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Endogenous Neuropeptide Y Prevents Recurrence of Experimental Febrile Seizures by Increasing Seizure Threshold

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Abstract

Febrile seizures (FSs) typically occur at the onset of fever and do not recur within the same febrile episode despite enduring or increased hyperthermia. Recurrent seizures during the same febrile episode are considered "complex," with potentially altered prognosis. A characterized immature rat model of FS was used to test the hypotheses that (1) a first FS influences the threshold temperature for subsequent ones, and (2) the underlying mechanisms involve the release and actions of the endogenous inhibitory hippocampal neuropeptide Y (NPY). Experimental FSs were induced two or three times, at 3- to 4-h intervals, and threshold temperatures measured. To determine the potential effects of seizure-induced endogenous NPY on thresholds for subsequent seizures, an antagonist of the major hippocampal NPY receptor (type 2) was infused prior to induction of the second seizure. As an indicator of NPY release, NPY expression was determined 4 and 24 h later. Threshold core and brain temperatures for hyperthermic seizures were consistent with those observed during human fever. Threshold temperatures for a second and third seizure were significantly and progressively higher than those required for the first. This "protective" effect involved induction of endogenous NPY because it was abolished by the NPY antagonist. In addition, NPY mRNA expression was increased in dentate gyrus, CA3 and CA1, after an experimental FS, consistent with peptide release. Collectively these data indicate that the absence of repetitive seizures during a febrile episode involves the inhibitory actions of endogenous NPY, suggesting that the signaling cascade triggered by this peptide might provide targets for therapeutic intervention.

Index Entries

Seizures; fever; febrile; neuropeptide; NPY; hippocampus; animal model; rat; immature

Introduction

Febrile seizures (FSs) constitute the most prevalent seizure type in infants and young children, involving 2–5% of children under age 5 (Nelson and Ellenberg, 1981; Verity and Golding, 1991; Stafstrom, 2002). Remarkably, the processes occurring during and immediately after these seizures are poorly understood. For example, the identity of neuronal circuits that are activated, and whether a single FS predisposes to—or protects from—the occurrence of further seizures, have remained unresolved. Obvious limitations on the study of the mechanisms, pathophysiology, and cellular consequences of FSs derive from the inability to predict their timing or induce them in the human (Toth et al., 1998; Dubé et al., 2000; Baram, 2002).

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Observational studies of FSs have led to their classification into simple and complex (International League Against Epilepsy, 1981). Simple FSs carry a benign prognosis (Nelson and Ellenberg, 1981; Annegers et al., 1987; Verity and Golding 1991; Offringa et al., 1994; Berg and Shinnar, 1996a; Stafstrom, 2002), and analyses of their typical features have contributed to enhanced precision of epidemiological prospective studies addressing their outcome (Annegers et al., 1987; Berg and Shinnar, 1996a). The key clinical features of simple FSs include the occurrence of a single ictal event during a febrile illness (American Academy of Pediatrics, 1999). Not uncommonly, the seizure may be the first indication of the presence of fever (Offringa et al., 1994; American Academy of Pediatrics, 1999), and several studies have documented the relative scarcity of repetitive seizures within a single febrile episode (Annegers et al., 1987; Offringa et al., 1994; Berg and Shinnar, 1996b). Thus, although core temperatures that suffice to induce a FS might persist for several hours or might even be exceeded, repeated seizures occur uncommonly for example, in 13.7% of cases (Berg and Shinnar, 1996a). The occurrence of repeated seizures during a single febrile episode is unusual enough to be considered a “complex” feature by the Commission of the International League Against Epilepsy (International League Against Epilepsy, 1981; American Academy of Pediatrics, 1999) and is associated with a higher risk of subsequent unprovoked seizures, that is, epilepsy (Annegers et al., 1987; Berg and Shinnar, 1996a).

The paucity of further seizures, despite enduring high core temperatures, might result from dissociation between the measured core temperature and the actual brain temperature. This type of divergence has been demonstrated during neuropathological states, including ischemia and trauma, in both humans (Schwab et al., 1997; Henker et al., 1998) and animal models (Colbourne et al., 1993). If, during fever (or hyperthermia), core temperature rises disproportionately to that of brain, then threshold brain temperatures for the induction of seizures may not be reached. Alternatively, the first FS in itself might modulate neuronal excitability in the involved circuits, increasing the threshold temperature required for triggering additional seizures. Strong candidates for mediating such hours-long protection are endogenous inhibitory neuropeptides that might be released during the seizures, because these neuromodulators tend to exert actions within this time frame (Hokfelt et al., 2000). Among characterized peptides, evidence is mounting for the involvement of neuropeptide Y (NPY) in modulation of neuronal excitability under both physiological and pathological conditions, including those occurring during seizures (Klapstein and Colmers, 1993; Gruber et al., 1994; Vezzani et al., 1996; Woldbye et al., 1997). NPY, expressed in specific neuronal populations in hippocampus (Kohler et al., 1986; Gruber et al., 1994), has been found to reduce limbic network excitability both in vivo and in vitro (Klapstein and Colmers, 1993; Woldbye et al., 1997; Richichi et al., 2004).

