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ORIGINAL RESEARCH REPORT

Enkephalin downregulation in the nucleus accumbens underlies chronic stress-induced anhedonia

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Abstract

Restraint and immobilization have been extensively used to study habituation of the neuroendocrine response to a repeated stressor, but behavioral consequences of this stress regimen remain largely uncharacterized. In this study, we used sucrose preference and the elevated-plus maze to probe behavioral alterations resulting from 14 days of restraint in rats. We observed a decrease in sucrose preference in stressed animals, particularly in a subgroup of individuals, but no alteration in anxiety behaviors (as measured in the elevated-plus maze) four days following the last restraint. In these low-sucrose preference animals, we observed a downregulation of the expression of preproenkephalin mRNA in the nucleus accumbens. Furthermore, we observed a strong correlation between enkephalin expression and sucrose preference in the shell part of the nucleus accumbens, with a lower level of enkephalin expression being associated with lower sucrose preference. Interestingly, quantification of the corticosterone response revealed a delayed habituation to restraint in the low-sucrose preference population, which suggests that vulnerability to stress-induced deficits might be associated with prolonged exposure to glucocorticoids. The induction of ΔFosB is also reduced in the nucleus accumbens shell of the low-sucrose preference population and this transcription factor is expressed in enkephalin neurons. Taken together, these results suggest that a ΔFosB-mediated downregulation of enkephalin in the nucleus accumbens might underlie the susceptibility to chronic stress. Further experiments will be needed to determine causality between these two phenomena.

Keywords

ΔFosB, corticosterone, opioid, resilience, sucrose preference, vulnerability

History

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Introduction

Stressful life events are one of the most important risk factors in the development of anxiety disorders and major depression (Kendler et al., 1999). In rodents, chronic stress has been used to induce many behavioral and physiological features related to depression, including pronounced weight loss, increased anxiety, dysfunctional sleep, as well as anhedonia (Krishnan & Nestler, 2008). This last feature is one of the core symptoms of depression and it can be experimentally induced by chronic unpredictable stress, which consists in a regimen of varying stressors applied for an extended period of time (Willner, 2005). In contrast to chronic unpredictable stress, the neuroendocrine response to a repeated and predictable stressor (i.e. restraint or immobilization) habituates overtime (Grissom & Bhatnagar, 2009; Márquez et al., 2004). There is an important inter-individual variability in habituation to repeated restraint, and presumably this variability can have repercussions on physiological and behavioral impacts of stress as well.

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The endogeneous opioid enkephalin (ENK) is a prime candidate for providing an interface between chronic stress and its ensuing behavioral deficits. ENK activates both μ and δ opioid receptors (OR) *in vivo* and it has been implicated in behavioral and neuroendocrine aspects of the stress response. ENK is derived almost exclusively from the precursor protein proenkephalin. ENK deficient mice are overly anxious and have an exacerbated stress response (Bilkei-Gorzo et al., 2004, 2008; König et al., 1996; Ragnauth et al., 2001). A reduction in striatal preproenkephalin (ppENK) mRNA density was observed following repeated immobilization (Lucas et al., 2007), and hypothalamic ppENK expression may be involved in habituation to repeated restraint stress (Dumont et al., 2000). Overall, ENK release is thought to attenuate the aversive and anxiogenic aspects associated with stress exposure.

ENK is highly expressed in the nucleus accumbens (ACC). ENK neurons have local collaterals, and also send heavy projections to the ventral pallidum (VP; van Dongen et al., 2005; Zahm & Heimer, 1990; Zhou et al., 2003). ENK release and μ-OR activation in the ACC and VP are associated with hedonic perception (Barbano & Cador, 2007; Smith & Berridge, 2005). For example, the μ-OR agonist DAMGO facilitates ''liking'' reactions to sucrose, as defined by the hedonic responses of tongue protusions and paw licking, when

injected locally in the ACC and VP (Smith & Berridge, 2007). ENK may well be the endogenous substrate implicated in the activation μ-OR as indicated by the proximity between ENK fibers and μ-OR immunoreactivity in the ACC (Svingos et al., 1996). In addition, there is accumulating evidence that the transcription factor FosB, and its truncated isoform ΔFosB, play an important role in the processing of natural rewards (Vialou et al., 2010; Wallace et al., 2008). In this study, we investigated potential behavioral deficits that could be induced by repeated restraint stress in a population of rats, and we then asked if ENK expression and ΔFosB could be implicated in the development of the observed behavioral deficit.

Material and methods

Animal care

Forty-four Sprague Dawley male rats (weighting 225–250 g; Harlan) were given *ad libitum* access to rat chow and water, and singly housed in a 12 h light on; 12 h light off facility. The investigation conformed to the guidelines of the Canadian Council on Animal Care and was approved by the institutional animal care committee. Upon arrival, rats were given access to a bottle of 1% sucrose solution and a bottle of water for 14 days. Bottles were switched side and weighed daily. Five days before beginning of the stress procedure, sucrose and water intake was used to determine the initial sucrose preference of each animal.

