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Chronic valproate normalizes behavior in mice overexpressing calcineurin

Claire J. Herzog [a], Stéphanie Miot [a], Isabelle M. Mansuy [b], Bruno Giros [a], Eleni T. Tzavara [a,*]

[a] INSERM U-513, 8 rue de Général Sarrail, 94000, Créteil, France
[b] Brain Research Institute, University of Zürich and Swiss Federal Institute of Technology, Zürich, Switzerland

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Abstract

Calcineurin (PP2B) is a Ca^{2+}-dependent protein phosphatase enriched in the brain that takes part in intracellular signaling pathways regulating synaptic plasticity and complex brain functions. We report here that when these pathways are activated by transgenic expression of calcineurin, locomotor activity of mice in response to novelty is increased, as well as the behavioral and molecular responses of the psychostimulant cocaine. We also observed that the anxious-like behavior is altered. These behavioral changes are indicative of a generally increased behavioral responsiveness and could be normalized by chronic treatment with the mood stabilizer valproate. These results provide proof of concept that calcineurin-dependent dephosphorylation plays an important role in behavioral reactivity and in the effects of mood regulators. Mice overexpressing calcineurin represent a novel tool to study affective responses related to psychiatric disorders.

Keywords: Calcineurin; Cocaine; Valproate; Behavioral reactivity; Affective disorders; Mood stabilizer

1. Introduction

There is an increasing shift from single neurotransmitter-based theories towards global network approaches, advocating an important role of signal-transduction pathways in multifaceted patterns of behavior and in the pathophysiology of psychiatric disorders. The underlying hypothesis is that psychiatric disorders have too complex a semiology to be attributed to a single neurotransmitter system dysregulation. Signaling pathways, on the other hand, broadly affect neuronal networks by dynamically regulating the activity of multiple neurotransmitter systems, as well as processes such as synaptic activity, receptor desensitization, cell survival, neuroplasticity, and cellular resilience. Minor variations in ubiquitous regulators of signaling pathways can affect complex functions (Charney and Manji, 2004) and the same second messenger system can be implicated in seemingly unrelated brain functions. For example calcium-stimulated-adenylyl-cyclase-dependent signal-transduction has been involved in drug dependence (Valverde et al., 1996; Tzavara et al., 2002), in circadian rhythms (Tzavara et al., 1996) and in learning and memory (Xia and Storm, 2005); all of which can be affected during the life-time of an individual suffering from schizophrenia or mood disorder. It has been suggested that the association for a given protein might lie not with a diagnostic category per se but with more specific aspects of the phenotype, such as affective symptoms and cognitive effects, which cross traditional psychiatric diagnostic boundaries (Craddock et al., 2006).

A central feature in signal-transduction is the dynamic balance between protein kinases and protein phosphatases that is essential for synaptic plasticity. Recent evidence shows that the expression and activity of various kinases/phosphatases or their downstream targets are modified (i) following psychoactive drug administration (Ron and Jurd, 2005), (ii) in animal models of psychiatric disorders (Angelucci et al., 2005) and (iii) in post-mortem samples from psychiatric patients (Albert et al., 2002). For instance, the protein kinase DARPP-32 (cAMP-regulated-phosphoprotein-of-Mr32,000) mediates effects of common psychotomimetics (Svenningsson et al., 2003) as well as antidepressants (Svenningsson et al., 2002). In the balance of kinase/phosphatase activity, the phosphatase calcineurin acts as a major negative regulator of protein phosphorylation. Interest-

* Corresponding author. Tel.: +33 1 49813572; fax: +33 1 49813685.
E-mail address: tzavara@im3.inserm.fr (E.T. Tzavara).

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ingly, mice in which calcineurin activity is increased experimentally by expression of an active form in the mouse forebrain (CN98 mice), show impaired synaptic plasticity (Winder et al., 1998). Using this transgenic mouse model, we recently showed that calcineurin is involved in the mechanisms of action of antidepressants (Crozatier et al., 2007).

