Disulfiram Administration Affects Substance P-like Immunoreactive and Monoaminergic Neural Systems in Rodent Brain*

(Received for publication, August 9, 1989)

James E. Marchand§§, Kenneth Hershman§§, M. S. Amarendra Kumar†, Michael L. Thompson§§, and Richard M. Kream§§

From the Departments of §Anesthesiology and §Pharmacology, Tufts University School of Medicine and the †Department of Anatomy, Tufts University School of Veterinary Medicine, Boston, Massachusetts 02111

The biosynthetic enzyme peptidylglycine α-amidating monooxygenase catalyzes the formation of a variety of biologically active α-amidated peptides from respective COOH-terminal glycine-extended peptide precursors. Peptidylglycine α-amidating monooxygenase activity is dependent on copper, ascorbate, and molecular oxygen and is inhibited by the relatively selective copper chelator N,N-diethyldithiocarbamate or its disulfide dimer disulfiram (Antabuse). In the present study, chronic disulfiram treatment (100 mg/kg/day, for 12–25 days) resulted in significant changes in several neurochemical parameters in the mouse central nervous system, including levels of substance P-like, unamidated substance P-Gly-like, and protease-generated substance P-Gly-Lys-like immunoreactivities (SP-LI, SP-G-LI, and SP-G-K-LI, respectively). Combined high performance liquid chromatography/radioimmunoassay analyses of the extracted SP-LI, SP-G-LI, and SP-G-K-LI species indicated very similar chromatographic and immunological behavior as demonstrated for chemically authentic peptide standards. Additionally, changes in levels of monoamines and their metabolites were observed after drug administration. Complementary immunohistochemical analyses using affinity-purified anti-SP-G sera localized these drug-induced changes in levels of immunoreactive unamidated precursor to neural elements that normally express SP. As a functional corollary to alterations in neurochemical parameters, we observed significant disulfiram-induced increases in pain thresholds, potentiated by capsaicin treatment. Overall, our results indicate that the observed changes in steady state levels of immunoreactive SP and of the immature COOH-terminal extended forms of SP may reflect compensatory biosynthetic and posttranslational processing events in SP-containing neural systems after pharmacological challenge.

Substance P (SP),1 a neuropeptide with widespread distribution throughout the brain, spinal cord, and autonomic nervous system of many mammalian species, is involved in transmission and modulation of sensory information, regulation of neuroendocrine processes, and integration of motor function (1). SP is typical of a variety of gut-brain peptides that contain an amidated COOH terminus, a posttranslational modification that is required for full biological and pharmacological activity (1–3). It has been demonstrated that precursor forms of amidated peptides are found as COOH terminal glycine-extended species (4–7), with glycine serving as the nitrogen donor in the amidation reaction (8, 9). Conversion of glycine-extended peptide precursors to biologically mature α-mediated peptides is catalyzed by peptidylglycine α-amidating monooxygenase with cofactor requirements of copper, ascorbate, and molecular oxygen (10). Previous studies have reported a time-dependent loss of peptide amidation in several primary cell culture systems, probably due to cofactor depletion (10, 11), suggesting that this terminal maturation reaction may be rate-limiting in peptide biosynthesis and susceptible to modulation by specific drugs. Additionally, it has been recently demonstrated that administration of the relatively selective copper chelator N,N-diethyldithiocarbamate or its disulfide dimer, disulfiram (Antabuse), produces a dose-dependent increase in glycine-extended immature forms of α-melanocyte-stimulating hormone and of joining peptide in the intermediate pituitary and in cultured mouse corticotropic tumor cells, via inhibition of peptidylglycine α-amidating monooxygenase activity (12). However, the effects of inhibitors of amidation on neuropeptide expression within the CNS have not been previously reported.

The analysis of COOH-terminal extended immature forms of SP may serve as a sensitive indicator of biosynthetic and posttranslational processing events in SP-containing neural systems. Previous work from this laboratory has provided an immunohistochemical and chromatographic characterization of the unamidated immediate precursor form to SP, i.e. SP-G-LI, by combined RIA-HPLC analyses of rodent nervous tissues, utilizing highly specific and sensitive anti-SP-G sera (5, 13). In the present study, we have examined the effects of disulfiram administration on several neurochemical parameters in mouse CNS, including levels of SP-LI, SP-G-LI, SP-LI, SP-like immunoreactivity.

* This work was supported by National Institutes of Health Grant R01 DA 04128 (to R. M. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom reprint requests should be addressed: Depts. of Anesthesiology and Pharmacology, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111.

1 The abbreviations used are: SP, substance P (RPKPQPPFGLM-NH2); CON, controls; DIS, disulfiram-treated; DIS/CAP1, disulfiram- and capsaicin-treated, short survival time; DIS/CAP2, disulfiram- and capsaicin-treated, long survival time; CAP, capsaicin-treated animals; NKA, neurokinin A (HKTDSVFGGLM-NH2); NKB, neurokinin B (DMHDFGGLM-NH2); OT, oxytocin (CYYQDCPLG-NH2); AVP, arginine vasopressin (CYPQCNFGPR-NH2); HPLC-ECD, electrochemical detection after HPLC; 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindoleacetic acid; PBS, phosphate-buffered saline; SP-LI, SP-like immunoreactivity.
monoamines, and their metabolites. In addition, levels of immunoreactive heterogenous SP precursor forms were quantified as protease-generated SP-G-K-LI determinants from extracted tissues of drug-treated and control animals. The same neurochemical parameters were measured in two additional groups of animals receiving combined drug treatments of disulfiram and the chemical irritant capsaicin, in order to promote depletion of mature SP. Capsaicin has been previously established as an agent that elicits SP release from primary afferent nerve terminals (14). The present study included complementary immunohistochemical analyses to localize changes in levels of unamidated precursor nerve terminal systems. Finally, pain thresholds were measured using the tail-flick assay, as a functional indication of changes in SP-LI expression in primary afferent pathways mediating nociceptive responses.

