Neuropsychopharmacology (2009) 34, 775–785

www.neuropsychopharmacology.org

Prodynorphin-Derived Peptides Are Critical Modulators of Anxiety and Regulate Neurochemistry and Corticosterone

Walter Wittmann[1,4], Eduard Schunk[1], Iris Rosskothen[1], Stefano Gaburro[2], Nicolas Singewald[2], Herbert Herzog[3] and Christoph Schwarzer*[,1]

[1]Department of Pharmacology, Innsbruck Medical University, Innsbruck, Austria; [2]Department of Pharmacology and Toxicology, Institute of Pharmacy and Center for Molecular Biosciences Innsbruck, University of Innsbruck, Innsbruck, Austria; [3]Neuroscience Program, Garvan Institute of Medical Research, Darlinghurst, NSW, Australia

Stress and anxiety are mainly regulated by amygdala and hypothalamic circuitries involving several neurotransmitter systems and providing physiological responses to peripheral organs via the hypothalamic–pituitary–adrenal axis and other pathways. The role of endogenous opioid peptides in this process is largely unknown. Here we show for the first time that anxiolytic parameters of explorative behavior in mice lacking prodynorphin were increased 2–4-fold in the open field, the elevated plus maze and the light–dark test. Consistent with this, treatment of wild-type mice with selective κ-opioid receptor antagonists GNTI or norbinaltorphimine showed the same effects. Furthermore, treatment of prodynorphin knockout animals with U-50488H, a selective κ-opioid receptor agonist, fully reversed their anxiolytic phenotype. These behavioral data are supported by an approximal 30% reduction in corticotropin-releasing hormone (CRH) mRNA expression in the hypothalamic paraventricular nucleus and central amygdala and an accompanying 30–40% decrease in corticosterone serum levels in prodynorphin knockout mice. Although stress-induced increases in corticosterone levels were attenuated in prodynorphin knockout mice, they were associated with minor increases in depression-like behavior in the tail suspension and forced swim tests. Taken together, our data suggest a pronounced impact of endogenous prodynorphin-derived peptides on anxiety, but not stress coping ability and that these effects are mediated via κ-opioid receptors. The delay in the behavioral response to κ-opioid receptor agonists and antagonist treatment suggests an indirect control level for the action of dynorphin, probably by modulating the expression of CRH or neuropeptide Y, and subsequently influencing behavior.
Neuropsychopharmacology (2009) **34,** 775–785; doi:10.1038/npp.2008.142; published online 17 September 2008

Keywords: amygdala; κ-opioid receptor; hypothalamic–pituitary–adrenal axis; stress; prodynorphin knockout; paraventricular nucleus

INTRODUCTION

Cerebral control of stress and anxiety depends on several brain areas including the amygdala and the hypothalamus. These nuclei use the hypothalamic–pituitary–adrenal (HPA) axis as common output pathway, regulating the physiological response via ACTH and corticosterone release. Besides the classical transmitter systems such as serotonin or catecholamines, several neuropeptide systems are also considered to be involved in generating symptoms of depression, anxiety, and stress. Corticotropin-releasing hormone (CRH) is thought to be a key player, with potential influence of other peptides such as neuropeptide Y (NPY), cocaine and amphetamine-regulated transcript (CART) and substance P. Intraventricular injection of CRH or overexpression of CRH is anxiogenic in mice (Stenzel-Poore *et al*, 1996). Inactivation of CRH receptor 1 reduces anxiety (Smith *et al*, 1998; Timpl *et al*, 1998). In contrast, deletion of CRH receptor 2 is anxiogenic without affecting basal HPA axis activity (Bale *et al*, 2000; Coste *et al*, 2000; Kishimoto *et al*, 2000). NPY is mostly reported as the counterpart of CRH, producing opposing effects and thereby potentially balancing the emotional state (for reviews see Heilig *et al*, 1994; Sajdyk *et al*, 2004). CART and substance P both elicit anxiogenic effects (for reviews see Ebner and Singewald, 2006; Stanek, 2006). One of the less understood players in emotional control is the opioid system. This depends partially on the complexity of the system comprised of a variety of neuroactive peptides derived from three independent gene products that all act on three different classical opioid receptors. In addition, also direct effects of opioids on NMDA receptors were proposed (Wollemann and Benyhe, 2004). Contradictive data from pharmacological studies may result from this complexity.

There is evidence that dynorphin modulates emotional control. Tsuda *et al* (1996) proposed an involvement of KOR in the anxiolytic action of diazepam. Chronic pain

*Correspondence: Dr C Schwarzer, Department of Pharmacology, Innsbruck Medical University, Peter-Mayr-Str. 1a, A-6020 Innsbruck, Austria, Tel: +43 512 9003 71205, Fax: +43 512 9003 73200, E-mail: schwarzer.christoph@i-med.ac.at
[4]Current address: Department of Anatomy and Structural Biology, University of Otago, Dunedin, New Zealand

induces anxiety in mice, which is associated with increased KOR-specific binding in the amygdala. On the other hand, Narita *et al* (2006) showed in the same study marked anxiolytic effects of KOR agonists. Also big dynorphin (a precursor peptide consisting of dyn A and B) was suggested as anxiolytic peptide (Kuzmin *et al*, 2006). In contrast Knoll *et al* (2007) proposed anxiolytic effects of KOR antagonists in rats. Dynorphins are released during stress and prodynorphin deletion influences stress-induced behavior (McLaughlin *et al*, 2003) and the κ-opioid receptor was made responsible for the dysphoric component of stress (Land *et al*, 2008). Very recently, Bilkei-Gorzo *et al* (2008) reported increased startle–response and somewhat reduced exploratory behavior on the zero-maze in dynorphin knockout mice, suggesting an anxiogenic phenotype. This was opposed by reduced stress-induced hyperthermia and unchanged explorative behavior in the light–dark test. In the same study Bilkei-Gorzo *et al* (2008) report control of hormonal stress reactivity by endogenous enkephalins and dynorphins, but suggested enkephalin as most important opioid peptide in anxiety control.

However, we still know only very little about the impact of endogenous dynorphin on emotional control. The distribution of prodynorphin in the brain overlaps with areas involved in emotional control (Lin *et al*, 2006; Marchant *et al*, 2007). In the present study, we undertook combined neurobiochemical, hormonal and behavioral investigations on prodynorphin knockout mice (dyn$^{(-/-)}$) to determine and clarify the role of endogenous prodynorphin-derived peptides in anxiety and stress behavior.

MATERIALS AND METHODS

Animals

The generation of dyn$^{(-/-)}$ mice was described recently (Loacker *et al*, 2007). Mice were backcrossed onto the C57Bl/6N background over 8–10 generations and wild-type littermates were used as controls. Partially commercial C57Bl/6N (Charles River, Sulzfeld, Germany) were used in pharmacological experiments. The data obtained from these animals were indistinguishable from wild-type mice of our breeding. For breeding and maintenance mice were group housed with free access to food and water. Temperature was fixed to 23°C and 60% humidity with a 12 h light–dark cycle (lights on 0700–1900 hours). Age and testing experience matched male mice at 3–8 months age were tested in all experiments. 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.

Physical Exam

To investigate the general constitution we performed a systematic basic characterization of the prodynorphin knockout mice in regard to their physical conditions, sensory abilities, and motor functions. This exam (Crawley, 1999; Karl *et al*, 2003) comprised the wire-hang test (1 min 30 cm elevated) to check muscular strength and RotaRod (20 s 0–10 r.p.m., subsequently increasing over 60 s from 10 to 30 r.p.m.) for motor coordination and balance. Visual cliff and acoustic response tests were performed for sensory abilities. Basic behavior was evaluated in an empty cage for 1 min, observing running, jumping, circling, freezing, rearing, defecation, urination, and grooming. Basal activity and circadian rhythm of wild-type and dyn$^{(-/-)}$ mice was monitored in their home cages using the Infra-Mot system (TSE, Bad Homburg, Germany). Animals were observed for two dark–light cycles after an initial accommodation phase of several hours.

Behavioral Testing

If not stated differently, mice were single housed 7 days before testing and transferred to the testing facility 24 h before commencement of experiments. Tests were performed between 0900 and 1300 hours. All tests were video monitored and evaluated by an experimenter blinded to the genotype of animals.

Open field. Open field behavior was tested over 10 min in a 50×50 cm flexfield box equipped with infrared rearing detection. Illumination was set to 150 lux. Animals were video monitored and their explorative behavior was analyzed using the Video-Mot 2 equipment and software (TSE-systems, Bad Homburg, Germany). Arenas were subdivided into border (up to 8 cm from wall), center (20×20 cm, ie 16% of total area), and intermediate area according to the recommendations of EMPRESS (European Mouse Phenotyping Resource of Standardised Screens; http://empress.har.mrc.ac.uk).

