EJN
European Journal of Neuroscience

European Journal of Neuroscience, Vol. 28, pp. 173–180, 2008 doi:10.1111/j.1460-9568.2008.06306.x

Behavioural profile of a new mouse model for NPY deficiency

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Keywords: anxiety, exploration, learning, motor activity, NPY knockout mouse, sex

Abstract

The abundantly expressed neuropeptide Y (NPY) plays an important role in anxiety and stress reactivity, as exogenous NPY administration reduces anxiety-like behaviour in rodents. However, unlike the potent effects of NPY seen in pharmacological studies, two independent examinations of a genetic mouse model for NPY deficiency have shown only subtle, inconsistent and task-dependent anxiety-related phenotypes for male mutants. Here we present results of a newly developed germline NPY-knockout model, which has been characterized behaviourally using a comprehensive multi-tiered phenotyping strategy. Mice of both sexes were investigated in locomotion and exploration tasks, anxiety-related paradigms, a hippocampus-dependent memory test and a battery of basic tasks screening for sensory and motor functions. Male and female NPY-deficient mice consistently demonstrated suppressed levels of locomotion and exploration. Furthermore, mutant mice exhibited a pronounced anxiogenic-like phenotype when tested in spatiotemporal anxiety-relevant paradigms (i.e. elevated-plus maze, open field and light–dark task). Importantly, this phenotype was more pronounced in male NPY mutants, revealing a moderate sexually dimorphic impact of NPY deficiency on behaviour. Interestingly, lack of NPY did not result in impaired learning and memory in either sex. Our carefully selected comprehensive behavioural phenotyping strategy revealed a consistent hypolocomotive and sex-dependent anxious-like phenotype. This new NPY-knockout mouse model reveals the importance of sex-specific testing. It also offers a potent new model for research into anxiety-related disorders and suggests potential treatment options for these conditions via the NPY system.

Introduction

Neuropeptide Y (NPY) is a highly conserved 36-amino-acid peptide which is widely expressed throughout the CNS (Adrian *et al.*, 1983). Its effects are mediated by at least five G-protein-coupled receptors, Y1, Y2, Y4 and Y5. Originally noted for its effects on feeding behaviour, NPY also regulates other important physiological processes including metabolism and anxiety (for review see Kask *et al.*, 2002; Lin *et al.*, 2004).

The anxiolytic-like action of exogenously administered NPY has been described for several rat models using a variety of anxiety paradigms (Heilig *et al.*, 1989; Broqua *et al.*, 1995; Sajdyk *et al.*, 1999). Intracerebroventricular NPY also suppressed open field (OF) and home cage activity (Heilig & Murison, 1987). Targeted micro-injection studies have demonstrated that the amygdala, particularly the basolateral nucleus, mediates the anxiety-related effects of NPY (Heilig *et al.*, 1993; Sajdyk *et al.*, 1999). Pharmacological testing using agonists and antagonists for NPY receptors have demonstrated that the Y1 receptor is the most likely candidate involved in NPY's anti-anxiety effects (Heilig *et al.*, 1993; Kask *et al.*, 1996). However, the Y5 and in particular the Y2 receptor also appear to play an important role in these effects (Naveilhan *et al.*, 1998; Sajdyk *et al.*, 2002; Redrobe *et al.*, 2003).

Thus, pharmacological studies suggest that NPY is involved in the regulation of anxiety; continuous low levels of NPY neurotransmission seem to be necessary to signal safety or the absence of threat (Kask *et al.*, 1998a, b). However, from a behavioural perspective, pharmacological investigations can be complicated by problems with solubility of the compound used, its side effects and issues associated with handling and necessary restraint during the injection process. Genetically engineered animal models lacking the gene of interest are therefore a valid alternative, circumventing some of these problems, although compensatory mechanisms have to be considered when using germline knockout (KO) models. It is crucial to apply a comprehensive behavioural phenotyping strategy when characterizing new genetic animal models. Furthermore, handling stress and other confounding factors (e.g. circadian rhythm) have to be identified and minimized to avoid false-positive or fragmentary results. Two independent behavioural examinations of NPY-KO mice developed by Palmiter *et al.* found an inconsistent and confounded behavioural phenotype: Palmiter reported increased anxiety-like behaviours in the elevated-plus maze (EPM) and increased learning abilities in the passive avoidance task (Palmiter *et al.*, 1998) whereas Bannon observed a wild-type (WT)-like performance for mice derived from the same line in both paradigms and an elevation in anxiogenic-like behaviours in the OF (Bannon *et al.*, 2000; Table 1). Importantly, both studies relied solely on motor activity-dependent measures of anxiety

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Received 30 October 2007, revised 28 April 2008, accepted 29 April 2008

TABLE 1. Anxiety- and learning-related phenotype of germline NPY-KO males developed by Palmiter *et al.* (1998) in two different studies

Behavioural task	Bannon *et al.*, 2000	Palmiter *et al.*, 1998
Elevated plus maze	WT-like	↑ Anxiety
Open field	↑ Anxiety	WT-like
Light-dark	Not tested	WT-like
Passive avoidance	WT-like	↑ Avoidance/learning

rather than including a locomotion-independent parameter (i.e. ratio of motor activity in an aversive area or risk assessment behaviour).

In order to clarify these inconsistencies, a thorough and targeted behavioural phenotyping strategy is necessary. Therefore, the current study examined a newly developed NPY-KO model using a comprehensive multi-tiered approach including a battery of tests for sensory, neurological and motor functions, for locomotion and exploration and for anxiety. We also investigated the higher functions of learning to more comprehensively characterize the behavioural effects of NPY deficiency. Importantly, sex-specific effects of NPY were also examined, adding to the increasing literature showing the importance of testing both male and female animal models to fully understand the impact a given neuropeptide might have on neurobehavioural domains.