Stress and blood sampling procedures

The experimental protocol consisted of daily sessions of 1 h restraint stress (between 08:00 and 10:00 h) in adjustable restraining cages (Centrap) for 14 days (Figure 1A). All animals were weighed daily and control animals were otherwise left undisturbed in the home cage. Four blood samples were taken through the saphenous vein on the first restraint session (day 1), on day 7 and on day 14. To minimize the stress induced by the sampling procedure, animals were handled 3 days before blood sampling. During handling, animal legs were shaved and animals were habituated to a short restraint. Blood samples were collected before the beginning of stress procedure, immediately after restraint session, and 45 minutes after animals were returned to their home cage. For blood sampling, animals were gently restrained in a piece of cloth, a 22-gauge needle was introduced into the saphenous vein, blood was withdrawn and collected in microhematocrit tube (CB300; Sarstedt, Montreal, Canada). After the blood was collected, bleeding was stopped by applying a small pressure over the area of the sampled. Preliminary data showed this blood sampling method to be minimally stressful for the animal, and to allow serial samplings without using anesthesia (Diehl et al., 2001). Blood was kept on ice until centrifugation and collection of serum. Radioimmunoassay for corticosterone analysis was performed according to the manufacturer protocol using an antiserum raised in rabbits against a corticosterone–BSA conjugate and ^{125}I corticosterone–BSA as tracer (MP Biomedical, Montreal, Canada).

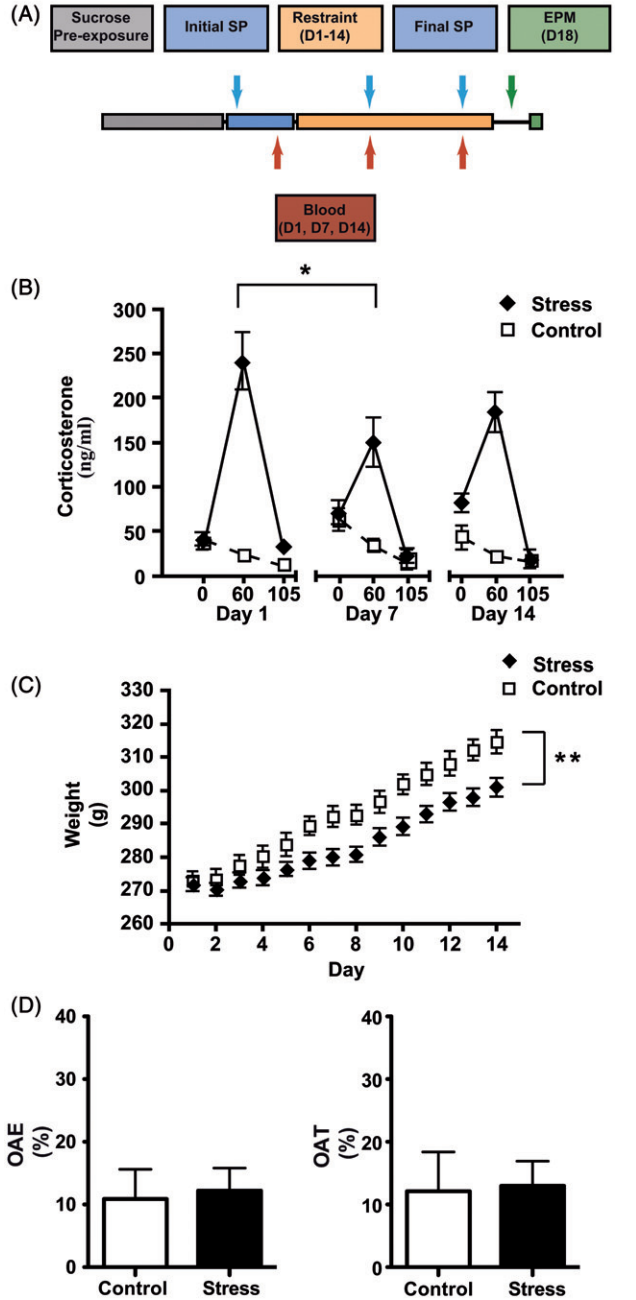

Figure 1. Physiological and behavioral consequences of repeated restraint. (A) Before stress exposure, animals were pre-expose to 1% sucrose solution and the initial sucrose preference was recorded. Sucrose preference was determined by measuring the amount of sucrose solution consumed, compared to water, over a 12 h period. The stress procedure consisted of daily 1 h restrain session for 14 consecutive days. Sucrose preference was measured during the stress protocol at two time points over two consecutive days (days 6–7; days 13–14). Blood was sampled before and after restraint on day 1, 7, and 14. Finally animals were exposed to the elevated-plus maze four days after the last restraint. (B) We observed a modest habituation of the corticosterone response between day 1 and day 7. We also observed a sensitization of basal corticosterone levels on day 14. (C) Stress had a prominent impact on weight gain. (D) No significant effect of stress was observed on the elevated-plus maze in the percentage of open-arm time ratio (OAT) and in the percentage of open-arm entries ratio (OAE).

Sucrose preference and elevated-plus maze

During the stress procedure, sucrose preference was measured on the 6th and 7th days, and on the 13th and 14th days.

The measure was taken by pre-weighing water and sucrose 1% solution filled bottles. Animals were given two bottles choice for 12 h, from 19h00 to 7h00 (during their active phase), and the bottles were weighed post-consumption. The position of the bottles was alternated between tests to avoid a place preference confound. Elevated-plus maze testing was done four days after the last restraint session according to previously published procedures (Bondi et al., 2008; Pellow et al., 1985; Walf & Frye, 2007). We used this timeframe because we did not want to measure acute anxiety generated by the last restraint session and/or the blood sampling procedure. Moreover, an anxiety inducing effect has previously been reported following chronic stress using a similar timeframe (Bondi et al., 2008). Four days following the last restraint, animals were transported into the testing room and left at least 15 minutes in their home cage. Testing was conducted under a 60 dB background white noise and 60 lux illumination as measured in the center of the maze. The elevated-plus maze consisted of four beige plastic arms (10 × 50 cm), two opposite arms being enclosed by 50 cm high walls (closed arms). The maze was elevated 50 cm from the floor and a camera was located about 2 meters above the maze. At the beginning of the testing session, the animal was put in the center of the maze and animal behaviors were recorded for 5 minutes. Animal behavior was tracked and analyzed with ANY-MAZE (Stoelting Inc., Wood Dale, IL) installed on a personal computer. The accuracy of ANY-MAZE tracking of behaviors was validated by the experimenter. The open-arm time percentage was calculated by taking the time spent in open arms of the maze, divided by the overall time spent in both open and closed arms. The open-arm entry percentage was calculated by dividing the number of entries in open arms of the maze by the overall number of entries.