**EXPERIMENTAL PROCEDURES**

**Animal Injections/Behavioral Measurements—Mice** (DGAF1/2, 2–6 months) were divided into five groups (n = 10 per group) consisting of: (1) vehicle-treated controls (CON), (2) disulfiram-treated (DIS), (3) disulfiram- and capsaicin-treated, short survival time (DIS/CAP1), (4) disulfiram- and capsaicin-treated, long survival time (DIS/CAP2), and (5) capsaicin-treated animals (CAP). Experimental groups (DIS, DIS/CAP1, DIS/CAP2, CON, and CAP) were subsequently injected under ether anesthesia with 50 mg/kg capsaicin, in 0.5% Tween 80/saline. Controls received vehicle alone. On day 10, animals in DIS/CAP1, DIS/CAP2, and CAP groups were subcutaneously injected, under ether anesthesia with 100 mg/kg disulfiram in 0.5% Tween 80/saline. Controls received vehicle alone. Within 3 h of capsaicin treatment, two animals in the DIS/CAP1 group and four animals in the CAP group died. No losses were sustained in the DIS/CAP2, CON, and DIS groups. On day 12, animals in DIS/CAP1 were killed. On day 18, the remaining animals were injected either with a second dose of capsaicin (50 mg/kg under ether anesthesia, DIS/CAP2, CAP) or with vehicle (DIS, CON). After capsaicin treatment, two animals in the DIS/CAP2 group and three animals in the CAP group died. Again, no losses were sustained in the CON and DIS groups. All remaining animals were killed on day 25. During the course of the study, pain thresholds were monitored every 2nd or 3rd day by latency of thermal nociceptive response using a focused light source to the tail. Experimental protocols were approved by Tufts University Animal Research Committee, protocol 89–88.

**Immunological Reagents: Further Characterization of Anti-SP-G-K and Anti-SP-G-K-L1 Antibodies**—The generation of the antisera to the synthetic peptides SP, SP-G, and SP-G-K has been described previously (5). In competitive binding analyses, each antiserum was demonstrated to be highly specific for both the reduced and sulffoxide forms of its homologous peptide antigen and displayed minimal recognition (<0.001% cross-reactivity) of heterologous SP-related peptide antigens and a variety of unrelated neuropeptides. Recently, the structures of two novel mammalian tachykinins, neurokinin A (NKA) and neurokinin B (NKB), have been elucidated (15). The COOH terminal of extended precursor forms of SP, NKA, and NKB share the identical sequence of Gly-Leu-Met-Gly-Lys and may all be immunologically cross-reactive species. Although we had previously demonstrated coelution of enzymatically generated SP-G-K-LI and SP-G-LI after extraction from rodent brain with synthetic standards by detailed HPLC-RIA analyses (5), we could not completely eliminate the possibility of coelution of heterogeneous forms of related tachykinins with these same standards. Therefore, in order to monitor any potential contributions of these related COOH-terminal extended tachykinin precursor forms in the RIAs by direct means, NKA-G and NKB-G were generated from the respective G-K-extended forms by carboxypeptidase B digestion by HPLC purification. The specificity of anti-SP-G-K and anti-SP-G-LI could be assessed towards COOH-terminal glycine-extended forms of related tachykinin species. In addition, we measured the cross-reactivities of SP-free acid and SP-Gly-Lys-Arg in our assays. Finally, matrix cross-reactivities of COOH-terminal extended forms of the unrelated peptides octotcin (OT) and arginine vasopressin (AVP) were measured. The AVP analogs AVP-Gly<sub>10</sub> (AVP-G), AVP-Gly<sub>10</sub>-Lys<sub>11</sub> (AVP-G-K), AVP-Gly<sub>10</sub>-Lys<sub>11</sub>-Arg<sub>12</sub> (AVP-G-K-R) and the homologous OT analogs OT-G, OT-G-K, and OT-G-K-R were generously provided by Drs. M. Altstein and H. Gainer of the NINCDS.

**Radioimmunoassays of SP-LI, SP-G-LI, and SP-G-K-LI—The RIA procedures** were performed essentially as described (5). In these analyses, anti-SP, anti-SP-G, and anti-SP-G-K sera were used at a final dilution of 1:240,000, 1:24,000, and 1:120,000, respectively, at which 35% radioiodinated tracer was specifically bound. RIAs utilized corresponding synthetic peptide standards whose concentrations had previously been determined by amino acid analyses. RIAs were radioiodinated forms of Bolton-Hunter-conjugated peptide analogs prepared according to our published procedures (5, 16). Initial incubation was for 24 h, followed by addition of 10,000–20,000 cpm of ¹²⁵I labeled tracer and another 24 h incubation. Finally, preincubation of the bound radioactive tracer was initiated by addition of goat anti-rabbit sera, followed by a 24-h incubation. After centrifugation, supernatants were aspirated and the pellets counted in a Beckman Gamma 4000 at greater than 70% efficiency. Nonspecific binding defined as precipitated radioactivity in the absence of primary antibody (1–2%), was subtracted from each assay tube. For unknown samples, peptide concentrations were quantified by interpolation from a log-log plot of the standard dose-response displacement curve. In these analyses, the lowest detectable doses were approximately 2 pg/tube, and the intra- and interassay coefficients of variation were less than 5 and 10%, respectively. For each tissue extract, at least two dilutions were assayed in triplicate. Levels of heterogenous SP precursor forms were quantified as protease-generated SP-G-K determinants in sample aliquots from extracted tissues after digestion with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (1 mg/ml, 16 h at 22° C), as described previously (5, 13).

