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Overexpression of neuropeptide Y decreases responsiveness to neuropeptide Y

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A B S T R A C T

Neuropeptide Y (NPY) is an endogenous neuropeptide that is abundantly expressed in the central nervous system. NPY is involved in various neurological processes and neuropsychiatric disorders, including fear learning and anxiety disorders. Reduced levels of NPY are reported in Post-Traumatic Stress Disorder (PTSD) patients, and NPY has been proposed as a potential therapeutic target for PTSD. It is therefore important to understand the effects of chronic enhancement of NPY on anxiety and fear learning. Previous studies have shown that acute elevation of NPY reduces anxiety, fear learning and locomotor activity. Models of chronic NPY overexpression have produced mixed results, possibly caused by ectopic NPY expression. NPY is expressed primarily by a subset of GABAergic interneurons, providing specific spatiotemporal release patterns. Administration of exogenous NPY throughout the brain, or overexpression in cells that do not normally release NPY, can have detrimental side effects, including memory impairment. In order to determine the effects of boosting NPY only in the cells that normally release it, we utilized a transgenic mouse line that overexpresses NPY only in NPY+ cells. We tested for effects on anxiety related behaviors in adolescent mice, an age with high incidence of anxiety disorders in humans. Surprisingly, we did not observe the expected reduction in anxiety-like behavior in NPY overexpression mice. There was no change in fear learning behavior, although there was a deficit in nest building. The effect of exogenous NPY on synaptic transmission in acute hippocampal slices was also diminished, indicating that the function of NPY receptors is impaired. Reduced NPY receptor function could contribute to the unexpected behavioral outcomes. We conclude that overexpression of NPY, even in cells that normally express it, can lead to reduced responsiveness of NPY receptors, potentially affecting the ability of NPY to function as a long-term therapeutic.

1. Introduction

Neuropeptide Y (NPY) is found abundantly throughout the central nervous system, primarily in a subset of GABAergic interneurons (Freund and Buzsáki, 1996). It has been shown to regulate physiological processes such as feeding behavior (Kamiji and Inui, 2007), locomotor activity (Heilig and Murison, 1987; Heilig et al., 1988), blood pressure (Lettgen et al., 1994), and circadian rhythms (van den Pol et al., 1996). NPY has also been implicated in neurological and neuropsychiatric disorders including epilepsy (Colmers and El Bahh, 2003), alcoholism (Thiele et al., 1998), and depression (Morales-Medina et al., 2010). In addition, it has been shown to regulate anxiety (Cohen et al., 2012; Heilig, 2004) and fear learning behavior (Tasan et al., 2016). Rodent models of stress-induced anxiety/post-traumatic stress disorder (PTSD) have shown a reduction in NPY levels in brain tissue (Cohen et al., 2012) and plasma (Li et al., 2017). Similarly, combat veterans with PTSD have been shown to have lower NPY levels in both blood plasma and cerebrospinal fluid (Sah et al., 2009, 2014). NPY has been proposed as a potential therapeutic for anxiety disorders including PTSD (Reichmann and Holzer, 2016) (clinicaltrials.gov, NCT 00748956), making it important to understand the long-term effects of NPY on brain function and behavior.

Increases in NPY through intranasal injection (Serova et al., 2013) or transgenic upregulation (Thorsell et al., 2000) have shown that NPY can decrease rodent sensitivity to stress-induced anxiety. Further, NPY applied directly to hippocampus has been shown to alleviate anxiety symptoms in a rodent model of PTSD (Cohen et al., 2012). However, the effect of increased NPY on baseline anxiety has been inconsistent across studies. Several studies have tested the effects of acute NPY application on baseline anxiety-like behavior in rodents. Intracerebroventricular (ICV) injection of NPY has been shown to reduce anxiety-like behavior in some studies (Broqua et al., 1995; Heilig et al., 1989; Karlsson et al., 2005, 2008), but not in others (Lach and de Lima, 2013). Direct injections of NPY to hippocampus likewise have shown inconsistency, with one study finding decreased anxiety behavior (Smiałowska et al., 2007), and another finding no anxiolytic effect on baseline anxiety behavior (Cohen et al., 2012).

Chronic overexpression of NPY has also produced mixed effects on baseline anxiety-like behavior. A transgenic rat model overexpressing NPY failed to show a change in baseline anxiety behavior (Thorsell

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et al., 2000) while viral-mediated NPY overexpression in hippocampus caused a mild anxiolytic effect (Lin et al., 2010). However, NPY is primarily expressed in a subset of GABAergic interneurons in the central nervous system, and it requires high frequency stimulation for release. The normal spatiotemporal patterns of NPY release are not replicated by administration of exogenous NPY, whether it is throughout the brain or only in hippocampus, nor by chronic NPY expression in other cell types (ectopic expression). Long-term administration of exogenous NPY has also been shown to have detrimental effects on cardiac function (Zhang et al., 2015) and non-specific overexpression can impair spatial memory (Sørensen et al., 2008a; Thorsell et al., 2000). It is therefore imperative to determine the effects of increasing NPY expression specifically from the cells in which it is normally expressed (Ste Marie et al., 2005; Thiele et al., 1998).

In this study, we use a mouse line (NPY-Tet) that exhibits entopic overexpression of NPY to determine the effects of global, chronic overexpression of NPY in NPY+ cells on anxiety and related behaviors. We use adolescent mice because of the prevalence of anxiety disorders at this age in humans (Beesdo et al., 2009; Britton et al., 2013; Garnefski et al., 2002; Kessler et al., 2012a, 2012b) as well as behavioral differences between adolescent and adult mice (Ishii et al., 2019; Lee et al., 2016; Moore et al., 2011, 2013). We find little to no change in anxiety-like measures in adolescent mice with chronic entopic NPY expression, although there is a deficit in nest-building behavior. There is no change in hippocampal-dependent fear learning. In addition, we observe a reduction in the effect of bath applied NPY on field potentials in hippocampal slices from NPY-Tet mice, indicating decreased NPY receptor responsiveness. Reduced NPY receptor responsiveness provides partial compensation that limits the long-term effects on behavior of chronically enhanced NPY expression, such that it may even prove to be detrimental.