Although the mechanisms preventing recurrent FSs are obviously important for the clinical management of infants and children with fever and FSs (Annegers et al., 1987; Offringa et al., 1994; American Academy of Pediatrics, 1999), they are difficult (if not impossible) to study in the human. Therefore, the goals of the current study were to utilize a characterized animal model in the immature rat (Toth et al., 1998; Dubé et al., 2000) for testing the hypothesis that a single hyperthermic seizure reduces the likelihood for a second seizure via activation of neuropeptide-mediated inhibition.

Materials and Methods

Experimental FS Paradigm

Sprague-Dawley-derived rats, born and maintained in quiet facilities under controlled temperatures (21–22°C), a 12-h light schedule, and unlimited access to food and water, were used for these experiments. All experimental procedures were approved by the Institutional

Animal Care Committee and conformed to the National Institutes of Health (NIH) guidelines for the care of experimental animals.

Experimental FSs were provoked as described previously (Toth et al., 1998; Dubé et al., 2000). Briefly, hyperthermia (i.e., increased body and brain temperatures) was induced in 10- to 11-d-old rats, using a warmed airstream directed ~30 cm above them. Core temperatures were measured before hyperthermia induction and at the onset of hyperthermia-provoked seizures. The correlation of behavioral and electrographic seizures that involve the hippocampal circuit has been documented (Dubé et al., 2000; Bender et al., 2003) and is shown in Fig. 1A. In general, there is excellent correlation between EEG and behavioral seizure duration. For evaluation of threshold temperatures, brain and/or rectal temperatures required for the induction of experimental FS were noted at the onset of seizures and hyperthermia was then terminated. For determination of NPY induction, hyperthermia was maintained for 30 min, resulting in seizure duration of approx 20 min (Dubé et al., 2000). For determination of threshold temperatures, seizures were stopped immediately after onset by discontinuing the hyperthermia. Animals were then placed on a cool surface and returned to their home cages. For determination of the effects of a single experimental FS on threshold temperatures of subsequent ones, seizures were induced two or three times, at 3- to 4-h intervals, to approximate the clinical situation of a febrile illness (Berg and Shinnar, 1996b).

Brain and Core Temperature Measurements

To verify the correlation of brain and core (rectal) temperatures associated with onset of seizures, brain temperatures were measured directly and validated using two separate methods. First, a group of immature rats ($n = 8$) were implanted with a stainless steel cannula positioned immediately above the dura (Baram et al., 1992), 24 h prior to the experiment. On the day of the experiment, baseline and threshold temperatures were obtained via the cannula, using a probe marked precisely to reach the dura. Each baseline measure was repeated in quintuplicate; threshold readings, by definition, were obtained once only. A second means for measuring brain temperature involved anesthetizing the skin of immature rats and directing the probe through the cranial suture to the dura ($n = 8$). Because the results were essentially identical to those obtained using the chronic cannula (35.62 ± 0.15°C vs. 35.54 ± 0.19°C, respectively), the latter method, associated with minimal discomfort, was chosen. It should be noted that permanent implants of temperature sensors are not feasible in the neonatal rat in the absence of sufficient subcutaneous tissue, as sensor implantation results in skin sloughing.

In Vivo Electrophysiology

To ascertain the concordance of the behavioral and electrophysiological seizures provoked by hyperthermia, a separate group of 9-d-old rats were implanted unilaterally in the dorsal hippocampus with bipolar twisted wire electrodes, as described previously (Baram et al., 1992; Dubé et al., 2000). On the following day, baseline hippocampal EEGs, as well as recordings during hyperthermia-induced seizures, were obtained in freely moving rats via long flexible wires, as described elsewhere (Baram et al., 1992; Dubé et al., 2000). Correct electrode placement was verified in all animals.

Determination of the Effects of NPY Y2 Receptor Antagonist on Subsequent Seizure Threshold Temperatures

To investigate the potential mechanisms underlying the increased resistance of limbic circuits to subsequent FSs, an NPY receptor type 2 (Y2R) antagonist was used (Malmstrom, 2001; Vezzani and Sperk, 2004). Specifically, BIIE0246 (courtesy of Dr. A. Vezzani) was dissolved in 25% polyethylene glycol 300 and administered at a dose of 100 nm/kg; 2.5 nm/μL, into the cerebral ventricle, 10 min before a second experimental FS was induced (4 h after the first).