Here we have assessed affective-like responses in CN98 mice. Mice were subjected to an open field (actimeter), elevated plus-maze and zero-maze test for the assessment of their behavioral reactions to novelty and stress. Since psychostimulant induced hyperlocomotion in rodents is predicted to reflect manic states in humans, we also assessed the effects of acute cocaine administration on locomotor activity in CN98 mice. In parallel we have measured c-fos expression upon acute cocaine injection. The expression of this immediate early gene is a marker of neuronal activation and is used widely to evaluate neuronal activation in response to psychostimulants (Graybiel et al., 1990; Steiner and Gerfen, 1993; Svenningsson et al., 2003). CN98 mice showed enhanced locomotor activity in response to novelty, reduced anxiety-like behavior on the elevated plus- and zero-mazes, and increased sensitivity to the locomotor stimulant actions of cocaine, accompanied by elevated c-fos expression in the cortex, striatum, and nucleus accumbens after acute cocaine injection. The increased behavioral reactivity that we observed in CN98 mice as compared to their wild type (WT) littermates, was reminiscent of the altered behaviors observed in animal models of emotional lability, mania or bipolar disorder (Gessa et al., 1995). Therefore, we evaluated weather the anticonvulsant/mood regulator valproate, a drug widely used in the clinical treatment of patients, was able to reverse the altered behavioral pattern of the CN98 mice.

2. Materials and methods

2.1. Animals

The CN98 transgenic mice that express a constitutively active form of calcineurin Aα subunit (Winder et al., 1998) were used in this study and their WT littermates were used as controls. This transgene is driven by the CAMKII (Ca^{2+}/calmodulin-dependent-protein-kinase) promoter, that restricts its expression to the forebrain and throughout the hippocampus and dentate gyrus (Winder et al., 1998). I. Mansuy provided heterozygous couples and a colony was established in our laboratory (Crozatier et al., 2007); breeding was maintained on a C57/Bl6 background in standard laboratory conditions (12-h light/dark cycle with light on at 07:30; room temperature 21 ± 1 °C, food and water *ad libitum*). All experiments were carried out in accordance with the European Communities Council Directive (86/809/EEC) and approved by the local ethical committee. Behavioral tests were performed by an experimenter blinded to genotype [wild type (WT) or transgenic (CN98) mice] and treatment. Except when effects of habituation were assessed, each mouse was tested only once, and for each experiment, and the numbers of animals utilized were 8–12 per experimental group, for both behavioral and *in situ* hybridization experiments.

2.2. Treatments

Drugs, purchased from Sigma (L'Isle d'Abeau Chesnes, France), were dissolved in 0.9%NaCl and injected intraperitoneally (10 ml/kg). Valproate (200 mg/kg; i.p.) was administered chronically, once daily for 21 days. Cocaine (5, 15, 30 or 45 mg/kg; i.p.) was injected acutely and locomotor activity was registered immediately after.

2.3. Behavioral tests

2.3.1. Locomotor activity

Locomotion was evaluated in activity boxes (20 × 15 × 25 cm) connected by an interface to a computer (Imetronic, Bordeaux, France) and located in a sound-attenuated experimental room. Ambulations (horizontal locomotion) were defined as the number of photocell beams (located 15 mm above the floor) broken per unit of time.

a. Locomotor activity in response to novelty: Reactivity to novelty was examined by placing naive mice, never exposed to the apparatus, in the box and measuring horizontal locomotor activity for 30 min. The same mice were used the following day to measure locomotor activity under habituated conditions.
b. Locomotor activity in habituated animals: The aforementioned mice were placed in the apparatus for 20 min (habituation period) and then locomotor activity was measured for 30 min.
c. Locomotor activity in response to cocaine: Another group of mice was used for pharmacological experiments. These mice were habituated to the apparatus for 30 min the day before the experiment. The day of the experiment mice were placed in the apparatus, cocaine or drugs were injected after a 20 min habituation period and locomotor activity measured for an additional 30 min period.

2.3.2. Elevated plus-maze test

The maze (Pellow and File, 1986; Linden et al., 2004) consisted of two open arms (37.5 × 5 × 0.3 cm) and two closed arms (37.5 × 5 × 15 cm) linked by a central platform (5 × 5 cm) and positioned 63 cm above the ground.

