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Preproenkephalin Knockout Mice Show No Depression-Related Phenotype

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Clinical, preclinical, and pharmacological studies have suggested that decreased enkephalin tone is associated with depression-like symptoms and increase in enkephalin signaling could have a therapeutic value in the treatment of depression. In this study we demonstrate that, surprisingly, animals lacking enkephalin (preproenkephalin, Penk1$^{-/-}$) showed no depression-related phenotype in the Porsolt forced swimming or tail suspension tests. Moreover, Penk1$^{-/-}$ mice had a lower frequency of depression-related behavior in stress-induced hypoactivity and ultrasonic vocalization models of depression, similar to animals treated with antidepressant drugs, although this effect was specific to the genetic background. In addition, there was no significant difference in the efficacy of antidepressant reference compounds in wild-type and knockout animals. Nialamide and amitriptyline were even slightly more effective in animals with genetic deletion of Penk1, whereas the minimal effective dose of imipramine and fluoxetine was the same in the two genotypes. The dual peptidase inhibitor RB-101 was also effective in Penk1$^{-/-}$ as well as in Penk1$^{-/-}$/Pdyn$^{-/-}$ animals, although its efficacy was somewhat reduced compared with wild-type animals. This result was also surprising because the antidepressant effects of RB-101 were thought to be due to the elevation of enkephalin levels.

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INTRODUCTION

Several independent studies suggested a role of enkephalins in depression. In the chronic mild stress model of depression, which has a high face validity, enkephalin levels in the nucleus accumbens were decreased (Dziedzicka-Wasylewska and Papp, 1996). Similarly, a blunted enkephalin release was found after exposing chronic mild stressed animals to a conspecies (Bertrand *et al*, 1997). These and other studies led to the conclusion that depressive behaviors, especially anhedonia, may be related to the reduced levels or responsiveness of enkephalin neurotransmission in the nucleus accumbens (Zangen *et al*, 2002). However, in contrast to these findings there was an increased enkephalin immunoreactivity in limbic structures (Primeaux and Holmes, 2000) and ventral striatum, but not in the nucleus accumbens (Holmes, 1999) in the bulbectomy model of depression. It was suggested that this increase, at least partly, contributes to the behavioral changes observed after bulbectomy since increase of enkephalin level through viral gene transfer in the ventral striatum mimicked some effects of bulbectomy (Primeaux *et al*, 2003).

Increased enkephalin levels after pharmacological blockade of enkephalin-degrading enzymes resulted in a reduction of depression-related behaviors in animal models (Tejedor-Real *et al*, 1993, 1995). This effect is probably mediated by an enhanced delta opioid receptor (DOPr) activity, because similar results have been obtained by application of DOPr agonists (Broom *et al*, 2002) and because DOPr knockout mice showed an increased frequency of depression-like behavior in the forced swimming test (Filliol *et al*, 2000).

It has been demonstrated that enkephalin levels are increased by the classical antidepressant imipramine (Dziedzicka-Wasylewska and Papp, 1996) through inhibition of enkephalin degradation (de Gandarias *et al*, 1999). This effect of imipramine may contribute to its therapeutic effects. Decrease of enkephalin signaling through the blockade of DOPr with naltrindole antagonized the antidepressant effect of agmatine in the forced swimming test (Zomkowski *et al*, 2005). Moreover, the antidepressant effect of imipramine or iprindole was potentiated using inhibitors of enkephalin degrading peptidases (de Felipe *et al*, 1989). There are two enzymes participating in the degradation of enkephalins, the neutral endopeptidase or

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enkephalinase (EC 2.4.24.11) and aminopeptidase N (EC 3.4.11.2). The analgesic and antidepressant properties of these drugs are thought to base on their ability to increase the enkephalin levels (Nieto *et al*, 2005). However, it should be noted that these enzymes also act on a number of other peptides such as substance P, CGRP, and angiotensin.

The aim of the present study was to elucidate the role enkephalin plays in depression-like behavior and in the mechanism of action of classical antidepressants and 'enkephalinase inhibitors' using preproenkephalin (Penk1) knockout animals. Since we have previously shown that the genetic background can interfere with the manifestation of the Penk1 mutant phenotype, we have included Penk1$^{-/-}$ mice on C57BL/6J and DBA/2J genetic backgrounds. These two strains represent different branches in the mouse family tree and differ in many behavioral traits (Witmer *et al*, 2003).

