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Differential distribution of synapsin IIa and IIb mRNAs in various brain structures and the effect of chronic morphine administration on the regional expression of these isoforms

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Abstract

Quantitative reverse transcriptase-polymerase chain reaction and in situ hybridization techniques were used to determine the regional distribution of synapsin IIa and IIb mRNAs in rat central nervous system and to assess the effect of chronic morphine administration on the gene expression of these two isoforms of synapsin II. These isoforms are members of a family of neuron-specific phosphoproteins thought to be involved in the regulation of neurotransmitter release. Our data demonstrate the widespread distribution, yet regionally variable expression, of synapsin IIa and IIb mRNAs throughout the adult rat brain and spinal cord. The ratios of the relative abundance of synapsins IIa and IIb differed by up to 4.5-fold among the various regions studied. Synapsin IIa and IIb mRNAs were shown to be highly concentrated in the thalamus and in the hippocampus, whereas lower concentrations were found in most other central nervous system structures. In this study, we show differential regulation by morphine of synapsins IIa and IIb in various regions of the brain. In the striatum, a 2.4-fold increase was observed in the levels of synapsin IIa mRNA following chronic morphine regime, whereas no change was found for synapsin IIb. On the other hand, mRNA levels of synapsin IIb in spinal cord of chronically treated rats were markedly decreased (by 62%), while no alterations were observed in synapsin IIa. Selective regulation by morphine has also been demonstrated in several other central nervous system structures. The opiate-induced regulation of the gene expression of synapsin II isoforms could be viewed as one of the cellular adaptations to the persistent opiate effects and may be involved in the molecular mechanism underlying opiate tolerance and/or dependence.

Keywords: Synapsin II; Opiate tolerance; Morphine; In situ hybridization; Reverse transcriptase-polymerase chain reaction (RT-PCR); Regional distribution

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1. Introduction

The synapsins comprise a family of four highly related neuron-specific phosphoproteins, synapsins Ia and Ib and synapsins IIa and IIb, which together account for ≈ 9% of total synaptic vesicle proteins. The synapsins are associated with the cytoplasmic surface of small synaptic vesicles, bind to the cytoskeleton and contribute to the regulation of neurotransmitter release [7,9,24,51,57]. They serve as major brain substrates for cAMP-dependent and Ca^{2+}/calmodulin-dependent protein kinases [26,52,62]. The phosphorylation state of the synapsins is increased under conditions that promote Ca^{2+}-dependent release of neurotransmitters, while dephosphorylation of these molecules provides inhibitory constraint for synaptic vesicle exocytosis [24].

Synapsins I and II are encoded by two different genes. The a and b isoforms of each protein derive from alternative splicing of the primary transcript. All four synapsins share a common NH_2-terminal domain. The differences among the four proteins are restricted to the C-terminal region. In contrast to synapsin I, the alternative use of two different exons leads to a more extended region of dissimilarity for synapsins IIa and IIb [57]. Both synapsins I and II are fairly abundant in the brain and are present in most brain regions. Synapsin II is less abundant than synapsin I at a ratio of 1 : 2 [19]. The ratio of synapsin IIa to IIb is 1 : 2 [67]. Compared to the current state of knowledge on synapsin I, much less is known regarding synapsin II function and regulation (for reviews, see [59,64]). Differential expression of synapsin II has been demonstrated in several regions of the central nervous system (CNS), in-

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cluding the retina [30,31], the cerebellar nuclei [57] and the olfactory bulb [56]. In addition, several reports have suggested a role for synapsin II in the formation and maintenance of synapses [18] and in the regulation of synaptic vesicle function [22]. Knockout experiments have revealed that synapsin II is essential for the acceleration of synaptic vesicle traffic during repetitive stimulation [51].

Activation of opiate receptors was shown to inhibit adenylate cyclase (AC) activity [3,10,54] and to reduce the activity of voltage-dependent Ca^{2+} channels [2,25]. Moreover, acutely applied opiates were shown to attenuate neurotransmitter release from various neuronal cells and tissues [40–42,48]. In view of the role played by opiates in inhibiting neurotransmitter release, we have previously shown that acute opiate agonist application reduces synapsin I phosphorylation in cocultures of rat spinal cord (SC) and dorsal root ganglion (DRG) neurons [44]. A similar result was described by Fleming et al. [21] using rat striatal membranes.

Prolonged exposure to opiates results in tolerance and dependence. These phenomena are accompanied by various cellular compensatory adaptations, including alterations in gene expression (for reviews, see [46,53]). In this regard, we have recently observed that chronic opiate treatment leads to an increase in synapsin I protein in SC-DRG cocultures, as well as to an increase in synapsin I mRNA in the locus coeruleus (LC), amygdala, central gray of the pons (CGP) and SC [34,43]. No information is currently available regarding the effect of opiate exposure on the expression of synapsin II. Here, we report that the mRNAs of synapsins IIa and IIb are differentially distributed throughout the brain and that chronic opiate exposure leads to differential effects on the levels of the two isoforms in various regions of the CNS.

2. Methods

2.1. Materials

pGEM3Z plasmids containing full length synapsin IIa and IIb cDNAs were kindly donated by Dr. G. Thiel (Institute for Genetics, University of Cologne, Germany). Restriction enzymes were supplied by Promega (Madison, WI, USA). In situ hybridization detection reagents and RNA transcription kit were from Boehringer, Mannheim, Germany. First-strand cDNA preparation kit and PCR reagents were obtained from Clontech (Palo Alto, CA, USA). All other chemicals were from Sigma (St. Louis, MO, USA).

2.2. Animals and tissue preparation

Adult male Wistar rats (150–200 g) were used in this study. Slow-release morphine pellets (75 mg each) (NIDA, Rockville, MD, USA) were transplanted subcutaneously, two on the first day and two on the following day. Control rats were implanted with placebo pellets using the same surgical procedure. Tolerance state was determined by the hot plate assay and dependence was assessed by naloxone-induced jump latency. On the fifth day, animals were decapitated and specific brain structures as well as SCs were removed and RNA was extracted (for reverse transcriptase-polymerase chain reaction (RT-PCR)). Alternatively, animals were perfused transcardially with 250 ml saline flush followed by 250 ml 4% paraformaldehyde in phosphate-buffered saline (PBS) (for in situ hybridization). Brains and SCs were removed and post-fixed in 4% paraformaldehyde for additional 48 h at room temperature. Paraffin blocks were prepared by standard methods. 5-μm coronal sections were cut and mounted on poly-L-lysine-coated slides which were stored at room temperature in a dust-free box [34].

2.3. Preparation of synapsin IIa and IIb DNA probes

Sequences unique either to synapsins IIa or IIb were chosen to be used as probes in order to ensure specific detection of the corresponding mRNAs. A 224-bp fragment (positions 1514-1737) of synapsin IIa and a 207-bp fragment (positions 1493–1699) of synapsin IIb were generated by PCR reactions. 10 ng of pGEM3Z plasmids containing the full length sequence of each isoform were linearized to be used as templates for the amplification reaction. PCR products were generated following 35 cycles that consisted of: 1.5 min denaturation at 94°C, 30 s annealing at 60°C, 45 s extension at 70°C. The following primers were used at a final concentration of 2.4 μM each: synapsin IIa sense – 5′ CGGGATCCCGCAACCCCAAG-GAATGCAGCC 3′; synapsin IIa antisense – 5′ GCTCTA-GAGCTGGGGCTTCTGTGGTGGAGCA 3′; synapsin IIb sense – 5′ CGGGATCCCGGCCCCCCAAGGTTGTT-TACAG 3′; synapsin IIb antisense – 5′ GCTCTA-GAGCAGCACTGAGAGGACACAG 3′. Primers were designed to contain BamH1 and Xba sequences in the 5′ end of the sense and of the antisense oligonucleotides, respectively. PCR products were digested with these two enzymes, purified on a 1.3% agarose gel, and ligated into Bluescript KS(−) employing the Boehringer's ligation kit. Following transformation into Escherichia coli DH5-α competent bacteria, the plasmids were extracted and processed for cRNA synthesis.