On the test day, animals were transferred to the testing room in their home cages and allowed to acclimate for at least 1 h prior to testing. A test session began by placing a mouse on the central platform facing the open arm to allow free exploration of the maze for 5 min. An arm entry was recorded when all four legs on the mouse were on the arm. The total amount of entries (in closed and open arms) was used to determine the locomotor activity of each tested mouse. After each test session the maze was cleaned before placing the next mouse.

2.3.3. Zero-maze test

The maze (Shepherd et al., 1994) consisted of a 7 cm wide ring (external diameter 47 cm) divided into four equal parts of alternating closed and open sections and positioned 50 cm above the ground. On the test day, animals were transferred to

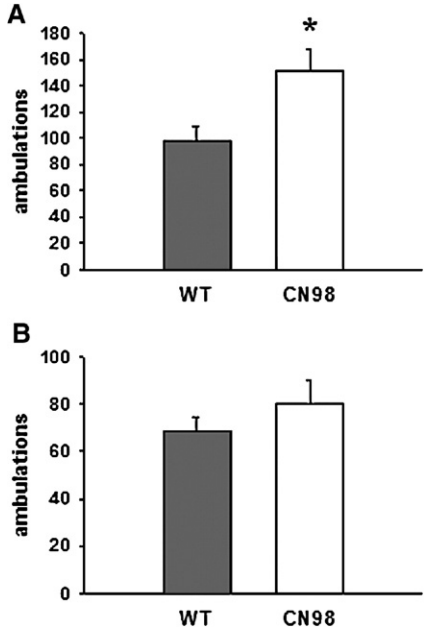

Fig. 1. Locomotor activity in mice overexpressing calcineurin. (A) Locomotor activity in response to novelty is increased in CN98 mice. Horizontal locomotor activity (ambulations) for WT (grey bars) and CN98 (white bars) mice placed for the first time in an actimeter. $n=8–10$ animals per group. *$P<0.05$ for WT versus CN98 animals. (B) No difference in locomotor activity in habituated CN98 and WT mice. Horizontal locomotor activity (ambulations) for WT (grey bars) and CN98 (white bars) mice habituated to the actimeter. $n=8–10$ animals per group. *$P<0.05$ for WT versus CN98 animals.

the testing room in their home cages and allowed to acclimate for at least 1 h prior to testing. Mice were individually placed in one of the closed compartments and their ambulatory behaviors were recorded with a with a video-track system. The time spent in the open compartment during the 5 min of the test session was measured. The total time each mouse was engaged in locomotor activity (in the closed and open compartments) was calculated as in index of locomotor activity. After each test session the maze was cleaned before placing the next mouse.

Each mouse was tested in the plus-maze or the open maze only once. Mice tested in the plus-maze (or the zero-maze) were either naïve to all manipulation or were previously subjected to a single actimeter session. With completely naïve mice or mice already subjected to a single actimeter session we observed identical results in the elevated plus-maze and zero-maze.

2.4. In situ hybridization

Fifteen minutes after cocaine injection (15 mg/kg), mice were sacrificed by decapitation and brains quickly removed and frozen. Coronal sections were taken at −20 °C and mounted on glass slides. c-fos specific antisense oligonucleotides (5′-GTT GAC AGG AGA GCC CAT GCT GGA GAA GGA GTC GGC TGG GGA ATG -3′) were labeled with [^{35}S]dATP, using terminal transferase (Amersham Biosciences), to a specific activity of 5×10^{-8} dpm/μg. Sections were covered with 100 μl of a solution containing 50% hybridization solution (Amersham Biosciences), 40% deionized formamide (Merck Eurolab, Strasbourg, France), 500 μg/ml poly(A) (Roche, Meylan, France), 100 mM 4-dithiothreitol, and $3–5\times10^{-5}$ dpm of each labeled oligonucleotide. The samples were incubated overnight at 42 °C, washed in saline sodium citrate, followed by PBS, and exposed to X-ray films (Biomax; Eastman Kodak, Rochester, NY) for 21 days.

