Elsevier Editorial System(tm) for Neuroscience
Manuscript Draft

Manuscript Number:

Title: Influence of gender and genetic background on anxiety-related and stress-induced behaviour of prodynorphin-deficient mice

Article Type: Research Paper

Section/Category: Cognitive, Behavioral and Systems Neuroscience

Keywords: anxiety; stress; amygdala; emotional control; kappa opioid receptor; prodynorphin knockout

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Abstract: The role of dynorphin/kappa opioid receptors in epilepsy and addiction are well accepted, but their function in emotional control is not yet fully understood. Data obtained from different strains of prodynorphin (pDyn)- and kappa opioid receptor (KOP)-deficient mice do not provide a consistent picture of the functions of Dyn/KOP in anxiety, suggesting the influence of testing conditions and/or genetic background. Therefore, we investigated the behaviour and neurochemistry of male and female pDyn KO mice on the balb/c and C57Bl/6N background. Consistent with our results obtained from male mice on the C57bl/6N background, we observed an anxiolytic phenotype in the elevated plus maze, open-field and light-dark test in male mice on the balb/c background. Female mice on the balb/c background displayed an anxiolytic phenotype; however these data reflect high trait anxiety and inter-individual differences. In contrast, female mice on the C57Bl/6N background displayed low trait anxiety and a paradigm-dependent anxiolytic phenotype. No differences were observed in the forced swim test, while balb/c pDyn KO mice displayed prolonged immobility in the tail suspension test. In line with our previous results, we observed reduced CRH mRNA in the central amygdala in all groups of mice. In contrast, the recently observed CRH mRNA reduction in the hypothalamic paraventricular nucleus appears restricted to male, but not female mice.
Our data support previous data suggesting a pronounced impact of endogenous prodynorphin-derived peptides on anxiety. Moreover, our data support the idea that the anxiolytic phenotype manifests only at elevated stress levels.

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Cover Letter

Dear Prof. Dr. Liisa Galea,

Please find enclosed a manuscript of Iris Kastenberger, Christian Lutsch, Herbert Herzog and myself entitled: Influence of gender and genetic background on anxiety-related and stress-induced behaviour of prodynorphin-deficient mice “ for consideration of publication in NEUROSCIENCE.
Presently, there are a number of investigations regarding the involvement of kappa opioid receptor (KOP) in dysphoria and addiction, exploring the use of KOP antagonists or partial agonists as therapeutic agents. However, there are a number of unequivocal data regarding the function of dynorphin and KOP in anxiety control. Bruchas and colleagues recently proposed that stress level of the testing conditions might cause such differences. Therefore, we now investigated prodynorphin deficient mice on two genetic backgrounds with markedly different stress sensitivity. Moreover, we tested both genders, as female rodents are generally seen as more stress resistant. The data we obtained strongly support the idea of stress-level dependent anxiogenic effects of endogenous dynorphin. We are positive that our data help to clarify this important point and support the development of anti-depressant and anti-addictive drugs by providing information on potential side-effects.

Yours sincerely
Christoph Schwarzer

***Highlights**

Male prodynorphin knockout mice display anxiolytic phenotype.

Female prodynorphin knockout mice display a paradigm dependent anxiolytic phenotype.

Prodynorphin knockout mice display gender differences in stress response.

The anxiolytic phenotype correlates to stress resistance of mouse strain and gender.

Influence of gender and genetic background on anxiety-related and stress-induced behaviour of prodynorphin-deficient mice

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Abstract

The role of dynorphin/kappa opioid receptors in epilepsy and addiction are well accepted, but their function in emotional control is not yet fully understood. Data obtained from different strains of prodynorphin (pDyn)- and kappa opioid receptor (KOP)-deficient mice do not provide a consistent picture of the functions of Dyn/KOP in anxiety, suggesting the influence of testing conditions and/or genetic background. Therefore, we investigated the behaviour and neurochemistry of male and female pDyn KO mice on the balb/c and C57Bl/6N background. Consistent with our results obtained from male mice on the C57bl/6N background, we observed an anxiolytic phenotype in the elevated plus maze, open-field and light-dark test in male mice on the balb/c background. Female mice on the balb/c background displayed an anxiolytic phenotype; however these data reflect high trait anxiety and inter-individual differences. In contrast, female mice on the C57Bl/6N background displayed low trait anxiety and a paradigm-dependent anxiolytic phenotype. No differences were observed in the forced swim test, while balb/c pDyn KO mice displayed prolonged immobility in the tail suspension test. In line with our previous results, we observed reduced CRH mRNA in the central amygdala in all groups of mice. In contrast, the recently observed CRH mRNA reduction in the hypothalamic paraventricular nucleus appears restricted to male, but not female mice.