2.4. Synthesis of cRNA probes

Bluescript KS(−) plasmids containing either the 224 bp of the synapsin IIa cDNA (positions 1514–1737) or 207 bp of the synapsin IIb cDNA (positions 1493–1699) were cut using BamH1(for antisense orientation) or Xba (for sense orientation). cRNAs were transcribed in vitro in the presence of digoxigenin–labeled UTP (Boehringer, Mannheim, Germany) with either T7 RNA polymerase (antisense) or T3 RNA polymerase (sense). These cRNA probes were used for in situ hybridization experiments.

2.5. In situ hybridization

Brain and SC paraffin sections were deparaffined, rinsed twice in 2 × SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0), and treated with proteinase K (25 μg/ml) for 30 min at 37°C. Each section was pre-hybridized with 100 μl of pre-hybridization buffer (50% formamide, 4 × SSC, 2 × Denhardt's solution, 500 μg/ml salmon sperm DNA and 0.1% SDS) for 1–3 h at room temperature. For hybridization, 30–50 ng of synapsin IIa or IIb digoxigenin-labeled antisense cRNA probes were applied in 30 μl of hybridization solution (equivalent to the pre-hybridization buffer with the addition of 10% dextran sulphate) for each section. Slides were coverslipped with parafilm and incubated at 42°C for 16 h in a humid chamber. Post-hybridization washes were as follows: 4 × SSC (two changes, 15 min each) followed by RNAse A treatment (50 μg/ml) for 30 min at 37°C, 2 × SSC + 0.1% SDS, 2 × SSC (twice), 1 × SSC (twice), for 10 min each. Following a blocking step (using blocking reagent supplied by Boehringer's DIG-detection kit), sections were incubated for 3–5 h at room temperature with antidigoxigenin antibody conjugated to alkaline phosphatase (diluted 1 : 300

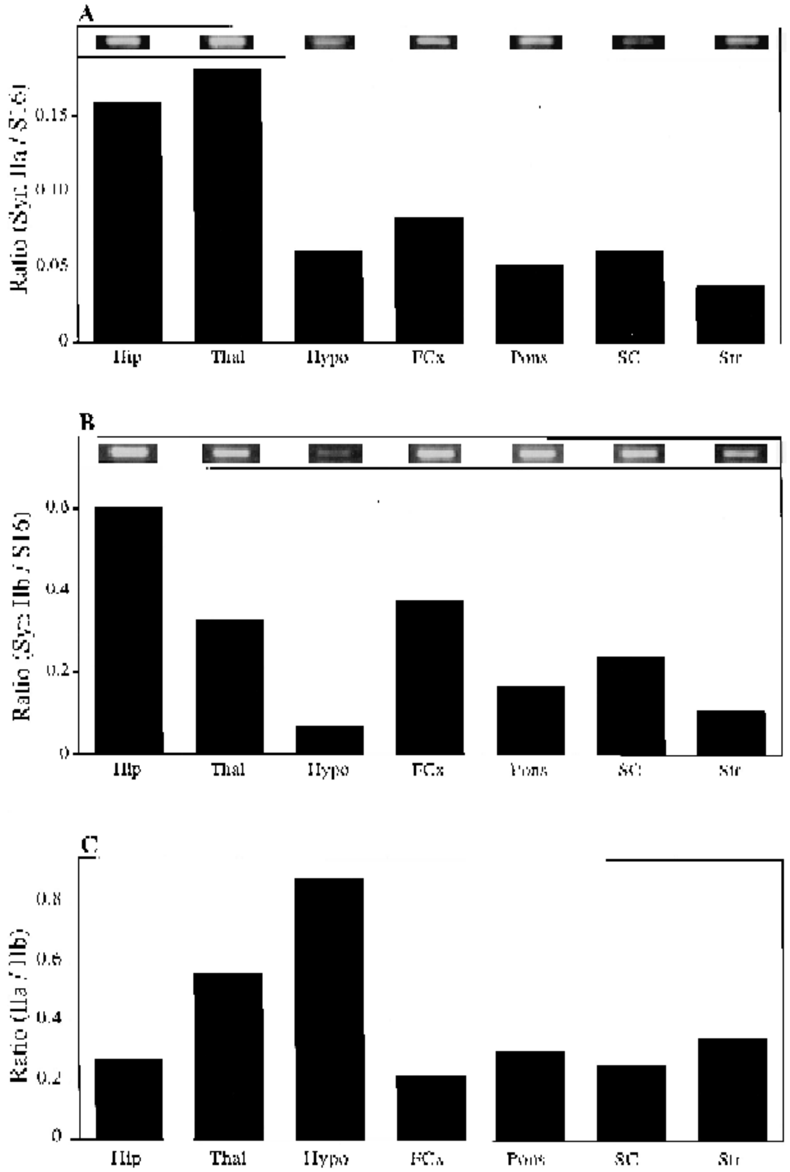


Fig. 1. RT-PCR analysis of the relative distribution of synapsins IIa and IIb in various CNS areas. mRNA levels of synapsins IIa and IIb have been determined by quantitative RT-PCR. A: the bands in the upper panel correspond to the 224-bp fragment amplified by RT-PCR on a template of full length synapsin IIa. The bar graph shows the ratio of the mRNAs of synapsin IIa to the S16 internal standard. B: the bands in the upper panel correspond to the 207-bp fragment representing synapsin IIb; the bar graph shows the ratio of synapsin IIb to S16. C: the ratio of synapsin IIa/synapsin IIb. Hippocampus (Hip); thalamus (Thal); hypothalamus (Hypo); frontal cortex (FCx); spinal cord (SC); striatum (Str).

in blocking solution). Color was developed overnight using a mixture of 5-bromo-4-chloro-3-indolylphosphate (0.17 mg/ml) and nitroblue tetrazolium salt (0.34 mg/ml). The reaction was stopped by washing with 10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, followed by ascending concentrations of alcohol, xylene and Permount mounting. Morphine-treated and control sections from parallel brain and SC areas were obtained under the same conditions and were hybridized and stained in parallel. Sections for in situ hybridization were taken from four control and four morphine-treated rats. At least six experiments were performed for each condition.

The in situ hybridization signals were viewed and photographed by bright-field microscopy. In order to ensure the specificity of the probes, we performed the following controls on adjacent sections: (1) hybridization with synapsin IIa and IIb digoxigenin-labeled sense RNA probes; (2) hybridization in the absence of cRNA probes; and (3) RNAse A treatment of the sections prior to hybridization with the antisense probe. Representative sections were chosen to demonstrate the distribution of each synapsin II isoform.

