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Dopamine is necessary to endogenous morphine formation in mammalian brain *in vivo*

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Abstract

Morphine, the most used compound among narcotic analgesics, has been shown to be endogenously present in different mammalian/invertebrate normal tissues. In this study, we used mice that cannot make dopamine due to a genetic deletion of tyrosine hydroxylase specifically in dopaminergic neurons, to test the hypothesis that endogenous dopamine is necessary to endogenous morphine formation *in vivo* in mammalian brain. When dopamine was lacking in brain neurons, endogenous morphine was missing in brain mouse whereas it could be detected in brain from wild type rodent at a picogram range. Our data prove for the first time that endogenous dopamine is necessary to endogenous morphine formation in normal mammalian brain. Morphine synthesis appears to be originated from dopamine through L-tyrosine in normal brain tissue. Morphine synthesis is not considered to occur inside the same neuron in normal tissue; released dopamine might be transported into morphinergic neuron and further transformed into morphine. A physiological role for endogenous morphine is suggested considering that dopamine could modulate thermal threshold through endogenous morphine formation *in vivo*. Thus, dopamine and endogenous opiates/opioid peptides may be interconnected in the physiological processes; yet, endogenous morphine may represent a basic link of this chain.

Keywords: biosynthetic pathway, dopamine, morphine, transgenic mouse.

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Morphine alkaloids in animal tissues were first demonstrated by immunological recognition (Gintzler *et al.* 1976). Subsequently, endogenous morphine-like compounds have been identified in mouse and calf brain as well as in human (Hazum *et al.* 1981; Killian *et al.* 1981). The molecular structure of the HPLC purified compound was confirmed as morphine by liquid and gas chromatographic retention times and mass spectrometry in various normal tissues such as rat and rabbit skin, mouse, bovine and primate brain, hypothalamus and adrenal glands, invertebrate and human tissues, mammalian lung and human plasma (Goldstein *et al.* 1985; Donnerer *et al.* 1986; Cardinale *et al.* 1987; Stefano *et al.* 1993; Liu *et al.* 1997; Guarna *et al.* 1998; Goumon and Stefano 2000; Neri *et al.* 2004; Muller *et al.* 2008). Endogenous morphine and morphine-6-glucuronide have been also detected in different human and animal cell lines (Poeaknapo *et al.* 2004; Zhu *et al.* 2005; Muller *et al.* 2008). Data on subcellular distribution showed that the major fractions of the alkaloid resides in a synaptosomal fraction (Donnerer *et al.* 1987).The presence of endogenous morphine was recently found in large dense core vesicles of SH-SY5Y cells (Muller *et al.* 2008). Studies have demonstrated that animals do contain the same morphine precursors and metabolites as opium poppy. Codeine, morphine and their conjugate have also been identified in human cerebrospinal fluid (Cardinale

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Abbreviations used: DD, dopamine deficient; DOPAC, dihydroxyphenylacetic acid; GC/MS, gas chromatography/mass spectrometry; HVA, homovanillic acid; MHPG, 3-methoxy-4 hydroxyphenylethylene glycol.

et al. 1987). Reticuline, thebaine, and codeine are some of the main intermediates of morphine biosynthesis in the poppy plant (Kutchan 1998) and these compounds also have been found in mammals (Kodaira *et al.* 1989; Hofmann *et al.* 1999; Zhu *et al.* 2003). In mammalian tissue some steps in the biosynthetic way were demonstrated by adding some intermediate precursors. Thebaine, an intermediate of morphine biosynthesis in the poppy plant, was transformed to oripavine, codeine, and morphine by rat liver, kidney and brain microsomes in the presence of an NADPH-generating system (Kodaira and Spector 1988). Morphine synthesis was demonstrated in healthy human white blood cells exposed to catecholamine (Zhu *et al.* 2005). Undifferentiated human cancer cells in culture (neuroblastoma cells SH-SY5Y) were capable of synthesizing morphine when exposed to different heavy isotope-labeled potential precursors (e.g., with ^{13}C, ^{2}H, and ^{18}O labels) administered to cell and incubated for a certain period (Boettcher *et al.* 2005). When [2,2-$^{2}H_2$] dopamine was added to SH-SY5Y, labeling was found in morphine molecule from cell pellet (Boettcher *et al.* 2005). In Parkinson's disease, after chronic L-DOPA exposure, elevated morphine levels were found (Matsubara *et al.* 1992). Considering these findings, dopamine might be proposed as starting point in morphine biosynthetic pathway in normal mammalian tissue *in vivo* (Fig. 1). To test this hypothesis, we used mice that cannot make dopamine to determine if morphine is produced. We demonstrate that this is indeed the case. In dopamine deficient (DD) mice, morphine formation could not be detected *in vivo* whereas endogenous morphine was detected in brain from controls.

