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Prodynorphin gene deletion increased anxiety-like behaviours, impaired the anxiolytic effect of bromazepam and altered $GABA_A$ receptor subunits gene expression in the amygdala

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Abstract

This study evaluated the role of prodynorphin gene in the regulation of anxiety and associated molecular mechanisms. Emotional responses were assessed using the light–dark test, elevated plus maze and social interaction tests in prodynorphin knockout and wild-type mice. Corticotrophin releasing factor and proopiomelanocortin gene expressions in the hypothalamus were evaluated after restraint stress using in situ hybridization. The anxiolytic efficacy of bromazepam and $GABA_A$ receptor subunits gene expression in the amygdala were also assessed in both genotypes. The deletion of prodynorphin increased anxiety-like behaviours and proopiomelanocortin gene expression in the arcuate nucleus (two-fold). Moreover, the anxiolytic action of bromazepam was significantly attenuated in the mutant mice. Decreased $GABA_A\gamma_2$ and increased $GABA_A\beta_2$ gene expression receptor subunits were found in the amygdala of prodynorphin knockout mice. These results indicate that deletion of prodynorphin gene is associated with increased anxiety-like behaviours, enhanced sensibility response to stress stimuli, reduced anxiolytic efficacy of bromazepam and altered expression of the $GABA_A$ receptor subunits.

Keywords

Anxiety, benzodiazepine, $GABA_A$ receptor, prodynorphin gene

Introduction

Dynorphins are endogenous opioid peptides that bind with high affinity to κ-opioid receptors although they also display significant potency at δ-opioid receptors (Mansour et al., 1988). The distribution of dynorphin peptides and prodynorphin gene expression in the brain and spinal cord suggest their involvement in a large number of conditions such as neuropathic pain, drug dependence, fibromyalgia, epilepsy and mood disorders (Akil et al., 1984, 1998; Dickenson, 1991; Jhamandas, 1984; Russell, 1998; Simonato and Romualdi, 1996; Vaccarino et al., 1999).

Dynorphins are closely involved in the regulation of stress and anxiety. The highest expression of the prodynorphin gene occurs in the supraoptic and paraventricular nucleus of the hypothalamus and in different regions of the hippocampus (Hollt et al., 1980). Indeed, prodynorphin and corticotrophin releasing factor genes are expressed in the same neurons of the paraventricular nucleus (Roth et al., 1983). These reports support the role of dynorphins in the modulation of stress and emotional control. Indeed, there is a large body of evidence for the involvement of dynorphins in the mechanisms regulating the response to several stressful stimuli. For instance, prodynorphin peptides are elevated in the hypothalamus and pituitary after certain stress stimuli, and decreased in the pituitary after forced swimming induced stress (Nabeshima et al., 1992), suggesting that these peptides might modulate the behavioural changes induced by stressors. Tsuda et al. (1996) found that the effects of the anxiolytic drug diazepam were abolished in mice pretreated with the κ-opioid receptor antagonist nor-binaltorphimine using a Vogel-type conflict paradigm, suggesting the potential participation of this

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receptor system in the anxiolytic action of benzodiazepines and the regulation of anxiety. Increased levels of anxiety-like behaviours were found in several paradigms (elevated plus maze test in rats and CD-1 mice and in fear potentiated startle paradigm in rats) pretreated with nor-binaltorphimine (Knoll et al., 2007; Wall and Messier, 2002), whereas the treatment with the κ-opioid receptor agonist U-50,488H resulted as anxiolytic in mice with a high level of anxiety induced by repeated experiences of social defeats (Kudryavtseva et al., 2004). Kuzmin et al. (2006) showed that the big dynorphin precursor peptide induced anxiolytic-like behaviour in the elevated plus maze test in mice. However, other studies found anxiolytic effects after the administration of κ-opioid receptor antagonists in unlearned and learned fear in rats (Knoll et al., 2007). Furthermore, it has been proposed that dynorphins play an important role in the regulatory mechanisms of aversive and dysphoric properties of stress (Land et al., 2008; McLaughlin et al., 2006a, 2006b) and that deletion of prodynorphin affected stress-induced behaviours (McLaughlin et al., 2003).

Recently, two studies used mice lacking the prodynorphin gene to evaluate the role of this opioid peptide in anxiety-like behaviours. The first study (Bilkei-Gorzo et al., 2008) showed an enhanced response to stress reactivity in mice lacking the prodynorphin gene in several paradigms evaluating anxiety. In contrast, the second study (Wittmann et al., 2009) described an anxiolytic behavioural phenotype in prodynorphin knockout mice with decreased basal levels of corticotrophin-releasing hormone (CRH) gene expression in the hypothalamic paraventricular nucleus and amygdala accompanied by decreased serum basal level of CRH. On the other hand, the deletion of the prodynorphin gene increased pro-opiomelanocortin (POMC) gene expression in the arcuate nucleus (Wittmann et al., 2009). The results of these studies are contradictory and consequently the role of prodynorphin in the basal regulation of anxiety and stress in mice remains unsettled.