2.6. Quantitative RT-PCR analysis

Total RNA was extracted from specific CNS areas of two control and two morphine-treated rats by the method of Chomczynski and Sacchi [12]. First-strand cDNA synthesis was carried out (using Clontech 1st-strand cDNA kit) under the following conditions: 2.5 μg of total RNA from each tested region was mixed with oligo(dT) primer and random hexamer. The volume was adjusted to 13.5 μl with diethylpyrocarbonate-treated water. The mixture was incubated for 5 min at 70°C. RNAse inhibitor, dNTPs and murine RT were then added (final volume of 20 μl). Following incubation at 43°C for 100 min, the RT was inactivated by heating the reaction for 5 min at 95°C and the volume was adjusted to 100 μl. For each area analyzed, 2.5 μl of cDNA synthesis reaction mixture was used for PCR, which was conducted in a final volume of 50 μl containing 0.16 mM of each dNTP, 1 μM of each primer and 0.65 U of Taq DNA polymerase. The PCR reaction was cycled as follows: 1-min denaturation at 93°C, 45 s annealing at 67°C, 50 s extension at 71.5°C, 45 s denaturation at 91.5°C, for 4 cycles. Then, 45 s each of: annealing at 64°C, extension at 71.5°C and denaturation at 91.5°C, for 3 cycles. The PCR was then continued as follows: synapsin IIa, 23 cycles of 45 s (each step) at 58°C (for annealing), at 71.5°C (for extension) and 91.5°C for denaturation. Synapsin IIb, 20 cycles of 45 s (each step) at 62°C (for annealing), extension at 71.5 and 91.5°C for denaturation. The last cycle was performed with 1.5 min extension at 71.5°C. In order to quantify the amount of input mRNAs of the various tested regions as well as of control vs. treated samples, coamplification of an internal control S16 ribosomal protein mRNA was performed. A 400-bp cDNA fragment was coamplified using the following primers: S16 sense – 5′ TAAACCCTGATCCTTGA-GACTGG 3′; S16 antisense – 5′ TGAGGAGCTCGT-GTTCATACTTG 3′. The final products were analyzed by 1.3% agarose gel. To eliminate the possibility of non-specific contamination, we performed the PCR reaction with the omission of cDNA templates. S16 was found to be an appropriate internal standard since quantitation of the amount of S16 mRNA in the different CNS regions revealed similar concentrations with a deviation of $< 10\%$ between samples. Moreover, the mRNA for S16 was found not to be affected by morphine treatment.

2.7. Quantitation and analysis

Quantitative analysis of the RT-PCR was performed with the aid of a computerized video-imaging system using the public domain NIH Image program. For each of the regions studied, the areas and the mean optical densities (OD) of the two synapsin II isoforms and of S16 PCR signals were determined. The background OD values were subtracted from the signal mean OD values. The data for synapsin II mRNAs are presented as mean OD and are expressed as ratio of synapsin over S16 (syn/S16).

At the beginning RT-PCR was conducted for each individual region. However, since it was found that the relative amounts of synapsins IIa and IIb in each region were consistent in different experiments and in different animals, the RNA samples of each area were pooled.

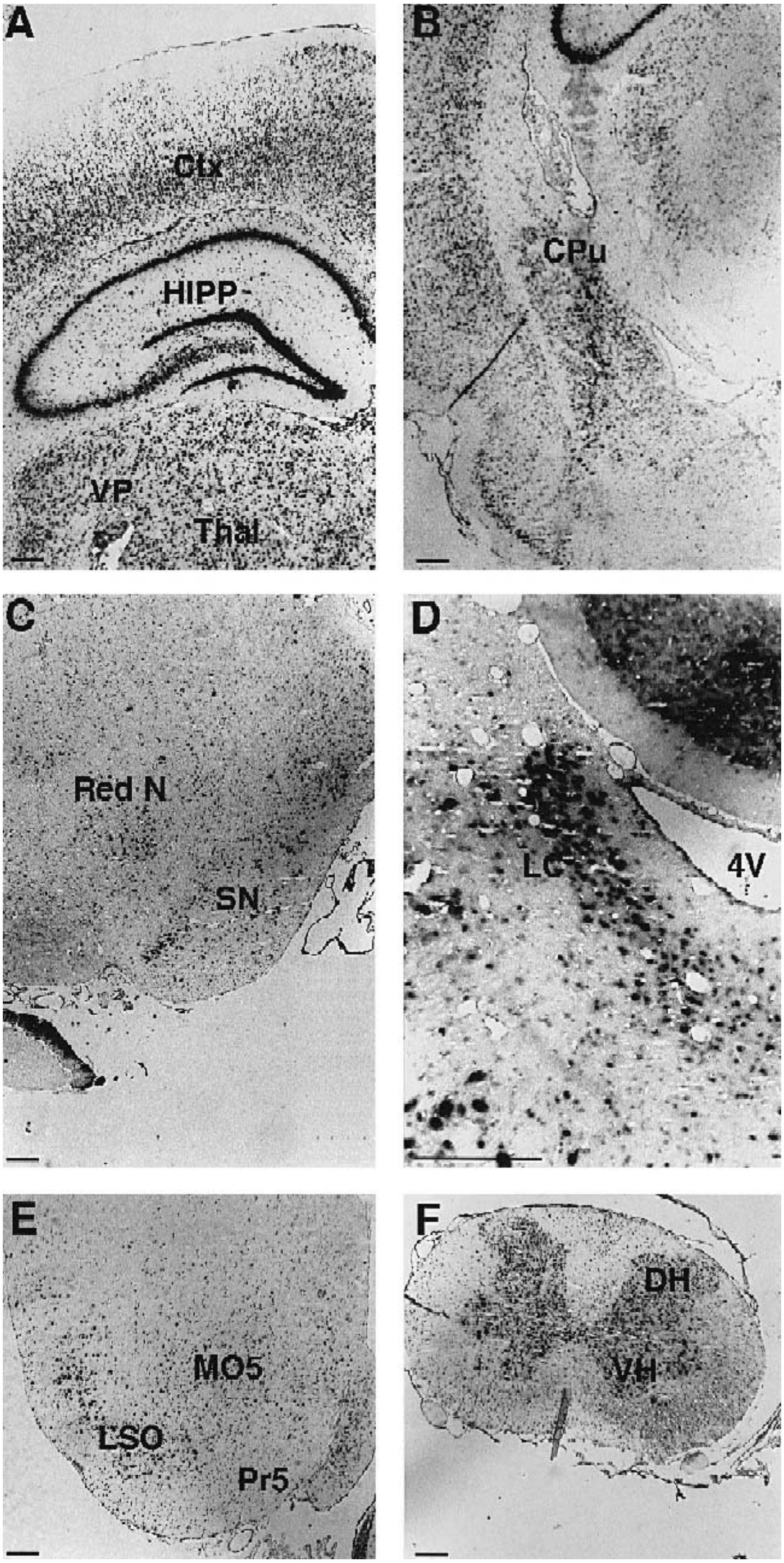
3. Results

3.1. Regional distribution of synapsin IIa and IIb mRNAs as determined by PCR

To determine the regional distribution of these two isoforms in the brain, we designed two PCR products corresponding to unique sequences which characterize each synapsin (positions 1514–1737 of synapsin IIa and 1494–1699 of synapsin IIb).

Fig. 2. In situ hybridization of synapsin IIa mRNA in discrete CNS areas. Coronal sections were hybridized with digoxigenin-labeled antisense cRNA probe for synapsin IIa, as described in Materials and methods. A: section showing hippocampus, thalamus, ventroposterior nucleus (VP) and cortex (Ctx). B: section showing caudate-putamen (CPu). C: section showing the substantia nigra (SN) and the red nucleus (Red N). D: high magnification of section showing locus coeruleus (LC) and location of the fourth ventricle (4V). E: section showing the principal sensory (Pr5) and the motor (Mo5) nuclei of the trigeminal nerve, and the lateral superior olive (LSO). F: section showing spinal cord, dorsal horn (DH) and ventral horn (VH). Bars, 250 μm.

From the regions studied by PCR (Fig. 1A), the highest amount of synapsin IIa mRNA was found to be present in the thalamus and in the hippocampus (87% of the thalamus). The amount of synapsin IIa in the frontal cortex (Fcx) is 45% of that present in the thalamus. Much lower levels were found in the hypothalamus, pons, SC and



striatum (33, 28, 34 and 33%, respectively, as compared to the thalamus). Of all CNS areas examined by RT-PCR, the hippocampus was found to contain the highest concentration of synapsin IIb mRNA. This isoform is much less abundant in other brain regions, in the following order: hippocampus (100%), Fcx (62%), thalamus (54%), SC (39%), pons (27%), striatum (18%), hypothalamus (11%) (Fig. 1B). Quantitative analysis of the ratio of synapsin IIa/synapsin IIb (Fig. 1C) reveals that synapsin IIb is more abundant in all the regions that were investigated. The highest ratio of IIa/IIb was found in the hypothalamus (0.88), an intermediate ratio was found in the thalamus (0.56), and the lowest ratios were found in the striatum (0.35), pons (0.30), hippocampus (0.27), SC (0.26), FCx (0.22). A more detailed analysis of synapsin mRNA distribution was performed employing in situ hybridization, the results of which are presented below.

