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Adult-onset hippocampal-specific neuropeptide Y overexpression confers mild anxiolytic effect in mice

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Abstract

The anticonvulsive properties of neuropeptide Y (NPY) are opening up opportunity for the development of NPY gene transfer as a therapy for epilepsy. In order to pursue the potential clinical translation of this approach, the effects of somatic NPY gene transfer on other hippocampal functions need to be assessed. The present study characterized the behavioral effects of recombinant adeno-associated viral vector (rAAV)-mediated hippocampal NPY overexpression in adult male mice and also Y1 receptor knockout mice. In wild-type mice, there were no obvious adverse effects on the general health, motor function and cognition following rAAV-NPY treatment. Moreover, hippocampal NPY overexpression induced a moderate anxiolytic effect in the open field test and elevated plus maze. Intriguingly, the treatment also increased depressive-like behavior in the tail suspension test. Elevated hippocampal NPY levels in the absence of Y1 signalling had no effects on anxiety or cognition and actually improved the depressive-like phenotype observed in the wild-type mice treated with rAAV-NPY.

1. Introduction

Neuropeptide Y is a 36 amino acid peptide abundantly expressed in the central nervous system with seizure-modulating effect (Vezzani et al., 1999). Seizures induce robust changes in the expression of NPY and its Y receptors in brain regions crucially involved in the initiation and propagation of seizures (Bellmann et al., 1991; Sperk et al., 1992). Intracerebral infusion of NPY suppresses epileptiform activity in various models of epilepsy (Smialowska et al., 1996; Woldbye, 1998; Woldbye et al., 1997), while agonists and antagonists of Y receptors also modulate susceptibility to experimentally-induced seizures (Gariboldi et al., 1998; Woldbye et al., 1997). Furthermore, transgenic rats over-expressing NPY showed reduced seizure susceptibility and epileptogenesis (Vezzani et al., 2002), whereas NPY knockout mice are more vulnerable to chemically- or electrically-

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induced convulsions (Baraban et al., 1997; Shannon and Yang, 2004). In hippocampal slices from epileptic patients, NPY has a potent and long-lasting inhibitory action on perforant path-evoked excitatory responses from dentate granule cells (Patrylo et al., 1999). Based on the extensive evidence of its anticonvulsant property, NPY has emerged as a promising candidate for epilepsy gene therapy. Using recombinant adeno-associated viral vectors (rAAV), we and others have previously shown that hippocampal NPY overexpression confers both anticonvulsant and antiepileptogenic effects (Foti et al., 2007; Lin et al., 2006; Richichi et al., 2004). Recently, the therapeutic efficacy using this approach was demonstrated in a rat model of temporal lobe epilepsy (TLE), where rAAV-NPY delivery in already epileptic rats leads to a remarkable decrease in the progression of seizures and spontaneous seizure frequency (Noe et al., 2008). The clinical potential of a rAAV-NPY based gene therapy for treatment of TLE is intriguing.

NPY acts through the G-protein coupled Y receptors (Y1, Y2, Y4, Y5 and Y6), of which the Y1, Y2 and Y5 receptors have been implicated in its seizure modulatory effect (Vezzani et al., 1999; Woldbye et al., 1997). In addition to its seizure-modulating action, NPY is also known to modulate a number of other physiological functions including cardiovascular regulation, metabolism, nociception, bone homeostasis, cognition, anxiety, depression and stress sensitivity (Baldock et al., 2005; Kask et al., 2002; Lin et al., 2004). Of these physiological processes, the hippocampus is critically involved in the modulation of cognition, anxiety and depression (Bird and Burgess, 2008; Drevets et al., 2008; Santarelli et al., 2003). Indeed, transgenic rats with hippocampal NPY overexpression were shown to exhibit attenuated stress sensitivity, absent fear suppression and impaired spatial memory acquisition (Thorsell et al., 2000). For eventual clinical application of hippocampal rAAV-NPY administration as a therapy for TLE, its potential effects on other hippocampal-mediated functions need to be addressed. To date, the behavioral effects of vector-mediated adult-onset hippocampal NPY overexpression has not been well characterized. Only one study reported a transient spatial learning deficit in a two-platform spatial discrimination water maze test (Sorensen et al., 2008a). This study therefore aimed to examine the behavioral effects of rAAV-mediated hippocampal NPY overexpression in mice, with particular focus on affective and cognitive parameters. Furthermore, since Y1 receptor is downregulated in human epileptic patients (Furtinger et al., 2002), we also studied the effect of NPY overexpression in Y1 receptor deficient mice.

2. Experimental methods

2.1. Animals

Generation of Y1 receptor knockout mice (Y1Δ) was described previously (Howell et al., 2003). Both Y1Δ and wild-type mice were maintained on a mixed C57BL/6-129/SvJ background. Mice were group-housed in 2–3 under a 12 h light/dark cycle (lights off at 1900 h), with food and water provided *ad libitum*. All animal work was conducted under approval of the "Garvan Institute/St. Vincent's Hospital Animal Experimentation Ethics Committee" and were in agreement with the "Australian Code of Practice for the Care and Use of Animals for Scientific Purpose".

2.2. Recombinant AAV vector production

The construction of Human NPY cDNA was subcloned into an AAV expression cassette consisting of the rat neuron-specific enolase (NSE) promoter, woodchuck post-transcriptional regulatory element (WPRE) and a bovine growth hormone polyA (bGHpA) signal flanked by AAV2 inverted terminal repeats (pAM/NSE-NPY-WPRE-bGHpA). The same expression cassette without the transgene (pAM/NSE-Empty-WPRE-bGHpA) was used as control.

High-titer chimeric AAV vectors expressing a mix of AAV serotype 1 and serotype 2 capsid proteins were generated as described previously (Richichi et al., 2004). Briefly, HEK 293 cells were transfected with the AAV plasmid, together with the AAV helper plasmids pH21, pRV1 and pFΔ6 by calcium phosphate transfection methods. Forty-eight hours following transfection, cells were harvested and the vector purified by heparin affinity columns as described (During et al., 2003). Genomic titers were determined using the Perkin-Elmer-Applied Biosystem Prism 7700 sequence detector system (Foster City, CA) as described previously (Clark et al., 1999) with primers against the WPRE sequence and vector titer normalized to 1×10^{13} genome copies/mL.

2.3. Vector administration

Male adult mice (25–35 g; 10–12 weeks old; $n = 10$–12 per experimental group) were anesthetized with a single dose of ketamine/xylazine (100 mg/kg and 20 mg/kg; i.p.) and placed on a Kopf stereotaxic frame. The injection coordinates for dorsal hippocampus were (from bregma): anterio-posterior, −1.7 mm; medio-lateral, ±0.8 mm; dorso-ventral, −2.2 mm; for ventral hippocampus: anterio-posterior, −2.7 mm, medio-lateral, ±3.0 mm; dorso-ventral, −3.0 mm (Franklin and Paxinos, 1997). 1 μL rAAV1/2 vector per injection site was delivered bilaterally into both dorsal and ventral hippocampus at a rate of 0.1 μL/min using a 10 μL Hamilton syringe attached to Micro4 Micro Syringe Pump Controller (World Precision Instruments Inc., Sarasota, USA). Animals were monitored post-surgery until recovery from anesthesia.