Our data support previous data suggesting a pronounced impact of endogenous prodynorphin-derived peptides on anxiety. Moreover, our data support the idea that the anxiolytic phenotype manifests only at elevated stress levels.

Keywords: amygdala, emotional control, kappa opioid receptor, prodynorphin knockout

1. Introduction

Anxiety is a fundamental part of the behaviour of animals and human beings. The proper response to anxiety cues prompts a state of defensive motivation. In contrast, improper responses lead to severe psychiatric problems. Classical transmitters like serotonin (Wise et al., 1970, Westenberg et al., 1987, Graeff, 2002) and noradrenaline (Vlachakis et al., 1974, Brunello et al., 2003), along with several neuropeptides have been proposed as modifiers of anxiety-related behaviour. Regulatory circuits involve a dense network of cortical, amygdalar, hypothalamic and brainstem nuclei. However, the basolateral and central nuclei of the amygdala and the paraventricular hypothalamic nucleus appear to be most relevant (for review see Lang et al., 2000).

The role of Dyn/KOP in anxiety control is not well understood. Data obtained from pDyn- and KOP-deficient mice are relatively rare and do not provide a consistent picture of the functions of Dyn/KOP in anxiety. In pDyn KO mice on a C57bl/6J background (Bilkei-Gorzo et al., 2008), zero-maze and startle response tests suggested an anxiogenic phenotype, while no effect was seen in the light-dark test. Femenia and colleagues (Femenia et al., 2010) observed reduced time spent in the light area of the light-dark test and less time spent on the open arm of the elevated plus maze in pDyn KO mice (C57Bl/6 non-specified). However in both tests the number of entries appeared to be unaffected or even increased, contradicting an anxiogenic phenotype. In our pDyn KO mouse line, maintained on a C57bl/6N background (Wittmann et al., 2009), a markedly anxiolytic phenotype was observed in three independent tests (open field, light-dark choice and elevated plus maze), which was reproduced in wild-type mice through treatment with the KOP antagonists

norBNI and GNTI. The pDyn KO phenotype was reversed by treatment with the selective KOP agonist U-50488H. While we found no differences in stress-induced hyperthermia, Bilkei-Gorzo et al. (2008) reported a delayed and subtle increase in stress-induced hyperthermia in their pDyn KO mice. In general the alterations are subtle and may be camouflaged by compensatory changes, as all models published to date are germ-line knockouts. Thus, up-regulation of both Mu and Delta opioid receptors was observed in anxiety-related brain nuclei of pDyn and KOP KO mice (Slowe et al., 1999, Clarke et al., 2003). In addition, anxiety testing is strongly influenced by epigenetic and environmental conditions including the social status of mice (Kudryavtseva et al., 2004). In line with this, Bruchas et al. (2009) showed that pDyn KO mice displayed a markedly stronger anxiolytic phenotype under pre-stressed compared to non-stressed conditions.

The complexity of anxiety control is also reflected in pharmacological experiments. Tsuda et al. (1996) proposed the involvement of KOP in the anxiolytic action of diazepam, and KOP agonists produced anxiolytic-like behaviour in the elevated plus maze (Privette and Terrian, 1995). In addition, big Dyn (a fragment containing Dyn A and Dyn B) was suggested to be an anxiolytic peptide (Kuzmin et al., 2006). The marked anxiolytic effects of KOP agonists were opposed by the finding of increased KOP-specific binding in the amygdala in chronic pain-induced anxiety in mice (Narita et al., 2006). In contrast, several other reports suggest pro-aversive effects in the elevated plus maze mediated by KOP agonists injected into the periaqueductal grey (Motta et al., 1995, Nobre et al., 2000). Moreover, blockade of KOP receptors abolished the anxiogenic effects of CRH (Bruchas et al., 2009). A synopsis of these studies suggests spatial and temporal differences in the response to KOP activation (Wittmann et al., 2009). This is supported by specific alterations in the transmitter systems of pDyn KO mice known to be involved in emotional control. Most

importantly, reduced expression of corticotropin-releasing hormone (CRH) was observed in the hypothalamic paraventricular nucleus independently in two different strains of pDyn KO mice (Wittmann et al., 2009, Femenia et al., 2010), which could be reproduced in wild-type mice by a single injection of 10 mg/kg norBNI 48 h before testing (Wittmann et al., 2009). Moreover, reduced CRH expression in the central amygdala support an anxiolytic phenotype of pDyn KO mice. Inhibition of synaptic transmission and LTP in the basolateral amygdaloid nucleus via activation of KOP stimulation was recently reported (Huge et al., 2009). This nucleus plays a crucial role in anxiety control (Heilig et al., 1994), and CRF1-receptor-mediated activation of the Dyn/KOP system in this nucleus was shown to cause anxiety-like behaviour in mice (Bruchas et al., 2009).

