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Role of the blood-brain barrier in differential response to opioid peptides and morphine in mouse lines divergently bred for high and low swim stress-induced analgesia

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Over 20 years ago, the Sadowski group separated two mouse lines, one with high (HA) and the other with low (LA) sensitivity to swim stress-induced analgesia (SSIA). Recently, we proposed that increased leakage of the blood-brain barrier (BBB) in the HA line created the difference in the response to SSIA. To search for further evidence for this hypothesis, differences in the levels of the BBB proteins occludin and claudin-5 were analysed. In addition, we sought to evaluate practical differences in BBB permeability by examining the antinociceptive levels in HA and LA mouse lines after i.v. administration of peptides that have limited access to the CNS. Western blot was used to analyse the differences between occludin and claudin-5. To evaluate the functional differences between the BBB of HA and LA mice, the antinociception levels of endomorphin I, biphalin and AA2016 (peptides with limited BBB permeabilities) in the tail flick test were examined. The expression levels of occludin and claudin-5 in the HA mouse line were lower than in the LA and control mice. Central antinociception of the opioid peptides were significantly higher in the HA line than in the LA and control lines. Our data support the hypothesis that BBB leakage is responsible for the differences between the HA and LA mouse lines. Although SSIA confirmed BBB differences between both lines, it is not limited to the opioid system and could be a useful model for studying the role of the BBB in molecular communications between the periphery and CNS.

Key words: blood-brain barrier, swim stress-induced analgesia, high analgesia mouse line, low analgesia mouse line, intravenous, intrathecal, tail-flick pain test

INTRODUCTION

Stress-induced analgesia is suppression of pain sensitivity upon exposure to a stressful stimulus. This behavioural phenomenon has been known for over 30 years in experimental animals (Akil et al. 1976) as well as in humans (Carr et al 1981). Over 20 years ago, a group headed by Sadowski separated two strains of mice, one with high sensitivity (HA) and the other with low (LA) sensitivity to stress-induced analgesia initiated by swim stressor (SSIA), followed by measurement of analgesia with the tail-flick test (Panocka et al. 1986a,b). Following this study, both strains were extensively screened for biochemical differences. Although these studies identified some phenotypic differences (Panocka et al. 1991, Marek et al. 1993, Sadowski and Panocka 1993, Sadowski and Konarzewski 1999, Kest et al. 1999, Sacharczuk et al. 2010a) none of them fully explained the differences in stress-induced analgesia.

Just recently, electron microscopy structural analysis (Gajkowska et al. 2011) proposed that increased leakage of the blood-brain barrier (BBB) in the HA line created the differences between the HA and LA lines in response to SSIA. The microvascular structure of the LA line is similar to the healthy, control group of Swiss-Webster mice, whereas the HA line is characterised by a leaky capillary wall. The BBB ultrastruc-

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tural differences between the HA and LA mice indicate that BBB leakage in the HA mouse could be a major reason for the high response to SSIA. It is well known that stress elevates peripheral endogenous opioid peptide levels (e.g., beta-endorphins). The SSIA phenomenon can be explained as a result of the influx of endogenous peripheral opioids into the CNS, which induces central analgesia.

Another example of the functional influx of a peripheral peptide into the CNS has been presented recently (Kastin and Pan 2010). In the current study, we present additional evidence that BBB leakage differentiates the HA and LA mouse lines. The permeability of the BBB is highly dependent on the functional integrity of the interendothelial junctional complex, especially tight junctions (Wilhelm et al. 2011). The principal transmembrane proteins of the cerebral endothelial tight junctions include occludin, claudins (mainly claudin-5) and junctional adhesion molecules (JAM) (Bauer et al. 2011, Liu et al. 2012). Low expression levels of these proteins can significantly contribute to an increase in BBB permeability (Krizbai et al. 2005). Therefore, we decided to investigate the expression of claudin-5 and occludin in mouse strains that show differential sensitivity to SSIA.

Because leakage of the BBB should influence the permeability of various circulating peptides, evaluation of the activity of peripherally applied opioid peptides characterised by limited CNS access was chosen. Here, we evaluate differences in the analgesic activity of intravenously administered endomorphin I, biphalin and AA2016 peptides in HA and LA mouse lines.