3.2. In situ hybridization studies of synapsin IIa

3.2.1. Forebrain

Of all structures of the forebrain, the Fcx showed the highest intensity of synapsin IIa hybridization signals. Very weak labeling (if any) was detected in the nucleus accumbens (NAc) (data not shown). This finding is interesting, taking into account that low labeling was also obtained for the ventral tegmental area (VTA), which projects to the NAc.

3.2.2. Telencephalon

In agreement with the results obtained by RT-PCR, it was clearly demonstrated that the most intense signals were present in the hippocampus. Very intense signals were obtained in all hippocampal areas, i.e. CA1-CA4, and the dentate gyrus (Fig. 2A). Synapsin IIa mRNA was also found in additional structures of the telencephalon, including the amygdala and the amygdalohippocampal area (AHi) (data not shown). Moderate to low hybridization signals were viewed in the basal ganglia (caudate-putamen) (Fig. 2B). This result is compatible with the RT-PCR detection of low synapsin IIa mRNA levels in the striatum.

3.2.3. Diencephalon

In the thalamus, signals were detected in all nuclei, although synapsin IIa mRNA signals were most profound in the ventroposterior nucleus (Fig. 2A). The hypothalamus showed a relatively low intensity of hybridization signals in all areas. Moderate signals were found in the medial habenular nucleus (MHb), which is part of the epithalamus (data not shown).

3.2.4. Brain stem

Brain stem structures were clearly stained for synapsin IIa mRNA. In the midbrain, synapsin IIa mRNA was observed in cell bodies of several nuclei including the substantia nigra and the red nucleus (Fig. 2C). Almost no signal was found in the VTA. Regarding the pons (Fig. 5C), the highest labeling was present in the LC, which is the major noradrenergic nucleus in the brain, located on the floor of the fourth ventricle (Fig. 2D). Intense to moderate signals were viewed in the dorsal tegmental nucleus (DTg), in the dorsal raphe nucleus (DRN) and in the medial and lateral superior olive (LSO) (Fig. 5C). Low labeling was detected in structures related to the principal sensory (Pr5) and motor (Mo5) nuclei of the trigeminal nerve (Fig. 2E).

3.2.5. SC

We studied the distribution of synapsin IIa mRNA in coronal sections of several subregions of the SC (cervical, dorsal and lumbar). The mRNA was predominantly located in the gray matter in the laminae of both the dorsal and ventral horns (Fig. 2F). High magnification revealed that the signals of the mRNA were localized in different cell types originating both from sensory and motor systems.

3.3. In situ hybridization studies of synapsin IIb mRNA

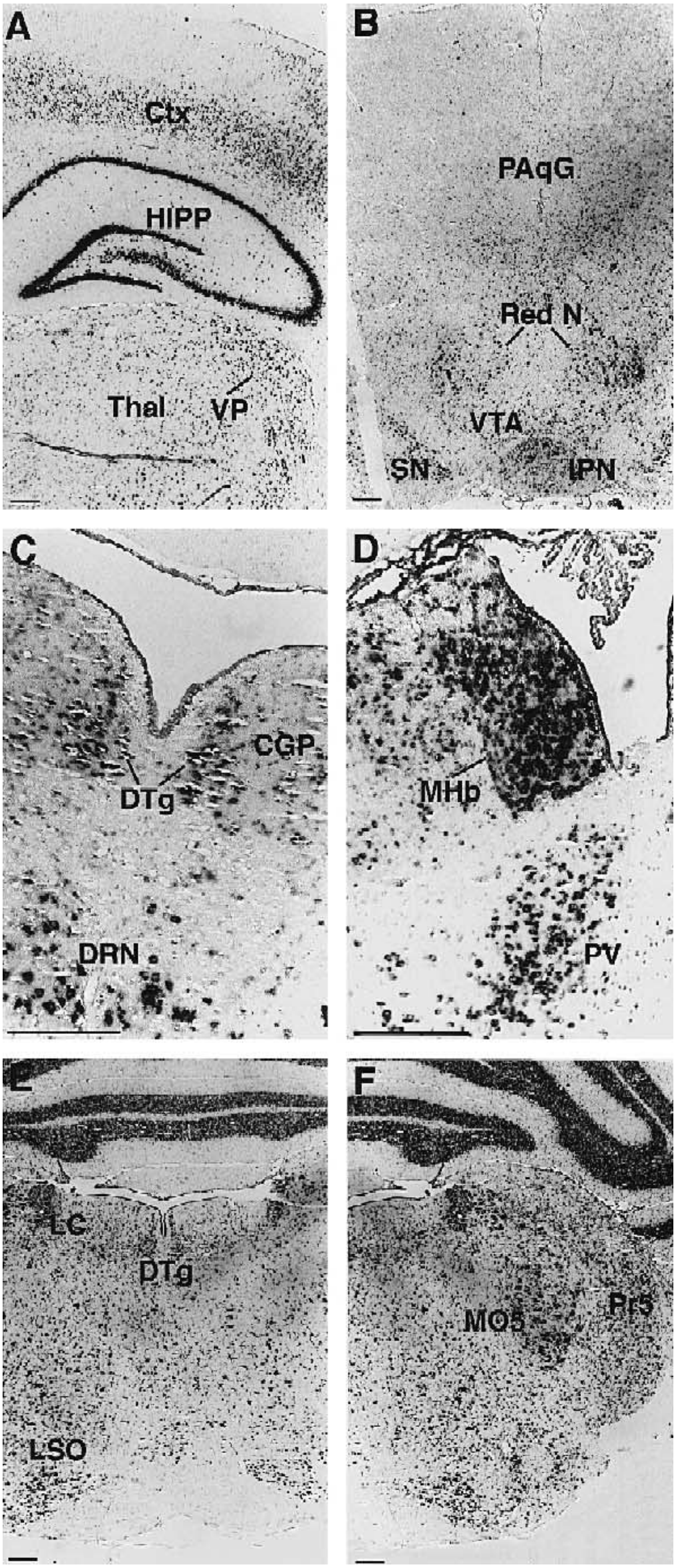
3.3.1. Forebrain

Scattered staining was observed in the outer layer of the cortex in the forebrain. The signals were more intense in the region which corresponds to the somatosensory cortex. Moderate signals were observed in the primary olfactory cortex, in the olfactory tubercle and in the islands of Calleja. Very weak labeling was found in the caudate-putamen, whereas similarly to synapsin IIa, no significant staining was obtained in the NAc (data not shown).

