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Increased stress-induced analgesia in mice lacking the proneuropeptide convertase PC2

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Abstract

Many neuropeptides involved in pain perception are generated by endoproteolytic cleavages of their precursor proteins by the proprotein convertases PC1 and PC2. To investigate the role of PC2 in nociception and analgesia, we tested wild-type and PC2-null mice for their responses to mechanical and thermal nociceptive stimuli, before and after a short swim in cold or warm water. Basal responses and responses after a cold swim were similar between the two groups. However, after a short forced swim in warm water, PC2-null mice were significantly less responsive to the stimuli than wild-type mice, an indication of increased opioid-mediated stress-induced analgesia. The enhanced analgesia in PC2-null mice may be caused by an accumulation of opioid precursor processing intermediates with potent analgesic effects, or by loss of anti-opioid peptides.

Keywords: Proprotein convertase; Stress-induced analgesia; Nociception; Opiates; Neuropeptides; Proteolytic processing

Proprotein convertase 2 (PC2) is a member of the subtilase family of proteinases implicated in the proteolytic maturation of precursor proteins by cleavages after selected pairs of basic residues [29]. PC2 is primarily expressed in neuronal and endocrine cells where, together with PC1 and carboxypeptidase E (CPE), it mediates the production of neuropeptides and hormones. The function of CPE is to trim off the C-terminal basic residues exposed by PC1 and PC2 cleavages [10]. Studies have shown that PC1 and PC2 often process the same substrates, at distinct cleavage sites or at the same sites with differing cleaving efficiency [3,17,27,31]. Thus, the pattern of peptides derived from the proteolytic processing of prohormones and proneuropeptides in neuroendocrine cells is partly determined by the relative abundance as well as the cleavage site preference of these two convertases.

Compared to wild-type (WT) mice, PC2 knockout (KO) mice maintained in our colony appeared to be relatively less defensive during ear-tagging. This casual observation led us to speculate that these mutant mice may be less sensitive to pain. Precursors to most nociceptive and analgesic neuropeptides are potential substrates for PC1 and PC2. These include precursors to substance P (SP), nociceptin/orphanin FQ (N/OFQ), cholecystokinin (CCK), corticotropin-releasing factor (CRF), enkephalins (ENKs), dynorphins (DYNs), β-endorphin (β-END), neurotensin/neuromedin N (NT/NN), somatostatin (SS), oxytocin (OT) and galanin. In the brain, PC2 is more abundant and more widely distributed than PC1 [38]. Molecular characterization of PC2 KO mice has confirmed the primary, albeit not exclusive, role of this enzyme in the proteolytic processing of most of these proneuropeptides [1,4,13,18,24,36].

In this study, we have examined the consequences of PC2 deficiency on nociception and analgesia in mouse, before and after a short swim stress.

PC2 KO mice [11] were received as heterozygotes that had been backcrossed for four generations into CD-1 genetic background. They were perpetuated by brother–sister heterozygous mating. They were housed in temperature-controlled rooms with 12-h dark:12-h light cycles and were provided with food and drink *ad libitum*. They were handled according to the guidelines of the Canadian Council on Animal Care. The mice used in this

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study were 8–12 months old, WT ($n = 7$) and PC2-KO ($n = 6$) males.

Mice were acclimatized to the test cage or chamber for a minimum of 30 min before the stress was applied and the tests conducted. Baseline threshold readings were taken two to three times for each mouse. Stress induction was induced by forcing the mouse to swim for 180 s in a 4-L beaker containing 2 L of water at 10 °C (cold swim) or at 33 °C (warm swim); it was removed from the water, patted dry with a towel, allowed to rest for 2 min and then tested 5 min and 30 min after swim. In a mechanical nociceptive test, the mouse was placed in a cage with a grid floor; Von Frey hairs of increasing force were applied in ascending order (1–15 g) to the plantar surface of hind paw until a flexion reflex was observed. In a tail-flick thermal nociceptive test, the mouse tail tip was placed over a beam of light set at an intensity which yielded baseline latencies of approximately 5 s, and the time elapsed before the mouse flicked its tail from the light source was measured. A 15-s cutoff time was implemented to prevent injury to the tail. In a hotplate thermal nociceptive test, the mouse was placed on a metal plate thermostatically maintained at 50 °C and the time elapsed before it licked its hind paws or jumped off the hot-plate was measured. A 40-s cutoff was implemented to prevent injury.

In a formalin nociceptive test, the mouse was placed into an observation cage consisting of a 30 cm × 30 cm × 30 cm Plexiglass box with a mirror below the floor at 45° angle to allow unobstructed view of the paws; it was given a 10-μl subcutaneous injection of 2.5% formalin into the plantar surface of one hindpaw to induce an acute inflammatory response. It was then monitored for 50 min and nociceptive behavioral categories were recorded every 5 min. These categories and nociceptive weights were as follows: category (1) weight 0, the injected paw is not favored; category (2) weight 1, the injected paw has little or no weight on it; category (3) weight 2, the injected paw is elevated and is not in contact with any surface; category (4) weight 3, the injected paw is licked, bitten or shaken. A weighted average nociceptive score, ranging from 0 to 3, was calculated by multiplying the time spent in each category by the category weight and dividing the product by the total time for each 5 min time block [9].