2.4. Behavioral characterization paradigm

Behavioral testing was conducted 4 weeks after the surgery when transgene expression has reached a stable optimal level as shown previously (Xu et al., 2001). Mice were handled for 2 min per day for 5 days prior to behavioral testing to reduce confounding handling-induced stress response. This was done by touching and picking up the mice in their home cage and allowing the mice to sniff the hands (with glove) of the experimenter.

The behavioral tests were carried out at least two days apart in the following order: (1) physical examination and reflexes, (2) motor function, (3) open field test, (4) hole-board test, (5) light–dark test, (6) elevated plus maze, (7) passive avoidance and (8) tail suspension test. At least 1 h prior to testing, mice were transported to the testing room for habituation. Experiments were performed during the light phase between 1300 h and 1630 h with the exception of the hole-board test which was conducted between 1400 h and 1800 h.

2.5. Physical examination and reflexes

A series of simple tests on the general health and reflexes were carried out prior to the behavioral test battery to ensure that the animal did not have major health problems or any sensory or motor deficits that might affect its performance in complex tasks (Crawley, 1999). Briefly, the following tests were performed. *Empty cage* — each mouse was individually placed in an empty cage for 3 min to record abnormal spontaneous behaviors (such as wild-running, excessive grooming, freezing). *Unknown object* — an unfamiliar object was placed in the mouse's home cage and any abnormal

behavior such as biting and attacking the object was recorded. *Visual cliff test* — for assessment of visual function, each mouse was placed onto the center of a small elevated platform (approximately 20 cm × 25 cm). The latency to reach the edge of the platform and the frequency of dipping the head over the edge was measured during 120 s. In another version of this task animals were individually placed onto a beam, which was mounted on the edge of a platform installed 10 cm above the bench. The time and side the mouse stepped down from the beam was recorded. Normally, the mouse would step down from the side onto the platform.

Balance reflex — each mouse was placed in an empty cage, which was rapidly moved from side to side and then up and down. The normal postural reflex is to extend all four legs in order to maintain an upright, balanced position. *Righting reflex* — the mouse was turned on its back and observed whether it could right itself to an upright position. *Eye blink reflex and ear twitch reflex* — these reflexes were examined by touching the eye of the mouse with a cotton-tip swab and by slightly pinching the tip of the ear with a tweezer. *Whisker-orienting reflex* — the whiskers of a freely moving mouse were touched lightly by a tweezer. The normal reflex is a pause in the continual moving of the whiskers and turning towards the stimulus.

2.6. Motor functions

2.6.1. Wire-hang test

The neuromuscular strength of mice was examined by the wire-hang test. The mouse was placed on a wire cage lid and the lid was gently waved so that the mouse grips the wire. The lid was then turned upside down approximately 50 cm above the surface of some soft bedding materials. The latency to fall onto the bedding was recorded with a cut-off time of 60 s.

2.6.2. Accelerod

Motor coordination, balance and ataxia were tested on an accelerating rotarod (Ugo Basile, Comerio VA, Italy). The mice were first trained to walk on the rotating rod at a constant speed (12 rpm) for 2 consecutive days, 1 trial of 120 s per training session. The latency and frequency to fall off the rotarod within this time period was recorded. Mice were placed with their body axis perpendicular to the rotation axis and their head was directed against the direction of the rotation so that the animal had to progress forward to maintain its balance. During the 120 s of the training trials, the animals were instantly replaced on the rotarod if they fell to ensure the amount of training received was consistent across all animals. Two hours after the second training session, mice were subjected to a single trial of accelerod testing. During this time, the rotation speed was constantly increased in 4 rpm increments (30 s for each rotation speed), i.e. from 4 rpm to 40 rpm over 4.5 min. The latency to fall off the rod and the actual rotating speed level were measured. The maximum duration of this test was 5 min.

2.7. Open field test (OF)

Mice were tested in an automated infrared photobeam controlled open field activity box of the dimension 43.2 × 43.2 cm (MED Associates Inc., St Albans, VT, USA) to analyse general motor activity. Each mouse was placed into the right front corner of the open field. The mouse was allowed 10 min in the arena during which time the various parameters including distance travelled, ambulatory activity and resting behavior in the center and peripheral zones were measured and recorded by the automated infrared beam array system (MED Associates Inc. software coordinates for central zone: 3/3, 3/13, 13/3, 13/13). At the end of the trial, mouse was removed from the activity box, returned to its cage and the box was cleaned by 70% ethanol to remove any odor cues influencing the behavior of the subsequent mouse to be tested.

2.8. Light—dark test (LD)

In the LD test the travelled distance and time spent in a brightly illuminated zone compared to a dark zone can be used to assess anxiety in rodents (Costall et al., 1989; Crawley, 1999). The same activity box used for OF was used for the LD test, with the addition of a dark box insert that divides the activity box into two equal sized light and dark compartments. An opening located in the center of the partition connects the two compartments. The mouse was placed in the light compartment facing the entrance to the dark compartment. The time spent in, entries into, and distance travelled in the differentially illuminated compartments were recorded for 10 min. The chamber was cleaned with 70% ethanol between trials.

2.9. Hole-board test (HB)

The HB test provides independent measures of locomotor activity and directed exploration. Furthermore, it can be used as a basic task for anxiety and basal screening for working memory (Karl et al., 2006; Ohl et al., 2003). The mouse was placed in the automated open field activity box, which is equipped with a hole-board floor insert for mice (MED Associates, Inc.; 16 holes; diameter 1.6 cm). Distance travelled and the number of head-dipping into the holes in a 7 minute test session were measured by infrared beams. Both an increase and a decrease in head-dipping has been associated with anxiety-like states (Ohl et al., 2003; Saitoh et al., 2006; Takeda et al., 1998) and can be reversed by treatment with anxiolytics (Do-Rego et al., 2006; Saitoh et al., 2006). Thus, this test is often used in combination with other anxiety-related tasks to provide additional independent measures to establish the anxiety-related behavioral phenotype. The ratio of head-dipping ('entries') into novel holes (i.e. holes that had not been explored, as determined by head-dipping) to total hole entries was used as a basic assessment of working memory.

2.10. Elevated plus maze (EPM)

The EPM is an ethologically-based approach—avoidance conflict test targeting the natural conflict between the tendency of mice to explore a novel environment and the tendency to avoid a brightly lit open area (Montgomery, 1955). The elevated plus maze consists of 4 arms in the shape of a "+" elevated 1 m above the floor. Two alternate arms are dark and enclosed while two alternate arms are open and lit. The open and enclosed arms of the plus maze generate exploratory behavior and the avoidance of elevated open arms is an indication of the intensity of anxiety. Each mouse was placed onto the center field of the "+" facing an open arm and was allowed to explore the maze for 5 min. The behavior and movement of each mouse was recorded by a video camera and subsequently scored by a blinded experimenter. Anxiety was indicated by the time spent on open arms as well as open arm entries. The number of total arm entries was also recorded as a measure of general motor activity. After each test, mouse was returned to its home cage and the maze was cleaned with 70% ethanol.