3.3.2. Telencephalon

The hippocampus (within all regions, including CA1-CA4 and the dentate gyrus) was the most heavily labeled structure. This observation is in line with the result obtained by RT-PCR analysis. It can clearly be seen that the cortex was stained mostly within the inner layer, and to a lesser extent than the hippocampus (Fig. 3A). It is worth noting that the signals are more extended in the somatosen-

Fig. 3. In situ hybridization of synapsin IIb mRNA in discrete CNS areas. Coronal sections were hybridized as described in Fig. 2. A: section showing hippocampus, thalamus, ventroposterior nucleus and cortex. B: section showing periaqueductal gray area (PAqG), interpeduncular nucleus (IPN), red nucleus, substantia nigra and ventral tegmental area (VTA). C: high magnification of section showing central gray of the pons (CGP), dorsal raphe nucleus (DRN) and dorsal tegmental nucleus (DTg). D: high magnification of section showing medial habenular nucleus (MHb) and paraventricular nucleus (PV). E: an overview of the pons area showing the locus coeruleus, the dorsal tegmental nucleus and the lateral superior olive. F: section showing the principal sensory and the motor nuclei of the trigeminal nerve. Bars, 250 μm.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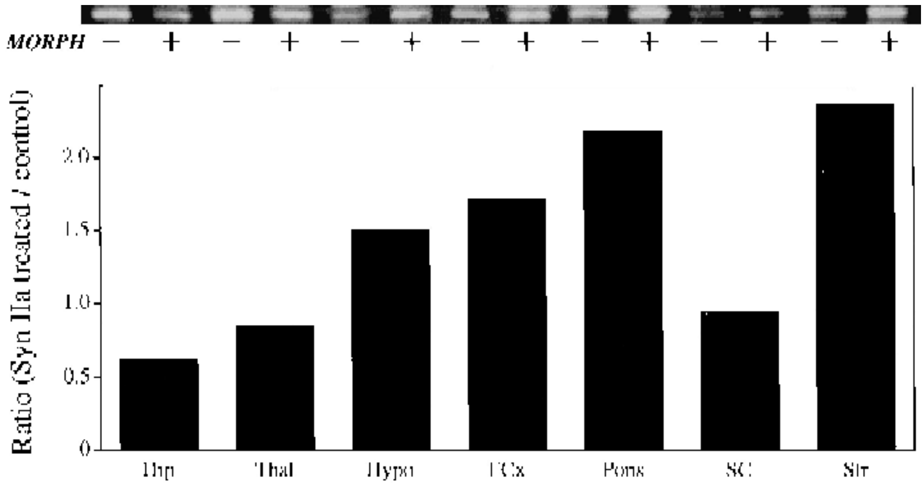

Fig. 4. Comparison by RT-PCR of synapsin IIa mRNA expression in various CNS regions of control and morphine-treated rats. Quantitative RT-PCR experiments were conducted in parallel on RNA extracted from various CNS areas of control and morphine-treated rats. For each sample, an S16 ribosomal PCR fragment was coamplified as an internal standard for normalization of synapsin IIa. No changes were obtained in the amount of S16 mRNA following morphine administration. The upper panel represents pairs of bands which correspond to a 224-bp fragment of synapsin IIa, taken from the indicated brain regions of control and morphine-treated rats. The bar graph represents the ratio of synapsin IIa mRNA in the indicated regions of treated vs. control.

sory cortex. Moderate to low synapsin IIb hybridization signals were observed in the caudate-putamen (data not shown), consistent with the results obtained by RT-PCR in this region. Considerable amounts of synapsin IIb mRNA were detected in: the AHi area, the primary olfactory cortex, the median eminence and the amygdala (Fig. 8A).

3.3.3. Diencephalon

Among the structures that comprise the diencephalon, the thalamus showed the most intense labeling (although to a much lesser extent than the hippocampus). Significant staining was observed in the ventroposterior thalamic nucleus (Fig. 3A) and to a lesser extent in the paraventricular thalamic nucleus (Fig. 3DFig. 7A). Moderate scattered signals were viewed in the dorsolateral and reticular thalamic nuclei. Intense signals were detected in the MHb nucleus (Fig. 3DFig. 7A). The nuclei of the hypothalamus showed relatively low hybridization signal, which was concentrated mainly in the arcuate nucleus and in the ventro- and dorsomedial hypothalamus (Fig. 7C).

3.3.4. Brain stem

In the midbrain, strong synapsin IIb mRNA hybridization signals could be observed in the red nucleus and in the substantia nigra pars reticulata, while a lower concentration was found in the pars compacta (Fig. 3B). Highly intense signals were observed in the interpeduncular nucleus and moderate staining in the superior colliculi and the lateral geniculate nucleus (data not shown). Low and inconsistent labeling was detected in the VTA and scattered staining was observed in the periaqueductal gray area (Fig. 3B). In the pons, similar to the distribution of synapsin IIa, the LC was heavily labeled and signals were found in the DTg, DRN and in the medial and LSO (Fig. 3C,E). In addition, signals were viewed in the CGP. Interestingly, unlike the findings for synapsin IIa, high hybridization signals for synapsin IIb were present in all structures related to the principal sensory and motor nuclei of the trigeminal nerve (Fig. 3F, Fig. 8C).

3.3.5. SC

The distribution of synapsin IIb in the SC was similar to that found for synapsin IIa. The mRNA was predominantly localized to the gray matter in the laminae of both the dorsal and ventral horns, which include cell bodies of sensory and motor neurons, respectively (data not shown).

3.4. Effect of chronic morphine on synapsin IIa mRNA

Exposure of rats to morphine pellets for 96 h leads to alterations in synapsin IIa mRNA expression in several brain areas. The most significant change was observed by RT-PCR for the striatum and the pons, in which chronic exposure to morphine caused a 2.4- and a 2.2-fold increase in synapsin IIa mRNA, respectively. Enhancement of synapsin IIa gene expression was also found in the FCx (1.7-fold) and in the hypothalamus (1.5-fold). On the other hand, chronic morphine treatment reduced the level of synapsin IIa mRNA in the hippocampus by 40% compared with control brains. A moderate decrease was observed in the thalamus, while no significant change was obtained in the SC of morphine-treated animals (Fig. 4), even though these areas are believed to be involved in opiate action. In situ hybridization experiments, which were performed in parallel on brain and SC sections taken from morphine-treated and control rats, showed similar results to those obtained by RT-PCR (Fig. 5A,B). It can clearly be seen that synapsin IIa mRNA is decreased in the hippocampus,

