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Chronic Maternal Morphine Alters Calbindin D-28k Expression Pattern in Postnatal Mouse Brain

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ABSTRACT The distribution pattern of calbindin (CB)-D28k-expressing neurons results to be altered in several brain regions of chronic morphine exposed adult mice. In this study, the influence of chronic maternal exposure to morphine on the distribution pattern of CB-D28k-expressing neurons in the brain of mouse offspring was investigated. Females of CD-1 mice were daily administered with saline or morphine for 7 days before mating, during the whole gestation period, and until 21 day post-partum. Their offspring were sacrificed on postnatal day 18, and the brains were examined by histology using cresyl violet and by immunohistochemistry using a rabbit polyclonal anti-CB-D28k antibody. Histology revealed no significant differences in the distribution pattern and the number of neurons between the offspring forebrain of the control group of mice and the two groups of mice treated with different doses of morphine. However, immunohistochemical analysis revealed that the number of CB-D28k-immunoreactive neurons remarkably decreased in the cingulate cortex, in the layers II–IV of the parietal cortex and in all regions of the hippocampus, while it increased in the layers V–VI of the parietal cortex and in the subicular region of the offspring brain of morphine treated mice. Overall, our findings demonstrate that maternal exposure to morphine alters the pattern of CB-D28k-expressing neuron pattern in specific regions of murine developing brain, in a layer- and dose-dependent way, thus suggesting that these alterations might represent a mechanism by which morphine modifies the functional aspects of developing brain. **Synapse 70:15–23, 2016.** © 2015 Wiley Periodicals, Inc.

INTRODUCTION

Morphine, extracted from *Papaver somniferum*, belongs to the 4,5-epoxymorphinan opiate class. As analgesic drug, it is administered for the treatment of mild to severe, acute, and chronic pain. Repeated morphine administrations may develop tolerance, physical dependence and addiction, the latter mainly attributed to the involvement of different brain regions and circuits (Pasternak and Pan, 2013). Both functional and gene expression studies have identified several proteins, including voltage-gated channels, adenyl cyclase, phospholipases, and many others that are involved in the in vivo adaptation to morphine and different effectors (Pasternak and Pan, 2013). In the central nervous system (CNS), morphine binds mainly μ-opioid receptors that are quite numerous in the limbic system where the antinociceptive action of opioids is high (Pasternak and Pan, 2013).

In the rodent hippocampus, morphine was found to affect the glutamatergic synaptic transmission both

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at pre- and post-synaptic sites, by modifying the expression levels of the cytoskeletal proteins involved in signaling and trafficking, and by altering post-synaptic density (Heidari et al., 2013; Morón et al., 2007). The immature brain is sensitive to the actions of opiates. They accumulate in the offspring nervous system due to the increased permeability of the blood-brain barrier affecting the CNS development. Infants exposed in uterus to opiates become physically dependent, and after parturition they suffer of nervous, gastrointestinal and respiratory dysfunctions (Richardson et al., 2006). In the rat, prenatal exposure to morphine resulted in altered physiological and behavioral processes, depending on the dose and the time of exposure (Chen et al., 2015; Niesink et al., 1999). Increased intrauterine death, significant deficits in sensory function, motor activity, exploratory behavior, learning and memory performances, delay opening of eye and ear, and reduced spontaneous activity were observed in the embryos of opiate treated rats (Fodor et al., 2014). These clinical manifestations are mainly attributable to opiate adverse effects on neuron migration and survival, leading to an overall inhibition of brain growth and CNS development. Increased mitosis, cell proliferation, and neuronal density as well as altered morphology of specific neurons were reported in some cortical and sub-cortical areas of the developing brain of opiate treated rats (Fodor et al., 2014; Lasky et al., 1977; Nosal, 1982; Zagon and McLaughlin, 1977). In contrast, maternal administration of cocaine or heroin to mice during pregnancy caused morphological alterations of pyramidal neurons in the somatosensory cortex, impairing short-term spatial memory in adult offspring (Lu et al., 2012). Offspring mice from maternal morphine administration in uterus showed only a light deficit in motor control, and a significant reduction of spontaneous activity during the first postnatal days (Castellano and Ammassari Teule, 1984). Changes in the functions of the endogenous opioid system as well as altered neuronal networks have been also reported in opiate exposed mice (Richardson et al., 2006).