Elevated plus maze. Behavior was tested over 5 min on an elevated plus maze 1 m above ground consisting of two closed and two open arms, each 50×5 cm in size. The test instrument was build from gray PVC, the height of closed arm walls was 20 cm. Illumination was set to 180 lux. Animals were placed in the center, facing to an open arm. Analysis of open and closed arm entries and time on open arm was automatically done with Video-Mot 2 equipment and software.

Light–dark test. Explorative behavior in a brightly lit area (150 and 400 lux) was investigated by insertion of a black box into the open field arena, covering one third of the space. Time spent and distance traveled was measured over a 10 min period in the open area. One small field directly at the entrance to the black box was assigned as transition zone. To reach the larger compartment assigned as open area, the mouse had to leave the dark area completely.

Forced swim test. Mice were tested for an initial 15 min trial and the next day in four repeats of 6 min trials. To increase stress we performed the test in 25°C water with 2 h interval for the second day trials. Immobility, defined as no activity for at least 2 s was evaluated from video clips for the final 4 min of each trial independently by two investigators blinded to the genotype of animals.

Tail suspension test. The tip (~1.0–1.5 cm) of the tail of 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 100 lux. Immobility (lasting over 2 s) and latency to the first immobile phase of the mice was evaluated. This test was performed in accordance to the recommendations of EMPRESS, with some adaptations for video monitoring instead of automatic evaluation.

Stress-induced hyperthermia. A temperature probe, lubricated with glycerol, was inserted into the rectum of the mouse for a depth of up to 2 cm. The temperature probe remained in the animal till a stable temperature was reached (maximum 10 s). Temperature measurement was repeated after 10 min and the rise in temperature between the first and second measurement was considered as stress-induced hyperthermia (Olivier *et al*, 2003).

Flinch test. Animals were placed in a chamber with a floor of metal bars and subjected to 1 s shocks of gradually increasing amperage starting from 0.05 mA and successive increment of 0.05 mA with 30 s inter-shock intervals. Mice were scored for their first visible response to the shock (flinch—1 paw lifted), their first severe motor response (run or jump), and their first vocalized distress (scream).

Opioid Receptor Pharmacology

The KOR antagonists norBinaltorphimine (norBNI) and 5′-guanidinyl-17-(cyclopropylmethyl)-6,7-dehydro-4,5α-epoxy-3,14-dihydroxy-6,7–2′,3′-indolomorphinan dihydrochloride (GNTI) and the selective KOR agonist *trans*-(–)-3,4-dichloro-*N*-methyl-*N*-(2-(1-pyrrolidinyl)cyclohexyl) benzeneacetamide hydrochloride (U-50488H) were purchased from Tocris Cookson. GNTI shows higher KOR selectivity and antagonist potency than norBNI (Jones and Portoghese, 2000). All drugs were dissolved in saline and pH was adjusted to 7.2. NorBNI (10 mg/kg) and U-50488H (2.5 mg/kg) were given intraperitoneally (i.p.) at different intervals. GNTI was given intracisternally (3 nmol in 3 μl) 20 h before testing under mild sevofluoran anesthesia. Drug doses and application times were chosen according to recent studies in mice (Jewett *et al*, 2001; Solbrig *et al*, 2006).

In Situ Hybridization

For *in situ* hybridization the following custom synthesized (Microsynth, Balgach, Switzerland) DNA oligonucleotides complementary to mouse mRNAs were used: NPY: 5′-GAGGGTCAGTCCACACAGCCCCATTCGCTTGTTACCTAG CAT-3′; CRH: 5′-CCGATAATCTCCATCAGTTTCCTGTTGC TGTGAGCTTGCTGAGCT-3′; Orexin: 5′-GAATCGTCTTTA TTGCCATTTACCAAGAGACTGACAGCGGCGAGC-3′; preprotachikinin A (PPTA): 5′-ATCGTTGGCATCGATTTCCTC TGCAAACAGTTGAGTGGAAACGAG-3′; CART: 5′-TCCTT CTCGTGGGACGCATCATCCACGGCAGAGTAGATGTCCAG G-3′; proopiomelanocortin (POMC): 5′-TGGCTGCTCTCC AGGCACCAGCTCCACACATCTATGGAGG-3′; agouti-related protein (AgRP): 5′-AGCTTGCGGCAGTAGCAAAAGG CATTGAAGAAGCGGCAGTAGCAC-3′; thyrotropin-releasing hormone (TRH): 5′-AACCTTACTCCTCCAGAGGTTCC CTGACCCAGGCTTCCAGTTGTG-3′; tyrosin-hydroxylase (TH): 5′-TGGATACGAGAGGCATAGTTCCTGAGCTTGTCC TTGGCATCACTG-3′; tryptophan-hydroxylase 2 (TPH2): 5′-TTCGACTTCAGAACTTCTTCGTCGGGACCTCCTGGATTC GATATG-3′: arginin-vasopressin (Avp): 5′-GGAGACACTG TCTCAGCTCCATGTCAGAGATGGCCCTCTT-3.

Oligonucleotides (10 pmol) were labeled with [^{35}S]-dATP (1300 Ci/mmol, NEN, Vienna, Austria) by reaction with terminal deoxynucleotidyltransferase (Roche, Mannheim, Germany). Incubations with different probes were performed on series of matching sections from knockout and wild-type mice. Incubation lasted for 16–18 h (52°C). Sections were washed four times with 1–2 × SSC (58°C), dried, and exposed to Kodak MR films (Amersham, Buckinghamshire, UK) for 2 days or 1 week, depending on the intensity of the signal. Subsequently sections were dipped into radiation-sensitive emulsion (Kodak NTB, Integra Biosciences, Fernwald, Germany) and exposed for another 4–20 days. Matching sections from the same brain level of knockout and control mice were analyzed together, as described previously (Schwarzer *et al*, 1998).

For evaluation of *in situ* hybridization, digitized images of the areas of interest were acquired from photo emulsion dipped and superficially Nissl counter-stained brain slices at 200 × magnification using a digital camera (Axiocam, Zeiss, Heidelberg, Germany) mounted onto a Zeiss Axiophot 2 microscope (Sainsbury *et al*, 2002). Silver-grain densities were evaluated by an experimentally blinded observer outlining single neurons and measuring percentage of area covered by silver grains (black grains in bright-field image, Image-J software). Percentage of silver-grain area compared to total area calculated for single neurons was averaged. Expression levels are given as mean percent of control.

Quantification of *in situ* hybridization signals on autoradiography films over distinct cell layers or entire small forebrain nuclei were performed as controls and yielded essentially the same alterations as measured from dipped sections (data not shown).

Serum Analyses

Animals were killed between 1200 and 1400 hours under deep CO_2 anesthesia by decapitation. Trunk blood was captured and serum was stored at −20°C until analyzed. Determination of corticosterone serum levels was done with a commercial radioimmunoassay (MP Biochemicals, Orangeburg, NY) according to manufacturer's suggestions. Each serum was analyzed in duplicates.

Analgesia

To disclose the influence of altered pain sensitivity in dyn$^{(-/-)}$ mice, in some experiments animals were injected with meloxicam (2 mg/kg; 30 min pretesting, i.p.). Meloxicam was chosen because it did neither display central nor locomotor effects at the dose applied (Engelhardt *et al*, 1996).

Statistical Analysis

For all comparisons of the two genotypes the Student's *t*-test was used. Comparison of more than two groups was done by one-way ANOVA, followed by Bonferroni *post hoc* test,

applying GraphPad Prism 4.0 software. *P*-values of <0.05 were accepted as statistically significant. For the large number of comparisons in *in situ* hybridization analysis we applied the Holm's step-down method (Holm, 1979) to adjust for multiple testing. All comparisons regarding mRNA levels were included. All data are given as mean ± SEM (*n*).

