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

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ABSTRACT

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

Introduction

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

Neuropeptide Y (NPY) is a 36-amino acid polypeptide transmitter abundantly expressed in the brain where it predominantly acts by binding to three G-protein coupled receptors (Y1, Y2, and Y5) (Berglund et al., 2003; de Quidt and Emson, 1986). NPY application exerts prominent seizure-suppressant effects in rodents *in vitro* and *in vivo* (Baraban et al., 1997; Klapstein and Colmers, 1997; Vezzani et al., 1999; Woldbye et al., 1996, 1997, 2005) and recombinant adeno-associated viral (rAAV) vector-mediated overexpression of NPY also suppresses seizures in both acute and chronic models of epilepsy (Foti et al., 2007; Noe et al., 2008, 2010; Richichi et al., 2004; Sørensen et al., 2009). Consequently, rAAV-mediated NPY gene therapy has been proposed as an alternative treatment strategy for patients suffering from intractable TLE (McCown, 2010; Riban et al., 2009).

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

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

Materials and methods

Animals

Adult male Wistar rats (Charles River, Germany) weighing 250–350 g at the beginning of the experiments were used. Animals were housed on a 12-h light/dark cycle with *ad libitum* access to food and water. All experiments were performed according to the Swedish Animal Welfare Agency guidelines and approved by the local Ethical Committee for Experimental Animals.

Viral vector injections

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

Transgene overexpression

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

Y5 receptor and NPY mRNA in situ hybridization

The procedure was performed as previously described (Woldbye et al., 2010) with only minor modifications. At the time of sacrifice, the rats were decapitated and their brains were quickly removed and frozen on dry ice. Subsequently, 15 µm thick coronal serial sections at the hippocampal level were cut on a cryostat and thaw-mounted on Superfrost glass slides, dried on a hotplate and stored at −80 °C. The slides were defrosted for 10 min at room temperature (R_T), subsequently fixed for 5 min in 4% PFA, and rinsed briefly and placed for 5 min in PBS. Then, the slides were transferred to 70% ethanol for 5 min and stored in 95% ethanol at 4 °C until hybridization. Synthetic antisense oligonucleotide DNA probes were used for *in situ* hybridization: Y5 receptor mRNA: 5′-CGA GTC TGT TTT CTT TGT GGG ACA ATC CAC AGC TTA TAC TCC TGC-3′; prepro-NPY mRNA: 5′-GTC-CTC-TGC-TGG-CGC-GTC-CTC-GCC-CGG-ATT-GTC-CGG-CTT-GGA-GGG-GTA-3′ (Mikkelsen and Woldbye, 2006). The oligoprobes were labeled at the 3′-end with [α^{35}S]dATP (1250 Ci/mmol; #NEG734H001MC; PerkinElmer, Denmark) using terminal deoxynucleotidyl transferase (Roche Diagnostics, Mannheim, Germany). Labeled probes were added with a specific activity of $1–3\times10^5$ cpm/100 µl to the hybridization buffer containing 50% formamide (v/v), 4× saline sodium citrate (SSC; 1× SSC = 0.15 M NaCl, 0.015 M NaCitrate–$2H_2O$, pH 7.0), 10% dextran sulfate (w/v) and 10 mM dithiothreitol. After adding a volume of 120 µl hybridization mixture to each slide, they were covered with Parafilm and left overnight at 42 °C in humidity boxes. The slides were then briefly rinsed in 1× SSC at R_T, washed for 30 min in 1× SSC at 60 °C, passed through a series of 1 min rinses in 1× SSC, 0.1× SSC, 70% ethanol, and 95% ethanol at R_T, and finally air-dried. The slides where exposed together with ^{14}C-microscales on ^{35}S-sensitive Kodak BioMax MR films (Amersham Biosciences, Denmark) for 3–8 weeks and developed in Kodak GBX developer.

Y5 receptor immunohistochemistry

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

NPY immunohistochemistry

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

Y5 receptor binding

Y5 receptor binding was performed as previously described (Woldbye et al., 2005). The slides were defrosted at R_T and subsequently preincubated for 20 min in binding buffer (pH 7.4), containing 25 mM N-(2-hydroxyethyl)-piperazine-N′-(2-ethanesulfonic acid) (HEPES), 2.5 mM $CaCl_2$, 0.5 g/l bacitracin, 0.5 g/l BSA. The slides were then incubated at R_T for 60 min in binding buffer containing 0.1 nM [^{125}I][Tyr^{36}]mono-iodo-PYY (4000 Ci/mmol; porcine synthetic, #IM259; Amersham Biosciences, Denmark) to which was added 100 nM BIBP3226 (Y1 receptor antagonist; #E3620, Bachem AG, Switzerland) + 100 nM BIIE0246 (Y2 receptor antagonist; #1700, Tocris Bioscience, UK) to visualize Y5 receptor binding. After a brief rinse, the slides were washed for 2×30 min in binding buffer at R_T