Morphine appears to mainly act on GABAergic inhibitory interneurons that express the Ca^{2+}-binding proteins Calbindin (CB-D28k), Parvalbumin, and Calretinin which buffer elevated intracellular levels of free calcium acting as modulators of short intracellular Ca^{2+} signals (Maharajan et al., 2000; Schwaller, 2010). The CB-D28k calcium-binding protein is expressed in many brain regions, and various neuronal types in rodents (Baimbridge and Miller, 1982; Celio, 1990). Altered CB-D28k expression levels may result in impaired Ca^{2+} homeostasis and/or Ca^{2+} signaling, ultimately leading to the loss of synapses and dysfunctions of the neural network (Heizmann and Braun, 1992). Homozygous CB-D28k-knock-out (KO) mice display motor coordination impairment, although they show a normal brain development, and no evident behavioral phenotype (Airaksinen et al., 1997). CB-D28k depletion contributes to neuronal deficits and cognitive dysfunction in mouse models, and persistent mitochondrial fission that could lead to synaptic damage, bio-energetic failure, and subsequent neurodegeneration (Kook et al., 2014). Evidence exists that CB-D28k blocks multiple pro-apoptotic pathways (Guo et al., 1998; Palop et al., 2003), and it is involved in neurofilament assembly, thus playing an important role in the regulation of synapse formation (Schwaller, 2010).

A previous immunohistochemical study on the brain of adult mice treated with morphine showed changes in the distribution pattern of CB-D28k-expressing neurons in several brain regions, particularly in the anterior cingulate and parietal cortical regions (Maharajan et al., 1998). In this study, we investigated the effect of maternal morphine treatment on the distribution pattern of CB-D28k-expressing neurons in the brain of offspring mice. To this purpose, female CD-1 mice were daily administered with different doses of morphine before mating, during the whole gestation period, and for 21 days post-partum. Histological and immunohistochemical analyses on the brains of the offspring mice sacrificed on postnatal Day 18 (P18) were performed using cresyl violet staining for histology, and a rabbit polyclonal anti-CB-D28k antibody for immunohistochemistry. Our results show that maternal morphine chronic treatment alters the pattern of CB-D28k-expressing neurons in the cingulate and parietal cortices, and the hippocampus of offspring brain, thus suggesting that maternal exposure to the opiate may alter some functional aspects of developing brain.

MATERIAL AND METHODS

Animals

Four month old adult females of CD-1 mice (Harlan, Italy) were provided with standard pellet diet and water ad libitum, and housed in a 12-h/12-h light/dark cycle at constant temperature (22°C) and humidity ($60 \pm 5\%$). Morphine treatment protocols were performed as previously described (Maharajan et al., 2000). Adult mouse females were divided into three experimental groups. Briefly, six adult healthy control mice (M0 group) were daily intraperitoneally (i.p.) injected with 3.3 mL/kg body weight of physiological saline, six adult mice (M30 group) were i.p. treated with 30 mg/kg body weight of morphine (SALARS, Como, Italy) in saline, and other six adult mice (M60 group) were i.p treated with 60 mg/kg body weight of morphine. After 7 days of treatment, the females of the three groups were allowed to mate with normal, untreated males. Successfully mated females (as attested by vaginal plug) continued to receive a daily treatment through the gestation

TABLE I. Influence of maternal morphine exposure on the number of neurons immunoreactive for CB-D28k in the cingulate cortex of 18-day postnatal mice

Group	Mean number of neurons/mm^2 ± SD
M0	509 ± 42
M30	303 ± 17 $P < 0.0001$*
M60	252 ± 101 $P < 0.0001$* $P = 0.1260$†

Test 1. Factor Analysis of Variance "Anova" with Fisher's Protected Least Significant Difference for 3 groups is used. (*) *P*-values against M0 and (†) *P*-values against M30.

period, and until 21 days postpartum. The neonates were allowed to suckle on their natural mothers until the postnatal Day 18 (P18), when they were sacrificed. Thus, the murine offspring were exposed to morphine, through their mothers, during 19 days of prenatal period and 18 days of postnatal life. The protocol procedure was approved by the Veterinary Scientific Committee of the University of Naples Federico II (art 3 D.LVO 116/92). The number of the animals was lowering as much as possible.

Sampling from offspring and tissue preparations

The P18 neonates were deeply anaesthetized by i.p. injection of Ketamine HCl (Ketalar, 60 mg/g body weight) and sodium pentobarbital (Nembutal, 17.5 μg/g body weight). The brains were fixed by transcardial perfusion using a fixative containing 1% paraformaldehyde, 0.2 picric acid and 1.5% glutaraldehyde in 0.01 M phosphate buffer, pH 7.4 (Goodman et al., 1993).

