Changes in ^3H -Substance P Receptor Binding in the Rat Brain After Kainic Acid Lesion of the Corpus Striatum

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Previous studies have indicated that the substantia nigra contains the highest concentration of substance P-like immunoreactivity (SPLI) in the brain. Paradoxically, it also appears to contain one of the lowest concentrations of substance P receptors in the brain. One possibility is that the massive amount of SPLI blocks the binding of the radioligand to the substance P receptor and/or "down-regulates" the number of substance P receptors present in this structure. Since greater than 95% of the SPLI within the substantia nigra originates from the corpus striatum, we have lesioned this area and measured the changes in substance P receptor concentration in the substantia nigra and other corpus striatal projection areas. A semiquantitative autoradiographic technique for measuring the binding of ^3H -substance P to substance P receptors was used in conjunction with tritium-sensitive film. ^3H -substance P binding was measured in both the corpus striatum and its projection areas after kainic acid lesion of the corpus striatum. At either 4 or 21 d after the lesion there was approximately a 90% loss of substance P receptors in the rostral striatum, a 74% loss in the globus pallidus, a 57% increase in receptor number in lamina I and II of the ipsilateral somatosensory cortex, and no apparent change in the number of receptors in the substantia nigra pars reticulata, superior colliculus, and central gray.

These findings suggest that the low concentration of substance P receptors found within the substantia nigra is not due to the massive SPLI innervation, since removal of greater than 95% of the SPLI had no measurable effect on the concentration of substance P receptors. An explanation of these results may be found in the previous reports that another mammalian tachykinin, substance K, is present in the substantia nigra and that substance K binding sites are also present here. These results may suggest that in some cases the specificity of peptide action is determined by the specificity of the postsynaptic receptor.

Substance P (SP) is an endopeptide which is a neurotransmitter candidate in the CNS. Within the mammalian CNS the highest concentration of substance P-like immunoreactivity (SPLI) is found in the substantia nigra (Brownstein et al., 1976; Cuello and Kanazawa, 1978; Kanazawa and Jessell, 1976). In the substantia nigra this SPLI is present in a dense fiber and terminal plexus located in both the pars reticulata and to a lesser extent the pars compacta (Ljungdahl et al., 1978). The corpus striatum, which is composed of the caudate, putamen, and glo-

bus pallidus, is the origin of more than 97% of the SPLI (Pet-tibone et al., 1980) found within the substantia nigra (Brownstein et al., 1977; Jessell et al., 1978; Mroz et al., 1977). No cell bodies intrinsic to the substantia nigra appear to contain SPLI (Ljungdahl et al., 1978). Thus, nearly all the SPLI found within the substantia nigra is localized in axons and synaptic terminals (Di Figlia et al., 1981; Duffy et al., 1975) whose cell bodies are located in the ipsilateral corpus striatum. Release studies have shown that SPLI is released within the substantia nigra during potassium-induced stimulation of the ipsilateral corpus striatum (Jessell, 1978; Michelot et al., 1979). Biochemical studies have also indicated that small amounts of SPLI can be synthesized within the corpus striatum and transported to the ipsilateral substantia nigra (Sperk and Singer, 1982; Torrens et al., 1983).

Recently, several groups have reported on the binding properties and distribution of SP receptors in the rat brain (Cascieri and Liang, 1983; Mantyh et al., 1984a; Perrone et al., 1983; Quirion et al., 1983; Rothman et al., 1984; Viger et al., 1983). From these studies there appears to be a single class of SP receptors in the rat brain, which have a relatively high affinity ($K_D = 1 \text{ nM}$) and a low overall binding concentration (14 fmol/mg wet weight tissue). The distribution of SP receptors is also distinctive; very high concentrations are found in the external plexiform layer of the olfactory bulb and the solitary nucleus, moderate concentrations in the striatum and central gray, and low concentrations in most parts of the cerebellum and dorsal thalamus (Mantyh et al., 1984a; Quirion et al., 1983; Rothman et al., 1984). Paradoxically, in several regions of the rat and cat brain there appears to be a dramatic mismatch between the levels of SPLI and the concentration of SP receptors (Mantyh et al., 1984a). One of these "mismatch" areas is the substantia nigra, which, although it contains the highest level of SPLI in the brain, appears to have one of the lowest concentrations of SP receptors in the CNS (Fig. 1).