To further clarify the role of the prodynorphin gene in anxiety and response to stress several experiments were performed in prodynorphin knockout (PDYN KO) and wild-type (WT) mice as follows: (1) the evaluation of different paradigms testing anxiety-like behaviours; (2) the hypothalamic gene regulation of CRH and POMC after acute restraint stress; (3) the anxiolytic efficacy of benzodiazepines, as well as potential alterations in $GABA_A$ receptor subunits gene expression.

Material and methods

Animals

Adult male C57BL/6 prodynorphin gene double mutant PDYN KO mice (Sharifi et al., 2001) and WT littermates were used in all the experiments. All mice were born in the animal facility at Neuroscience Institute of Alicante (Spain) from breeding pairs PDYN (+/−) initially provided by Dr Ute Hochgeschwender (University of Oklahoma, USA) and backcrossed throughout eight generations. WT and PDYN KO mice were matched for similar age (2–3 months) and weight (25–35 g) and used in every experiment only once. Mice were maintained at a constant temperature of $23 \pm 2^{\circ}C$ and in a 12 h dark–light cycle (light from 08:00 to 20:00), with free access to food and water. All experiments were in accordance with guidelines established by the European Council Directive (86/609/EEC) and were approved by the Institutional Animal Care Committee.

Drug

Bromazepam (Sigma-Aldrich, Madrid, Spain) was dissolved in water (50 and 100 μg/kg) and administered (p.o., 30 min) to WT and PDYN KO mice. The dose and pattern of administration were based on previous results from our laboratory (Urigüen et al., 2004).

Behavioural paradigms

Open field. The open field consisted of a transparent squared cage (25 cm × 25 cm × 25 cm) with a white Plexiglas floor. Mice (WT and PDYN KO) were individually placed in the centre of the apparatus. Sessions were recorded with a video camera and analysed with the SMART program (Spontaneous Motor Activity Recording & Tracking) v.2.0 software system (Panlab, Barcelona, Spain). Total, central and peripheral distance covered (cm) in a 30-minute interval was measured.

Anxiety-like behaviours. The light–dark test was carried out as previously described (Bourin and Hascoet, 2003; Crawley and Goodwin, 1980). The apparatus comprised two chambers (20 cm × 20 cm × 15 cm), a dark and enclosed box and an illuminated box (60 W/400 lux desk lamp placed 25 cm above the light box) connected by a 4 cm × 4 cm black and enclosed passageway. The time (s) spent by mice in the light box and the number of transitions between the light and dark chamber were measured during a single trial of 5 min.

The elevated plus maze test was carried out as previously described (Handley and Mithani, 1984; Lister, 1987; Pellow et al., 1985). The apparatus consisted of two opposite open arms (30 cm × 5 cm) without side walls and two enclosed and black horizontal perpendicular arms (30 cm × 5 cm × 15 cm). The apparatus was elevated 75 cm above the floor and the room was illuminated with two white bulbs (40 W/40 lux) located at the top. The time (s) spent in the open arms as well as entries into the open and closed arms were measured during a period of 5 min.

The social interaction test is a behavioural paradigm in which two mice from different home cages that have not established territory are placed together in a small chamber (20 cm × 40 cm × 10 cm). The time that mice socially interact (sniffing, following, grooming, kicking, crawling under or over the partner, and touching or nearly touching their faces) was measured for 5 min (File and Hyde, 1978).

Stress procedure

Unanaesthetized mice (WT and PDYN KO) were confined in an acrylic cylindrical tube (inner size 10 cm × 3 cm × 3 cm), with small holes to allow breathing, for a period of 30 min.

After this period of restraint stress, the animals were removed from the tubes and placed in their home cages. Non-stressed mice (WT and PDYN KO) remained in their home cages undisturbed. After 150 min both groups of mice were killed by decapitation.

Histology

The brains of WT and PDYN KO mice were rapidly removed, fresh frozen and stored immediately at −80°C until use. The brains were subsequently cut into fine slices (12 µm) with a cryostat to study hypothalamic gene expression of POMC in the arcuate nucleus (ARC) and that of corticotrophin releasing factor (CRF) in the paraventricular nucleus of the hypothalamus (PVN) by in situ hybridization. Brain sections of WT and PDYN KO mice were cut in the cryostat at 500 µm and dissected as described previously, with some modification (Palkovits, 1983) to obtain the amygdala (AMY). These sections were identified according to Paxinos and Franklin (2001).

In situ hybridization histochemistry

The in situ hybridization histochemistry (ISHH) was performed as described previously (Corchero et al., 1999; Young et al., 1986) using synthetic oligonucleotide probes complementary to POMC mRNA (bases 96–134; Thermo-Scientific, Barcelona, Spain) and CRF mRNA (bases 496–543; Thermo-Scientific). Each set of slides (four slides/mouse) was apposed to the same film (Kodak BioMax MR-1, Sigma-Aldrich, Madrid, Spain) in individualized cassettes for 20 days (POMC and CRF).