Perfusion and tissue processing

Rats were anesthetized with intraperitoneal ketamine/xylazine solution (80 and 10 mg/kg, respectively) and perfused transcardially with 300 ml of saline solution (0.9% NaCl), followed by 500 ml of 4% paraformaldhehyde (PFA) in 0.1 M borax buffer (pH 9.5). All steps and solutions used in perfusion and tissue processing were at 4 °C unless mentioned otherwise. Brains were subsequently removed and postfixed 90 min in 4% PFA. After postfixation, the brains were cryoprotected by a 36 h immersion in 20% sucrose diluted in the fixative solution, frozen on dried ice and stored for further processing. Frozen brains were sectioned at 30 μm using a sliding microtome in the coronal plane. Five one-in-five serial sections were collected in cold cryoprotectant solution and stored at −20 °C.

Radioactive *in situ* hybridization

For *in situ* hybridization, individuals representative of each group (control $n = 8$; low-sucrose preference $n = 6$; high-sucrose preference $n = 9$) were chosen. Protocols for riboprobe synthesis and *in situ* hybridization were adapted from previous investigations in this and other laboratories (Simmons et al., 1989; Poulin et al., 2006, 2008). Sections were mounted onto positively charged slides (Surgipath, Richmond, IL) and dried in vacuum. The prehybridization treatment included permeabilization in proteinase K (10 g/mL at 37 °C; Roche, Indianapolis, IL), acetylation in 0.25% acetic anhydride in 100 mM TEA, and finally dehydration in graded concentration of ethanol (EtOH).

Radioisotope-labeled antisense RNA copies were synthesized by incubating 250 ng of linearized plasmid in transcription buffer, 10 mM dithiothreitol, 0.2 mM ATP/GTP/CTP, 100 uCi of 35S-UTP, 40 U of RNase inhibitor (Roche), and 20 U of T7 RNA polymerase for 60 min at 37 °C. The cRNA probe directed against ppENK (938 bp) was generated from a plasmid generously provided by Dr Sabol (Yoshikawa et al., 1984) and target nucleotide −104 to 832. The radioactive riboprobes were purified with a RNA column (Roche). The probe (10^7 cpm) was mixed into 1 ml of hybridization solution [518 l of formamide, 62 l of 5 M NaCl, 10 l of 1 M Tris (pH 8.0), 2 l of 0.5 M EDTA (pH 8.0), 20 l of 50× Denhardt's solution, 207 l of 50% dextran sulfate, 50 l of 10 mg/ml tRNA, 10 l of 1 M dithiothreitol, and completed to final volume with RNAse-free water.

Hybridization was completed overnight on slide warmers set at 56 °C and 100 μL of the hybridization solution was put on each slide. Post-hybridization washes included 4× standard saline citrate (SSC), RNase A (20 g/mL, 37 °C), 2× SSC, 1× SSC, 0.5× SSC, 30 min 0.1 × SSC at 60 °C, and dehydrated in graded ETOH concentrations. After drying under vacuum, the sections were exposed on film for 16 h. Sections were then defatted in xylene and dipped in NTB2 nuclear emulsion (Kodak, Rochester, NY). After 7 days of exposition, the slides were immersed in D19 developer (Kodak), rinsed in H_2O, and fixed in a fixative solution (Kodak). Finally, slides were counterstained in thionin, dehydrated in ethanol, cleared in xylene and coverslipped with DPX (BDH Laboratories Supply, Toronto, Canada).

Immunohistochemistry for ΔFosB

ΔFosB was revealed using the avidin-biotin method on adjacent sections of the brains used for the ISH. Brain sections were washed in PBS, blocked for 30 min in PBS with 0.3% triton-X and 1% BSA. A rabbit polyclonal antibody raised against ΔFosB (SC-48; Santa Cruz Biotechnology, Dallas, TX) was incubated overnight at a concentration of 1:5000. This antibody recognizes both FosB and ΔFosB, but because immunostaining was carried 24 h after the elevated-plus maze, and 5 days after the last stress, we considered the protein detected to be the stable isoform ΔFosB in accordance with from the Nestler laboratory (Perrotti et al., 2004; Wallace et al., 2008). The biotinylated secondary antibody from Jackson Immunoresearch laboratories (1:1000) staining was followed by avidin-biotin amplification method (Vector Lab, Burlingame, CA). The immunohistochemical reaction was revealed with DAB substrate and slides were coverslipped with DPX (BDH Laboratories Supply). ΔFosB-positive cells were counted in the same regions quantified in the ISH experiment. Quantification was done according to an automated procedure. We first took photographs of the sections under identical conditions for all animals using the 20× objective of a DM4200 Leica microscope equipped with a MicroPublisher camera (Qimaging, Surrey, BC). The whole images (rectangle of 430 μm × 320 μm) were quantified for at least four sections per animal at different rostrocaudal level of

the nucleus accumbens with a proprietary automation on Openlab (Improvision). This automation selected positive objects according to their size and intensity of labeling. Every section counted by the automation was verified by an experimenter blind to the experimental conditions. This procedure was found to be unbiased, reproducible and correlated well with manual counting (less than 10% difference).