RESULTS

Physical Exam and Basic Behavior

No obvious differences in fertility, body weight, sensory functions or fur, and whiskers condition were observed between wild-type and dyn$^{(-/-)}$ animals. Basic behavior was normal, lacking circling, excessive running, or jumping behavior. Noteworthy, in the empty cage, dyn$^{(-/-)}$ mice differed significantly in the number of freezing periods (0.77 ± 0.18 (25); $p = 0.0306$) compared to dyn$^{(+/+)}$ (1.47 ± 0.29 (17)) defined by complete immobility including the face (Karl *et al*, 2003)) and defecation frequency (0.65 ± 0.15 (17) in dyn$^{(+/+)}$ *vs* 0.27 ± 0.09 (25) in dyn$^{(-/-)}$; $p = 0.0252$). In addition, in the hang wire test the duration spent on the wire was significantly longer in dyn$^{(+/+)}$ (57.4 ± 2.65 (17) *vs* 45.0 ± 3.23 (25 s) in dyn$^{(-/-)}$, $p = 0.0088$; Supplementary Figure S1A). Home-cage activity was indistinguishable between the two genotypes. Animals displayed normal behavior, showing highest activity in the first hours of the dark cycle and lowest activity in the light cycle (Supplementary Figure S1B). Arbitrary activity units counted over 48 h also did not differ (dyn$^{(+/+)}$ 25455 ± 2250 (8); dyn$^{(-/-)}$ 22424 ± 2574 (8)).

Behavioral Testing

To determine explorative behavior 14 dyn$^{(+/+)}$ *vs* 14 dyn$^{(-/-)}$ mice were tested in the open field test (Figure 1a). Dyn$^{(-/-)}$ mice showed significantly increased ambulation in both, the center and the intermediate zone of the open field. Overall motor activity was increased in dyn$^{(-/-)}$ mice (3.28 ± 0.18 m in WT *vs* 4.28 ± 0.41 m in KO; $p = 0.0377$). This phenotype could be fully reversed by treatment with the specific KOR agonist U-50488H (2.5 mg/kg; 2 days and 24 h before testing; Figure 1b). A single injection of 2.5 mg/kg U-50488 30 min before testing did not attenuate the difference between the genotypes (Figure 1b). Blockade of KOR receptors by 3 nmol GNTI (20 h) or 10 mg/kg norBNI (48 h) in wild-type mice resulted in an open field behavior comparable to that of dyn$^{(-/-)}$ animals. Mice tested 1 h after norBNI were indistinguishable form saline-treated mice (Figure 1c).

In the elevated plus maze test, dyn$^{(-/-)}$ mice spent over 3.5-fold more time on the open arms ($2.13 \pm 0.57\%$ (17) in dyn$^{(+/+)}$ *vs* $8.28 \pm 1.48\%$ (25) in dyn$^{(-/-)}$; $p = 0.0020$; data represent % of total time spent in open arm) during twice as much visits (19.4 ± 2.76 (17) in dyn$^{(+/+)}$ *vs* 30.6 ± 2.72 (25) in dyn$^{(-/-)}$; $p = 0.0085$; data represent % of total entries into open arm) compared to wild-type littermates (Figure 2a). In contrast, the number of closed arm entries did not differ significantly between genotypes, suggesting unchanged locomotor activity. Consistent with our observations in the open field, 2-day treatment with the KOR-specific agonist U-50488H reversed this phenotype (Figure 2b). The phenotype of dyn$^{(-/-)}$ mice was also mimicked by long-lasting blockade of KOR receptors applying GNTI or norBNI in wild-type mice (Figure 2c).

At both illumination levels tested in the light–dark test, 150 and 400 lux, dyn$^{(-/-)}$ mice spent significantly more time in the lit area than dyn$^{(+/+)}$ (160 ± 16.8 (12) *vs* 288 ± 28.1 s (14) and 113 ± 13.0 (19) *vs* 195 ± 25.3 s (16), respectively; Figure 3)) and traveled a longer distance than wild-type littermates (0.9 ± 0.11 m (12) *vs* 2.1 ± 0.21 m (14) and 0.8 ± 0.13 m (19) *vs* 1.3 ± 0.17 m (16), respectively). Interestingly, the number of entries into the lit compartment differed only at 400 lux light intensity (13.2 ± 1.8 (19) dyn$^{(+/+)}$ *vs* 21.1 ± 2.75 (16) dyn$^{(-/-)}$; Figure 3).

In the flinch test dyn$^{(-/-)}$ mice responded later to the electrical footshocks. Flinch response was detected at about 20% higher amperage (0.23 ± 0.012 mA (11) *vs* 0.28 ± 0.018 mA (11); $p = 0.0461$ in dyn$^{(+/+)}$ and dyn$^{(-/-)}$, respectively) jump/run (0.27 ± 0.024 mA (11) *vs* 0.35 ± 0.035 mA (11); $p = 0.0676$ in dyn$^{(+/+)}$ and dyn$^{(-/-)}$, respectively), and scream responses (0.34 ± 0.037 mA (11) *vs* 0.46 ± 0.027 mA (11); $p = 0.0172$ in dyn$^{(+/+)}$ and dyn$^{(-/-)}$, respectively) at approximately 30% higher amperage as in dyn$^{(+/+)}$ controls.

Significant differences were also observed between the two genotypes in depression-like behavior and stress–response-related tests. In the repeated modified Porsolt swim test, both groups displayed an increase of immobility from initially 115 to finally 180 s in the last test (Figure 4a). However, in dyn$^{(-/-)}$ mice this increase occurred earlier, resulting in significant differences in immobility as well as active struggling in the third test (Figure 4a). In unstressed animals, immobility in the tail suspension test was increased in dyn$^{(-/-)}$ mice (131 ± 14.3 s (11); $p = 0.0269$; Figure 4b) as compared to wild-type animals (86.7 ± 12.3 s (14)). This was accompanied by a reduced latency to the first immobility phase (67.7 ± 7.90 s (11) in dyn$^{(-/-)}$ *vs* 101 ± 10.2 s (14) in dyn$^{(+/+)}$ mice; $p = 0.0222$). This difference was completely abolished in mice tested 1 day after the repeated forced swim test (135 ± 8.54 s (9) for dyn$^{(-/-)}$; 132 ± 13.7 s (9) for WT). Importantly, dyn$^{(-/-)}$ mice did not respond with any increase in immobility on the prestressing. Prodynorphin deficient mice display test-dependent mild hyperalgesia (Wang *et al*, 2001), but respond later to electrical footshocks (see above). In addition, they lack stress-induced analgesia (McLaughlin *et al*, 2003). Hyperalgesia may influence the behavior of mice in the tail suspension test through pain induced by hanging on the tail tip. Therefore, we repeated this test 40 min after i.p. injection of 2 mg/kg meloxicam. Under these conditions, dyn$^{(-/-)}$ mice (117 ± 10.2 s (12); $p = 0.0410$ *vs* wild type) displayed significantly less immobility than dyn$^{(+/+)}$ mice (148 ± 10.0 s (14); Figure 4b).

Basal body temperature and stress-induced hyperthermia induced by insertion of an anal probe did not differ significantly between non prestressed animals of the two genotypes (Figure 4c).

Central Alterations in Response to Prodynorphin Gene Deletion

To clarify the role of dynorphin peptides in the central regulation of anxiety, we investigated changes in expression