MATERIALS AND METHODS

Animals

The generation of Penk1 mutant mice on C57BL/6J and DBA/2J congenic backgrounds has been described (Konig *et al*, 1996; Bilkei-Gorzo *et al*, 2004). Mice lacking Penk1$^{-/-}$ and preprodynorphin (Pdyn$^{-/-}$) were generated by crossing homozygous Pdyn$^{-/-}$ animals on a congenic C57BL/6J genetic background (Zimmer *et al*, 2001) with homozygous C57BL/6J-Penk$^{-/-}$ mice. The resulting double heterozygous Penk1$^{+/-}$/Pdyn$^{+/-}$ animals were intercrossed to obtain the homozygous double Penk1$^{-/-}$/Pdyn$^{-/-}$ mutants and wild-type Penk1$^{+/+}$/Pdyn$^{-/-}$ controls. Male Penk1$^{-/-}$ and Penk1$^{+/+}$ mice (3–5 months old) were kept in a reversed light–dark cycle at least 2 weeks before testing and housed in groups of 3–5. Each animal was used only once and was naïve to the model. Experiments were carried out in the active (dark) phase between 10 and 16 h. The experimenter was blind to knockout genotype. Animal procedures followed the guidelines of the German Animal Protection Law.

Forced Swimming Test

Animals were placed in a Plexiglas cylinder (10 cm internal diameter, 50 cm high) filled with 22–23°C water (10 cm height). Duration of the experiment was 6 min, the behavior of the animals was evaluated between the 2nd and 6th minute for 4 min. The immobility time was measured using a stopwatch. A mouse was judged to be immobile when it remained floating in the water, making only those movements necessary to keep its head above the water (Porsolt *et al*, 1977; Porsolt, 2000). Means and standard errors were calculated for each group. Wild-type and knockout animals with the same genetic background were compared using the Student's *t*-test.

Tail Suspension Test

Mice were suspended by their tails from a metal rod, which was fixed 50 cm above the surface of a table covered with soft cloth in a sound-isolated room. The tip of the tail was fixed using adhesive Scotch tape; the duration of the test was 6 min. The immobility time was determined with a stopwatch (Steru *et al*, 1985). This behavioral model was carried out only on animals on a DBA/2J genetic background, since majority of C57BL/6J mice climb up their tail in the tail suspension test situation (Mayorga and Lucki, 2001). Mean and standard error values were calculated, and groups were compared using the Student's *t*-test.

Stress-Induced Hypomotility

The animals were placed into an open-field arena with a grid floor (Conducta, Experimetria Ltd, Hungary) and their motor activity was registered for 5 min. Just after this period, mice received 10 inescapable foot shocks (2 s, 500 mA) with 30 s intervals. About 24 h later we placed the animals back to the same arena and registered their motor activity for 5 min. Hypomotility was calculated as difference in distance traveled before and after the stress exposure. Data were analyzed using two-way ANOVA (within factor: time; between factors: genotype) followed by LSD test separately in both genetic backgrounds.

Ultrasonic Vocalization

About 5–7-day-old pups were removed from the dam, placed individually into a glass jar, and put into a sound-isolated box kept at room temperature (23 ± 1°C). The duration and number of ultrasonic calls was registered using the Ultravox system (Noldus, The Netherlands) for 5 min. Groups were compared using Mann–Whitney test.

Antidepressant Treatment

Animals were treated with vehicle or drug either intraperitoneally 30 min before, or intravenously 5 min before the test. A separate control (vehicle-treated) group was used for each drug in each genotype. The MAO inhibitor nialamide was suspended in saline with the help of a drop of Tween 20 and tested in the dose range of 25–150 mg/kg. The tricyclic uptake inhibitors amitriptyline and imipramine were dissolved in saline and were used in a dose range of 1–20 mg/kg. The specific serotonin reuptake inhibitor fluoxetine was dissolved in saline and tested in the doses of 10 and 20 mg/kg. The forced swimming test was carried out as described above. RB-101 was dissolved in ethanol (10%), Cremofor EL (10%) and distilled water (80%). Animals were injected through the tail vein with the help of a restrainer with the vehicle or the drug intravenously and tested 10 min after the treatment as described above. The efficacy of drugs was evaluated using one-way ANOVA, the effect of individual doses using Dunnett's test.

Effect of the Delta Receptor Antagonist Naltrindole on the Antidepressant Activity of RB-101

Mice were first injected s.c. with 3 mg/kg naltrindole or vehicle. About 30 min later, the animals were treated with RB101 or vehicle and tested in the forced swimming paradigm as described above. Data were analyzed using one-way ANOVA followed by Bonferroni test.