To eliminate potential con-founders resulting from the infusion procedure, sham infusions were carried out also before the first seizure.

In Situ Hybridization Histochemistry and Analysis for NPY mRNA

In situ hybridization histochemistry (ISH) was performed on brain sections from animals sacrificed 4 or 24 h after experimental FS induction ($n = 6$ per group), as described previously (Brunson et al., 2001). Briefly, 20-μm coronal sections were mounted on gelatin-coated slides and stored at −80°C. Sections were thawed, air-dried, fixed in paraformaldehyde, dehydrated and rehydrated through graded ethanols, and exposed to 0.25% acetic anhydride in 0.1 *M* triethanolamine and dehydrated. Prehy-bridization (for 1 h) and hybridization (overnight) were performed at 40°C in a humidified chamber. The NPY deoxyoligonucleotide probe (Genosys) complementary to the coding region of NPY mRNA, was 3′-end-labeled with ^{35}S-dATP. Sections were washed as described (Brunson et al., 2001) and apposed to film (Hyperfilm β-Max; Amersham, Airlington Heights, IL) for 7 d.

For analysis of data, six anatomically matched sections, containing the dorsal hippocampus (anterior [A] 2.3–2.6 mm in reference to the interaural line [Sherwood and Timiras, 1970]), per animal, were used. Optical density of hybridization signal was measured over the regions of interest, without knowledge of treatment and by subtracting the density of corpus callosum for background, and compared with ^{14}C calibration standards, as described in detail in Eghbal-Ahmadi et al. (1999) and Brunson et al. (2001).

Data Analysis

Data are presented as mean ± S.E. The significance ($p < 0.05$) of observed differences among experimental and control groups was evaluated using ANOVA or Student's *t*-test with Welch's correction, as appropriate.

Results

Experimental FSs Involve the Hippocampus and Are Elicited at Physiological Temperatures

As described previously, hyperthermia-induced seizures were characterized by an arrest of heat-induced hyperactivity, body flexion, and biting of an extremity, occasionally followed by clonus (Dubé et al., 2000; Bender et al., 2003). The hippocampal electrographic (EEG) correlates of these seizures are shown in Fig. 1A. As apparent in the figure, the low-amplitude multiple-frequency background activity (top trace) was converted by the hyperthermia into rhythmic high-voltage-spike and spike-wave trains. Seizure threshold temperature (measured rectally) in this group of animals was 40.88 ± 0.3°C.

Brain and Core Temperatures in Immature Rats Are Correlated Both at Seizure Threshold and During Normothermic Conditions, but the Correlation Is Temperature Dependent

Repeated measurements of core and brain temperatures for each individual rat were tightly clustered and reproducible. Core values measured at 5-min intervals over a 25-min period in animals maintained on a euthermic pad were within 0.1°C for a given 10- to 11-d-old rat and averaged 32.88 ± 0.07°C ($n = 10$), as is shown in Fig. 1B. These temperatures were virtually identical to those obtained in rats upon removal from home cages (data not shown). Under the same conditions, brain temperatures for an individual rat were also highly consistent (Fig.1C). Interestingly, mean euthermic brain temperatures were 2.8°C higher than mean core values: The divergence of brain and core temperature was consistent among individual animals. In addition, this divergence was temperature dependent and approached zero at temperature ranges provoking seizures. Thus, at the onset of hyperthermic seizures (i.e., at threshold temperatures), brain temperatures averaged 40.7 ± 0.2°C ($n = 29$) compared with core

temperatures of 40.88 ± 0.3°C (n = 31). These data indicate that within the confines of the model and methods used here, core measurements provide an adequate approximation of brain threshold temperatures for experimental FSs.

A Single Hyperthermia-Induced Seizure Increases Threshold Temperature to a Subsequent One

To determine whether sustaining a single hyperthermic seizure influences the probability of having a second, core temperatures required to generate a FS were evaluated for rats subjected to hyperthermia once, twice, or three times at 3- to 4-h intervals. As shown in Fig. 2, baseline core temperatures of immature rats did not differ prior to each hyperthermia session (repeated measure ANOVA, p=0.991). However, threshold temperatures were significantly higher for the second seizure, compared with the first, and were elevated even further for the third seizure (repeated measure ANOVA, p = 0.0015; F = 7.53, degrees of freedom = 2). These data indicate that an experimental FS "protects" from developing another in response to subsequent hyperthermia by raising the temperature required to provoke a seizure.

Threshold Elevation for a Second Experimental FS Is Abolished by NPY Y2R

As shown in Fig. 3, administration of the selective antagonist of NPY Y2R abolished the increased threshold temperature for a second seizure compared with the first. (Threshold for the second seizure after NPY Y2R antagonist was 40.8 ± 0.2°C compared with 41.6 ± 0.2°C in animals treated with vehicle; $p < 0.05$; Fig. 3B). These findings are consistent with the involvement of endogenous NPY in the mechanisms underlying the increased resistance of limbic circuits to a second experimental FS.