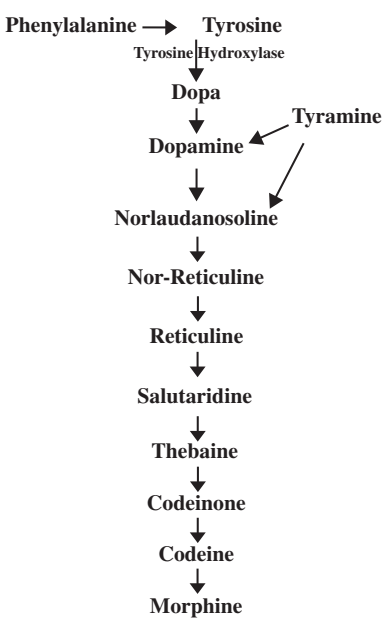

Fig. 1 Putative biosynthetic pathway for endogenous morphine in vertebrate and invertebrate tissue.

Materials and methods

Dopamine deficient mice

Mice unable to synthesize dopamine specifically in dopaminergic neurons (DD mice) were created by inactivating the tyrosine hydroxylase (*Th*) gene then restoring *Th* function in noradrenergic cells. Briefly, DD ($Th^{-/-}$ $Dbh^{Th/+}$) mice carrying two inactive tyrosine hydroxylase alleles, one functional dopamine β-hydroxylase (Dbh^{+}) allele, and one *Dbh* allele that drives expression of *Th* (Dbh^{Th}) were generated as described (Zhou and Palmiter 1995). Controls included animals that carry at least one intact *Th* allele and one intact *Dbh* allele. Dopamine deficient (DD) mice are hypoactive and aphagic, and die 3–4 weeks postnatally if not rescued by daily injections of the synthetic dopamine precursor L-3,4-dihydroxyphenylalanine (L-dopa), which temporarily restores dopamine synthesis and allows dopaminergic neuron functions (Zhou and Palmiter 1995; Szczypka *et al.* 1999). Dopamine levels in the striatum of DD mice reach about 9% of wild-type levels 3 h after L-dopa treatment, but fall to < 1% within 24 h (Szczypka *et al.* 1999). In our experiments, DD mice were maintained for 68 h without synthetic L-Dopa. All mice were treated in accordance with guidelines established by the National Institutes of Health and the University of Washington Animal Care Committee.

Determination of dopamine and noradrenaline level in mouse brain

Mice used for these experiments were anesthetized with CO_2, cervically dislocated, decapitated and the brain dissected, placed on dry ice and then stored at −80°C. Various brain areas from control and DD mice were dissected on a cold plate. The tissue samples were homogenated by ultrasonication in deionized water at 4°C (w/v 1 : 10), and dissolved in an equal volume of 0.2 N perchloric acid and centrifuged at 25 000 *g* for 10 min at 4°C. The supernatant was analysed by using high-pressure liquid chromatography (HPLC) using electrochemical detection as described previously (Guevara-Guzman *et al.* 1994) for dopamine and noradrenaline. Dopamine metabolite dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and noradrenaline metabolite 3-methoxy-4 hydroxyphenyl-ethylene glycol (MHPG) were determined in the same brain regions.