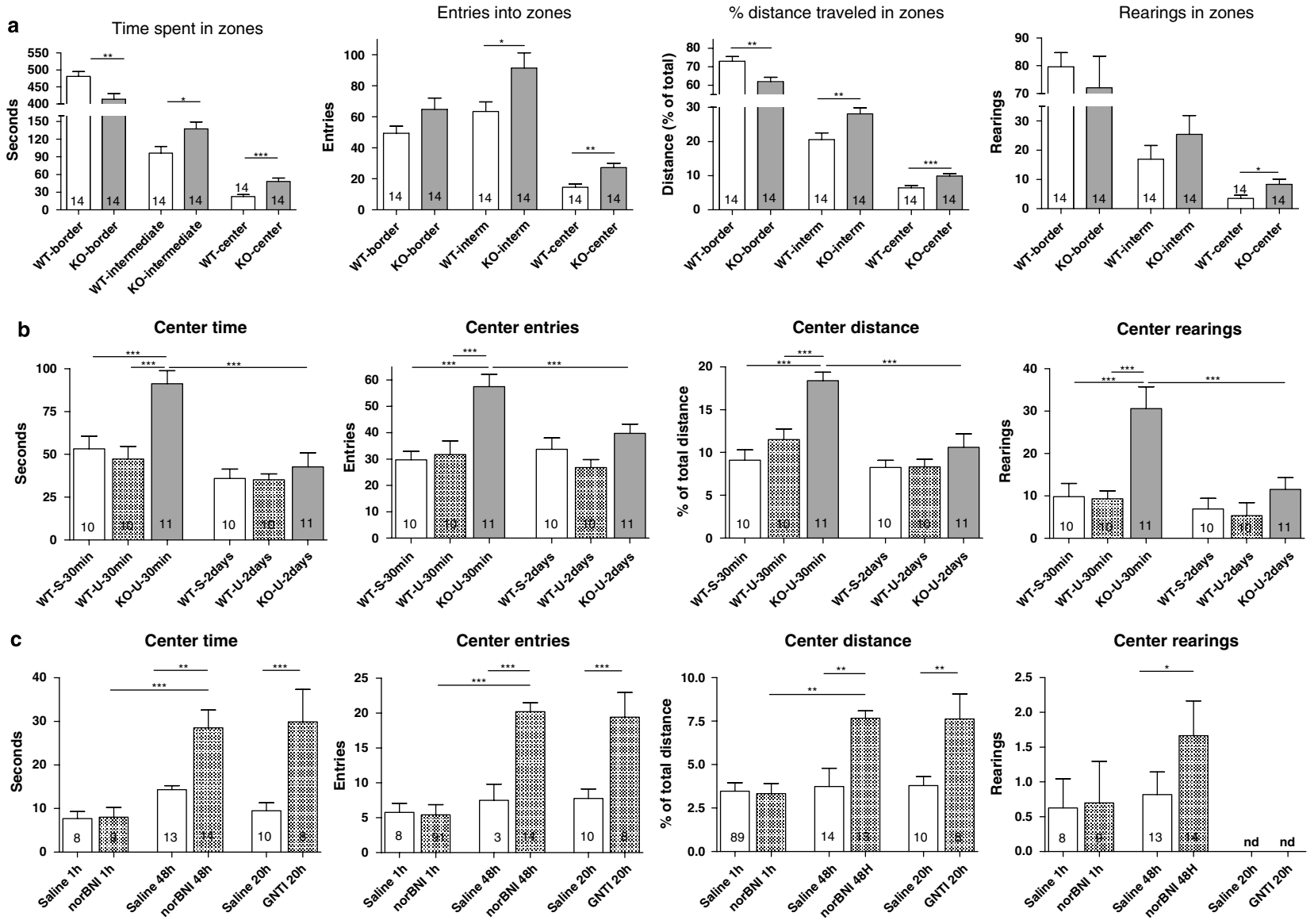

Figure 1 Ambulation in the open field was assessed over 10 min for dyn$^{(+/+)}$ and dyn$^{(-/-)}$ mice. Dyn$^{(-/-)}$ mice more often entered the intermediate and central zone and spent significantly more time and traveled longer distances in these compartments. In contrast, dyn$^{(+/+)}$ mice preferentially remained in the border zone (a). Intraperitoneal injection of the KOR-specific agonist U-50488H did not affect the behavior of mice 30 min after treatment but reversed the anxiolytic phenotype of dyn$^{(-/-)}$ mice after 2 days (b). In line with this, treatment of dyn$^{(+/+)}$ mice with specific KOR antagonists norBNI (10 mg/kg, i.p.) and GNTI (3 nmol, i.c.) mimicked the phenotype of dyn$^{(-/-)}$ mice after 48 and 20 h, respectively (c).
*$p<0.05$; **$p<0.01$; ***$p<0.001$ *vs* respective control.

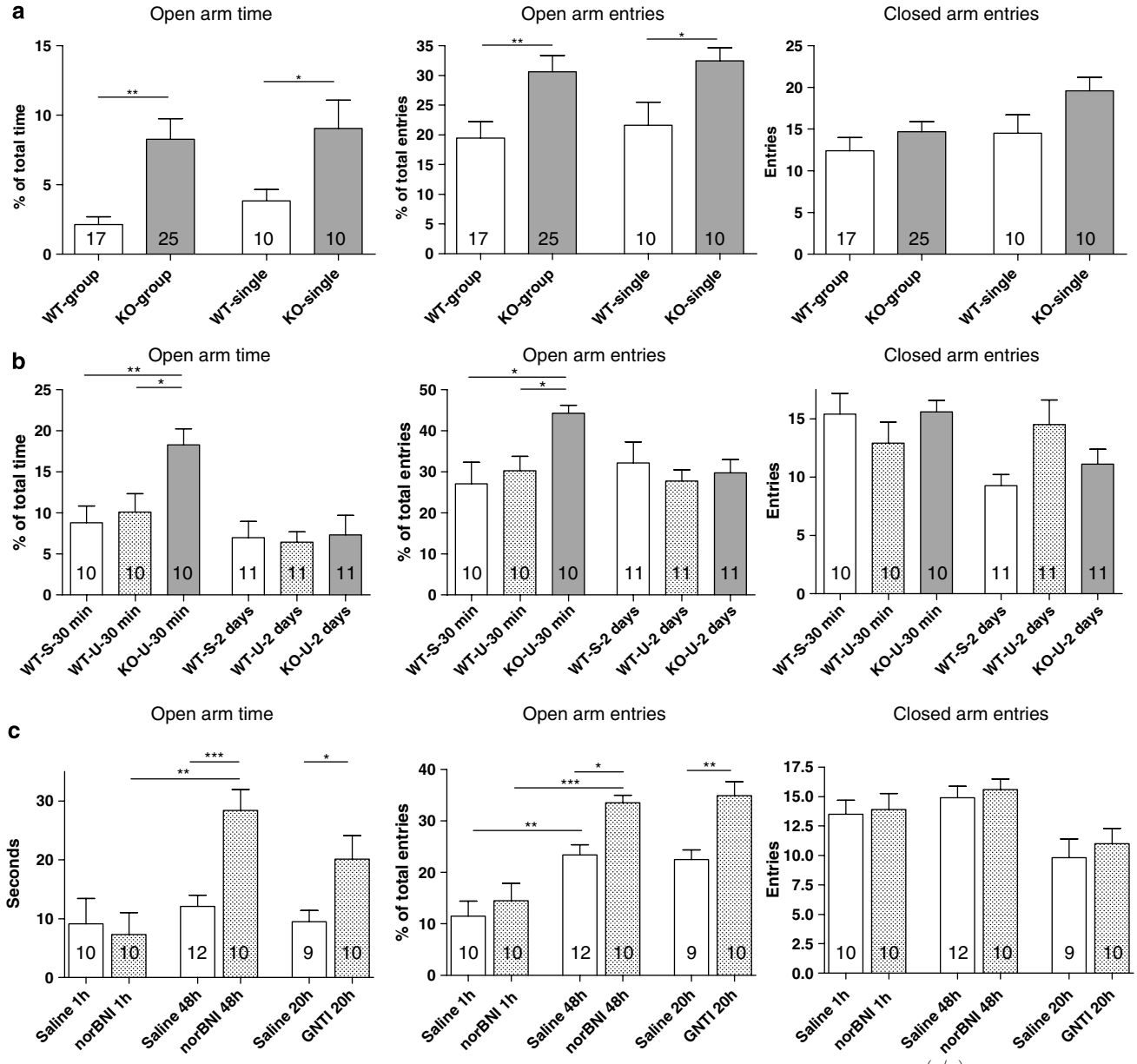

Figure 2 Explorative behavior was tested in group- and single-housed mice (a). Independent of housing conditions, dyn$^{(-/-)}$ mice more often entered the open arms and spent significantly more time on the open arms than dyn$^{(+/+)}$ mice. No differences were observed in the number of closed arm entries, suggesting unchanged motor activity (a). Intraperitoneal injection of the KOR-specific agonist U-50488H did not affect the behavior of mice 30 min after treatment but reversed the anxiolytic phenotype of dyn$^{(-/-)}$ mice after 2 days (b). In line with this, treatment of dyn$^{(+/+)}$ mice with specific KOR antagonists norBNI (10 mg/kg, i.p.) and GNTI (3 nmol, i.c.) mimicked the phenotype of dyn$^{(-/-)}$ mice after 48 and 20 h, respectively (c).
*$p<0.05$; **$p<0.01$; ***$p<0.001$ *vs* respective control.

levels of neurotransmitters known to involved in anxiety and emotional control (Table 1). Thus, in the central amygdala, CART and CRH mRNAs were decreased in dyn$^{(-/-)}$ mice (by 23 and 33%, respectively), whereas neurokinin B mRNA was increased by 15%. In the paraventricular nucleus of the hypothalamus CRH and TRH, mRNAs were decreased by 27 and 24% (Table 1), respectively. In contrast PPTA mRNA was increased by 35%. No changes of TRH expression were observed in the lateral hypothalamic area. NPY and CART mRNAs were increased in the medial amygdala of dyn$^{(-/-)}$ mice by 45 and 19% (Table 1), respectively. In this nucleus PPTA and chromogranin B mRNAs were decreased by about 13%. In the arcuate nucleus, AgRP and NPY mRNAs were decreased (by 38 and 26%, respectively), in parallel, CART and POMC mRNAs were increased by 18 (nonsignificant) and 21% (Table 1), respectively. In this nucleus, a strong increase (by 77%) of NKB mRNA expression was measured. Chromogranin B mRNA was unchanged in hypothalamic nuclei, but somewhat reduced in the medial amygdala. Although most minor changes lost statistical significance when correction for multiple comparisons was performed, the functionally important changes of NPY and CRH in the amygdala and paraventricular nucleus of the hypothalamus were solid (shaded fields in Table 1).