Experimental FSs Result in an Enhanced NPY Expression

For endogenous CNS neuropeptides, release typically triggers synthesis (Hokfelt et al., 2000), so that although direct measurement of peptide release at individual synapses of neonatal hippocampus may not be feasible (because of subfemtomolar quantities and spatial constraints [Merali et al., 1998]), increased mRNA expression of a given peptide provides an adequate measure of its prior secretion. As shown in Fig. 4, ISH analysis of NPY expression within the hippocampal formation demonstrated increased steady-state NPY mRNA levels over the granule cell layer of the dentate gyrus (DG) already by 4 h after the seizures, and in DG, CA3 and CA1 areas, by 24 h. This finding is consistent with release-coupled synthesis of NPY, that is, with seizure-evoked release and actions of this endogenous inhibitory neuropeptide. In addition, NPY mRNA levels increased at 24 h after a 20-min hyperthermic seizures also in the medial amygdala nucleus but not in the prefrontal cortex (data not shown).

Discussion

The principal findings of these studies are: (1) In the immature rat model, having "a first FS" leads to increased threshold for additional ones; (2) this effect lasts for several hours and is augmented further after a second seizure; and (3) the mechanisms for this "protective" effect of experimental FSs involve the actions of the endogenous inhibitory peptide NPY. Taken together, these findings provide a neurobiological basis for the prevalence of a single seizure per febrile episode in children and suggest that a flurry of FSs might imply significant breakdown of protective inhibitory mechanisms in the limbic circuit.

A Single Experimental FS Decreases the Probability of Having Another

The current experiments demonstrate that a single hyperthermic seizure leads to a significant elevation of temperatures required to elicit a second (or third) seizure. This finding recapitulates the typical situation in the human. In 80–90% of human FSs, only a single seizure occurs within

a febrile episode. This is true even when the fever is sustained or fluctuates with antipyretics. In several relatively large epidemiological studies of FSs, the total number of complex seizures accounted for 20–35% of all FSs (Annegers et al., 1987; Berg and Shinnar, 1996a).Within the group of complex FSs, repetitive seizures comprised about 50%, occurring in 13.7% (Berg and Shinnar, 1996a), 14.7% (Berg et al., 1992), 16% (Berg and Shinnar, 1996b), or 19% (Annegers et al., 1987) of all seizures. These data indicate that the majority of infants and children are resistant to having more than one seizure during a given febrile episode. The basis for this clinically observed resistance is difficult to determine, in part because of the inability to measure true “threshold temperatures” in febrile children: A delay between seizure onset and evaluation of the child's temperature is almost universal, and this delay is highly variable (Berg, 2002).

Reproducing the human phenomenon of “one seizure per febrile episode” within the immature rat model permitted the designing of experiments to determine the mechanisms involved. It should be noted that the same animal model shares several key features of human FS: age specificity, a physiologically relevant threshold temperature range that is concordant with high fever in children (Berg et al., 1992); and minimal morbidity (Dubé et al., 2000; Baram, 2002). This model has suggested that even clinically nonfocal hyperthermia-induced seizures involve hippocampal formation (Toth et al., 1998; Dubé et al., 2000; Baram, 2002) (and see Fig. 1A) and has facilitated in vivo and in vitro studies to define the short- and long-term consequences of FSs, as well as the mechanisms involved.

In the studies reported here, a higher temperature was required to induce seizures following a single hyperthermic seizure. This effect was progressive with additional seizures, whereas baseline core temperatures did not differ before each seizure. In addition, no significant dissociation occurred between core and brain temperatures at onset of the first seizure or a subsequent one (data not shown), indicating that the resistance to a second seizure was not an artifact of the fact that core temperature did not reflect brain temperatures at seizure onset. What, then, might be the mechanism(s) by which a single experimental FS results in elevation of the temperature required to elicit further seizures?

The Mechanism for Seizure-Induced Increase of Threshold Temperature, Lasting for Hours, Might Involve Hippocampal Neuropeptides

The rarity of repetitive FSs during a single fever episode might result from an alteration of the excitability of neuronal circuits involved in their generation. Strong candidates for mediating protective effects in the time frame of hours are endogenous neuropeptides (see Introduction). A candidate peptide is corticotropin-releasing hormone (CRH), which is abundant in developing hippocampus (Chen et al., 2001), excites hippocampal neurons (Aldenhoff et al., 1983; Hollrigel et al., 1998), and precipitates seizures in immature rodents (Baram et al., 1992; Baram and Hatalski, 1998). Thus, FS-induced reduction of CRH levels in hippocampal synapses might be anti-excitatory. However, experimental FSs increase CRH mRNA expression in hippocampal formation (Hatalski et al., 2000), consistent with enhanced release of the endogenous peptide, thus excluding involvement of CRH in seizure-induced “protection” from further hyperthermic seizures.

