Imidazoline Receptor Proteins Are Decreased in the Hippocampus of Individuals with Major Depression

John E. Piletz, He Zhu, Gregory Ordway, Craig Stockmeier, Ginny Dilly, Donald Reis, and Angelos Halaris

Background: A downregulation of I$_2$-imidazoline binding sites has been reported in frontal cortices of depressed suicide victims, according to I$_2$-radioligand binding and confirmed by Western blotting. We now report Western blots of imidazoline receptor proteins in hippocampi of subjects with and without depression at the time of death.

Methods: Postmortem diagnoses were obtained from 17 cases of Axis I major depressive disorder and 17 cases without Axis I psychopathology. No psychotropic compounds were found in body fluids. Hippocampi were removed, sectioned, and assessed histologically. Throughout the analysis, each major depressive disorder sample was paired with a sample from a psychiatrically healthy subject based on equivalent life spans and postmortem delays. The antiserum was identical to that used in previous studies that reported a downregulation of cortical 29/30-kd imidazoline receptor–binding proteins in depression.

Results: A triad of imidazoline receptor–binding protein bands (40–50 kd) was detected in the human hippocampus. Subjects with major depressive disorder had significantly less intensity in each imidazoline receptor–binding proteins band compared with control subjects ($p < .01$ for overall bands).

Conclusions: The present results can be aligned with previous reports of downregulation of I$_2$-radioligand binding sites in both cortices and platelets of depressed patients. Biol Psychiatry 2000;48:910–919 © 2000 Society of Biological Psychiatry

Key Words: Imidazoline receptors, monoamine oxidases, immunoreactivity, hippocampus, major depression, suicide

Introduction

Imidazoline receptors (more conservatively called I-sites) and $\alpha_2$-adrenoceptors ($\alpha_2$ARs) are distinct membrane proteins that share high affinity for imidazoline compounds such as clonidine and idazoxan (Ernsberger et al 1995). I-sites are not adrenergic in nature because they lack appreciable (nanomolar) affinities for all known monoamines. There are two subtypes of I-sites (I$_1$ and I$_2$), and these are differentially distributed throughout the brain (DeVos et al 1994). Recently, an imidazoline receptor–binding protein (IRBP) was cloned from human hippocampus and shown to have I$_1$-like properties (Ivanov et al 1998b; Piletz et al 2000). The function of the hippocampal IRBP remains controversial (Guyenet 1997), but studies of brainstem nuclei have linked an I$_1$ site to the central control of blood pressure (Bousquet et al 1984; Molderings 1997).

Additionally, I$_2$ sites are of interest to psychiatry because they are physically associated with monoamine oxidases A (MAO-A) and B (MAO-B; Tesson et al 1995). An I$_2$ binding site has been localized within a 73 amino acid sequence (K149–M222) of MAO-B (Raddatz and Lanier 1997; Raddatz et al 1997). This I$_2$ site lies outside the catalytic domain proposed for MAO-B enzymatic inhibition, but a point mutation (T158$^*$A of MAO-B) within the same region (K149–M222) causes complete loss of MAO-B activity (Cesura et al 1996). On the other hand, not all I$_2$ sites are accounted for by MAO-A or MAO-B molecules. In fact, a relatively small subpopulation of MAO-A or MAO-B molecules is actually accessible for I$_2$ radioligand binding, and this varies in a tissue-dependent manner (Raddatz and Lanier 1997; Raddatz et al 1997). The source and significance of this subpopulation of MAO molecules remains unknown.

Both I$_1$ and I$_2$ sites have been found to be altered in depressed patients (Piletz et al 1994; Sastre et al 1995; Sastre and Garcia-Sevilla 1997), as have $\alpha_2$AR agonist binding sites for clonidine (Callado et al 1998). First, an increase in radioligand binding density ($B_{\text{MAX}}$) of I$_1$ sites on platelet plasma membranes of depressed patients was reported (Piletz et al 1994, 1996a, 1996b) relative to...
healthy control subjects. Then, I₂ sites were shown to be decreased in platelet internal membranes of depressed patients (Piletz et al 1994) and in frontal cortices of suicide victims (Sastre et al 1995; Sastre and Garcia-Sevilla 1997) compared with control subjects. Furthermore, studies with radiolabeled clonidine (or its analogs) have consistently revealed an up-regulation of α₂AR agonist binding sites in platelets of depressed patients (Garcia-Sevilla et al 1981; Piletz et al 1994) and in some brain regions of suicide victims (Callado et al 1998; Meana et al 1992; Ordway et al 1994). Thus, numerous studies have now revealed alterations in I-sites, α₂AR agonist sites, or both in depression.

The first purification of an IRBP was reported by Wang et al (1992) using bovine adrenal chromaffin cells. A human hippocampal homologue of this protein was subsequently cloned by Ivanov et al (1998b; Piletz et al 2000). Based on retentions of the bovine IRBP by two affinity chromatography resins and selective elutions from those columns by an imidazoline displacing agent, this protein was reported (Wang et al 1992) to possess a combination of I₁ and I₂ binding properties. This protein was further shown to run as a single band on a SDS gel with a molecular weight (MW) of 70 kd (Wang et al 1992). The same protein was later used as an immunogen to produce polyclonal antiserum, designated IRBP antiserum (Wang et al 1993), which we have used in our study.