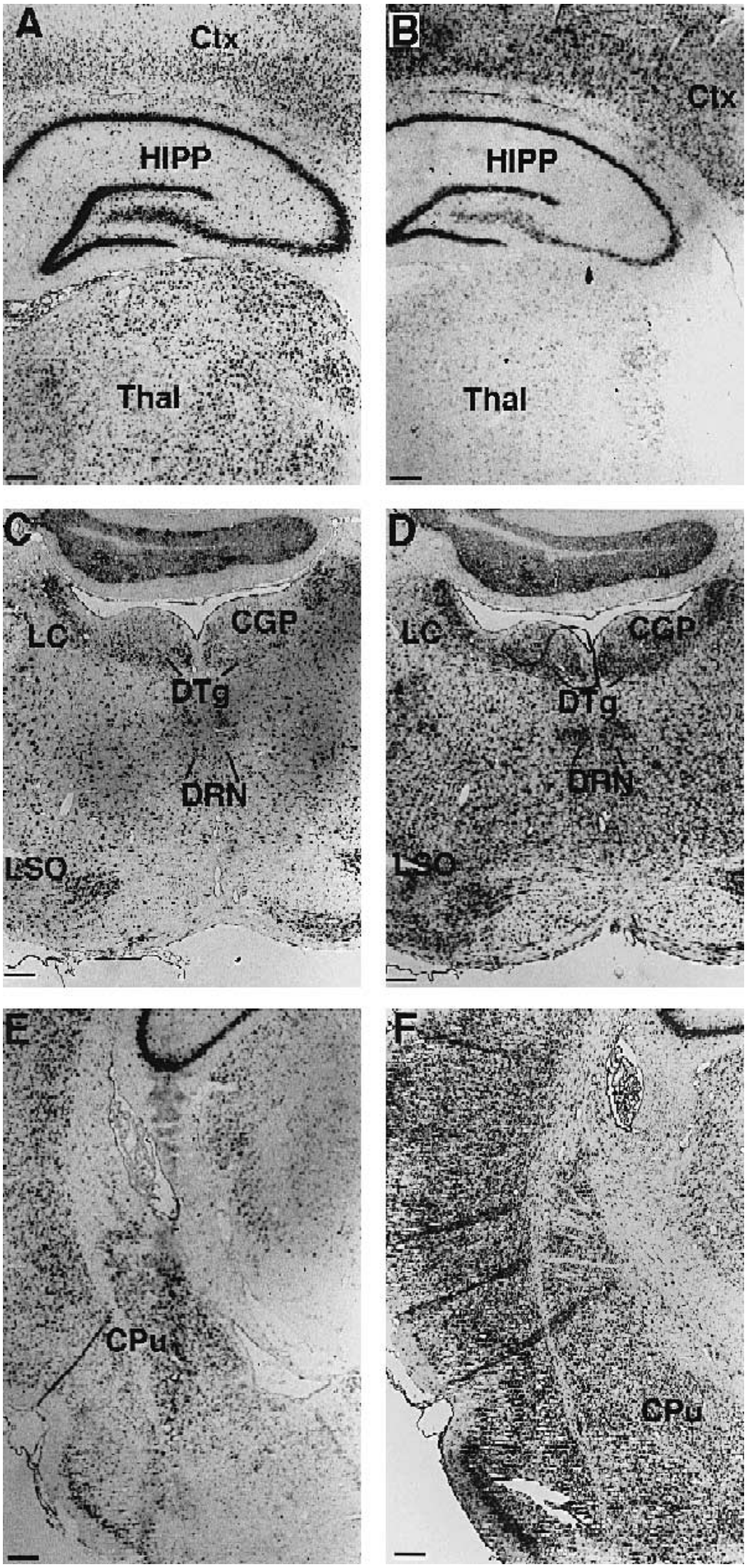

Fig. 5. In situ hybridization of synapsin IIa mRNA in CNS sections of control and chronic morphine-treated rats. A,C,E: sections from control rats. B,D,F: sections from morphine-treated rats. A,B: an overview of the hippocampus area. C,D: an overview of the pons showing the central gray of the pons, dorsal raphe nucleus, dorsal tegmental nucleus, locus coeruleus and lateral superior olive. E,F: an overview of the striatum showing the caudate-putamen nucleus. Bars, 250 μm.

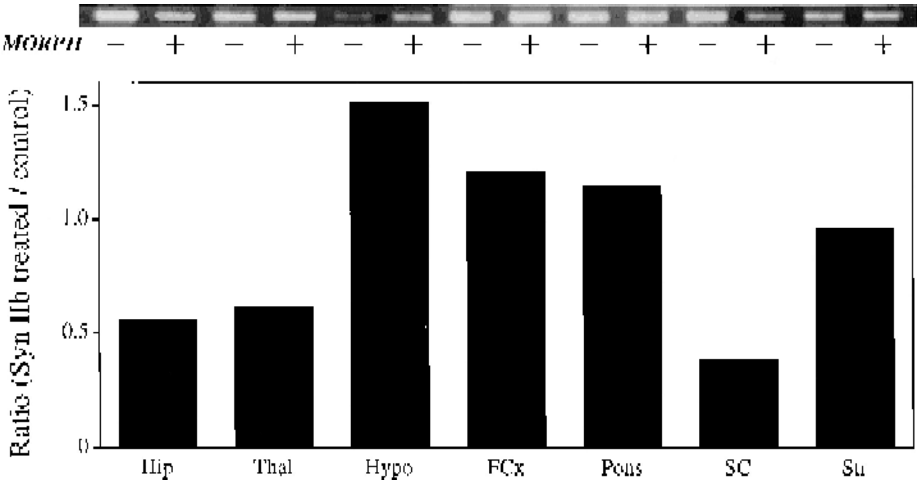

Fig. 6. Comparison by RT-PCR of synapsin IIb mRNA expression in various CNS regions of control and morphine-treated rats. Quantitative RT-PCR experiments were conducted as described in Fig. 4. The upper panel represents pairs of bands, which correspond to a 207-bp fragment of synapsin IIb, taken from the indicated brain regions of control and morphine-treated rats. The bar graph shows the ratio of synapsin IIb mRNA in treated vs. control rats.

whereas an increase is evident in the cortex. In a similar manner, the levels of synapsin IIa mRNA in the pons are elevated following continuous morphine exposure in comparison to control (Fig. 5C,D). These results are in agreement with the RT-PCR analysis. As shown in Fig. 5C,D, several subregions such as the LC, DTg, DRN and CGP were specifically affected. Intense hybridization signals for synapsin IIa mRNA were also present in the striatum of chronically treated rats as compared with controls (Fig. 5E,F).

3.5. *Effect of chronic morphine on synapsin IIb mRNA*

Comparison of the amounts of synapsin IIb mRNA in various brain structures of control and morphine-treated rats showed that this mRNA is affected by opiates in a different manner than IIa. A moderate increase of 1.5-fold in synapsin IIb mRNA levels was observed in the hypothalamus, whereas a marked decrease (of 71%) was found for the levels of this mRNA in the SC of treated rats. Less profound reductions were viewed in the hippocampus (44%) and in the thalamus (39%). No significant changes were found for the FCx, pons or striatum (Fig. 6). Employing in situ hybridization, we were able to confirm the RT-PCR results. Hybridization of synapsin IIb antisense cRNA probe to coronal sections of various brain subregions demonstrated a decrease in the amount of synapsin IIb mRNA in the hippocampus and in the thalamus of morphine-exposed rats. A decrease was also found in the MHb nucleus (Fig. 7A,B). On the other hand, an increase in the in situ hybridization signals was observed in the hypothalamus of opiate-treated rats (Fig. 7C,D). These increases were evident in the arcuate nucleus as well as in the dorso- and ventromedial hypothalamus. In situ hybridization demonstrated a morphine-induced increase in synapsin IIb mRNA in the amygdala, as well as in the principal sensory and motor nuclei of the trigeminal nerve (Fig. 8A–D). Interestingly, opiates have been shown to play a role in mediating pain sensation in the trigeminal nerve region. In agreement with the RT-PCR data, a robust reduction in synapsin IIb mRNA levels was found in the SC (Fig. 7E,F).

4. Discussion

The present study shows that synapsins IIa and IIb, two isoforms of synapsin II, have different regional distributions in the brain. Specific brain areas differed in their synapsin IIa mRNA content by up to 5-fold and synapsin IIb mRNA levels varied by up to 9-fold.

The structural diversity and differential distribution of synapsins I and II [30,31,36,56,57] suggest common and different roles of each in the integration of distinct signal transduction pathways that modulate neurotransmitter release in various types of neurons. It has been proposed that synapsin II plays a more important role in the long term regulation of neurotransmitter release, while short term changes are accomplished by synapsin I [51,59]. Stone and coworkers suggested that synapsin II isoforms are involved in processing pre-synaptic information [56]. Fried and Han [22] have recently shown that transfection of synapsin IIb into NG108-15 neuroblastoma X glioma hybrid cells resulted in an increase in the number of small clear and large dense core vesicles, an increase in the number of varicosities per neurite, as well as elevated levels of all four isoforms of the synapsins and of synaptophysin [22]. Inhibition of synapse formation and maintenance was shown to occur following synapsin II suppression in hippocampal slices treated with antisense oligonucleotides [18]. On the other hand, by employing synapsin II knockout experiments, Rosahl and colleagues concluded that synapsin II is