Noteworthy, orexin mRNA, which is colocalized with dynorphin mRNA in neurons of the lateral hypothalamic area was unchanged (Table 1). Significant changes in the

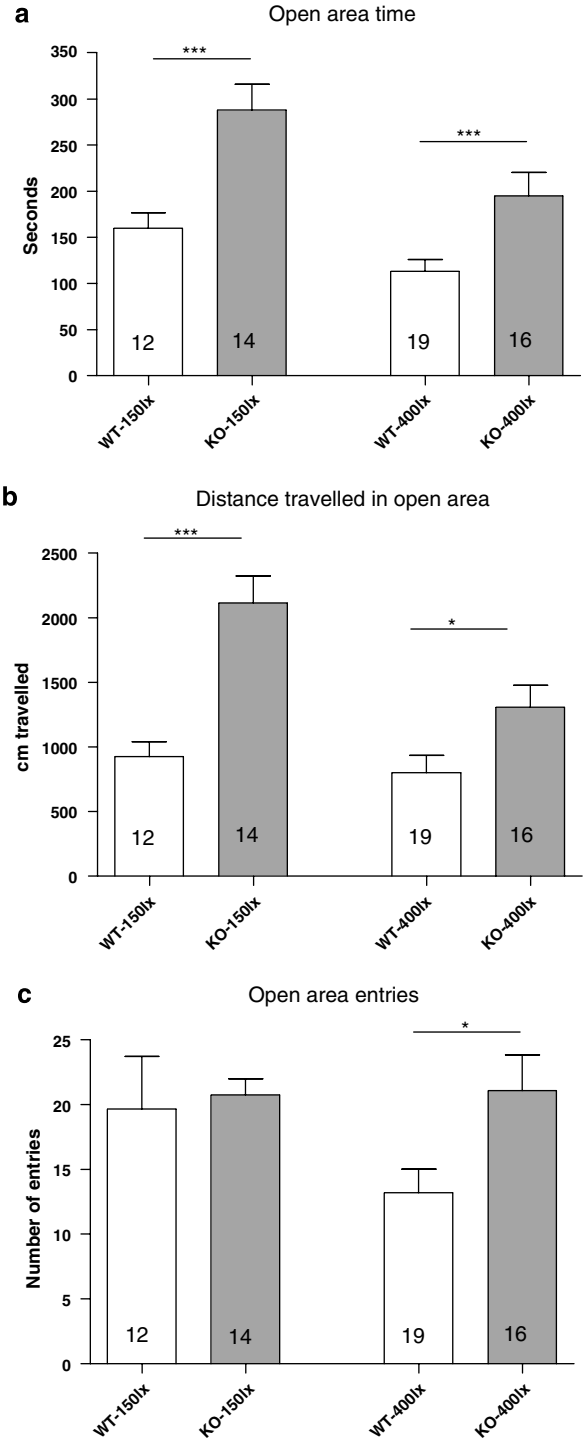

Figure 3 At 150 and 400 lux illumination, $dyn^{(+/+)}$ mice displayed less explorative behavior in the lit area, as reflected by lower time (a) and lower distance (b) measured in the open compartment. Interestingly, open area entries differed only at the higher aversive 400 lux (c).
$^{*}p<0.05$; $^{***}p<0.01$ *vs* respective control.

mRNA expression were neither detected for TH in the locus coeruleus nor for TPH2 in the dorsal raphe nucleus (Table 1).

Interestingly, the reduction of CRH mRNA in the paraventricular nucleus and the central amygdala was also observed in $dyn^{(+/+)}$ animals injected with 10 mg/kg norBNI 48 h before analysis (Figure 5).

Peripheral Alterations in Response to Prodynorphin Gene Deletion

To investigate how the ablation of prodynorphin could influence anxiety-related behaviors, we measured key parameters in the serum of these mice. Consistent with the reduced anxiety in $dyn^{(-/-)}$ mice serum corticosterone levels were significantly reduced (74.4 ± 13.8 ng/ml (6); $p<0.05$) as compared to $dyn^{(+/+)}$ mice (125 ± 7.9 ng/ml (5)). Similarly, wild-type mice treated with 10 mg/kg norBNI 48 h before analysis showed attenuated corticosterone levels (80.3 ± 27.6 ng/ml (6)). Stress-induced (i.p. saline injection 1 h before testing) increases in corticosterone serum levels of wild-type mice (226 ± 16.3 ng/ml (6) were significantly attenuated in $dyn^{(-/-)}$ mice (167 ± 4.6 ng/ml (7); $p<0.05$ *vs* $dyn^{(+/+)}$ saline; ANOVA with Dunnett's *post hoc* test; Figure 4d).

DISCUSSION

This study demonstrates a clear-cut anxiogenic role of prodynorphin-derived peptides as $dyn^{(-/-)}$ mice display markedly increased explorative behavior in three independent anxiety tests. In contrast, we observed longer duration of immobility in the tail suspension test and a faster increase in immobility in the repeated forced swim test. This phenotype is accompanied by marked alterations of the expression levels of neuropeptides (CRH and NPY) known to play a critical role in the regulation of emotionality in key areas of the amygdala and the hypothalamus. Consistent with this serum levels of corticosterone are also decreased in these animals (Shepard *et al*, 2000). Importantly, the increased exploratory behavior could be reversed by treatment with the specific KOR agonist U-50488. Blockade of KOR in wild-type mice by applying the specific antagonists norBNI and GNTI revealed an increase in exploratory behavior and a reduction of corticosterone serum levels. The delay in both, behavioral and corticosterone level response to KOR blockade in wild type or KOR stimulation in $dyn^{(-/-)}$ mice suggests an indirect effect of prodynorphin-derived peptides on anxiety control. Our data partially contradict a recently published study, reporting inconsistent results of dynorphin knockout mice from different anxiety tests (Bilkei-Gorzo *et al*, 2008). In this study, the zero-maze and the startle–response test suggested anxiolytic effects of endogenous dynorphins, although no effect was seen in the light–dark test at very high illumination (1000 lux). This is contradicted by their finding of decreased stress-induced hyperthermia, which would indicate an anxiogenic effect of endogenous dynorphins. However, decreased stress-induced hyperthermia was accompanied by increased basal body temperature, which was not reported from other strains of $dyn^{(-/-)}$ mice. Importantly, they report differences in hormonal stress response (amplitude and duration) in $dyn^{(-/-)}$ mice *vs* controls. Therefore, the discrepancies between their and our data may be based beside differences of the genetic background also on different housing conditions leading to differences in basal stress levels. In any case, we observed consistent data from three independent tests, which were reproducible with pharmacological approaches and are