2. Results

2.1. Adolescent NPY-tet mice have increased NPY expression but no change in weight

We used an established mouse line that contains a doxycycline-regulated cassette in the NPY locus, resulting in entopic overexpression of NPY (only in cells that normally express NPY) in the absence of doxycycline (NPY-Tet) (Ste Marie et al., 2005). We first evaluated the NPY-Tet mouse line to confirm overexpression of NPY in relevant brain regions. We measured NPY protein levels with an enzyme-linked immunosorbent assay (ELISA). We observed an increase in NPY in whole hippocampus (Fig. 1A), amygdala (Fig. 1B), prefrontal cortex (Fig. 1C), and brain stem (Fig. 1D) from NPY-Tet mice compared to wild-type controls (NPY-WT). NPY levels were increased in both male and female NPY-Tet mice, and there were no sex-specific difference in NPY levels in any of the brain regions tested. There was a trend towards reduced NPY in hippocampus of female NPY-WT mice, as has previously been reported in rats (Rugarn et al., 1999), but the difference was not statistically significant. Overall, these results confirm that more NPY is being produced in the NPY-Tet mice. Because NPY has been shown to regulate feeding behavior and cause weight gain (Zheng et al., 2013), we measured the weight of male and female NPY-WT and NPY-Tet mice. However, we found no genotype-dependent difference in weight between adolescent NPY-Tet mice and controls (Fig. 1E), similar to the result from adult NPY-Tet mice (Ste Marie et al., 2005). We did observe the expected sex-dependent difference in body weight at this age.

2.2. Overexpression of NPY leads to a change in some baseline anxiety-related measures

Since the effect of acute application of NPY has previously yielded inconsistent results on baseline anxiety-like behavior (Karlsson et al., 2008; Serova et al., 2013), we next sought to determine if chronic overexpression of NPY only in NPY+ cells would lead to changes in baseline anxiety-related behavior in naïve mice. To assess this, we first tested for differences in anxiety-like behavior using the elevated plus maze (EPM). We found a significant genotype-dependent decrease in the time spent in the open arms (Fig. 2A) and an increase in the anxiety index (Fig. 2D) in NPY-Tet mice compared to NPY-WT mice. However, there was no genotype-dependent change in the percentage of open arm entrances (Fig. 2B) or total arm entrances (Fig. 2C). These data indicate that there may be a mild anxiogenic effect of increased NPY with no change in overall locomotor activity. To further assess this, mice were tested for thigmotaxis in the open field. There was no statistically significant alteration in the percent of center distance traveled (Fig. 2E) in NPY-Tet mice or the number of entrances into the central zone (Fig. 2F), indicating no effect of genotype on anxiety-like behavior in this test Further, there was not a change in locomotor activity in NPY-Tet mice as shown by no difference in total distance traveled (Fig. 2G), total resting time (Fig. 2H), or velocity (Male NPY-WT 11.71 ± 0.67 cm/s, Female NPY-WT 13.33 ± 0.58 cm/s, Male NPY-Tet 11.32 ± 0.55 cm/s, Female NPY-Tet 11.64 ± 0.64 cm/s; Genotype: $F_{(1,67)} = 2.7$, $p = 0.11$; $n = 13, 20, 20, 15$). Taken together, these data indicate that overexpression of NPY in the NPY-Tet mice does not cause the expected reduction in anxiety-like behavior, and even leads to changes in some anxiety-dependent measures that are consistent with a mild increase in anxiety-like behavior.

2.3. Overexpression of NPY does not change fear learning or extinction

Acute application of NPY has been shown to inhibit fear learning and facilitate fear extinction (Tasan et al., 2016). We used the contextual fear conditioning protocol to measure hippocampal-dependent fear learning and fear extinction in adolescent NPY-WT and NPY-Tet mice. Specifically, we measured the amount of freezing behavior following an aversive stimulus (3 footshocks) (Fig. 3A, top schematic). The freezing behavior increases as mice learn to anticipate a footshock. Both the NPY-WT and NPY-Tet mice were able to learn at a similar rate in the contextual fear conditioning paradigm (Fig. 3B, C). Mice were placed back into the context 24 h later, and both NPY-WT and NPY-Tet mice showed increased freezing behavior compared to baseline freezing, demonstrating fear learning (Fig. 3D, E; baseline vs Day 1). We further examined the extinction behavior in these mice. As mice are re-exposed to the context (with no aversive stimuli), the fear memory is extinguished and no longer associated with the original context following a contextual fear conditioning protocol. We found that there was no significant difference in the ability of adolescent NPY-Tet mice to extinguish the fear memory compared to wild-type controls (Fig. 3D, E). Together, our results show that chronic overexpression of NPY in NPY + cells does not alter fear learning or extinction.

2.4. Overexpression of NPY leads to deficits in nest building behavior

We evaluated the innate, non-learned behavior of nest-building (Deacon, 2006), a test of cognitive well-being (Jirkof, 2014). The effects of increased NPY expression on nest building have not previously been evaluated. To study this, NPY-Tet and NPY-WT mice were each given a pre-weighed cotton nestlet and allowed to construct a nest during the 12 h dark cycle and the nests evaluated the next morning. Using predetermined criteria described in detail in a previous study (Corder et al., 2018; Deacon, 2006), nests were scored for the amount of material shredded and overall nest construction. Scores range from 1 to 5. A score of 1 is given to an undisturbed nestlet. A nest with a score of 5 consists of a completely shredded nestlet, a dome-like shape with an obvious crater, and walls higher than the height of the mouse as it lays in its nest. We found that the nest scores of NPY-Tet mice were significantly lower than those of NPY-WT (Fig. 4), consistent with the conclusion that there is a gross change in brain function, particularly in hippocampus and/or prefrontal cortex. This could potentially indicate a decrease in cognitive well-being in mice expressing elevated NPY.