Double immunofluorescence for ΔFosB and fluorescent *in situ* hybridization

In order to reveal the colocalization of ΔFosB with preproenkephalin mRNA, we performed immunofluorescence combined with fluorescent *in situ* hybridization on floating sections (Travers et al., 2005). First, free-floating sections were washed with SSC 2× and incubated for 60 min in prehybridization buffer at 55 °C, composed of the hybridization solution (see previous protocol) with molecular grade salmon sperm DNA (250 ng/mL) and tRNA (250 ng/mL), under gentle agitation. Hybridization was carried out overnight at 55 °C with 100 ng/mL of digoxigenin-labeled RNA probe. Posthybridization treatments included 3 washes in SSC 2×, 30 min of SSC 2× in 50% formamide (55 °C), 10 min in SSC 1×, 10 min SSC 0.5×, 10 min SSC 0.1×, 30 min SSC 0.1× in 50% formamide (55 °C). Immunofluorescent detection of ΔFosB was carried out with a block in PBS, 0.3% triton-X and 0.5% normal donkey serum and incubation in the primary antibodies (1:5000; SC-48 and anti-DIG-POD) overnight in the blocking solution. Following washes with PBS + 0.05% Tween-20, DIG-labeled probe was visualized with Cy3-tyramide using the tyramide amplification kit (PerkinElmer) according to the manufacturer's instruction. Following more washes, sections were incubated in Alexa-488 donkey anti-rabbit (1:100; Invitrogen) for 2 h and the sections were coverslipped with Vectashield mounting medium (Vector Laboratories). No labeling was observed with the antisense probe, which was included in every experiment as a control for *in situ* hybridization. In addition, *in situ* hybridization labeling with this protocol was identical to what we obtained in prior *in situ* hybridization studies from out group (Poulin et al., 2008). Immunofluorescent labeling for ΔFosB was very similar to the immunohistochemistry protocol, although perhaps with lower sensitivity.

Results

Animals were given free access to both a 1% sucrose solution bottle and a water bottle upon arrivals for fourteen days. Before the first restraint session, rats were randomly assigned to either the control ($n = 12$) or the experimental ($n = 32$) group. There was no initial difference in weight or in sucrose preference between the two groups. Fourteen days of restraint stress had a profound impact on weight with a 30.8% reduction in weight gain observed in the experimental group as measured on day 14 (Figure 1C). Blood samples were taken at different time points on day 1, day 7, and day 14 on all animals. We report habituation of the corticosterone response to repeated restraint stress (Figure 1B; two-way ANOVA, $F_{(4,\ 276)} = 3.59$, $p < 0.05$). On day 1, 60 min of restraint increased blood corticosterone concentration to 241 ng/mL, which was significantly lower on day 7 (150 ng/mL) and day 14 (183 ng/mL). We did not observe any difference between control and stress groups in the open-arm time ratio (OAT; Figure 1D), open-arm entry ratio (OAE; Figure 1D), or for the distance traveled in the maze (data not shown).

Repeated restraint induced a sucrose preference deficit

Fourteen days of daily restraint induced a sucrose preference deficit (Figure 2A; *t*-test with Welch's correction for unequal variances; $p < 0.05$) in the stressed group (77.2%) compared to the control group (82.1%). We observed an important inter-individual variability in preference sucrose on day 14 in the stress group that was absent on the initial sucrose preference measure. Whereas the majority of stressed individuals had a sucrose preference similar to the control group, some individuals developed an important sucrose preference deficit (Figure 2A). In order to differentiate these two populations (low versus high-sucrose preference), we have set the cut-off criteria to two standard deviations (5.5%) lower than the control group mean (82.1%). thus all individuals with a sucrose preference $\leq$71% were defined as low-sucrose preference. No control animal had a sucrose preference on day 14 lower than the cut-off criteria. Since no initial sucrose preference values were below this value, it is presumed that the stress regimen is responsible for reducing sucrose preference in some vulnerable individuals. Only six individuals (18.8%) were found to have a sucrose preference below the cut-off criteria, and thus composed our low-sucrose preference population. These animals were found to have a similar initial preference for sucrose (82.8%) compared to high-sucrose preference (86.5%) and control (86.6%; Figure 2B; no significant difference between the three populations were found on day 1 or day 7). Low-sucrose preference individuals were also found to take longer to habituate to restraint as revealed by an elevated corticosterone concentration on day 7 (285.9 ng/mL) compared to high-sucrose preference individuals (130.5 ng/mL; Figure 2C). There is no difference in weight gain between the high and low-sucrose preference groups. In addition, looking back at anxiety-like behaviors difference between high and low-sucrose preference groups, we found a significant difference in open-arm time (OAT) ratio between high- and low-sucrose preference group (Figure 2D; ANOVA $F_{(2,\ 41)} = 3.70$, $p < 0.05$). In addition, we observed a significant difference in open-arm entry (OAE) ratio between low-sucrose preference group, and unstressed control and high-sucrose preference group (Figure 2E; ANOVA $F_{(2,\ 41)} = 5.97$, $p < 0.05$). No significant difference was found in the total number of entries (Figure 2F; ANOVA $F_{(2,\ 41)} = 3.21$, $p > 0.05$).

