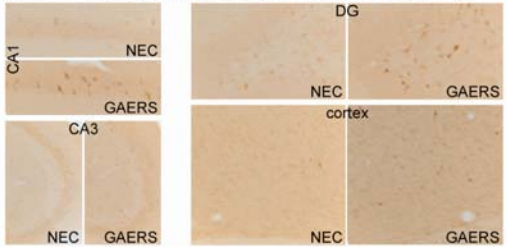


B Quantification of NPY-positive neurons in adult animals



Quantification of NPY-positive neurons in different brain regions of **juvenile** (A; n=6) and **adult** (B; n=3) GAERS expressed as percentage of levels measured in age-matched NEC. Representative images of NPY-immunoreactivity in several brain regions of **juvenile** animals (C).

C NPY-immunoreactivity in several brain regions of juvenile animals



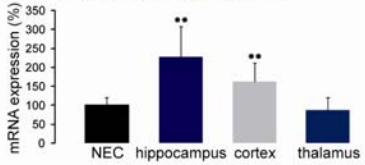
The number of NPY-positive neurons is highly elevated in the three hippocampal subregions and in somatosensory cortex of juvenile as well as adult GAERS compared to age-matched NEC.

In contrast, there is no difference in expression levels between GAERS versus NEC in striatum.

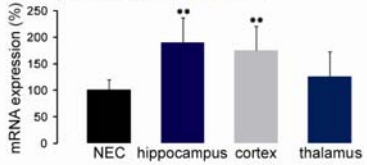
NPY-immunopositive neurons are absent in the thalamus of either juvenile or adult GAERS and NEC.

2. NPY mRNA expression is highly increased in whole hippocampus and somatosensory cortex of juvenile as well as adult GAERS as compared to age-matched NEC.

A NPY mRNA expression in juvenile animals

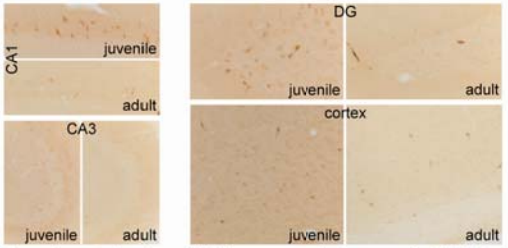


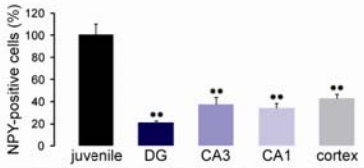
B NPY mRNA expression in adult animals



NPY mRNA expression in different brain regions of **juvenile** (A) and **adult** (B) GAERS expressed as percentage of appropriate levels measured in age-matched NEC (n=10).

3. NPY expression is more pronounced in all brain regions of juvenile as compared to adult GAERS.





Number of NPY-positive neurons in different brain regions of **adult GAERS** compared to expression levels in **juvenile GAERS**. Data are expressed as percentage of levels measured in juvenile GAERS. Representative images of NPY-immunoreactivity in juvenile and adult **GAERS** are shown on the left side.

A prominent reduction of NPY-immunoreactivity occurs in GAERS with maturation and occurence of SWD.

Conclusions

1. **Major decreases in NPY expression correlate with the occurrence of absence seizures in GAERS.**
2. **The appearance of SWD is associated with a significant decrease of NPY expression in the somatosensory cortex and in the three subregions of the hippocampus analyzed.**
3. **It was already shown that i.c.v. application of NPY and of a selective Y_2 agonist suppresses SWD in adult GAERS** (Morris et al., 2007, European Journal of Neuroscience, 25, 1136-1143).
4. **Thus, our data strongly suggest that NPY is an endogenous antiepileptic factor preventing SWD.**
5. **Furthermore, our results support the concept that the primary somatosensory cortex and not the thalamus initiates SWD.**

Outlook

Functional studies to identify the NPY receptor subtype/s involved in the protection against absence seizures are in progress.