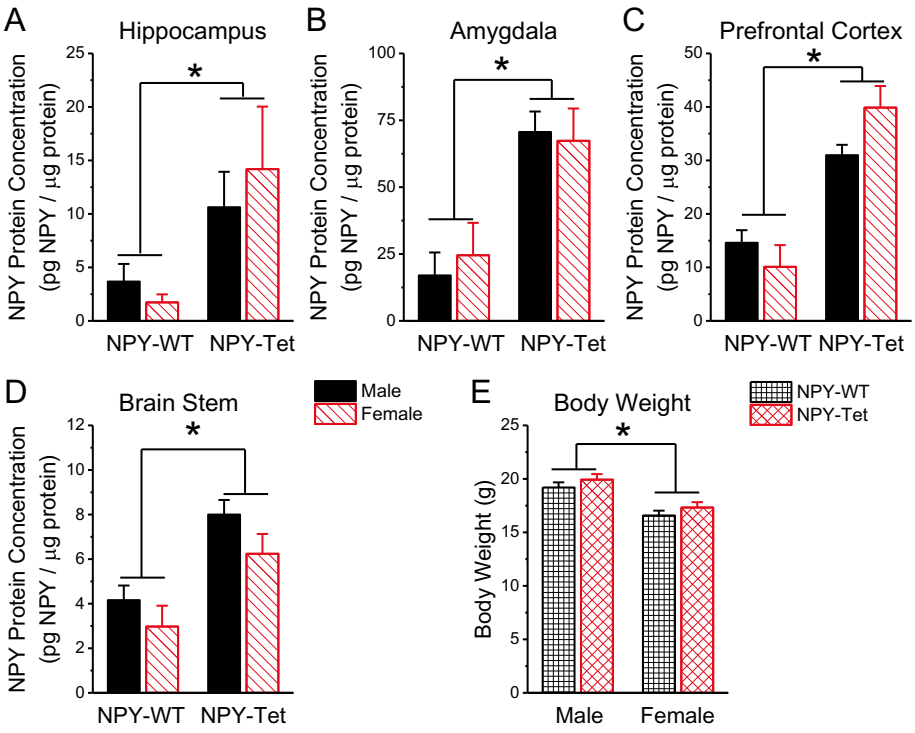

Fig. 1. NPY-Tet mice have increased NPY expression and no change in body weight.
(A) NPY-Tet mice have significantly increased levels of NPY protein in hippocampus ($F_{(1,39)} = 66.5$, $p < 0.001$; Male NPY-WT, n = 11; Female NPY-WT, n = 6; Male NPY-Tet, n = 13; Female NPY-Tet, n = 10). (B) NPY-Tet mice also had significantly increased levels of NPY in amygdala ($F_{(1,25)} = 21.82$, $p < 0.001$; Male NPY-WT, n = 8; Female NPY-WT, n = 4; Male NPY-Tet, n = 10; Female NPY-Tet, n = 4). (C) NPY-Tet mice also had significantly increased levels of NPY in Prefrontal Cortex ($F_{(1,27)} = 50.39$, $p < 0.001$; Male NPY-WT, n = 9; Female NPY-WT, n = 3; Male NPY-Tet, n = 13; Female NPY-Tet, n = 3). (D) NPY-Tet mice also had significantly increased levels of NPY in brainstem ($F_{(1,36)} = 22.81$, $p < 0.001$; Male NPY-WT, n = 12; Female NPY-WT, n = 6; Male NPY-Tet, n = 12; Female NPY-Tet, n = 7). (E) NPY-Tet mice maintain a similar weight to wild-type controls, though there was a sex-dependent difference (Genotype: $F_{(1,147)} = 2.31$, $p = 0.13$; Sex: $F_{(1,147)} = 27.12$, $p < 0.001$). Statistics for A-D were conducted using a two-way ANOVA. There were no sex-specific effects in NPY for any of the brain regions analyzed (A-D). Body weight was analyzed using a two-way ANCOVA with age as a covariate (Age: $F_{(1,148)} = 6.59$, $p = 0.01$).

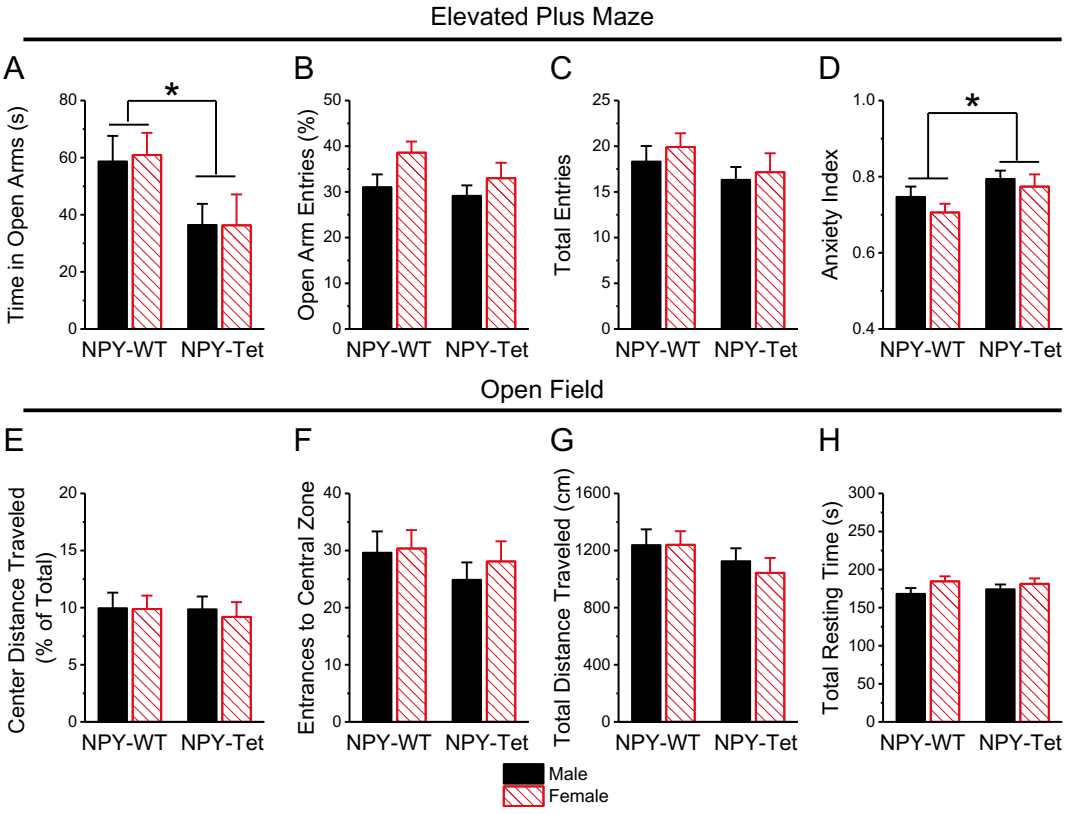

Fig. 2. NPY-Tet mice have increased anxiety-like behavior compared to wild-type controls.
(A-D) Elevated plus maze showed NPY-Tet mice to have a decrease in (A) open arm time (Genotype: $F_{(1,84)} = 7.04$, $p = 0.01$). There was no genotype-dependent change in (B) percent of open arm entries, though there was a sex-dependent effect (Sex: $F_{(1,84)} = 4.32$, $p = 0.04$). There was no genotype-dependent change in (C) total entrances. NPY-Tet mice had an increased (D) anxiety index (Genotype: $F_{(1,84)} = 4.82$, $p = 0.03$). All statistics for elevated plus maze were run with a two-way ANOVA (Male NPY-WT, n = 19; Female NPY-WT, n = 25; Male NPY-Tet, n = 28; Female NPY-Tet, n = 13). (E-H) In the open field, there was no difference in (E) the percent of center distance traveled in NPY-Tet mice, (F) center zone entrances, (G) total distance traveled, or (H) total resting time. All statistics for open field were run with a two-way ANCOVA with age as a covariate (Age: $F_{(1,67)} = 7.80$, $p = 0.007$; Male NPY-WT, n = 13; Female NPY-WT, n = 20; Male NPY-Tet, n = 20; Female NPY-Tet, n = 15).