2.11. Passive avoidance test (PA)

In the training session, mouse was placed in the light chamber of the two-chamber apparatus (MED Associates Inc., St Albans, VT, USA) and the door to the dark chamber was opened. The latency to enter the dark chamber was measured as a control for visual ability and preference for the dark chamber. Immediately after the mouse entered the dark chamber, the door between the two chambers was closed and a 0.3 mA foot shock was delivered for 1 s. The animal was left in the dark chamber for a further 10 s to allow the formation of an association between the dark chamber and the foot shock. After

10 s, the mouse was removed from the dark chamber and returned to its home cage. In the retention test session 24 h later, the mouse was placed in the light chamber and the latency to enter the dark chamber was measured. Upon entering the dark chamber, the mouse was removed from the chamber and returned to its home cage. The cut-off time for each trial was 5 min, which was recorded as the latency if the mouse failed to enter the dark chamber within the cut-off time. The test chamber was cleaned by 70% ethanol between animals.

2.12. Tail suspension test (TST)

The apparatus consisted of a horizontal 25 cm metal wire elevated approximately 25 cm above the bench by two plastic poles at each end of the wire. Mouse was suspended in the air by taping the distal end of the tail onto the wire with Scotch adhesive tape. The test session was video-recorded and the length of time the mouse assumed an immobile posture during the 6 min testing period was scored by a blinded experimenter.

2.13. Immunohistochemistry

To confirm NPY overexpression in the hippocampus, mice were sacrificed at the end of behavioral characterization by sodium pentobarbitone overdose (15 μL Nembutal, i.p.) and perfused transcardially with 1× PBS followed by 4% PFA. Following cryoprotection in 30% sucrose, coronal brain sections of 40 μm were cut for immunohistochemistry. Briefly, sections were rinsed in PBS-Triton before being incubated in 1% (v/v) H_2O_2 in 50% (v/v) methanol for 30 min to remove endogenous peroxidase. Following 2×5 min rinses in PBS-Triton, sections were incubated overnight at room temperature with a polyclonal NPY primary antibody (1:250 dilution; Auspep Pty Ltd., Victoria, Australia). Sections were then washed with PBS-Triton and anti-rabbit biotinylated secondary antibody (1:250 dilution; Cell Signaling) was applied. Following a 3-hour incubation, sections were washed with PBS-Triton and treated with ExtrAvidin Peroxidase (1:250 dilution; Sigma) for 2 h before a final wash in PBS and stained with diaminobenzidine (DAB). Sections were mounted onto slides and left to dry overnight before being dehydrated in ascending concentrations of ethanol, immersed in xylene and coverslipped. Immunostained brain sections were photographed using a digital camera attached to a Zeiss Axiophot microscope, and images captured using Irfanview 4.23 Software.

2.14. Serum corticosterone assay

For serum corticosterone assay, mice were anesthetized by isoflurane and tail blood was collected from WT-YFP and WT-NPY mice (n=6 per group) 3 weeks after vector injection at 1000 h. Serum was isolated by centrifugation and corticosterone level was determined using Enzyme Immunoassay Kit at 1:200 dilution according to the manufacturer's instruction (Assay Designs, Inc., Ann Arbor, MI, USA).

2.15. Statistical analysis

Statistical analysis was performed using JMP software (SAS Institute Inc., Cary, NC, USA). Two-way analysis of variance (ANOVA) was used to assess the main effects 'genotype' and 'vector' and their interaction, followed by pair-wise comparison by Student's t test when genotype and/or vector effect reach statistical significance. Significant difference between rAAV-NPY treated group vs. rAAV-Empty group of the same genotype was denoted by *P<0.05 or **P<0.01. Significant difference between genotypes that received the same vector (e.g. Y1Δ-Empty vs. WT-Empty) was denoted by #P<0.05 or ##P<0.01. Statistical significance was set at P<0.05. All data are presented as means±standard error of the mean (S.E.M.).

3. Results

3.1. rAAV-mediated hippocampal NPY overexpression

Vector delivery and successful NPY overexpression was confirmed by immunohistochemistry for NPY. As shown in Fig. 1, dramatically increased NPY immunostaining was observed in the hippocampus of rAAV-NPY injected mice as compared to the rAAV-Empty injected controls. Particularly high NPY immunoreactivity was observed in the dentate hilus, CA1 and CA3 subfields of rAAV-NPY injected mice.

3.2. Physical examination and reflexes

General health of all treated mice appeared normal. None of the different experimental groups exhibited aberrant behaviors in the empty cage or toward an unknown object. All mice showed normal neurological reflexes and sensory abilities (balance, righting, eye blink, ear twitch and whisker-orientation reflexes). In the visual cliff beam test, there was no statistical difference between the latencies mice stepped down from the beam (WT-Empty: 8.0±1.7; WT-NPY: 6.9±2.1; Y1Δ-Empty: 9.3±2.1; Y1Δ-NPY: 8.4±3.1). Similarly, in the alternative version of the test, the latency to reach the edge of the

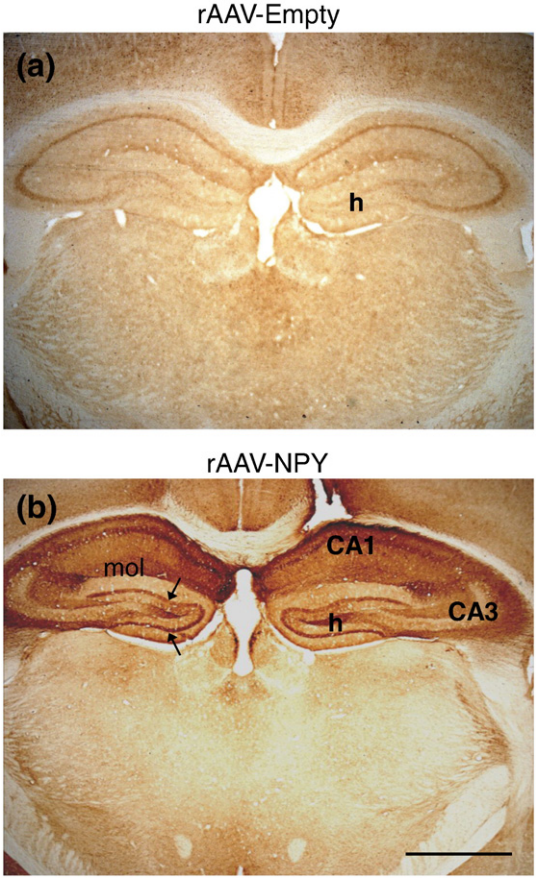

Figure 1 Immunohistochemical staining of mouse brain injected with (a) rAAV-Empty or (b) rAAV-NPY. Arrows depict a dense immunoreactive band in the inner molecular layer of the rAAV-NPY injected hippocampus. h, dentate hilus; mol, molecular layer. Scale bar: 1 mm.

visual platform was comparable between the different groups (WT-Empty: 6.9±2.6; WT-NPY: 8.0±1.1; Y1Δ-Empty: 3.8±0.8; Y1Δ-NPY: 5.9±1.6). However, a significant genotype effect was observed for the number of head-dipping (genotype effect: $F_{1,38}=23.84$, $P<0.001$), with the Y1 knockout (Y1Δ) mice exhibiting significantly more head-dipping behaviors than wild-type mice (Y1Δ-Empty: 19.8±2.6 vs. WT-Empty: 6.9±1.8, $P<0.001$). There were no differences between the rAAV-NPY groups (WT-NPY: 6.1±2.0; Y1Δ-NPY: 15.7±2.8) and their respective rAAV-Empty controls of the same genotype (geno-type×vector interaction: $F_{1,38}=0.51$, $P=0.480$; vector effect: $F_{1,38}=1.14$, $P=0.292$).