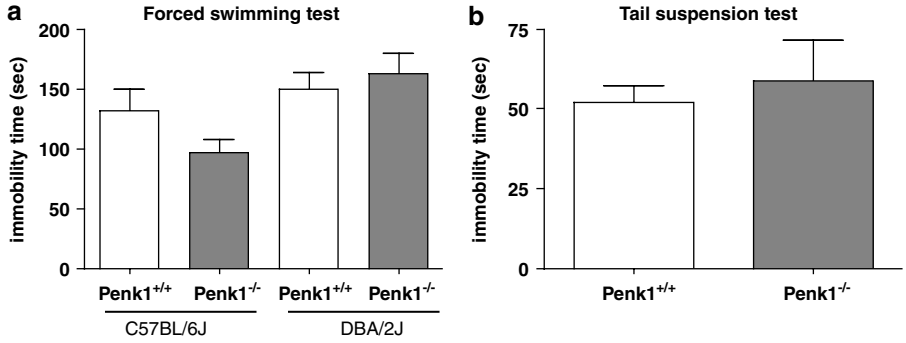

Figure 1 (a) Time spent with immobility did not differ in wild-type and Penk1$^{-/-}$ mice on a C57BL/6J ($t_{18} = 1.63$, not significant) or DBA/2J ($t_{18} = 0.56$, not significant) genetic background. (b) Deletion of Penk1$^{-/-}$ did not influence the behavior of animals in the tail suspension test ($t_{18} = 0.48$, not significant). Columns represent column means (+SEM) ($n = 10$).

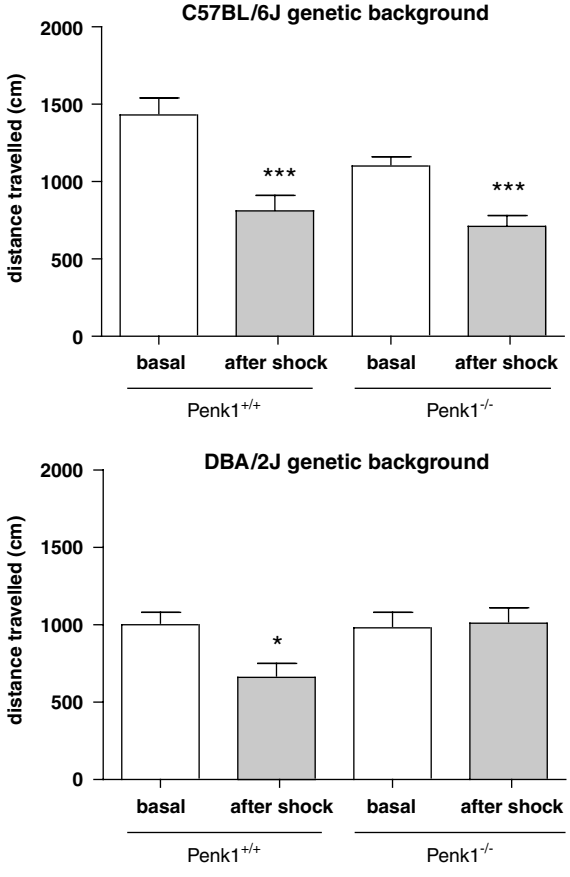

Figure 2 Horizontal activity of animals was measured in an open field just before and 24 h after receiving 10 electric foot shocks. Hypomotility was not present in Penk1$^{-/-}$ mice on a DBA/2J genetic background, whereas knockout mice on a C57BL/6J background had the same stress reactivity as wild-type mice. Columns represent group means (+SEM) ($n = 10$). *$p < 0.01$; ***$p < 0.001$ two-way ANOVA followed by LSD *t*-test.

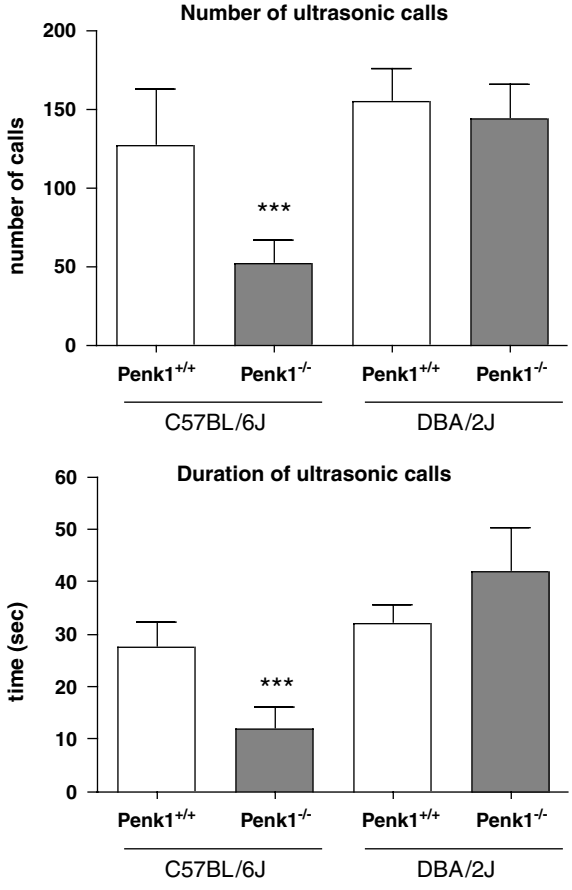

Figure 3 The number and duration of ultrasonic calls in mouse pups was significantly lower in Penk1$^{-/-}$ mice compared to wild-type animals on a C57BL/6J ($U = 194.5$; $p < 0.001$ for the number and $U = 145{,}0$; $p < 0.001$ for the duration) but not on a DBA/2J genetic background. Each column represents the mean value (+SEM) of 23–28 animals. ***$p < 0.001$ unpaired Student's *t*-test.