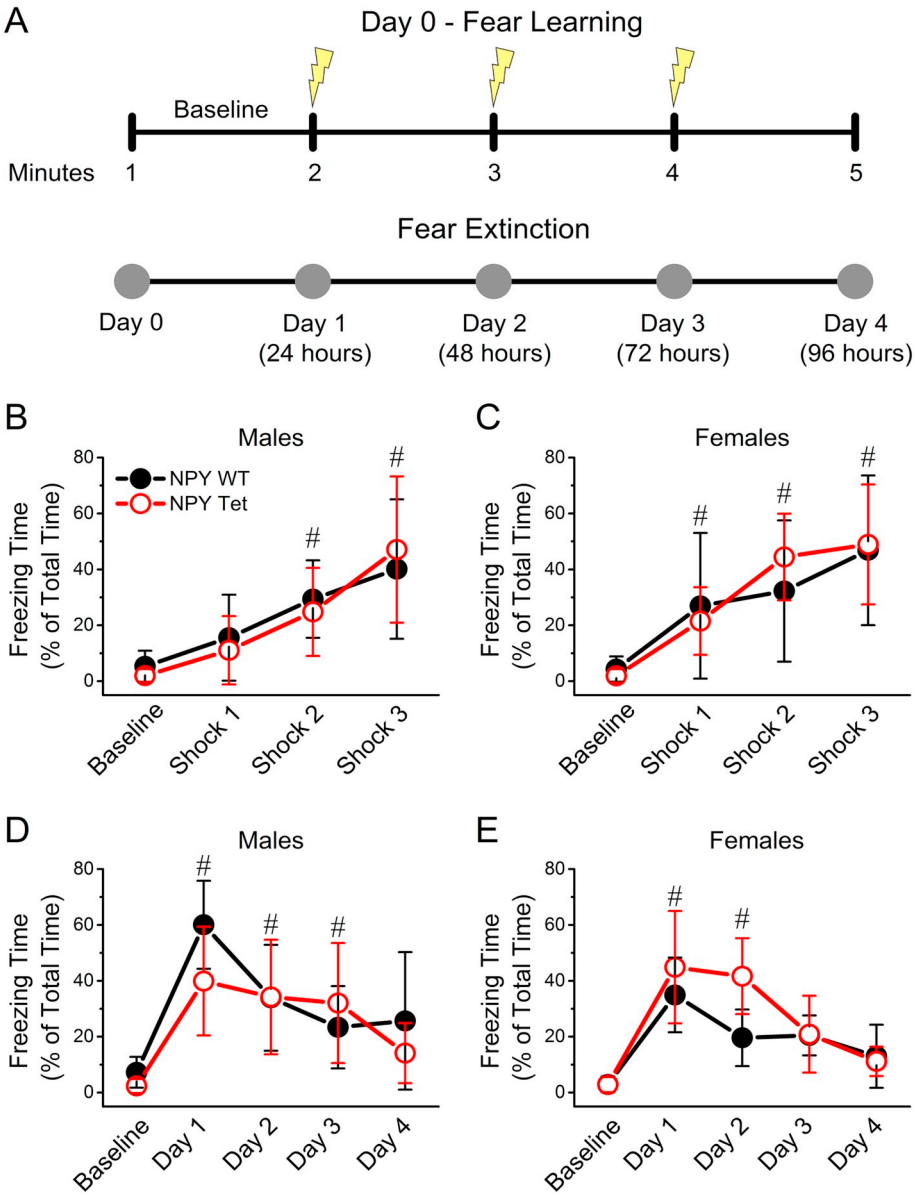

Fig. 3. NPY-Tet mice have no change in contextual fear learning.
(A) Schematic of fear conditioning protocol. *Re*-exposure to the fear conditioning box with no footshock was conducted for four days in a row following footshock exposure. (B-C) NPY-Tet mice showed comparable fear learning behavior as shown by comparable freezing behavior between genotypes over the course of the footshock exposure. Shock number was the only significant factor (Male NPY-WT, n = 7; Female NPY-WT, n = 8; Male NPY-Tet, n = 10; Female NPY-Tet, n = 8). (D-E) NPY-Tet mice extinguished fear memories at the same pace as wild-type controls. Time was the only significant factor. (Male NPY-WT, n = 5; Female NPY-WT, n = 5; Male NPY-Tet, n = 8; Female NPY-Tet, n = 5). Statistics were conducted using a three-way repeated measures ANOVA to compare sex, genotype, and shock number or time followed by a Tukey post-hoc test. # signifies statistical significance from baseline freezing.

2.5. NPY-tet mice have decreased sensitivity to NPY in the temporoammonic pathway of hippocampal CA1

Because we did not see the expected reduction in anxiety-like behavior, and saw no alterations in fear learning, we sought to determine if there was circuit compensation that could potentially account for these surprising behavioral results. The temporoammonic (TA) pathway in the CA1 region of hippocampus is a pathway that has been shown to be sensitive to stress (Yang et al., 2006), and implicated in fear learning and anxiety behavior (Kallarackal et al., 2013). The TA pathway is located in the stratum lacunosum-moleculare (SLM) layer of CA1 and originates from layer III of the entorhinal cortex (Fig. 5A). It provides excitatory input onto the CA1 pyramidal cells, which serve as the primary output of hippocampus. NPY+ cells are abundantly expressed in CA1 (Armstrong et al., 2012), and the TA pathway innervates NPY+ cells in both SLM and stratum radiatum to induce NPY release (Li et al., 2017). We have previously shown that both bath-applied and endogenously released NPY modulate synaptic transmission from the temporammonic pathway onto CA1 pyramidal cells (Li et al., 2017), and NPY release in the temporammonic pathway is impaired by stress-induced anxiety (Li et al., 2017). We therefore focused on the temporammonic pathway in CA1 to test for effects of chronic NPY overexpression on circuit function.

We first asked whether there is an overall change in TA circuit function due to the increased expression of NPY. We used extracellular recordings and measured field postsynaptic potentials (fPSPs) from the TA pathway in acute slices from ventral hippocampus. We found no change in synaptic transmission, as evidenced by no change in the input/output curve (Fig. 5B) or in the paired-pulse ratio (Fig. 5C) in slices from NPY-Tet mice compared to NPY-WT mice. This indicates that chronic overexpression of NPY in the cells that normally express NPY does not alter basal synaptic transmission in the temporoammonic pathway in CA1.

We next sought to determine if there was some compensation via changes in receptor expression or sensitivity associated with NPY overexpression. Bath application of NPY caused a dose-dependent reduction in the extracellular excitatory postsynaptic potential (fEPSP) slope in NPY-WT slices (Fig. 6B), as previously observed (Li et al., 2017). However, we found that there is impairment of the response of the TA pathway to bath applied NPY in the NPY-Tet slices (Fig. 6B), with no significant reduction in fEPSP at doses of NPY up to 1.25 μM. This indicates a reduction in the responsiveness of NPY receptors in NPY-Tet mice.