Image analyses quantification

The optical densities were calculated from the uncalibrated mode of the Image Program (see http://rsb.info.nih.gov/nih-image) by subtracting from each measurement its corresponding background and expressed in greyscale values. The background measurement was taken from an area of the slice with the lowest non-specific hybridization signal and subtracted from the hybridization signal measurement in the same slice. The results were averaged for each experimental group and were expressed considering mean control values from WT non-stressed mice as 100%.

Evaluation of $GABA_A$ receptor subunits gene expression in the amygdala

RNA was extracted from microdissected AMY of WT and PDYN KO mice using the Mini RNA Isolation I Kit (Zymo Research, Orange, CA, USA). The reverse transcription was carried out following the instructions of the manufacturer of a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA). The standard reaction for amplification of $GABA_A$ receptor subunits was carried out as follows: 2.5 µl of cDNA products were amplified with 2.5 U of GoTaqPlus (Promega, Madrid, Spain) in the buffer provided by the manufacturer, which contains $MgCl_2$, and in the presence of the specific primers together with the GAPDH primers (Table 1), used as an internal control, as previously described (Warnault et al., 2007) with some modifications. The ratio between each RNA target/GAPDH was quantified with the Imager TM gel analyser and NIH Image analysis software. Mean and standard error of all experiments performed were calculated after normalization to GAPDH.

Table 1. Specific primer sequences used for RT-PCR $GABA_A$ receptor subunits

Gene	Primer sequence
$GABA_{A\alpha 2}$	forward: 5′-AAAAGAGGATGGGCTTGGGA-3′
	reverse: 5′-ACGGGATGTTTTCTGCCTGTAT-3′
$GABA_{A\beta 2}$	forward: 5′-GGAGTGACAAAGATTGAGCTTCCT-3′
	reverse: 5′-GTCTCCAAGTCCCATTACTGCTTC-3′
$GABA_{A\gamma 2}$	forward: 5′GTGAAGACAACTTCTGGTGACTATGTGGT-3′
	reverse: 5′ CATATTCTTCATCCCTCTCTTGAAGGTG-3′
GAPDH	forward: 5′TGAAGGTCGGTGTGAGABAACGGATTTG-3′
	reverse: 5′-CATGTAGGCCATGAGGTCCACCAC-3′

Statistical analyses

Results are expressed as mean values ± SEM. Behavioural and gene expression results were analysed with the program Sigma Stat 3.1. A two-tailed Student's *t*-test was used when comparing two groups, and one- and two-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test when comparing three or four groups, or when comparing two groups and two different factors. The level of statistical significance was chosen as $p < 0.05$.

Results

Spontaneous motor activity

In the open field, no significant differences were found in the travelled distance (cm) between PDYN KO and WT mice when considering the total time period in peripheral, central and total areas (Figure 1). Student's *t*-test: peripheral distance ($t = 1.201$, $p = 0.251$), central distance ($t = 0.953$, $p = 0.358$), total distance ($t = 1.69$, $p = 0.113$).

Anxiety-like behaviours analyses

The light–dark test revealed that PDYN KO mice spent significantly less time in the light area ($t = 4.577$, $p < 0.001$) compared with WT animals (Figure 2A). No differences ($t = 0.885$, $p = 0.395$) were found between PDYN KO and WT mice in the number of transitions (Figure 2B).

In the elevated plus maze, the time spent in the open arms was significantly decreased in PDYN KO ($t = 3.029$, $p = 0.013$) compared with WT mice (Figure 2C). No significant differences ($t = -1.748$, $p = 0.111$) were found in the number of entries into the open arms between the two groups (Figure 2D).

The time of social interaction was significantly decreased ($t = 4.842$, $p < 0.001$) in PDYN KO mice compared with WT mice (Figure 2E).

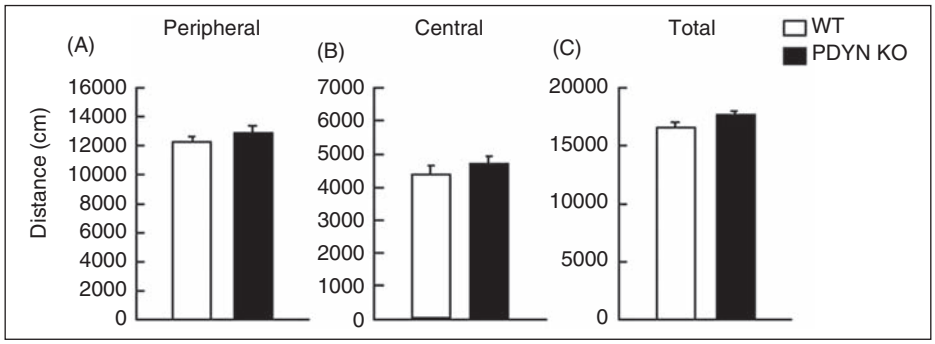

Figure 1. Motor activity of WT and PDYN KO mice in the open field. Columns represent the means and vertical lines ± S.E.M of distance (cm) ($n = 8$) measured in total 30-minute session. (A) peripheral distance, (B) central distance, and (C) total distance in the open field.