Sample pre-treatment and GC/MS analysis of endogenous morphine

Before gas chromatography/mass spectrometry (GC/MS) analysis, the brain tissue was pretreated by coupling a classical deproteinization method with solid-phase extraction as previously described (Felby *et al.* 1974; Lubli *et al.* 1990; Guarna *et al.* 1998, 2004). Three groups each of six pooled mouse brains were used. Briefly, deproteinization was achieved by adding to the homogenate a (1/1.2 v/v) saturated ammonia sulphate solution, followed by acidification with 1 mL of 3N HCl and heating at 121°C for 30 min. The sample was cooled, filtered through Buchner funnel using Whatman n°1 filter paper. The solution was adjusted to pH 9 with phosphate buffer at pH 9 and then submitted to morphine extraction by solid phase on Bond Elut Certify columns (Varian, Harbor city, CA, USA). The extraction steps were: 1) activation of the extraction phase with 4 mL of methanol and 4 mL of distilled water; 2) passing of the sample through the column (flow rate about 2 mL/min); 3) cleaning up of the sorbent phase using 4 mL of distilled water; 4) changing of the phase pH by adding 1 mL of 100 mM acetate buffer at pH 4; 5) cleaning up

of the phase by adding 2 mL of methanol; 6) drying and 7)eluting with 4 mL of methylene chloride/isopropyl alcohol/ NH_4OH (80/20/2 by volume). New glassware was used for each experiment and blank samples were also extracted in order to check for contamination of both reagents and solvents. Extraction solvents were collected into 120 × 80 glass tubes. Simultaneously, 1 mL of standard solution, containing morphine (Carlo Erba, Milano, Italy) in concentrations ranging from 0.5 ng/mL to 10 ng/mL, was put into similar glass tubes. Nalorphine (100 µL of 250 ng/mL; S.A.L.A.R.S, Como, Italy) in methanol was added, as an external standard, to the samples and to the standard solutions. Immediately before analysis, sample extracts and standard solutions were derivatized by adding 50 µL of a solution of N-Methyl-N-trimethyl-silyltrifluoroacetamide (Pierce, Rockford, IL, USA) 20% in toluene; 1 µL of each derivatized sample was submitted to GC/MS analysis which was performed using Agilent Technologies 6890 N gas chromatograph coupled with a Agilent Technologies 5975 B inert MS detector and equipped with a Agilent Technologies-5MS column (30 m × 0.25 mm × 0.25 µm film thickness). The temperatures were respectively 250°C for the injector and 280°C for the detector. The oven temperature was programmed at 50°C for 2 min, up to 280°C at a heating rate of 35°C/min, the final temperature being held for 5 min. Helium was used as the carrier gas, the head pressure was 2 psi and the flow rate of the carrier was 0.7 mL/min. The injection mode was splitless, the purge valve was open after 1 min. Derivatized morphine and nalorphine retention times (RT) were respectively 11.26 min and 11.96 min. The analysis was performed in selected ion monitoring mode; derivatized morphine was identified by fragments 429 (quantifier ion), 401 and 414 (qualifier ion) and derivatized nalorphine by fragments 455 (quantifier ion), 440 and 414 (qualifier ion). Previous GC/MS analysis of standard solutions of derivatized morphine and nalorphine did validate both the retention times and the characteristic ions for these substances (Pfleger *et al.* 1992; Don-Liang *et al.* 1995). Morphine identification using GC/MS-selected ion monitoring was achieved if retention time was the same as the standard solution one, peaks of the chosen ions grew simultaneously, the heights of the peaks of the chosen ions showed reciprocal abundance similar as those of a standard solution. Morphine quantification was based on a multipoint calibration curve constructed using fragment 429 for morphine and 455 for nalorphine. The extraction recovery for morphine was established as previously described (Guarna *et al.* 1998) and was estimated as 82–86%. The minimal detectable amount of derivatized morphine was 10 pg (signal-to-noise ratio > 5).