While some information exists on the importance of testing conditions, little information is available on the influence of the genetic background or gender on the effects of pDyn KO on anxiety. Therefore, we backcrossed our pDyn KO mice onto the balb/c background over eight generations and investigated their anxiety-related phenotype and neurochemistry together with female pDyn KO mice on the C57Bl/6N background.

2. Material and Methods

2.1 Animals

The generation of pDyn KO mice has been described elsewhere (Loacker et al., 2007). Mice were backcrossed onto the balb/c and C57Bl/6N backgrounds over eight and 10 generations, respectively. For breeding and maintenance mice were group-housed with free access to food and water. Temperature was maintained at 23 °C with 60% humidity and a 12 h light-dark cycle (lights on 7 am to 7 pm). Mice were

tested at 3 to 6 months of age in all experiments. Age- and testing experience-matched wild-type littermates of the respective background were used as controls. Tests were performed in the fixed order: open-field, elevated plus maze, light-dark choice, forced swim, and tail suspension test. All procedures involving animals were approved by the Austrian Animal Experimentation Ethics Board in compliance with the European convention for the protection of vertebrate animals used for experimental and other scientific purposes ETS no. 123. Every effort was taken to minimize the number of animals used.

2.2 Behavioural testing

Unless stated otherwise, mice were group-housed before testing and transferred to the ante-room of the testing facility 24–72 h before the commencement of experiments, where they were given free access to food and water. The climate and light-dark cycle remained constant. Tests were performed between 9 am and 1 pm. Test settings were in accordance with the recommendations of EMPRESS (European Mouse Phenotyping Resource of Standardised Screens; http://empress.har.mrc.ac.uk) where available. Due to the high trait anxiety of balb/c mice, the illumination in all anxiety tests was reduced to 50 lux (C57Bl/6N: 150 in the open-field, 180 in the elevated plus maze and 400 lux in the light-dark test). All tests were video monitored and evaluated by an experimenter blinded to the genotype of the animals.

Open field: Open-field behaviour was tested over 10 min in a 50 x 50 cm flexfield box equipped with infrared rearing detection. Illumination was set to 150 lux for C57Bl/6N mice and reduced to 50 lux for balb/c mice according to their high trait anxiety level. Explorative behaviour was analysed using Video-Mot 2 equipment and

software (TSE-systems, Bad Homburg, Germany). Arenas were subdivided into the border (up to 8 cm from wall), centre (20 x 20 cm, i.e. 16% of total area), and intermediate area.

Elevated plus maze: Behaviour was tested over 5 min in an elevated plus maze 0.7 m above the ground, consisting of two closed and two open arms, each 50x5 cm in size. The test instrument was built from grey PVC, the height of the closed arm walls was 20 cm, while the open arms were enclosed by a rim of 3 mm. Illumination was set to 50 lux for balb/c and 180 lux for C57Bl/6N mice. Animals were placed in the centre, facing an open arm. Analysis of open and closed arm entries and time spent on the open arm was conducted automatically using Video-Mot 2 equipment and software. Entry into the open arm was recorded only when all four legs of the mouse left the neutral central area.

Light-dark test: Explorative behaviour in a (brightly) lit area (50 lux for balb/c; 400 lux for C57Bl/6N) was investigated by insertion of a black box into the open-field arena, covering one third of the space. One small field directly at the entrance to the black box was assigned as the transition zone. To reach the larger compartment assigned as the open area, the mouse had to completely leave the dark area. The number of entries, time spent and distance travelled in the light area were evaluated for C57Bl/6N mice. For balb/c mice entry or lack thereof into this field was recorded, as the number of entries was too low to statistically evaluate the time spent and distance travelled in this compartment in wild-type mice.

Forced swim test: This test was performed in a single 15-min trial. To increase stress we performed the test in water heated to 25 °C. Immobility, defined as no activity for at least 2 sec, was independently evaluated from video clips for the final 4 min of the test by two investigators blinded to the genotype of the animals.