**Analyses of Biogenic Amines—Monoamines and their metabolites** were quantified by electrochemical detection after HPLC (HPLC-ECID), in an adaptation of the method of Mefford (17), and Kilpatrick and co-workers (18). The system included an LKB Model 2150 pump, Rheodyne 7010 injector with either 20- or 50-μl loop, BAS LC-4B amperometric detector connected to a Hewlett-Packard 3390A integrator. Biogenic amines were fractionated by a C18 Microsorb 3-micron Short One column, at a flow rate of 1.5 ml/min. The mobile phase consisted of 0.01 M NaOH, 0.110 M citric acid, 1.6% (w/v) Na<sub>2</sub>EDTA, 3.3% (w/v) sodium dodecyl sulfate, 0.85% (v/v) diethyamine, 12% (v/v) acetonitrile, pH 3.15. Amperometric responses were measured after oxidation of analytes at 0.7 V. These included responses to dopamine and its major metabolite 3,4-dihydroxyphenylalanine.

**Table 1**

| Peptide          | Anti-SP-G | Anti-SP-G-K |
|------------------|-----------|-------------|
| SP-Gly-Lys-Arg   | 0.05      | 0.80        |
| SP-free acid (RPKQPQFGFLM) | 0.03 | 0.04       |
| NKA-Gly          | 0.16      | <0.001      |
| NKA-Lys          | <0.001    | <0.001      |
| NKB-Gly          | <0.001    | <0.001      |
| NKB-Lys          | <0.001    | <0.001      |
| OT-Gly           | <0.001    | <0.001      |
| OT-Lys           | <0.001    | <0.001      |
| OT-Lys-K-G       | <0.001    | <0.001      |
| AVP-Gly          | <0.001    | <0.001      |
| AVP-Gly-Lys      | <0.001    | <0.001      |
| AVP-Gly-Lys-Arg  | <0.001    | <0.001      |
acetic acid, epinephrine and norepinephrine, 5-hydroxytryptamine (5-HT) and its major metabolite 5-hydroxyindoleacetic acid (5-HIAA), and the internal standard 3,4-dihydroxybenzylamine. Typically, a complete HPLC-ECD analysis was completed in 15–18 min. The detector response was linear from 0.05 to 2 mg of standard compounds. Standard curves were constructed twice daily by plotting detector responses in units of peak height versus known amounts of monoamines for a minimum of three concentrations of standards.

Sample Preparation for Combined Biogenic Amine and Peptide RIA Analyses—Levels of biogenic amines were quantified by HPLC-ECD and levels of peptides by RIA from the same tissue samples using the extraction procedure, as described here. Animals were killed by decapitation after asphyxiation in 100% CO₂. Dissections were performed in a modification of the rat procedure of Glowinski and Iversen (19), and included spinal cord, medulla,pons,medial basal hypothalamus, preoptic area, and main olfactory bulb. Tissue samples were quick-frozen on dry ice followed by storage at −80 °C. Samples were homogenized while deep frozen by sonic disruption in 40 volumes of monoamine HPLC-ECD mobile phase (see above) containing 0.125 or 0.250 ng of 3,4-dihydroxybenzylamine per 40 µl. After homogenization, 10% of the total sample was reserved for HPLC-ECD analyses of monoamines and rapidly replaced with an equivalent volume of glacial acetic acid. After delipidation with an equal volume of chloroform, the aqueous upper layers from these extractions were combined. After delipidation and concentration with equal volumes of chloroform, the aqueous upper layers from these extractions were further purified and concentrated by adsorption and elution from C18 Sep-Pak Cartridges (Waters) in 3 ml of 70% acetonitrile, 0.1% trifluoroacetic acid. Eluates were then treated with 3 ml of chloroform, and the concentrated aqueous upper layers (approximately 1 ml per extract) were reserved and stored at −20 °C. Prior to HPLC fractionation, samples were diluted 1:5 with 40 mM phosphoric acid, pH 3.5, and filtered through a 0.22-µm nylon disc. The injection volume was 1 ml, and 0.5-mI fractions were collected and stored for peptide RIAs. Blank runs consisted of injections of 1 ml of 40 mM potassium phosphate, pH 3.5, were performed between sample runs to bring the detector calibration of standard retentions times. RIA analyses of aliquots from fractions collected during blank runs indicated no measurable peptide immunoreactivities over the fractionation range. SP-LI, SP-G-L1, and SP-G-K-L1 were monitored by RIA performed directly on duplicate lyophilized aliquots from each fraction. RIA standard curves were generated in the presence of equivalent aliquots of lyophilized HPLC mobile phase (40 mM potassium phosphate, pH 3.5, containing 30% acetonitrile). However, antibody binding and competitive displacement were found to be unaffected by residual HPLC mobile phase. Finally, HPLC-RIA analyses of SP-G-L1 were performed on tissue extracts from both control and disulfiram-treated animals before and after trypsin digestion, as follows. Two hundred µl of each sample extract was lyophilized and reconstituted in 1 ml of 50 mM Tris, pH 7.6, 2 mM CaCl₂, 1 µg/ml trypsin, and incubated for 18 h at 25 °C, followed by a 10-min incubation at 100 °C.