METHODS

The animals were handled according to the guidelines of the local ethics committee for experimentation on animals. They also conform to the International Association for the Study of Pain (Zimmerman 1983).

Animals

Swiss-Webster mice selectively bred towards high (HA) and low (LA) analgesia (Panocka et al. 1986b) were obtained from the Institute of Genetics and Animal Breeding at the Polish Academy of Sciences. Briefly, outbred mice were exposed to 3-min of swimming in 20°C water.Two minutes after completion of the swim, latency of the nociceptive reflex on a hot plate (at 56°C) was measured. Males and females displaying the longest (50–60 s) and the shortest (<10 s) post-swim latencies of the hind paw flick or lick response were chosen as progenitors of the HA and the LA line, respectively. A similar procedure was repeated in each offspring generation, and only subjects displaying the longest and the shortest post-swim hotplate latencies were mated to maintain the HA and the LA line, respectively. Unselected Swiss-Webster mice were used as the control group (C).

Western blotting

The samples for the western blots of occludin and claudin-5 from the brains of HA, LA and control animals (A) were prepared from one-half of the mouse brains (5 specimens from each of the three mouse cell lines). The proteins were electrophoresed using standard denaturing SDS-PAGE procedures and blotted on polyvinylidene difluoride (Pall, East Hills, NY, USA) or nitrocellulose (GE Healthcare, Waukesha, WI, USA) membranes. Blocking of the nonspecific binding capacity of the membranes was carried out at room temperature for 30 min in TBS-T (Tris-buffered saline with 0.1% Tween 20) containing either 5% milk (for occludin and β-actin) or 3% bovine serum albumin (for claudin-5). The blots were incubated with the following primary antibodies (diluted in TBS-T): anti-β-actin (Sigma): 1:5 000; anti-claudin-5 (Invitrogen): 1:500 and anti-occludin (TransLabs, Lexington, KY, USA): 1:1 000. After washing, the blots were incubated at room temperature with an anti-mouse IgG secondary antibody (TransLabs, Lexington, KY, USA) diluted 1:4 000 in TBS-T for 30 min and then washed again in TBS-T. The immunoreaction was visualised using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) on X-ray film (Agfa, Mortsel, Belgium).

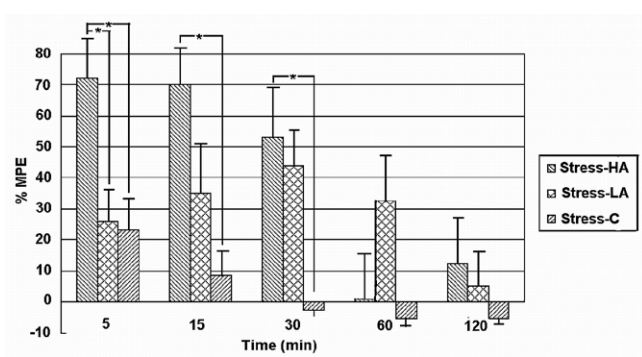

Fig. 1. Stress induced analgesia in HA, LA and C mouse lines

Drugs

Morphine was purchased from Polfa-Warszawa, Poland. Endomorphine I, biphalin and the AA2016 peptide were synthesised in house usingthe "in-solution" method described previously (Lipkowski et al. 1982, Lukowiak et al. 2009). All of the substances were in the hydrochloride form. On the day of an experiment, the tested substances were dissolved in 0.9% sterile saline and administered intravenously (IV). Morphine, endomorphine I and the AA2016 peptide were administered at a dose of 4 mg/kg, whereas biphalin was delivered at a dose of 2 mg/kg.

Antinociception

Antinociception was quantified using a radiant-heat tail flick nociception assay (Hau et al. 2002). The baseline latency was set at 2–3 seconds with a maximum exposure to heat (cut-off time) of 7 seconds to avoid thermal damage to the tail. The degree of antinociception was expressed as a percentage of the maximum possible effect (% MPE), calculated as % MPE = [(post-treatment latency − baseline latency)/(cut-off latency − baseline latency)] × 100. All of the experimental groups consisted of six mice. The drugs were administered intravenously (IV) into the tail vein. All of the substances were dissolved in 0.9% sterile saline and tested in doses previously established. Analgesia was assessed at time points of 5, 15, 30, 60 and 120 minutes. Naloxone (10 mg/kg IP; 100 mL bolus) was administered to confirm opioid receptor affinity of the peptides (not presented).