Tail suspension test: The tip (c.a. 1.0–1.5 cm) of the tail of the mice was securely fastened with medical adhesive tape to a metallic surface. Mice were suspended for 6 min approximately 30 cm above the surface. The illumination on the floor of the table was about 100 lux. Immobility (lasting over 2 sec) and latency to the first immobile phase of the mice was evaluated.

2.3 In situ hybridization

In situ hybridization was carried out as described elsewhere (Wittmann et al., 2009). The following custom-synthesized (Microsynth, Balgach, Switzerland) DNA oligonucleotides complementary to mouse mRNAs were used: NPY: 5´-GAGGGTCAGTCCACACAGCCCCATTCGCTTGTTACCTAGCAT-3´; CRH: 5´-CCGATAATCTCCATCAGTTTCCTGTTGCTGTGAGCTTGCTGAGCT-3´.

To evaluate *in situ* hybridization, digitized images of the areas of interest were acquired from photo-emulsion dipped and superficially Nissl counter-stained brain slices at 200x magnification using a digital camera (Axiocam, Zeiss, Heidelberg, Germany) mounted onto a Zeiss Axiophot 2 microscope. The density of silver grains was evaluated by an experimentally blinded observer by outlining single neurons and measuring the percentage of area covered by silver grains (black grains in bright-field image, Image-J open source software available from imagej.nih.gov/ij/download/). The values of at least 30 single neurons were used to calculate the mean value for each animals. Thus, n represents the number of animals. Expression levels are given as mean percent of control.

2.4 Serum analyses

Animals were killed by decapitation between 12.00 and 14.00 hours while under deep CO_2 anaesthesia. Trunk blood was captured and serum was stored at -20 °C until

analysis. Corticosterone serum levels were measured using a commercial radioimmunoassay (MP Biochemicals, Orangeburg, NY) according to the manufacturer's instructions. Each serum was analysed in duplicate.

2.5 Statistical analysis

The Student´s t test was used for all comparisons of the two genotypes, with the exception of the analysis of the behaviour of male balb/c mice in the light-dark test. Here we applied a Chi^2 test to the distribution frequency of classification data. Comparison of more than two groups was carried out by One-Way-ANOVA, followed by Bonferroni´s multiple comparison test, using GraphPad Prism 5.0 software. P-values of < 0.05 were accepted as statistically significant. All data are given as mean ± SEM (n).

3. Results

3.1. Male balb/c mice

Open-field test: pDyn KO mice spent significantly more time (intermediate 11 ± 3.9 sec (15) vs. 47.7 ± 13.8 sec (12); centre 9.7 ± 3.0 sec (15) vs. 28.4 ± 7.3 sec (12); WT vs. pDyn KO, respectively) and travelled further (intermediate 3.2 ± 0.8% (15) vs. 10.0 ± 2.5% (12); centre 2.7 ± 0.5% (15) vs. 6.2 ± 1.4% (12); WT vs. pDyn KO, respectively) in the intermediate and central areas of the open field than WT mice. In addition, the number of entries into these areas was higher in pDyn KO mice (intermediate 10.8 ± 3.6 (15) vs. 34.2 ± 9.7 (12); centre 3.3 ± 0.9 (15) vs. 11.2 ± 3.8 (12); WT vs. pDyn KO, respectively). Due to high inter-individual differences statistical significance was only reached for the border and intermediate areas, and not for the centre (Fig. 1A).

Elevated plus maze: pDyn KO mice displayed more exploratory behaviour on the open arm than WT mice (Fig. 2). This is reflected by the significantly longer distance travelled (1.98 ± 0.47% (n=13) vs. 3.86 ± 0.67% (n=10); p=0.0277 WT vs. pDyn KO, respectively) and time spent on the open arms (6.2 ± 1.38 sec. (n=13) vs. 12.3 ± 2.53 sec. (n=10); p= 0.0353; WT vs. pDyn KO, respectively). The number of visits to both the closed (33.5 ± 4.83 (n=13) vs. 29.7 ± 6.14 (n=10); p= 0.6264; WT vs. pDyn KO, respectively) and open arms (7.7 ± 2.14 (n=13) vs. 9.7 ± 1.46 (n=10); p= 0.4879; WT vs. pDyn KO, respectively) did not appear to differ (Fig. 2).

Light-dark choice test: Balb/c mice are known to be very shy in the light-dark test. In fact, balb/c WT mice very rarely exit the dark compartment. Therefore, the usual evaluation of entries, time and distance in the light area could not be applied. However, we still observed a significantly higher number of animals entering the light area in the pDyn KO group (9 out of 15) as compared to WT (1 out of 20) animals (p = 0.0004; Chi^2 test).