Statistical Analysis—The number of surviving animals in the CAP group (2) was too small for statistical analysis of results from tailflick, RIA, and monoamine assays. Tailflick latencies were subjected to repeated measures analysis of variance to assess significant differences in the trend of the data across the 2 weeks of treatment. Post hoc testing used Dunnett’s t test to compare treatment groups to the control group in terms of differences in tailflick latency on any one particular day. RIA data for each brain region were subjected to one-way analysis of variance, again using Dunnett’s t test for post hoc testing of both treatment groups to the reference control group. Significance level for all tests was p < 0.05.

RESULTS

Tissue Levels of SP-LI, SP-G-L1, and SP-G-K-L1 in Mouse CNS after Drug Treatment—In the present study, we have examined the effects of an inhibitor of peptidylglycine α-amidating monooxygenase activity, i.e. disulfiram, on neurochemical and physiological parameters in the mouse, including peptide and monoamine levels and pain thresholds. In addition, two of the disulfiram-treated groups were also injected with capsaicin in order to promote increased peptide turnover in peripheral afferents as a consequence of SP depletion from terminals (14). We observed that levels of SP-LI were generally reduced in CNS areas of experimental animals as compared to controls (Fig. 1A). Most notably, levels of SP-LI were significantly reduced by approximately 30% in the spinal cords of drug-treated animals as compared to controls. In contrast, levels of SP-G-L1 were dramatically increased at a level of statistical significance of p < 0.01 in four brain and spinal cord areas of all experimental groups, as compared to vehicle-treated controls (Fig. 1B). Concentrations of SP-G-L1 in the main olfactory bulb and pons of control and experimental groups were found to vary at the
Effects of Disulfiram on SP-LI and Monoaminergic Systems 267

FIG. 1. Effect of drug treatments on CNS peptide levels. Mice were treated with disulfiram, or disulfiram in combination with capsaicin, as described under "Experimental Procedures." Levels of SP-LI (A), SP-G-L1 (B), and SP-G-K-L1 (C) were quantified by selective RIAs from extracted brain areas in control (CON), disulfiram-treated (DIS), and combined disulfiram/capsaicin-treated (DIS/CAP1 and DIS/CAP2) animals. Brain areas are preoptic area (POA), medial basal hypothalamus (MBH), pons (PONS), medulla (MED), and spinal cord (SPCD). SP-G-K-L1 levels were quantified after treatment of extracted tissue with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (TRYP), as described. *, \( p < 0.05; **, p < 0.01 \). Graphed values represent mean \( \pm \) S.E.

limits of detection of the SP-G RIA and could not be accurately quantified. Thus, these values are not included in the presented data. The elevations in levels of SP-G-L1 ranged from approximately 5- to 17-fold in experimentals versus controls with an average increase of 11.7-fold \( \pm 1.4 \) (S.E.). To monitor changes in unamidated precursor relative to mature peptide, SP-G-L1:SP-LI ratios were plotted. With coordinate reductions in levels of SP-LI and increases in levels of SP-G-L1, the SP-G-L1:SP-LI ratios were markedly increased in treated versus control animals. In control animals, levels of SP-G-L1 expressed on a normalized basis ranged from 0.38 to 2.3% of the levels of SP-LI across CNS regions. After disulfiram treatment, normalized levels of SP-G-L1 ranged from 4.2 to 21% of the levels of SP-LI across CNS regions, with an average value of 14.3% \( \pm 1.8 \) (S.E.) (Fig. 2A). On a regional basis, the smallest increases in SP-G-L1 were observed in the medial basal hypothalamus of drug-treated animals versus controls, whereas the largest increases in SP-G-L1 were noted in the medulla and preoptic area of these same animals. In addition, there were no statistically significant differences in the magnitude of increase of the SP-G-L1:SP-LI ratio among the three treatment groups DIS, DIS/CAP1, and DIS/CAP2 across CNS areas. Interestingly, levels of heterogeneous SP precursors, normalized as SP G K determinants and monitored by RIA of trypsin-treated extracts, were increased in several brain areas of animals concomitantly treated with both drugs, i.e. the DIS/CAP1 group (Fig. 1C). These changes were effectively realized as SP-G-K-L1:SP-LI ratios and were

FIG. 2. Effects of drug treatments on normalized ratios of CNS peptides. SP-G-L1:SP-LI ratios (A) and SP-G-K-L1:SP-LI ratios (B) are expressed on a percentile basis. Details and symbols are exactly as presented in Fig. 1.
shown to reach levels of statistical significance in the pons, preoptic area, and spinal cord of animals in the DIS/CAP1 group (Fig. 2B). Unlike the dramatic 10-fold increases in SP-GK:SP-LI ratios observed in CNS areas of drug-treated animals, SP-G-K:SP-LI ratios were modestly increased by approximately 2-fold in the CNS areas mentioned above.

Finally, there was an observable trend toward increased extended precursor versus mature SP, normalized as SP-GK:SP-LI ratios, in the brain stems and spinal cords of animals treated with disulfiram alone (DIS group).