2.5. Data analysis

Data represent mean±S.E.M. of the indicated number of animals per group. Results were analyzed with one, two or three-

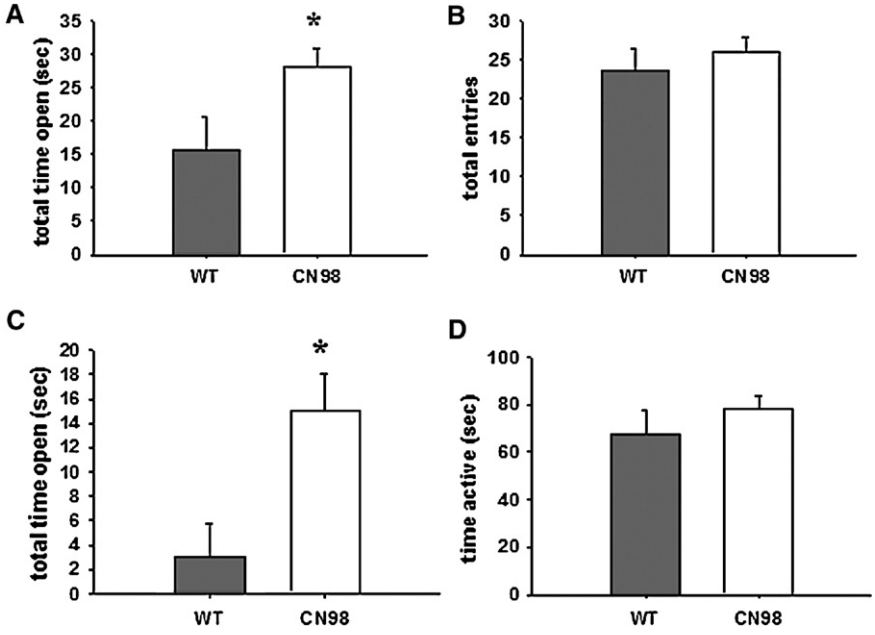

Fig. 2. Reduced behavioral inhibition and anxiety-like behavior in CN98 mice. (A, B) Elevated plus-maze: Time spent in the open arms (A) and total entries (i.e. sum of entries in open and closed arms) (B) for WT (grey bars) and CN98 (white bars) mice. (C, D) Zero-maze: Time spent in the open compartments (C) and total time engaged in locomotor activity (i.e. time active in all compartments) (D) for WT (grey bars) and CN98 (white bars) mice. $n=8–12$ animals per group. *$P<0.05$ for WT versus CN98 animals.

way ANOVA, followed by Duncan's post-hoc test when appropriate. All analyses were run with the Statistica Software.

3. Results

We evaluated locomotion in CN98 upon exposure to novelty. When mice were introduced in a novel environment (first exposure to the actimeter cage) we observed a higher exploratory activity in CN98 than WT mice ($F(1,15)=5.37$, $P<0.05$; one-way ANOVA, Fig. 1A). This was however no longer observed upon subsequent exposure to the actimeter ($F(1,15)=0.95$; n.s.; Fig. 1B), indicating a selective hyperlocomotor effect of novelty in CN98 and not a general increase in locomotor activity.

Behavioral inhibition and anxiety-like behaviors were investigated in the elevated plus-maze and zero-maze tests (Ognibene et al., 2005; Olausson et al., 2001; Zorner et al., 2003). In the plus-maze, CN98 mice showed reduced anxiety and a behavioral disinhibition expressed as an increase in the time spent in open arms compared to WT mice ($F(1,22)=4.56$ $P<0.05$, one-way ANOVA, Fig. 2A). Total entries (i.e. the sum of entries in the open and closed arms) were not different between CN98 and WT mice ($F(1,22)=0.7$ n.s., one-way ANOVA, Fig. 2B). Similarly in the zero-maze, CN98 mice spent significantly more time in the bright (open) compartment than did WT mice ($F(1,7)=6.22$, $P<0.05$, one-way ANOVA, Fig. 2C) and engaged in more exploratory activity in this compartment