Acknowledgement

This work was supported by grants of „Schweizerische Liga gegen Epilepsie", „Stiftung Emilia Guggenheim-Schnurr der Naturforschenden Gesellschaft Basel" and „Freiwillige Akademische Gesellschaft Basel".

7.2.2 38th Annual Meeting of the Society of Neuroscience

Washington DC, USA, November 15-19, 2008

Neuropeptide Y is an endogenous antiepileptic factor against absence seizures.

Landweer S.[1,3], Otten U.[1], Nitsch C.[2], Juhasz G.[3], Boehrer A.[4] and R. Bernasconi[1].

[1]Institute of Physiology, University of Basel, Switzerland
[2]Institute of Anatomy, University of Basel, Switzerland
[3]ELTE Budapest, Hungary
[4]INSERM U 596 Strasbourg, France

Using juvenile and adult male Genetic Absence Epilepsy Rats from Strasbourg (GAERS) we investigated the role of brain-derived neurotrophic factor (BDNF) and neuropeptide Y (NPY) in generalized non-convulsive epilepsy. Goal of this study was to demonstrate the impact of these neuromodulators on the occurrence of spike-and-wave discharges (SWDs). Expression levels were determined using Western blot analyses, real-time polymerase chain reaction and stereological counting of NPY immunopositive neurons. Our results indicate that in the somatosensory cortex elevated neuronal activity in adult GAERS is accompanied by an increase in BDNF protein levels. In contrast, hippocampal BDNF is already elevated in juvenile GAERS which do not yet display SWDs. Moreover, we found highly increased NPY mRNA expression in the somatosensory cortex and hippocampus of both, juvenile and adult GAERS as compared to age-matched non-epileptic control animals (NEC). In addition, quantitative immunohistochemical analyses revealed more NPY-positive cells in the hippocampal subregions CA1, CA3 and dentate gyrus of GAERS as compared to NEC. In both cortex and hippocampus, NPY expression is more pronounced in juvenile than in adult animals. NPY-immunopositive neurons were absent in the thalamus of either juvenile or adult GAERS and NEC.
Our results demonstrate that major changes in NPY expression correlate with the occurrence of absence seizures in GAERS. Appearance of SWDs associated with a significant decrease of NPY expression suggests a protective mechanism for this peptide against absence seizures in juvenile GAERS. Furthermore, these results support the concept that the primary somatosensory cortex and not the thalamus initiates SWDs. Functional studies to identify the NPY receptor subtype/s involved in the protection against absence seizures are in progress.

Neuropeptide Y is an endogenous antiepileptic factor against absence seizures.

Landweer S.[1,3], Otten U.[1], Nitsch C.[2], Juhasz G.[3], Boehrer A.[4] and R. Bernasconi[1]

[1] Institute of Physiology and [2] Institute of Anatomy, University of Basel, Switzerland; [3] ELTE Budapest, Hungary; [4] INSERM U596 Strasbourg, France

449.14

s.landweer@unibas.ch

Aim of the study

Define the impact of NPY and BDNF on the occurrence of spike-and-wave discharges.

Introduction

Absence seizures are associated with the abrupt occurrence of bilaterally synchronous 3 Hz spike-and-wave discharges (SWDs) over wide cortical areas. They arise from an aberration of the interplay between the cortex and thalamus. Site of generation has been proposed within the deep layers of the facial somatosensory cortex (Polack et al., 2007, Journal of Neuroscience, 27, 6590-6599). Mechanisms involved in absence epilepsy have not yet been identified.

Using juvenile and adult male Genetic Absence Epilepsy Rats from Strasbourg (GAERS) we studied the role of neuropeptide Y (NPY) and brain-derived neurotrophic factor (BDNF) in generalized non-convulsive epilepsy.

Methods

Experiments were performed in juvenile (<30 days, seizure free) and adult (>3 months, frequent spontaneous SWDs) GAERS and age-matched non-epileptic control animals (NEC).