Values are expressed as means ± standard errors of means (S.E.M.). Student's *t*-test was applied to compare non-paired groups for a single independent variable; two- and three-way ANOVA to compare data sets with multiple variables. $p < 0.05$ was considered significant.

The baseline thresholds readings (column I) were similar between the two genotypes in mechanical tests (panel A: 1.48 ± 0.08 g in WT; 1.5 ± 0.16 in KO), in tail-flick thermal tests (panel B: 5.01 ± 0.34 s in WT; 4.8 ± 0.15 s in KO), and in hotplate thermal tests (panel C: 17.01 ± 1.79 s in WT; 18.00 ± 0.67 s in KO).

After a cold swim (column II), readings at 5 min significantly increased above baseline to comparable levels between genotypes: 8–10-fold in mechanical tests (panel A: 12.84 ± 1.39 g in WT; 15 ± 0.00 g in KO), 2–3-fold in tail-flick tests (panel B: 9.76 ± 1.65 s in WT; 15.00 ± 0.00 s in KO), 1.5–2-fold in the hotplate (panel C: 24.6 ± 4.75 s in WT; 40.00 ± 0.00 s in KO). At 30 min, the readings returned to baseline levels in mechanical tests (panel A: 1.91 ± 0.40 g in WT; 2.98 ± 0.83 s in KO) and in tail-flick tests (panel B: 6.13 ± 0.76 s in WT; 8.04 ± 0.35 s in KO); in hotplate tests (panel C), there were 2-fold below baseline in WT mice (8.60 ± 1.77 s) and 1.5-fold above baseline in KO mice (27.00 ± 0.23 s).

After a warm swim (column III), WT readings at 5 min in mechanical, tail-flick and hotplate tests were 1.91 ± 04 g, 4.57 ± 0.44 s, 14.04 ± 1.41 s, respectively and were all not significantly different from their respective baseline; in contrast, KO readings in the three tests increased 6 ($p < 0.0001$), 2 ($p < 0.001$) and 1.5 ($p < 0.01$)-fold to 12 ± 1.93 g, 11.83 ± 0.66 s, and 26.00 ± 1.26 s, respectively. At 30 min, the readings for both genotypes were not significantly different from their respective baseline in mechanical tests (panel A: 2.98 ± 0.82 g in WT; 4.10 ± 2.18 g in KO) and tail-flick tests (panel B: 3.37 ± 0.24 s in WT; 6.89 ± 1.03 s in KO); in hotplate tests, the readings decreased 1.5–2-fold below baseline in both genotypes (panel C: 7.89 ± 0.76 s in WT and 11.26 ± 1.82 s in KO).

Nociceptive behavior scores of WT and KO mice following formalin injection are shown in Fig. 1D. Despite a trend towards an attenuated second phase nociceptive response of PC2 KO mice, the differences between the two groups were not significant.

Stress induces two types of analgesia in animals: opioid-mediated and non-opioid-mediated analgesia. Stress caused by a short swim in water induces opioid-mediated analgesia when the water is warm and NMDA-induced analgesia when it is cold [16,22,34]. Tests conducted in this study indicate that, relative to WT mice, PC2-KO exhibit increased opiate-mediated stress-induced analgesia, at both the spinal (tail-flick test) and supraspinal (mechanical and hotplate tests) levels. However, the sustained analgesia demonstrated by these mutant mice in the hotplate test, 30 min after a cold swim suggested that supraspinal non-opioid response mechanisms may also be affected. Interestingly, opioid mediated analgesia was not induced by warm swim stress in WT mice, as their mechanical and thermal thresholds were unchanged or lower relative to baseline. This may be due to the age, the gender or the genetic background of the mice used in the study. Indeed, these parameters are known to influence pain perception in mice [20,21,23,32].

Brain regions associated with SIA include the pituitary, the hypothalamus, the amygdala, the periaqueductal gray, the nucleus raphe magnus, the nucleus raphe pallidus, and the medial preoptic area [5,35]. Several neuropeptides found in these regions have been implicated in opioid-dependent and opioid-independent SIA: βEND, ENKs, DYNs, NT/NN, and SP promote it, whereas CCK, OFQ/N, FMRFamide and neuropeptide FF (NPFF) oppose it [6,19,25,30]. These neuropeptides are generated by endoproteolytic cleavages of larger precursors at PC recognition sites. The production of several of them has been examined in PC2-KO mice. Compared to WT mice, these mutant mice contained less βEND 1–31 [1], Met-ENKs and Leu-ENKs [18], OFQ/N [1], DYNs A-8, A-9 and B-13 [4], NN [36] and CCK-8 [37] in their brain extracts (Table 1). The fact that, except for DYN(1–8), all these neuropeptides were detectable suggests that PC1 or other convertases can cleave the