Immunohistological procedures

The brains were either processed for histology using cresyl violet-staining or underwent to immunohistological procedure as previously described (Maharajan et al., 1998). Briefly, coronal sections of brain, cut with a vibratome, were incubated as free floating sections with the rabbit primary anti-CB-D28k antibody (dilution 1:2000; Swant, Switzerland). The revelation system consisted of biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA), avidin-biotin-peroxidase complex, and diaminobenzidine as a chromogen. The stained sections were dehydrated, mounted in Entellan, and observed under a light microscope (Nikon Eclipse E600). Photographs were taken by a digital camera DXM1200F. The CB-D28k-immnoreactive cells present in specific brain areas were counted as previously described (Maharajan et al., 2000), under a 25× objective, using camera lucida and suitably calibrated rectangles. The number of cells was expressed as the mean cell number per mm^2. At least eight readings taken per group of mice were evaluated for their statistical significance.

Statistical analysis

Test 1 factor Analysis of Variance (Anova) followed by Fisher's Protected Least Significant Difference (LSD) was applied for the 3 mouse groups (M0, M30, M60), and the *P*-values of M30 versus M0 and those of M60 versus M30 were calculated. Statistical significance was set at $P < 0.05$.

RESULTS

Histological observations

Brain sections from P18 neonatal mice showed no significant changes in the distribution pattern and number of neurons between the control (untreated) group of mice (M0) and the two groups of mice treated with different amount of morphine, 30 mg/kg body weight (M30) and 60 mg/kg body weight (M60), respectively, as revealed by cresyl violet staining (data not shown).

Calbindin (CB-D28k) immunohistochemistry

The majority of data regarding CB-D28k immunohistochemical neuronal patterns mainly refers to neonatal rat brains (Alcantara et al., 1993, 1996, Goodman et al., 1993; Sànchez et al., 1992), whereas only few studies have been reported in the murine brain (Blatow et al., 2003; Carretta et al., 2003; Kook et al., 2014). Our study shows that in specific areas of the forebrain the pattern of CB-D28k-expressing neuronal population in P18 neonatal mouse brains of M30 and M60 groups of mice exposed to maternal treatment of morphine is remarkably different from that of M0 group of untreated mice. The results of the quantitative analysis in the cingulate and parietal cortical regions are shown in Tables I and II.

The cingulate cortex

In the M0 mouse group, about 509 ± 42 neurons of the cingulate cortex resulted to be CB-D28k-immunoreactive (Table I). CB-D28k-immunoreactivity (CB-D28k-IR) occurred with different intensity in numerous nerve cell bodies lying at the layer II (Figs. 1A and 1D). CB-D28k-immunoreactive neuronal distribution decreased through the distal layers (Figs. 1A and 1D). Neurons lying within the layers III to VI were mainly multipolar with intense CB-D28k-IR of their cellular processes (Figs. 1D and 1G). In the M30 mouse group (Fig. 1B), and to a larger extent in M60 mouse group (Fig. 1C), the intensity of CB-D28k-IR of neurons in the layer II decreased (Figs. 1E and 1F). The total number of CB-D28k-labeled neurons also decreased in M30 and M60 groups as compared to M0 mouse group (Table I). Several neurons in the layers III–VI showed multipolar and bipolar shapes (Figs. 1E, 1F, 1H, and 1I). The intensity of CB-D28k-IR in the neuronal cell processes of

TABLE II. Influence of maternal morphine exposure on the number of neurons immunoreactive for CB-D28k in various layers of the parietal cortex of 18-day postnatal mice

	Mean number of neurons/mm2 ± SD per layer			
Group	Layers II–III	Layer IV	Layer V	Layer VI
M0	1570 ± 340	1691 ± 191	123 ± 58	84 ± 33
M30	992 ± 94	1775 ± 286	232 ± 41	144 ± 22
	$P < 0.0001$*	$P < 0.4358$*	$P < 0.0005$*	$P < 0.0002$*
M60	950 ± 131	1204 ± 116	283 ± 59	126 ± 25
	$P < 0.0001$*	$P = 0.0001$*	$P < 0.0001$*	$P = 0.0053$*
	$P = 0.7031$†	$P < 0.0001$	$P = 0.0694$†	$P = 0.1925$†

Test 1. Factor Analysis of Variance "Anova" with Fisher's Protected Least Significant Difference for 3 groups is used. (*) P-values against M0 and (†) P-values against M30.

offspring brain did not change at any concentration of maternal morphine treatment (Figs. 1D–1I).