In the present report we have performed semiquantitative autoradiography to determine whether changes occur in the distribution of ^3H -SP receptor binding sites in the rat CNS following a kainic acid lesion of the corpus striatum. Using this technique we will address the possibility that high levels of ^3H -SP receptor binding sites are normally not observed in the substantia nigra because the massive amounts of SPLI present interfere with the binding of the radioligand and/or "down-regulate" the number of SP receptors present in this structure. To examine these possibilities we have used two survival times (4 and 21 d) after kainic acid lesion of the corpus striatum to address the former and latter hypothesis, respectively. The present experiments will also address the question of what proportion of the ^3H -SP receptors within the corpus striatum is present on intrinsic neurons or fibers of passages and axonal terminals.

Materials and Methods

Male Sprague-Dawley rats (150–250 gm) were anesthetized with equithesin and mounted stereotactically. Injections of kainic acid were then

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Figure 1. Series of photomicrographs illustrating the differences in the distribution of substance P-like immunoreactivity (SPLI) and SP receptors in coronal sections of the midbrain of the rat. *a*, Light-field photomicrograph of SPLI, where the dark reaction indicates the presence of SPLI. *b*, Dark-field photomicrograph of SP receptor binding sites in a comparable section of the midbrain as in *a*, where the light areas correspond to concentrations of SP receptors. *c*, Dark-field photomicrograph showing the nonspecific ^3H -SP binding in a serially adjacent section to *b*. Note that in some areas of the brain there is a correlation between the distribution of SPLI and SP receptors. However, in other brain areas, such as the substantia nigra pars reticulata (SNr), there is little obvious correlation between the levels of the neurotransmitter and the levels of its receptor. The substance P immunohistochemistry in *a* was performed using the avidin-HRP technique with the primary antiserum directed against the C-terminal end of SP as previously described (Hunt and Mantyh, 1984). Bar, 1 mm.

made ($4.0 \mu\text{g}$ in $4 \mu\text{l}$ of sterile saline injected with a 30 gauge Hamilton syringe) 1 mm anterior to the bregma, 2.8 mm lateral to the midline, and 5.7 mm down from the surface of the cortex. At either 4 (4 animals) or 21 (4 animals) d after the kainic acid injection the animals were killed by decapitation, the brains rapidly removed, blocked in the coronal

plane, placed on brass microtome chucks, and frozen in dry ice. The brains were then serially sectioned ($20 \mu\text{m}$) and thaw-mounted on gelatin-coated microscope slides and stored at -20°C in boxes of desiccant for 48 hr.

To label the substance P receptors we used the ^3H -substance P (^3H -

SP) receptor binding protocol previously described (Mantyh et al., 1984a). The slide-mounted 20 μ m tissue sections were allowed to come to room temperature and then placed in a preincubation medium (19°C for 10 min) of 50 mM Tris-HCl, pH 7.4, containing 0.005% (vol/vol) poly-ethylenimine (Sigma). The slide-mounted sections were then incubated at 19°C for 10 min in a solution of 50 mM Tris-HCl, pH 7.4, containing BSA (200 mg/liter; Sigma), chymostatin (2 mg/liter; Sigma), leupeptin (4 mg/liter; Sigma), bacitracin (40 mg/liter; Sigma), and 2 nM 3 H-SP [2-L-prolyl-3,4- 3 H(N)] (New England Nuclear; specific activity, 26–28.5 Ci/mmol). This was done by placing the slides on a flat surface and covering the sections with 1.5 ml of the incubation medium. To estimate the nonspecific binding, paired serial sections were incubated as described above except that 1 μ M substance P (Peninsula Labs) was added to the incubation solution. Following this incubation the slides were rinsed with 4 washes of ice-cold 50 mM Tris-HCl (pH 7.4, 4°C, 5 sec each) and 4 washes of dH₂O (4°C, 5 sec each) and then quickly dried in the cold room using a stream of cold air. Sections were left for a further 3 hr to dry in the cold and then stored in desiccant-filled boxes overnight at –20°C.

