Proteomic Analysis of Embryonic Stem Cell–Derived Neural Cells Exposed to the Antidepressant Paroxetine

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Antidepressant drugs can have significant effects on the mood of a patient suffering from major depression or other disorders. The pharmacological actions of these drugs generally affect the uptake or metabolism of the neurotransmitters serotonin, noradrenalin, and, to a lesser extent, dopamine. However, many aspects of antidepressant action are not understood. We conducted a proteomic analysis in a neuronal cell culture model in an attempt to identify molecules important to the operation of pathways functionally relevant to antidepressant action. The model involved generating cultures containing mixed neural and glial cells by controlled differentiation of mouse embryonic stem cells, followed by exposure to 1 μM paroxetine for 14 days. After antidepressant exposure, we observed increased expression or modification of sepiapterin reductase (SPR), heat shock protein 9A, RAS and EF-hand domain containing, and protein disulfide isomerase associated 3 and decreased expression or modification of creatine kinase, actin, prohibitin, a T-cell receptor α chain, defensin-related cryptdin 5, and the intermediate filament proteins glial fibrillary acidic protein and vimentin. SPR, the most strongly up-regulated protein observed, controls production of tetrahydrobiopterin, an essential cofactor for the synthesis of many neurotransmitters including serotonin, making it a plausible and intriguing candidate protein for involvement in mood control and antidepressant drug action. SPR and the other proteins identified may represent links to molecular processes of importance to mood dysregulation and control, and their respective genes may be novel candidates for the study of antidepressant pharmacogenetics.

Key words: antidepressants; sepiapterin reductase; tetrahydrobiopterin; prohibitin; heat shock proteins; 2-D gel electrophoresis; intermediate filaments

Antidepressant drugs are widely used in the treatment of mood disorders and other conditions. None of the several effective antidepressants available work for all patients, and it is not possible to predict which patient will respond to which drug. It can take several weeks for an appropriate therapeutic response, if there is one, to become apparent; during this period a patient continues to suffer morbid feelings and has an increased risk of suicide. Therefore, choosing the right antidepressant for each patient is a significant problem in psychiatry (Wells et al., 1989). An additional problem is that 10%–20% of patients are treatment refractory—no effective antidepressant treatment is ever found (Benmansour et al., 1999; Spigset and Martensson, 1999).

Although the efficacy of antidepressants in the treatment of depression has been proven by many studies, their mechanism of action is not completely understood (Benmansour et al., 1999; Schafer, 1999; Harlan et al., 2006). The most enduring theory about the pathological basis of depression and the action of antidepressants is the monoamine hypothesis, which suggests that the brains of depressed patients have a deficit in monoamine transmission (Maes and Meltzer, 1995; Benmansour et al., 1999). However, the monoamine hypothesis does not explain the delay in the therapeutic effect of antidepressants, which occurs despite monoamine being elevated within hours (Schafer, 1999), or the lack of monoaminergic activity of several substances with antidepressant effects (Schwaninger et al., 1997).

A growing body of evidence has now extended the understanding of depression and antidepressant function beyond the monoaminergic synapse to include the concepts of neuronal plasticity and neurogenesis (Jacobs et al., 2000; Duman et al., 2001; Harlan et al., 2006). Reduced hippocampal volume and other subtle changes in brain structure have been observed in patients with...
depression, implicating loss of neurons as one cause or effect of the illness (Bremner et al., 1995; Gurvits et al., 1996; Sheline et al., 1996; Steffens et al., 2000; Duman et al., 2001). Some antidepressants appear to stimulate neurogenesis in the hippocampus and perhaps other regions of the brain (Benmansour et al., 1999; Malberg et al., 2000; Manev et al., 2001; Czeh and Lucassen, 2007); moreover, in rodent models the behavioral effects of antidepressants are not manifested if neurogenesis is inhibited (Santarelli et al., 2003). A related observation is that antidepressant treatment can prevent structural changes in the brain caused by chronic stress (Czeh and Lucassen, 2007). This links to the strong body of evidence supporting disturbance of the hypothalamic-pituitary-adrenal (HPA) axis as a key component of the pathophysiology of depression (File, 1990; Barden 1996; Sullivan et al., 1997; Stout et al., 2002) and the importance of normalizing these defects in order to achieve a clinical response to medication (Holsboer and Barden, 1996; Binder et al., 2004). Processes such as these require changes in neuronal receptor density, morphology, survival, and growth that are likely to occur over durations more compatible with the delayed therapeutic effects of antidepressant drugs (Benmansour et al., 1999; Sapolsky, 2000; Jacobs et al., 2000; Duman et al., 2001; Manev et al., 2001).