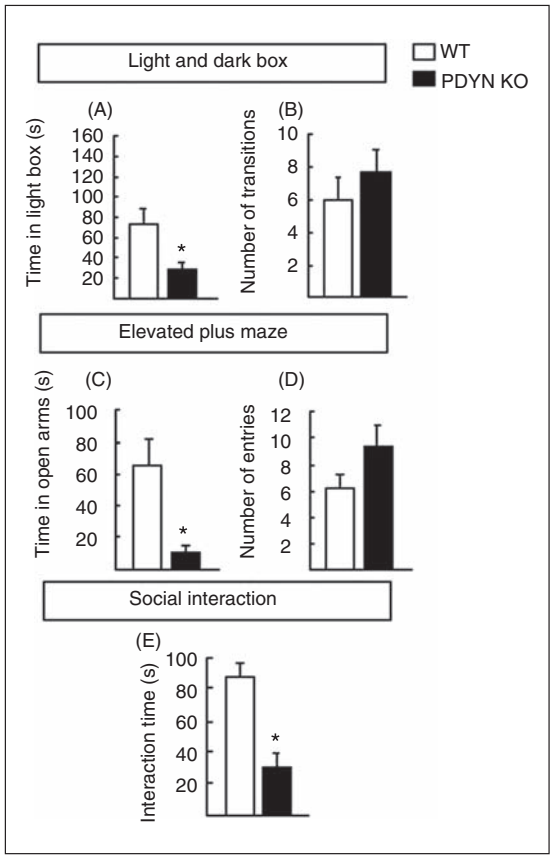

Figure 2. Light–dark test: time (s) spent in light area (A) and number of transitions (B) of WT and PDYN KO mice for a period of 5 min ($n = 7$). Columns represent the means and vertical lines ± SEM *Values from PDYN KO mice that are significantly different ($p < 0.001$, Student's t-test) from WT mice. Elevated plus maze test: time (s) in open arms (C) and number of entries into open arms (D) was evaluated for a period of 5 min ($n = 7$) in WT and PDYN KO mice. Columns represent the means and vertical lines ± SEM. *Values from PDYN KO mice that are significantly different ($p = 0.013$, Student's t-test) from WT mice. Social interaction: time (s) of social interaction was evaluated in WT and PDYN KO mice for a period of 5 min ($n = 8$) (E). Columns represent the means and vertical lines ± SEM *Values from PDYN KO mice that are significantly different ($p < 0.001$, Student's t-test) from WT mice.

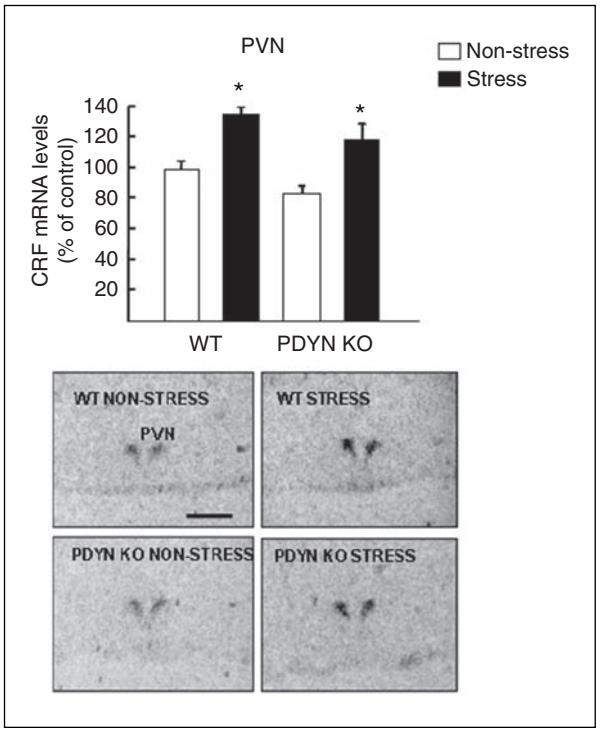

Figure 3. Effects of restraint stress on CRF gene expression in the PVN of the hypothalamus. Columns represent the means and vertical lines ± S.E.M of CRF mRNA levels ($n = 10$). The lower panels show representative autoradiograms of coronal brain sections at the level of PVN. Bar represents 1 mm.
*Values from CRF gene expression levels that are significantly different from WT no stress ($p < 0.05$, two-way ANOVA).