Several related polypeptides react with IRBP antiserum on Western blots. These include its progenitor 70-kd immunogen from bovine adrenal chromaffin cell membranes (Wang et al 1993), an 85-kd protein from freshly prepared rat brain membranes (Ivanov et al 1998a; 1998c), and several breakdown polypeptides (Ivanov et al 1998c). Correlations have been established between the intensities of doublet 29/30-kd IRBP bands versus Bₘₐₓ values for I₂ sites across several rat and human tissues and conditions (Escriba et al 1994; Garcia-Sevilla et al 1995). Conversely, 33- and 45-kd bands detected by IRBP antiserum have been correlated with I₁ sites in human tissues (Garcia-Sevilla et al 1999; Ivanov et al 1998a). IRBP antiserum fails to cross-react with MAO-A, MAO-B, or any α₂AR subtypes (Escriba et al 1999; Ivanov et al 1998a). In summary, there appear to be multiple subtypes of I-sites:

1. One I₂ site represents a subpopulation of MAO molecules encoded by the K149–M222 sequence within MAO-B, but it cannot be detected by IRBP antiserum.
2. Another I₂ site is related to a 29/30-kd doublet protein in brain and to a 70-kd protein in bovine adrenal chromaffin cells according to cross-reactivity with IRBP antiserum.
3. An I₁-like site is related to a 45-kd protein in brain according to cross-reactivity with IRBP antiserum.
4. Another I₁-like site is related to a 33-kd protein in platelets according to cross-reactivity with IRBP antiserum (Ivanov et al 1998a).

We have proposed the latter three subtypes may be breakdown products of a common 85-kd precursor protein (Ivanov et al 1998c). They may also all be derived from the same cloned hippocampal IRBP (Piletz et al 2000).

In our study, IRBP antiserum has been used to quantify Western blots of hippocampi from suicide subjects who were retrospectively diagnosed with MDD at the time of death, compared with paired control subjects who lacked a major (Axis I) psychiatric diagnosis. Based on the previous studies of Garcia-Sevilla et al (1996), we hypothesized that hippocampi from depressed suicide victims might be low in 29/30-kd IRBP bands relative to control subjects, but a 45 kd band might be increased in MDD subjects compared with control subjects.

Methods and Materials

Brain Tissue

Human brains were obtained at the time of autopsy by the Medical Examiner’s Office of Cuyahoga County, Ohio, in accordance with an approved institutional review board protocol. Cadavers were immediately refrigerated when arriving at the medical examiner’s office. The coroner determined the cause of death. Brain sections were dissected, coded to protect the subject’s identity, and frozen (−82°C) in tightly sealed containers. Samples from both groups of subjects were stored for comparable lengths of time before thawing.

Information on lifetime events and the recent (within the last month of life) psychiatric status of all subjects was obtained from next-of-kin during structured clinical interviews by a trained interviewer. The interviews were according to the Schedule for Affective Disorders and Schizophrenia: Lifetime Version (SADS-L) supplemented by questions from the Diagnostic Interview Schedule (DIS-III-R) to make diagnoses compatible with the DSM-IV (Rush and Weissenburger 1994). The SADS has obtained adequate validity when comparing the patient report with that of an informant (Andreasen et al 1977). Evaluations of drug and alcohol abuse and dependency were assessed using the DIS-III-R (Kelly et al 1998). Axis I diagnoses were made by a psychiatrist and a clinical psychologist, based on the data gathered from the structured interview and, when available, hospital and medical records.

Hippocampi were collected from 17 subjects diagnosed with MDD (Table 1) and 17 healthy control subjects. Ages ranged from 25 to 86 years (mean ± SEM, 55 ± 5 years) for subjects with MDD and from 23 to 82 years (53 ± 4 years) for control subjects. Postmortem delay until autopsy was 17 ± 1 hour for MDD subjects and 17 ± 1 hour for control subjects. Among the 17 subjects diagnosed with MDD (5 women, 12 men), two had comorbid Axis I diagnoses of alcohol depen-
Table 1. Demographic Information for Subjects with Major Depression