It is clear there is a lack of fundamental knowledge about the biochemical, cellular, and physiological determinants of mood and its dysfunction and that this is the stimulus for new experimental approaches to the biology of major depression and its treatment. Improved understanding of the molecular and cellular processes affected by exposure to antidepressant drugs may help to answer some questions about the actions of these drugs and perhaps will add to the body of knowledge about the biology of mood and mood disorders. We used a proteomic approach to examine the effects of paroxetine, the most potent and selective serotonin reuptake inhibitor (SSRI), in a model in vitro system composed of neural cells differentiated in a controlled fashion from a mouse embryonic stem (ES) cell line (Lee et al., 2000). We chose this differentiated state (stage 1) by inclusion in the medium of 1,400 U/mL leukemia inhibitory factor (LIF; Chemicon International Inc., Temecula, CA). Embryoid bodies were allowed to form by dissociating the ES cell colonies into a single-cell suspension at a density of $2.5 \times 10^3$ cells/cm$^2$ in the ES cell medium without LIF (stage 2). After 4 days, the embryoid bodies were plated on gelatin-coated plates in DMEM/F12 containing insulin, transferrin, selenium, and fibronectin (ITSFn) supplement, from GIBCO (stage 3). The medium was replenished every 2 days for 10–12 days, at which time the outgrowing neural progenitor cells were dissociated with 0.05% trypsin/EDTA and plated at a concentration of $2 \times 10^3$ cells/cm$^2$ on plates precoated with 15 mg/mL poly(l-ornithine) and 1 μg/mL laminin (Sigma, St. Louis, MO) in N2 medium (Johe et al., 1996) supplemented with 10 ng/mL basic fibroblast growth factor and 1 μg/mL laminin, all from GIBCO (stage 4). Neural progenitor cells were expanded for 6 days, at which time they were differentiated into neurons and other neural cells by replacing bFGF from stage 4 medium with 1 μM dibutyryl cAMP and 200 μM ascorbic acid (both from Sigma, St. Louis, MO). The cells were allowed to differentiate for 7 days before use, with replacement of the medium every second day (stage 5).

RNA Extraction and Semiquantitative Reverse-Transcriptase PCR

Total RNA was isolated at various stages of ES cell neural differentiation using TRIzol Reagent (Invitrogen, Carlsbad, CA). cDNA was generated using Superscript II First-Strand Synthesis enzyme (Invitrogen, Carlsbad, CA) and Oligo (dT)$_{12-18}$ primers, according to the enzyme supplier’s instructions. For each reverse-transcribed (RT) sample, a parallel “minus RT” reaction containing 2 μl of DEPC water instead of the reverse transcriptase enzyme was performed. After cDNA production, the RNA was digested with RNase H (Invitrogen, Carlsbad, CA), water was added to make a final volume of 100 μl, and the cDNA was stored at $-20^\circ$C. For control purposes, RNA was extracted from the liver and midhindbrain regions of 2-week-old C57Bl/6 mice, and cDNA was prepared as described above. Furthermore, the RT-PCR experiments used several replicates of the different culture stages and the replicates used in the proteomic experiments.

Before analyzing relative expression of mRNA at various stages of the ES cell–derived neural culture system, cDNA was normalized to the signal from beta-2 microglobulin (B2m) mRNA, as estimated on agarose gels (Fig. 1). The B2m signal was measured using Quantity One densitometry software (Bio-Rad Laboratories Inc., Hercules, CA), and cDNA volumes for subsequent reactions were adjusted accordingly. PCR was carried out with an appropriate volume of each cDNA, 1 μl of 10 × HotMaster Taq (1 U/μl; Eppendorf), and the supplier’s buffer in a final volume of 10 μl. Cycling parameters were 94°C for 15 sec, annealing between 53°C and 68°C for 30 sec (depending on the primer set), and elongation at 68°C for 45 sec. The number of cycles varied between 28 and 35. Primer sequences are available from the authors on request.