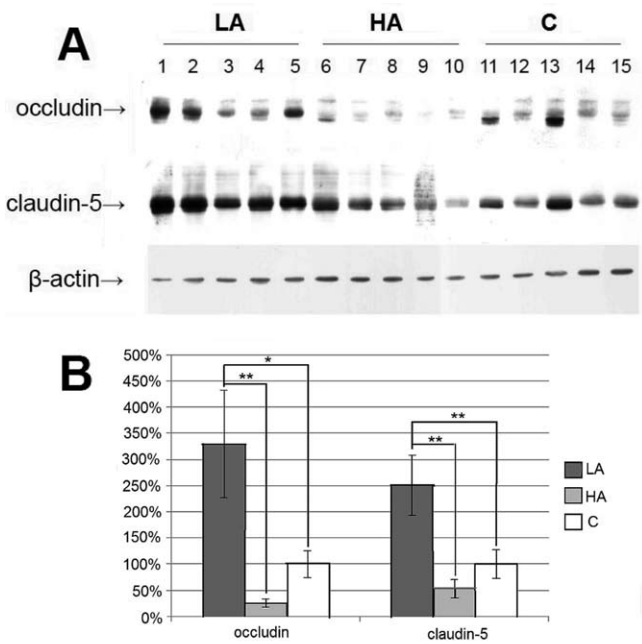

Fig. 2. Western blot of occludin and claudin-5 from brains of HA, LA and C mice lines (A) and densitometric analysis of the blots (B). Due to posttranslational modifications (phosphorylation on serine, threonine and tyrosine residues) occludin presents a series of bands between 62 and 82 kDa on the blots (Gonzalez-Mariscal et al. 2003). The bands used for quantification have been marked with "→(". In case of claudin-5 only one band of 23 kDa (marked with "→") has been analysed.

Fluorescence activity of mouse brain extracts

AA2016 (4 mg/kg) was injected intravenously into the HA, LA and C mouse lines. After 0.5 h, the animals were decapitated and the brain was removed. Samples were prepared from the mouse brains from 5 homogenate specimens from each mouse line. The homogenates were mixed with 4 volumes of ethanol and centrifuged. The fluorescence of the supernatants was measured at 400 nm. The differences in the emission intensities are presented in Results.

Statistics

The data were analysed either with a one-way ANOVA or a two-way RM ANOVA followed by the Bonferroni *post-hoc* test for multiple comparisons. The data were presented as the mean ± SEM. Statistically significant differences are shown as a value equal to or exceeding (*) $P<0.1$ and (**) $P<0.05$.

RESULTS

Our experiments showed that swim stress significantly differentiates between the lines we examined in terms of SSIA magnitude, as assessed with the TF test (Fig. 1). The amount of SSIA in the NaCl-injected groups was considerably greater in the HA mice compared to the LA or C mice. This result indicates a short-lived, but substantial increase in the magnitude of SSIA with an SSIA peak at 5 min after the swim. The LA line expressed some elevation in the SSIA magnitude, but this was not statistically significant from the control group. Moreover, the effect of time was non-significant in the LA line, which indicates that the low SSIA values remained relatively stable across the measured time points. However, the low SSIA in the LA mice was substantially higher than in the C mice.

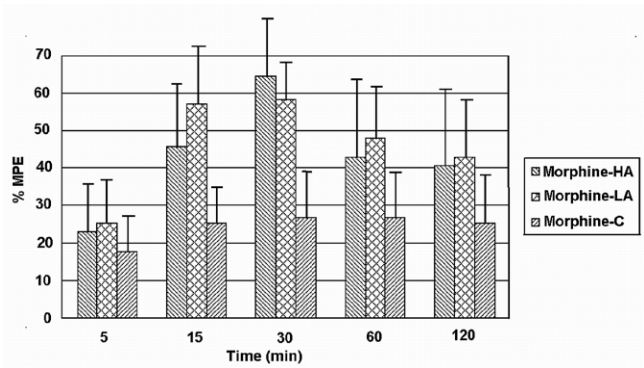

Fig. 3. Antinociception of morphine (4 mg/kg) in HA, LA and C mice. No significance were observed between mice lines.