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Race/Sex</th>
<th>Cause of death</th>
<th>PMD (hours)</th>
<th>Toxicology</th>
<th>Axis I</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Suicide</td>
<td>48 W/M</td>
<td>SIGSW, slashed wrists</td>
<td>18.5</td>
<td>Flurazepam</td>
</tr>
<tr>
<td>2</td>
<td>Suicide</td>
<td>30 B/M</td>
<td>SIGSW</td>
<td>18</td>
<td>Ethanol</td>
</tr>
<tr>
<td>3</td>
<td>Suicide</td>
<td>54 W/M</td>
<td>CO poisoning</td>
<td>23</td>
<td>Phenobarbital, phenytoin</td>
</tr>
<tr>
<td>4</td>
<td>Suicide</td>
<td>50 W/F</td>
<td>Hanging</td>
<td>23</td>
<td>NDD</td>
</tr>
<tr>
<td>5</td>
<td>Suicide</td>
<td>86 W/M</td>
<td>Self-inflicted stabbing</td>
<td>21</td>
<td>NDD</td>
</tr>
<tr>
<td>6</td>
<td>Suicide</td>
<td>78 W/F</td>
<td>Blunt force trauma</td>
<td>25</td>
<td>NDD</td>
</tr>
<tr>
<td>7</td>
<td>Suicide</td>
<td>25 W/F</td>
<td>Hanging</td>
<td>17</td>
<td>NDD</td>
</tr>
<tr>
<td>8</td>
<td>Suicide</td>
<td>43 W/M</td>
<td>Hanging</td>
<td>21</td>
<td>NDD</td>
</tr>
<tr>
<td>9</td>
<td>Suicide</td>
<td>45 W/M</td>
<td>Self-inflicted stabbing</td>
<td>8</td>
<td>NDD</td>
</tr>
<tr>
<td>10</td>
<td>Suicide</td>
<td>83 W/F</td>
<td>Slashed wrists</td>
<td>21</td>
<td>NDD</td>
</tr>
<tr>
<td>11</td>
<td>Suicide</td>
<td>62 W/M</td>
<td>SIGSW</td>
<td>20</td>
<td>NDD</td>
</tr>
<tr>
<td>12</td>
<td>Suicide</td>
<td>42 W/M</td>
<td>SIGSW</td>
<td>21</td>
<td>NDD</td>
</tr>
<tr>
<td>13</td>
<td>Suicide</td>
<td>73 W/M</td>
<td>SIGSW</td>
<td>18</td>
<td>Diazepam, codeine</td>
</tr>
<tr>
<td>14</td>
<td>Suicide</td>
<td>29 W/M</td>
<td>Hanging</td>
<td>9</td>
<td>Cocaine, ethanol</td>
</tr>
<tr>
<td>15</td>
<td>Suicide</td>
<td>47 W/M</td>
<td>SIGSW</td>
<td>11</td>
<td>Ethanol</td>
</tr>
<tr>
<td>16</td>
<td>Suicide</td>
<td>75 W/F</td>
<td>CO poisoning</td>
<td>29</td>
<td>NDD</td>
</tr>
<tr>
<td>17</td>
<td>Suicide</td>
<td>68 W/M</td>
<td>CO poisoning</td>
<td>4</td>
<td>NDD</td>
</tr>
</tbody>
</table>

PMD, postmortem delay; W, white; M, male; SIGSW, self-inflicted gunshot wound; B, black; CO, carbon monoxide; F, female; NDD, no drugs detected.

*Coroner’s ruling.

dence, and two had histories of alcohol abuse (Table 1). Subjects in the control group consisted of 4 females and 13 males, and the causes of death in this group were: cardiovascular failure (n = 11), gunshot (n = 1), pulmonary embolism (n = 1), aneurysm (n = 1), pancreatitis (n = 1), lightning strike (n = 1), and bike accident (n = 1). All control subjects were assessed retrospectively through structured interviews with family members and had no active major psychiatric diagnoses (Axis I; DSM-IV) at the time of death. Among the control subjects, one had experienced an episode of adjustment disorder with depressed mood 5 months before death, and one had a history of alcohol abuse 7 years before death.

A toxicologic screen of blood, bile, and urine from all subjects was performed at the county coroner’s office. Qualitative and quantitative analyses were performed to detect the following compounds or classes of compounds: ethanol, barbiturates, benzodiazepines, sympathomimetic drugs, and antidepressant and antipsychotic drugs and their metabolites. The toxicology results from MDD subjects are shown in Table 1. Postmortem records revealed that within the month before death, three MDD subjects had received antidepressant drug prescriptions (one for fluoxetine, one for nortriptyline, and one for sertraline); however, these substances were not found in body fluids at the time of death (an exclusion criteria was evidence of antidepressants or antipsychotics in the toxicologic screens). The toxicologic screens of six control subjects revealed the following: two subjects had low but detectable levels of ethanol, one had ethanol plus a low level of cocaine, one had flurazepam, one had phenobarbital plus phenytoin, and one had diazepam plus codeine in the blood. They were not excluded from the study.

Dissection
At the time of autopsy, the brain was dissected into small tissue blocks. Particular care was taken to maintain gross morphology during freezing. Tissue containing the right hippocampus was placed on hard cardboard and then dipped into isopentane (−50°C) for 5 sec. Blocks of hippocampus were placed on dry ice for 10 min, and then stored at −82°C. Frozen blocks were then mounted on a specimen chuck of a cryostat microtome (Leica, Cryocut 1800, Reichert-Jung, Deerfield, IL). All brain tissue surrounding Ammon’s horn of the hippocampus was dissected away with a razor blade. Tissue sections through Ammon’s horn (including the dentate gyrus) were cut at −16°C, and sections (60 μm thick) from each subject were placed in an ice-cold microcentrifuge tube. The adjacent section (40 μm thick) was cut for morphology, dried at room temperature, and stained with cresyl violet. Histologic sections were used to confirm anatomic equivalence of the hippocampal sections for all MDD subjects and their paired control subjects.