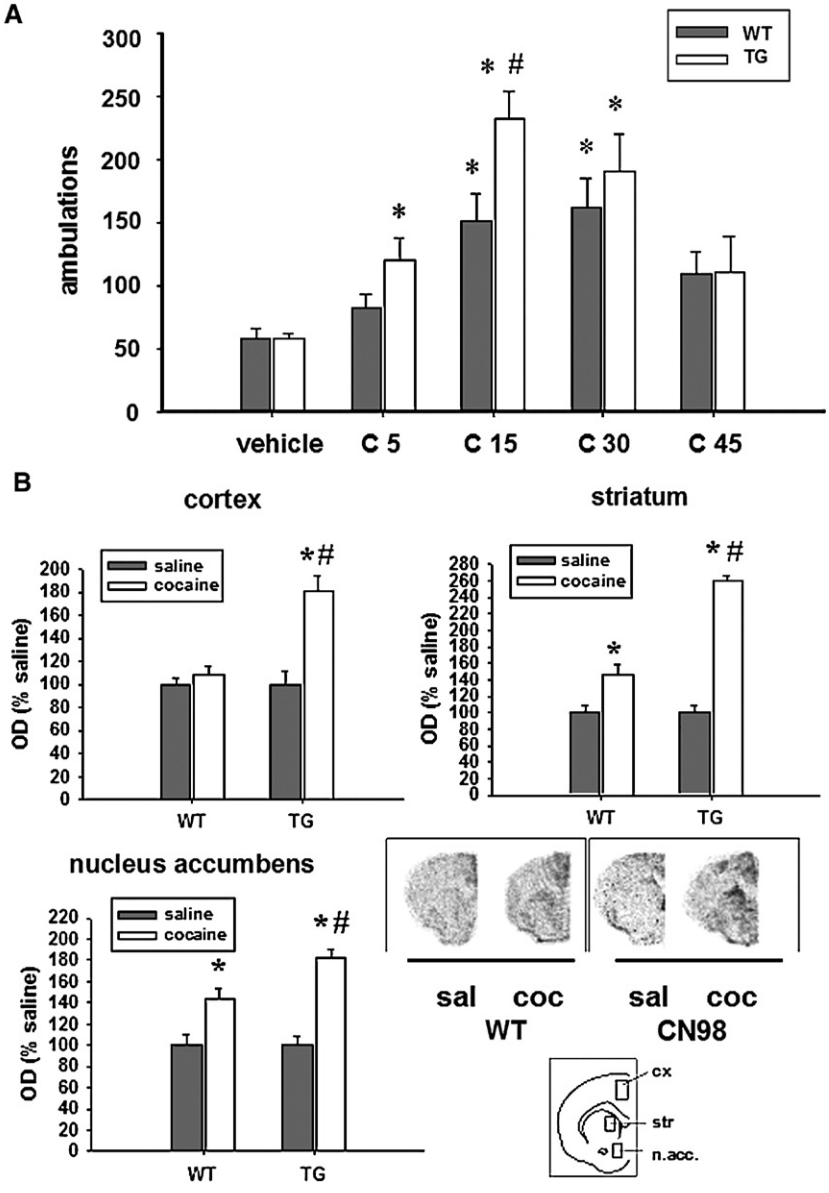

Fig. 3. Response to cocaine is increased in CN98 mice. (A) Cocaine-induced hyperactivity is increased in CN98 mice. Horizontal locomotor activity (ambulations) measured in an actimeter after an acute injection of vehicle or 5, 15, 30 or 45-mg/kg cocaine in WT (grey bars) and CN98 (white bars) mice. $n=8$–12 animals per group. *$P<0.05$ for animals treated with cocaine versus animals of the same genotype treated with vehicle, $^{\#}P<0.05$ for WT versus CN98 animals under the same treatment. (B) C-fos expression in response to cocaine is increased in CN98 mice. Expression of the immediate early gene c-fos in striatum, nucleus accumbens, and frontal cortex after administration of vehicle or 15 mg/kg cocaine in WT (grey bars) or CN98 (white bars) mice. $n=8$–12 animals per group. *$P<0.05$ for animals treated with cocaine versus animals of the same genotype treated with vehicle, $^{\#}P<0.05$ for WT versus CN98 animals under the same treatment.