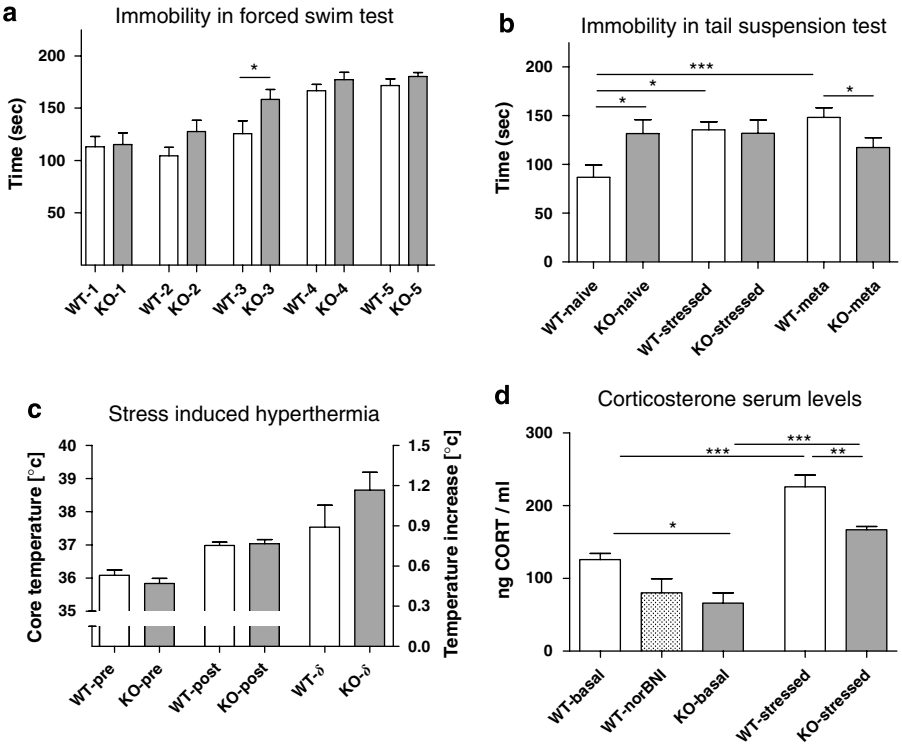

Figure 4 Time spent immobile did not differ between dyn$^{(+/+)}$ and dyn$^{(-/-)}$ mice in the first and last trial of the repeated forced swim test. However, immobility times increased earlier in dyn$^{(-/-)}$ mice, reaching statistical significance at trial 3 (a). Non prestressed dyn$^{(+/+)}$ mice displayed less immobility in the tail suspension test than dyn$^{(-/-)}$ mice. Interestingly, in prestressed animals, no difference was observed, which was due to increased immobility in dyn$^{(+/+)}$ mice (b). Immobility was reduced in dyn$^{(-/-)}$, but not in dyn$^{(+/+)}$ mice under meloxicam anesthesia (b). This is in line with reports on mild hyperalgesia and lack of stress-induced analgesia in dyn$^{(-/-)}$ mice. No differences between the genotypes were observed in stress-induced hyperthermia (c). Basal corticosterone serum levels were reduced in both dyn$^{(+/+)}$ mice pretreated with 10 mg/kg norBNI and dyn$^{(-/-)}$ animals. Stress-induced corticosterone levels were also augmented in dyn$^{(-/-)}$ mice (d).
*$p<0.05$; **$p<0.01$; ***$p<0.001$ *vs* respective control.

supported by neurochemical and hormonal changes observed in dyn$^{(-/-)}$ mice.

In line with reports on other prodynorphin-deficient mouse lines (Sharifi *et al*, 2001), no obvious influence of the deletion on basal behavior or physiological parameters (ie home-cage activity, fertility, body weight, and core temperature) was observed. However, we measured a significantly reduced holding time in the wire-hang test. This stands in contradiction to the performance on the rotarod and the increased activity in the open field test. Noteworthy, during the wire-hang test, the animals did not loose hold, but actively explored the distance to the ground and voluntarily jumped down. Thus, the reduced holding time might rather reflect the anxiolytic phenotype than reduced physical strength. This is also supported by the reduced number of freezing events and reduced defecation in the empty cage observation. On the other hand, an effect of prodynorphin deficiency on motor performance cannot be excluded as dynorphin is involved in the regulation of dopamine release in rat striatum (Schoffelmeer *et al*, 1997). However, this did not translate into changed home-cage activity.

Regulation of anxiety behavior involves several neurotransmitter systems. Beside the classical transmitters serotonin (Wise *et al*, 1970; Westenberg *et al*, 1987; Graeff, 2002) and noradrenalin (Vlachakis *et al*, 1974; Brunello *et al*, 2003), also several neuropeptides were proposed as modifiers of anxiety-related behavior.

The minor effects of short-term treatment of dyn$^{(-/-)}$ mice with KOR agonists or of wild-type mice with KOR antagonists argue against a direct interaction with anxiety control. This is in line with reports on functions of dynorphin agonists and antagonists in the control to anxiety (Kuzmin *et al*, 2006; Knoll *et al*, 2007), but may also be due to strong influences of test paradigms and social status of mice (Kudryavtseva *et al*, 2004). On the basis of our findings of delayed effects on anxiety-related behavior induced by KOR agonist treatment of dyn$^{(-/-)}$ mice and KOR antagonists treatment in dyn$^{(+/+)}$ mice, we suggest an indirect modulation of anxiety control circuits in dyn$^{(-/-)}$ mice. This is supported by neurochemistry in dyn$^{(-/-)}$ mice detecting specific alterations in transmitter systems known to be involved in emotional control. Interestingly, dyn$^{(-/-)}$ mice show no difference in the expression of tyrosine- or tryptophan-hydroxylase mRNA, suggesting unchanged levels of serotonin and catecholamines. However, several neuropeptide systems within amygdalar and hypothalamic nuclei displayed adaptations that may be of relevance for the observed anxiolytic phenotype. The key features are increased NPY expression in the basolateral amygdala and concomitant reduction in CRH expression in the central amygdala and the PVN, which could be reproduced in wild-type mice by a single injection of 10 mg/kg norBNI 48 h before testing. These changes may reflect alterations in the regulatory circuit of NPY in the basolateral amygdala

Table 1 Alterations in Neuropeptide mRNA Levels in Prodynorpin KO Mice

Area	Nucleus	mRNA	WT	KO
Amygdala	Central	CART	100 ± 5.14 (6)	76.8 ± 5.99 (6)*
		CRH	100 ± 4.06 (6)	66.5 ± 5.11 (6)***
		NKB	100 ± 2.47 (6)	115 ± 2.96 (6)**
		PPTA	100 ± 3.51 (6)	111 ± 5.51 (6)
	Medial	CART	100 ± 4.09 (5)	119 ± 6.24 (6)*
		CgB	100 ± 3.31 (5)	86.8 ± 3.51 (6)*
		NPY	100 ± 3.04 (6)	145 ± 5.89 (6)***
		PPTA	100 ± 3.51 (6)	86.9 ± 4.38 (6)*
	Basolateral	NPY	100 ± 6.71 (6)	133 ± 5.45 (5)**
Hypothalamus	Paraventricular	CgB	100 ± 4.45 (5)	100 ± 3.96 (6)
		CRH	100 ± 6.62 (9)	73.0 ± 4.79 (10)**
		PPTA	100 ± 3.84 (6)	135 ± 7.30 (6)**
		TRH	100 ± 2.54 (4)	76.2 ± 2.41 (5)***
	Arcuate	AgRP	100 ± 8.35 (5)	61.7 ± 6.82 (5)**
		CART	100 ± 3.99 (6)	118 ± 7.33 (5)
		CgB	100 ± 8.98 (3)	97.5 ± 8.12 (5)
		NKB	100 ± 3.32 (6)	177 ± 1.43 (6)***
		NPY	100 ± 3.97 (6)	73.9 ± 3.17 (6)***
		POMC	100 ± 2.18 (5)	121 ± 5.20 (6)**
	Lateral area	Orexin	100 ± 1.71 (6)	98.0 ± 4.25 (6)
		NKB	100 ± 6.77 (6)	128 ± 6.84 (6)*
		TRH	100 ± 6.70 (6)	99.0 ± 4.98 (6)
Mesencephalon	Dorsal raphe nucleus	TPH2	100 ± 6.06 (7)	110 ± 5.65 (6)
Brain stem	Locus coeruleus	TH	100 ± 3.74 (7)	95.5 ± 3.60 (6)

Data are given as percent of control and represent mean ± SEM (number of animals).
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ *vs* respective control obtained by Student's *t*-test.
Gray shading indicates statistically significant differences confirmed after adjustment for multiple testing applying the Holm's step-down method.

suppressing CRH expression in the central nucleus (Heilig *et al*, 1994; Sajdyk *et al*, 2004). Increasing evidence shows a crucial role of NPY and Y-receptors in anxiety-related behavior (for review see (Kask *et al*, 2002)). Thus, injection of NPY into the amygdala was shown to be anxiolytic. Y1-receptors were proposed to mediate these anxiolytic effects (Wahlestedt *et al*, 1993). This was recently confirmed in Y1-receptors-deficient mice (Karlsson *et al*, 2008). In addition, NPY is seen as the major counterpart of CRH, mediating mostly opposing effects and thereby balancing the emotional state (for reviews see (Heilig *et al*, 1994; Sajdyk *et al*, 2004)). Furthermore, intraventricular injection of CRH or over-expression of CRH is anxiogenic in mice (Stenzel-Poore *et al*, 1996) and inactivation of CRH receptor 1 reduces anxiety (Smith *et al*, 1998; Timpl *et al*, 1998), whereas deletion of CRH receptor 2 is anxiogenic (Bale *et al*, 2000; Coste *et al*, 2000; Kishimoto *et al*, 2000).