CRF and POMC gene expression in the hypothalamus after acute stress

Acute restraint stress markedly increased CRF gene expression in the PVN of WT (34%, $p = 0.001$) and PDYN KO (42%, $p < 0.001$) mice when the effect of acute restraint stress is compared with non-stressed mice from the same genotype after a post-hoc conducted test (Figure 3). No significant differences were found between both genotypes under

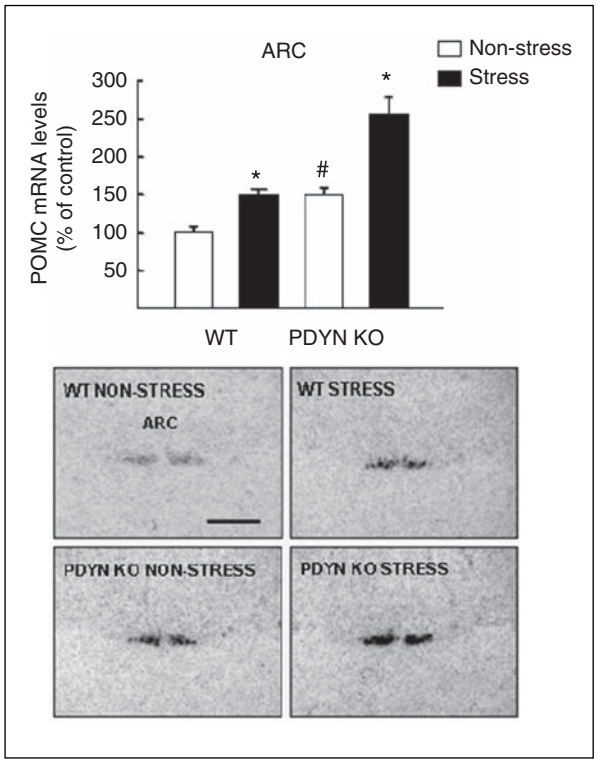

Figure 4. Effects of restraint stress on POMC gene expression in the ARC of the hypothalamus in WT and PDYN KO by in situ hybridization. Columns represent the means and vertical lines ± SEM of POMC mRNA levels ($n = 10$). The lower panels show representative autoradiograms of coronal brain sections at the level of ARC. Bar represents 1 mm.
*Values from POMC mRNA levels in PDYN KO mice and PDYN WT mice that are significantly different from WT non-stressed mice ($p < 0.05$, two-way ANOVA).
#Values from POMC mRNA basal levels in PDYN KO that are significantly different ($p < 0.05$, two-way ANOVA) from basal levels of WT non-stressed mice.

basal (non-stress) conditions (Figure 3). Two-way ANOVA analyses revealed significant changes in the genotype [($F_{(1,27)} = 5.962$, $p = 0.022$) and stress ($F_{(1,27)} = 28.056$, $p = 0.001$) but not in the genotype × stress ($F_{(1,27)} = 0.0006$, $p = 0.981$)].

Basal POMC gene expression in the ARC of PDYN KO mice was significantly higher (45%, $p < 0.05$) than that in WT mice (Figure 4). The two-way ANOVA revealed that acute restraint stress increased POMC gene expression in the ARC of both genotypes [genotype ($F_{(1,27)} = 23.244$; $p = 0.001$), stress ($F_{(1,27)} = 26.332$; $p = 0.001$), genotype × stress ($F_{(1,27)} = 4.089$; $p = 0.050$)] but this effect was more pronounced in PDYN KO mice (72%, $p < 0.001$) than in WT mice (45%, $p < 0.047$).

Effect of bromazepam on anxiety-like behaviours

In WT mice, the one-way ANOVA ($F_{(2,20)} = 15.93$, $p < 0.001$) revealed that the acute administration of bromazepam (50 and 100 µg/kg) significantly increased the time spent in the lit area in the light–dark box test (50–56%, $p < 0.001$); however, no differences were found between the two doses (Figure 5A). In PDYN KO mice the one-way ANOVA ($F_{(2,21)} = 16.71$, $p < 0.001$) revealed that only the higher dose of bromazepam (100 µg/kg) significantly increased the time spent in the lit area (87%, $p < 0.001$) (Figure 5B). Bromazepam (50 and 100 µg/kg) did not alter the number of transitions between the light and the black chambers in WT ($F_{(2,20)} = 0.498$, $p = 0.616$) and PDYN KO mice ($F_{(2,21)} = 0.556$; $p = 0.582$) (Figure 5C and D).

$GABA_A$ receptor subunits gene expression

The deletion of the PDYN gene significantly decreased (30%, $t = 2.245$, $p = 0.044$) the expression of $GABA_{A\gamma 2}$ receptor subunit in the AMY of KO mice compared with that in WT animals (Figure 6). In contrast, the $GABA_{A\beta 2}$ receptor subunit was found markedly increased (2.3-fold) in the same brain region of PDYN KO mice compared with WT mice ($t = 2.543$, $p = 0.025$) (Figure 6). No significant differences between genotypes were observed for the $GABA_{A\alpha 2}$ receptor subunit in the AMY ($t = 0.341$, $p = 0.740$) (Figure 6).