RESULTS

Behavioral Despair Models

We evaluated two behavioral despair models: Porsolt's forced swim and the tail suspension tests. In these tests, antidepressant drugs reduce the time, animals remain motionless (behavioral despair), and conversely, increase the time in which animals struggle trying to escape. First we analyzed mice on a DBA/2J genetic background. Deletion of Penk1 gene did not change the behavior of animals in the forced swimming test (Figure 1a). Because the genetic background may influence the manifestation of the phenotype (Bilkei-Gorzo *et al*, 2004), we repeated the

experiment with wild-type and knockout animals on a C57BL/6J background. Animals with genetic deletion of the Penk1 gene showed again a same depression-related behavior as wild-type mice (Figure 1a). DBA/2J mice of both genotypes also behaved similar in the tail suspension test (Figure 1b). We did not use mice from the C57BL/6J background in this assay, because C57BL/6J mice are known to climb up on their own tails (Mayorga and Lucki, 2001).

Stress-Induced Hypomotility

This paradigm evaluates the activity of animals in an open field system, in which they were exposed previously to inescapable foot shocks. The motility of the animals was significantly lower 24 h after the electric foot shock both in wild-type and knockout mice on the C57BL/6J genetic background shown by the lack of interaction between stress and genotype ($F_{1,18} = 0.59$; $p > 0.05$). The amplitude of reduction was −43.2% in Penk1$^{+/+}$ ($t_9 = 6.14$; $p < 0.001$) and −35.5% in Penk1$^{-/-}$ ($t_9 = 8.34$; $p < 0.001$) animals. However, Penk1$^{+/+}$ and Penk1$^{-/-}$ mice on a DBA/2J genetic background showed significantly different reactivity to foot shock stress ($F_{1,18} = 7.10$; $p < 0.05$). LSD *post hoc* test revealed that stress-induced hypomotility was present in Penk1$^{+/+}$ (−30.3%; $p < 0.05$), but not on Penk1$^{-/-}$ animals (+3.00%; $p > 0.05$) (Figure 2).

Ultrasonic Vocalization

Mouse pups placed on a hard and cold surface isolated from the dam emitted a series of ultrasonic calls. The number and the duration of the calls were significantly lower in Penk1$^{-/-}$ animals on a C57BL/6J genetic background (Figure 3). In contrast, knockout animals on a DBA/2J