Results

Dopamine, noradrenaline and their metabolite content in brain from control and DD mice

Dopamine and noradrenaline levels were determined in several different brain regions from control and DD mice (Fig. 2a and b). The noradrenaline levels were not significantly different in four regions from control and DD mice (Fig. 2a).There were higher levels of dopamine in the striatum and lower levels in other brain regions of control mice; however, dopamine was undetectable in all these brain regions

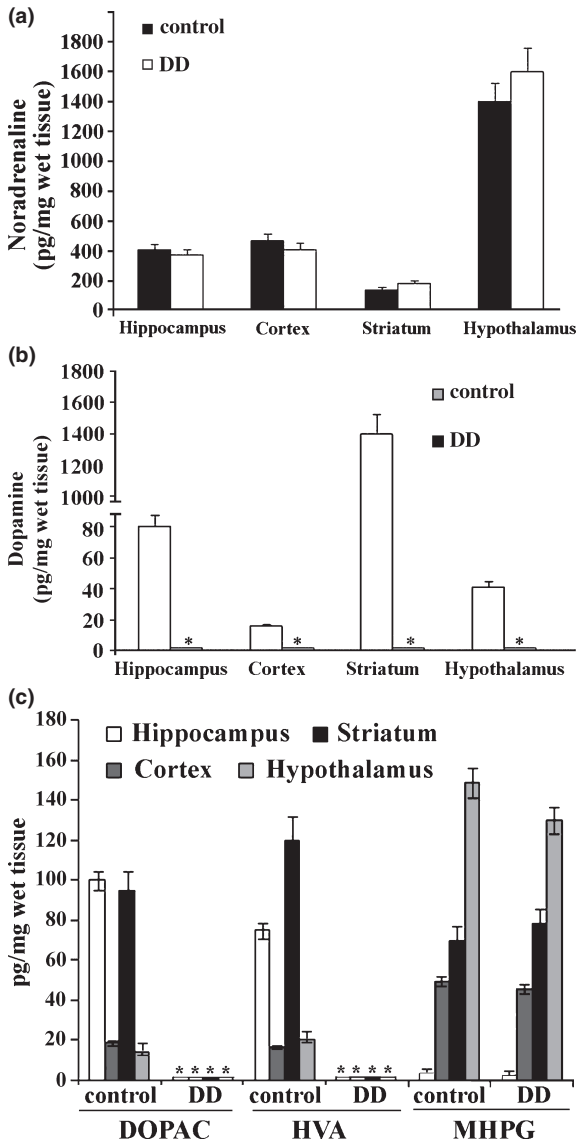

Fig. 2 Noradrenaline, dopamine, DOPAC, HVA and MHPG level in mouse brain. Noradrenaline (a) and dopamine (b) level was measured in hippocampus, cortex, striatum and hypothalamus from control and DD mice. DOPAC, HVA and MHPG level were measured in the same regions from control and DD mice (c). Each mean level is obtained from six mice. *Significant level (α) less than 0.01 in one-way ANOVA, DD versus control.

of DD mice (Fig. 2b). DOPAC and HVA level in different brain regions show that dopamine metabolites are formed from control mice whereas are lacking in dopamine deficient mice (Fig. 2c). Noradrenaline metabolite MHPG was formed in brain regions from both control and DD mice (Fig. 2c).

Endogenous morphine content in brain from control and DD mice

Brain content of endogenous morphine quantified by GC/MS from three independent experiments was 140 ± 12 pg/g (wet