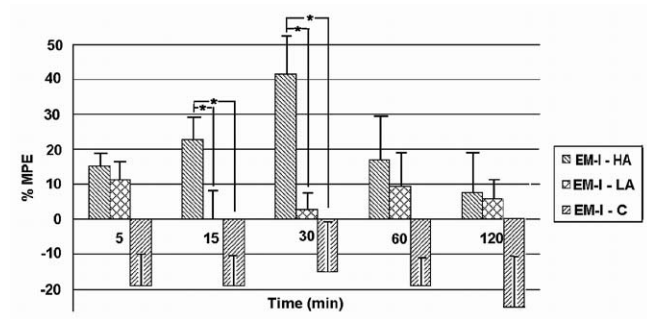

Fig. 4. Antinociception of endomorphin I (4 mg/kg) in HA, LA and C mice lines (*P<0.05, **P<0.001).

Western blots of occludin and claudin-5 from the brains of HA, LA and C animals showed a differential expression pattern (Fig. 2A). Densitometry analyses of the blots (Fig. 2B) showed that both occludin and claudin-5 concentrations are lower in the HA lines compared to the LA and C lines (Fig. 2B). Expression of both proteins was significantly lower in the HA mice compared to the LA.

Morphine given intravenously to the HA, LA and control lines resulted in the same level of antinociception in all three mouse lines (Fig. 3). In contrast, endomorphin I (at a dose of 4 mg/kg, IV) given to the control and LA mice did not induce any antinociception. However, the same dose induced significant antinociception in the HA mice (Fig. 4). Similar results were obtained in the case of intravenous administration of biphalin. The antinociceptive effect in the HA line was very strong and prolonged (Fig. 5). An antinociceptive effect in the LA line was also observed at the dose applied but was significantly lower. The antinociceptive effect of AA2016 was also strong in the HA line and significantly higher than the observed antinociception in the LA and control mice (Fig. 6). All antinociceptive effects were reversed by naloxone that confirmed opioid receptors involvement. The concentrations of AA2013, estimated as fluorescence activity of the ethanol extract in the brain homogenates, showed higher values in the HA line compared to the LA line and the control animals (Fig. 7).

DISCUSSION

The BBB is located at the interface of the CNS and the periphery. Blood circulation into the BBB regulates the passage of signalling molecules from the periphery into the CNS, as well as from the CNS into the periphery. Differences in BBB permeability are implicated in the pathogenesis of a considerable number of neurological disorders (Neuwelt 2004, Persidsky et al. 2006, Friedman et al. 2009) and also play important roles in individual responses to various behavioural and environmental factors (e.g., Banks and Kastin 1996, Lossinsky and Shivers 2004, Hawkins and Egleton 2008). Our recent analysis indicated that a pathological structure of the BBB in the HA mouse

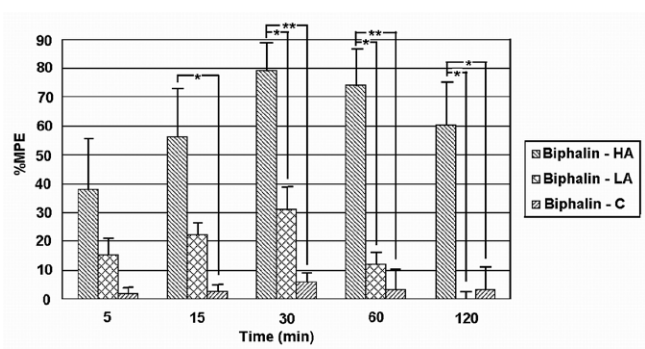

Fig. 5. Antinociception of biphalin (2 mg/kg) in HA, LA and C mice lines (*P<0.05, **P<0.001)