Discussion

The results of the present study demonstrated a relevant role of the PDYN gene in the regulation of emotional behaviours and associated molecular mechanisms. This was confirmed by the following facts: (1) the absence of the PDYN gene increased the vulnerability to experience anxiety-like behaviours in a variety of experimental paradigms evaluating different anxiogenic stimuli; (2) the deletion of PDYN disrupted, at least in part, the homeostatic balance of the hypothalamic–pituitary–adrenal (HPA) axis since increased sensitivity in POMC gene expression was observed in response to restraint stress; and (3) the anxiolytic action of bromazepam is reduced and $GABA_A$ receptor subunits are altered in the AMY of PDYN KO mice.

The analyses of motor activity consisted in the evaluation of travelled distance in a total period of 30 min. The results revealed that deletion of the PDYN gene failed to modify total motor activity in travelled distance. The overall lack of motor alterations in PDYN KO mice was consistent with the results reported by Bilkei-Gorzo et al. (2008). In contrast, another report (Wittmann et al., 2009) found that deletion of the PDYN gene resulted in increased ambulatory behaviour. These discrepancies may be explained by differences in the specific method used to evaluate motor activity, the genetic construction used to delete the PDYN gene and the specific strain of mice.

The distribution of PDYN gene in brain areas closely involved in the regulation of emotional behaviours (Lin et al., 2006; Marchant et al., 2007) suggests a relevant role in anxiety and stress responses. Indeed, several studies suggested that pharmacological modulation of the dynorphin κ-opioid system resulted in anxiety-like or anxiolytic behaviour (Knoll et al., 2007; Kudryavtseva et al., 2004; Wall and Messier, 2002). Dynorphin peptides and the κ-opioid receptor have been involved in the regulatory mechanisms of aversive and dysphoric properties of stress (Land et al., 2008; McLaughlin et al., 2006a) and the dynorphin genetic ablation affected stress induced behaviours (McLaughlin et al., 2003). In the present study, the evaluation of anxiety-like behaviours

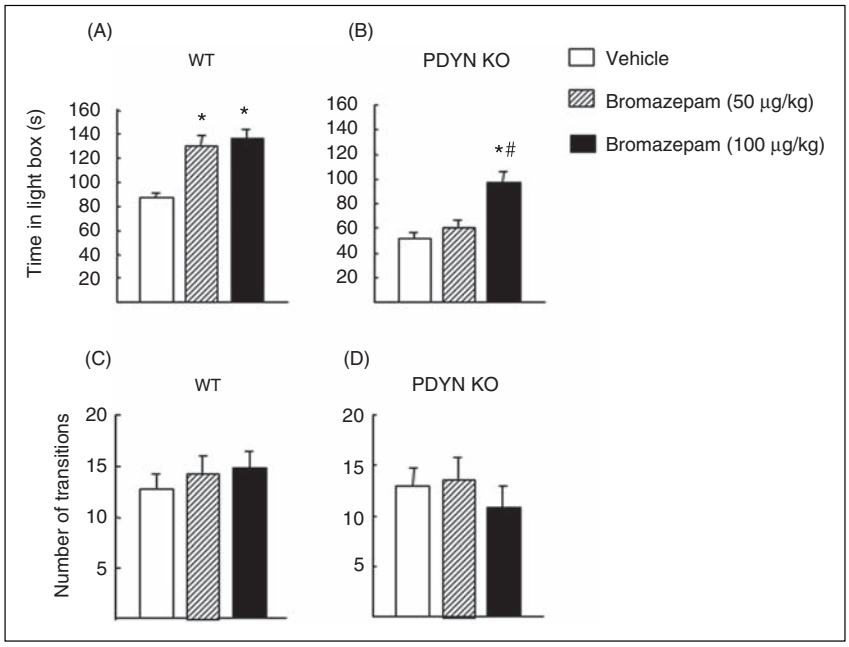

Figure 5. Effect of bromazepam in the light–dark test in PDYN KO and WT mice ($n = 10$). Mice received 50 or 100 μg/kg p.o. or its water vehicle (0.3 ml) and 30 min later were exposed to the light–dark box test for 5 min. Columns represents the mean values and vertical lines ± S.E.M of (A, B) time (s) spent in the lit area and (C, D) the number of transitions.

*Values from drug-treated PDYN KO or WT mice that are significantly different (ANOVA, Student–Newman–Keuls, $p < 0.05$) from its corresponding vehicle-treated mice.

#Values from drug-treated PDYN KO mice that are significantly different (ANOVA, Student–Newman–Keuls, $p < 0.05$) from the 50 μg/kg dose.