Interesting to note is also the moderate decrease in expression of PPTA mRNA, the precursor of substance P, in the medial amygdala. In recent years, increased efflux of substance P under stressful conditions was shown in the amygdala (Ebner *et al*, 2004). Mainly acting via NK1 receptors, substance P produces anxiogenic effects when injected into the dorsal periaqueductal gray, lateral septum, or medial amygdala (Aguiar and Brandao, 1996; Gavioli *et al*, 1999; Ebner *et al*, 2004). Interestingly, NK1 receptor activation can increase the expression of the anxiogenic CRH receptor 1, as shown in cell culture (Hamke *et al*, 2006). In addition, in dyn$^{(-/-)}$ mice, CART mRNA was reduced in the central, but increased in the medial amygdala and in the arcuate nucleus of hypothalamus. Although CART was mostly investigated in relation to feeding behavior, there is some evidence that CART is also involved in the regulation of anxiety. Thus, Kask *et al* (2000) demonstrated dose-dependent anxiogenic effects of icv-injected CART in the elevated plus maze. However, the mechanism by which CART elicits its anxiogenic effects remains unknown (Stanek, 2006). On the other hand the decreases in PPTA and CART expression are moderate compared to the alterations in CRH expression and may be compensated by concomitant increases in other nuclei. Thus, a major role of substance

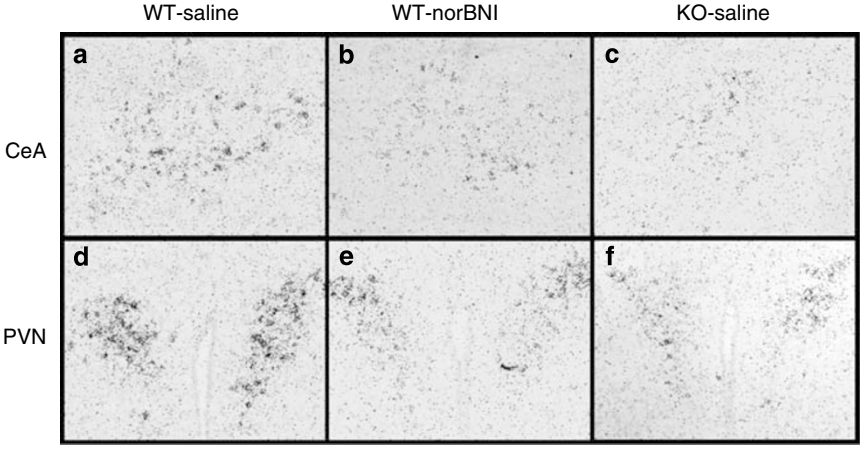

Figure 5 High-resolution *in situ* hybridization for CRH mRNA in the central amygdala (upper panel) and the paraventricular nucleus (lower panel). Photomicrographs obtained from 20 μm sections are depicted for wild-type mice 48 h after saline (a, d) or 10 mg/kg norBNI (b, e) injection and saline-injected dyn$^{(-/-)}$ mice (c, f). Note the marked drop in signal after norBNI treatment, which is comparable to the labeling seen in dyn$^{(-/-)}$ animals.

P or CART in the anxiolytic phenotype of dyn$^{(-/-)}$ mice is rather unlikely.

Interestingly, dyn$^{(-/-)}$ mice display a prolonged period of elevated corticosterone plasma levels after the zero-maze test, which causes mild stress–responses in mice. Therefore, a delayed termination of the stress–response was proposed in these mice (Bilkei-Gorzo *et al*, 2008). This supports our findings in the repeated forced swim test, where dyn$^{(-/-)}$ mice show a faster increase in immobility and a decrease of struggling in the second and third repeat on day 2. At first glance, our data contradict the findings of McLaughlin *et al* (2003) who did not find increasing immobility. However, they used 30°C warm water and a test interval of 7–12 min on day 2. In contrast, we used 23–25°C water and an interval between trials of about 2 h. Lower water temperature reduces floating behavior of mice in the forced swim test (Bachli *et al*, 2008). The increased test interval may influence the progression of immobility especially if one group of mice shows delayed stress termination, as reported for the dyn$^{(-/-)}$ mice (Bilkei-Gorzo *et al*, 2008). Noteworthy, our final immobility times are very similar to those observed by McLaughlin *et al* (2003).

In line with partially increased depressive-like behavior in the forced swim test, we also observed increased immobility in the tail-suspension test in naive dyn$^{(-/-)}$ animals. However, this difference was abolished in prestressed mice. Interestingly, this was due to increased immobility in wild-type mice, whereas there was no difference in immobility between naive and stressed dyn$^{(-/-)}$ mice. One potential explanation could be the mild hyperalgesia, which was reported for dyn$^{(-/-)}$ mice (Wang *et al*, 2001). In addition, dyn$^{(-/-)}$ mice lack stress-induced analgesia (McLaughlin *et al*, 2003). Indeed, we could reduce immobility in dyn$^{(-/-)}$, but not wild-type mice by pretreatment with the nonsteroidal analgesic meloxicam. Noteworthy, we also observed higher thresholds to electrical footshocks in dyn$^{(-/-)}$ mice. However, this might reflect more the anxiolytic than a hypoalgesic phenotype.

CONCLUSIONS

Our data provide clear evidence that prodynorphin-derived peptides acting on KOR are important in anxiety control. KOR signaling modulates the neurochemistry of brain nuclei involved in control of emotionality through the regulation of the expression of other main players in anxiety, CRH, and NPY.

ACKNOWLEDGEMENTS

We thank Cornelia Aigner and Inge Kapeller for excellent technical assistance. We also thank Dr Gerald Obermair for statistical advice.

DISCLOSURE/CONFLICT OF INTEREST

This work was supported by the Austrian Science Fund grants P18471-B5 and P20107-B5 to Christoph Schwarzer. Other authors have nothing to disclose.

REFERENCES

Aguiar MS, Brandao ML (1996). Effects of microinjections of the neuropeptide substance P in the dorsal periaqueductal gray on the behaviour of rats in the plus-maze test. *Physiol Behav* **60**: 1183–1186.

Bachli H, Steiner MA, Habersetzer U, Wotjak CT (2008). Increased water temperature renders single-housed C57BL/6J mice susceptible to antidepressant treatment in the forced swim test. *Behav Brain Res* **187**: 67–71.

Bale TL, Contarino A, Smith GW, Chan R, Gold LH, Sawchenko PE *et al* (2000). Mice deficient for corticotropin-releasing hormone receptor-2 display anxiety-like behaviour and are hypersensitive to stress. *Nat Genet* **24**: 410–414.

Bilkei-Gorzo A, Racz I, Michel K, Mauer D, Zimmer A, Klingmuller D *et al* (2008). Control of hormonal stress reactivity by the endogenous opioid system. *Psychoneuroendocrinology* **33**: 425–436.

Brunello N, Blier P, Judd LL, Mendlewicz J, Nelson CJ, Souery D *et al* (2003). Noradrenaline in mood and anxiety disorders: basic and clinical studies. *Int Clin Psychopharmacol* **18**: 191–202.

Coste SC, Kesterson RA, Heldwein KA, Stevens SL, Heard AD, Hollis JH *et al* (2000). Abnormal adaptations to stress and impaired cardiovascular function in mice lacking corticotropin-releasing hormone receptor-2. *Nat Genet* **24**: 403–409.

Crawley JN (1999). Behavioral phenotyping of transgenic and knockout mice: experimental design and evaluation of general health, sensory functions, motor abilities, and specific behavioral tests. *Brain Res* **835**: 18–26.

Ebner K, Rupniak NM, Saria A, Singewald N (2004). Substance P in the medial amygdala: emotional stress-sensitive release and modulation of anxiety-related behavior in rats. *Proc Natl Acad Sci USA* **101**: 4280–4285.

Ebner K, Singewald N (2006). The role of substance P in stress and anxiety responses. *Amino Acids* **31**: 251–272.

Engelhardt G, Homma D, Schlegel K, Schnitzler C, Utzmann R (1996). General pharmacology of meloxicam—Part I: effects on CNS, gastric emptying, intestinal transport, water, electrolyte and creatinine excretion. *Gen Pharmacol* **27**: 673–677.

Gavioli EC, Canteras NS, De Lima TC (1999). Anxiogenic-like effect induced by substance P injected into the lateral septal nucleus. *Neuroreport* **10**: 3399–3403.

Graeff FG (2002). On serotonin and experimental anxiety. *Psychopharmacology (Berl)* **163**: 467–476.