The concentration of SP peptide was determined by amino acid analysis, and the purity of substance P was determined using reverse-phase high-pressure liquid chromatography (HPLC). All 3 H-SP batches were also analyzed periodically by HPLC to determine the purity and stability of the stock 3 H-SP. To determine the stability of 3 H-SP in the incubation medium we performed HPLC analysis on the 2 nM 3 H-SP incubation solution before and after the 15 min incubation.

For autoradiographic analysis of 3 H-SP binding sites the slides were placed in apposition to either emulsion-coated coverslips or LKB tritium-sensitive film. After 4–16 weeks the emulsion-coated coverslips or LKB films were developed in D-19 developer, washed, and fixed. Sections were then placed in Carnoy's fixative for 3 hr, Nissl-stained, and mounted with Depex. Dark- and light-field photomicrographs were then taken of the silver grains and Nissl stain, respectively. Controls for chemographic artifacts were made by performing the binding exactly as described above except that the 3 H-SP was omitted from the incubation medium. Background labeling was defined as the 2 nM 3 H-SP binding that was not displaced in the presence of 1 μ M substance P.

To estimate the density of 3 H-SP binding sites semiquantitatively, microdensitometry combined with tritium-sensitive film was performed as previously described (Rainbow et al., 1984). Thus, after development and fixation as described above, the optical densities of the autoradiograms were determined by projecting the autoradiograms at 20 $\times$ on a white horizontal surface and quantifying the density of the projected image with a photocell connected to a digital voltmeter. This densitometer consisted of a Sharp BS-500A silicon blue photodiode connected to a Radio Shack voltage amplifier and a 15 V DC power supply. At 20 $\times$ the resolution of this device corresponded to a region 50 μ m in diameter on the projected sections. Previous experiments had established that LKB film does not respond linearly to a linear increase in radioactivity. We therefore constructed a series of standards, exposed these to the LKB film, developed and fixed the film, measured this film densitometrically, and used these values with the Texas Instruments curve-fitting program (Hewlett-Packard) to obtain an equation that described the film characteristics. Raw density values for the concentration of 3 H-SP binding sites were then obtained from a minimum of five separate readings and placed in this equation to correct for the nonlinearity of the film. In addition, we have corrected for regional tritium quenching by using the regional quenching coefficients for tritium given by Geary and Wooten (1985).

Results

Binding of 3 H-SP to rat brain

In a previous paper (Mantyh et al., 1984a) we reported on some of the binding characteristics of 3 H-SP to slide-mounted tissue sections, and in general these results were in good agreement with other SP binding studies (Cascieri and Liang, 1983; Perrone et al., 1983; Quirion et al., 1983; Viger et al., 1983). Briefly, 3 H-SP appeared to bind to one class of receptor with a K_D of 1.1 nM and a B_{max} of 14.7 fmol/mg tissue wet weight. All binding in the present experiments was conducted as previously reported, where HPLC analysis showed that the stock solution was 98% 3 H-SP prior to the incubation and 95% 3 H-SP after the incubation. In testing for chemographic artifacts, no positive

or negative chemography was observed in any area of the brain.

The distribution of 3 H-SP binding sites in the normal rat brain has been described in detail in a previous paper (Mantyh et al., 1984a). Comparisons of this material with brain areas contralateral to the striatal lesion in the present study revealed an identical distribution of 3 H-SP binding sites. Thus, in the present study control binding will refer to areas of the same brain contralateral to the corpus striatal lesion.