All mice exhibited normal muscular strength in the wire-hang test and were able to grasp the wire for the 2 min duration of the test. Although the NPY-overexpressing wild-type mice appeared to have a shorter latency to drop from the accelerod and at slower rotation speed (Fig. 2), this effect was not statistically significant (genotype×vector interaction: $F_{1,38}=3.29$, $P=0.078$; genotype effect: $F_{1,38}=0.07$, $P=0.787$; vector effect: $F_{1,38}=2.21$, $P=0.145$).

3.3. Open field test

Distances travelled in the OF, as a measure of locomotion and exploratory activity, were comparable between all groups (Fig. 3a).

In addition to its utility in evaluating the general motor activity of animals, this test also mimics the natural conflict in mice between the tendency to explore a novel environment and

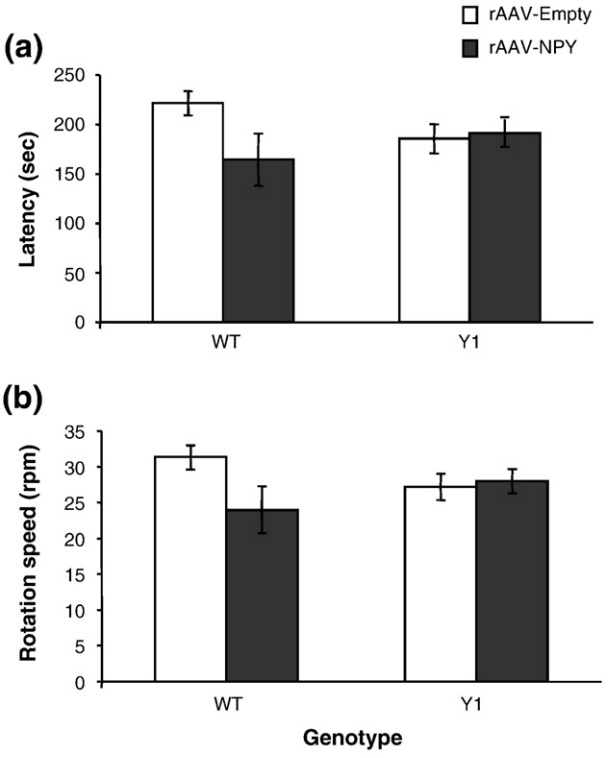

Figure 2 Mice performance on the accelerod. (a) Latency to fall from accelerod. (b) Maximum rotation speed achieved on the accelerod. All data are presented as means±standard error of the mean (S.E.M.).

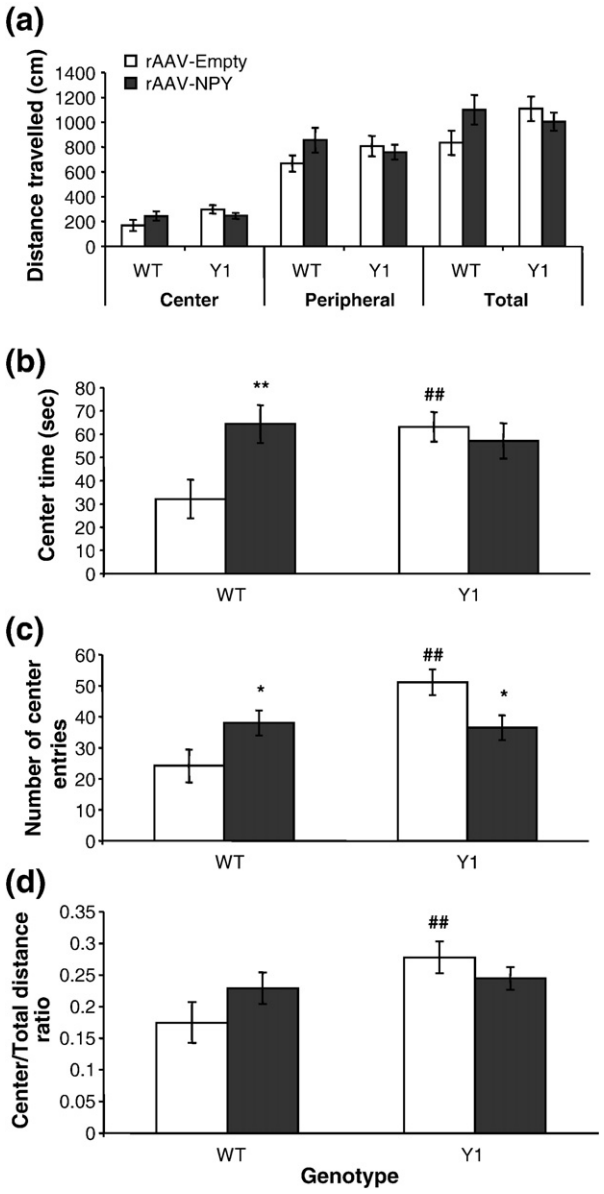

Figure 3 Mice performance in the open field test. (a) Distance travelled in the different regions of the open field and total distance travelled. (b) Time spent in the center of the open field. (c) Number of entries into center of the open field. (d) Ratio of distance travelled in the central area of the open field. Significant difference between rAAV-NPY treated group vs. rAAV-Empty group of the same genotype was denoted by *$P<0.05$ or **$P<0.01$. Significant difference between WT and Y1Δ mice that received the same treatment was denoted by ##$P<0.01$. All data are presented as means±standard error of the mean (S.E.M.).

to avoid an exposed open area (Crawley, 1985; Defries et al., 1966) and is now routinely used as a test to screen for changes in anxiety level (Belzung and Griebel, 2001; Holmes et al., 2002). NPY overexpression exerted a differential effect on the OF center time (genotype×vector interaction: $F_{1,38}=6.03$, $P<0.05$; Fig. 3b). While hippocampal NPY overexpression lead to a twofold increase in the center time in the wild-type mice