Materials and methods

Generation of NPY-KO mice

A detailed description of the generation of this KO line will be given elsewhere. In short, a 130-kb mouse genomic BAC clone from a 129SVJ line was mapped and various fragments were subcloned. A 10-kb *Eco*RI fragment containing a 6-kb 5′-flanking sequence as well as exons 1 and 2 of the *NPY* gene and an 11-kb *Sac*I fragment containing exons 2, 3 and 4 were chosen for the construction of an *NPY-Cre* knock-in construct. The linearised version of that clone was transfected into ES cells. Two positive clones were injected into C57BL/6 blastocysts and chimeric mice were bred to generate heterozygous mice; subsequently, homozygous *NPY-Cre* knock-in mice were bred.

Animals

Germline NPY-KO and WT-like control mice from a colony maintained at the Biological Testing Facility of the Garvan Institute of Medical Research were used for the experiments. Male and female age-matched (males, ± 5 days; females, ± 12 days) mice (n = 8–10 per genotype and sex) of similar genotype and sex were pair-housed in Macrolon cages provided with cellulose paper as nesting material, and received food and water *ad libitum*. Cages were held in a temperature- and humidity-controlled room (22°C; 55–60% relative humidity) with a 12 : 12 h light : dark cycle (light phase, ~70 lx white light; dark phase, < 2 lx red light). Microbiological monitoring revealed no infection of the SPF facility, with the exception of the pathogens commonly found in commercial and research facilities, *Pasteurella pneumotropica* and *Helicobacter* spp. Mice were transferred to the experimental room 1 h prior to testing to allow habituation. Equipment was cleaned with 30% ethanol solution before each animal was tested. Unless otherwise stated, testing was commenced 1 h after the onset of the light phase (see also Table 2 for test schedule). All research and animal care procedures were approved by 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 Purposes.

TABLE 2. Test schedule

	Age of test animals (days)	
Behavioural paradigm	Male	Female
Hole board	71	76
LD	78	79
EPM	81	82
OF	85	85
Physical exam	88	86
Accelerod	95–98	88–91
Passive avoidance	115	100

The test schedule shows the order in which tests were completed, including the average test age (males, ± 5 days; females, ± 12 days) of the animals. Handling-intensive tasks (i.e. physical exam and accelerod) and tests using an aversive stimulus (i.e. electrical foot-shock in passive avoidance) were performed at the end of the experimental schedule to avoid confounding the stress response of test animals.

Physical exam

General health, sensory abilities, neurological motor reflexes, and motor function and coordination have a huge impact on animals' behavioural performance, and aberrations in these basic functions can be misinterpreted as alterations in more complex behavioural domains (Crawley & Paylor, 1997; Crawley, 1999). A wide range of basic tasks were used to evaluate all mice for such basic sensory and motor abilities including the accelerod test (for details of test paradigms see Karl *et al.* 2003). Tests were performed after completion of all other handling-sensitive paradigms (see Table 2) during the light phase of the circadian cycle.

Open field (OF)

In this test, the conflict between the drive to explore a new environment and a natural aversion to illuminated open areas is used to examine both anxiety and motor activity (Crawley, 1985). Mice were tested in an automated, photobeam-controlled OF, 43.2 × 43.2 cm (MedAssociates Inc., Vermont, USA). The arena was divided into a central and a peripheral zone (central zone dimensions: software coordinates 3/3, 3/13, 13/3, 13/13). Mice were placed in a corner of the arena (illumination level 20 lx) and were allowed to explore the arena for the following 10 min, while their activity was measured automatically (software settings: box size, four; ambulatory trigger, two; resting delay, 1500 ms). Measures of anxiety included the time spent in the central area of the OF and distance travelled in the centre as a ratio of overall distance travelled. These anxiety measures are considered to be largely independent of overall activity levels. The defecation score was also recorded as an indicator of anxiety. Distance travelled, time spent in ambulation, time spent 'resting' (no photobeam-detectable movement) and small motor movements (photobeam breaks without ambulation) were all included as measures of motor activity. Vertical activity (rearing) was used as a measure of exploration.

Elevated-plus maze (EPM)

One of the most well-validated anxiety tasks, the EPM, induces a conflict between the animal's desire to examine a new environment

and its preference for 'safe' enclosed arms over the aversive exposed and elevated open arms (Montgomery & Monkman, 1955; File, 1993). The grey (PVC) plus-maze consisted of two open (35 × 6.0 × 0.4 cm) and two closed (35 × 6 × 28 cm) arms extending from a central platform (6 × 6 cm), raised 70 cm above the ground (illumination on open arms, 70 lx). Mice were placed on the central platform, facing an enclosed arm, and were allowed to explore the maze for 5 min. The frequency of entries (defined as more than half the animal's body length entering the arm) into and time spent on open and closed arms, as well as frequencies and latencies of rearing, grooming, stretch–attend postures and head-dipping over the edge of the open arms were recorded online. Measures of anxiety included latency to enter an open arm, duration of time spent on open arms, open arm entries as a proportion of total entries (entry ratio), and frequency of stretch–attend postures. The time spent on open arms and ratio of open: total arm entries provide measures of anxiety that are relatively independent of overall activity levels. Measures of motor activity included the frequency of arm entries (enclosed and total) and explorative-like rearing.

Hole-board

The hole-board test measures directed exploration and can also be used as an initial basic screen for working memory (Boissier & Simon, 1962; Makanjuola *et al.*, 1977). The OF chamber was fitted with a hole-board floor insert for mice (MED Associates Inc.; 16 holes of diameter 1.6 cm). Testing of male mice took place from 1 h after the onset of the dark phase (illumination at floor level < 2 lx). Each mouse was placed in the centre of the arena and was left to explore the environment. The infrared photobeams provided automated measures of the distance travelled, ambulatory frequency, head dipping frequency and working memory ratio (number of head dips into novel holes divided by total number of head dips) in a 7-min test session (Lister, 1987).