There is a downregulation of enkephalin expression in the nucleus accumbens of low-sucrose preference rats

In order to understand what might differentiate low-sucrose preference individuals, we measured the expression of ppENK mRNA using quantitative in situ hybridization in the caudate putamen and nucleus accumbens

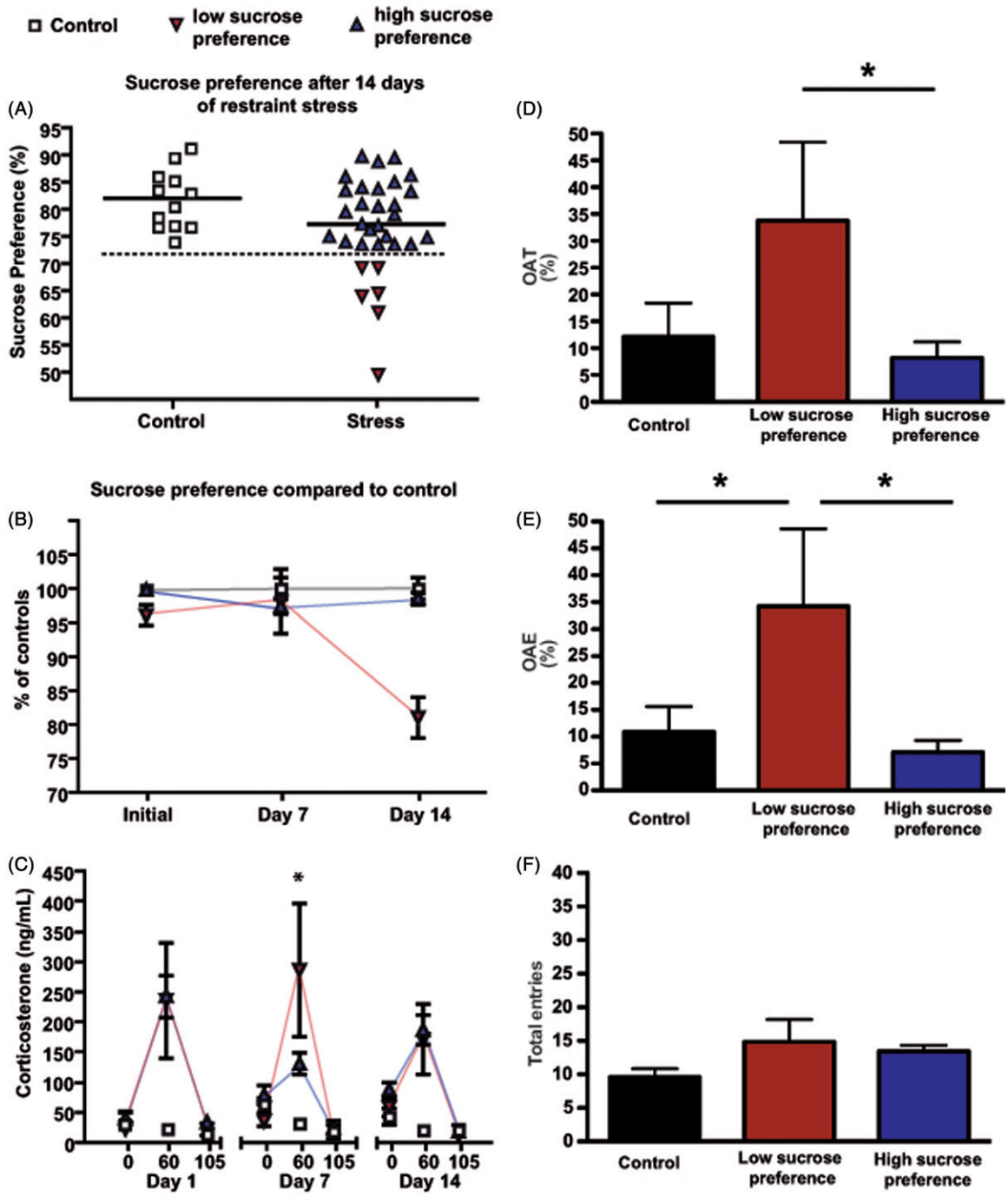

Figure 2. Repeated restraint induces a sucrose preference deficit. (A) Repeated restraint stress induced a significant reduction of sucrose preference on day 14 ($p<0.05$). Most individuals were not affected by stress, but 18% ($n=6$) of rats developed a severe deficit in sucrose preference. (B) There was no significant difference between the three groups initially, and on day 7. (C) On day 7, the corticosterone response to restraint of low-sucrose preference individuals was significantly higher than high-sucrose preference animals (*$p<0.05$), which suggest that low-sucrose preference individuals were more exposed to corticosterone. (D) The open-arm time ratio (OAT) shows a significant difference between low-sucrose and high-sucrose preference group. (E) Similar results are observed with the open-arm entries ratio (OAE). (F) No significant difference between groups are observed in the total number of entries.

(ACC; Figure 3A). We observed a significant reduction in ppENK mRNA expression in the shell and core parts of the ACC of the low-sucrose preference population compared to both control and high-sucrose preference (Figure 3B; ANOVA $F_{(2,\ 19)}=3.84$, $p<0.05$). Most interestingly, there is a strong correlation between the final sucrose preference level and the ppENK mRNA density in the ACC of all groups. This correlation reached significance in the shell (Figure 3C; $r=0.534$, $p<0.01$), as well as in the core (data not shown; $r=0.533$, $p<0.01$), where animals with the weakest preference for sucrose were found to have the weakest ppENK mRNA density. There was no correlation with initial sucrose preference showing that the chronic stress regimen in the low-sucrose preference population is likely to responsible for the reduction of ENK expression. In addition, we observed no reduction of ppENK expression in caudate putamen, as well as in the amygdaloid complex (data not shown), which shows the specificity of ppENK mRNA diminution to the ACC.