Histology of the lesioned brains

The caudate putamen, globus pallidus, and substantia nigra all displayed a marked atrophy on the side of the lesion. A loss of neuronal nuclei and Nissl substance was observed in the corpus striatum, with an increase in small glial nuclei. In most animals the lesion was purposely made large so as to destroy nearly all the neurons in the corpus striatum that project to the substantia nigra (Fig. 2). These lesions extended throughout the entire caudate putamen and globus pallidus and usually included layer VI of the overlying cerebral cortex while sparing the adjacent septum (Fig. 2). The density of small glial nuclei was also increased in the substantia nigra pars reticulata ipsilateral to the lesion. All other areas of the midbrain appeared normal.

3 H-SP binding in rats 4 d after a unilateral lesion of the corpus striatum

Areas involved in the lesion site generally had decreased 3 H-SP receptor binding sites compared to the unlesioned contralateral control side. Thus, 3 H-SP binding was decreased to $10 \pm 3\%$ of control values in the rostral striatum, $16 \pm 5\%$ in the middle striatum, $1 \pm 1\%$ in the caudal striatum, and $26 \pm 5\%$ in the globus pallidus compared to the side contralateral to the lesion (Figs. 2, 4). Areas outside the lesion area, such as layer I of the cerebral cortex ipsilateral to the lesion, had a $126 \pm 11\%$ rise in 3 H-SP binding sites, whereas other areas of cerebral cortex showed no change. In the midbrain no significant change in 3 H-SP binding was observed in the substantia nigra, ventral tegmental area, superior colliculus, or the central gray (Figs. 3, 4).

3 H-SP binding in rats 21 d after a unilateral lesion of the corpus striatum

No significant differences were noted between the results obtained from rats that survived 4 d or 21 d after the unilateral lesion of the corpus striatum.

Discussion

The corpus striatum, composed of the globus pallidus, caudate nucleus, and putamen, has been of interest to neuroscientists because of its suspected involvement in a variety of neurological diseases, including Parkinson's disease, striatonigral degeneration, and Huntington's chorea (Kanazawa et al., 1977; Matthews and Miller, 1979). Because of this intense interest, both the connectivity and putative neurotransmitters utilized by some of the striatonigral projection neurons are relatively well known. Using histochemical techniques it has been shown that many of the neurons giving rise to the striatonigral projection are of the medium spiny type (Grofova, 1975; Preston et al., 1980), some of which contain GABA or SPLI (Brownstein et al., 1977; Gale et al., 1977; Hong et al., 1977; Kanazawa et al., 1977; Nagy et al., 1978).

One approach to investigating the role of various neurotransmitters in striatal function is to measure changes in neurotransmitters and their receptors after striatal lesions (Pan et al., 1983; Penney and Young, 1982). One such lesion, produced by injecting kainic acid into the rat striatum, has been proposed as a neurochemical model for Huntington's disease (Coyle and Schwarcz, 1976). Kainic acid, a potent analog of glutamate, has been reported to be an excitotoxin that primarily destroys intrinsic cell bodies, leaving axon terminals of extrinsic origin

Figure 2. Two dark-field photomicrographs showing the effects of an ipsilateral striatal kainic acid lesion (4 d survival) on the concentration of ^3H -SP receptor binding sites in coronal sections of rat forebrain. Note that there is a large depletion of SP receptors in the caudate putamen (CPu) on the lesioned (L) as compared to the control (C) side. Also note that there is an increase in SP receptors in layer 1 of the cerebral cortex (arrows) on the lesioned side.

Figure 3. Two dark-field photomicrographs showing the distribution of SP receptors in the rat midbrain after the ipsilateral striatal kainic acid lesion shown in Figure 2. Section *a* is a coronal brain section that is slightly caudal to section *b*. Note that in both sections there appears to be little difference in the concentration of SP receptors in the substantia nigra (SN) between the lesioned (L) and control (C) sides.