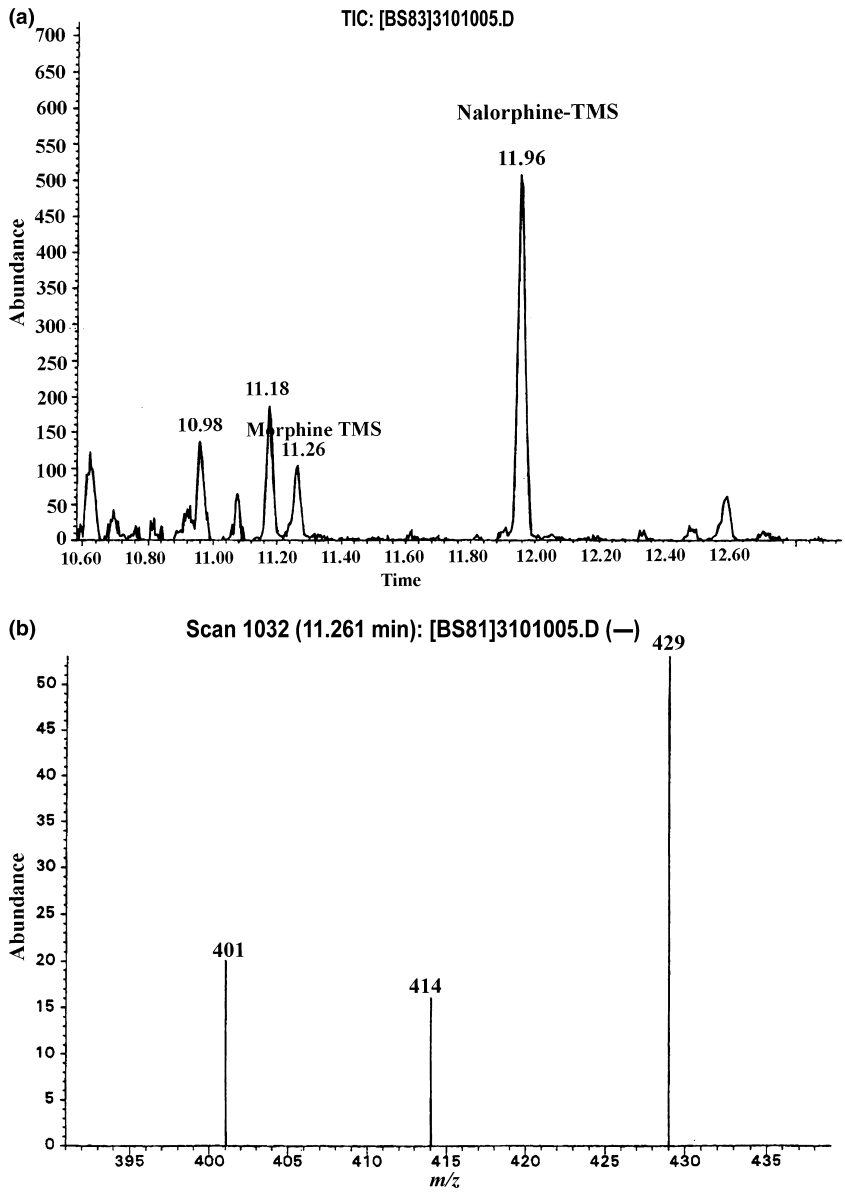

Fig. 3 Chromatographic plot of control mouse brain morphine content A representative chromatographic plot of a pooled brain sample obtained from six mice is shown in (a) after derivatization. The retention times of derivatized morphine and nalorphine were respectively 11.26 and 11.96 min. Ion abundance for morphine is reported in (b).

weight) in control mice (Figs 3–6). Analysis of the peak at morphine retention time (11.26 min) was done using as characterizing ions for morphine fragments 401, 414 (qualifier) and 429 (quantifier). Both retention time and ions' reciprocal abundances similar to those of a morphine standard solution identified the substance present in the sample as morphine (Fig. 3a and b). Reciprocal distinctive ion abundance was not respected in mass spectrum of the peak at 11.27 retention time in brain sample from DD mice (Fig. 4). Detailed analysis of the three fragments with respect to morphine distinctive ions in DD sample does not fulfill the identification criteria for morphine as previously described. In fact, the heights of the peaks of the chosen ions do not show reciprocal abundance as for morphine and the chosen ions do not grow simultaneously (Fig. 5). Ion abundance for morphine in mouse brain from three independent experiments show that there is a significant difference for each considered ion between DD and control mice (Fig. 6). The blank samples showed no morphine contamination as noted by the absence of morphine positive material (data not shown). Morphine contamination was also not found in Purina 5LJ5 diet as previously shown (Guarna *et al.* 1998).