Forced swim test: The forced swim test was performed in a single 15-min session. No marked differences in immobility were observed over the last 4 min of this test (68.1 ± 15.7 sec. (n=10) vs. 71.7 ± 23.6 sec. (n=7); WT vs. pDyn KO, respectively).

Tail suspension test: Statistically significant differences were observed for both the time spent immobile and the latency to the first immobile phase between WT and pDyn KO mice. While WT mice spent 15.8 ± 6.0 sec (n=9) immobile, pDyn KO mice were immobile for an average of 56.4 ± 10.8 sec (n=7) (p = 0.0038). The onset of the first immobile period was observed in WT mice after 239 ± 23.6 sec (n=9), while pDyn KO mice displayed the first immobile phase after 149 ± 117 sec (n=7; p=0.1411). One of the WT, but none of the pDyn KO mice climbed along their tail to the bar and had to be removed from evaluation.

Neurochemistry: *In situ* hybridization revealed a reduction of CRH mRNA in the central amygdala (100 ± 11.5 (n=4) vs. 78 ± 8.7 (n=5); p=0.0154; WT vs. pDyn KO, respectively) and in the paraventricular nucleus (100 ± 8.2 (n=5) vs. 82 ± 8.8 (n=5); p=0.0088; WT vs. pDyn KO, respectively) of balb/c pDyn KO mice. NPY mRNA was increased in neurons of the basolateral amygdala of pDyn KO mice (100 ± 6.5 (n=5) vs. 117 ± 3.3 (n=5); p=0.0008; WT vs. pDyn KO, respectively), but not in the central amygdaloid nucleus (100 ± 3.0 (n=4) vs. 99 ± 5.1 (n=5); p=0.7406; WT vs. pDyn KO, respectively). Data represent the mean percent of control ± SD (n).

Corticosterone serum levels: No marked differences in serum corticosterone levels were observed between pDyn KO and WT mice (73.3 ± 34.4 ng cort./ml (n=11) vs. 52.0 ± 23.3 ng cort./ml (n=11); p= 0.1046; WT vs. pDyn KO, respectively).

3.2 Female mice:

Female mice on C57Bl/6N and balb/c backgrounds were tested. However, valid data for all tests were only obtained from the C57Bl/6N mice. Balb/c mice were very shy, even at reduced light intensities, and therefore allowed only limited evaluation.

Open-field test: female mice on the balb/c background rarely left the border zone, and therefore statistical analysis of ambulation in the intermediate and central zones did not reveal significant differences. On the other hand, pDyn KO mice spent less time (588 ± 2.21 sec (n=12) vs. 546 ± 17.4 sec (n=8); p=0.0102; WT vs. pDyn KO, respectively) and travelled a shorter distance (95 ± 2.79% (n=12) vs. 87 ± 3.07% (n=8); p=0.0019; WT vs. pDyn KO, respectively) in the border zone, reflecting increased exploration of non-border areas (9.0 ± 1.92 sec (n=12) vs. 49.4 ± 16.7 sec (n=8); p=0.0065 and 4.9 ± 0.81% (n=12) vs. 13.1 ± 3.07% (n=8); p=0.0065; WT vs. pDyn KO, respectively; Fig. 1B). Female pDyn KO mice on the C57Bl/6N background

did not behave markedly differently from WT mice. However, the number of transitions between the border and intermediate area increased significantly by 35% (Fig. 1C).

Elevated plus maze: female pDyn KO mice on the C57Bl/6N background displayed significantly increased ambulation on the open arms compared to WT mice (Fig. 2). This was reflected by increased time (9.8 ± 1.93 sec (n=16) vs. 23.4 ± 3.91 sec (n=18); p=0.0051; WT vs. pDyn KO, respectively), increased distance (3.4 ± 0.79 m (n=16) vs. 9.6 ± 1.58 m (n=18); p=0.0019; WT vs. pDyn KO, respectively) and more entries (2.6 ± 0.43 (n=16) vs. 5.4 ± 0.86 (n=18); p=0.0079; WT vs. pDyn KO, respectively) onto the open arms (Fig. 2). Entries into closed arms did not appear to differ (11.5 ± 1.22 (n=16) vs. 13.9 ± 1.75 (n=18); p=0.2805; WT vs. pDyn KO, respectively). Balb/c mice rarely entered the open arms even in low light conditions, and therefore we evaluated the number of pokings into the open arm. This was done by introducing a small field (5 x5 cm) in the initial segments of the open arms. To enter this field, mice did not need to completely leave the neutral central zone and therefore it was less aversive than the open arms themselves. pDyn KO mice explored this “poking area” more frequently (5.4± 1.09 entries (n=14) vs. 22.1 ± 9.06 entries (n=8); p=0.0245; WT vs. pDyn KO, respectively) and for longer (3.7 ± 1.02 sec (n=14) vs. 19.3 ± 9.03 sec (n=8); p=0.0332; WT vs. pDyn KO, respectively) than wild-type mice.