Hamke M, Herpfer I, Lieb K, Wandelt C, Fiebich BL (2006). Substance P induces expression of the corticotropin-releasing factor receptor 1 by activation of the neurokinin-1 receptor. *Brain Res* **1102**: 135–144.

Heilig M, Koob GF, Ekman R, Britton KT (1994). Corticotropin-releasing factor and neuropeptide Y: role in emotional integration. *Trends Neurosci* **17**: 80–85.

Holm S (1979). A simple sequentially rejective multiple test procedure. *Scand J Stat* **6**: 65–70.

Jewett DC, Grace MK, Jones RM, Billington CJ, Portoghese PS, Levine AS (2001). The kappa-opioid antagonist GNTI reduces U50,488-, DAMGO-, and deprivation-induced feeding, but not butorphanol- and neuropeptide Y-induced feeding in rats. *Brain Res* **909**: 75–80.

Jones RM, Portoghese PS (2000). 5′-Guanidinonaltrindole, a highly selective and potent kappa-opioid receptor antagonist. *Eur J Pharmacol* **396**: 49–52.

Karl T, Pabst R, von Horsten S (2003). Behavioral phenotyping of mice in pharmacological and toxicological research. *Exp Toxicol Pathol* **55**: 69–83.

Karlsson RM, Choe JS, Cameron HA, Thorsell A, Crawley JN, Holmes A *et al* (2008). The neuropeptide Y Y1 receptor subtype is necessary for the anxiolytic-like effects of neuropeptide Y, but

not the antidepressant-like effects of fluoxetine, in mice. *Psychopharmacology (Berl)* **195**: 547–557.
Kask A, Harro J, von Horsten S, Redrobe JP, Dumont Y, Quirion R (2002). The neurocircuitry and receptor subtypes mediating anxiolytic-like effects of neuropeptide Y. *Neurosci Biobehav Rev* **26**: 259–283.
Kask A, Schioth HB, Mutulis F, Wikberg JE, Rago L (2000). Anorexigenic cocaine- and amphetamine-regulated transcript peptide intensifies fear reactions in rats. *Brain Res* **857**: 283–285.
Kishimoto T, Radulovic J, Radulovic M, Lin CR, Schrick C, Hooshmand F *et al* (2000). Deletion of crhr2 reveals an anxiolytic role for corticotropin-releasing hormone receptor-2. *Nat Genet* **24**: 415–419.
Knoll AT, Meloni EG, Thomas JB, Carroll FI, Carlezon WA (2007). Anxiolytic-like effects of {kappa}-opioid receptor antagonists in models of unlearned and learned fear in rats. *J Pharmacol Exp Ther* **323**: 838–845.
Kudryavtseva NN, Gerrits MA, Avgustinovich DF, Tenditnik MV, Van Ree JM (2004). Modulation of anxiety-related behaviors by mu- and kappa-opioid receptor agonists depends on the social status of mice. *Peptides* **25**: 1355–1363.
Kuzmin A, Madjid N, Terenius L, Ogren SO, Bakalkin G (2006). Big dynorphin, a prodynorphin-derived peptide produces NMDA receptor-mediated effects on memory, anxiolytic-like and locomotor behavior in mice. *Neuropsychopharmacology* **31**: 1928–1937.
Land BB, Bruchas MR, Lemos JC, Xu M, Melief EJ, Chavkin C (2008). The dysphoric component of stress is encoded by activation of the dynorphin kappa-opioid system. *J Neurosci* **28**: 407–414.
Lin S, Boey D, Lee N, Schwarzer C, Sainsbury A, Herzog H (2006). Distribution of prodynorphin mRNA and its interaction with the NPY system in the mouse brain. *Neuropeptides* **40**: 115–123.
Loacker S, Sayyah M, Wittmann W, Herzog H, Schwarzer C (2007). Endogenous dynorphin in epileptogenesis and epilepsy: anticonvulsant net effect via kappa opioid receptors. *Brain* **130**: 1017–1028.
Marchant NJ, Densmore VS, Osborne PB (2007). Coexpression of prodynorphin and corticotrophin-releasing hormone in the rat central amygdala: evidence of two distinct endogenous opioid systems in the lateral division. *J Comp Neurol* **504**: 702–715.
McLaughlin JP, Marton-Popovici M, Chavkin C (2003). Kappa opioid receptor antagonism and prodynorphin gene disruption block stress-induced behavioral responses. *J Neurosci* **23**: 5674–5683.
Narita M, Kaneko C, Miyoshi K, Nagumo Y, Kuzumaki N, Nakajima M *et al* (2006). Chronic pain induces anxiety with concomitant changes in opioidergic function in the amygdala. *Neuropsychopharmacology* **31**: 739–750.
Olivier B, Zethof T, Pattij T, van Boogaert M, van Oorschot R, Leahy C *et al* (2003). Stress-induced hyperthermia and anxiety: pharmacological validation. *Eur J Pharmacol* **463**: 117–132.
Sainsbury A, Schwarzer C, Couzens M, Herzog H (2002). Y2 receptor deletion attenuates the type 2 diabetic syndrome of ob/ob mice. *Diabetes* **51**: 3420–3427.
Sajdyk TJ, Shekhar A, Gehlert DR (2004). Interactions between NPY and CRF in the amygdala to regulate emotionality. *Neuropeptides* **38**: 225–234.
Schoffelmeer AN, Hogenboom F, Mulder AH (1997). Kappa1- and kappa2-opioid receptors mediating presynaptic inhibition of dopamine and acetylcholine release in rat neostriatum. *Br J Pharmacol* **122**: 520–524.
Schwarzer C, Kofler N, Sperk G (1998). Up-regulation of neuropeptide Y-Y2 receptors in an animal model of temporal lobe epilepsy. *Mol Pharmacol* **53**: 6–13.
Sharifi N, Diehl N, Yaswen L, Brennan MB, Hochgeschwender U (2001). Generation of dynorphin knockout mice. *Brain Res Mol Brain Res* **86**: 70–75.
Shepard JD, Barron KW, Myers DA (2000). Corticosterone delivery to the amygdala increases corticotropin-releasing factor mRNA in the central amygdaloid nucleus and anxiety-like behavior. *Brain Res* **861**: 288–295.
Smith GW, Aubry JM, Dellu F, Contarino A, Bilezikjian LM, Gold LH *et al* (1998). Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development. *Neuron* **20**: 1093–1102.
Solbrig MV, Adrian R, Baratta J, Lauterborn JC, Koob GF (2006). Kappa opioid control of seizures produced by a virus in an animal model. *Brain* **129**: 642–654.
Stanek LM (2006). Cocaine- and amphetamine related transcript (CART) and anxiety. *Peptides* **27**: 2005–2011.
Stenzel-Poore MP, Duncan JE, Rittenberg MB, Bakke AC, Heinrichs SC (1996). CRH overproduction in transgenic mice: behavioral and immune system modulation. *Ann NY Acad Sci* **780**: 36–48.
Timpl P, Spanagel R, Sillaber I, Kresse A, Reul JM, Stalla GK *et al* (1998). Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. *Nat Genet* **19**: 162–166.
Tsuda M, Suzuki T, Misawa M, Nagase H (1996). Involvement of the opioid system in the anxiolytic effect of diazepam in mice. *Eur J Pharmacol* **307**: 7–14.
Vlachakis ND, De Guia D, Mendlowitz M, Antram S, Wolf RL (1974). Hypertension and anxiety. A trial with epinephrine and norepinephrine infusion. *Mt Sinai J Med* **41**: 615–625.
Wahlestedt C, Pich EM, Koob GF, Yee F, Heilig M (1993). Modulation of anxiety and neuropeptide Y-Y1 receptors by antisense oligodeoxynucleotides. *Science* **259**: 528–531.
Wang Z, Gardell LR, Ossipov MH, Vanderah TW, Brennan MB, Hochgeschwender U *et al* (2001). Pronociceptive actions of dynorphin maintain chronic neuropathic pain. *J Neurosci* **21**: 1779–1786.
Westenberg HG, den Boer JA, Kahn RS (1987). Psychopharmacology of anxiety disorders: on the role of serotonin in the treatment of anxiety states and phobic disorders. *Psychopharmacol Bull* **23**: 145–149.
Wise CD, Berger BD, Stein L (1970). Serotonin: a possible mediator of behavioral suppression induced by anxiety. *Dis Nerv Syst* **31**(Suppl): 34–37.
Wollemann M, Benyhe S (2004). Non-opioid actions of opioid peptides. *Life Sci* **75**: 257–270.

Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)