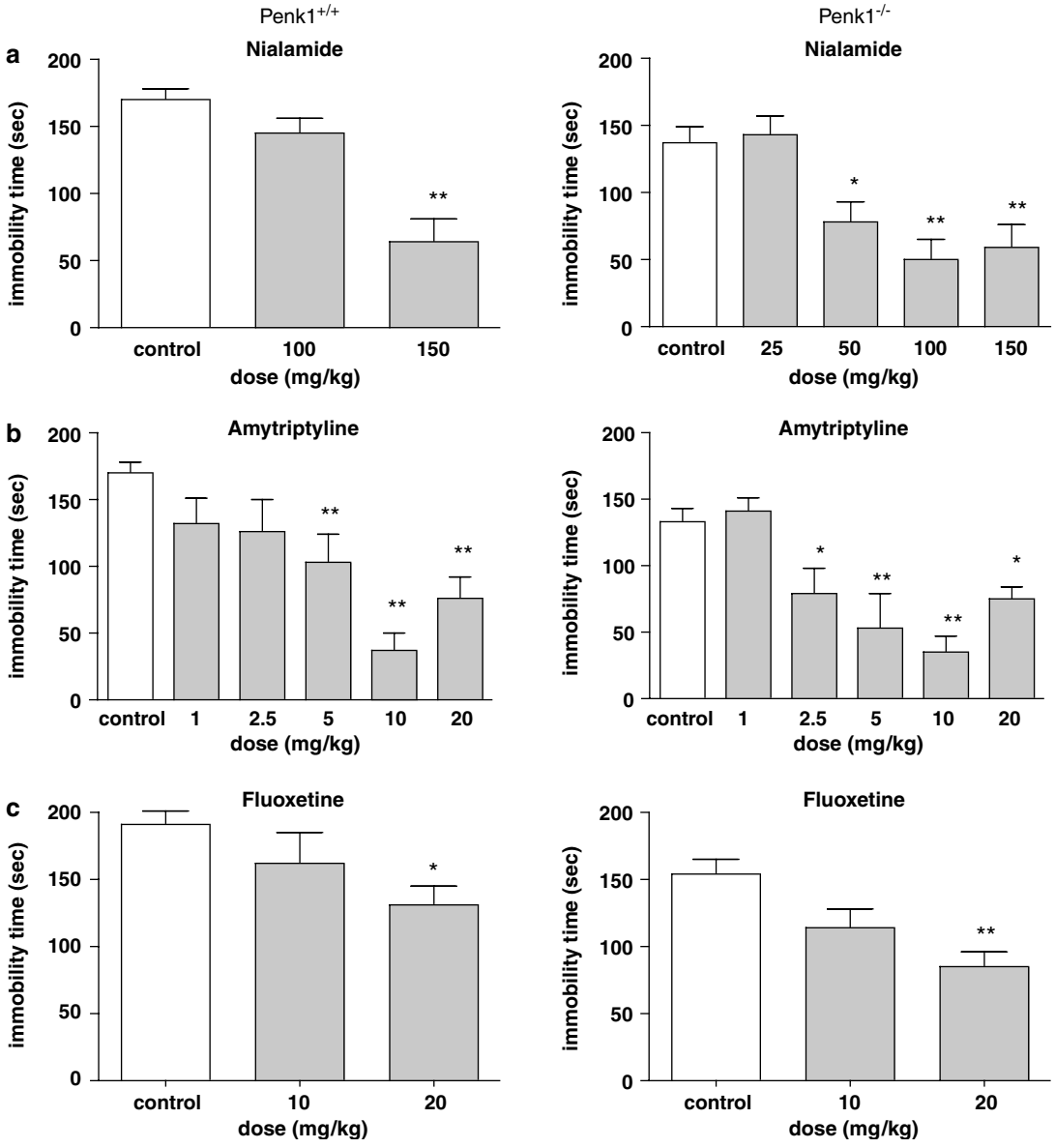

Figure 4 Activity of antidepressant reference compounds in forced swimming test in wild-type and Penk1$^{-/-}$ mice. Each column represents the mean value (+SEM) of 8–10 animals. *$p < 0.05$; **$p < 0.01$; one-way ANOVA followed by Dunnett's test.

genetic background did not show alteration in the ultrasonic vocalization (Figure 3).

Effect of Antidepressants in the Forced Swimming Test

Reference drugs and RB101 were first tested in wild-type and Penk1$^{-/-}$ mice on a DBA/2J genetic background. The MAO inhibitor nialamide significantly reduced the time spent with immobility both in wild-type and in knockout mice, but its efficacy was higher in the later group since the minimum effective dose (MED) value was 150 mg in wild-type and 50 mg/kg in the Penk1$^{-/-}$ animals (Figure 4a). The tricyclic uptake inhibitor, amitriptyline, was slightly more effective in Penk1$^{-/-}$ mice (2.5 mg/kg) compared with Penk1$^{+/+}$ animals (5 mg/kg, Figure 4b), whereas fluoxetine was effective in the highest dose tested (20 mg/kg) in mice of both genotypes (Figure 4c). The antidepressant effects of imipramine and RB-101 were studied in wild-type and Penk1 knockout mice on both C57BL/6J and DBA/2J genetic backgrounds. Imipramine and RB-101 had a similar effect in wild-type mice and animals with genetic deletion of Penk1 gene regardless of the genetic background (Figures 5 and 6). Because enkephalins may also be produced from Pdyn, one cannot exclude the possibility that enkephalins are still present in the absence of a functional enkephalin gene, even though we failed to detect any residual enkephalins in striatal extracts of Penk1$^{-/-}$ mice (Konig *et al*, 1996). Therefore we tested the activity of RB-101 on Penk1$^{-/-}$/Pdyn$^{-/-}$ double knockout animals. RB-101 remained active also in the absence of Penk1 and Pdyn (Figure 6).

Effect of the Delta Receptor Antagonist Naltrindole on the Antidepressant Activity of RB-101

Naltrindole had no effect on the immobility time of wild-type DBA/2J mice, but significantly reduced the antidepressant effect of RB-101 (Figure 7). Naltrindole remained inactive when administered alone in the Penk1$^{-/-}$ mice, but it partially antagonized the effect of RB-101. The immobility time of mice treated with naltrindole and RB-101 did not differ from the immobility times measured in control or RB-101-treated animals (Figure 7).