Light-dark choice test: statistically significant differences between WT and pDynKO mice on the C57Bl/6N background were observed for the number of entries into the light area (3.4 ± 0.52 (n=16) vs. 6.1 ± 1.14 (n=18); p=0.0473; WT vs. pDyn KO, respectively), but not for the distance travelled in this area (298 ± 42.2 cm (n=16) vs. 344 ± 67.2 cm (n=18); p=0.5773; WT vs. pDyn KO, respectively). Only 4 out of 22

mice on the balb/c background entered the light area, and therefore, this test could not be evaluated.

Forced swim test: The forced swim test was performed in a single 15-min session. No marked differences in immobility were observed over the last 4 min of this test between WT and pDyn KO mice on the C57Bl/6N (89 ± 49.8 sec (n=17) vs. 78 ± 44.2 sec (n=19); p=0.4952; WT vs. pDyn KO, respectively) or on the balb/c (91 ± 31.8 sec (n=11) vs. 94 ± 43.0 sec (n=15); p=0.8319; WT vs. pDyn KO, respectively) background.

Tail suspension test: pDyn KO mice on the balb/c background spent a prolonged time immobile (43.2 ± 28.9 sec (n=10) vs 74.5 ± 40.8 sec (n=15); p=0.0474; WT and pDyn KO respecitively), while the delay to to onset of the first immobile phase did not reach statistical significance (127 ± 60,4 sec (n=10) vs 88 ± 60.7 sec (n=15); p=0.1266; WT and pDyn KO respecitively). No statistically significant differences were observed for either the time spent immobile or the latency to the first immobile phase between WT and pDyn KO mice on the C57Bl/6N background. While WT mice spent 73 ± 49.9 sec (n=22) immobile, pDyn KO mice were immobile for an average of 74 ± 43.1 sec (n=32) (p = 0.8954). The onset of the first immobile period was observed in WT mice after 81 ± 39.6 sec (n=22), while in pDyn KO mice the first immobile phase occurred after 78 ± 33.7 sec (n=32; p=0.7240).

Neurochemistry: *In situ* hybridization revealed a reduction in the levels of CRH mRNA in the central amygdala (100 ± 5.8% (n=10) vs. 74 ± 3.8% of control (n=10); p=0.0014; WT vs. pDyn KO, respectively) but not in the paraventricular nucleus (100 ± 3.2% (n=10) vs. 92 ± 4.2% of control (n=10); p=0.1472; WT vs. pDyn KO, respectively) of C57Bl/6N pDyn KO mice. Similar results were obtained from Balb/c mice (CeA: 100 ± 2.6% (n=6) vs. 85 ± 1.35% of control (n=4); p=0.0029; WT vs. pDyn KO, respectively; PVN: 100 ± 4.7% (n=6) vs. 96 ± 5.0% of control (n=4);

p=0.5874; WT vs. pDyn KO, respectively). Levels of NPY mRNA were unchanged in neurons of the basolateral amygdala of female mice on both backgrounds (97 ± 5.0% (n=10) and 93 ± 3.1% of control (n=6); C57Bl/6N and Balb/c, respectively)..

4. Discussion

Investigation of the involvement of dynorphin in anxiety control has produced conflicting results to date. The phenotypes observed in different pDyn KO mouse lines range from markedly anxiolytic (Wittmann et al., 2009) over paradigm-dependent anxiolytic (Bilkei-Gorzo et al., 2008, Bruchas et al., 2009) to anxiogenic (Femenia et al., 2010). These studies used different strains of pDyn KO mice, on partially different genetic backgrounds, and applied different setups for behavioural testing. To investigate the influence of gender and genetic background, we investigated female C57Bl/6N and both genders of balb/c pDyn KO mice under identical housing conditions and testing arenas as used previously (Wittmann et al., 2009).