($P<0.01$), there was no significant difference between Y1Δ-Empty and Y1Δ-NPY groups. However, compared to wild-type controls, Y1Δ-Empty mice spent significantly more time in the center zone of the OF ($P<0.01$). It is interesting to note that the time spent in the center zone was also significantly higher in the Y1Δ-NPY mice compared to wild-type control mice ($P<0.05$). Similarly, NPY overexpression significantly increased ambulatory time in the center zone in the wild-type mice but not in Y1Δ mice (genotype × vector interaction effect: $F_{1,38}=5.19$, $P<0.05$; WT-Empty vs. WT-NPY, $P<0.05$; Y1Δ-Empty vs. Y1Δ-NPY, $P=0.244$; Table 1), although ambulatory time was already longer in the Y1Δ-Empty as compared to WT-Empty ($P<0.01$). The differential effect was even more evident in the number of entries mice made into the OF center zone (genotype × vector interaction effect: $F_{1,38}=9.93$, $P<0.01$; genotype effect: $F_{1,38}=7.94$, $P<0.01$; Fig. 3c). Pair-wise comparison revealed a significant difference between the genotypes, with Y1Δ-Empty made significantly more center zone entries compared to WT-Empty ($P<0.01$). Furthermore, while NPY overexpression increased center zone entries in the wild-type mice ($P<0.05$), it had the opposite effect in Y1Δ mice ($P<0.05$). Another measure used to examine anxious behaviors in the OF is the proportion of distance that the mouse travelled in the center zone (center/total distance), which allows for the adjustment of difference in basal locomotion between groups. In this parameter, there was a significant genotype effect ($F_{1,38}=5.02$, $P<0.05$), with Y1Δ mice exhibited higher center/total distance compared to wild-type mice ($P<0.01$; Fig. 3d). Pair-wise comparison showed significant difference between the rAAV-Empty injected groups ($P<0.01$) but the rAAV-NPY injected groups were comparable.

3.4. Light–dark test

In the LD test, there was a slight vector effect in the distance travelled in the dark zone (vector effect: $F_{1,38}=5.13$, $P<0.05$), due to a reduction in the Y1Δ-NPY group compared to Y1Δ-Empty controls ($P<0.05$; Fig. 4a). Vector treatment did not affect the distance travelled in dark zone in wild-type mice. There was no difference between the treatment groups in terms of light zone and total distance travelled (Fig. 4a). Activities in the light zone as measures for anxious behaviors, such as the time and distance travelled in the light zone and light zone to total distance ratio were unaffected by hippocampal NPY overexpression (Fig. 4, Table 1). However, Y1Δ-Empty mice exhibited an increase in light zone entry compared to wild-type mice (genotype effect: $F_{1,38}=7.70$, $P<0.01$; Y1Δ-Empty vs. WT-Empty, $P<0.01$; Fig. 4c).

Ambulatory activity in the dark zone was altered by NPY overexpression in the Y1Δ mice (Table 1). Time and frequency of ambulatory activities in the dark zone were reduced in Y1Δ-NPY mice compared to Y1Δ-Empty (vector effect: $F_{1,38}=6.09$, $P<0.05$ and $F_{1,38}=6.59$, $P<0.05$, respectively; pair-wise comparison, $P<0.05$ for both parameters). The reduction normalized a statistically non-significant increase in ambulatory activity by Y1 receptor deletion (Y1Δ-Empty vs. WT-Empty, dark zone ambulatory time, $P=0.10$; dark zone ambulatory episodes, $P=0.06$), to that of the wild-type mice level.

Table 1 Mice behavior in the OF, LD and HB tests.

	WT-Empty	WT-NPY	Y1Δ-Empty	Y1Δ-NPY
Open field test				
Center zone ambulatory time (s)	15.1±3.7	23.6±2.5*	26.8±3.2##	21.7±1.7
Center zone ambulatory episodes	131.5±35.1	188.4±28.3	232.8±25.4	188.7±16.8
Center zone resting time (s)	11.1±4.9	26.4±6.2	18.1±2.6	21.5±4.8
Peripheral zone ambulatory time (s)	61.7±5.7	86.6±9.4	70.8±7.9	69.1±5.7
Peripheral zone ambulatory episodes	371.3±40.5	494.7±63.6	472.2±50.4	432.3±38.7
Peripheral zone resting time (s)	412.4±15.1	361.2±8.8**	366.6±12.1#	375.3±12.6
Total vertical count	26.9±4.9	34.5±7.4	36.1±3.9	36.7±4.5
Total vertical time (s)	17.5±3.6	25.8±5.4	26.9±5.2	24.9±2.9
Light–dark test				
Light zone ambulatory time (s)	45.7±5.0	53.5±5.7	55.3±3.6	52.6±7.5
Light zone ambulatory episodes	369.3±44.9	374.4±43.8	447.3±32.8	419.3±64.4
Light zone resting time (s)	108.5±29.3	119.8±12.3	118.9±9.4	95.3±13.0
Dark zone ambulatory time (s)	92.8±8.6	83.6±6.3	108.3±5.4	84.3±5.0*
Dark zone ambulatory episodes	672.8±73.7	577.9±58.1	831.7±48.9	634.6±46.6*
Dark zone resting time (s)	214.5±24.9	210.6±18.7	176.7±9.8	239.6±24.6
Hole-board test				
Distance travelled (cm)	798.8±78.9	734.4±110.0	901.1±86.8	754.6±75.6
Ambulatory time (s)	78.7±7.6	73.1±11.3	90.2±9.6	75.1±7.7
Ambulatory episodes	486.8±53.9	434.0±82.2	583.7±68.8	466.8±51.3
Resting time (s)	261.6±12.4	259.2±12.7	246.2±9.8	258.1±10.0
Latency to first hole (s)	45.9±18.4	65.4±29.5	37.5±9.3	36.3±13.1

Significant difference between rAAV-NPY treated group vs. rAAV-Empty group of the same genotype was denoted by *$P<0.05$ or **$P<0.01$. Significant difference between WT and Y1Δ mice that received the same treatment was denoted by #$P<0.05$ or ##$P<0.01$. All data are presented as means±standard error of the mean (S.E.M.).