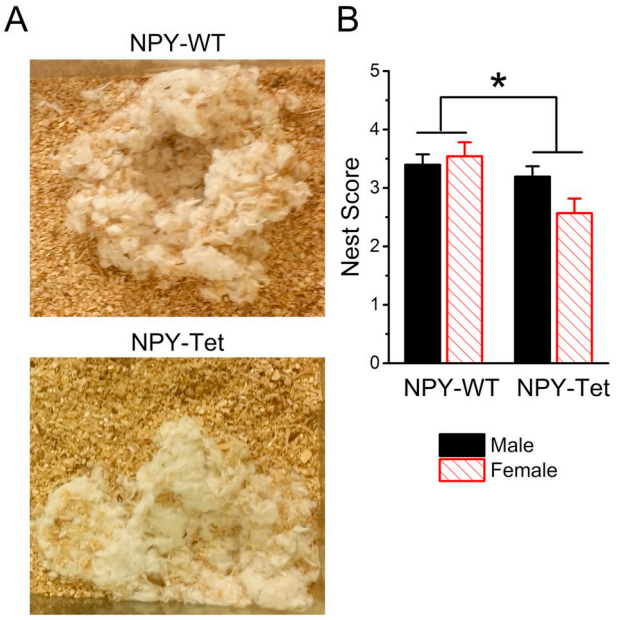

Fig. 4. NPY-Tet mice build inferior nests compared to those of wild-type controls.
(A) Example nests are shown for female NPY-WT and NPY-Tet mice. (B) Average nest scores were significantly different between genotypes (Genotype: $F_{(1,66)} = 7.57$, $p = 0.008$; Male NPY-WT, n = 22; Female NPY-WT, n = 12; Male NPY-Tet, n = 22; Female NPY-Tet, n = 11).

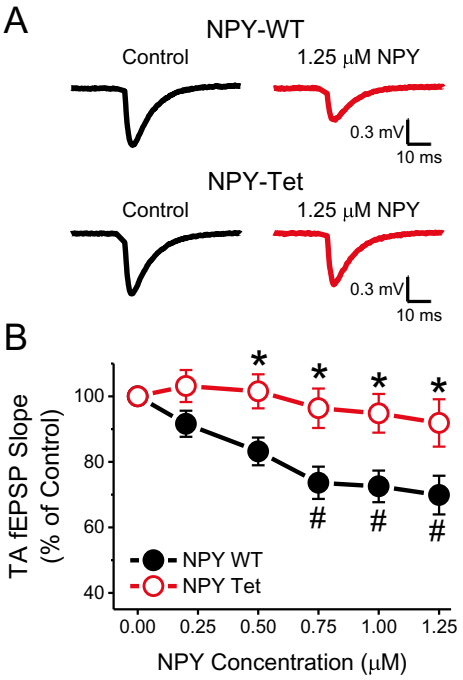

Fig. 6. NPY-Tet mice have decreased sensitivity to NPY in the temporoammonic pathway.
(A) Example traces from NPY-WT and NPY-Tet mice with and without 1.25 μM NPY. (B) Excitatory extracellular field potentials were not reduced in NPY-Tet mice and the curve was significantly different between genotype. * indicates a statistically significant difference from NPY-WT ($F_{(1,40)} = 8.62$, $p = 0.02$, NPY-WT n = 6, NPY-Tet n = 4). # indicates a statistically significant difference from baseline ($p < 0.05$) as determined by a one-way ANOVA followed by a Tukey's post-hoc test.

3. Discussion

In this study we determined the effects of chronic cell-type appropriate increases in NPY on behavior and synaptic function in adolescent mice. Our primary finding is that mice with chronic overexpression of NPY driven by the NPY promoter (NPY-Tet) have a decreased responsiveness to NPY in the TA pathway of hippocampal CA1. In addition, adolescent NPY-Tet mice showed an unexpected decrease in nest building behavior, and no change in fear learning behavior. Furthermore, adolescent NPY-Tet mice failed to have the predicted reduction in anxiety-like behavior. Overexpression of NPY in the NPY-Tet mice also had no effect on body weight or locomotion, both of which have been shown to be regulated by acute enhancement of NPY (Heilig and Murison, 1987; Heilig et al., 1988; Kamiji and Inui, 2007). A possible explanation for the unexpected behavioral results is the reduced NPY receptor responsiveness to its ligand.

Bath application of NPY decreases excitatory field potentials in the TA pathway in wild-type slices, as previously shown (Li et al., 2017). The acute effects of NPY on TA synapses were lost in slices from NPY-Tet mice, indicating an impairment of the NPY receptors regulating

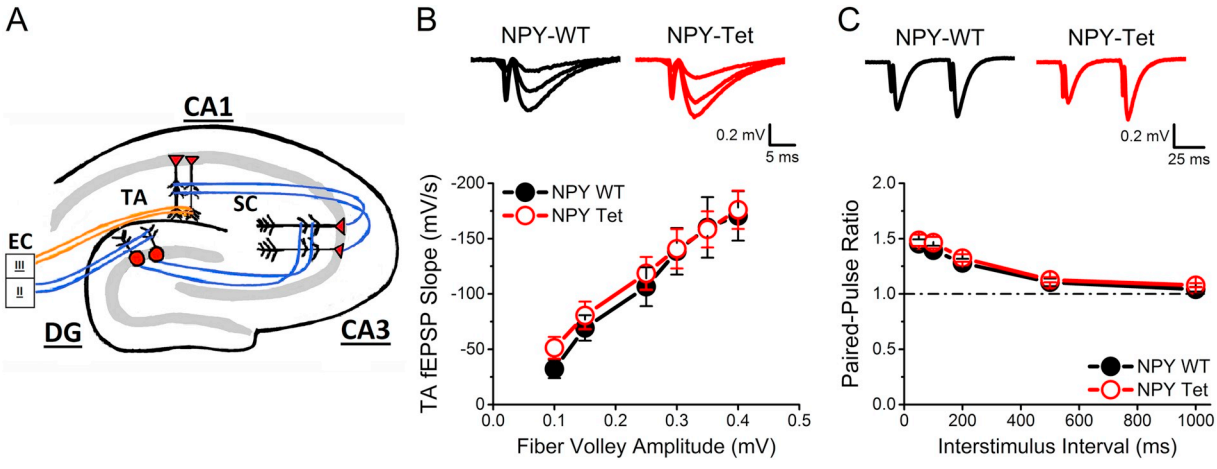

Fig. 5. Synaptic transmission is unchanged in the temporoammonic pathway of hippocampal CA1 of NPY-Tet mice.
(A) Simplified schematic of hippocampal primary circuits. The temporoammonic (TA) pathway from entorhinal cortex (EC) layer III is shown in orange. (B) NPY-Tet mice show no change in the input-output curve of extracellular field potentials recorded in CA1 SLM in response to stimulation of the TA pathway ($F_{(1,151)} = 0.09$, $p = 0.99$; NPY-WT n = 10, NPY-Tet n = 16). Inset: Example traces of fPSPs in response to increasing stimulus intensities for NPY-WT and NPY-Tet mice. (C) Likewise, there was no change in the paired-pulse ratio between genotypes ($F_{(1,208)} = 0.09$, $p = 0.98$; NPY-WT n = 15, NPY-Tet n = 27). Inset: Example traces of fPSPs at a 50 ms paired-pulse interval from NPY-WT and NPY-Tet mice.