Our present data confirm an anxiogenic role for prodynorphin-derived peptides. Thus balb/c pDyn KO mice displayed increased ambulation in all three anxiety-related tests, which is in line with results obtained for mice on the c57bl/6N background (Wittmann et al., 2009). However, the high trait anxiety level of balb/c mice was reflected in the results of a number of our tests. Thus, the increase in ambulation in aversive areas in the open-field test was significant only in the intermediate zone, which is less aversive than the centre. In contrast, C57Bl/6N mice entered the central area more readily than balb/c mice entered the intermediate zone, although the brightness of illumination was 50 lux for the balb/c and 150 lux for the C57Bl/6N mice. Evaluation of the elevated plus maze and light-dark choice test was also

adapted to this high trait anxiety. Wherever evaluation was feasible, pDyn KO mice were statistically significantly less anxious than wild-type mice.

In contrast to balb/c mice of both genders and male C57Bl/6N mice, female pDyn KO C57Bl/6N mice only constantly displayed increased ambulation in aversive zones in the elevated plus maze, and not in the open-field or light-dark choice tests. This may be due to the lower overall anxiety observed in female rodents (Steenbergen et al., 1990, Johnston and File, 1991), as the open-field test and the light-dark test are seen as less challenging than the elevated plus maze. This would be also consistent with the proposal of Bruchas et al. (2009), who proposed that the anxiolytic phenotype of pDyn KO mice is only detectable under highly challenging conditions.

In a recent study (Wittmann et al., 2009), we suggested that decreased expression of CRH in the central amygdala might represent an important feature of the anxiolytic phenotype of pDyn KO mice. This alteration in neurochemistry was observed in all mice strains and genders investigated, suggesting that down-regulation of CRH expression in the central amygdala is actually induced by the lack of pDyn.

In line with our recent results obtained from C57Bl/6N mice, male pDyn KO balb/c mice also displayed longer lasting and earlier immobility than wild-type mice in the tail suspension test. This phenotype was much weaker in female balb/c mice and was not observed in female pDyn KO C57Bl/6N. In contrast, no marked differences were observed in the forced swim test, regardless of genetic background or gender. It has been suggested that endogenous dynorphins play a role in stress-induced analgesia (Starec et al., 1996, McLaughlin et al., 2003). In fact, differences in immobility between the genotypes were abolished by analgesic treatment in our recent study (Wittmann et al., 2009). The gender differences in the tail suspension test may at least in part reflect the increased stress resistance of female mice. It is noteworthy

that potential influences of Dyn on the hypothalamic-pituitary-adrenal axis, as suggested by the reduction in CRH expression in the hypothalamic paraventricular nucleus in male pDyn KO mice (Wittmann et al., 2009), were confirmed in male balb/c but not female mice in the present study. However, decreased basal corticosterone serum levels did not reach statistical significance either in male or female balb/c or female C57Bl/6N mice. This is consistent with the study of Bilkei-Gorzo et al., who reported alterations in stress response duration, but not basal corticosterone serum levels in their pDyn KO mice (Bilkei-Gorzo et al., 2008).

Conclusions

Based on the behavioural and neurochemical data of our recent and present studies and the data obtained by other groups, we suggest that the influence of dynorphin on anxiety control predominantly depends on the relationship between the trait anxiety and stress resistance of mice and the level of aversion in the test setup. Thus, pDyn KO on the balb/c background with high trait anxiety displayed anxiolytic-like phenotypes in all tests and both genders. In contrast, pDyn KO on the less anxious C57Bl/6N background displayed gender differences. Female mice (which display lower overall anxiety than males) only appeared less anxious in the elevated plus maze test. It is noteworthy that male pDyn KO on this background only displayed increased entry into the light area of the light-dark choice test at 400, and not at 150 lux (Wittmann et al., 2009). In line with this, Bruchas et al. (2009) reported that pDyn KO mice displayed a clear anxiolytic phenotype in the elevated plus maze at high, but not low, illumination levels. Under low brightness (40 lux) and in a small open-field arena (25 x 25 cm), male pDyn KO mice on the C57Bl/6 background showed no anxiolytic-like behaviour (Femenia et al., 2010).

Acknowledgements

This work was supported by the Austrian Science Fund (FWF; P20107-B05). We wish to thank Inge Kapeller and Christa Stichlberger for excellent technical support.

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Legends to figures:

Figure 1: Ambulation in the open field by pDyn KO (shaded bars) and WT (white bars) mice was measured for 10 min. Time spent (upper row), distance travelled (mid row) and number of entries (lower row) into the different zones are given for male (left column) and female (mid column) balb/c and female C57Bl/6N (right column) mice. Data represent mean ± SD of the number of animals indicated in the bars. Statistical analysis was performed by one-way ANOVA with Bonferroni´s multiple comparison test for comparison of the two genotypes in the different zones of the open-field test. * … $p < 0.05$; ** … $p < 0.01$; *** … $p < 0.001$.