Light–dark (LD)

Anxiety is measured in this test by comparing the animal's activity and time spent in a brightly illuminated area with that in a dark compartment (Hascoet *et al.*, 2001). The OF chambers were equipped with dark box inserts covering half the arena, with a small opening to allow movement between the two areas (MedAssociates Inc.). The black Plexiglas insert was opaque to visible light, but allowed photobeams to pass through for automatic recording of the animal's movements. Mice were placed into the illuminated area (20 lx), facing the dark compartment (< 2 lx), and were allowed to explore the environment freely for 10 min. Proportion of time spent and distance travelled in the light relative to the dark compartment were taken as measures of anxiety. Vertical activity (rearing) in the light compartment can also be indicative of reduced anxiety. Overall distance travelled and rearing in the dark compartment were taken as measures of motor activity.

Passive avoidance

In this basic hippocampus-dependent learning test, the avoidance of a naturally less aversive dark compartment after it is paired with an electrical foot-shock indicates the retention of this memory (Bovet *et al.*, 1969). The behavioural performance of rodents in this task is also influenced by their general stress response (i.e. fear of highly illuminated areas and aversive stimuli such as electrical foot-shock) and nociception. In the training session mice were placed in a highly illuminated compartment (illumination 70 lx; Shuttle Box System: TSE Systems, Bad Homburg, Germany). After 10 s, the door to a dark chamber was opened. Once the mouse moved into the dark chamber (illumination < 2 lx) the door was closed and a single foot-shock (0.4 mA for 2 s) was delivered. Mice were kept in the dark chamber for another 60 s to allow the formation of an association between the location and the foot-shock. In the retention session 24 h later mice were again placed in the light compartment and 10 s later the door connecting light and dark chambers was opened. The latency to enter the dark chamber on each trial was measured, and increased entry latency on the second day indicated memory of the aversive stimulus.

Statistical analysis

Results were analysed using two-way (main factors: genotype and sex) and one-way ANOVA with the exception of passive avoidance, which was analysed using ANOVA for repeated measurements. Fisher's PLSD was used for *post hoc* comparisons, if appropriate. Differences were regarded as significant when $P < 0.05$. All data are presented as means ± SEM. Figures show means + SEM and significant *post hoc* effects vs. WT-like mice are indicated by '*' for males and by '#' for females (see specific details in each figure legend).

Results

The physical examination and the accelerod testing confirmed that all mice independently of genotype and sex were in good general health, with intact sensory and motor functions and coordination, neurological reflexes and neuromuscular strength (data not shown).

Locomotion

NPY deficiency suppressed motor activity in male as well as female mice, as indicated by a reduction in overall distance travelled in the OF paradigm [two-way ANOVA for factor genotype, $F(1,32) = 9.6$, $P = 0.004$; one-way ANOVA for males, $F(1,16) = 6.5$, $P = 0.02$; and for females, $F(1,16) = 3.8$, $P = 0.07$] and the LD test [two-way ANOVA for factor genotype, $F(1,32) = 13.7$, $P < 0.001$; one-way ANOVA for males, $F(1,16) = 7.8$, $P = 0.01$; and for females, $F(1,16) = 6.2$, $P = 0.02$] compared to WT control mice of the same sex (Fig. 1). A significant effect of sex was also found for this parameter in both tasks, with female mice showing increased locomotion compared to male mice [two-way ANOVA: OF test, $F(1,32) = 22.0$, $P < 0.001$; LD test, $F(1,32) = 7.3$, $P = 0.01$]. Although NPY-deficient mice showed a clear reduction in general locomotion, mutant animals nevertheless explored less frequently the test arenas including the aversive zones, as confirmed by the distance travelled in the light compartment of the LD test (WT male, 724.7 ± 61.1; NPY-KO male, 313.4 ± 69.3; WT female, 815.5 ± 42.7; NPY-KO female, 513.1 ± 103.4 cm).

Conversely, NPY-KO mice spent more time resting than did control mice of the same sex, both overall [two-way ANOVA for factor genotype, $F(1,32) = 28.4$, $P < 0.001$; one-way ANOVA for males, $F(1,16) = 21.4$, $P = 0.0003$; and for females, $F(1,16) = 8.1$, $P = 0.01$; Table 3] and in the less aversive peripheral zone of the OF [two-way ANOVA for factor genotype, $F(1,32) = 43.6$, $P < 0.001$; one-way ANOVA for males, $F(1,16) = 26.1$, $P < 0.001$; and for females, $F(1,16) = 17.6$, $P < 0.001$; data not shown]. Sex had a significant impact, with males demonstrating increased time spent resting compared to females [two-way ANOVA for overall, $F(1,32) = 19.0$, $P < 0.001$; for peripheral zone, $F(1,32) = 26.9$, $P < 0.001$; Table 3].

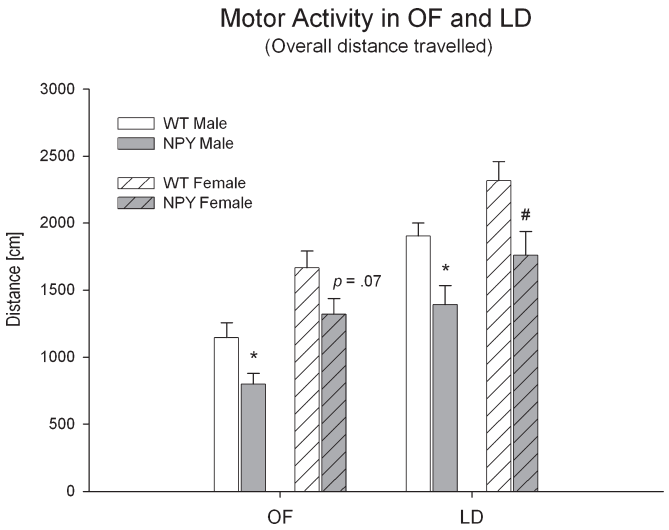

FIG. 1. Motor activity. Overall distance travelled (cm) as an automated measure of motor activity (locomotion) in the OF and LD. Means + SEM are shown. Significant *post hoc* effects for male NPY-KO vs. male WT mice are indicated by asterisks (*$P < 0.05$) and for females by '#' ($^{\#}P < 0.05$).