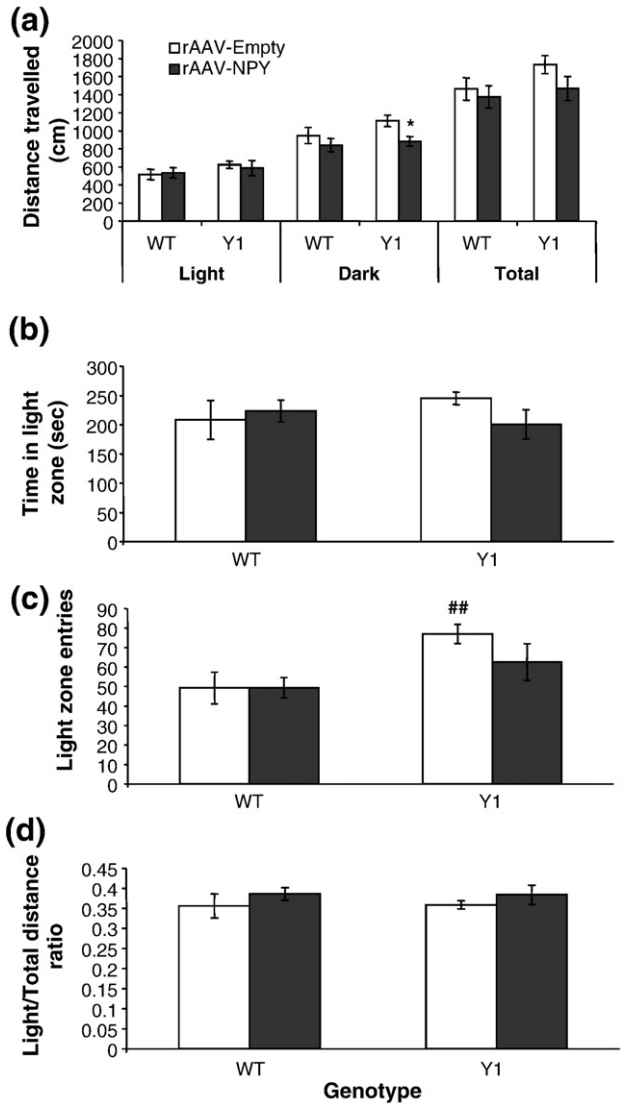

Figure 4 Mice performance in the light–dark test. (a) Distance travelled in the light, dark compartments and the total distance travelled. (b) Time spent in the light compartment. (c) Number of entries made into the light compartment. (d) Ratio of distance travelled in the light compartment. Significant difference between rAAV-NPY treated group vs. rAAV-Empty group of the same genotype was denoted by $*P<0.05$. Significant difference between WT and Y1Δ mice that received the same treatment was denoted by $\#\#P<0.01$. All data are presented as means ± standard error of the mean (S.E.M.).

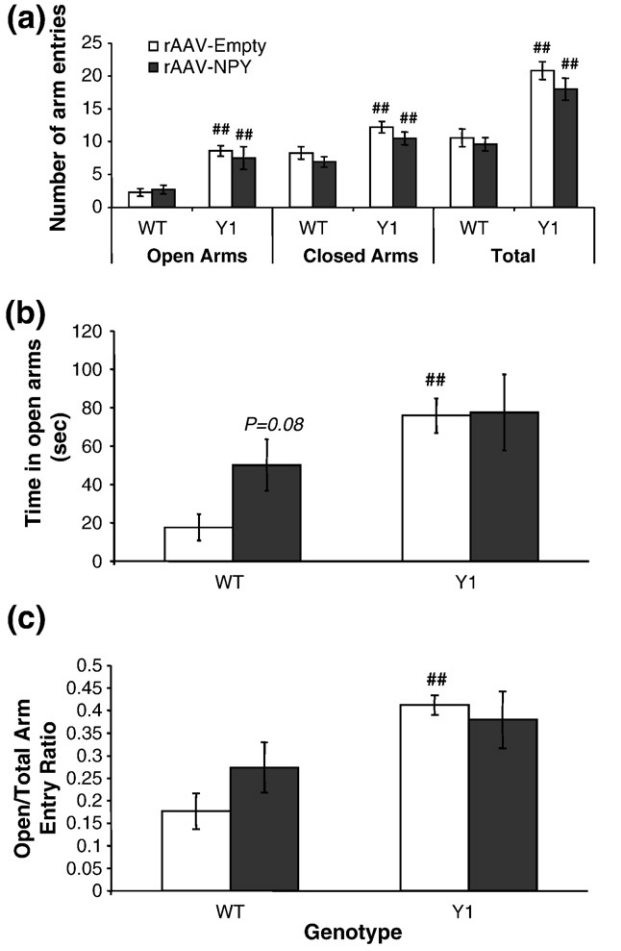

Figure 5 Mice performance in the elevated plus test. (a) Number of entries made into the open or closed arms of the EPM and total arm entries made. (b) Time spent in the open arms of the EPM. (c) Ratio of entries made into the open arms of the EPM. Significant difference between WT and Y1Δ mice that received the same treatment was denoted by $\#\#P<0.01$. All data are presented as means ± standard error of the mean (S.E.M.).

3.5. Elevated plus maze

Y1 receptor knockout mice exhibited more than threefold increase in the number of open arm entries (genotype effect: $F_{1,37}=29.50$, $P<0.001$), 1.5-fold increase in closed arm entries (genotype effect: $F_{1,37}=17.35$, $P<0.001$) and twofold increase in the total arm entries (genotype effect: $F_{1,37}=46.08$, $P<0.001$; no significant genotype × vector interaction for all three parameters; Fig. 5a). rAAV-NPY groups performed comparably to their corresponding rAAV-Empty groups of the same genotype.

Similarly, Y1 receptor deletion had a significant effect on the time the mouse spent in the open arms (genotype effect: $F_{1,37}=10.85$, $P<0.01$; Fig. 5b), with Y1Δ-Empty mice spent approximately fourfold more time on the open arms than the WT-Empty mice ($P<0.01$). Notably, WT-NPY mice spent much longer time in the open arms than the WT-Empty mice although this difference did not reach statistical significance (WT-Empty: 17.6 ± 6.9 s vs. WT-NPY: 50.1 ± 13.4 s, $P=0.08$; Fig. 5b).

As for the open field test, the ratio of open arm to total arm entries can be used as a locomotor-independent measure of anxiety-like behavior. A significant genotype effect was observed, with Y1Δ mice exhibiting higher numbers in open arm to total arm entries (genotype effect, $F_{1,37}=12.77$, $P=0.001$; Fig. 5c). No significant vector effect was observed for this parameter.

3.6. Hole-board test

In the HB test, there were no differences in parameters assessing activity or exploration (distance travelled, ambulatory

time and episode, resting time, Table 1), parameters assessing anxiety (latency to first head-dipping, total head-dipping, Fig. 6a), or parameter assessing working memory (novel entry ratio, Fig. 6b).

3.7. Passive avoidance test

As an indication of the memory of an aversive experience, the increase in latency to enter the dark chamber in which the mice received a mild foot shock upon entering on the training day was calculated (day 2 latency − day 1 latency). Despite large variations between the groups, in particular the Y1Δ-Empty and WT-Empty, these differences did not reach statistical significance due to variability within each group (genotype × vector interaction: $F_{1,38}=1.98$, $P=0.168$; genotype effect: $F_{1,38}=2.96$, $P=0.094$; vector effect: $F_{1,38}=0.01$, $P=0.934$; Fig. 6c).

3.8. Tail suspension test

There was no significant interaction effect between genotype and vector (genotype × vector interaction: $F_{1,38}=1.30$, $P=0.262$) in the TST. However, a significant difference was observed between the genotype (genotype effect: $F_{1,38}=14.60$, $P<0.001$) and also between the vector treatment groups (vector effect: $F_{1,38}=6.73$, $P=0.01$). Pair-wise comparison revealed a significant increase in immobility time in the rAAV-NPY injected wild-type mice compared to its corresponding control group ($P<0.05$; Fig. 6d). Genotype effect was mainly due to the significantly longer immobility time in the WT-NPY mice compared to the Y1Δ mice ($P<0.001$ vs. Y1Δ-Empty and $P<0.01$ vs. Y1Δ-NPY), although a subtle effect was observed between the WT-Empty and Y1Δ-Empty mice ($P=0.06$).