Figure 4. Histogram of ^3H -SP binding four days after a kainic acid lesion of the corpus striatum similar to that shown in Figure 2. Results from rats with a lesion of the corpus striatum with a 21 d survival gave similar results to those with a 4 d survival. Note that the kainic acid lesion included all areas of the corpus striatum known to contain neurons that project to the substantia nigra (globus pallidus, caudate, and putamen). All areas involved in the lesion showed a significant loss in the concentration of SP receptors. Note also that while layer I and II of the cerebral cortex showed a significant increase in SP receptor density, other areas, such as the substantia nigra, which are known receive a massive SPLI input from the corpus striatum, showed no change. The results are expressed as the means $\pm$ SEM of five determinations from two separate experiments. In all cases we have corrected for the non-linearity of the LKB film's response to increasing radioactivity (Rainbow et al., 1985) and regional tritium quenching by using the regional quenching coefficients for tritium as given by Geary and Wooten (1985). *, $p < 0.001$ (Student's t test). $\square$, control; $\square$ with diagonal lines, kainic acid lesion of the ipsilateral corpus striatum.

and fibers of passage relatively intact (Schwab et al., 1980). In the present experiments, we used this technique, together with semiquantitative SP receptor binding autoradiography, to examine the regulation of SP receptors in the striatonigral system. Previous attempts at labeling SP receptors have been hampered because substance P adsorbs extensively on both glass and certain kinds of plastics and because of the commercial unavailability of ^3H -SP. In this work, we overcame both of these difficulties by using polyethylenimine to block the nonspecific binding of SP to glass and a recently introduced commercial ^3H -SP.

In the striatum, most SP receptors appear to be on intrinsic striatal neurons and not fibers of passage or incoming terminals, since kainic acid lesions (hypothesized to destroy only intrinsic neurons) produce approximately a 90% decrease in the concentration of SP receptors. In the substantia nigra the very low levels of SP receptors normally observed appeared unchanged after striatal lesion. Previous studies have shown that the substantia nigra has the highest concentration of SPLI in the brain (Brownstein et al., 1976; Kanazawa and Jessell, 1976) and that 97% of this SPLI arises from the striatonigral projection (Brownstein et al., 1977; Jessell et al., 1977). Thus, the removal of 97% of the SPLI in the substantia nigra after kainic acid lesions of the striatum had no effect on SP receptor levels. In the present study we have addressed the possibilities that the SP receptors in the substantia nigra may either be down-regulated or the ^3H -SP ligand effectively blocked from binding by the massive amounts of the SPLI normally present in this structure, as shown in the present study. However, in light of the present data, neither of these hypotheses appears tenable, since removal of 97% of the SPLI from the substantia nigra did not change the concentration of SP receptor binding sites.

Areas that did show an unexpected increase in SP receptor

binding after striatal lesion were layers I and II of the ipsilateral cerebral cortex. Why this increase should occur is unclear, although previous binding studies that examined changes in the concentration of GABA receptors after striatal lesion revealed several changes that appeared to be due to transsynaptic effects (Pan et al., 1983). Since the substantia nigra is known to project to the thalamus (Beckstead et al., 1979) and the thalamus to the outer half of layer I of the cortex (Herkenham, 1980; Jones and Leavitt, 1974), this increase in SP receptors may also be due to a transsynaptic effect. However, equally plausible possibilities include a direct kainic damage to the cortex or a post-synaptic rise after loss of intracortical projections from deep to superficial layers.

Previous studies have shown that while GABA and dopamine receptors increase in the substantia nigra after kainic acid lesions of the striatum, cholinergic (muscarinic) and opiate receptors remain unchanged (Pan et al., 1983; Waddington and Cross, 1980). Since there are striatonigral projection neurons that contain either GABA or SPLI, it would appear that only certain classes of receptors in the substantia nigra respond to a loss of their presumed presynaptic neurotransmitter. Other classes of receptors, such as the SP receptors in the substantia nigra, do not show any measurable increase in receptor concentrations after withdrawal of the native ligand, at least at the time points we examined.