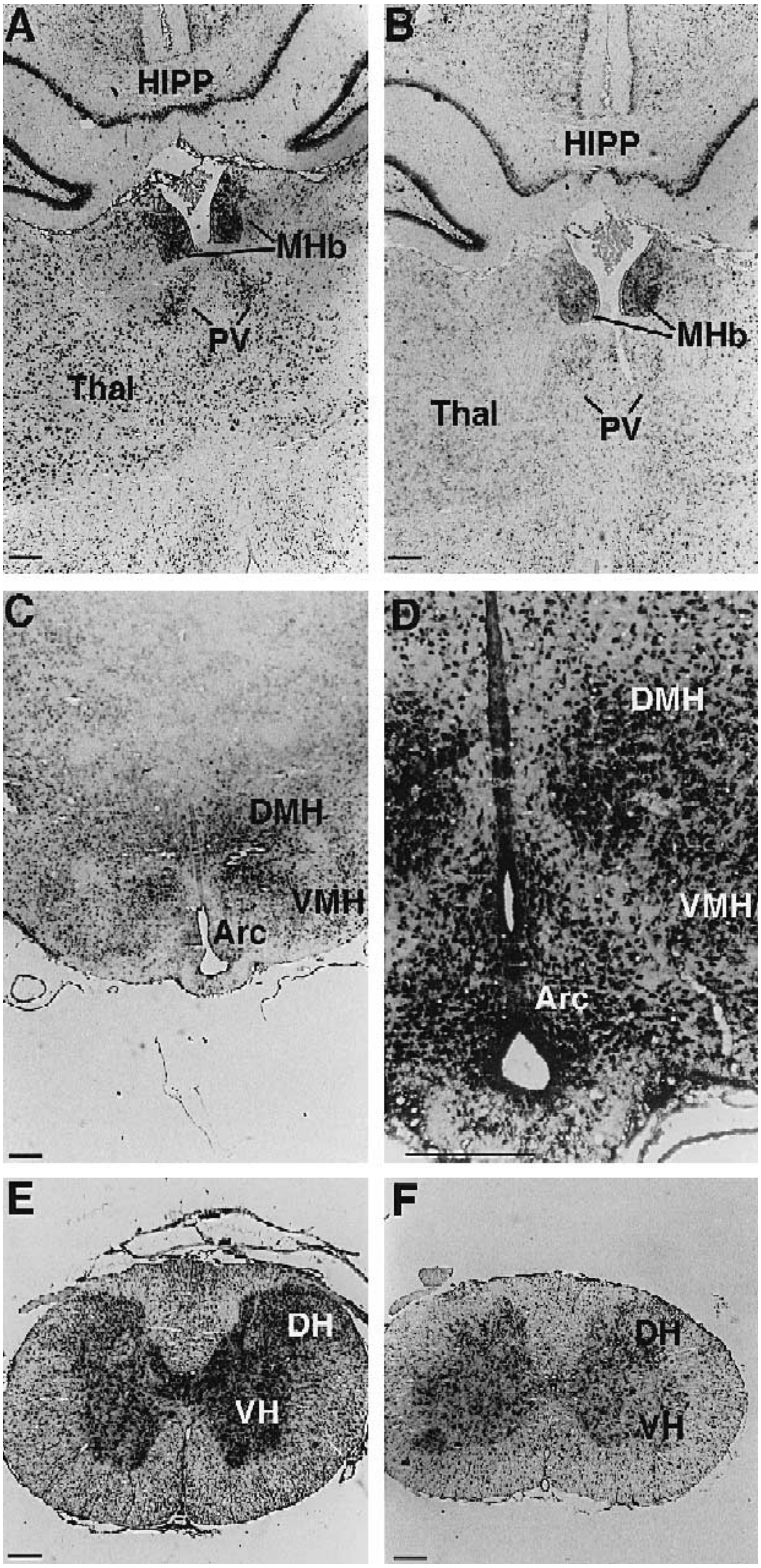

Fig. 7. In situ hybridization of synapsin IIb mRNA in CNS sections of control and chronic morphine-treated rats. Micrographs showing in situ hybridization staining, as was described in Fig. 5. A,C,E: control sections. B,D,F: sections from morphine-treated rats. A,B: an overview of the hippocampus area and the diencephalon. C,D: an overview of the hypothalamus showing the arcuate nucleus (Arc), dorsomedial hypothalamus (DMH) and ventromedial hypothalamus (VMH). E,F: an overview of the SC showing the dorsal horn and ventral horn. Bars, 250 μm.

not required for neurite outgrowth or synaptogenesis, but appears to be essential for acceleration of synaptic vesicle traffic during repetitive stimulation at physiological frequencies [51]. Defined functional differences have not yet been established for synapsins IIa and IIb. The fact that synapsins are substrates of multiple kinases suggests that they represent a point of convergence of various signal transduction pathways involving calcium, cAMP and growth factors.

The studies described above were aimed at assessing the role of synapsins IIa and IIb in the complex events leading to the development of opiate dependence. We have focused on the adaptive changes in the expression of these two isoforms in the brain and SC following chronic opiate treatment. In this regard, we have previously demonstrated that chronic exposure to opiate agonists resulted in increased levels of synapsin I protein in SC-DRG cocultures [43] and in increased mRNA levels of synapsin I in SC and several areas of the brain (including LC, amygdala and CGP) of chronic morphine-treated rats [34]. We have suggested that these increases might be part of a compensatory mechanism which attempts to overcome the attenuation of neurotransmitter release caused by opiates. Here, we report that prolonged administration of morphine dif-

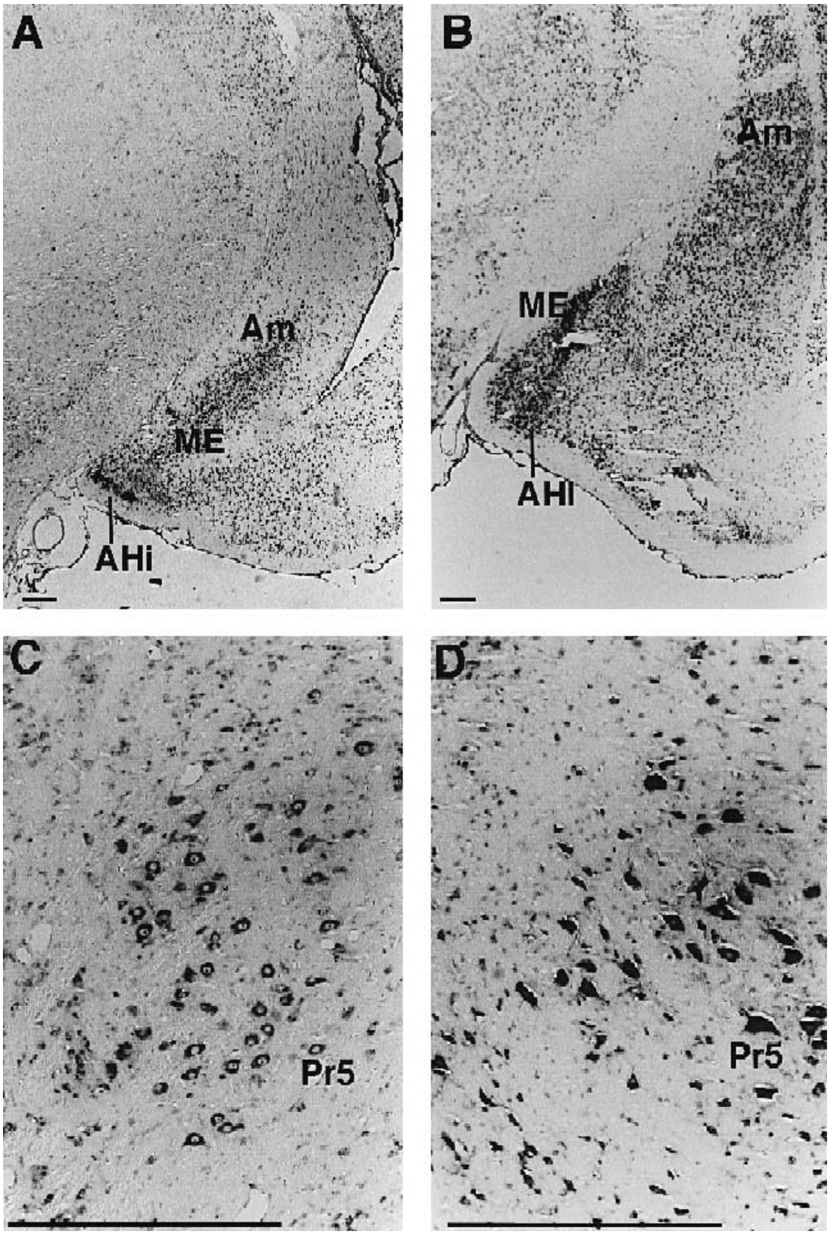

Fig. 8. In situ hybridization of synapsin IIb mRNA in the amygdaloid complex and in the principal sensory nucleus of the trigeminal nerve of control and chronic morphine-treated rats. Micrographs showing in situ hybridization staining, as was described in Fig. 5. A,C: control sections. B,D: sections from morphine-treated rats. A,B: an overview of the amygdala area showing the amygdala (Am), amygdalohippocampal area (AHi) and median eminence (ME). C,D: high resolution micrographs of cells within the principal sensory nucleus of the trigeminal nerve. Bars, 250 μm.