3.9. Serum corticosterone

WT mice injected with rAAV-NPY or rAAV-YFP into the hippocampus exhibited comparable levels of serum corticosterone 3 weeks after vector administration (WT-YFP: 46.7 ± 13.4 ng/mL; WT-NPY: 65.8 ± 22.9 ng/mL; Student's *t* test, $P=0.49$), suggesting no adverse effects on stress levels in these mice due to the treatment.

4. Discussion

In this study, a comprehensive assessment of the functional effects of adult-onset hippocampal NPY overexpression following somatic gene transfer was performed by utilising different behavioral tests of exploratory activity, anxiety, depression and learning and memory. In addition, this was compared to a situation of Y1 signalling deficiency common to that of TLE patients with reduction in hippocampal Y1 receptor expression (Furtinger et al., 2002). Evaluation of the effect of rAAV-NPY in Y1 receptor knockout mice was particularly relevant in determining the safety of rAAV-NPY as a treatment for TLE because the anticonvulsant effect of NPY is thought to be mediated by the Y2 and Y5 receptors (Lin et al., 2006; Vezzani et al., 1999; Woldbye et al., 1997),

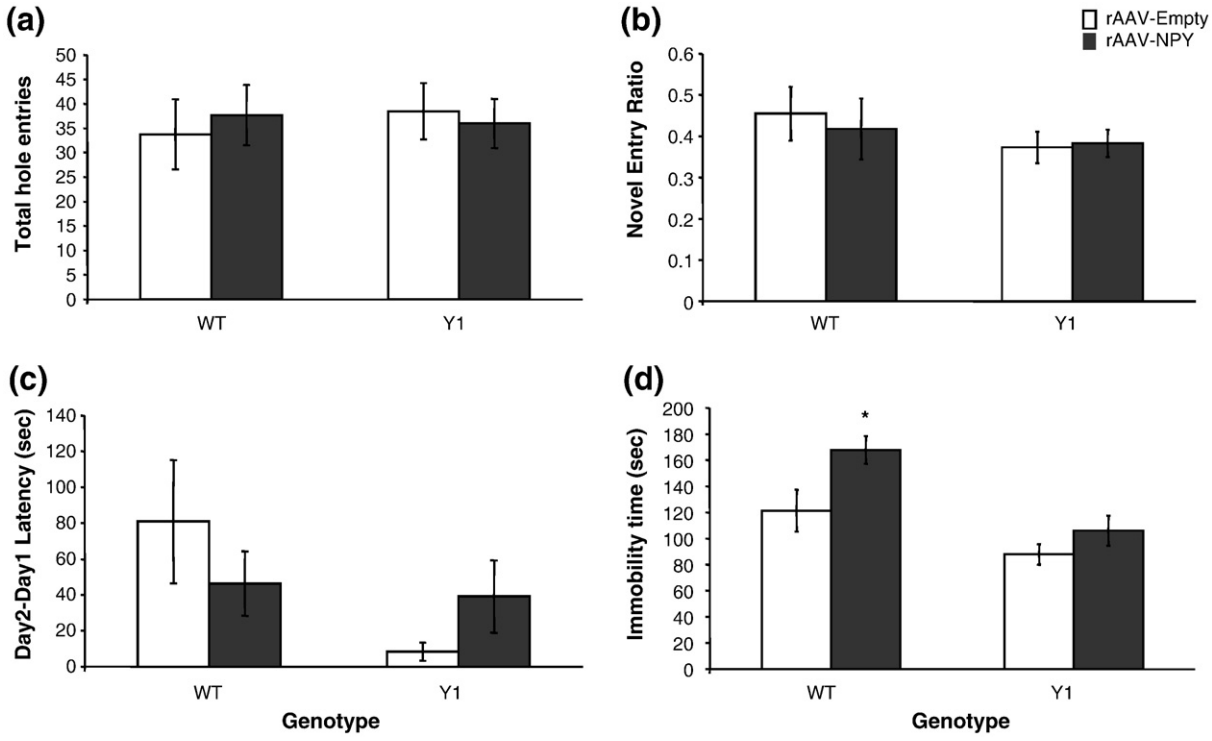

Figure 6 (a) Total hole entries mice made in the hole-board test. (b) Novel entry ratio [novel entries/total entries] as an indication of working memory in the hole-board test. (c) Increase in latency to enter dark chamber on the testing trial in the passive avoidance test. (d) Time mice assumed an immobile posture in the tail suspension test. Significant difference between rAAV-NPY treated group vs. rAAV-Empty group of the same genotype was denoted by *$P<0.05$. All data are presented as means ± standard error of the mean (S.E.M.).

which are the major receptors that were activated in the Y1 receptor knockout mice by hippocampal rAAV-NPY treatment in this study.

Our results showed that increased expression of NPY in the hippocampus did not cause any overt effects in the general health, neurosensory reflexes and motor function of the experimental animals. Locomotor activity and exploration was not affected by hippocampal NPY overexpression as demonstrated in the total distance travelled in the OF, LD and HB, and also the total arm entries in the EPM.

The observed increases in EPM arm entries and visual cliff test head-dipping behavior exhibited by the Y1 knockout mice suggest enhanced locomotion and explorative behavior, consistent with a previous report on these mice (Karl et al., 2006). The mechanism underlying the increased exploration is unclear and studies so far on the involvement of Y1 receptor in this behavioral domain reported conflicting data (Karl et al., 2006; Karlsson et al., 2008; Pedrazzini et al., 1998). While the inconsistency in the data could be due to genetic background difference of the different mutant mice developed, another explanation may be the complexity of the involvement of Y1 receptor in this behavioral parameter, which has been shown to be highly context-specific and sensitive to stress (Karl et al., 2006). Notably, both NPY and Y1 receptors are expressed in the peripheral nervous system including the adrenal glands and NPY was shown to regulate catecholamine synthesis and secretion via Y1 receptors. Both the adrenal and plasma levels of catecholamines (norepinephrine and epinephrine) were shown to be higher in Y1 receptor knockout mice (Cavadas et al., 2006). Thus the altered adrenosympathetic tone in the Y1 receptor knockout mice may play a role in their observed change of motor activity and explorative behaviors.

In the OF test, the NPY-overexpressing wild-type mice exhibited increased entries and time spent in the center zone, parameters suggestive of an anxiolytic effect by hippocampal NPY. Although this effect was not apparent in the LD and HB tests, the strong trend for increased open arm time in the WT-NPY group compared to the WT-Empty group in the EPM is consistent with the anxiolytic action by NPY overexpression observed in the OF test. Our finding is in line with previous studies demonstrating the anxiolytic effect of central NPY. Intracerebroventricular administration of NPY has been shown to reduce anxiety in rodents in a wide range of paradigms (Kask et al., 2002), whereas mice deficient of NPY exhibit an anxiogenic phenotype (Bannon et al., 2000; Karl et al., 2008; Palmiter et al., 1998). In addition, transgenic rats with hippocampal-specific NPY overexpression were insensitive to the anxiogenic effect of restraint stress on the elevated plus maze and also showed absent fear suppression in the punished drinking test, indicating a stress-protective role of hippocampal NPY (Thorsell et al., 2000). Interestingly, these NPY overexpressing rats showed comparable corticosterone levels to control rats both at baseline and after restraint stress (Thorsell et al., 2000), similar to our observation in the rAAV-NPY mice. Although serum corticosterone is a stress hormone marker often used to indicate level of anxiety, various studies and paradigms have shown that behavioral and endocrine stress responses can be dissociated (Benaroya-Milshtein et al., 2004; Koob et al., 1993; Moncek et al., 2004).