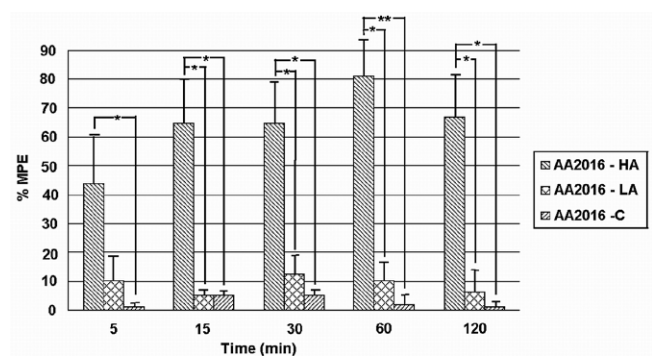

Fig. 6. Antinociception of AA2016 (4 mg/kg) in HA, LA and C mice lines (*P<0.05, **P<0.001).

line differentiates the two mouse lines (HA and LA), which have different sensitivity to SSIA. We hypothesised that the significantly stronger analgesia observed in the HA strain is a result of penetrating endogenous opioids from the periphery into the CNS (Gajkowska et al. 2010). The observed small levels of SSIA in the LA mice indicated that even in this line, stress slightly influenced BBB permeability. Further analysis of occludin and claudin-5 concentrations showed significant decreases in the concentration of both proteins.

These data provide further evidence for BBB impairment of the HA mouse. Additional arguments were provided using *in vivo* studies of peripherally applied morphine and opioid peptides. The morphine, which has quite well BBB permeability, applied intravenously expressed similar high antinociceptive effects for all HA, LA and C mouse lines. Endomorphin I is a tetrapeptide that is a very potent analgesic after central administration (ICV or IT), but its lack of effective BBB permeability results in loss of activity after systemic (IV) application (Zadina et al. 1997, Mallareddy et al. 2012). In our studies, we confirmed that intravenous administration of endomorphin I at a dose of 4 mg/kg did not express significant antinociception in the tail-flick test in both the C and LA groups. On the contrary, the same dose produced significant antinociception effects in the HA mouse line, confirming a more effective influx through the BBB.

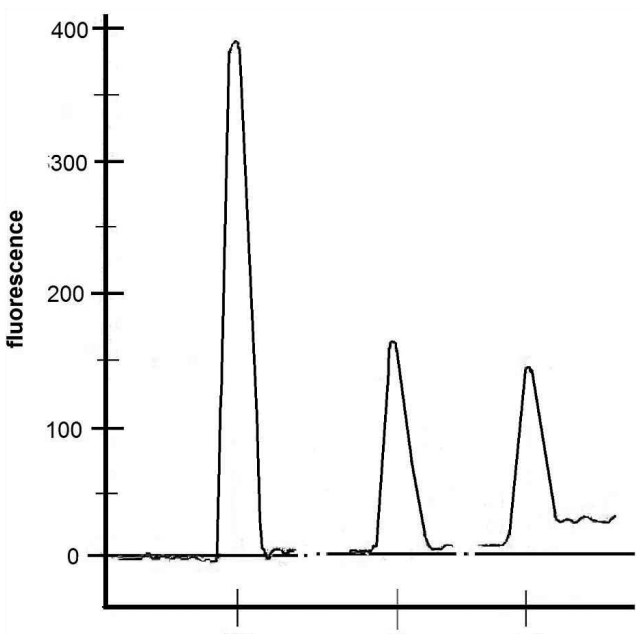

Fig. 7. Level of AA2016 in whole brain homogenate in HA, LA and C mice lines

Biphalin is a dimeric enkephalin analogue that induces antinociception similar to morphine after intravenous administration. However, after application directly to the CNS, it induced over 100-times higher antinociception than morphine (Kosson et al. 2008), which indicates the limitation of BBB permeability. Therefore, the significantly higher antinociception in the HA compared to the LA line provides evidence for differences in BBB functional effectiveness. Similar differences have been observed during the intravenous administration of the fluorescent opioid peptide analogue AA2016 (Lukowiak at al. 2009). The differences between observed antinociception in HA and LA and C lines well correlate with fluorescence differences of brain extracts. Although the proportion of the analgesic effects between the intravenous administration of opioid peptides in the HA and LA mouse are significant, their levels did not reach the activity levels of peptides applied directly to the CNS (IT or ICV). These data may suggest that the BBB in the HA line is only deviated rather than fully open. Higher fluorescence of the HA brain extracts compared to the LA and control lines additionally confirms that the BBB is more permeable to opioid peptides in the HA line. The provided data support our previous hypothesis that BBB leakage is a major difference between the two mice lines divergently bred for high and low SSIA. The functional differences of the BBB in the HA and LA mouse lines are summarised in Figure 8.