**Immunochemical Characterization of SP-LI, SP-G-LI, and SP-G-K-LI Extracted from Mouse Brain Samples**—Extensive HPLC/RIA analyses of extracted brain tissues from both control and drug-treated animals yielded very similar chromatographic and immunocromatic behavior for SP-LI, SP-G-LI, and SP-G-K-LI, as demonstrated for the chemically authentic peptide standards. After fractionation by gradient reverse phase HPLC, the major immunoreactive peaks of SP-LI, SP-G-LI, and SP-G-K-LI were coeluted with authentic standards (Fig. 3). In brain extracts of drug-treated animals only, small peaks of SP-LI and SP-G-LI were coeluted with synthetic sulfoxide derivatives of SP and SP-G, respectively (Fig. 3, A–D). Normalized SP-G-LI:SP-LI molar ratios as monitored by RIA of HPLC-fractionated brain extracts from control and drug-treated animals were calculated to be 2.9 and 25.7%, respectively. These values are consistent with average 10-fold changes monitored by RIA of individual brain and spinal areas (Fig. 1). In addition, our analyses demonstrated the presence of very low steady state levels of SP-GK-LI, normalized as 0.21 and 0.44% of the steady state levels of SP-LI in control and drug-treated animals, respectively (Fig. 3, E and F). Here we observed that approximately 70% of the total SP-GK-LI found in drug-treated animals was coeluted with authentic SP-G-K and its sulfoxide derivative, whereas 30% of the total immunoreactivity was eluted at earlier retention times (Fig. 3F). Consistent with our previous work (5, 13), SP-G-KLI in protease-treated extracts was increased approximately 75-fold, compared with untreated samples (Fig. 3, G and H). In the analyses of trypsin-treated samples from both control and drug-treated animals, the major peaks of SP-G-K-LI were coeluted with authentic SP-GK, with small but significant peaks of SP-G-K-LI (5–10% total immunoreactivity) eluted at retention times intermediate of SP-GK and its sulfoxide derivative. The minor peak of SP-G-K-LI may represent an amino-terminal-extended SP-G-K determinant resulting from incomplete trypsin digestion, or a previously uncharacterized molecular variant containing the COOH-terminal epitope of SP-G-K. Normalized SP-G-K-LI:SP-LI molar ratios were 15.4 and 24.7% in extracts from control and drug-treated animals, respectively. Thus, disulfiram treatment resulted in an apparent 70% increase in immunoactivity corresponding to heterogeneous forms of SP precursors monitored by RIA of SP-G-K determinants after trypsin treatment. Overall, the accuracy and precision (92–96% total recovery of chromatographed immunoreactivity) of the HPLC-RIA analyses have demonstrated the feasibility of our method as a general approach for quantification of changes in levels of immunoreactivity corresponding to mature and immature molecular forms of SP after pharmacological or physiological challenge.

**HPLC-ECAD Analysis of Biogenic Amine Levels**—It has been previously demonstrated that the enzyme responsible for noradrenaline biosynthesis, i.e., dopamine β-hydroxylase, is inhibited by disulfiram administration via chelation of copper cofactor (21). Thus, the present study included parallel analyses of monoamines, in part to monitor efficacy of drug administration via changes in CNS levels of norepinephrine. Here norepinephrine levels were modestly reduced in a variable manner in CNS areas of drug-treated animals as compared to controls (Fig. 4A). Average drug-induced reductions in norepinephrine levels ranged from 42 to 11% of control values in main olfactory bulb and pons, respectively. In addition, norepinephrine levels were reduced in a statistically significant manner in main olfactory bulb, preoptic area, medial basal hypothalamus, and spinal cord of all drug-treated groups, as compared to controls. In medulla and pons, noradrenaline levels were significantly reduced only for the DIS/CAP1 group, as compared to controls.

Statistically significant increases in 5-HT turnover, as monitored by the 5-HIAA:5-HT ratio, were observed in CNS regions of drug-treated animals, as compared to controls (Fig. 4D). These increases in 5-HT turnover were generally attributable to elevated levels of 5-HIAA in most CNS areas of drug-treated animals (Fig. 4C). In groups DIS/CAP1 and DIS/CAP2, observed decreases in 5-HT levels with concomitant elevations in 5-HIAA levels yielded dramatic increases in 5-HT turnover of 50–100% versus controls in medulla and medial basal hypothalamus, respectively.

Finally, dopamine levels in main olfactory bulb, preoptic area, and medial basal hypothalamus exhibited an inconsistent pattern of change in all the drug-treated groups, as compared to controls (data not shown). A statistically significant increase in dopamine levels was observed only in the medial basal hypothalamus of animals treated with disulfiram alone (DIS group). However, dopamine turnover as monitored by the 3,5-dihydroxyphenylacetic acid:dopamine ratio was reduced in a statistically significant manner in the preoptic area of all animals in drug-treated groups (data not shown).