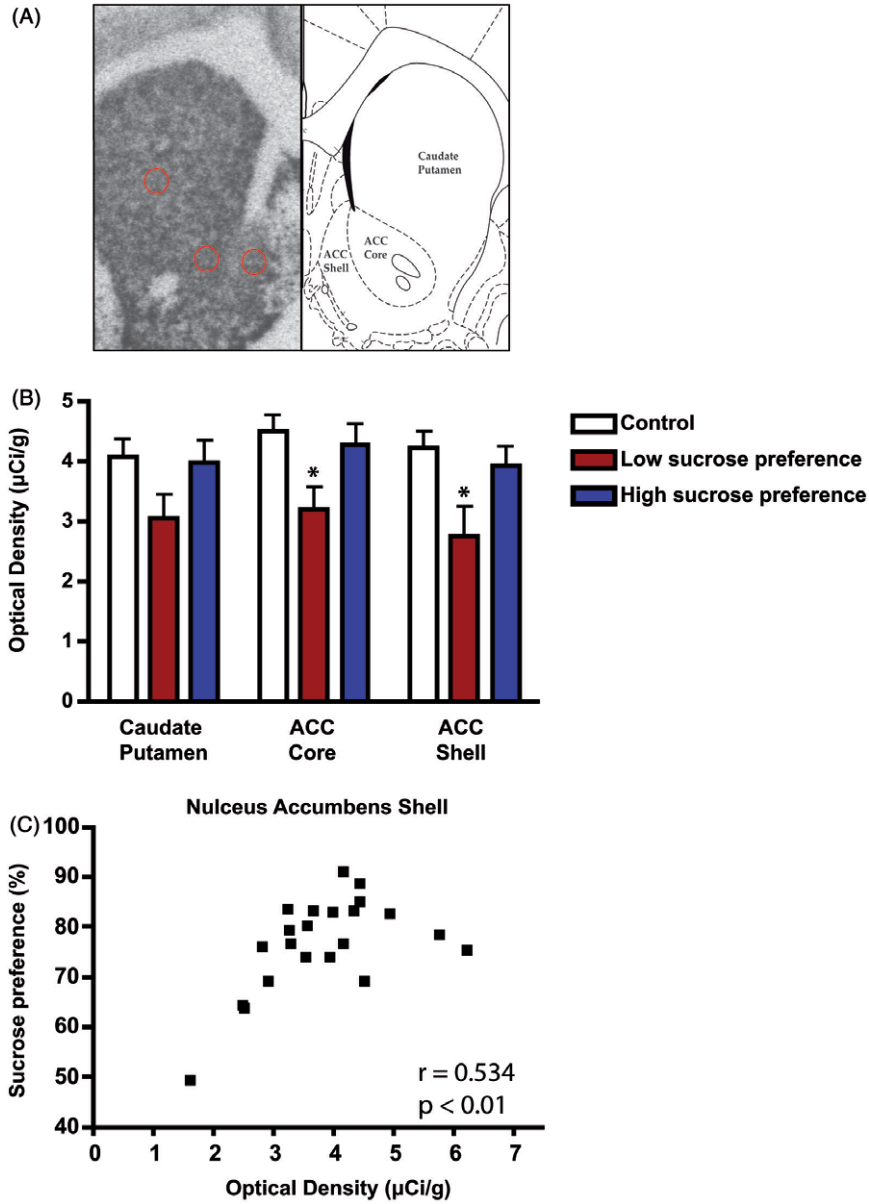

Figure 3. Enkephalin expression in the nucleus accumbens is downregulated in low-sucrose preference individuals. (A) Quantitative *in situ* hybridization of the caudate putamen, core and shell parts of the nucleus accumbens (ACC). (B) We observed a significant reduction of enkephalin expression in low-sucrose preference individuals compared to both control and high-sucrose preference individuals in the ACC ($^*p < 0.05$). (C) Most interestingly, sucrose preference on day 14 correlated strongly with enkephalin expression in the ACC shell ($r = 0.534$; $p < 0.01$). High density of ppENK mRNA is associated with an elevated sucrose preference.

Low-sucrose preference rats have a reduced induction of ΔFosB in the nucleus accumbens

We investigated the possible implication of ΔFosB in the downregulation of enkephalin expression. The transcription factor ΔFosB has been implicated in the stress response (Berton et al., 2007; Perrotti et al., 2004) and incentive values of drugs of abuse (Nestler, 2008). More recently, an increased in ΔFosB expression in the ACC has been associated with the consumption of natural rewards, and the overexpression of ΔFosB in the ACC using a viral vector was shown to increase sucrose preference (Wallace et al., 2008). Indeed, we observed a reduced number of ΔFosB positive cells in the ACC shell of the low-sucrose preference population (Figure 4; ANOVA $F_{(2,\ 17)} = 4.026$, $p < 0.05$). However, contrary to the ENK mRNA density, we did not observe a correlation between the number of ΔFosB positive cells in the ACC and sucrose preference. To verify if ΔFosB was indeed expressed in the ENK neurons of the ACC, we performed an immunofluorescence for ΔFosB combined to a fluorescent *in situ* hybridization for ppENK mRNA. We found that ΔFosB was expressed in ENK neurons of the ACC shell with a similar percentage in all groups (Figure 5; Control = 30.1%, low-sucrose preference = 31.4%, high-sucrose preference = 40.0%; ANOVA $F_{(2,\ 18)} = 1.682$, $p = 0.21$). We also observed similar percentages in the ACC core (Control = 46.7%, low-sucrose preference = 45.0%, high-sucrose preference = 45.3%). These results suggest that a reduced induction of the transcription factor ΔFosB, which is expressed in ENK neurons, might be responsible for the downregulation of ENK.