Immunodetection of Imidazoline Receptor Proteins
Frozen hippocampal sections were sonicated for 5 sec in ice-cold 220 μL of 5 mmol/L Tris-HCl buffer, pH 7.4, containing 0.25 mol/L sucrose and 1 mmol/L MgCl2, plus a previously described cocktail of eight protease inhibitors (Ivanov et al 1998c). Aliquots were assayed for total protein according to the Lowry-Biuret reagent kit from Sigma (St. Louis). Remaining homogenates were flash frozen. Samples were thawed and diluted to 0.8 μg protein/μL with ice-cold 40 mmol/L Tris-HCl buffer, pH 6.8, plus 4% SDS. Samples (12 μg) were denatured and electropho-
resed through two identical 13.7% polyacrylamide gels containing SDS (SDS-PAGE) according to our previous studies (Ivanov et al 1998c). Platelet total membrane proteins (1.0, 2.5, and 5.0 μg) were prepared from a common bag of platelet-rich plasma (Mississippi Blood Services, Jackson, MS) and run as standards on each gel. The proteins were electrotransferred onto nitrocellulose membranes (Hybond ECL, Amersham, Arlington Heights, IL.). After electrotransfer, the blots were blocked with milk and incubated with IRBP antiserum (1:3000 dilution in TBST/10% milk) at room temperature (Ivanov et al 1998c). Using an Amersham ECL detection system, IRBP-immunoreactive bands were detected by exposure to film for 2 to 6 min (Amersham ECL Hyperfilm). To detect β-actin as a standard, the same blots were stripped (Ivanov et al 1998c) and incubated with a 1:9000 dilution of β-actin antiserum (Chemicon International, Temecula, CA). Detection by ECL was performed identically with β-actin antiserum except using 1:3000 dilution of anti-mouse IgG antibody (Amersham), and the films were exposed for 30 sec to develop the bands. Samples from subjects with MDD were loaded beside paired control subjects, and each sample was run into two SDS-PAGE gels (duplicate Western blots).

A Microcomputer Controlled Imaging Device (MCID model M2; Imaging Research, St. Catharines, Canada) was used for densitometry. Optical densities (ODs) of sample bands were compared with those of standard platelet membranes on each blot. A standard curve from each blot assured that sample OD values were within the linear response range for each film and therefore useful for comparison to previous publications (Ivanov et al 1998c). We standardized OD values using an optical density step-wedge (Imaging Research). Coomassie blue staining of duplicate gels was also quantified using a Molecular Dynamics Personal Densitometer (Molecular Dynamics, Sunnyvale, CA). Coomassie blue intensity provided verification that comparable amounts of protein were loaded onto each lane and that there was sample integrity (i.e., no generalized proteolysis). Finally, OD values were obtained from the Western blots for β-actin and used to normalize IRBP OD values (subject by subject). As with IRBP, the OD values of β-actin bands were derived from two blots, and the values were averaged.

Note on IRBP Antiserum
The IRBP antiserum used herein is identical to that described repeatedly by Garcia-Sevilla’s group (e.g., Garcia-Sevilla et al 1996). Despite that group’s emphasis on a 45-kd band in human brain, both our laboratory and their laboratory have obtained comparable results when analyzing fresh rat cerebral cortex. With rat cortex, their method yielded 29/30-kd (60–63%), 45-kd (31–33%) and 84/85-kd (4–9%) bands, compared to our method’s 29/30-kd (47–56%), 45-kd (39–48%), and 84/85-kd (5–6%) bands (Garcia-Sevilla, personal communication, 1996). Thus, our study and theirs (Garcia-Sevilla et al 1996) are likely to differ only in tissue sampling, rather than methodologically (also see Discussion).

Statistics
Statistical analyses were performed using GraphPAD InStat (GraphPAD Software, San Diego). All results are expressed as mean ± SEM. Student’s paired t tests (two-tailed) were performed on the raw data and on their logarithms. The test of significance was p ≤ .05. A paired t test was used because samples from MDD subjects were paired for age and postmortem interval and because the pairs were processed together and run side by side on the same gels.