NPY mRNA expression was investigated using qRT-PCR with SYBR green as fluorescent dye and beta-actin as internal standard. Quantification was performed using the comparative C_t method.

Quantitative immunohistochemical analysis with stereological counting of NPY-immunopositive neurons was carried out using the avidin-biotin-immunoperoxidase method with diaminobenzidine as chromogen.

NPY-2R protein levels were measured using Western blot technique with a chemiluminescence system for detection. Anti-glyceraldehyd-3-phosphate dehydrogenase (GAPDH) was used as housekeeping-protein for normalization of protein loading.

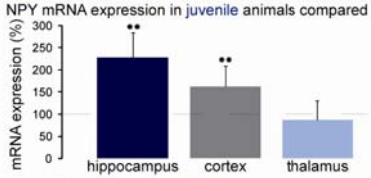
Functional studies were performed in adult GAERS by i.c.v. injection of BDNF. EEG was recorded using 4 cortical screw electrodes and a reference and ground electrode over the cerebellum.

Statistical analysis was performed using the Student's t-test and significant differences indicated $p<0.05$ (*) or $p<0.01$ (**).

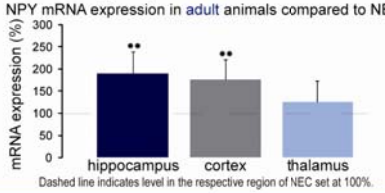
Results

1. NPY mRNA is markedly increased in parietal cortex and hippocampus of both, adult and juvenile GAERS.

A NPY mRNA expression in juvenile animals compared to NEC



B NPY mRNA expression in adult animals compared to NEC

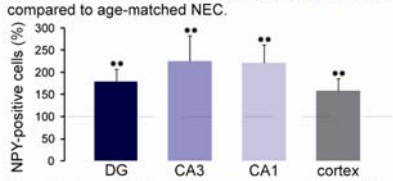


Dashed line indicates level in the respective region of NEC set at 100%.

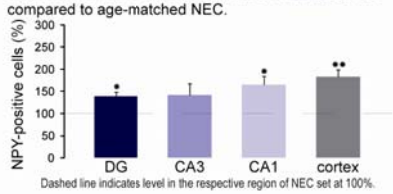
Results

2. Number of NPY-positive cells is markedly elevated in juvenile and adult GAERS compared to age-matched NEC. A marked decrease occurs with maturation.

A Quantification of NPY-positive neurons in juvenile GAERS compared to age-matched NEC.

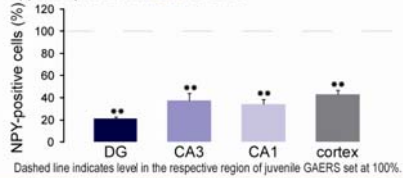


B Quantification of NPY-positive neurons in adult GAERS compared to age-matched NEC.



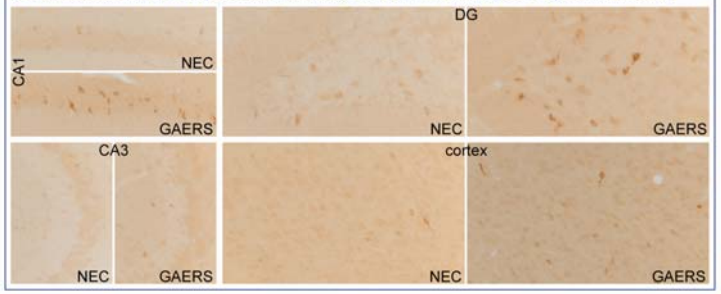
Dashed line indicates level in the respective region of NEC set at 100%.

C NPY expression is more pronounced in juvenile cortex and hippocampus than in adult GAERS.

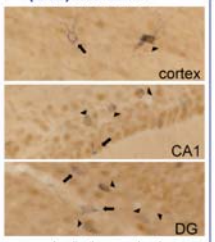


Dashed line indicates level in the respective region of juvenile GAERS set at 100%.