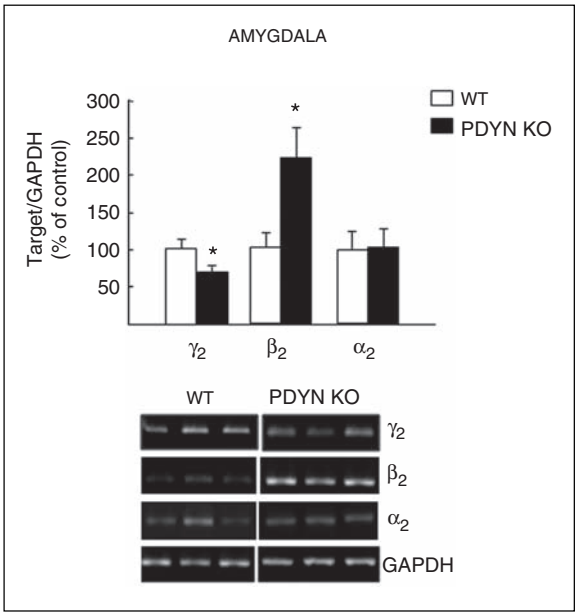

Figure 6. Evaluation of $GABA_A$ α_2, β_2 and γ_2 subunits receptor gene expression in the AMY of WT and PDYN KO ($n = 8$). Columns represent the mean of each RNA target/GAPDH ratio. PDYN KO levels are expressed relative to WT mice, expressed as a percentage. The lower panels represent a sample of the bands' intensity from the PCR products obtained of the different subunits analysed and the standard GAPDH in WT and PDYN KO mice.

*Values from PDYN KO mice that are significantly different ($p < 0.001$, Student's t-test) from WT mice.

was carried out by using three experimental paradigms (light–dark test, elevated plus maze and social interaction) that challenged the mice to different type of anxiogenic stimuli. In all of these tests, the deletion of PDYN markedly increased anxiety behaviours (reduction in the exploratory behaviour and time of interaction). These results are, in part, in agreement with those reported by Bilkei-Gorzo et al. (2008) that found in PDYN KO mice marked anxiety-like reactivity in the zero maze test although no significant differences were observed in the light–dark box test. In contrast, the study by Wittmann et al. (2009) showed a two- to four-fold increase of anxiolytic parameters in the open field, light–dark test and elevated plus maze tests. The reasons that may account for these contradictory results may be related to the specific genetic construction used to generate the mice lacking the PDYN gene (Loacker et al., 2007; Sharifi et al., 2001; Zimmer et al., 2001), or variations in the specific experimental conditions (previous acclimatization of mice, intensity of illumination, time and mode of detection or measurement, size of the apparatus, precise experimental paradigm or type of anxiogenic stimuli). Transgenic and knock out mice provide a powerful new tool for the elucidation of biological functions; however, since natural strain differences in behavioural traits occur in different types of strains, the genetic background of the inbred mouse strains must be carefully considered in the interpretation of behavioural phenotypes of knock out mice (Crawley, 1996; Gerlai, 1996). The phenotype of a mutant mouse is not only the result of the targeted gene, but it also reflects interactions with background genes and other unknown mutations in the genetic background

(Crawley et al., 1997), even the specific procedure to perform the genetic construction and generation of mutant mice. The genetic background should be as carefully controlled as any other experimental variable because molecular events do not run in isolation but involve large biochemical complexes and cascades, where each step is directly dependent upon many other simultaneous molecular events. Thus, it is important to take into account that the behavioural features, due to the lack of a particular ligand or receptor in knock out mice, should not be comparable to the effects caused by pharmacological modulation of this receptor in wild type mice.

The pronounced anxiety-like behavioural alterations induced by the deletion of PDYN gene may be associated to neuroendocrine and molecular 'neuroadaptations' in key elements regulating emotional behaviours. The HPA axis is known as the major brain circuitry that regulates the neuroendocrine response to stress. The POMC processes several proteins, ACTH and β-endorphin among others (Eipper and Mains, 1980; Mains and Eipper, 1980). In the arcuate nucleus of the hypothalamus, the β-endorphin is the main end product of POMC gene activity (Emeson and Eipper, 1986; Gramsch et al., 1980). Several authors have found increases in POMC gene expression after different stress paradigms, particularly in the mediobasal hypothalamus of rat after acute immobilization stress (Baubet et al., 1994) and within the ARC after restraint stress (Larsen and Mau, 1994). This circuit is regulated by stimulatory actions from 5-HT and NA neurons and inhibitory signals from GABA and β-endorphins (Calogero, 1995; Tsagarakis et al., 1990). The hormonal release (corticotrophin and corticosterone) that comprises HPA is also regulated by the end product inhibition. The stress has shown to increase the CRF gene expression in the PVN of the hypothalamus (Hollt et al., 1986). The integrity of this complex circuit is essential in order to maintain an adequate regulation to stress response. In this study, restraint stress significantly increased CRF gene expression in both WT and PDYN KO mice, suggesting that the deletion of this gene failed to produce disruption of the HPA axis at the level of the PVN. In contrast, POMC gene expression in the ARC markedly increased (two-fold) in PDYN KO compared with WT. Interestingly, basal levels of POMC were also significantly enhanced in PDYN KO, a result that is in agreement with a previous report (Wittmann et al., 2009) showing increased POMC gene expression in the ARC of PDYN KO mice. These findings suggest that deletion of the PDYN gene increased the sensitivity to stress at the level of POMC gene expression in the ARC nucleus. Furthermore, it is possible to hypothesize that the fact that POMC was increased under basal conditions in PDYN KO mice points to dynorphins possibly tonically inhibiting this gene. The deletion of the PDYN gene would result in increased POMC gene expression in the ARC.