**Anatomical and Behavioral Studies**—Complementary immunohistochemical analyses using affinity-purified anti-SP-G sera revealed markedly increased staining at the cellular level in CNS sections from DIS-treated animals, as compared to controls (Fig. 5). In drug-treated cases, immunohistochemical staining of SP-G-LI-containing neural elements was prominent in those areas of the brain exhibiting dense labeling in analyses using anti-SP sera, i.e., ventral pallidum, substantia nigra, and dorsolateral medulla and the substantia gelatinosa of the spinal cord. Areas of the brain that exhibited moderate to light labeling in analyses using anti-SP sera, i.e., preoptic area, medial basal hypothalamus, periaqueductal gray, nucleus solitarius, and ventral spinal cord, exhibited very light labeling in analyses using anti-SP-G sera. Pronounced staining of SP-G-LI-containing terminal fields was observed in the substantia gelatinosa of the spinal cord (Fig. 5A) and in the trigeminal nucleus caudalis of the medulla (Fig. 5C) of a DIS-treated animal. The general pattern of staining was similar to that seen in spinal cord and medulla in analyses using anti-SP sera (Fig. 5, D and E, respectively). However, some differences in the distribution of reaction product were observed in comparative analyses using the two antisera. With anti-SP sera, dense labeling was observed in the dorsal longitudinal fasciculus, a fiber tract located ventrolateral to the substantia gelatinosa. In sections treated with anti-SP-G sera, little or no reaction product was found in the dorsal longitudinal fasciculus. This difference was also observed in a medullary area, along the ventromedial border of the nucleus caudalis, which was stained in analyses using anti-SP sera but not in those employing anti-SP-G sera. Finally, analyses using anti-SP-G sera performed on CNS sections of vehicle-treated controls or animals administered only CAP yielded no observable immunohistochemical stain-
Fig. 3. Immunochemical characterization by combined HPLC/RIA analyses of SP-LI, SP-G-LI, and SP-G-K-LI extracted from mouse brain samples. HPLC elution profiles of SP-LI (A and B), SP-G-LI (C and D), and SP-G-K-LI (E and F) from extracted brain tissues of control (CON) and disulfiram-treated (DIS) animals. HPLC elution profiles of SP-G-K-LI (G and H) after trypsin treatment of extracted brain extracts (see “Experimental Procedures”) from CON and DIS animals. Arrows denote retention times of authentic SP, SP-G, SP-G-K, and their respective sulfoxide derivatives (SP-O, SP-G-O, SP-G-K-O), which are also recognized by the three antisera. For each analysis, the respective input immunoreactivity and percent recovery are as follows: A, 33.4 ng of SP, 96%; B, 34.1 ng of SP, 92%; C, 0.99 ng of SP-G, 95%; D, 8.3 ng of SP-G, 96%; E, 67.3 pg of SP-GK, 93%; F, 282.1 pg of SP-GK, 96%; G, 5.1 ng of SP-GK, 96%; H, 8.3 ng of SP-GK, 92%. Symbols are as in Fig. 1.

As a functional consequence of pharmacological challenge, pain thresholds, as monitored by thermal nociceptive response, were significantly altered in drug-treated animals. We observed a gradual increase in tailflick latency in disulfiram-treated animals versus controls, with statistical significance (p < 0.05) reached by the 16th day of treatment (data not shown). Combined disulfiram and capsaicin treatments were observed to produce the most dramatic increases in tailflick latency (p < 0.01) in experiments versus controls after 16 days of treatment. The disulfiram/capsaicin-induced elevation of pain thresholds may be explained by the additive effects of both drugs in significantly lowering SP levels in the spinal cord. These data are complemented by analyses of spinal cord from disulfiram/capsaicin-treated animals, which reveal significant elevations in SP-G-LI:SP-LI ratios, suggesting marked functional down-regulation of SP expression.
Finally, we observed that disulfiram pretreatment produced a dramatic protective effect on capsaicin-induced mortality in experimental animals. Rates of survival were shown to increase by approximately 300% in the disulfiram/capsaicin groups, as compared to the animals treated with capsaicin alone (data not shown). The biochemical mechanism of this protective effect is not immediately evident, but the recent availability of capsaicin for use in pain therapy (25) warrants its further investigation.

**DISCUSSION**

In the present study, we have demonstrated significant drug-induced changes in several neurochemical parameters in mouse CNS, including levels of immunoreactivity corresponding to mature and immature forms of SP, monoamines, and their metabolites. Regulatory events in the maturation processes of neuropeptides are not well understood (4). Our data suggest that the observed changes in steady state levels of immunoreactivities corresponding to mature SP and to the immature COOH-terminal extended forms of SP, i.e. SP-G and SP-G-K, are reflective of genuine compensatory biosynthetic and posttranslational processing events in SP-containing neural systems after pharmacological challenge. In addition, these data reflect established functional interrelationships between SP-containing and monoaminergic neural systems in sensory and integrative areas throughout the neuroaxis (1, 26, 27).

Although amino acid sequence data cannot be conclusively confirmed by combined chromatographic and immunochromatographic data, we are confident that the accuracy and precision of our analytical methodology have yielded strong presumptive evidence supporting the existence of mature SP and of amidated SP precursors in nervous tissues. To a best approximation, the dramatic increases in steady state levels of SP-G-L1 accurately reflect modulation of expression of a COOH-terminal glycine-extended precursor form of a CNS peptide after administration of an established inhibitor of the biosynthetic enzyme peptidylglycine α-amidating monoxygenase. In complementary immunohistochemical analyses, these neurochemical changes were localized at the cellular level to neural elements that normally express SP. Disulfiram (Antabuse) is the disulfide dimer of N,N-diethyldithiocarbamate, to which it is rapidly converted via a reductive mechanism (22, 23). The presumptive biologically active form of disulfiram, i.e. N,N-diethyldithiocarbamate, is a potent and relatively specific copper chelator that has been shown to reversibly inhibit pituitary peptidylglycine α-amidating monoxygenase activity in vitro (10) and in vivo (12). The ability of N,N-diethyldithiocarbamate to chelate copper is also thought to be responsible for its inhibition of the cuproprotein, dopamine β-hydroxylase (21), resulting in decreased levels of noradrenaline and epinephrine.