The hypoactive phenotype of NPY mutants was confirmed in the LD task, in which these mice displayed more time resting than did WTs [two-way ANOVA for factor genotype, $F(1,32) = 11.0$, $P < 0.01$; one-way ANOVA for males, $F(1,16) = 7.8$, $P = 0.01$; and for females, $F(1,16) = 6.2$, $P = 0.07$; Table 3].

Exploration

Consistent with the impact of NPY deficiency on locomotion, exploratory activity was also reduced in NPY-deficient animals, as two-way ANOVA for the factor genotype revealed a significant decrease, compared to WT mice, in vertical activity in the OF [$F(1,32) = 45.5$, $P < 0.001$; one-way ANOVA for males, $F(1,16) = 20.7$, $P < 0.001$; and for females, $F(1,16) = 25.0$, $P < 0.001$] and the LD [$F(1,32) = 23.8$, $P < 0.001$; one-way ANOVA for males, $F(1,16) = 11.3$, $P = 0.004$; and for females, $F(1,16) = 13.7$, $P = 0.002$; Fig. 2a]. No sex-specific differences were detected for this parameter. No significant differences were detected in explorative-like tendencies in the hole-board paradigm [i.e. total number of head dips; two-way ANOVA for factor genotype, $F(1,32) = 0.008$, n.s.; WT male, 34.9 ± 4.2; NPY-KO male, 33.7 ± 6.5; WT female, 36.3 ± 3.6; NPY-KO female, 38.4 ± 5.6]. However, NPY-deficient mice exhibited a decrease in the overall rearing frequency in the EPM [two-way ANOVA for factor genotype, $F(1,32) = 19.1$, $P < 0.001$; one-way ANOVA for males, $F(1,16) = 8.8$, $P = 0.009$; and for females, $F(1,16) = 10.5$, $P = 0.005$; Fig. 2b]. A reduction in exploration levels of NPY mutants was also evident in the less aversive areas of the test arenas, for example within the periphery of the OF test [two-way ANOVA for factor genotype, $F(1,32) = 39.0$, $P < 0.001$; one-way ANOVA for males, $F(1,16) = 17.6$, $P < 0.001$; and for females, $F(1,16) = 21.6$, $P < 0.001$; Table 3].

Anxiety

Increased levels of anxiety-related behaviours were detected consistently in NPY-deficient mice, compared to WT control mice, using various measures across different test paradigms. Importantly, in certain tasks this phenotype was more pronounced in male than in female NPY mutants (i.e. in EPM and LD). NPY-deficient mice avoided the centre of the OF and spent less time in this anxiety-inducing exposed area [zone time; two-way ANOVA for factor genotype, $F(1,32) = 25.8$, $P < 0.001$; one-way ANOVA for males, $F(1,16) = 52.1$, $P < 0.001$; and for females, $F(1,16) = 14.7$, $P = 0.002$; Fig 3a]. Similarly, these mice travelled less distance in the centre of the OF relative to the total arena [center ratio; two-way ANOVA for factor genotype, $F(1,32) = 14.3$, $P < 0.001$; one-way ANOVA for males, $F(1,16) = 17.6$, $P < 0.001$; and for females, $F(1,16) = 3.3$, $P = 0.09$; Fig 3b]. We also found a significant effect of sex on both OF parameters [two-way ANOVA for factor sex: zone time, $F(1,32) = 11.0$, $P = 0.002$; center ratio, $F(1,32) = 7.5$, $P = 0.01$] with males showing increased anxiety compared to female mice (Fig. 3a and b).

A pronounced sex-dependent anxiogenic phenotype was seen in the EPM, as only male NPY-deficient mice were significantly slower to enter open arms [two-way ANOVA for latency to enter open arms: for factor genotype, $F(1,32) = 8.8$, $P = 0.006$; factor sex, $F(1,32) = 4.1$, $P = 0.04$; genotype × sex interaction, $F(1,32) = 5.9$, $P = 0.02$; one-way ANOVA for males, $F(1,16) = 12.1$, $P = 0.003$; and for females, $F(1,16) = 0.18$, $P =$ n.s.; Fig. 4] and spent significantly less time within these exposed areas than did WT mice [two-way ANOVA for factor genotype, $F(1,32) = 4.1$, $P = 0.06$; for factor sex, $F(1,32) = 4.2$, $P < 0.05$; one-way ANOVA for males, $F(1,16) = 7.8$, $P = 0.01$;

TABLE 3. Results of behavioural tests

	Male		Female	
Behavioural paradigm	WT	NPY-KO	WT	NPY-KO
OF				
Small motor movements (*n*)	585.5 ± 6.5	371.1 ± 33.2***	630.7 ± 17.1	479.8 ± 24.1$^{\#\#\#}$
Vertical activity in periphery (*n*)	52 ± 6.8	14.7 ± 5.8***	56.9 ± 6.6	17.4 ± 4.8$^{\#\#\#}$
Resting duration (s)	381.6 ± 12.7	462.7 ± 11.9***	347.2 ± 11.3	393.3 ± 11.3$^{\#}$
LD				
Resting duration (s)	259.5 ± 9.9	332.5 ± 22.5*	245.8 ± 11.8	290.5 ± 21.5†
EPM				
Defecation score (*n*)	2.1 ± 0.8	3.4 ± 0.7	0.1 ± 0.1	2.8 ± 1.0$^{\#\#}$
Stretch–attend postures	2.5 ± 0.6	0.8 ± 0.3*	5.5 ± 0.6	2.3 ± 0.9$^{\#\#}$