Discussion

This study aimed at investigating inter-individual variations in behavioral deficits induced by chronic stress. We have used repeated restraint instead of chronic unpredictable stress for two reasons. First, we and others have noticed an important inter-individual variability in habituation to repeated restraint, and we hypothesized that this variability would have

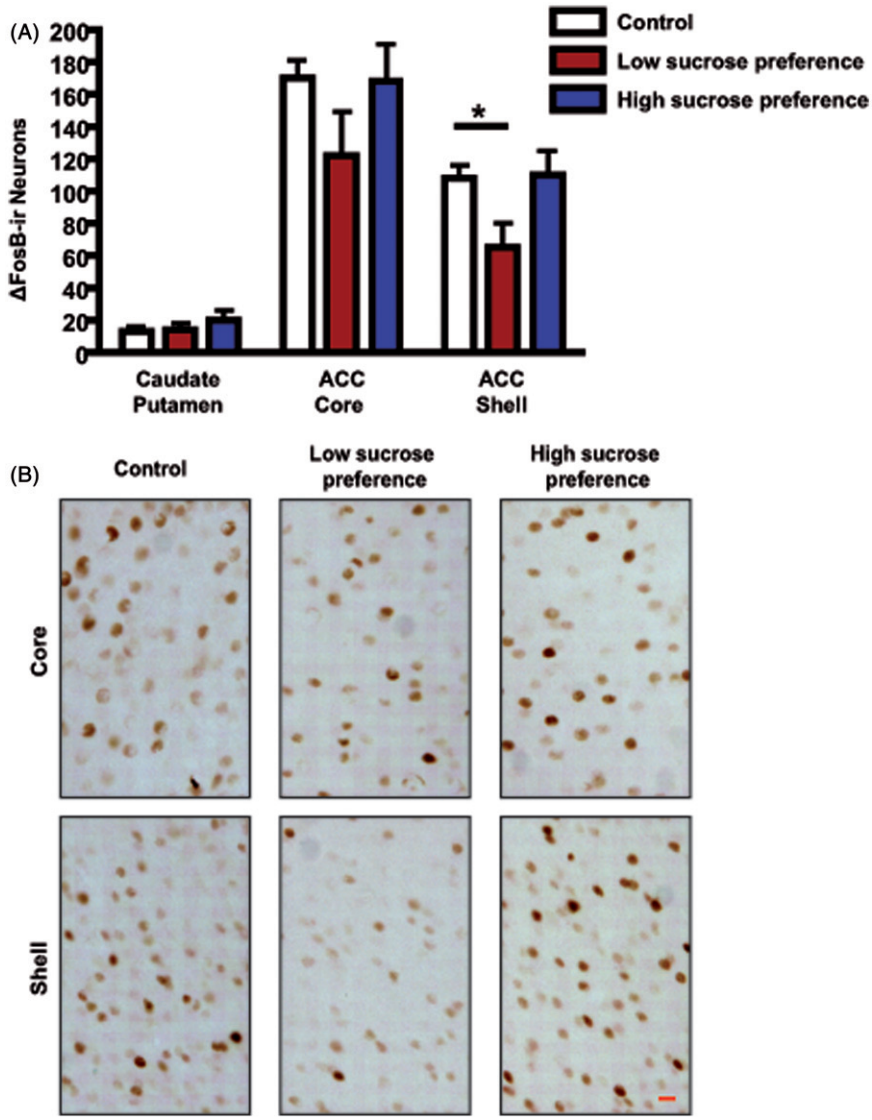

Figure 4. ΔFosB immunoreactivity is reduced in the nucleus accumbens shell. We observed a reduced number of ΔFosB positive neurons in the nucleus accumbens (ACC) shell in low-sucrose preference individuals compared to control (*$p < 0.05$), but no significant difference in the caudate putamen and ACC core. Scale bar = 12 μm.

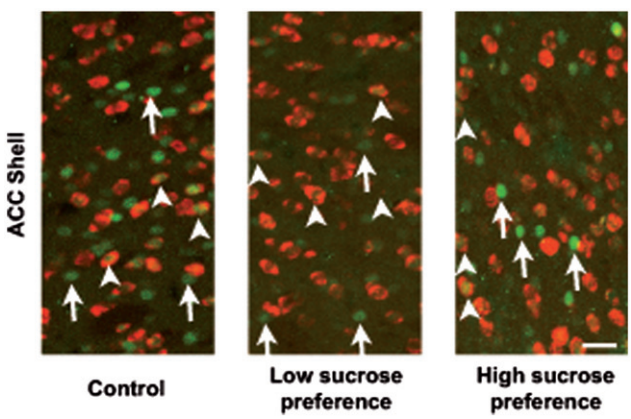

Figure 5. ΔFosB is expressed in enkephalinergic neurons of the nucleus accumbens. Confocal images displaying ΔFosB labeled nuclei (green) in neurons positive for ppENK mRNA (red) in the nucleus accumbens, with example of single-labeled neurons for ΔFosB indicated by arrows while examples of double-labeled neurons shown by arrowheads. About 35% of ΔFosB colocalized with ppENK mRNA in all three groups despite the decreased number of ΔFosB neurons in low-sucrose preference individuals (ANOVA $F_{(2,\ 18)} = 1.682$, $p = 0.21$). Scale bar = 25 μm.