What remains particularly perplexing about the striatonigral SPLI projection is why there is such a mismatch between the levels of SPLI and SP receptor in the substantia nigra. In previous papers we have demonstrated that the SP binding sites revealed by these autoradiographic binding techniques appear to correspond to a functionally coupled receptor (Mantyh et al., 1984b). Using electrophysiologically active vibratome sections of various brain areas (including the substantia nigra/ventral

tegmental area), it was found that the rate of inositol phospholipid hydrolysis was proportional to the number of ^3H -SP binding sites found in specific brain regions. Since the rate of SP-induced inositol phospholipid hydrolysis appears to be a measure of receptor occupancy in the CNS, these results suggest that the specific ^3H -SP receptor binding sites revealed by autoradiography do correspond to functional SP receptors. As we have noted before, this mismatch between the levels of a neurotransmitter and its receptor exists in other areas of the brain. Notable examples of this are substance P in the olfactory bulb, cerebral cortex, and globus pallidus (Mantyh et al., 1984a); thyrotropin-releasing hormone in the cerebral cortex, amygdala, and dorsal horn of the spinal cord (Mantyh and Hunt, 1985; Palacios, 1983); neurotensin in the entorhinal cortex, amygdala, striatum, and hypothalamus (Goedert et al., 1984; Young and Kuhar, 1981); enkephalin in the caudate putamen, cerebral cortex, amygdala, and spinal cord (Simantou et al., 1977); and nor-adrenaline in the striatum (Alexander et al., 1975). As previously discussed (Goedert et al., 1984; Mantyh et al., 1984a), there are several possible explanations for this mismatch, including differential distribution of neurotransmitter degrading enzymes, different rates of neurotransmitter turnover, and possible receptor subtypes that, using one set of incubation conditions, might bind to only one receptor subtype. One hypothesis is that the SP receptors in the substantia nigra may be a low-affinity subclass. However, this possibility has not been confirmed in previous binding studies (Cascieri and Liang, 1983; Mantyh et al., 1984a; Perrone et al., 1983; Quirion et al., 1983; Viger et al., 1983), where only one high-affinity SP receptor was observed in the rodent brain. In the case of neuropeptides, there may also be different molecular forms of the neuropeptide that are not recognized by either radioimmunoassay or immunohistochemistry but still interact with the receptor.

Several of the above-mentioned possibilities may be important in explaining the "mismatch" between SPLI and SP receptor levels in some areas of the CNS. Recent studies have shown that substance P is only one of several tachykinins present in the mammalian CNS (Kanagawa et al., 1983; Kimura et al., 1983; Maggio et al., 1983; Nawa et al., 1983). By definition, all tachykinins share the common C-terminus amino acid sequence Phe-X-Gly-Leu-Met-NH₂ (Erspamer, 1981). Since most antisera raised against SP are directed at its C-terminal end, there is a strong possibility that some of the previously reported SPLI may have been due to cross-reactivity with these other newly discovered mammalian tachykinins. Preliminary studies have shown that SP and another mammalian tachykinin, substance K, appear to have a different distribution of receptor binding sites in the rodent CNS and that substance K binding sites are present in the rodent substantia nigra (Mantyh et al., 1984c; Quirion and Dam, 1985). In addition, substance K has been reported to excite both dopaminergic and non-dopaminergic neurons in the rat substantia nigra (Innis et al., 1985). In light of the reports showing that substance P and substance K are in some cases encoded in the same gene (Nawa et al., 1983) and also appear to be present in the same ratio in all areas of the brain examined (Maggio and Hunter, 1984), the receptor binding data suggest that the presence of the proper receptor may determine whether a particular tachykinin system is functional. Other reports have demonstrated that the rate of SPLI turnover in striatonigral projection neurons is extremely slow compared to other classical neurotransmitter systems (Sperk and Singer, 1982; Torrens et al., 1983). These data further suggest that one cannot judge the importance of a neuropeptide in a neuronal system simply by measuring the amount of neuropeptide present, but rather its functional importance is dependent on the proper receptor being present and how much and often the neuropeptide is used. Thus, while the present study has shown that there is a poor correlation between SPLI and SP receptor levels in some neuronal systems, it is clear that the striatonigral

system should prove to be an excellent model system to investigate what regulates the expression of a family of neuropeptides (the tachykinins) and their receptors in the CNS.

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