these synapses. The overabundance of NPY could lead to desensitization of NPY receptors, which is common for GPCRs in the presence of abundant ligand (Gainetdinov et al., 2004). This can be caused by reduced receptor surface expression in the neuron (Böhm et al., 1997), and/or a decrease in receptor signaling efficiency (Gainetdinov et al., 2004). Interestingly, a previous study showed downregulation of Y1 receptor binding, but not Y2 receptor binding, in hippocampus of rats overexpressing NPY (Thorsell et al., 2000). This suggests subtype-specific functional changes of NPY receptors following overexpression. There may also be region-specific or pathway-specific differences in the effects of NPY overexpression on receptors, since the expression level of the different types of NPY receptors is not homogenous throughout the brain. It is not yet known which subtype of NPY receptors modulates the effects of NPY in the TA pathway. Y2 receptors have been shown to regulate the other major input to CA1, the Schaffer collateral pathway (El Bahh et al., 2002), however Y1 receptors mediate NPY's effects in the dentate gyrus (Sperk et al., 2007). Future studies are needed to investigate if other circuits and brain regions have reduced sensitivity to NPY in the NPY-Tet mice and determine which receptor subtypes are involved. However, decreased receptor responsiveness to NPY following overexpression is likely to limit the therapeutic effects of long-term increases in NPY.

We find that nest-building is impaired in the adolescent NPY-Tet mice. Nest building is temperature dependent, such that nest scores are reduced at higher ambient temperatures (Gaskill et al., 2013). NPY regulates energy expenditure and thermoregulation (Loh et al., 2015), and can cause either hypothermia or hyperthermia depending on the dose and injection location (Bouali et al., 1995). NPY-Tet mice have reduced adiposity (Ste Marie et al., 2005), and may be more sensitive to ambient temperatures. It is therefore possible that the impaired nest building in NPY-Tet mice results from altered thermoregulation. Nest-building is also considered a measure of cognitive well-being (Jirkof, 2014), and depends on hippocampus (Deacon and Rawlins, 2005; Deacon et al., 2002) and prefrontal cortex (Corder et al., 2018; Kolb and Whishaw, 1985). Previously we showed that reduction of GAD67 in NPY+ GABAergic cells improved nest building in adolescent mice (Corder et al., 2018), possibly indicating increased compulsive behavior (Mitra et al., 2017). Together, these results indicate that both outputs of NPY cells, GABA and NPY, regulate nest building behavior, with effects that are bidirectional.

NPY has anti-anxiety properties (Broqua et al., 1995; Heilig et al., 1989; Karlsson et al., 2005, 2008; Smiałowska et al., 2007), so it is surprising that there is a significant decrease in the open arm time of the elevated plus maze. This results in a modest (but statistically significant) increase in the anxiety index, suggesting a mild anxiety-like phenotype. This could be caused by reduced responsiveness of NPY receptors. However, there was no change in other anxiety-related measures, such as the number of open arm entries in the EPM, or of anxiety-related measures in the open field in adolescent NPY-Tet mice, consistent with little or no change in anxiety levels. Increased NPY can also decrease rodent sensitivity to stress-induced anxiety (Cohen et al., 2012; Serova et al., 2013; Thorsell et al., 2000). Future studies will be needed to determine whether NPY-Tet mice show protection against stress-induced anxiety in PTSD models such as predator scent stress that cause reductions in NPY levels (Cohen et al., 2012) and NPY release (Li et al., 2017).

Previous studies using acute increases in NPY or long-term NPY overexpression that was not cell type specific have shown differential results on anxiety. A rat model of NPY overexpression found no effect on baseline anxiety (Thorsell et al., 2000), but a model of adult-onset enhancement of NPY showed a decrease in anxiety-like behaviors (Lin et al., 2010). It is possible that these differences could be due to the level of NPY overexpression. Interestingly, the effects of acute NPY application are dose dependent, with higher doses reducing anxiety-like behavior (Broqua et al., 1995; Nakajima et al., 1998), while lower doses had either no effect (Lach and de Lima, 2013) or actually induced anxiety-like behavior (Nakajima et al., 1998). It is possible that the level of NPY released in NPY-Tet mice was comparable to low dose acute applications. However, given our results showing decreased responsiveness to bath-applied NPY, it is more likely that NPY signaling is low because of reduced NPY receptor function. It is possible that a smaller elevation of NPY levels might prevent this loss of receptor responsiveness and actually enhance NPY receptor signaling, resulting in reduced anxiety-like behavior. The levels of NPY expression in NPY-Tet mice can be reduced by doxycyline administration (Ste Marie et al., 2005), and heterozygous NPY-Tet mice could produce an intermediate level of NPY expression, since they have an intermediate mRNA level (Ste Marie et al., 2005), potentially providing better models for the therapeutic effects of chronic NPY exposure.

In addition to affecting anxiety, acute application of NPY has been demonstrated to directly modulate fear learning and memory (Gøtzsche and Woldbye, 2016). For example, enhancing NPY levels through ICV injection reduced fear learning and consolidation, while facilitating fear extinction (Karlsson et al., 2005; Tasan et al., 2016). However, we observed no change in contextual fear conditioning or extinction in adolescent NPY-Tet mice. In addition, although prolonged ectopic expression of NPY in hippocampus caused a delay in the ability of the animals to learn (Sørensen et al., 2008a), the NPY-Tet mice were able to learn at the same level as wild-type mice. The observed decrease in NPY receptor responsiveness is the most likely explanation as to why there was no effect of NPY overexpression on fear learning and extinction in the adolescent NPY-Tet mice. However, it is possible that other types of learning tasks will be modulated in the NPY-Tet mice, and these mice might help us to better understand the role that enhancing or decreasing NPY signaling has on learning and memory.

Hippocampus, and in particular ventral hippocampus, has been strongly linked to both anxiety and fear learning behavior (Bannerman et al., 2003; Engin and Treit, 2007; Goosens, 2011; Kim and Cho, 2017). The TA pathway in CA1 has been associated with memory consolidation and has been further directly linked to fear learning behavior (Kallarackal et al., 2013). It has also has been shown to be sensitive to stress (Yang et al., 2006) and modulated by endogenously released NPY (Li et al., 2017). We found no change in the input/output relationship in the TA pathway in NPY-Tet slices, suggesting that chronic NPY overexpression does not alter overall synaptic transmission. The lack of effect on synaptic transmission could be due to the reduced responsiveness of the receptors to its ligand. However, under normal conditions, release of NPY requires high frequency stimulation (Ledoux et al., 2009; Li et al., 2017), and is not released during low frequency paired-pulse stimulation. In ectopic overexpression models, where NPY is expressed in cells such as pyramidal cells that do not normally release NPY, it has been shown that NPY can be released by stimulation that does not normally cause NPY release (Sørensen et al., 2008a, 2008b), including low frequency paired-pulse stimulation (Sørensen et al., 2008a). It is unknown whether entopic overexpression of NPY in the NPY-Tet mice causes NPY release during low frequency-stimulation.