behavioral repercussions (unpublished observation; Marquez et al., 2004). Second, repeated restraint is considered a mild stressor, and therefore individuals developing behavioral deficits with this stress protocol would constitute the most vulnerable end of the population. Indeed, only 18% of stressed rats in this study developed a sucrose preference deficit. These low-sucrose preference rats had an amplified stress response on day 7, but not on day 1 and day 14, which shows that these animals took longer to habituate to restraint and were likely exposed to more corticosterone. Interestingly, chronic administration of high corticosterone has been used as a depression model and can induces several behavioral deficits (David et al., 2009), a finding in agreement with our data.

Is enkephalin the missing link between chronic stress and anhedonia?

Endogenous opioids have long been implicated in the modulation of the stress and ENK is thought to attenuate behavioral and hormonal responses to stress. In this study, we observed a reduction of ENK mRNA specifically in the shell

and core of the ACC of low-sucrose preference rats. Similar results were observed by Sweis et al. (2013) following repeated restraint stress. In addition, there is a growing list of evidence implicating endogenous opioids in hedonic perception, or what is called the ''liking'' aspect of natural rewards. For instance, local injections of a μ-OR antagonists in the ACC shell was shown to blunt ''liking'' reaction to sucrose and to reduce sucrose drinking (Kelley et al., 1996; Smith & Berridge, 2007). In contrast, μ-OR agonist injections in the ACC shell increases eating of palatable food and injection in this area also exacerbates ''liking'' reactions (Peciña & Berridge, 2005; Zhang & Kelley, 1997). Similar results were obtained with the V), a main target of accumbal ENK neurons (Smith & Berridge, 2007). Moreover, units recording in the VP correlate directly with the hedonic value associated with a particular stimulus (Tindell et al., 2006).

Neurochemical and behavioral data point to ENK as a likely natural substrate responsible for the μ-OR modulation of hedonic perception. ENK is highly expressed in the ACC and these neurons send heavy projections to the VP (Zahm, 2000). The tonically activated ventral striato-pallidal ENK pathway appears to be implicated in the attribution of hedonic values to rewarding stimuli. This is supported by the fact that ENK deficient mice have a reduced instrumental responding for palatable food under a progressive ratio (Hayward et al., 2002). This pathway could be implicated in the maintenance of a basal hedonic state as well. For instance, systemic injections of the non-specific opioid antagonist naloxone induce a robust conditioned place aversion, which is absent in ENK deficient mice and MOR deficient mice (Skoubis et al., 2001, 2005). Moreover, local injections of naloxone in the VP can induce a conditioned place avoidance (Skoubis & Maidment, 2003). Put together, these results clearly indicate that ENK release in the ACC and VP have a positive impact on hedonic homeostasis, whereas decreased ENK release in these structures, which would result from a downregulation of ENK expression in the ACC, could thus yield a low-sucrose preference state. Alternatively, individual rats with reduced expression of ENK in the ACC may perceive restraint stress as more aversive, which could potentiate the behavioral and endocrine consequences of restraint. This interpretation of the results is in agreement with the function of the ACC to retune the valence of stimuli in accordance to the environment (Reynolds & Berridge, 2008). Further experiments are needed to differentiate between these two hypotheses.

ΔFosB is potentially involved in the downregulation of enkephalin expression

ΔFosB is a truncated and unusually stable isoform of the immediate early gene FosB, and it tends to accumulate overtime in the brain following chronic stimulation. The functional roles of ΔFosB have been most studied with drugs of abuse, where it was shown to accumulate in dynorphin neurons of the ACC following chronic use of drugs. In this context, the induction of ΔFosB in the ACC increases sensitivity to the rewarding effects of drugs of abuse (Kelz et al., 1999). In addition, ΔFosB is induced in the ACC by the consumption of a sucrose solution, and overexpression of ΔFosB in this region was shown to increase sucrose preference (Wallace et al., 2008). Moreover, ΔFosB is specifically reduced in the nucleus accumbens in models of depression, but remain elevated in resilient animals (Vialou et al., 2010). To date no study addressed the transcriptional role of ΔFosB in ENK accumbal neurons, but our results suggest this transcription factor could modulate ENK transcription in accumbal neurons. Indeed, FosB can dimerize with Jun proteins to form the transcription complex AP-1 (Eferl & Wagner, 2003), a transcription complex shown to bind *in vitro* to CRE-2 site, located in the *Penk* gene promoter region (Comb et al., 2008; Kobierski et al., 1991). Members of the Fos family were shown to have the ability to modulate positively or negatively ppENK mRNA transcription. However, these studies were conducted *in vitro* and further experiments are needed to demonstrate a role for ΔFosB in the modulation of ENK expression *in vivo*.

Conclusion

In this manuscript, we report the induction of a sucrose preference deficit in a subpopulation of rats following 14 days of repeated restraint stress. These low-sucrose preference individuals had a significantly lower density of ppENK mRNA in the ACC, and we observed a strong correlation between ENK expression in this structure and sucrose preference. Although further work is needed to confirm the causal relationship, our results suggest an implication of the transcription factor ΔFosB in the stress-induced downregulation of ENK.

Declaration of interest

The authors declare no competing financial interests.

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