Discussion

Endogenous morphine could be detected in brain of control mice (picogram/gram wet weight) at a level in agreement with previous results; however, a lower morphine level (140 pg/g) was observed in control mice with respect to previously determined basal level from wild Swiss albino

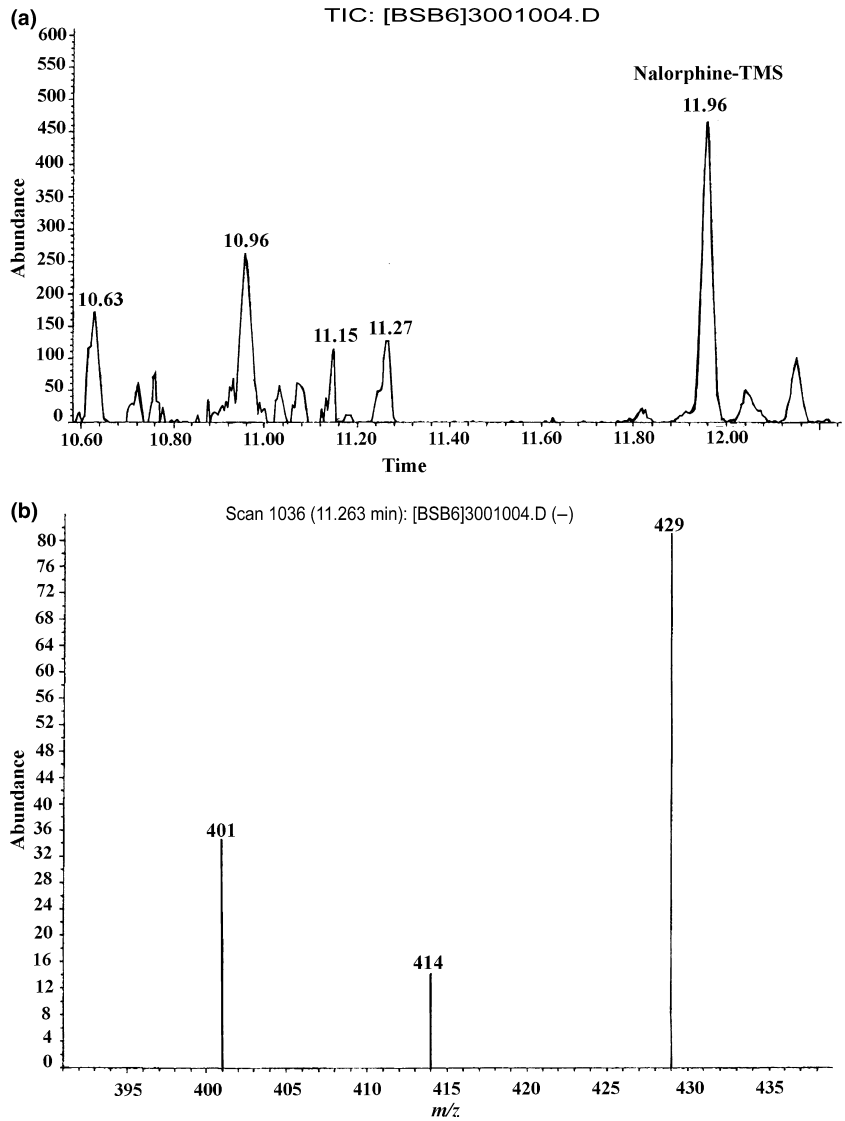

Fig. 4 Chromatographic plot of DD mouse brain morphine content. A representative chromatographic plot of a pooled brain sample obtained from six mice after derivatization is reported (a). Ion abundance for morphine is not respected as clearly visible in (b).