Here, the reduced seizure susceptibility after an experimental FS was attributable to actions of the inhibitory hippocampal peptide NPY. Abundant expression of NPY in hippocampal formation, typically in the interneuron, has been found (Deller et al., 1990). The peptide reduces excitability within the hippocampal network during seizures (Woldbye et al., 1996), acting presynaptically via Y2Rs (Schwarzer et al., 1998; El Bahh et al., 2002 but see- Woldbye et al., 1997) to reduce glutamate release onto hippocampal pyramidal cells. The information for the presence, location, and pharmacology of NPY and its receptors prompted us to employ a Y2R blocker in concentrations considered relatively specific, and this antagonist abrogated the

progressive increase in seizure threshold, suggesting that activation of these receptors by endogenous NPY contributed to the augmented seizure threshold.

Independent support for release of endogenous hippocampal NPY after experimental FS in the current studies was provided by increased NPY mRNA levels in hippocampus within hours after such seizures. This increased peptide production (Fig. 4), reported also in other seizure models (Vezzani et al., 1996;Schwarzer et al., 1998), is typical of release-coupled synthesis, described for many CNS peptides (Hokfelt et al., 2000). Taken together, the data indicate a role for endogenous NPY in the mechanisms by which an experimental FS "protects" the hippocampus from the occurrence of a second.

Relevance of These Studies to Human FSs

The current study documents that a single FS leads to enhanced inhibitory processes in the normal hippocampal circuit, which protect it from ensuing fever-provoked seizures within hours of the original ictus. The studies clarify the underlying mechanisms, providing a neurobiological basis for the "single-seizure-per-febrile-episode" principle operating in most children. The studies also imply that if recurrent seizures do occur within a single febrile episode, they may indicate that the underlying antiexcitatory processes are not "normal" and encourage a search (e.g., EEG, MRI) for predisposing factors that might have rendered the individual more susceptible to fever-induced seizures. In addition, recognizing that a FS leads to "protective" elevations of temperatures required for further seizures might influence management decisions: the scenario of repetitive seizures within a febrile episode with stable temperatures should raise the concern that these seizures might be provoked by an insult beyond the fever itself. A vigorous search for more malignant causes for the fever, (acute infection, hemorrhage), which may elicit breakdown of normal protective mechanisms, should be considered.

In summary, in the immature rat model, a single FS leads to enhanced release and actions of an inhibitory neuropeptide that serves to protect against the occurrence of additional seizures. These findings carry significant implications for the evaluation and management of repetitive FSs in infants and children.

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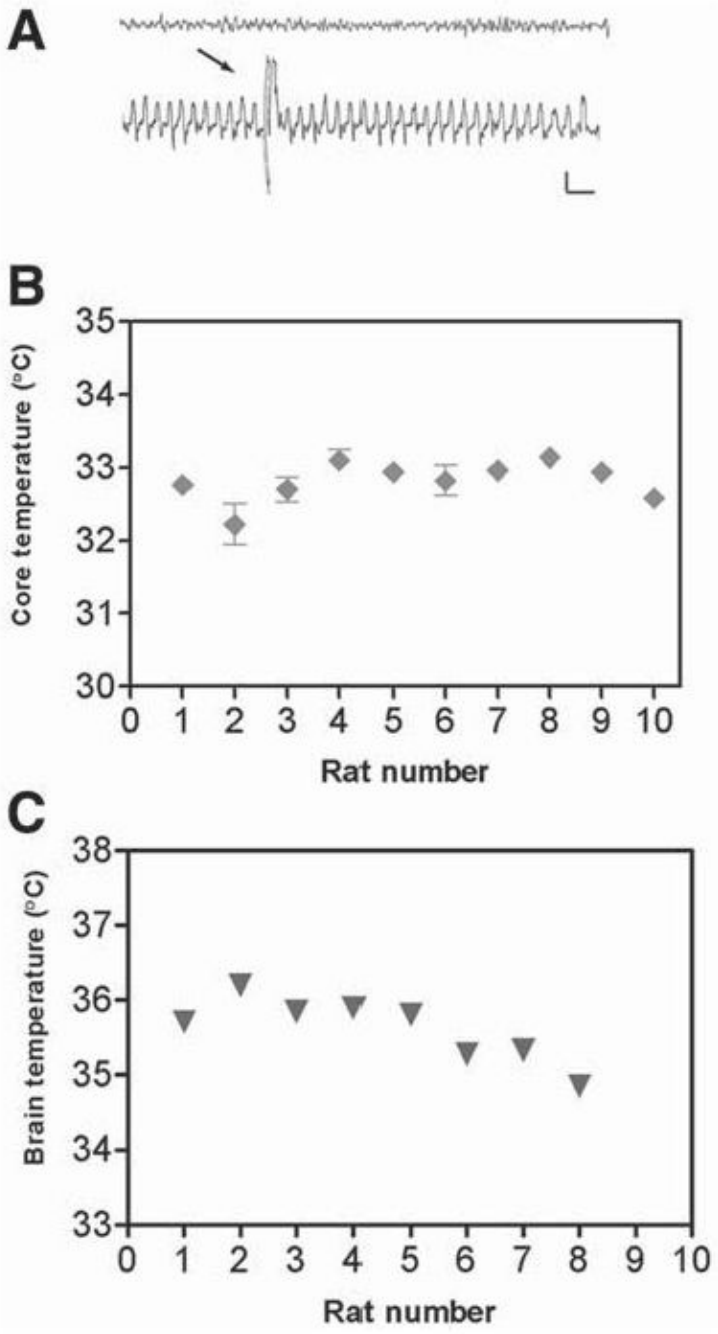