($F(1,7)=7.18$, $P<0.05$, not shown). Total time engaged in locomotor activity (i.e. the sum of activity in all compartments) was not different between CN98 and WT mice ($F(1,7)=1.7$, n.s., one-way ANOVA, Fig. 2D). It should be noted that unlike the actimeter experiment, locomotor activity, as reflected by total arm entries, in the plus-maze and zero-maze did not differ between CN98 and WT mice. However, as seen in the literature when testing genetically modified mice or when testing pharmacological effects of compounds in anxiety and locomotion tests, total arm entries in the plus-maze do not always coincide with conventional measures of locomotion (ambulations) in activity tests. Total arm entries in the plus-maze sometimes fail to picture changes in locomotor activity that are observed with an open field or an actimeter (see for instance Kuzmin et al., 2006; Ferguson et al., 2004).

When injected with cocaine (5, 15, 30 or 45 mg/kg), CN98 mice habituated to the actimeter exhibited a significantly higher hyperlocomotion than WT mice (effect of treatment $F(4,61)=15.09$, $P<0.001$ and genotype $F(1,61)=5.19$, $P<0.05$, two-way ANOVA). At the 45 mg/kg dose appearance of massive stereotypic movements parasitized locomotor activity and interfered with horizontal locomotion measurements. When we analyzed the effects of cocaine in CN98 and WT mice we show a significant interaction between genotype and treatment ($F(3,53)=2.81$; $P<0.05$, two-way ANOVA). Subsequent post-hoc analysis shows that while in WT mice cocaine had no effect on locomotion at 5 mg/kg, it did increase activity by 100% in CN98 mice. At 15 and 30 mg/kg, cocaine increased locomotion in both groups, but this increase was more pronounced in CN98 than WT animals (Fig. 3A).

In another set of CN98 and WT mice, c-fos mRNA expression was examined in the striatum, nucleus accumbens, and frontal cortex, major targets of dopaminergic inputs after a single cocaine injection (15 mg/kg). Interestingly, both the pattern and extent of c-fos increase were different in CN98 as compared to WT mice. In WT mice, c-fos mRNA expression was increased only in the striatum and nucleus accumbens (47%±11 and 44%±10 respectively). In CN98 mice, it was increased in the striatum, nucleus accumbens, but also in the frontal cortex; the increase was significantly higher in all structures (striatum 159%±8, $F(1,71)=37$ $P<0.001$, nucleus accumbens, 83%±8, $F(1,77)=4.3$ $P<0.05$, frontal cortex 81%±13, $F(1,54)=10.91$ $P<0.01$, two-way ANOVA for genotype and treatment) (Fig. 3B).

Finally, we investigated whether the mood stabilizer valproate, a drug widely used in the clinical treatment of patients, could reverse behavioral hyperactivity observed (i) upon first exposure to an actimeter, (ii) after a single injection of cocaine or (iii) after exposure to the elevated plus-maze in CN98 mice. Chronic valproate treatment prevented the increase in locomotor activity induced by novelty (Fig. 4A; genotype × chronic treatment (valproate or vehicle) interaction $F(1,46)=4.4$, two-way ANOVA). Chronic valproate also prevented cocaine-induced hyperlocomotion in CN98 mice (significant genotype × chronic treatment (valproate or vehicle) × acute treatment (cocaine or saline) interaction $F(1,74)=4.57$, $P<0.05$, three-way ANOVA). Chronic vehicle treatment had no such effect and