Peptidylglycine α-amidating monoxygenase has been shown to catalyze the formation of a terminal carboxyamide moiety and glyoxylate from a variety of peptidylglycine substrates (8–10, 24). SP-G is probably the major immediate precursor to SP, although we cannot rule out an alternative processing pathway via amidation of large molecular mass glycine-extended forms, followed by endoproteolytic cleavage at paired dibasic residues and release of mature SP (28). We have detected low steady state levels of SP-G-L1 in all CNS areas examined from both mice and rats.2 These areas included olfactory bulbs, preoptic area, medial basal hypothalamus, lateral septum, substantia nigra, interpeduncular nu-

---

2 J. E. Marchand and R. M. Kream, manuscript in preparation.
Effects of Disulfiram on SP-LI and Monoaminergic Systems

FIG. 5. Immunohistochemistry of brain sections from normal and drug-treated animals using antisera to SP and SP-G. Photomicrographs of sections of spinal cord and medulla from disulfiram-treated animals reacted with antisera to SP-G (A and C) and with antisera to SP (B and E). Reaction product demonstrating immobilized antigen is localized to substantia gelatinosa (A and B) and nucleus caudalis trigeminalis (C and E). Photomicrograph of medulla section from an animal with no disulfiram treatment reacted with antisera to SP-G demonstrating no reaction product in nucleus caudalis trigeminalis (D).

The presence of relatively modest steady state levels of SP-G-L1 in SP-containing neural systems that are dramatically altered by drug challenge suggests to us that terminal peptide amidation may represent a rate-limiting step in SP maturation and expression. By comparison, steady state levels of SP-G-K-L1 quantified without prior trypsin digestion from extracted brain of control animals were found to be 14 times lower than those of SP-G-L1 and were increased only approximately 2-fold in brain from drug-treated animals (Fig. 3, E and F, respectively). Free SP-G-K-L1 probably represents a short-lived processing intermediate in SP maturation found in very low steady state concentrations in CNS; our data strongly suggest that the neuronal carboxypeptidase responsible for converting SP-G-K-L1 into SP-G-L1 is not a likely
candidate for selective inhibition by pharmacological agents. Apart from serving as an immediate precursor to SP, SP-G-L1 may be involved in an independent regulatory role, because changes in levels of immunoreactive heterogenous SP precursors quantified as protease-generated SP-G-L1 determinants were shown to co-vary with levels of SP-G-L1 in these experiments. Thus, selective inhibition of terminal peptide amidation may indirectly affect other cellular biosynthetic processes, such as transcriptional or translational activity, or posttranslational peptide processing.

The present study provided quantification of an effective panel of neurochemical parameters from the same tissue samples in drug-treated and control animals, including neuronal monoamines. As predicted, levels of norepinephrine were modestly reduced in most CNS areas of drug-treated animals, consistent with inhibition of the biosynthetic enzyme, dopamine β-hydroxylase (21). To our knowledge, the effects of disulfiram administration on 5-HT utilization within the CNS have not been previously reported. The statistically significant increases in 5-HT turnover, as monitored by the 5-HIAA:5-HT ratio, observed in several CNS areas of drug-treated animals as compared to controls are most likely a secondary effect to the reductions in levels of neuronal norepinephrine. It has been demonstrated that nor-epinephrine-containing and 5-HT-containing neural systems are functionally interrelated in several CNS areas. For example, the median raphe region of the brainstem, containing numerous 5-HT-positive cell bodies, is innervated by an extensive noradrenergic terminal field originating from the locus ceruleus (29). Furthermore, jontophoresis of norepinephrine has been shown to alter the rate of firing of 5-HT-containing neurons (30). In pharmacological studies, injections of noradrenergic agonists and selective antagonists into the median raphe area were observed to produce respective decreases and increases in levels of 5-HIAA throughout the brain (31), consistent with our findings. Finally, drug-induced changes in dopamine utilization were observed to be opposite to those of 5-HT, as monitored by a significantly reduced dopamine:3,4-dihydroxyphenylacetic acid ratio in the preoptic area of drug-treated animals. Overall, these data suggest that disulfiram administration mediates diverse changes in transmitter metabolism, albeit to varying degrees, in central monoaminergic neurons.

In humans, alterations in neurological (32), neuroendocrine (33), physiological (34), psychological, and psychosexual (35) functions have been associated with chronic Antabuse administration, despite a rather extensive recent Danish clinical study (36) that concluded that use of Antabuse produces no side effects. In our study, the ability of the disulfiram dose employed (100 mg/kg/day) to perturb, but not to dramatically impair, the functional integrity of interactive peptidergic and monoaminergic neural systems reflects the strong homeostatic mechanisms involved in regulation of transmitter expression in neurons. In this regard, we conclude that the practical use of disulfiram or N,N-diethyldithiocarbamate for treatment of pain at the spinal level would require additional pharmacological agents. We observed that disulfiram-induced increases in pain threshold were potentiated by capsaicin, an agent known to cause SP depletion in primary afferents (14). The apparent elevation of pain thresholds may be explained by the additive effects of both drugs in significantly lowering SP levels in the spinal cord, with concomitant elevations in SP-G-L1:SP-L1 ratios (37). Interestingly, the drug-induced increases in SP-G-L1:SP-L1 ratios observed in the spinal cord were of a decidedly lesser magnitude than those observed in other brain areas such as the medulla and hypothalamus (Fig. 2A), strongly suggestive of marked functional down-regulation of SP expression in this area via diminished biosynthesis. The role of endogenous norepinephrine in the modulation of spinal nociceptive processes is well documented (38, 39).

In the present study, it is possible that the statistically significant decreases in levels of norepinephrine in the spinal cords of drug-treated animals (Fig. 5A) resulted in physiological antagonism to the analgesic effects of reduced spinal SP levels. Subsequent studies to evaluate the efficacy of Antabuse or N,N-diethyldithiocarbamate as a spinal analgesic may include restorative or even subthreshold doses of adrenergic agents such as the α2 agonist clonidine (40). An innovative pharmacological approach for treatment of chronic pain at the spinal level may include Antabuse pretreatment in combination with capsaicin (25), followed by low doses of α2 agonists and/or narcotic agents (41).