The parietal cortex

Remarkable differences in the CB-D28k-immunoreactive pattern and number of neurons in M0, M30, and M60 groups of mice were seen (Table II and Fig. 2). In the M0 group of mice, the majority of CB-D28k-immunoreactive neurons were strongly stained (Fig. 2A); they were more numerous in the layers II–III and IV, while their number decreased in the layers V and VI (Table II). CB-D28k-immunoreactive neurons localized in the layers II–IV varied in the intensity of their IR that was confined to their cell body (Figs. 2A and 2D). Neurons of the layers V–VI showed CB-D28k-IR both in their cell bodies and their thin, sometimes ramified, cellular processes (Fig. 2G). A significant decreased number of CB-D28k-containing neurons was found in the layers II–III in M30 mouse group (Table II; Figs. 2B and 2E), and in the layers II, III, and IV (Table II; Figs. 2C and 2F) in M60 mouse group as compared to M0 mouse group. In contrast, the number of CB-D28k-expressing neurons increased in the layers V and VI in M30 and M60 groups of mice as compared to M0 mouse group (Table II). Bipolar and multipolar CB-D28k-immunoreactive cells were recognized by their cellular processes in the tissues of offsprings from both maternal control and treated mice (Figs. 2G–2I).

The hippocampus

Some quantitative and qualitative differences in the immunoreactive structures were observed in different regions of the hippocampal regions of P18 mouse brain. In the dentate gyrus of M0 (Fig. 3A) and M30 (Fig. 3B) groups of mice, the granule cells and mossy fibers were strongly CB-D28k-immunoreactive, while IR intensity decreased in M60 mouse group (Fig. 3C). In M0 mouse group, the stratum oriens (SO), radiatum (SR), and sublacunosum moleculare (SLM) harbored several interneuronal cell bodies with strongly CB-D28k-immunoreactive cellular processes (Fig. 3D); in contrast, pyramidal cells in the CA1, CA2, and CA3 zones were only weakly CB-D28k-immunoreactive. In M30 group of mice, a slight decrease of neuronal CB-D28k-IR intensity was observed within the SO and SR zones (Figs. 3B and 3E). In M60 group of mice, a drastical reduction of CB-D28k-IR was observed in the SR interneurons and their cellular processes, while a few strong immunoreactive interneurons were still seen in the SO (Figs. 3C–3F). A similar pattern was observed in the subicular area. In M60 group of mice, an increased number of labeled cells as compared to M0 group of mice was observed; these cells were deeply stained and showed augmented dendritic systems (data not shown).

DISCUSSION

This study, while confirming previous observation that the chronic maternal morphine treatment does not modify the total number of neuronal population in neonatal forebrain (Maharajan et al., 2000), for the first time demonstrates that morphine maternal exposure affects CB-D28k expression in the cingulate cortex, parietal cortex, and hippocampus of P18 mouse brain, and that the opiate action is region-, layer-, and dose-dependent.

Cingulate cortex

The cingulate cortex is a part of the limbic system involved in emotion and affective responses, and memory processing that occurs shortly after training (Farr et al., 2000). Differently from humans, rodents lack a cingulate sulcus, thus their cingulate cortex comprises an anterior and middle cingulate cortical area, but misses the posterior cingulate cortex, while their posterior cortex area is occupied by the retrosplenial area (Vogt and Paxinos, 2014). Furthermore, the rodent cingulate cortex anatomically differs from that of humans in its intracingulate projections as well as in the projections to brain and spinal cord. Functional differences also exist between the rodent and human cingulate cortex, since in the rodents the dorsal area connects with sensorimotor and neocortical areas, while the ventral area has extensive connections with amygdala, temporal and limbic cortices, septum, medial preoptical and hypothalamic areas,