The anxiolytic effect of NPY is thought to be mediated by Y1 receptors. Microinjection of NPY and Y1 receptor agonist, but not the Y2 receptor agonist, into the central nucleus of the amygdala reproduced the anxiolytic effect of ICV NPY with high potency (Heilig et al., 1993). Moreover, the anxiolytic effect of intra-amygdaloid NPY was blocked by co-administration with Y1 receptor antagonist (Sajdyk et al., 1999) or intraventricular Y1 antisense administration (Heilig, 1995), which alone is anxiogenic (Wahlestedt et al., 1993). Our data suggest that Y1 receptor may also play a role in mediating the anxiolytic effect of NPY in the hippocampus since hippocampal rAAV-NPY failed to confer anxiolytic action in the Y1 receptor deficient mice. However, it should be noted that the Y1 knockout mice already exhibited an anxiolytic-like phenotype, therefore it remains possible that a 'ceiling' effect exist in these mice in terms of anxious behavioral measures and that additional anxiolytic effect was difficult to detect. While the reduced anxiety observed in Y1 receptor knockout mice may seem paradoxical to the supposedly anxiolytic effect of Y1 receptors, this is likely due to extra-hippocampal and extra-amygdaloid effects of Y1 receptors as they are widely expressed both in the central and peripheral nervous system (Balasubramaniam, 1997; Dumont et al., 1998). Future studies combining the rAAV-NPY treatment with specific down-regulation of Y1 receptor in the hippocampus either by conditional recombination method or antisense oligonucleotide may circumvent the peripheral effect of Y1 receptor deletion.

The mechanism underlying the effect of NPY on anxiety-like behaviors is still not entirely clear and is likely to be complex considering the involvement of different Y receptors. Even in the hippocampus alone, it was shown that NPY may mediate its anxiolytic effect by different Y receptor depending on the specific sub-regions and that a receptor may confer sub-region specific effect on anxiety (Smialowska et al., 2007). One potential mechanism by which hippocampal Y1 receptors may regulate emotionality is its role in adult hippocampal neurogenesis. NPY stimulates neuroproliferation in the hippocampus via Y1 receptors (Howell et al., 2005) and recently it was demonstrated that impairments in adult hippocampal neurogenesis lead to increased anxiety-related behaviors (Revest et al., 2009). This is supported by studies showing manipulations that increase adult hippocampal neurogenesis such as environmental enrichment, physical exercise and neurotrophin administration also reduce anxiety (Duman et al., 2008; Fox et al., 2006; Perez et al., 2009; Salam et al., 2009). Nevertheless, the present study does not rule out involvement of other Y receptors and further studies are required to fully elucidate the effects and mechanisms by which the different Y receptors affect anxiety response in the hippocampus.

Surprisingly, we observed depressive-like phenotype in the rAAV-NPY treated wild-type mice in the TST. This observation was unexpected as numerous studies have suggested NPY to be antidepressive. Animal models of depression and human postmortem study showed that NPY is significantly reduced in the brains of depressed subjects (Caberlotto and Hurd, 1999; Caberlotto et al., 1999; Heilig and Widerlov, 1990). Interventions with antidepressant efficacy, such as electroconvulsive shock stimulation (ECS) and antidepressant drugs, increase central NPY expression (Husum et al., 2000; Mikkelsen et al., 1994; Wahlestedt

et al., 1990). Furthermore, infusion of NPY into the cerebral ventricles or hippocampus produces an antidepressant effect (Ishida et al., 2007; Redrobe et al., 2002; Stogner and Holmes, 2000). While the reason for the observed depressive-like behavior in the rAAV-NPY wild-type mice is unclear, it should be noted that NPY overexpression did not induce depressive-like phenotype in the Y1 knockout mice, which showed comparable immobility scores to the wild-type mice.

Since the effect of rAAV-NPY gene transfer on spatial learning and memory has already been evaluated (Sorensen et al., 2008a; Sorensen et al., 2008b), we used two different tests to further assess the effect of NPY overexpression on other aspects of cognitive function. Using the novel to repeat hole ratio in the hole-board test as an indication of working memory (Baiardi et al., 2007; Karl et al., 2006) and the passive avoidance as a test for associative memory, we found no evidence of adverse effect by NPY overexpression on working memory and associative learning. This is consistent with previous report on the behavioral phenotype of NPY deficient mice, demonstrating the lack of effects by NPY in cognitive parameters measured by the same paradigms (Karl et al., 2008). Our data are also consistent with that reported by Ishida et al. (2007), showing no deficit in passive avoidance test after intrahippocampal NPY infusion. Sorensen et al. (2008a) showed that rAAV-NPY treated rats exhibited delayed learning in the two-platform spatial discrimination water maze test, but achieved comparable learning to the control rats at the end of the 7 day testing period. The same study demonstrated that long-term potentiation in the CA1 area was partially impaired by transgene NPY acting via Y2 receptors, likely by an inhibition of glutamate release onto pyramidal cells (Sorensen et al., 2008a). However, a follow up study showed that rAAV-mediated NPY overexpression did not affect short-term synaptic plasticity nor did it further compromise LTP in kindled animals, a model for epilepsy (Sorensen et al., 2009). Together these data suggest that rAAV-NPY have limited impact on cognition, with spatial learning more sensitive to its effect. However, in epileptic subjects with already impaired LTP (Beck et al., 2000) and memory function (Elger et al., 2004; Helmstaedter et al., 2003), rAAV-NPY treatment is unlikely to cause further impairment.

In summary, our data suggest that hippocampal NPY overexpression confers a moderate anxiolytic effect, possibly in part mediated by the Y1 receptors. Although hippocampal NPY overexpression induced a moderate increase in depressive-like behaviors, this effect is likely to be absent or diminished in chronic epileptic subjects with reduced hippocampal Y1 receptor expression. In the two cognitive tests used, hippocampal NPY overexpression did not alter cognitive functions. In conclusion, our study suggests that focal NPY overexpression are unlikely to result in significant adverse effects on mood regulation or learning and memory, and may be a safe therapeutic alternative for the treatment of drug-resistant temporal lobe epilepsies. Nevertheless, the potential subtle effect on mood regulation should be monitored.

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Contributors

E-J.D.L. designed the study, conducted most of the experiments and wrote the manuscript. S.L. and A.A. conducted the histochemical analysis. M.J.D. provided the vectors and contributed to the manuscript. H.H. supervised the project, participated in the data interpretation and edited the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of interest

E-J.D.L. and M.J.D. have consulted for Neurologix Inc. All other authors declare that they have no conflicts of interest.

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