ferentially affects synapsin IIa and IIb mRNA expression in various brain regions and in SC.

Unlike the relatively rapid changes that occur in response to acute opiate administration, prolonged drug exposure results in gradual functional alterations in the brain [46,53]. During the last several years, biochemical, pharmacological and molecular biology studies have shown that continuous exposure to opiates leads to the development of tolerance to most of the acute actions of opiates. Sharma et al. [54] were the first to report opposing effects of acute and chronic opiate exposure on cAMP levels in NG108-15 cells. This finding provided the basis for the notion of cAMP involvement in opiate addiction. Many other reports describing alterations in opiate signal transduction components have pointed to these changes as possible mechanisms for the development of tolerance. Among these, up-regulation of the cAMP system has been well established and widely documented. Elevated levels of AC activity have been reported in rat LC and striatum following chronic opiate treatment as well as in several cell culture models [5,10,14,16,54,65]. It was recently reported that the mRNA for type VIII AC was increased in a region-specific manner in rat brain after such treatment [33]. Moreover, chronic morphine exposure has been reported to increase the levels of various other signaling proteins such as cAMP-dependent protein kinase [46], β-adrenergic receptor kinase (β-ARK) [58] and cAMP response element binding protein (CREB) [69]. Alterations in immediate-early genes (IEGs) [29,60] and in the levels of the α-subunit of $G_{i/o}$-proteins were also reported [1,45,46,66].

Chronic administration of morphine is shown here to regulate the expression of synapsin IIa and IIb mRNAs in brain and SC. A particularly large increase was observed in the levels of synapsin IIa in the striatum, a region which is believed to be important for motivational aspects of drug reinforcement [53]. AC V mRNA has been reported to be nearly exclusively expressed in medium sized striatal neurons, which also express D1-dopaminergic receptors [23,38]. In this regard, it was demonstrated that prolonged exposure to morphine enhances AC activity in the striatum [14]. Moreover, we have recently shown that the activity of AC V in transfected COS-7 cells is increased following chronic opiate exposure [6]. It would be reasonable to speculate that the increase in AC activity and cAMP production in the striatum of morphine-treated rats could be responsible for the enhanced expression of synapsin IIa mRNA seen in this brain region. Elevations in synapsin IIa mRNA were also observed in other brain structures, although to a lesser extent (e.g. pons, FCx and hypothalamus). We cannot exclude the possibility that the AC and cAMP-activated systems are up-regulated in these areas as well. Indeed, at least for LC, NAc and VTA, increased AC and cAMP-dependent protein kinase activities were described [16,46,53]. In the case of synapsin IIb, an increase of 1.5-fold was observed in the hypothalamus, but a marked decrease was found in SC and modest decreases were observed in the thalamus and in the hippocampus. This finding is intriguing and may imply one of several things.

One possibility is the involvement of opposing opiate effects in various CNS areas. In this regard, it has been shown that opiate receptors could exert stimulatory effects and stimulate locomotor activity [15,27], as well as excite SC motor neurons [28]. In addition to their known inhibitory effect, relatively little is known about the mechanism underlying the excitatory opiate effects. However, several hypotheses have been raised: (1) the direction of the response to opiates depends upon the receptor subtype which is activated within specific brain regions [39]; (2) excitatory opiate effects can be achieved by inhibition of GABAergic pathways [47]; and (3) opiates could inhibit K^+ conductance [8,13,17,20,55]. In agreement with this possibility, we have previously reported that the mRNAs for two subtypes of the delayed rectifier K^+ channels in the SC are predominantly located in motor neurons and are significantly elevated following chronic morphine regime [35].

As described above, it is possible that these variations could be due to opiate receptor subtype-specific effects. Rat SC contains mainly the κ- and μ-receptor subtypes [4], striatum and the FCx contain mainly δ-receptors, whereas the pons is rich in the μ-receptor subtype [32]. However, this implication cannot stand as a sole cause for the opposing effects of opiates on synapsin IIb mRNA expression, since the hypothalamus (in which a 1.5-fold increase in synapsin IIb levels was observed) contains mainly κ-receptors [32].

Finally, various regions of the CNS may be differentially regulated by opiate treatment. Indeed, region-specific alterations for several proteins have been described and are discussed by Nestler et al. [46]. Moreover, it has been shown that opiates stimulate the activity of AC type II, while they inhibit other types of AC (Avidor-Reiss, T., Nevo, I., Bayewitch, M. and Vogel, Z., in preparation) [61]. Hence, we cannot exclude the possibility that a reduction in synapsin IIb levels after chronic exposure may reflect an attempt to compensate for the persistent opiate effect.

The transcriptional regulation of synapsins IIa and IIb and how it is modulated by opiates are not yet clear. The apparent association between the changes in the cAMP system and the alterations in synapsin II isoforms suggest that the up- or down-regulation of AC and subsequent cAMP production may regulate the expression of synapsin II via cAMP-regulated factors. Indeed, promoter mapping of synapsin II revealed putative binding sites for the transcription factors sp1, AP2 and NGFLA [11]. Recently, it has been reported that short term morphine treatment induces IEGs in the NAc [29]. On the other hand, Nestler et al. [46] described a reduction in the levels of both mRNA and protein of c-fos in the LC of rats chronically treated with opiates. It is thus conceivable that region-specific regulation by morphine of IEGs may lead to differential expression of synapsin II isoforms following chronic morphine treatment. Zif268 is a transcription factor which binds to a distinct response element but is regulated as an IEG product in a fashion similar to fos- and jun-like proteins. Petersohn et al.[50] have shown that this factor specifically binds to synapsin II promoter DNA. In addition, there is accumulating evidence that cAMP mediates some transcriptional factors that are not related to CREB and bind to a site different than CRE, e.g. AP-2 [46,49]. Indeed, morphine regulation of other cAMP-regulated transcription factors has recently been described by Meyer and Habener [37].

The reduction in synapsin IIa and IIb levels in several areas could result, according to Ferreira et al. [18], in an extensive loss of synapses. This could eventually lead to impaired signaling. It is also possible that the decrease in synapsin IIa and IIb mRNA levels in the hippocampus is in a way related to memory impairments that are known to occur following continuous opiate administration [63].

Several cellular adaptations were shown to accompany the development of tolerance and dependence following frequent utilization of opiates. In an attempt to regain homeostasis, these adaptations restore normal brain function during opiate exposure, but upon removal of the drug, the elevated levels of these cellular components are expected to produce enhanced neurotransmitter release, a process that is known to occur during withdrawal [68]. In this study, we provide evidence that continuous exposure to morphine results in region-specific alterations of synapsin IIa and IIb gene expression. We suggest that these changes could be part of the molecular adaptations that occur during chronic opiate exposure.

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