Frequency [*n*] of small motor movements and peripheral vertical activity in OF, time spent in resting behaviour [s] in OF LD test as well as defecation score [*n*] and frequency of stretch-attend postures [*n*] in the EPM are shown as means ± SEM. Significant *post hoc* effects vs. WT are indicated by '*' for males (*$P < 0.05$ and ***$P < 0.001$) and by '#' for females ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$); $^{\dagger}P = 0.07$.

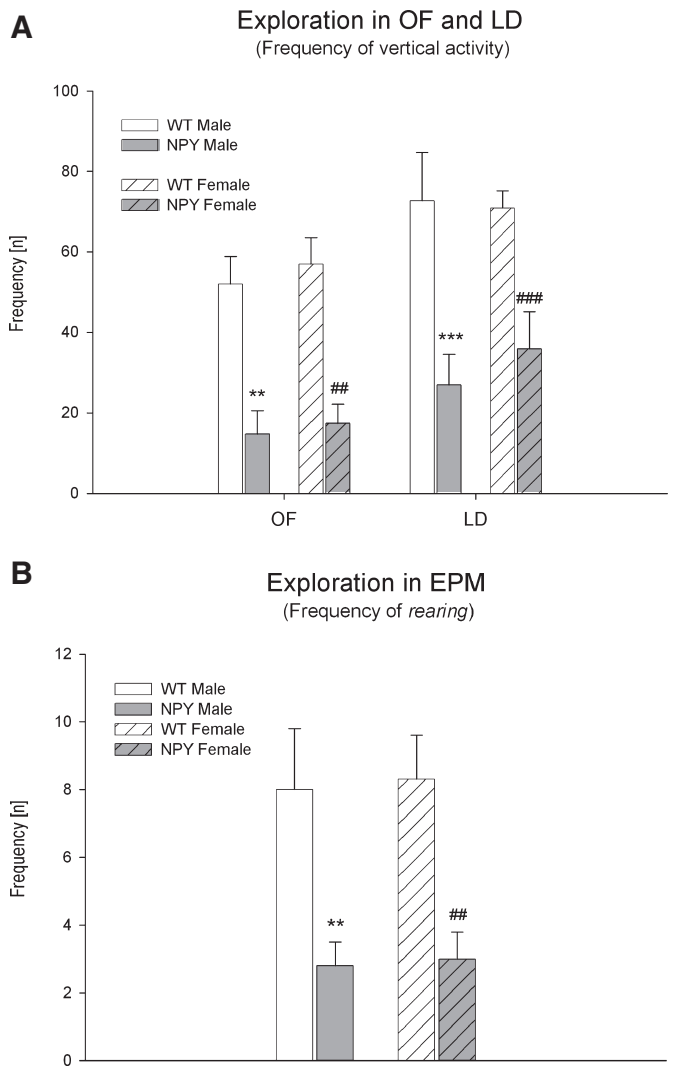

FIG. 2. Exploration. (A) Frequency of vertical activity (*n*) as an automated measurement of rearing in the OF and LD, and (B) frequency of rearing in the EPM. Means + SEM are shown. Significant *post hoc* effects for male NPY-KO vs. male WT mice are indicated by asterisks (**$P < 0.01$ and ***$P < 0.001$) and for females by '$^{\#}$' ($^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$).

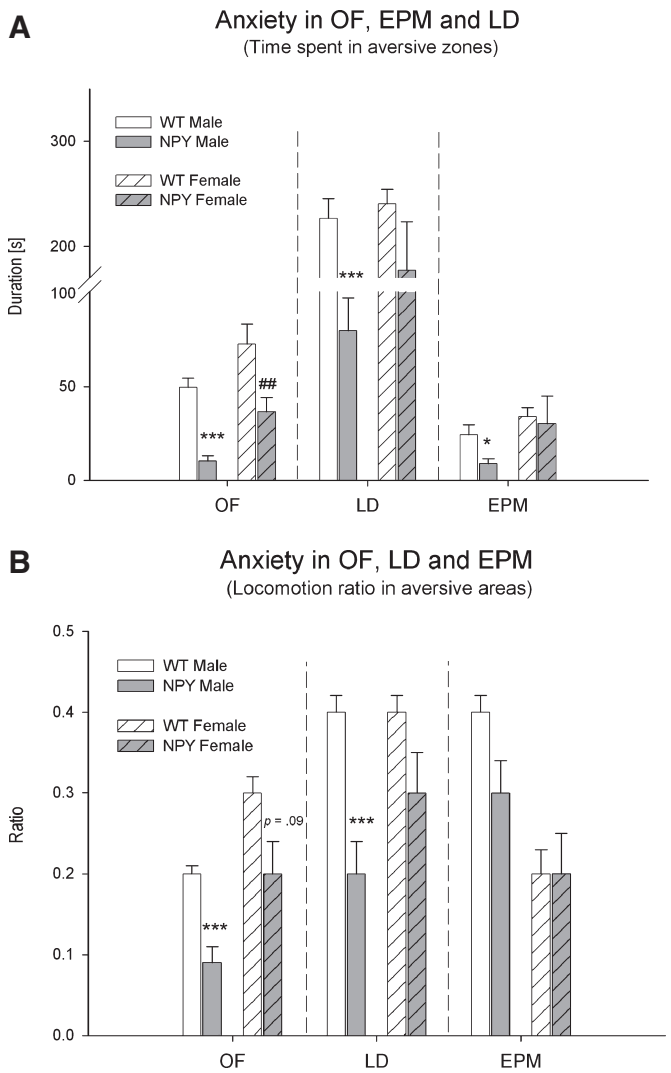

FIG. 3. Anxiety in the OF, LD and EPM. (A) time spent (s) in aversive zones (i.e. OF, central arena; LD, illuminated compartment; EPM, open arms), and (B) ratio of distance travelled (to total distance travelled) in the same zones are presented. Means + SEM are shown. Significant *post hoc* effects for male NPY-KO vs. male WT mice are indicated by asterisks (*$P < 0.05$ and ***$P < 0.001$) and for females by '$^{\#}$' ($^{\#\#}P < 0.01$).