Figure 2: Ambulation on the elevated plus maze by pDyn KO (shaded bars) and WT (white bars) mice was measured for 5 min. Time spent (upper row), distance travelled (mid row) and percentage of entries (lower row) onto the open arms are given for male balb/c (left column) and female C57Bl/6N (right column) mice. Data represent

mean ± SD of the number of animals indicated in the bars. Student´s t test was used to compare the two genotypes. * … $p < 0.05$.

Figure

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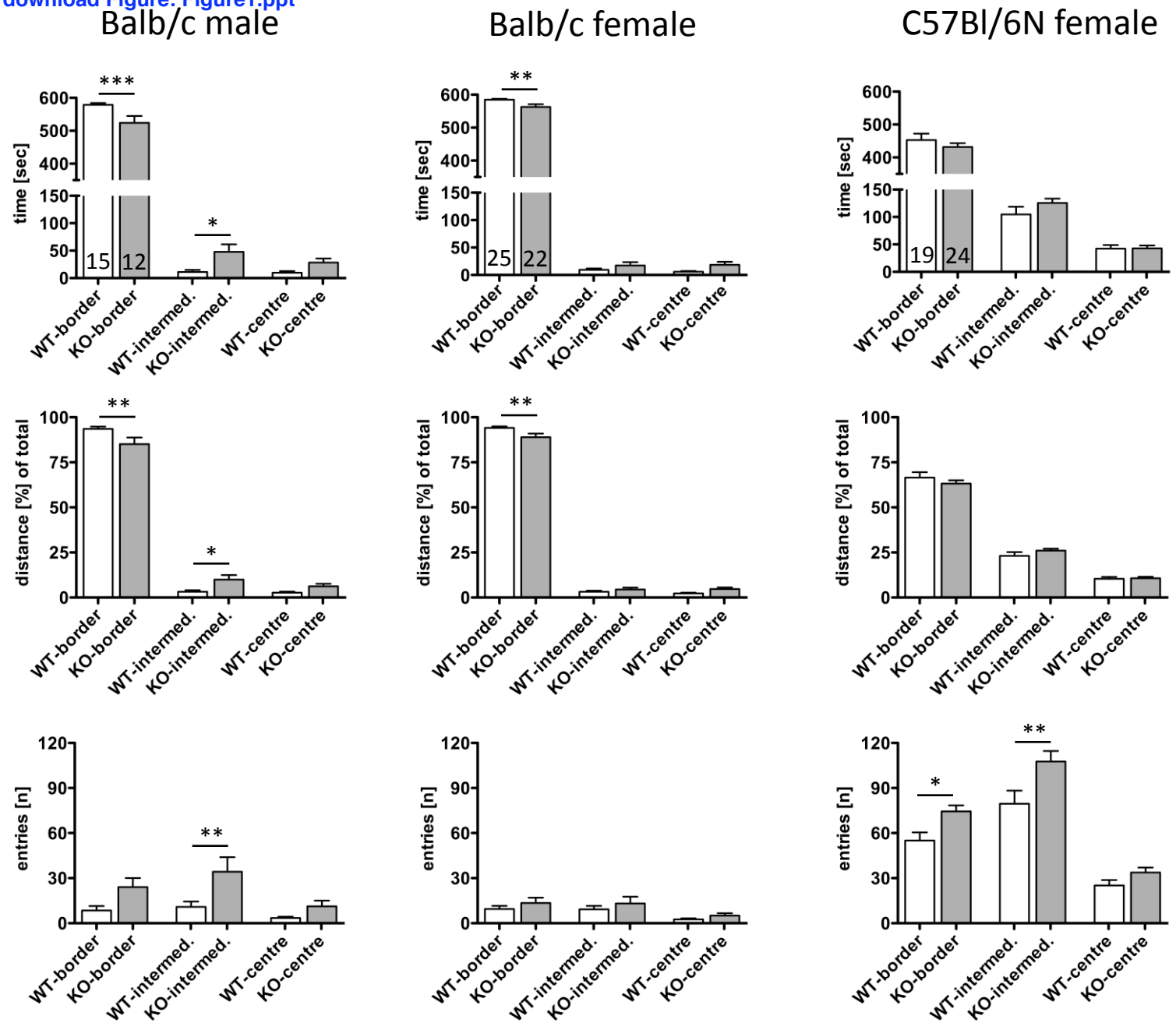

Figure
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