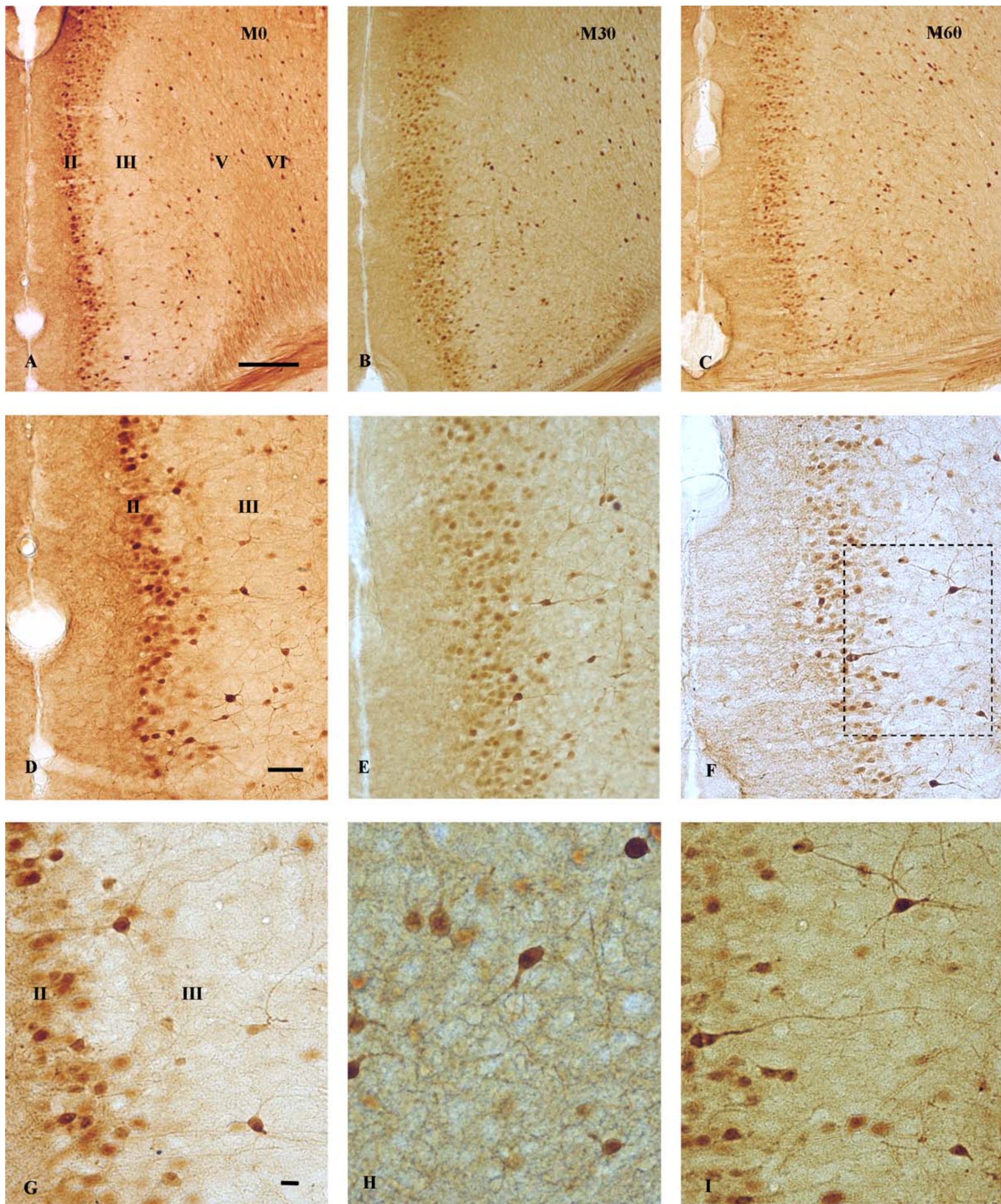

Fig. 1. Coronal sections of P18 mice cingulate cortex immunoreactive for CB-D28k. **A**: In M0, CBD28k-IR occurs with different intensity in numerous nerve cell bodies lying at the layer II. CBD28k–immunoreactive neurons decrease in number through the distal layers. **B**: In M30 and (**C**) in M60, CBD28k-immunoreactive neurons decrease in number and intensity of staining in layer II (**D–F**). Higher magnification of layers II and III in M0, M30, and M60: CB-D28k-IR in the cell processes of offspring brain is unaffected by maternal morphine treatment. (**G–I**) CB-D28k-immunoreactive neurons in layers III–VI are mainly multipolar in M0, M30, and M60, a feature of juvenile neurons. Calibration bars: A–C 10 μm; D–F 20 μm; G–I 40 μm.

and with brainstem monoaminergic cells. Both anterior and middle cortices also have different interactions with the visual cortex (Vogt and Paxinos, 2014). In the cingulate cortex of M0 group of mice (offspring of untreated females), the most numerous CB-D28k-immunoreactive interneurons lie at layer II and their number significantly decreases in M60 group of mice (offspring of females treated with the highest dose