The ability of disulfiram to modify CNS levels of immunoreactive unamidated precursor to SP makes it a useful probe for examining the role of terminal processing of SP in diverse biological processes. Further studies to evaluate the effects of disulfiram or N,N-diethyldithiocarbamate administration on CNS levels of peptides and of preprotachykinin mRNAs are in progress.

REFERENCES
1. Pernow, B. (1983) Pharmacol. Rev. 35, 86–138
2. Regoli, D., Drapeau, G., Dion, S., and Couture, R. (1988) Trends Pharmacol. Sci. 9, 290–295
3. Tatetomo, K., and Mutt, V. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 6065–6070
4. Mains, R. E., Eipper, B. A., Glembotski, C. C., and Dores, R. M. (1983) Trends Neurosci. 6, 229–235
5. Kream, R. M., Schoenfeld, T. M., Mancuso, R., Clancy, A. N., El-Bermani, W., and Macrides, F. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 4655–4658
6. Sugano, K., Aponte, G. W., and Yamada, T. (1985) J. Biol. Chem. 260, 11724–11729
7. Glembotski, C. C., Eipper, B. A., and Mains, R. E. (1983) J. Biol. Chem. 258, 7299–7304
8. Bradbury, A. F., Finnie, M. D. A., and Smyth, D. G. (1982) Nature 298, 666–668
9. Bradbury, A. F., and Smyth, D. G. (1983) Biochem. Biophys. Res. Commun. 113, 373–377
10. Eipper, B. A., Mains, R. E., and Glembotski, C. C. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 5144–5148
11. Paquette, T. L., Gingerich, R., and Sharp, D. (1981) Biochemistry 20, 7403–7406
12. Mains, R. E., Park, L. P., and Eipper, B. A. (1986) J. Biol. Chem. 261, 11998–11941
13. Kream, R. M., Kumar, M. S. A., El-Bermani, W., and Thompson, M. L. (1987) Soc. Neurosci. Abstr. 13, 1257
14. Jessell, T. M., Iversen, L. L., and Cuello, A. C. (1978) Brain Res. 152, 183–188
15. Nakashii, S. (1987) Physiol. Rev. 67, 1117–1141
16. Kream, R. M., Davis, B. J., Kawano, T., Margolis, F. L., and Macrides, F. (1984) J. Comp. Neurol. 222, 140–154
17. Mefford, I. N. (1981) J. Neurosci. Methods 3, 207–224
18. Kilpatrick, I. C., Jones, M. W., and Phillipsion, O. T. (1986) J. Neurochem. 46, 1865–1876
19. Glowinski, J., and Iversen, L. L. (1986) J. Neurochem. 13, 655–669
20. Hus, S. M., Raine, L., and Fanger, H. (1981) J. Histochem. Cytochem. 29, 577–580
21. Goldstein, M. A., and Lauber, E. (1964) Life Sci. 3, 763–767
22. Mussachio, J., Kopin, I. J., and Snyder, S. (1964) Life Sci. 3, 769–755
23. Faiman, M. D. (1979) in Biochemistry and Pharmacology of Ethanol (Majchrowicz, E., and Noble, E. P., eds) Vol 2., pp. 373–377
24. Kizer, J. S., Busby, W. H., Jr., Cottle, C., and Youngblood, W. W. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 3228–3232
25. Janes, G., and Lynn, B. (1987) Clin. J. Pain 3, 123–126
26. Bannon, M. J., Elliott, P. J., Alpert, J. E., Goedert, M., Iversen, S. D., and Iversen, L. L. (1983) Nature 306, 791–792
27. Baruch, P., Artaud, F., Godeheu, G., Barbeito, L., Glowinski, J., and Cheramy, A. (1988) Neuroscience 25, 889–898
28. Harmar, A. J., Armstrong, A., Lee, C. M., and Emson, P. C. (1984) Brain Res. 323, 342–344
29. Swanson, L. W., and Hartman, B. K. (1975) J. Comp. Neurol. 162, 47–50
30. Baraban, J. M., Wang, R. Y., and Aghajanian, G. K. (1978) Eur. J. Pharmacol. 52, 27–36
31. Placzek, A., Danysz, W., Kostowski, W., Bidziński, A., and Hauptmann, M. (1983) Pharmacol. Biochem. Behav. 19, 27–32
32. Borrett, D., Ashby, P., Bilbao, J., and Carlen, J. (1985) Ann. Neurol. 17, 396–399
33. Cavalleri, A., Polatti, F., and Bolis, P. F. (1978) Scand. J. Work Environ. Health 4, 66–72
34. Volier, L., and Nelson, K. L. (1984) Arch. Intern. Med. 144, 1294–1296
35. Jensen, S. B. (1984) Acta Psychiatr. Scand. 69, 543–549
36. Christensen, J. K. (1984) Br. J. Clin. Pract. Symp. Suppl. 38, 21–28
37. Otsuka, M., and Yanagisawa, M. (1987) Trends Pharmacol. Sci. 8, 506–510
38. Yaksh, T. L. (1985) Pharmacol. Biochem. Behav. 22, 845–858
39. Fang, I. H., and Vasko, M. R. (1986) Brain Res. 370, 269–279
40. Spaulding, T. C., Venafro, J. J., Ma, M. G., and Fielding, S. (1979) Neuropharmacology 18, 103–108
41. Yaksh, T. L. (1981) Pain 11, 293–346