and for females, $F(1,16) = 0.1$, P = n.s.; Fig. 3a]. In addition, there was a trend towards a reduced ratio of open to total arm entries in the EPM in NPY-deficient mice [two-way ANOVA for factor genotype, $F(1,32) = 3.3$, $P = 0.08$; Fig. 3b]. Two-way ANOVAs for defecation [factor genotype, $F(1,32) = 7.6$, $P < 0.01$; factor sex, $F(1,32) = 3.5$, $P = 0.07$] and the frequency of stretch–attend postures [factor genotype, $F(1,32) = 16.5$, $P < 0.001$; factor sex, $F(1,32) = 13.3$, $P < 0.001$] confirmed that NPY deficiency results in elevated arousal levels as shown by an increased defecation score and a drop in risk assessment behaviour (Table 3).

The LD test confirmed this sex-specific increase in anxiogenic behaviours as only mutant males spent significantly less time in the aversive illuminated compartment [two-way ANOVA for factor genotype, $F(1,32) = 17.0$, $P < 0.001$; one-way ANOVA for males, $F(1,16) = 31.8$, $P < 0.001$; and for females, $F(1,16) = 2.1$; P = n.s.; Fig. 3a] and exhibited a lower ratio of distance travelled in the illuminated area than did WT males [two-way ANOVA for factor genotype, $F(1,32) = 14.9$, $P < 0.001$; one-way ANOVA for males, $F(1,16) = 16.3$, $P < 0.001$; and for females, $F(1,16) = 2.3$, P = n.s.; Fig. 3b].

Learning and memory

NPY deficiency had no effect on learning and memory in the passive avoidance task, as all mice independently of genotype learned to avoid the dark chamber. Repeated-measures ANOVA revealed a significant effect over time, indicating that all mice, independent of their genotype, had learnt the association of foot-shock and dark chamber [latency to enter dark chamber over time, $F(1,32) = 33.7$, $P < 0.001$; Fig. 5]. Interestingly, we found a significant interaction for latency over time × sex [$F(1,32) = 20.0$, $P < 0.001$]. Female mice exhibited a significantly reduced delay to re-enter the dark chamber during the test session compared to male mice, although this latency to move between chambers was increased for mice of both sexes on the test day. However, NPY deficiency had no impact on this performance in mice

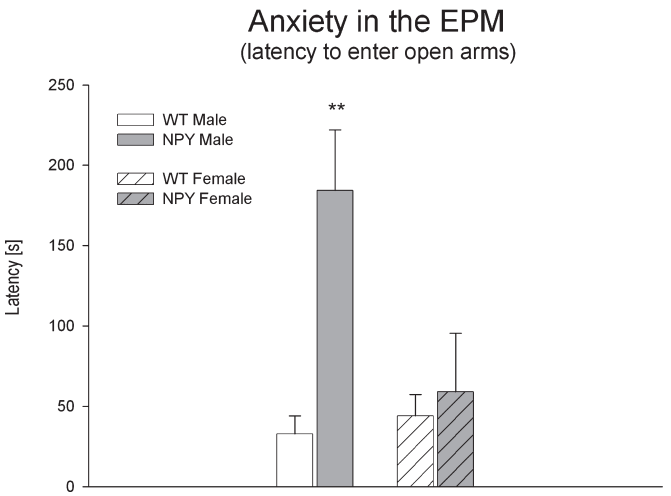

FIG. 4. Anxiety in the EPM. Latency (s) to enter open arms is shown as mean + SEM. Significant *post hoc* effects for male NPY-KO vs. male WT mice are indicated by asterisks (**$P < 0.01$).

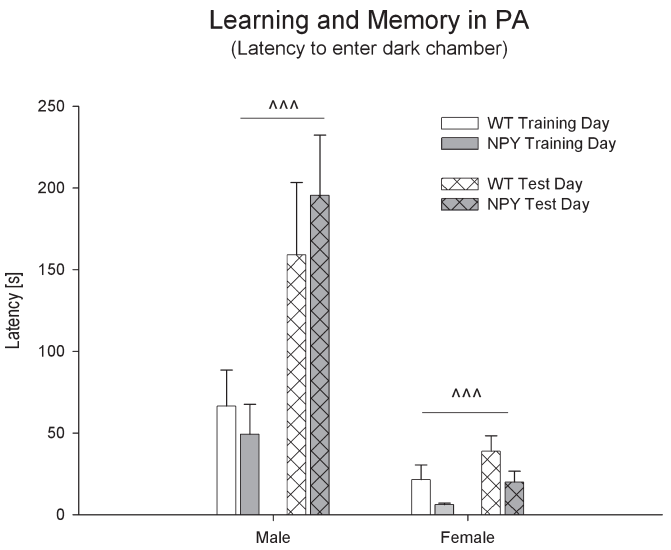

FIG. 5. Learning and memory in the passive avoidance task. Latency (s) to enter the dark compartment on day 1 (training day) and day 2 (test day) are shown as means + SEM. Significance level of repeated-measures ANOVA for latency over time is indicated by '^' (^^^$P < 0.001$).

of either sex [repeated-measures ANOVA for factor genotype, $F(1,32) = 0.03$, n.s.; Fig. 5]. Similarly, the hole-board task revealed no working memory deficits in NPY-KO mice, as indicated by the ratio of novel holes to total holes visited [two-way ANOVA for factor genotype, $F(1,32) = 0.007$, n.s.; WT male = 0.4 ± 0.04; NPY-KO male = 0.4 ± 0.04; WT female = 0.4 ± 0.02; NPY-KO female = 0.4 ± 0.04].