Results
Western blots revealed at least three IRBP-immunoreactive bands in the human hippocampus. These bands ranged in size between 40 and 50 kd (Figure 1A). In some subjects, the smaller of this triad (40 kd) appeared as a closely aligned doublet, but it could not be fully resolved (Figure 1A, section C2). In a few subjects, there was an additional band of ~55 kd (Figure 1A, sections D2, D3, C4). By extending the ECL reaction time to 15 hours and redeveloping the films, it was also possible to visualize a weak 30-kd band and an even weaker 33-kd band similar to that found in platelets (data not shown). The OD values for the 30- to 33-kd bands were close to background levels and therefore deemed unreliable. After stripping the blots of IRBP antiserum, a sharp band was also detected with β-actin antiserum at MW = 43 kd (Figure 1B). Densitometry was performed on all prominent bands (individually). Although the 40-kd IRBP-immunoreactive band appeared as a doublet in some cases, it was traced as a singlet to maintain uniformity across samples.

The IRBP-immunoreactive bands were normalized per β-actin protein in each sample as a means of internally correcting for slight variances in protein loading. No significant differences in β-actin were observed between MDD subjects compared with matched control subjects (p = .390, paired t test, two-tailed). Further verification of the uniformity of protein loading was seen on gels stained with Coomassie blue. Each lane was almost identical in Coomassie staining (data not shown).

Western blots revealed a significant decrease in the ratio of IRBP/β-actin in IRBP bands between 40 and 50 kd in MDD subjects compared with paired control subjects (Figure 2). Greater statistical significance was achieved using log-transformed values, indicating that relative differences were more consistent than absolute differences. From higher MW to lower MW, the significance levels for log-transformed IRBP data were as follows (by paired t tests, two-tailed): 50-kd band, p = .007; 45-kd band, p = .009; and 40-kd band, p = .033. The nontransformed p values are given in the legend to Figure 2. For the 55-kd bands, the OD values that appeared in a limited number of samples did not differ statistically between patients and control subjects, but this was probably unreliable because of wide variance in the 55-kd band between subjects. When the values of all prominent bands were pooled
together, an overall decrease was verified in depressed patients compared with matched control subjects ($p = .01$ for log-transformed data; Figure 2D). Thus, 14 of the 17 pairs of subjects had total IRBP immunodensities lower in those with MDD than in the paired control subjects (Figure 2D).

Finally, postmortem delay was found to be a significant covariable (Table 1). All IRBP bands, regardless of diagnostic group, were found positively correlated in intensity with postmortem delay (Figure 3). From higher MW to lower MW, the $p$ values and $r^2$ values for correlating IRBP immunodensities with postmortem delays were as follows: 50-kd band, $p = .0430$, $r^2 = .115$; 45-kd band, $p = .0030$, $r^2 = .229$; and 40-kd band, $p < .0001$, $r^2 = .407$. The result of pooling all three bands together is shown in Figure 3 ($p = .0010$, $r^2 = .264$). The effects of postmortem delay were not significantly different between diagnostic groups (i.e., nearly identical slopes).

**Discussion**

In our study, the ODs of IRBP-immunoreactive bands were found decreased by $\approx 25\%$ in the hippocampi of subjects with MDD compared with paired control subjects lacking a psychiatric diagnosis (Figures 1 and 2). This finding was significant using untransformed data (Figure 2), but for 45-kd and 50-kd bands, it was even more statistically significant when comparing log-transformed values. There was also an effect of postmortem delay to increase IRBP bands (Figure 3), but because both patients and control subjects had identical postmortem delays, this did not change the conclusion. Thus, imidazoline receptors appear to be less abundant in the hippocampi of depressed suicide victims compared with control subjects matched for age and postmortem delay.

Previous studies have shown that IRBP-immunoreactive bands are susceptible to proteolysis even in fresh brain tissue (Ivanov et al 1998c) and cultured cells (Ivanov et al 1998a). Therefore, a critical aspect of our experimental design was pairing of subjects with MDD and control subjects for identical postmortem delays. Our samples were homogenized in a cocktail of eight protease inhibitors to avoid proteolysis (Ivanov et al 1998a). Nonetheless, much intersubject variability in the intensity of IRBP bands could be due to postmortem delay (Table 1 and Figure 3).

It is debatable whether our findings relate better to the I$_1$ or I$_2$ subtype. To date, three studies from our laboratory...
Piletz et al (1990, 1996a, 1996b) have been consistent in revealing an elevation in the density of platelet I1 binding sites in depressed patients compared with healthy control subjects. Normalization (downregulation) of platelet I1 binding sites also has been documented 6 weeks after antidepressant treatment with either desipramine or fluoxetine (Piletz et al 1996a, 1991, 1996b). Rats injected with imipramine for 3 weeks also have been shown to downregulate brainstem I1 binding sites (Zhu et al 1997). Reports also exist that IRBP-immunodensities of platelet 33-kd bands (Zhu et al 1999) and 45-kd bands (Garcia-Sevilla et al 1996) are elevated in depressed patients. Various antidepressants can also downregulate the IRBP-immunoreactive bands in platelet membranes of depressed patients after antidepressant treatments (Garcia-Sevilla et al 1996; Zhu et al 1999). In one study, Garcia-Sevilla’s group reported (1996) a 45-kd band in frontal cortices from suicide victims was increased compared with sudden-death control subjects lacking a history of psychopathology. Thus, an abundance of evidence has accumulated that I1 sites, in conjunction with 33- and 45-kd IRBP-immunoreactive bands, are elevated in depressed patients.