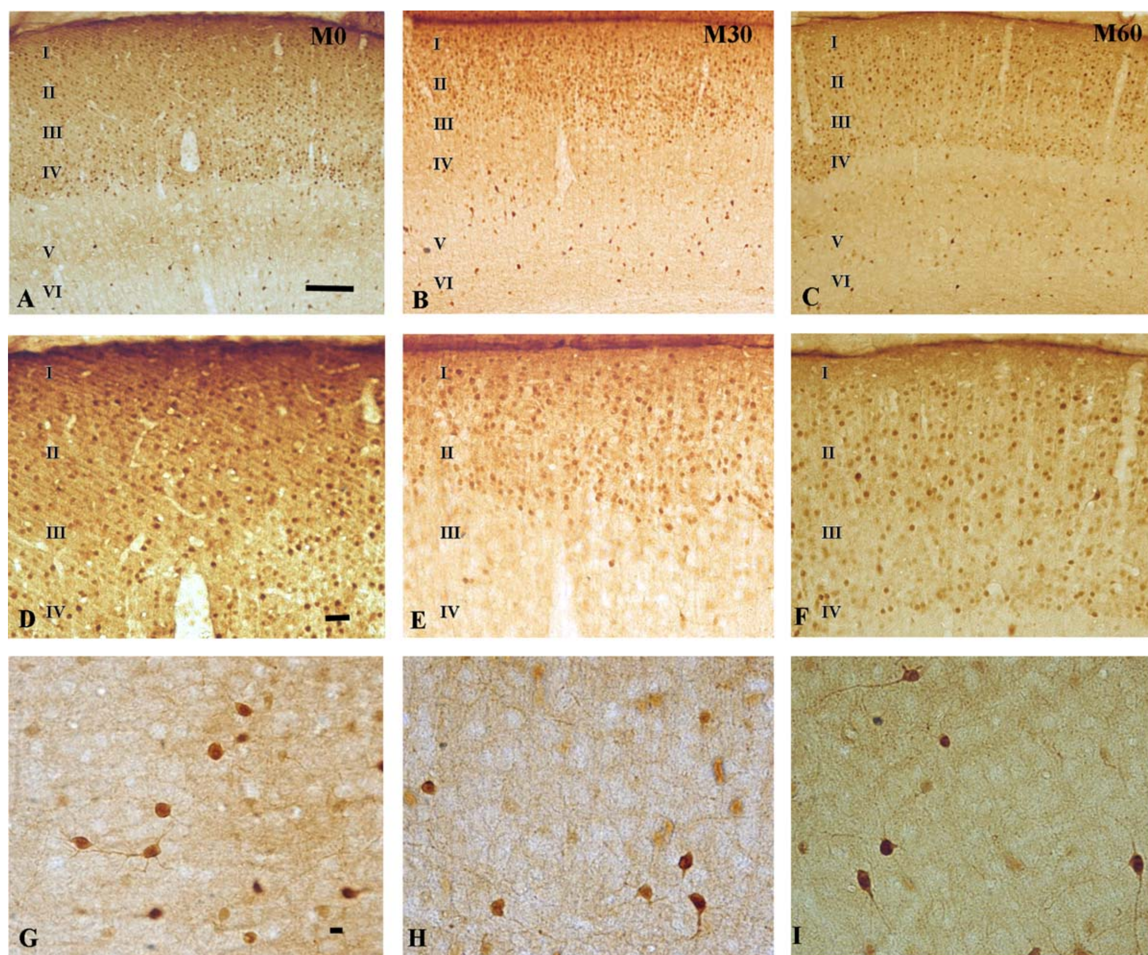

Fig. 2. Coronal sections of P18 mice parietal cortex immunoreactive for CB-D28k. **A**: In M0, CB-D28k-IR occurs with different intensity in numerous nerve cell bodies lying at the layers II–IV. CB-D28k-immunoreactive neurons decrease in number within the layers V and VI. **G**: In these last layers, several multipolar CB-D28k-immunoreactive neurons are visible. **B–E**: In M30 and (**C–F**) in M60, CB-D28k-immunoreactive neurons decrease in number and intensity of staining in layers I–IV, while increase in layers V–VI. **H**: CB-D28k-IR is present in the cellular processes of nerve cell bodies within layers V–VI in M30 and (**I**) in M60. G: Higher magnification of CB-D28k-immunoreactive nerve cell bodies and their cellular processes in M0. CB-D28k-IR in the cell processes of offspring brain is unaffected by maternal morphine treatment in (H) M30 and in (I) M60. Calibration bars: A–C 10 μm; D–F 20 μm; G–I 40 μm.

morphine), while no significant changes are detected in the remaining layers which comprise the pyramidal corticospinal projecting neurons, thus resembling the pattern seen in the adult mice (Maharajan et al., 1998).