Fig. 1.

(A) Electrophysiological features of hyperthermia-induced seizures in immature rats. EEGs were recorded using bipolar electrodes from dorsal hippocampus of 10-d-old rats. Top: Typical hippocampal activity in a euthermic animal, consisting of low-amplitude, nonrhythmic awake pattern (Dubé et al., 2000; Bender et al., 2003). Bottom: Rhythmic, high-amplitude epileptiform spike waves indicate a seizure. During this electrographic seizure, the animal was essentially motionless, with an occasional jerk (arrow) or oral automatisms. Calibration bars: 1 s; 50 μV. **(B,C)** Validity and correlation of brain and core temperatures in the euthermic immature rat. (B) Core temperatures of individual rats are tightly clustered within and among individuals. Symbols denote mean ± S.E. of five measurements obtained over 25 min from

individual animals kept on a euthermic pad. These were identical to temperatures obtained in the home cage and represent normothermic values for the 10-d-old rat. (Values for rat no. 1 were too clustered to visualize error bars). (C) Brain temperatures, obtained under the same conditions, by direct dural measurements (see Materials and Methods). These temperatures were, on average, 2.8°C higher than core temperatures.

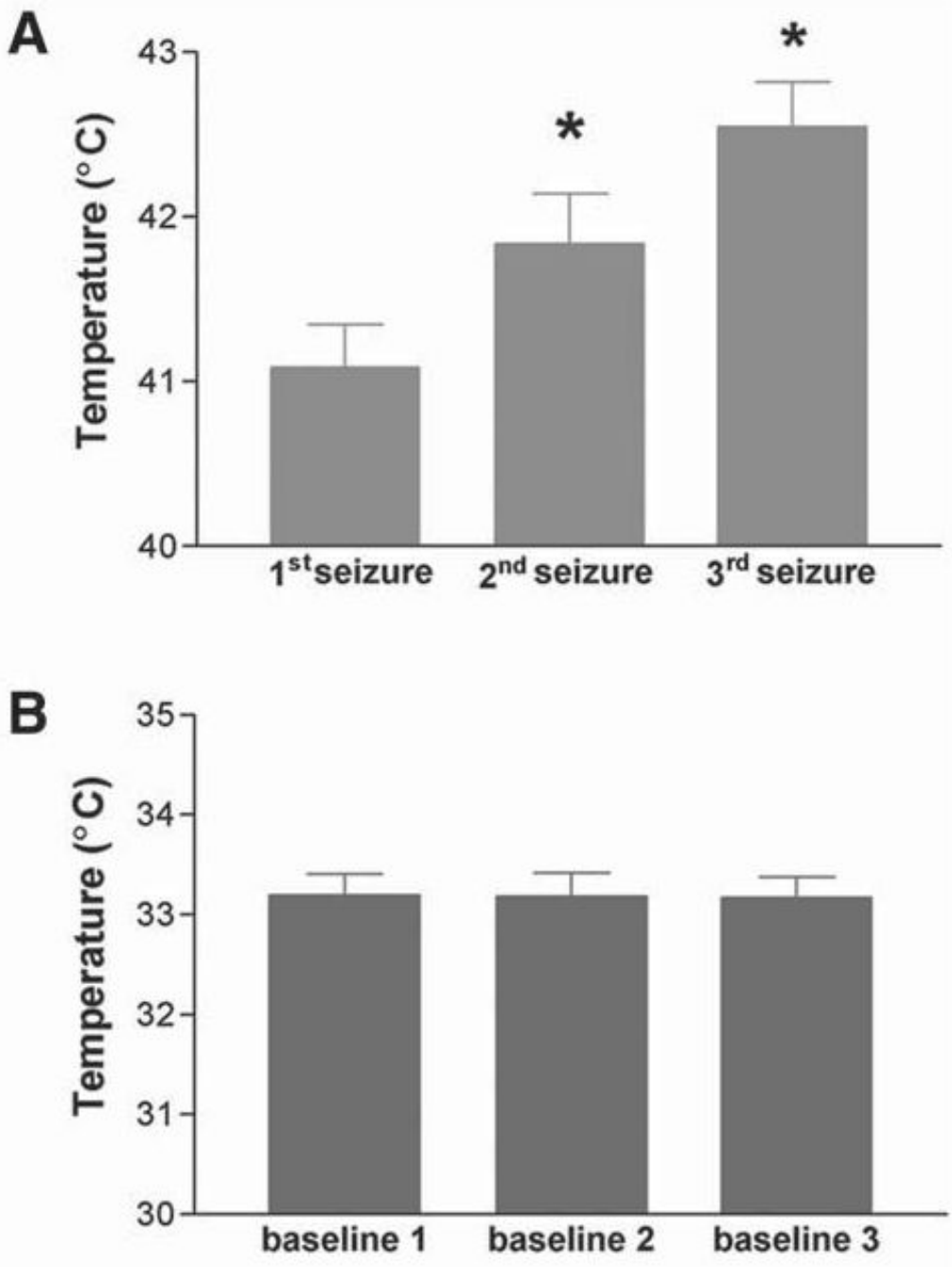

Fig. 2.

Progressive increase of temperatures required for further seizure provocation after an experimental FS in the immature rat. (A) Threshold temperatures (measured at seizure onset) were significantly higher for the second seizure and elevated even further for the third (repeated measure ANOVA, $p = 0.0015$). As explained in the text, at this temperature range, brain and core temperatures converged. (B) Baseline core temperatures, measured immediately prior to induction of hyperthermia, were not influenced by previous seizures, elicited 3–4 h earlier.