DISCUSSION

Previous studies suggested a correlation between enkephalin levels and depression-related behavior, as alterations in brain enkephalin levels were detected in the chronic mild stress (Dziedzicka-Wasylewska and Papp, 1996) and bulbectomy (Primeaux and Holmes, 2000) models of depression. Moreover, activation of the enkephalin system either with DOPr agonists or agents blocking the degradation of enkephalin yielded a decrease in depressive symptoms (Tejedor-Real *et al*, 1998). The results from these pharmacological studies were confirmed by the analysis of mice with genetic deletion of DOPr. These animals displayed a significant increase in immobility time, which was interpreted as a sign of increased proneness to depression-related behavior (Filliol *et al*, 2000). On the basis of this evidence, we have hypothesized that enkephalin deficient mice would also display more depression-related behaviors. However, the reactivity of Penk1$^{-/-}$ animals either did not

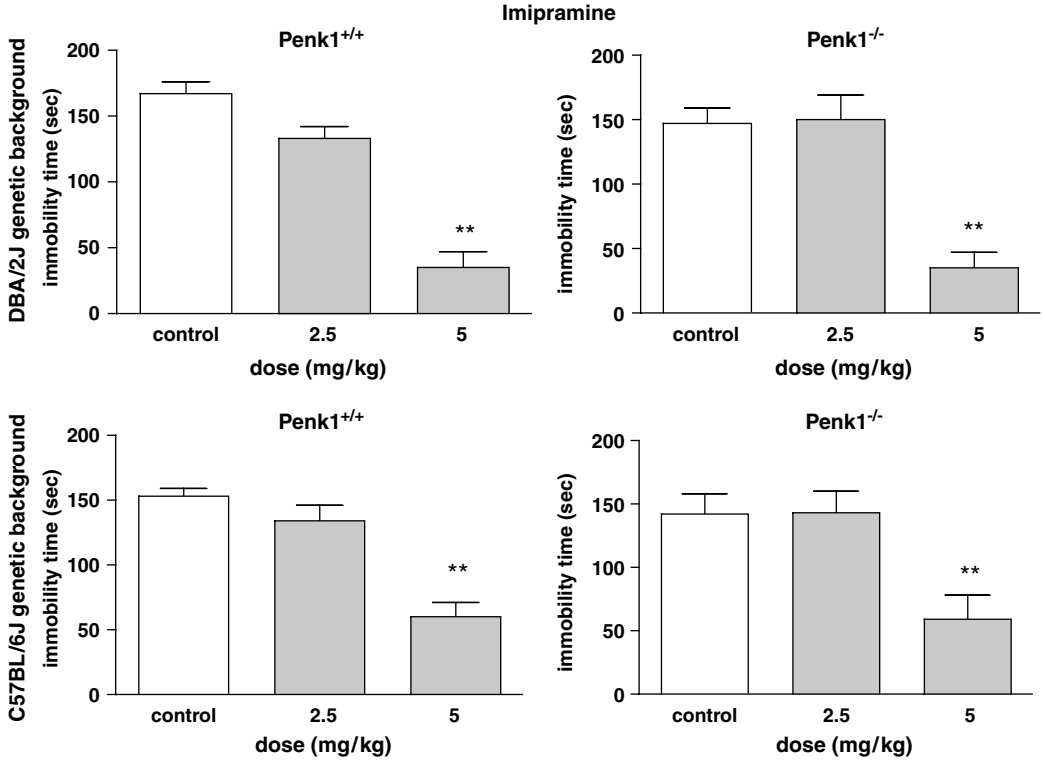

Figure 5 Activity of imipramine in forced swimming test in wild-type Penk1$^{-/-}$ mice on C57BL/6J and DBA/2J genetic backgrounds. Each column represents the mean value (+SEM) of 7–10 animals. $^{**}p<0.01$ one-way ANOVA followed by Dunnett's test.