Nonetheless, previous evidence also has suggested that I2 sites are changed in a reciprocal fashion with I1 sites in depression. Research findings support a lower density of I2 binding sites in depressed patients, with a normalization (up-regulation) following treatment with fluoxetine (Piletz et al 1994). Reciprocity with I1 sites was also observed in a 25-day treatment study of rats with imipramine, which led to up-regulation of I2 binding sites in the midbrain (Zhu et al 1996). Furthermore, 29/30-kd doublet bands associated with I2 sites were reported (Escriba et al 1994) to be downregulated in frontal cortices of suicide victims, whereas a 45-kd band related to I1 sites was increased in

![Figure 2. Imidazoline receptor–binding protein (IRBP) optical density (OD) values. IRBP OD values were normalized to β-actin from Western blots of hippocampi from subjects with major depressive disorder (MDD) at the time of death compared to matched control subjects (CTRL). Values represent integrated OD units from repeat analyses of IRBP bands (a.u., arbitrary units taken from the standard curve of platelet membranes on each blot) divided by the same parameters from each sample’s β-actin band. Bars represent group means derived from 17 pairs of matched MDD subjects and control subjects. The individual matches are shown by the dashed lines. (A) Densitometric analysis of the 50-kd band detected by IRBP antiserum for MDD and control subjects, p = .015 (untransformed data). (B) Densitometric analysis of the 45-kd band detected with IRBP antiserum for MDD and control subjects, p = .020 (untransformed data). (C) Densitometric analysis of the 40-kd band detected with IRBP antiserum for both MDD and control subjects, p = .033 (untransformed data). (D) Densitometric analysis of the combined 48- to 55-kd bands detected by IRBP antiserum for both MDD and control subjects, p = .010 (untransformed data). There was no significant difference in amount of β-actin loaded between MDD subjects and control subjects (p = .400).]
suicide victims relative to control subjects (Garcia-Sevilla et al 1996). None of these findings were directly related to MAO-B because MAO-B sites labeled by $[^3H]$-lazabemide were not altered in depressed suicide victims (Sastre and Garcia-Sevilla 1997). Thus, a number of previous studies have demonstrated reciprocal changes in densities of I$_1$ sites (higher) and of I$_2$ sites (lower) in depressed patients.

In our opinion, the results under discussion align closest with two previous studies of I$_2$ sites by Garcia-Sevilla et al (1996; Sastre and Garcia-Sevilla 1997). They reported 19% lowered IRBP-immunoreactivity for a 29/30-kd doublet in frontal cortices (Broadmann’s area 9) of suicide victims compared with sudden-death controls. $[^3H]$-idazoxan binding to I$_2$ sites in frontal cortices of suicide victims was also reported (Sastre and Garcia-Sevilla 1997) to be lowered (−40%) in suicide victims compared with sudden death control subjects. In human cortex, the 29/30-kd doublet is associated with I$_2$ sites labeled by $[^3H]$-idazoxan as a function of human aging (parallel changes; Garcia-Sevilla et al 1995). Furthermore, our results compare favorably with our earlier study (Piletz et al 1994) wherein lowered (−43%) I$_2$ radioligand binding sites on internal membranes of platelets were found in depressed patients; the platelet I$_2$ site also normalized (i.e., upregulated) following 6 weeks of treatment of depressed patients with fluoxetine (Piletz et al 1994). Thus, our present results seem in best agreement with previous studies of I$_2$ sites in depression (Garcia-Sevilla et al 1996; Piletz et al 1994; Sastre and Garcia-Sevilla 1997).

The 45-kd IRBP band may be of particular interest because Greney and coworkers (Greney et al 1997; Vonthron et al 1998) identified a 45-kd protein as a candidate for the I$_1$ receptor protein. Moreover, Garcia-Sevilla et al (1996) reported an elevation in the immunoreactivity (+51%) of a 45-kd IRBP band in frontal cortices of depressed suicide victims. Furthermore, platelet 33-kd (Zhu et al 1999) and 45-kd IRBP bands (Garcia-Sevilla et al 1996) have been reported to be higher (+40%) in depressed patients relative to healthy control subjects. In this light, the middle band in our hippocampal triad of IRBP bands (Figure 1) is identically sized as the 45-kd candidate I$_1$ protein found in frontal cortex (Garcia-Sevilla et al 1996). Thus, we cannot exclude that I$_1$ sites, rather than or in addition to I$_2$ sites, might be the subtype that is decreased in the hippocampi of depressed patients.