The variability in the effects of NPY overexpression on behavior could depend upon the specific receptor subtype, region, and/or pathway, especially as different NPY receptors have been linked to specific behavioral outcomes. For example, a previous study showed impaired nest-building behavior in an Y1 receptor knockout mouse (Baldock et al., 2007), which is consistent with the deficit seen in the NPY-Tet mice. However, Y1 receptors have also been shown to be involved in anxiety (Karl et al., 2006), fear memory (Lach and de Lima, 2013) and extinction (Verma et al., 2012). Because some expected effects of Y1 receptor activation were not seen in the NPY-Tet mice, and effects consistent with Y1 receptor blockade were seen, our data could be indicative of down-regulated Y1 receptor activity. There is likely some functionality of Y2 receptors in the NPY-Tet mice, as it was previously shown that overexpression of NPY protected against kainate-induced seizures (Nakajima et al., 1998; Ste Marie et al., 2005). Future studies are needed to confirm changes in receptor expression, sensitivity, and/or functionality for both Y1 and Y2 receptors in NPY-Tet mice.

Many rodent behavioral experiments use littermates, although other studies do not (Baier et al., 2009; Corder et al., 2018; Davis et al., 2012; Wu et al., 2017). The NPY-Tet and NPY-WT mice were bred as separate lines, and mice used in behavioral experiments were age-matched and from the same genetic background. A caveat of our study is that possible differences in parental care or home-cage environment could potentially translate into differences in the behavior of the pups. The NPY-Tet mice show a minor enhancement in anxiety-like behavior, which has been shown to correlate with differences in rodent maternal behavior (Caldji et al., 1998; Liu et al., 1997). However, it is unknown whether NPY-Tet mice have differences in specific maternal behaviors, or whether this influences anxiety or nest-building behaviors in their offspring, although this could be tested in future studies.

Previous studies have shown that NPY is regulated by the female sex hormone estradiol, which increases the amount of NPY in presynaptic boutons in hippocampus (Ledoux et al., 2009; Nakamura et al., 2007). Sex-dependent differences in behavior have been shown in mice with NPY deficiency, where the anxiogenic effect was more pronounced in male mice (Karl et al., 2008). We tested both male and female mice in all behavioral experiments, and found no differences based on sex. There was a sex-dependent difference in body weight in both NPY-WT and NPY-Tet mice, though no genotype-dependent difference as might be expected because of NPY's influence on feeding behavior (Zimanyi et al., 1998). One caveat in our study was that we did not control for estrous cycle in our female animals. However, a previous study found no differences in anxiety behavior across the estrous cycle in young adult female mice (Meziane et al., 2007). In addition, our experiments used adolescent mice, an age where sex-dependent effects may not yet be fully apparent. It is possible that there could be sex-dependent differences in the effects of chronically overexpressed NPY at older ages.

The NPY-Tet mice have enhanced levels of NPY throughout adolescence and into adulthood. It is possible that the behavioral effects of overexpressing NPY observed in the adolescent NPY-Tet mice could be different in adults, especially as NPY receptor levels change throughout development (Neveu et al., 2002). For example, cognitive deficits observed in 5-month old rats overexpressing NPY (Thorsell et al., 2000) were not observed in rats at one year of age (Carvajal et al., 2004), suggesting there were age-dependent compensatory mechanisms. Additionally, there could be differential effects of NPY overexpression depending on the timing of the onset; the reduced NPY receptor responsiveness might be prevented by delaying the onset of NPY overexpression until adulthood. In addition to the central nervous system, NPY is also expressed in the periphery, including the sympathetic nervous system (Ekblad et al., 1984) and adipocytes (Yang et al., 2008). It is not yet known whether NPY levels in the periphery are enhanced in the NPY-Tet mice, nor whether this contributes to any of the behavioral effects.

In conclusion, cell-type specific overexpression of NPY causes an increase in some behavioral measures that are consistent with a mild increase in anxiety-like behavior, and a deficit in nest-building behavior in adolescent NPY-Tet mice, but no significant change in other NPY-dependent behaviors tested, including fear learning and extinction. Though we did not directly test for a mechanism here, we hypothesize that this difference from expected outcomes is due to compensatory mechanisms, such as the reduced responsiveness of NPY receptors. Because there were some behavioral effects in the NPY-Tet mice, it is possible that certain types of NPY receptors and/or regions are more sensitive to the overexpression of NPY. The fact that there is impaired NPY receptor function in response to chronic increases in NPY levels is an important factor when considering the potential of NPY as a therapeutic target for the prevention or treatment of anxiety disorders and PTSD.

4. Methods

4.1. Animals

All experimental protocols conducted were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham (APN 20119). All experiments were conducted in accordance to the Guide for the Care and Use of Laboratory Animals adopted by the National Institutes of Health.

Mice were group housed with 3–7 same-sex littermates/cage after they were weaned (p24-p28). Mouse colonies were maintained at 21 ± 2 °C with food and water ad libitum on a 12 h light/dark cycle. The initial NPY-Tet$^{+/-}$ mouse line was obtained from Jackson Laboratory (B6;129S4-*Npy*^{tm2Rpa}/J; stock no. 007585) (Ste Marie et al., 2005) and maintained on a C57BL/6 background. This mouse line contains a tetracycline regulatory cassette knock-in into the neuropeptide Y gene resulting in overexpression of NPY. To ensure that we had maximum NPY levels, we used homozygous mice for the tetracycline regulatory cassette knock-in in our studies, which will be referred to as NPY-Tet mice. Heterozygous breeding resulted in approximately 0–2 animals/group/litter to be used for experiments. NPY-Tet and NPY-WT (no tetracycline cassette) mice were therefore bred homozygous from the same parental lines to generate greater experimental numbers. All mice were bred concurrently under similar conditions. Experiments were conducted with age-matched male and female mice at 1–2 months of age (p28 – p78) unless otherwise noted. Estrous cycle was not accounted for in females.

4.2. Behavior

All animals used for behavior experiments were handled by the experimenter for a minimum of 2 days for 4–5 min/day prior to testing. Any mice that were individually housed for the behavior task were allowed to habituate to individual cages for a minimum of 24 h prior to experiments. Mice were allowed to habituate to the testing room for a minimum of 45 min before the onset of behavioral testing. All behavioral experiments were conducted between 6:00 am and 4:00 pm unless otherwise noted. Most subjects did not undergo more than one behavioral test with the exception of open field tests, which were conducted with animals from the elevated plus maze cohorts. Light levels were measured using a Sekonic Speedmaster L-858D-4 and the noise levels were measured using Sound Meter, an android application by KTW Apps.