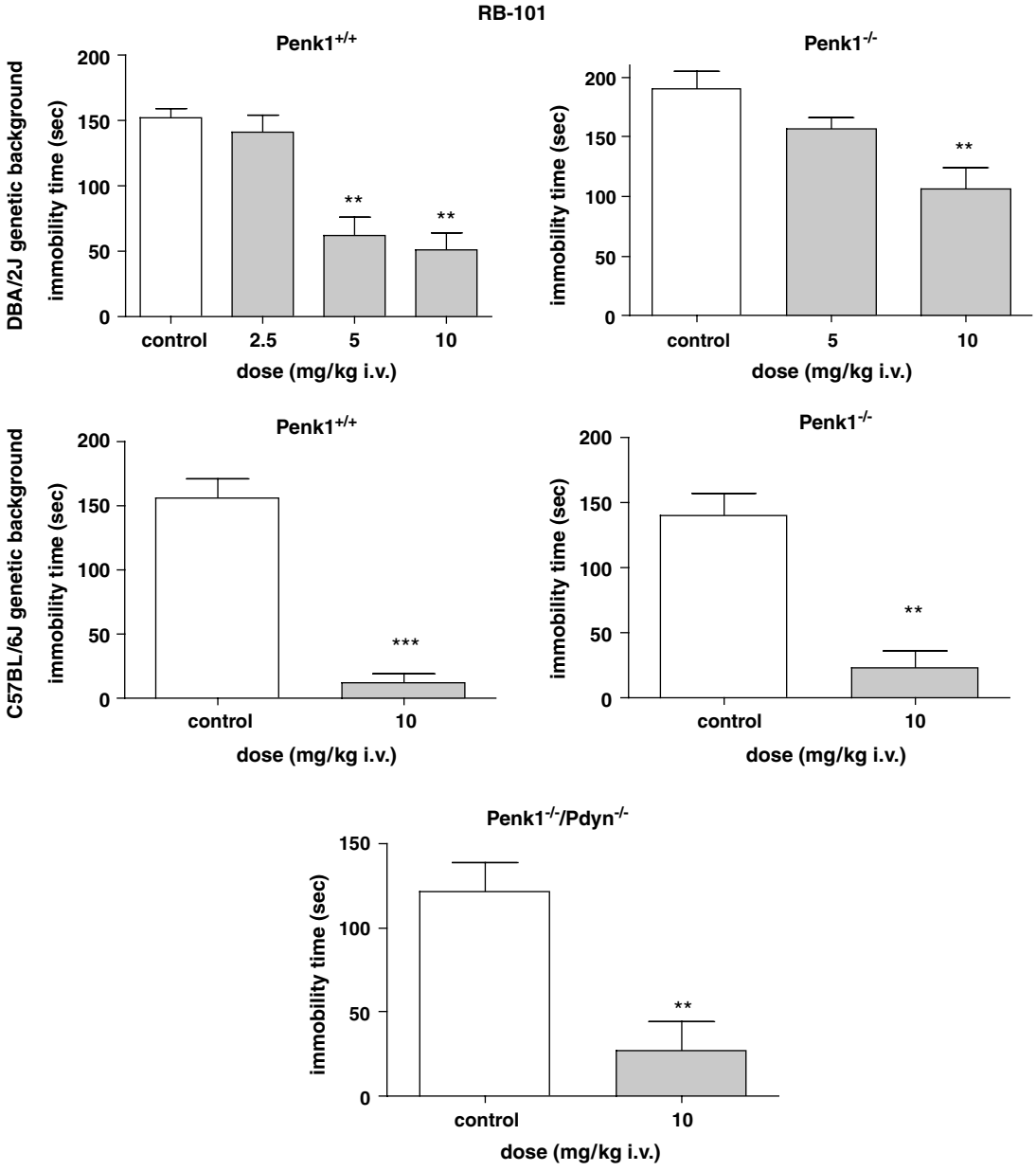

Figure 6 Activity of dual inhibitor RB-101 in forced swimming test in wild-type, Penk1$^{-/-}$, and Penk1$^{-/-}$/Pdyn$^{-/-}$ mice. Each column represents the mean value (+SEM) of 8–10 animals. $^{**}p<0.01$; $^{***}p<0.001$ one-way ANOVA followed by Dunnett's test.

differ from wild-type animals in two different models of depression or showed even a reduction in depression-related behavior dependent on the genetic background. Although C57BL/6J and DBA/2 mice are known to differ substantially in several animal models of depression (Ripoll *et al*, 2003; Ducottet and Belzung, 2005), the genetic background did not influence the depression-related phenotype, unlike to what we have observed previously with anxiety or pain phenotypes in Penk1$^{-/-}$ mice (Bilkei-Gorzo *et al*, 2004). Thus, behavioral analysis of Penk1 knockout animals does not support the hypothesis that a decreased enkephalin signaling contributes to an increase in depression-related behaviors.

One reason of the differences in the depression-related phenotypes between the DOPr (Filliol *et al*, 2000) and Penk1 knockout animals could be a compensatory change in the absence of enkephalins. Indeed, we have previously shown that the lack of enkephalin leads to an upregulation of opioid receptors in limbic areas (Brady *et al*, 1999; Clarke *et al*, 2003). It also seems possible that ambient stress levels may have influenced the different outcomes of these studies. The endogenous opioid system has a relatively low tonicity, but becomes activated upon stress exposure. Chronic mild stress can induce a depression-like state in rodents (Willner, 1997) and affect opioid signaling through the modulation of enkephalin and opioid receptor levels (Drolet *et al*, 2001).

Several clinical studies that addressed the relationship between depression and the enkephalin system also failed to provide a clear-cut picture. Shortly after the endogenous