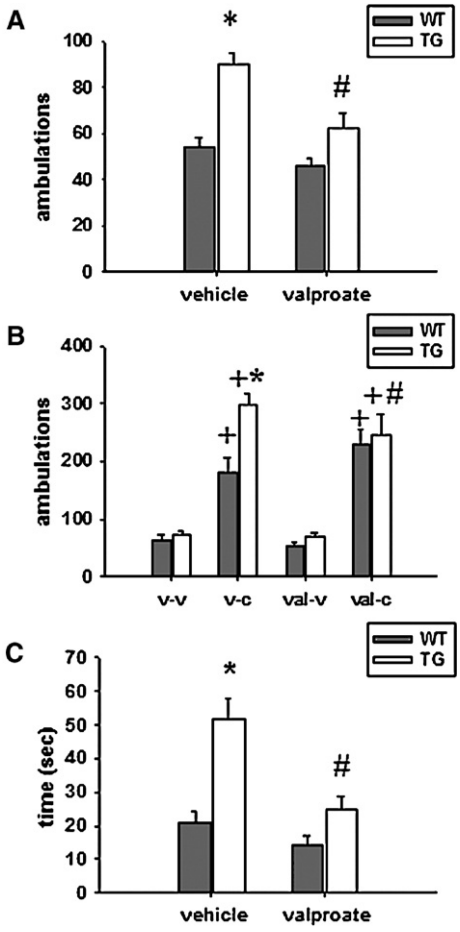

Fig. 4. The mood regulator valproate normalizes behavior in CN98 mice. (A) Horizontal locomotion (ambulations) upon a first exposure to the actimeter, for WT (grey bars) and CN98 mice (white bars) pre-treated chronically with vehicle (v) or valproate (val). $n=8$–12 animals per group. $^{*}P<0.05$ for CN98 versus WT animals; $^{\#}P<0.05$ for animals treated chronically with valproate versus animals of the same genotype treated with vehicle, (B) locomotor activity in response to an acute cocaine injection (15 mg/kg), for WT (grey bars) and CN98 mice (white bars) pre-treated chronically with vehicle (v) or valproate (val). $n=8$–12 animals per group. $^{+}P<0.05$ for animals treated acutely with cocaine versus animals of the same genotype and pretreatment treated acutely with saline; $^{*}P<0.05$ CN98 versus WT animals under the same pretreatment and treatment; $^{\#}P<0.05$ for animals treated chronically with valproate versus animals of the same genotype and acute treatment that were treated chronically with vehicle, (C) total time spent in the open arms of the plus-maze, for WT (grey bars) and CN98 mice (white bars) pre-treated chronically with vehicle (v) or valproate (val). $n=8$–12 animals per group. $^{*}P<0.05$ for CN98 versus WT animals; $^{\#}P<0.05$ for animals treated chronically with valproate versus animals of the same genotype treated with vehicle.

cocaine-induced hyperlocomotion was still markedly enhanced in CN98 animals when compared to WT ($P<0.001$) (Fig. 4B). In the elevated plus-maze, chronic valproate treatment normalized the time CN98 mice spent in open arms (genotype × chronic treatment (valproate or vehicle) interaction $F(1,35)=5$ $P<0.05$, two-way ANOVA) (Fig. 4C). In addition, valproate treated CN98 mice made fewer entries in the open arms of the elevated plus-maze as compared to vehicle treated CN98 mice (11±1 entries for vehicle treated versus 6±1 for valproate treated CN98 mice).

4. Discussion

The aim of this study was to assess affective-like responses in CN98 mice, assess their behavioral and c-fos response to an acute cocaine challenge, and evaluate whether chronic valproate treatment can reverse the altered behaviors of CN98 mice. CN98 mice displayed a stronger locomotor response to novelty and spent more time in the open arms of the elevated plus-maze and zero-maze than WT mice did. They also expressed increased locomotor response to cocaine injection, accompanied by an altered magnitude and pattern of c-fos mRNA expression in response to cocaine. We also show that chronic valproate treatment attenuated the hyperresponsiveness of CN98 mice to novelty and cocaine injection, and reduced the time spent in open arms of the plus-maze. Thus, towards a comprehensive assessment of behavioral reactivity and disinhibition, we suggest that CN98 mice display a context dependent hypersensitivity that is normalized by the mood regulator valproate.

Increased activity upon exposure to novelty, and reduced anxiety-like behaviors could be relevant to behavioral disinhibition, namely reduced neophobia, increased risk-taking behavior and augmented agitation in response to contextual changes. Such a behavioral pattern has been described in putative genetic models of affective disorders (Roybal et al., 2007; Rozeboom et al., 2007; White et al., 2007). In CN98 mice acute administration of cocaine had more pronounced stimulatory effects on c-fos expression in the dorsal and ventral striatum, as well as in the cortex. In this later region cocaine induced c-fos expression only in CN98 mice. The behavioral response to cocaine was also increased in these mice. The parallel increases in c-fos expression and locomotor activity that we observed are in agreement with previous studies having established c-fos as a reliable marker of neuronal activation in response to psychostimulants (Svenningsson et al., 2003). Psychostimulant induced hyperlocomotion in rodents is thought to reflect manic state in humans, and enhanced biochemical and behavioral responsiveness to psychostimulants is at the basis of pharmacological models of mania (Lenox et al., 2002; Machado-Vieira et al., 2004).