Discussion

The behavioural profile of this new genetic mouse model for NPY deficiency is characterized by a pronounced and consistent sex-independent hypoactive phenotype and an anxiogenic phenotype, which was more pronounced in male NPY mutants. No impact of NPY on learning and memory was found for either sex. Females exhibited increased levels of motor activity and exploration as well as diminished levels of anxiety compared to male mice. Our comprehensive multi-tiered evaluation of this germline NPY-KO model provides the first consistent genetic replication of findings from pharmacological studies, confirming the importance of NPY in the regulation of motor activity, exploration and, in particular, anxiety. This study also highlights the importance of screening male and female test animals to clarify possible sex-specific behavioural alterations in genetic animal models.

Lack of NPY diminished motor activity, as mutant mice of both sexes showed both reduced locomotor activity and increased resting behaviour across several tests, compared to control animals. Although exposure to stress has been found to suppress motor activity (Takeda *et al.*, 1998), our study demonstrates that the motor-suppressant effect of NPY deficiency is relatively independent of stress, as this reduced activity was seen across different zones of the various paradigms, including the less aversive and therefore less stressful areas. Preliminary data from our lab confirmed the phenotype for mutant mice, which was also evident when male NPY mutants were tested in the OF under less aversive dim red light conditions. This motor activity-inhibiting characteristic of genetic NPY deficiency confirms the importance of both genetic and pharmacological strategies, as pharmacological studies have described the opposite, with suppression of motor activity, both in the OF and in the home cage (Heilig & Murison, 1987), and an increase in ethanol-induced sedation (Gilpin *et al.*, 2004) following central NPY administration. Inconsistencies between pharmacological and genetic studies on NPY are well-documented, as e.g. NPY-KO mice show no abnormalities in food intake or body weight whereas exogenous NPY significantly increases food intake (Morley *et al.*, 1987; Bannon *et al.*, 2000). Furthermore, whereas pharmacological studies have demonstrated a consistent anxiolytic effect of Y1 receptor activation, characterization of the Y1 receptor-KO mouse revealed a much more complex stress- and circadian rhythm-dependent anxiety-related phenotype (Karl *et al.*, 2006). This dichotomy between pharmacological and genetic studies confirms the importance of combining the two strategies when investigating the behavioural impact of neurotransmitters and/or neuropeptides.

Interestingly, a reduction in rearing and general vertical activity in mutant NPY mice of both sexes indicated NPY's involvement not only in general motor activity but also in explorative-like behaviours. The suppression of motor activity and exploration in NPY-KO mice was not due to any gross motor abnormalities, as all mice showed intact motor functions during the physical examination and accelerating rotarod.

The newly developed NPY-KO mice consistently avoided aversive regions of the OF, LD and EPM test arenas, as indicated by the time spent in these areas. Male NPY-deficient mice demonstrated a more potent and consistent anxiogenic phenotype than female NPY-KO mice, suggesting moderate sex-specific effects of NPY deficiency. The potent anxious-like phenotype of NPY-deficient mice cannot simply be attributed to hypoactivity as the anxiogenic-like phenotype was confirmed by more locomotion-independent parameters (i.e. ratio of distance travelled in aversive areas of the OF and LD). In this context it is important to note that hypoactive NPY mutants did not show a complete absence of explorative-like activity, as this was only reduced to 40–50% of WT levels (e.g. in the LD test). Further stress-dependent measurements such as risk assessment behaviour (i.e. stretch–attend postures) and defecation score in the EPM also support an anxiogenic-like phenotype for NPY-deficient mice.

The increased anxiety levels seen at baseline in NPY mutants is consistent with the extensive pharmacological data showing that NPY

acts as an anxiolytic, and provides further evidence that NPY is involved in anxiety regulation and stress reactivity. In contrast, neither of the transgenic rodent models available for NPY overexpression exhibit altered anxiety levels under baseline conditions, suggesting that either the low level of overexpression seen in these models (Thiele *et al.*, 1998; Thorsell *et al.*, 2000; Carvajal *et al.*, 2004) is not effective in this regard or that lack of NPY is a better model for exploring this behavioral domain, adding additional value to our new NPY-KO model.

NPY's involvement in core mechanisms of emotionality and behavioural stress response is mainly mediated via the amygdala and the hippocampus. Stressors such as anxiety-provoking behavioural paradigms initiate a rapid release of corticotropin releasing factor (CRF) within the amygdala, which regulates the various components of the endogenous stress response (Heilig, 2004). NPY release during a later phase mediates the adequate termination of the acute stress response and regulates costly anxiety-related defense behaviours (Kask *et al.*, 2002). This stress-inhibiting mechanism is most probably blocked in NPY-deficient mice, and the ensuing imbalance between NPY and CRF activity in the amygdala could lead to the apparent anxiogenic-like behaviour of these mice.