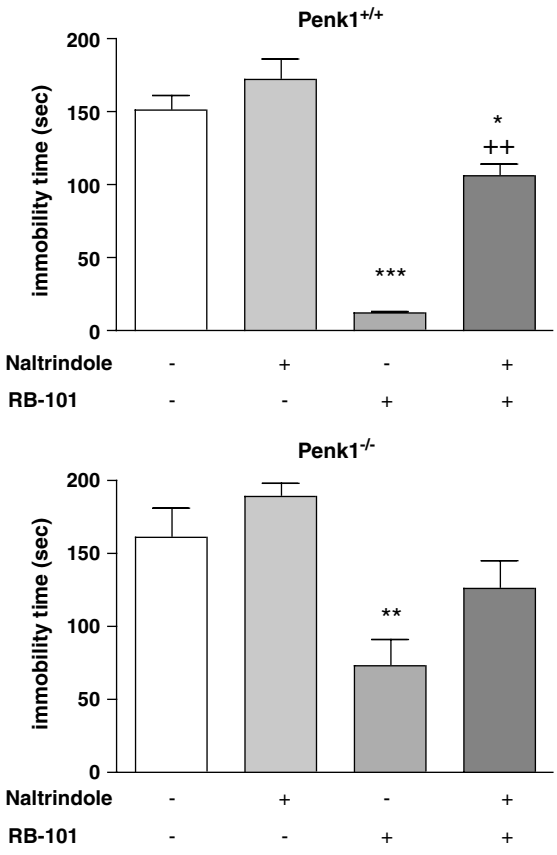

Figure 7 Effect of the DOPr blocker naltrindole on the antidepressant effect of RB-101 in wild-type and Penk1$^{-/-}$ mice. Each column represents the mean value (+SEM) of 8–10 animals. *$p<0.05$; **$p<0.01$; ***$p<0.001$ *vs* vehicle-treated group; ++$p<0.01$ *vs* RB-101-treated group using one-way ANOVA followed by Bonferroni's test.

opioid system was discovered it was even suggested that depression is an enkephalin deficiency syndrome (Ehrenpreis, 1982). This early hypothesis was not supported by a later clinical study when depressive patients were treated with an enkephalin analog. An alleviation of the symptoms was only observed after the first treatment (Jungkunz *et al*, 1983). Genetic linkage analysis also did not provide a comprehensive result: one early study excluded the association between Penk1 (Curtis *et al*, 1993) or neutral endopeptidase (Comings *et al*, 2000) and depression, but a more recent study found an association between proenkephalin gene and depression (Ogden *et al*, 2004).

It has been proposed that endogenous opioids, particularly enkephalins, contribute to the antidepressant effect of reference compounds, because their efficacy was antagonized by the non-specific opioid receptor antagonist naloxone (Tejedor-Real *et al*, 1995) or the DOPr-specific antagonist naltrindole (Zomkowski *et al*, 2005). Interestingly, the tricyclic catecholamine uptake inhibitor imipramine increased brain enkephalin level by antagonizing the activity of the neutral endopeptidase (de Gandarias *et al*, 1999). Although this effect was suggested to contribute to the antidepressant activity of the drug, our results do not support this hypothesis. In fact, none of four reference compounds evaluated in this study (nialamide, imipramine, and amitiptyline fluoxetine) showed a reduced efficacy in animals lacking enkephalin. On the contrary, the MED of nialamide and amitriptyline was even lower in the knockout mice. Thus these antidepressants maintained their antidepressant activity in animals with genetic deletion of enkephalin. However, our findings do not exclude the possibility of beneficial effects of increased enkephalin levels in the treatment of depression.

The most surprising result of our experiments was the residual efficacy of the enkephalinase inhibitor RB 101 in animals without enkephalin. The analgesic and antidepressant effect of RB 101 was thought to be based entirely on the elevation of enkephalin levels (Nieto *et al*, 2005). Nevertheless, RB 101 significantly decreased the time spent with immobility in the forced swimming test, albeit with a reduced efficacy when compared with wild-type animals. The lack of influence of deletion of Penk1 on the antidepressant effect of imipramine and RB-101 was the most surprising, hence we tested these drugs on mice on a C57BL/6J genetic background. Lack of Penk1 gene did not influence the antidepressant effect of the drugs on mice from the C57BL/6J strain, therefore we can exclude that a background gene effect hides the existence of the interaction.

Because it has been reported that enkephalins can also be produced from dynorphin precursors (Brownstein, 1980), we considered the possibility that enkephalin is still present in the absence of Pdyn, and perhaps elevated by the RB101 treatment, although we failed previously to detect any enkephalins in striatal extracts from Penk1 mice using highly sensitive radioimmunoassays (Konig *et al*, 1996). However, RB-101 still had an antidepressant effect in Penk1$^{-/-}$/Pdyn$^{+/+}$ double knockout animals, which conclusively demonstrates that this drug effect cannot be exclusively dependent on the elevation of enkephalin levels. Furthermore, pharmacological blockade of the DOPrs with naltrindole reduced the effect of RB-101 significantly, but not completely. Together these data suggest that RB-101 either also acts on another substrate, or protects another neuropeptide with antidepressant activity from degradation by the neutral endopeptidase and/or aminopeptidase N. This neuropeptide may even compensate for the lack of enkephalins in Penk1$^{-/-}$ animals and contribute to the absence of a depression-related phenotype in Penk1$^{-/-}$ mice. The identification of this peptide could have a significant impact in depression research.

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