A variety of speculations might be considered to fully reconcile our findings with those of Garcia-Sevilla and colleagues (1996). Modern theories about psychiatric illnesses have invoked “neuronal circuitry” mechanisms, rather than unidirectional alterations in neurotransmitter concentrations or in receptor regulations in all brain regions. One relevant example comes from postmortem studies of schizophrenic brains, where completely opposite changes in dopaminergic neuronal density have been observed when comparing prefrontal cortices to hippocampi from the same schizophrenic subjects (Selemon and Goldman-Rakic 1999). Similar to the reciprocal change noted for neurons in different brain regions in schizophrenia, IRBP regulation might also be expressed reciprocally in prefrontal cortex versus hippocampus in depression. If this hypothesis proves true, then levels of the same IRBP (i.e., the 45-kd IRBP) could be increased in the prefrontal cortex (Garcia-Sevilla et al 1996) and decreased in the hippocampus of depressed patients (the study under discussion).

Our criterion for subject selection was more rigorous than that of Garcia-Sevilla and coworkers (1996). The psychiatric status of our subjects before death was evaluated via postmortem interviews with next of kin to establish firm retrospective diagnoses (Table 1). All depressed subjects in our study had experienced an active phase of MDD at the time of their deaths. By contrast, only 4 of 13 suicide victims in the study of Garcia-Sevilla et al (1996) had any historic evidence of MDD. Secondly, the average postmortem delay in our study was 17 ± 1 hour (identical for both groups of subjects); however, the postmortem delays in the study of Garcia-Sevilla et al (1996) were 26 ± 3 hours for suicide victims and 30 ± 4 hours for the control subjects. Blood, urine, and bile were also analyzed in our study at the time of autopsy to confirm the absence of antidepressants or antipsychotic compounds (Table 1). No biochemical screening of psychoactive substances was reported by Garcia-Sevilla et al.
(1996), except for ethanol. Ethanol was found in 8 of 13 suicide victims and 9 of 11 control subjects in the study of Garcia-Sevilla et al (1996). This high incidence of ethanol could have caused changes in IRBP in the study by Garcia-Sevilla et al. It should also be noted that neither of our studies were able distinguish whether changes in IRBP bands were due to the depressive illness itself or to committing suicide.

Our laboratory has reported (Ivanov et al 1998a) that two endogenous brain substances, agmatine and norepinephrine, are capable of up-regulating IRBP bands when these agents are applied to cultured cells. We also recently reported (Halaris et al 1999) an elevation in plasma agmatine concentration in depressed patients. Agmatine is a metabolite of arginine, formed in the brain by arginine decarboxylase (Reis and Regunathan 1998). Agmatine competes for radioligand binding to I-sites (Li et al 1994; Piletz et al 1995). In rats' hippocampi the concentration of agmatine was reported (Feng et al 1997) to average 2857 pmol/g wet weight, which is well in excess of its affinity constant (K\textsubscript{d} range of two studies = 33–127 nmol/L) at the high-affinity state of I\textsubscript{1} binding sites (Piletz et al 1995). Furthermore, following a 6-hour treatment with 10 μmol/L agmatine, IRBP immunodensity in a human cell line (MEG-01) was reported (Ivanov et al 1998a) to be upregulated on cell membranes (i.e., ligand-induced up-regulation). Conceivably, if hippocampal concentrations of agmatine were low in patients with MDD, this might provide a mechanism to downregulate IRBP immunoreactivity (Halaris et al 1999).

Endogenous norepinephrine (NE) has also been proposed as a regulator of I-sites in vivo (Piletz et al 1998). In healthy women of reproductive age, a positive correlation was found between steady-state plasma NE concentrations and platelet I\textsubscript{1} binding site densities (Piletz et al 1998). Moreover, in a study of tissue culture effects (Ivanov et al 1998a), IRBP-immunoreactivity was upregulated in response to treating cultured cells with 10 μmol/L NE for 6 hours. Thus, changes in brain NE, as well as agmatine, could underlie the observed downregulation of IRBP in postmortem hippocampi of depressed subjects.

Finally, the possible physiologic significance of a downregulation of imidazoline receptors in the hippocampus of depressed patients should be discussed. Beyond some role in the pharmacologic modulation of blood pressure via brainstem nuclei (Molderings 1997), nothing is certain about the function of imidazoline receptors in other brain regions. Although a subpopulation of MAO-B proteins possess I\textsubscript{2} binding sites (Tesson et al 1995), it is not known if other I-sites also have a relationship with enzymes that metabolize monoamines. Neuronal inhibition of NE release in pulmonary arteries is also ascribed to a peripheral subtype of imidazoline receptors, but this site may possess neither I\textsubscript{1} nor I\textsubscript{2} pharmacologic properties (Fuder and Schwarz 1993; Molderings and Gothert 1995). It is also believed that I\textsubscript{1} sites reside on neurons (Ernsberger et al 1995). A deficiency in, or dysregulation of, monoaminergic neurons in the central nervous system remains the prevailing hypothesis for the etiology of depression. Thus, tentative associations of I-sites with monoaminergic neurons might signify some role for imidazoline receptors in the pathophysiology or treatment of depression.