In mice, stress induces the release of endogenous peptides into the bloodstream. In the HA mouse, the leaky BBB allows the penetration of systemic circulating endogenous opioid peptides into the CNS to stimulate antinociception, described as SSIA. Opioid peptides given intravenously behave similarly to endogenous opioids, effectively penetrating into the leaky BBB in the HA line. The permeability of the BBB in the LA line is proportional to the peptide properties but is generally decreased in comparison to the HA line. On the contrary, the BBB permeability of morphine is similar in the three mouse lines (C, HA and LA).The lack of significant differences in non-peptidic (morphine or SN80) BBB permeability was most likely the reason behind the failure to obtain conclusive results after years of searching for differences between the HA and LA mouse lines.

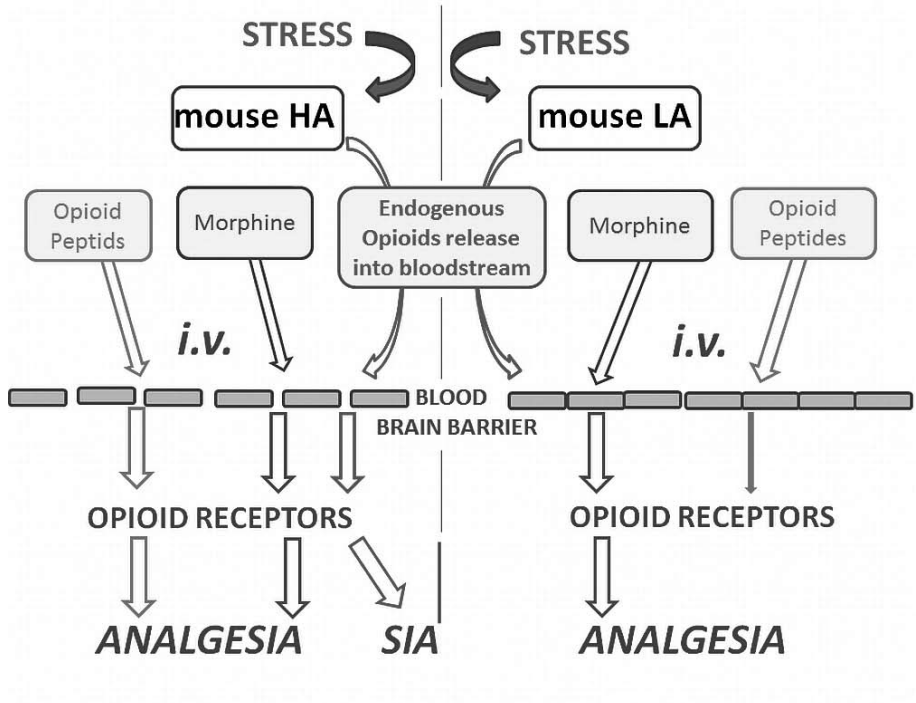

Fig. 8. Cartoon presentation of BBB function in LA and HA mouse lines

CONCLUSIONS

Our results indicate that an altered BBB is the basis for differences in the stress response between the HA and LA mouse lines. Endogenous opioid peptides released by stress in the periphery can penetrate more easily into the CNS in the HA animals to induce central analgesia. Similarly, exogenous peptide applied to the periphery (IV) can penetrate the BBB and induce central analgesia. In contrast, the BBB in the LA line and control mice limits the permeability of endogenous and exogenous peptides, resulting in reduced central analgesia. The presented results are focused on opioids and stress-induced analgesia. However, the HA/LA two mouse line model may serve as a general natural model of pathological impairment of BBB permeability in studies of communication between the CNS and periphery.

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