type brain (290 pg/g) (Guarna *et al.* 2004). Control mice mostly carry only one tyrosine hydroxylase allele and have a tissue content of brain dopamine lower than the corresponding value found in wild mouse type (Chourbaji *et al.* 2004). Dopamine metabolite DOPAC and HVA could be formed from control mice whereas were undetectable in DD mice. Noradrenaline metabolite MHPG was found in both DD and control mice at a similar concentration. Endogenous morphine concentration in mouse brain appears to be correlated with dopamine concentration suggesting that the difference in endogenous morphine concentration between control and wild mouse originate from dopamine level. When dopamine was lacking in mouse brain neurons due to a lack of tyrosine hydroxylase gene, endogenous morphine was undetectable. Our data prove for the first time that dopamine is necessary for morphine formation *in vivo* in mouse brain. Dopamine can also be synthesized from tyramine which constitutes an alternative route to dopamine formation. But the fact that DD mice had no dopamine or morphine indicates that this pathway is not relevant to endogenous morphine formation in mouse. Brain dopamine content could be only partially restored by L-dopa administration to DD mice. At the peak of activity, 3 h after the standard dose of 50 mg/kg L-DOPA added with carbidopa, the highest dopamine content was found in the striatum, but it was only 9.1% of the wild type levels (Szczypka *et al.* 1999). Although morphine biosynthesis might be restored in L-dopa treated DD-brain, morphine levels were undetectable at the level of our methodology's sensitivities.

It is also important to understand where morphine synthesis occurs in rodent brain. Different regions from rodent brain have a appreciable content of opiate alkaloids at the range of pmol/g tissue as shown by different authors (Donnerer *et al.* 1987; Lee and Spector 1991; Muller *et al.*

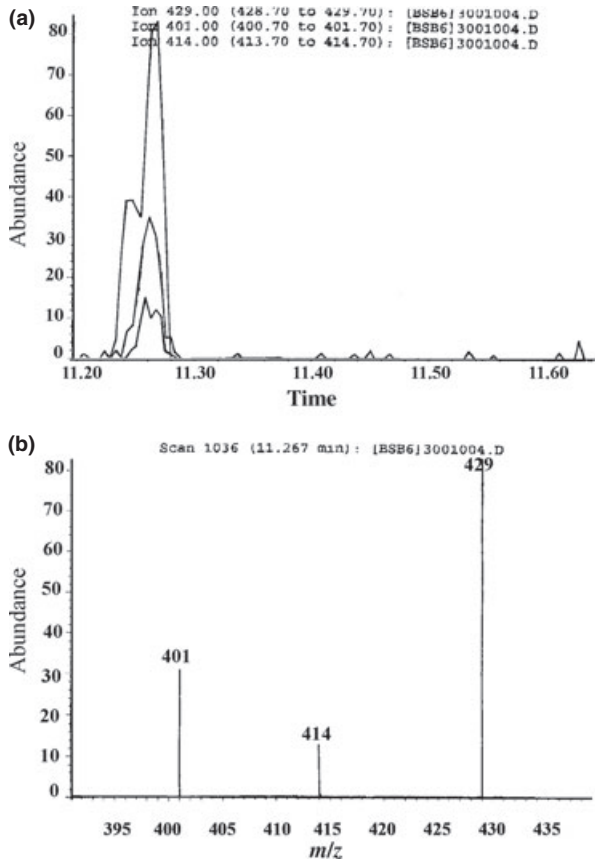

Fig. 5 Analysis of peak at morphine retention time from DD mouse brain. The analysis of peaks originated from the three fragments (a) and their abundance (b) is represented in figure for case reported in Fig. 4.