Parietal cortex

In the M0 group of mice, CB-D28k-expressing neurons are more numerous in the layers II–IV and less in the layers V–VI. The chronic maternal morphine exposition differently affects the number of CB-D28k-containing neurons in the offspring brain as compared to what seen in the adult mice (Maharajan et al., 1998). In the M30 group of P18 mice, the number of CB-D28k-expressing neurons increases in the layer V like in M30 adult mice. In the M30 group of mice, the number of CB-D28k-containing neurons significantly decreases in the layers II–III, doesn't significantly change in the layer IV, and increases in the layer V. A slightly increase of CB-D28k-expressing neurons is detected in the layer VI of both M30 and M60 mice as compared to the control group of mice, and in contrast to the adult mice where the number of CB-D28k-expressing neurons remained unchanged at both doses of morphine treatment (Maharajan et al., 1998).

The cingulate and parietal cortical regions of M0 (control) P18 mice show more numerous CB-D28k-containing neurons than adulthood (Maharajan et al., 1998). Our data are in agreement with those from Kook et al. (2014) and Alcantara et al. (1993, 1996) who showed that in rat the number of cortical CB-D28k-expressing neurons increases up to 8th–11th day after birth, while decreases after 11th day, and

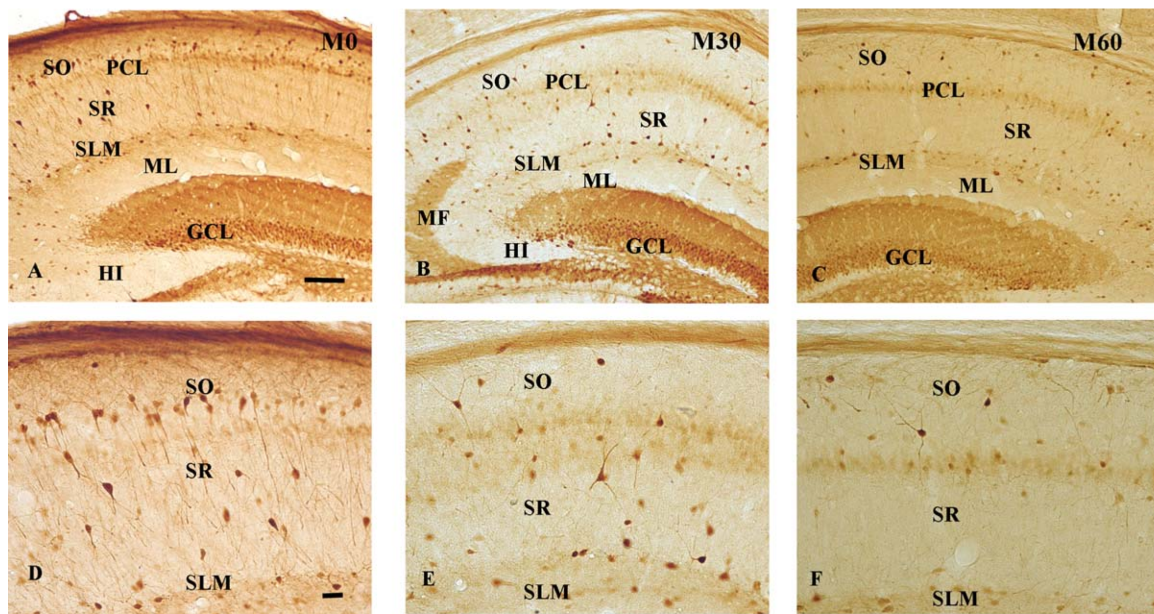

Fig. 3. Coronal sections of P18 mice hippocampus immunoreactive for CB-D28k-IR. **A**: In MO, CB-D28k-IR occurs in interneurons of SO, SR, SLM, GCL, and MF. **D**: Higher magnification of SO, SR, and SLM zones. **B**: In M30, CB-D28k-IR is not changed in neurons of GCL and MF zones. **E**: Higher magnification of SO, SR, and SLM zones: CB-D28k-IR is decreased in interneurons of SO and SR zones. **C**: In M60, CB-D28k-IR is strongly decreased in interneurons of the SR, and GCL. **F**: Higher magnification of SO, SR, and SLM zones: note the almost absence of CB-D28k-immunoreactive interneurons in the SR zone. Calibration bars: A–C 10 μm; D–F 20 μm. SO, stratum oriens; SR, stratum radiatum; SLM, stratum sublacunosum moleculare; ML, molecular layer; GCL, granular cell layer; HI, hilus; MF, mossy fibers.

the adult pattern is achieved by the end of the third postnatal week. Such findings have been attributed to the shift from calbindin to parvalbumin (PV) content of cortical developing nonpyramidal neurons (Alcantara et al., 1993, 1996). Such event probably occurs in mice as well: a small number of PV-immunolabeled neurons have been detected in P18 control mice resulting more numerous at the layer IV (Maharajan et al., 2000), although less numerous than the CB-D28k-immunoreactive cells observed in this study. Whether some of these CB-D28k containing neurons coexpress PV as a sign of conversion or constitute two different subpopulations remains to be established.