Benzodiazepines (BZDs) acting through their binding on the interface of α and γ subunits of $GABA_A$ receptor complex are well known to act as anxiolytics promoting the inhibitory actions of GABA neurotransmitter in the central nervous system (Da Settimo et al., 2007). The role of dynorphin peptides in the anxiolytic action of BZD remains unknown. In animal models evaluating anxiety-like behaviours, BZD tends to increase the exploratory activity of rodents in plus maze and light–dark test paradigms (Crawley and Goodwin, 1980; Gonzalez and File, 1997; Guy and Gardner, 1985; Hascoet and Bourin, 1998). In this study, the administration of bromazepam (50 and 100 μg/kg) presented anxiolytic action in WT mice. In contrast, in PDYN KO mice the dose of 50 μg/kg was without effects and only the higher dose (100 μg/kg) of bromazepam resulted in anxiolytic action although the response was reduced compared with WT mice. The impaired anxiolytic action of BZD was also previously observed in rats treated with κ-opioid receptor antagonists (Tsuda et al., 1996) and in other animal models showing increased anxiety (Bailey and Toth, 2004; Commissaris et al., 1990; Sibille et al., 2000; Urigüen et al., 2004). Furthermore, several reports have shown a variable sensitivity in the response to BZD treatment in the human population (Domino et al., 1989; Gupta et al., 1997), particularly in those groups of patients with anxiety disorders presenting diminished response to benzodiazepine treatment (Cowley et al., 1997; Kaschka et al., 1995; Nutt and Glue, 1989; Schlegel et al., 1994). These findings suggest that alterations in $GABA_A$ receptor functional activity may be underlying the impaired anxiolytic response to benzodiazepines (Bailey and Toth, 2004; Bremner et al., 2000; Crestani et al., 1999; Kaschka et al., 1995; Malizia et al., 1998; Rainnie et al., 1992; Schlegel et al., 1994; Tiihonen et al., 1997). Interestingly, the PDYN KO mice showed significant decreased expression in $GABA_A\gamma_2$ receptor subunit and increased expression in the $GABA_A\beta_2$ in the AMY. In vivo electrophysiological studies proposed an obligatory role of the γ_2 subunit for BZD action, suggesting that the presence of this subunit may be necessary for high affinity BZD binding and functional modulation of the receptor by BZD (Pritchett et al., 1989; Sigel et al., 1990). In fact, KO homozygous mice for the γ_2 subunits resulted in a severe deficit in BZD binding site and showed diminished effects for diazepam (Gunther et al., 1995), whereas KO heterozygous mice for γ_2 subunits with reduced γ_2 protein levels displayed anxiety-like behaviours (Crestani et al., 1999). These findings may explain, at least in part, the high levels of anxiety observed in PDYN KO mice as well the impaired response to BZD treatment. The $GABA_A\beta_2$ subunit has also been involved in the regulation of anxiolytic actions, specially for non-BZD drugs (Hamon et al., 2003). Probably the marked increase observed in the $GABA_A\beta_2$ receptor subunit in the AMY of PDYN KO mice may be a compensatory up-regulation that may occur because of the impaired function of $GABA_A\gamma_2$ receptor subunit. Thus, the absence of PDYN gene may alter the expression of γ_2 and β_2 subunits in the AMY producing an impaired anxiolytic action of BZD. The effects of PDYN gene in different $GABA_A$ receptor subunits in other brain regions remain to be further explored. The $GABA_A\alpha_2$ receptor subunit has been largely involved in the anxiolytic action of BZD (Low et al., 2000; McKernan et al., 2000; Rudolph et al., 1999); however, in this study the results did not reveal significant differences between WT and PDYN KO mice. Similarly, other mutant mice (B6-R-/-) with a high level of anxiety did not present functional alterations in $GABA_A$ α_1 or α_2 receptor subunits (Bailey and Toth, 2004). In contrast, mice lacking $5\text{-}HT_{1A}$ receptor showed decreased $GABA_A$ α_1 or α_2 receptor subunit levels in several regions of the brain including the

AMY (Bailey and Toth, 2004; Sibille et al., 2000). These findings point to some phenotypes displaying high levels of anxiety possibly presenting unaltered the expression of the α subunit, and suggest that this subunit may not be necessary to modulate the anxiolytic response of BZD (Pritchett et al., 1989; Sigel et al., 1990).

In summary, the results of this study support a pivotal role of the prodynorphin gene in the regulation of anxiety-like behaviours and in the pharmacological efficacy of benzodiazepines as anxiolytic drugs.

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