This project was supported in part by NIH Grant Nos. MH57601 (AH), MH46922 (GO), MH45488 (CS), and MH65183 (JP). We thank Dr. Herbert Meltzer and Dr. James Overholser for their expert assistance during the postmortem diagnoses at Case Western Reserve University, Cleveland, Ohio. We also appreciate Mr. Josh Farley and Dr. Violetta Klimek (University of Mississippi Medical Center) for help in sectioning tissues and with densitometric measurements.

References


Fudier H, Schwarz P (1993): Desensitization of inhibitory
prejunctional α2-adrenoceptors and putative imidazoline
receptors on rabbit heart sympathetic nerves. Naunyn
Schmiedebergs Arch Pharmacol 348:127–133.

Garcia-Sevilla JA, Zis AP, Hollingsworth PJ, Greden JF, Smith
Ordway GA, Widdowson PS, Streator-Smith K, Halaris A
918 J.E. Piletz et al
BIOL PSYCHIATRY
(1998): Alpha2-adrenoceptors and I1-imidazoline binding
sites: Relationship with catecholamines in women of repro-

Piletz JE, Chikkala DN, Ernsberger P (1995): Comparison of the
properties of agmatine and endogenous clonidine-displacing
substance at imidazoline and α2-adrenergic receptors. J Pharma-


I1-imidazoline binding sites are elevated in depression but not

lowers 3H-para-aminoclonidine binding in platelets of de-
pressed patients. Arch Gen Psychiatry 48:813–820.

3H-para-aminoclonidine binding to platelet purified plasma
membranes from depressed patients. Neuropsychopharma-

Piletz JE, Halaris AE, Chikkala D, Qu YS (1996b): Platelet I1
imidazoline binding sites are decreased by two dissimilar
antidepressant agents in depressed patients. J Psychiatr Res

Piletz JE, Ivanov TR, Sharp JD, Ernsberger P, Chang C-H,
differs from I1 and I2-imidazoline binding sites.
Arch Pharmaco Exp Ther 32:441–452.

Kelly B, Raphael B, Judd F, Perdices M, Kernutt G, Burrows

Li G, Regunathan S, Barrow CJ, Eshraghi J, Cooper R, Reis DJ
(1994): Agmatine: An endogenous clonidine-displacing sub-

Meana JJ, Barturen F, Garcia-Sevilla JA (1992): Alpha2-adreno-
ceptors in the brain of suicide victims: Increased receptor
density associated with major depression. Biol Psychiatry
31:471–490.

Molderings GH, Gothert M (1995): Inhibitory presynaptic imi-
dazoline receptors on sympathetic nerves in the rabbit aorta
differ from I1 and I2-imidazoline binding sites. Naunyn
Schmiedebergs Arch Pharmacol 351:507–516.

Molderings GJ (1997): Imidazoline receptors: Basic knowledge,
recent advances and future prospects for therapy. Drug

Ordway GA, Widdowson PS, Streator-Smith K, Halaris A
(1994): Agonist binding to α2-adrenoceptors is elevated in the
locus coeruleus from victims of suicide. J Neurochem 63:
617–624.

Piletz JE, Andrew M, Zhu H, Feng YZ, Rains J, Halaris A
(1998): Alpha2-adrenoceptors and I1-imidazoline binding
sites: Relationship with catecholamines in women of repro-

Piletz JE, Chikkala DN, Ernsberger P (1995): Comparison of the
properties of agmatine and endogenous clonidine-displacing
substance at imidazoline and α2-adrenergic receptors. J Pharma-


I1-imidazoline binding sites are elevated in depression but not

lowers 3H-para-aminoclonidine binding in platelets of de-
pressed patients. Arch Gen Psychiatry 48:813–820.

3H-para-aminoclonidine binding to platelet purified plasma
membranes from depressed patients. Neuropsychopharma-

Piletz JE, Halaris AE, Chikkala D, Qu YS (1996b): Platelet I1
imidazoline binding sites are decreased by two dissimilar
antidepressant agents in depressed patients. J Psychiatr Res

Piletz JE, Ivanov TR, Sharp JD, Ernsberger P, Chang C-H,
differs from I1 and I2-imidazoline binding sites.
Arch Pharmaco Exp Ther 32:441–452.

Kelly B, Raphael B, Judd F, Perdices M, Kernutt G, Burrows

Li G, Regunathan S, Barrow CJ, Eshraghi J, Cooper R, Reis DJ
(1994): Agmatine: An endogenous clonidine-displacing sub-

Meana JJ, Barturen F, Garcia-Sevilla JA (1992): Alpha2-adreno-
ceptors in the brain of suicide victims: Increased receptor
density associated with major depression. Biol Psychiatry
31:471–490.

Molderings GH, Gothert M (1995): Inhibitory presynaptic imi-
dazoline receptors on sympathetic nerves in the rabbit aorta
differ from I1 and I2-imidazoline binding sites. Naunyn
Schmiedebergs Arch Pharmacol 351:507–516.

Molderings GJ (1997): Imidazoline receptors: Basic knowledge,
recent advances and future prospects for therapy. Drug
imidazoline receptor. *Naunyn Schmiedebergs Arch Pharmacol* 358:R69.