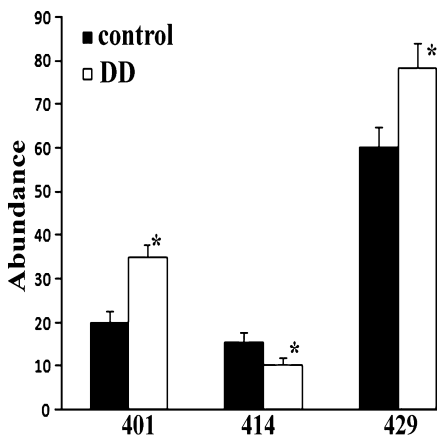

Fig. 6 Ion abundance for morphine in DD and control mouse brain. Abundance for ion 401, 414 and 429 in brain sample from DD and control mice is represented as the mean value of three independent experiment. *Significant level (α) less than 0.01 in one-way ANOVA, DD versus control.

2008). Spinal cord (Donnerer *et al.* 1987), hippocampus (Muller *et al.* 2008) and amygdala (Zhu *et al.* 2004) appear to be enriched of morphine. Regarding other brain areas as cortex, midbrain, pons/medulla and cerebellum, there doesn't seem to be anyone area that has a much higher concentration than the others. Morphine immunoreactivity was found in different areas from rodent brain. Morphine reactive neurons or processes were found in brainstem and cerebellum (Gintzler *et al.* 1978). Neuron perikaria, fibers and dendrites immunoreactive for morphine were found in cortex, hippocampus, cerebellum, striatum, brainstem by different authors (Bianchi *et al.* 1993, 1994; Muller *et al.* 2008) showing that endogenous morphine is stored in specific brain neurons of different brain areas. Otherwise, dopaminergic neurons are localized in restricted brain areas as substantia nigra or ventrotegmental area. These considerations speak against the colocalization of endogenous dopamine and morphine inside the same neuron. Dopaminergic nucleus project to various brain areas as striatum, cortex, limbic and cerebellum where morphine immunoreactive neurons have been localized (Seroogy *et al.* 1989; Nelson *et al.* 1997). It is possible that dopamine could be released and taken up by these neurons with by means of either dopamine or noradrenaline transporter and then converted to morphine similarly as exogenous dopamine, added to the medium, is taken up by culture cell to synthesize morphine (Boettcher *et al.* 2005). According to this hypothesis, the density of neurons able to synthesize and store morphine in a brain area constitutes the limiting step in morphine formation. Previous *in vitro* studies showed that endogenous morphine can be released from nerve cell further supporting that dopamine could be taken up, converted to morphine and then released. Depolarization due to high potassium concentrations increased the release of the alkaloid from rodent brain nerve terminals many fold with respect to the basal value, and the release was dependent on the presence of calcium in the medium showing that endogenous morphine can be released in a Ca^{++} dependent manner (Guarna *et al.* 1998). Recent finding showed in SH-SY5Y cells that endogenous morphine is secreted in response to nicotine stimulation via a Ca^{++} dependent mechanism substantiating a neurotransmitter role for the endogenous alkaloid (Muller *et al.* 2008).

The physiological significance of endogenous morphine is another issue of importance. We have shown that dopamine is necessary to endogenous morphine synthesis *in vivo*. Mammalian normal organism is capable of synthesizing one compound which is exogenously used as potent analgesic; however, the endogenous role played by morphine found in nervous tissue might be different. DD mice are more sensitive to thermal stimulus. Dopamine-deficient mice, submitted to a tail-flick at different temperatures, withdrew their tails more quickly after saline injection with respect to control, indicating that they are more sensitive to pain under basal conditions (Hnasko *et al.* 2005). In Parkinson patients after acute L-dopa withdrawal, heat and cold pain threshold were significantly lowered (Slaoui *et al.* 2007). Endogenous morphine immunoneutralization decreased thermal response

latency in mice showing that endogenous morphine modulates thermal threshold in mice (Guarna *et al.* 2002). We suggest that dopamine modulates thermal threshold through endogenous morphine formation *in vivo*.

These data shed a new light on relationship between morphine and dopamine. Thus, dopamine and endogenous opiate/opioid peptides may be interconnected in the physiological processes; yet, endogenous morphine may represent a basic link of this chain.

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