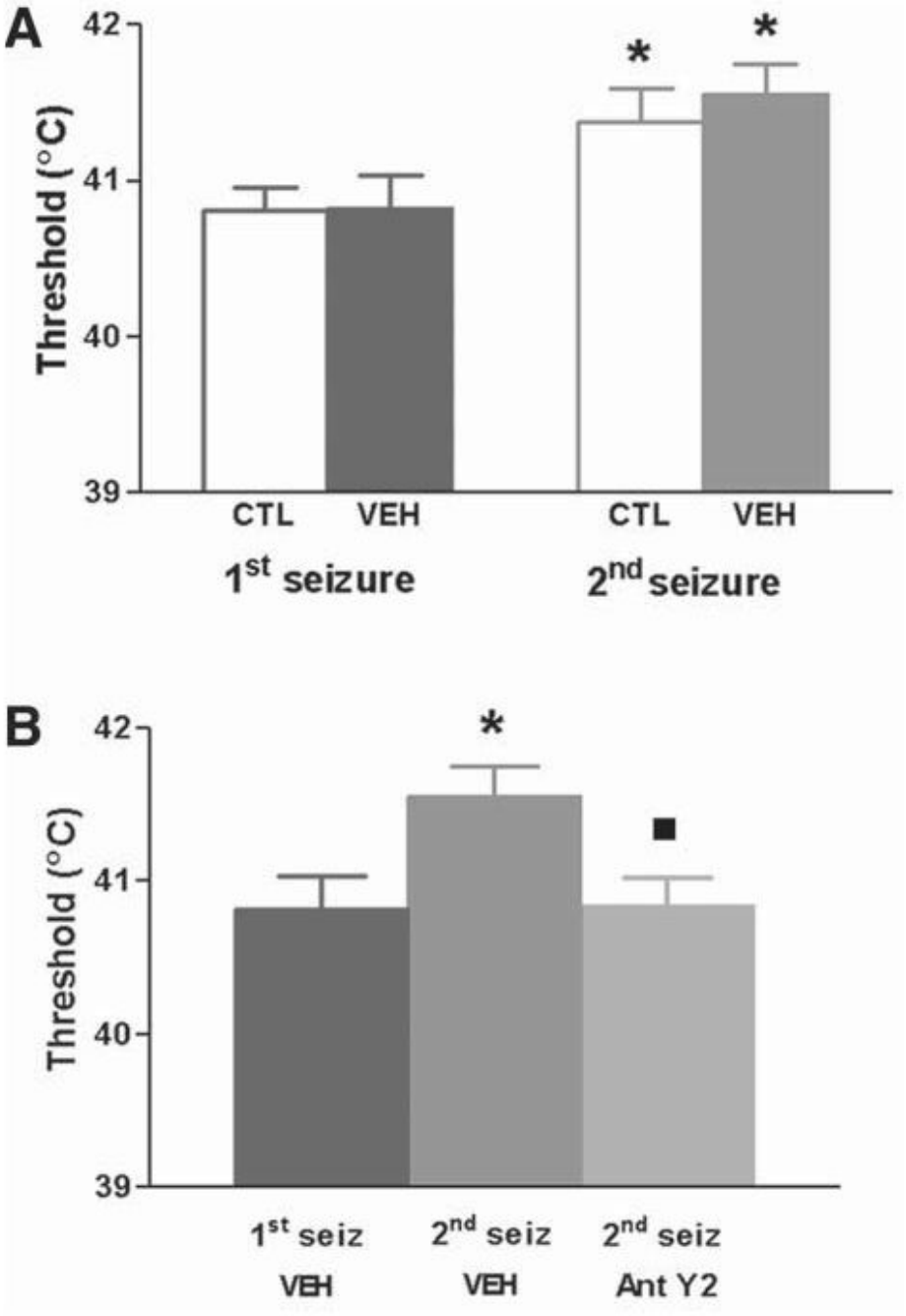

Fig. 3.

The progressive increase of threshold temperature for a second experimental FS was abolished by a selective antagonist of NPY Y2Rs. (A) Comparison of threshold for a first (1st) and second (2nd) seizure in untreated control rats (CTL) and those infused with vehicle (VEH). The asterisk (*) denotes a significant difference from the first seizure threshold. (B) Comparison of threshold for a first and second seizure in rats infused with vehicle and those infused with NPY receptor antagonist prior to the second seizure. The solid square indicates significantly lower threshold vs that for the second seizure in the vehicle-infused group.

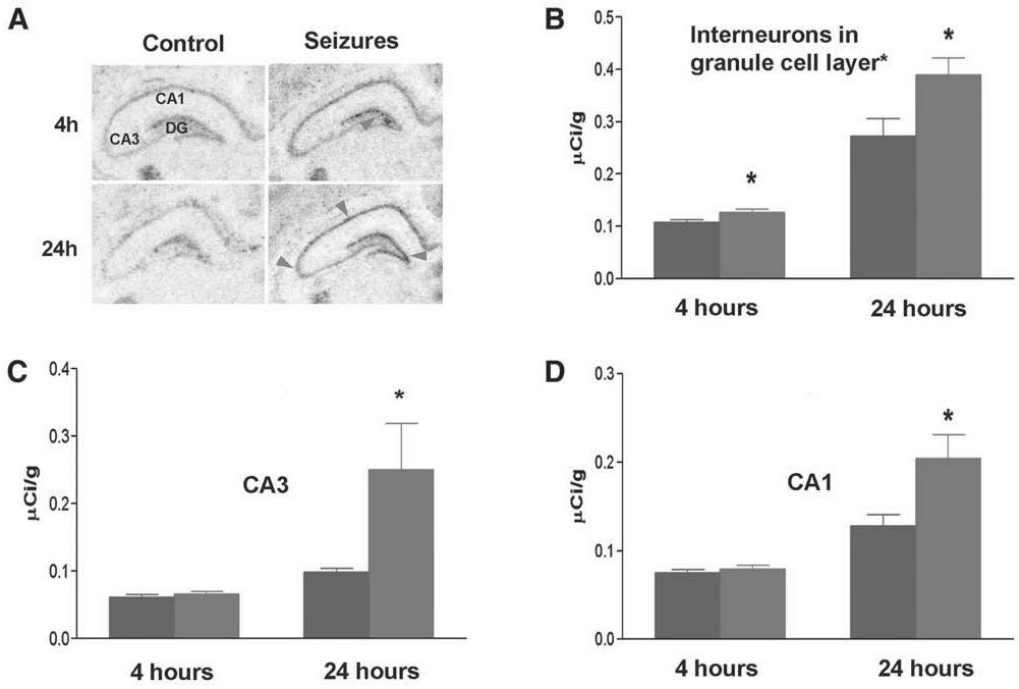

Fig. 4.

Experimental FSs induce NPY gene expression in immature rat hippocampus. **(A)** Hippocampal sections from naive and seizure-experiencing immature rats were subjected to ISH for NPY mRNA (see Materials and Methods). Scale bar = 1.3 mm. **(B)** Quantitative analysis of the NPY mRNA signal demonstrates increased expression in dentate gyrus interneurons by 4 h after the seizures, and in dentate gyrus, CA3 and CA1 by 24 h. Values are means with standard error bars. * denotes statistically significant difference compared with the naive (control) group. Note that for neuropeptides in general, increased secretion typically results in enhanced expression, so that the latter can be used as an indicator of endogenous peptide release.