3. Visualization of NPY expression differences in several brain regions of juvenile animals



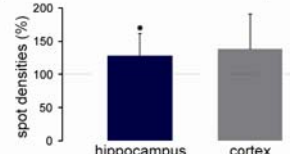
4. Colocalisation of NPY (blue) and BDNF



colocalisation: arrowheads
NPY only: arrows

5. NPY-2R protein levels are slightly elevated in hippocampus and cortex of adult GAERS compared to NEC.

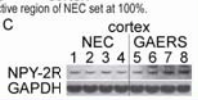
A



Dashed line indicates level in the respective region of NEC set at 100%.

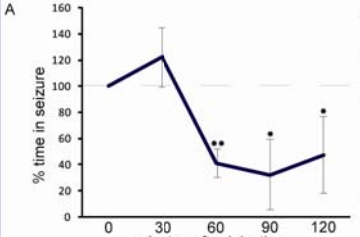
B

C



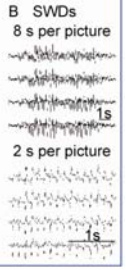
6. I.c.v. application of BDNF (1µg) suppresses absence seizures similar as already shown for NPY.

A



Dashed line indicates baseline SWDs set at 100%.

B SWDs



Conclusions

1. **NPY mRNA levels are markedly increased in parietal cortex and hippocampus of adult and juvenile GAERS.**
2. **The marked decrease in NPY-positive cells in adult GAERS coincides with the occurrence of SWDs.**
3. **NPY-2R protein levels are slightly elevated in hippocampus and parietal cortex of adult GAERS.**
4. **The majority of NPY-immunoreactive neurons also contain BDNF, whereas more than 50% of the BDNF-positive neurons lack NPY.**
5. **I.c.v. application of NPY suppresses SWDs in adult GAERS** (Morris et al., 2007, European Journal of Neuroscience, 25, 1136-1143)**, a similar but delayed effect is seen after intracerebral application of BDNF.**
6. **Thus, our data strongly suggest that NPY is an endogenous antiepileptic factor preventing SWDs.**
7. **Investigation of the effects of different NPY antagonists in juvenile GAERS is in progress.**

Abbreviations

BDNF, brain-derived neurotrophic factor; CA, hippocampal cornu ammonis; DG, dentate gyrus; GAERS, Genetic Absence Epilepsy Rats from Strasbourg; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; i.c.v., intracerebroventricular; NEC, non-epileptic control rats; NPY, neuropeptide Y; NPY-2R, NPY receptor Y_2; qRT-PCR, quantitative real-time polymerase chain reaction; SWDs, spike-and-wave discharges

Acknowledgement

This work was supported by grants of „Schweizerische Liga gegen Epilepsie", „Stiftung Emilia Guggenheim-Schnurr der Naturforschenden Gesellschaft Basel", „Freiwillige Akademische Gesellschaft Basel" and „Senglet Stiftung zur Förderung des Pharmazeutischen Nachwuchses in Basel".

7.3 Curriculum vitae of Svenja Landweer

Education

01.2006 – 05.2009	PhD thesis “Role of neurotrophins and neuropeptides in Genetic Absence Epilepsy Rats from Strasbourg (GAERS), a model for human generalized absence seizures” Molecular Neurobiology Research Group, Institute of Physiology, University of Basel, Switzerland
06.2008 – 10.2008	Doctoral research internship Institute of Biology, Eötvös Lorand University of Budapest, Hungary
10.2000 – 09.2005	Study of Pharmaceutical Sciences, University of Basel, Switzerland 09.2005 Final Examen, grade 5.6 09.2004 Diploma Examen, grade 6
05.2004 – 09.2004	Diploma thesis “Role and function of neurotrophins and IL-6 in the pathogenesis of Morbus Alzheimer” Institute of Molecular Pharmacy, University of Basel, Switzerland
08.1996 – 08.2000	Kantonsschule Heerbrugg SG, Switzerland, Typus B