The current study is the first to describe a sex-specific behavioural phenotype in a genetic animal model for NPY deficiency. The more potent anxiogenic-like phenotype in male NPY mutants could be due to NPY's interaction with hormones such as gonadotropin-releasing hormone (GnRH) and luteinizing-hormone releasing hormone (LHRH) as NPY is considered one of the key molecular links between the metabolic and reproductive pathways (Crown *et al.*, 2007). GnRH has anxiogenic-like properties whereas LHRH has anxiolytic-like properties (Aikey *et al.*, 2002). Importantly, females exhibit an oestrus-dependent expression of GnRH and LH is responsible for the production of testosterone. Interestingly, NPY expression was found to be lower in several brain regions of female compared to male rats, including the hippocampus, hypothalamus and striatum (Rugarn *et al.*, 1999). This might explain the less prominent behavioural impact of NPY deficiency in female mice. Furthermore, variations in NPY levels are seen across the oestrus cycle, with increased expression during the proestrus in female rats (Bauer-Dantoin *et al.*, 1992), suggesting that the sex-specific differences in the stress response may be caused by cyclic variations in NPY levels in female but not male mice (i.e. oestrus-dependent decrease in central NPY release in WT females may have reduced the overall difference in NPY levels compared to mutant females). Importantly, all females were kept in close proximity within one holding room, so a synchronized oestrus cycle for female test mice can be assumed (i.e. Lee–Boot effect: Lee & van der Boot, 1955). However, it should be noted that heightened anxiety was still seen in female mutant NPY mice, albeit less pronounced, confirming the overall important role of this neuropeptide in the regulation of anxiety-related behaviours.

Compensatory developmental features are evident in most germline genetic animal models. Trivedi *et al.* (2001) described a dramatic increase (60–400%) in Y2 receptor mRNA expression in the hippocampus and amygdaloid brain structures (i.e. postomedial cortical amygdaloid nucleus and nucleus accumbens) of the NPY-KO mouse developed by Palmiter *et al.* (1998). Importantly, Y2-KO mice exhibit a potent anxiolytic-like phenotype (Redrobe *et al.*, 2003; Tschenett *et al.*, 2003). It is also known that the Y2 receptor is predominantly located presynaptically, mediating feedback inhibition of neurotransmitter release such as GABA (via inhibition of Ca^{2+} channels) and glutamate (Tschenett *et al.*, 2003). An upregulation of Y2 receptors in our NPY-deficient mouse model would down-regulate the release of GABA and glutamate (Qian *et al.*, 1997), which are both implicated in the pathophysiology and treatment of anxiety disorders, and would induce an anxiogenic-like response. Interestingly, Sainsbury *et al.* (2002) reported a correlation between Y2 receptor levels and CRF mRNA expression. An increased Y2 receptor expression in NPY-mutant mice would therefore result in elevated levels of CRF, thereby producing a further increase in anxiogenic-like behaviours (Sajdyk *et al.*, 2004) in these mice.

Y1 and Y5 receptor expression levels of NPY-deficient mice seem to be relatively unaltered in brain areas linked to the manifestation of anxiety and stress-related behaviours such as the amygdala (Trivedi *et al.*, 2001; Lin *et al.*, 2004). Based on these findings, rather subtle compensatory changes within these two receptor systems could have accounted for some of the observed behavioural alterations in anxiety-related domains in NPY mutants.

NPY and its Y2 receptor have previously been implicated in learning and memory (Flood *et al.*, 1987; Redrobe *et al.*, 2004). However, we found no evidence of altered working memory in the hole board or hippocampus-dependent passive avoidance task. Importantly, passive avoidance learning is reliant on the animal associating a naturally less aversive dark compartment with a painful electrical foot-shock. The NPY-KO mouse line back-crossed from the NPY mouse model developed by the Palmiter group has previously been shown to exhibit reduced nociception (Bannon *et al.*, 2000), which would affect the learning outcome in the passive avoidance by limiting the aversion to the dark chamber. Further screening of these mice in a wider variety of tasks for learning and memory is therefore necessary to determine the full extent of NPY's effect on this domain. Interestingly, a reduced delay to enter the passive avoidance dark chamber on the test day indicates less robust learning and memory in females. Although several studies have demonstrated better reference memory in male than in female rats, the sex imbalance in learning and memory performance of mice is less consistent (Jonasson, 2005).

In conclusion, using a new genetic animal model for NPY deficiency we have demonstrated a potent and consistent effect of genetic NPY depletion on a variety of behavioural domains in both sexes, as shown by suppressed motor activity and exploration, and increased anxiety. Importantly, the anxiogenic phenotype was more prominent in male NPY-deficient mice. The hypoactive and anxious phenotype observed in our new NPY-KO mouse model was revealed by a variety of measures across several comprehensive well-validated paradigms, which also incorporated tests for basic sensory and motor functions. The hypoactive phenotype of the NPY-KO mouse model demands motor-activity-insensitive anxiety paradigms for the future. Our findings demonstrate the importance of combining pharmacological and genetic studies, as well as considering sex-specific characteristics, when exploring the behavioural profile of any given neuropeptide. Moreover, the NPY-deficient mice represent a unique opportunity to investigate NPY's impact on anxiety-related disorders as it is the only genetic NPY model providing a consistent anxiety-related phenotype under baseline conditions.

Acknowledgements

This work was supported by the Schizophrenia Research Institute, utilizing infrastructure funding from NSW Health, by the Sylvia and Charles Viertel Charitable Foundation, by a Bill Ritchie Postdoctoral Research Fellowship (awarded to T.K.) and by the National Alliance for Research on Schizophrenia and Depression (NARSAD Young Investigator Award awarded to T.K.). We thank the biological testing facility staff (J. Fisher, M. Pickering and K. Kerr) for their support. The critical comments by Jerry Tanda on the manuscript are gratefully acknowledged.

Abbreviations

CRF, corticotropin releasing factor; EPM, elevated-plus maze; KO, knockout; LD, light–dark; NPY, neuropeptide Y; OF, open field; WT, wild-type.

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