In our study, the behavioral supersensitivity of the CN98 mice was reversed by chronic treatment with valproate, an anticonvulsant with antimanic and mood stabilizer properties. Mood regulators, (lithium or the anticonvulsants valproate and carbamazepine) are first line treatments for affective disorders. In animal models of bipolar disorder, mood regulators reduce psychostimulant or novelty induced hyperlocomotion (Arban et al., 2005;Shaldubina et al., 2002). Interestingly, it has been proposed that mood regulators could act by resetting intracellular kinase/phosphatase equilibrium (Du et al., 2004). The fact that valproate reduced cocaine hypersensitivity in CN98 and normalized their emotional reactivity to novelty further implicates calcineurin and its downstream targets in the mechanism of action of mood regulators and in the pathophysiology of affective disorders.

Our results confirm and extend previous studies suggesting that calcineurin dysfunction could be involved in molecular networks associated with response to stress, memory of aversive experiences (Lin et al., 2003), antidepressant mechanism of action (Crozatier et al., 2007) and psychotic behavior (Miyakawa et al., 2003). It was shown that mice invalidated for calcineurin show increased basal locomotor activity. Our results may seem surprising in the light of this study and of previous reports showing that PKA activation (which is antagonistic to calcineurin activation) mediates behavioral and biochemical effects of psychostimulants (Svenningsson et al., 2003). Phosphorylation/dephosphorylation dependent cascades show bidirectional plasticity (Bhalla et al., 2002). For instance it is well known that calcineurin dephosphorylates downstream targets of PKA and therefore calcineurin overexpression is predicted to inhibit cyclic AMP/PKA signaling. However the recent discovery of calcineurin activated adenylyl cyclase in the hippocampus (Chan et al., 2005) establishes a putative mechanism by which calcineurin overexpression may increase cyclic AMP signaling. Because of such bidirectional plasticity signal-transduction-cascades were proposed as key mediators in affective disorders, in which both positive and negative affective responses can coexist (Manji et al., 2003). It is also important to note that the calcineurin knockout mice utilized by Miyakawa and collaborators (Miyakawa et al., 2003; Zeng et al., 2001) are forebrain specific knockout of the CNB1 subunit of calcineurin, which is the regulatory subunit of calcineurin in the brain. In our model, the CN98 mouse over-express CNAa, the catalytic subunit of calcineurin. Even if the regulatory and catalytic calcineurin subunits participate in the same molecular cascade, the knockout mice used by Miyakawa and collaborators and the CN98 mice that we use here cannot totally be considered as mirror models. Finally it should be noted that such apparently contradictory results were obtained with CN98 versus calcineurin knockouts in learning and memory paradigms. Thus, in CN98 mice, the expression of an active form of CNA in the mouse brain modestly impairs synaptic plasticity, learning and memory (Mansuy et al., 1998a,b: Winder et al., 1998). In contrast, a modest reduction in calcineurin activity enhances synaptic plasticity and cognitive functions in the mouse (Malleret et al., 2001). However, full inhibition by pharmacological intervention or plain knockout was found to impair rather than improve performance (Zeng et al., 2001; Gerdjikov and Beninger, 2005).

In conclusion, congruent with recent studies that relate calcineurin and its inter-player DARPP-32 to psychosis in animal models (Miyakawa et al., 2003) and in humans (Gerber et al., 2003), our results suggest that genetic variations of calcineurin could predispose to a higher responsiveness to stimuli and could be related to the greater vulnerability to environmental situations that more easily trigger a pathological emotional state in patients that suffer from affective disorders. This study provides proof that manipulation of calcineurin, a candidate signal-transduction protein for affective disorders, results in relevant behavioral changes, which are corrected by the mood regulator valproate. CN98 mice might be helpful for the investigation of neurobiological substrates underlying the vulnerability to affective disorders and the identification of novel therapeutic targets.

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