4.2.1. Elevated plus maze

The elevated plus maze (EPM) was used to measure anxiety behavior. The apparatus is a cross plexiglass platform raised one meter above the floor with two closed arms (76.2 cm × 6.35 cm), two open arms (76.2 cm × 6.35 cm), and a central hub. Low light (~9 lx) and white noise (~64.8 dB) were maintained throughout the test. Each animal was placed in the central hub, facing an open arm, at the beginning of each experiment. Mice were allowed to explore the maze for 5 min with various parameters (including time spent and entrances into each arm) tracked via automated software (Med Associates, St. Albans, VT). The anxiety index values were determined using a previously published equation (Cohen et al., 2012).

4.2.2. Open field

The Open Field Test (OF) was used to measure locomotor activity and assess anxiety-like behavior via thigmotaxis (Corder et al., 2018). The OF apparatus is a square (27.9 cm^3) box with plexiglass sides with 48 infrared beams and tracking software (Med Associates, St. Albans, VT). OF experiments were conducted in a lighted room (~ 140 lx) with a white noise machine (~ 73 dB). All mice were placed in the same corner of the box at the beginning of the test. Total time and distance ambulatory was measured in both the center and periphery of the box for 5 min.

4.2.3. Fear conditioning

Fear conditioning experiments were conducted in a 30.5 cm × 24.1 cm × 21 cm box with a clear plexiglass front, an electrifiable grid

floor and a sound generator (Med Associates, St. Albans, VT). Freezing behavior was automatically tracked via video tracking software (Video Freeze; Med Associates, St. Albans, VT).

Contextual Fear Conditioning was used to assess hippocampal-dependent fear learning behavior. Subjects were allowed a 2 min, free exploration period followed by a 0.5 mA footshock for 1 s followed by a minute long rest period. This was repeated 2 more times for a total of 3 footshocks. Twenty-four hours later the subjects were placed back in the unaltered box for 5 min to monitor their freezing behavior. Extinction of fear conditioning was studied by placing the subjects back in the same context for 30 min each day for 4 days following the initial footshock (Day 0, Fig. 3A).

4.2.4. Nest building behavior

Nest building behavior was used as a measure of cognitive well-being (Deacon and Rawlins, 2005; Deacon et al., 2002; Jirkof, 2014). Mice were placed in a clean cage containing 0.5 cm of bedding and pre-weighed, soft cotton nesting material (4 g ± 0.1 g; Ancare) 2 h before the onset of the dark cycle. Nests were scored the following morning, 2 h after the onset of the light cycle. Nests were scored using pre-determined criteria (Bartley et al., 2015; Brady et al., 2016; Deacon, 2006) that included percent of nesting material shredded and overall construction quality. Nests were scored from 1 to 5, using the following criteria: 1, nestlet barely touched; 2, nestlet partially torn up (50–85% remains intact); 3, nestlet mostly shredded but often no identifiable nest site (< 85% shredded); 4, an identifiable, but flat nest; or 5, a (near) perfect nest with clear nest crater (> 85% shredded). Photographs were taken and nests were scored by 2–3 independent scorers who were blind to the genotype and sex of the mice. These scores were averaged for each subject.

4.3. Electrophysiology recordings

Mice were anesthetized using isoflurane and decapitated with a guillotine. The brain was then rapidly removed and 400 µm thick coronal slices of hippocampus were made with a vibrating microtome (Campden) using standard methods (Li et al., 2017). Slices from ventral hippocampus were collected and CA3 was removed from each slice. Dissection solution was kept ice cold (1–3 °C) and contained the following (in mM): 120 NaCl, 3.5 KCl, 0.7 $CaCl_2$, 4.0 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 glucose, bubbled with 95% O_2/5% CO_2, pH 7.35–7.45. Slices were allowed to recover at room temperature in the dissection solution for > 1 h prior to recording. Recordings were conducted in a submersion recording chamber perfused with external recording solution, a custom artificial cerebrospinal fluid, containing (in mM): 120 NaCl, 3.5 KCl, 2.5 $CaCl_2$, 1.3 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$ and 10 glucose, bubbled with 95% O2, pH 7.35–7.45. Recordings were conducted at 22–24^{0}C.

Field postsynaptic potential (fPSP) were measured in the temporoammonic (TA) pathway in stratum lacunosum-moleculare (SLM). A recording electrode (glass micropipette filled with external recording solution; 2–5 MΩ) and a bipolar tungsten stimulating electrode (FHC, Bowdoinham, ME) were placed in SLM. The synaptic response was measured as the initial slope of the fPSP. Paired-pulse stimulation was applied using 50, 100, 200, 500, and 1000 ms intervals. A 15–20 min stable baseline was obtained before the onset of each experiment by setting the stimulation at an intensity that generated 50–75% of the maximum synaptic response (the largest fPSP before population spikes are generated). Paired-pulse ratios were calculated as the slope of response 2/slope of response 1. The input/output curve was determined as the slope of the fPSP plotted against the amplitude of the fiber volley. The neuropeptide Y dose response curve was plotted with slopes at each dose normalized to the baseline.

All electrophysiology experiments were conducted in the presence of the NMDA antagonist D-2-amino-5-phosphonovalerate (D-APV, 50 µM) to prevent long-term potentiation. The NPY dose response curve was also measured in the presence of the $GABA_A$ receptor antagonist picrotoxin (100 µM) to block inhibition so that the effect of NPY on excitation could be directly measured. Neuropeptide Y (Tocris, 1176/200 U) was freshly resuspended in external recording solution before each experiment.

4.4. Enzyme-linked immunosorbent assay

Neuropeptide Y levels were measured with commercially available ELISA kit (EMD Millipore, EZHNPY-25 K). Mice were anesthetized with isoflurane and euthanized by decapitation. Hippocampus, Amygdala, Prefrontal Cortex, and/or Brain Stem were collected and flash frozen with dry ice. Samples were homogenized in NP-40 homogenization buffer (10 mM Tris pH 7.5,10 mM NaCl, 3 mM $MgCl_2{\cdot}6H_2O$, 1 mM EDTA, 0.5M of 10% NP-40) and centrifuged at 16,000 × *g* for 10 min at 4 °C. Supernatant was collected and stored at −80 °C. A Bradford Assay was conducted to ensure uniform total protein level loading (1 µg/ 1 µL) into the ELISA assay.

4.5. Statistical analysis

All statistics were performed with Origin software (Origin Lab Corporation, 2002) or SPSS Statistics (IBM, 2015). Comparisons were made using two-way ANOVA, using genotype and sex as independent variables, unless otherwise noted, and Tukey's post hoc test. There were no sex-specific differences except where noted. If an age difference was found between groups (as determined with two-way ANOVA) then age was run as a covariate. These instances are noted in figure legends. Samples sizes (n) refer to slice number in electrophysiological experiments and animal number in all other experiments. Statistics for all experiments are shown in the table in Supplementary Data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.npep.2019.101979.

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