Educated by

Aebi, U.; Anselmetti, D.; Arber, S.; Beier, K.; Betz, G.; Bickle, T.A.; Bienz, K.A.; Boelsterli, U.; Boller, T.; Bongartz, G.; Brenner, A.; Brenner H.R.; Dopfer, O.; Drewe, J.; Eberle, A.; Engel, A.; Engel, J.; Erb, P.; Ernst, B.; Folkers, G.; Gehring, W.J.; Gescheidt, G.; Guentert, T.W.; Güntherodt, H.J.; Grzesiek, S.; Hauri, H.P.; Hauser, P.; Hersberger, K.; Huber, H.; Huwyler, J.; Imanidis, G.; Im Hof, H.C.; Jenal, U.; Keller, W.; Kessler, M.; Kiefhaber, T.; Körner, C.; Krähenbühl, S.; Kunz, D.; Leuenberger, H.; Lüdin, E.; Lüthi, A.; Marrer, S.; Mayans, O.; Meier, B.; Meier, C.; Meier, T.; Melchers, F.; Meyer, J.; Meyer, U.A.; Monard, D.; Mühlebach, S.; Müller, H.J.; Müller, J.; Müller, S.; Neri, D.; Otten, U.; Pfleiderer, G.; Rehmann-Sutter, C.; Reichert, H.; Rolink, A.; Rüegg, M.; Schaffner, W., Schirmer, T.; Schlienger, R.; Schmid, B.J.; Schmid, V.; Schönenberger, C.; Scholer, A.; Seelig, J.; Seelig-Löffler, A.; Séquin, U.; Sick, I.; Spiess, M.; Spornitz, U.M.; Stoeckli, E.; Strazewski, P.; Vedani, A.; Wessels, H.P.; Wormser, R.; Zaugg, C.; Zuberbühler, A.

Work experience

10.2005 – 04.2009	Temporary job as pharmacist in public pharmacy Hard-Apotheke, Birsfelden BL, Switzerland
05.2005 – 08.2005	Assistance in hospital pharmacy Kantonsspital, Bruderholz BL, Switzerland
10.2004 – 05.2005	Assistance in public pharmacy Hard-Apotheke, Birsfelden BL, Switzerland
07.2003 – 09.2003	Traineeship in Molecular Biology and Biochemistry Institute of Molecular Pharmacy, University of Basel, Switzerland
02.2003	Traineeship in hospital pharmacy Kantonsspital St. Gallen, Switzerland
02.2001 & 10.2001	Traineeship in public pharmacy Sternen-Apotheke, Altstätten SG, Switzerland

Publications

Landweer S *et al.* (2010) On the protective role of neuropeptide Y against spike-and-wave discharges in a genetic rat model of absence epilepsy.
The manuscript is submitted for publication.

Landweer S *et al.* (2010) Involvement of the brain-derived neurotrophic factor – tyrosine kinase receptor B – neuropeptide Y cascade in suppressing absence seizures in a genetic rat epilepsy model.
The manuscript is submitted for publication.

Landweer S (2008) Role of Neurotrophins in Epilepsy - a Progress Report. *Epileptologie* **25**, 35-42

Schnydrig S, Korner L, **Landweer S** *et al.* (2007) Peripheral lipopolysaccharide administration transiently affects expression of brain-derived neurotrophic factor, corticotropin and proopiomelanocortin in mouse brain. *Neuroscience Letters* **429**, 69-73

Grants/Awards

„Stipendium der Senglet-Stiftung zur Förderung des pharmazeutischen Nachwuchses in Basel“ 2008
„Förderbeitrag der Freiwilligen Akademischen Gesellschaft Basel“ 2008
„Förderbeitrag von der Stiftung Emilia Guggenheim-Schnurr“ 2007
„Forschungsförderungsbeitrag der Schweizerischen Liga gegen Epilepsie“ 2006
„Amedis Förderpreis für ApothekerInnen“ 2004