In this study, the maternal chronic morphine treatment causes a decrease of the CB-D28k expression in neurons from the layers II–V of the parietal cortex, while PV-immunoreactive neurons were reported to increase in the same layers (Maharajan et al., 2000). During the postnatal life, CB-D28k-expressing neurons change their phenotype being mainly multipolar in the early postnatal period (Alcantara et al., 1993; Hof, 1999), and becoming bipolar or fusiform shaped at the end of the third postnatal week (Hof et al., 1999, present study). A previous study (Maharajan et al., 2000) reported an increased number of PV-labeled dendrites in the cingulate and parietal cortical regions of neonatal mouse brain under chronic prenatal morphine exposure. Another study showed that the treatment of pregnant mice with the opioids heroin and cocaine causes a decrease or an increase of dendrite outgrowth, respectively, in the pyramidal neurons of the somatosensory cortex of murine offsprings (Lu et al., 2012; Fodor et al., 2014). All these findings provide evidence that prenatal exposition to opiates can affect the development of neuronal connections by delaying or accelerating neural outgrowth.

Hippocampus

Hippocampus is the region of learning and memory that involve plastic changes of synapses throughout the life. The CB-D28k-immunoreactive neuronal distribution pattern in the hippocampus of M0 (control) P18 mice resembles that observed in the adult mice and rats (Celio, 1990; Maharajan et al., 1998; Mattson and Chan, 2001; Palop et al., 2003): CB-D28k-IR occurs in the granular cells of the dentate gyrus, in both few pyramidal cells and interneurons of CA1, 2, and 3 zones, and in the mossy fibers that project to CA1. New granule cells are generated throughout the postnatal life in rat (van Praag et al., 2002): they appear first at the border of the ilus and later colonize the outer parts of the granular layer (Cameron et al., 1993). The newborn granule cells do not contain CB-D28k (Rami et al., 1987): thus, the weak Ca^{2+}-buffer capacity of young granule cells would facilitate the induction of synaptic plasticity. During

this stage, the CB-D28k content increases up to the mature levels and 2–3 week postnatal granule neurons are considered mature cells (Müller et al., 2005). In this study, we observed a significant reduction of the CB-D28k-immunoreactive mossy fibers, interneurons of the layered Ammon's horn, and of the staining intensity of the granular neurons only in the M60 treated mouse group. In contrast, no changes were observed in the number and IR intensity in PV-containing neurons both in the M30 and M60 P18 murine groups compared to the controls (M0 group) in a previous study carried out under the same experimental conditions (Maharajan et al., 2000). In the adult female mice too, no differences were evident in the total number of CB-D28k-immunoreactive neurons among M0, M30, and M60 groups of mice (Maharajan et al., 1998). Thus, overall previous data (Richardson et al., 2006) and our findings strengthen the evidence that the opioid system may differently act in adult and young subjects.

Learning deficits in adult mice expressing familial AD-mutant hAPP have been ascribed to decreased levels of CB-D28k in the granule cells of the dentate gyrus (Palop et al., 2003). We observed reduced CB-D28k-IR in the same region of P18 mice from females exposed at the highest doses of morphine. Since drug addiction is an abnormal form of learning and adaptation of the memory system to the new condition (Eisch et al., 2000), the decreased expression of CB-D28k in this region produced by morphine may probably affects the capacity of learning and memory processes of mice that might become apparent later in life.

In conclusion, our results demonstrate that chronic morphine treatment of mice at prenatal and postnatal stages affects, in a layer- and dose-dependent fashion, the expression pattern of a subpopulation of CB-D28k-immunoreactive neurons, mainly interneurons localized in the cingulate and parietal cortical regions, and hippocampus of P18 mouse brain. This opiate action might lead to learning and memory, behavioral, and mood disorders later in the life. Such results differ from those observed in the adult subjects exposed to the same opiate treatment, and can be attributed to the immaturity of the neuronal system in the juvenile mice.

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AUTHOR CONTRIBUTIONS

FF contributed to animal manipulation, mouse treatment, and brain collection. PM and VM prepared sections and performed histology and immunohistochemistry. AC, VM, and RDM contributed to the experimental design, the interpretation of results, and the writing of the paper draft. All the authors discussed the results and contributed to write the final version of the manuscript. All the authors approved the final version of the manuscript.

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