**MATERIALS AND METHODS**

**ES Cell–Derived Neural Culture**

The differentiation of ES cells into neural cells was carried out as previously described (Okabe et al., 1996; Lee et al., 2000) with minor modifications. R1 ES cells (Nagy et al., 1993) were maintained on gelatin–coated tissue culture plates in an ES cell medium (DMEM supplemented with 10% FCS, MEM nonessential amino acids, nucleosides, β-mercaptoethanol, 1-glutamine, and antibiotics, all from GIBCO, Auckland, New Zealand). Cells were maintained in an undifferentiated state (stage 1) by inclusion in the medium of 1,400 U/mL leukemia inhibitory factor (LIF; Chemicon International Inc., Temecula, CA). Embryoid bodies were allowed to form by dissociating the ES cell colonies into a single-cell suspension at a density of $2.5 \times 10^3$ cells/cm$^2$ in the ES cell medium without LIF (stage 2). After 4 days, the embryoid bodies were plated on gelatin-coated plates in DMEM/F12 containing insulin, transferrin, selenium, and fibronectin (ITSFn) supplement, from GIBCO (stage 3). The medium was replenished every 2 days for 10–12 days, at which time the outgrowing neural progenitor cells were dissociated with 0.05% trypsin/EDTA and plated at a concentration of $2 \times 10^3$ cells/cm$^2$ on plates precoated with 15 mg/mL poly(l-ornithine) and 1 μg/mL laminin (Sigma, St. Louis, MO) in N2 medium (Johe et al., 1996) supplemented with 10 ng/mL basic fibroblast growth factor and 1 μg/mL laminin, all from GIBCO (stage 4). Neural progenitor cells were expanded for 6 days, at which time they were differentiated into neurons and other neural cells by replacing bFGF from stage 4 medium with 1 μM dibutyryl cAMP and 200 μM ascorbic acid (both from Sigma, St. Louis, MO). The cells were allowed to differentiate for 7 days before use, with replacement of the medium every second day (stage 5).
Cell Dosing and Sample Preparation

Paroxetine hydrochloride hemihydrate, a gift from Glaxo Smith Kline (Brentford, UK), was dissolved in water as a 1 mM stock. After the stage 5 neural cells were allowed to differentiate for 7 days, the cultures were incubated in ES cell medium containing either 1 μM paroxetine hydrochloride or vehicle only for 14 days, with regular replacement of the medium. Cells were then rinsed with ice-cold PBS, harvested using a cell-scaper, recovered by centrifugation, and frozen at −80°C. For extraction of total protein, the pellet was dissolved in 0.5 mL of lysis buffer containing 8M urea, 2M thiourea (Sigma, St. Louis, MO), 4% 3-{(3-chloramidopropyl)dimethylammonio}-1-propane-sulfonate (CHAPS; Roche, Basel, Switzerland), 20 mM Tris, 130 mM DTT, 500 μL of Pharmalyte (pH 3–10; Pharma Biotech), and 125 mM Pefabloc (Roche). The samples were then purified using a PlusOne 2-D Clean-Up kit (Amersham Biosciences, Auckland, New Zealand) prior to 2-D gel electrophoresis. The protein was assayed using the nitric acid method (Bible et al., 1999), and concentrations were visually checked using 1-D gel electrophoresis.

2-D Gel Electrophoresis

Immobilized pH gradient gel strips (IPGs, 17 cm, pH 4–7; Bio-Rad Laboratories Inc., Hercules, CA) were each rehydrated overnight with 100 μg of protein sample suspended in 300 μL of rehydration buffer, which is the lysis buffer minus the Pefabloc. The strips were transferred to an isoelectric focusing (IEF) tray and focused using a Protean IEF system (Bio-Rad Laboratories Inc., Hercules, CA). The program used for IEF was 300 V, 1 hr; 1,000 V, 1 hr; 3,000 V, 1 hr; 6,000 V, 75,000 Vh, and then reduced to 500 V indefinitely. IPG strips were equilibrated for 10 min in 5M urea, 0.05M Tris–HCl (pH 6.8), 0.3M glycerol, 0.03M SDS, and 0.05M DTT. The strips were then reequilibrated for 10 min in this solution without the DTT but with 0.24M iodoacetamide (AppliChem GmbH, Darmstadt, Germany) and 0.1% (w/v) bromophenol blue. Strips were placed on a 12.5% SDS polyacrylamide gel with a 2-D SDS-PAGE Standard (Bio-Rad Laboratories Inc., Hercules, CA) and electrophoresed at 10 mA for 18 hr. The gels were fixed in 40% (v/v) ethanol and 0.1M glacial acetic acid for 30 min and then transferred to sensitizing solution [30% (v/v) ethanol, 0.125% (v/v) glutaraldehyde, 8 mM sodium thiiosulfate, 0.5M sodium acetate] for 30 min before being placed in silver solution (0.01M silver nitrate, 5 mM formaldehyde) for 20 min. Gels were developed with 0.24M sodium carbonate, 2.5 mM formaldehyde for 8–10 min, followed by the addition of 0.04M EDTA to

Fig. 1. Characterization by RT-PCR analysis of ES cell-derived neural cells. Agarose gel electrophoresis of semiquantitative reverse-transcribed PCR products for various genes in the ES cell-derived neural model. Stage 4 cells were not included as they were limited in number and critical for production of stage 5 neural cells. A mouse mid-hindbrain tissue sample provided a control for neural cell types, and a mouse liver sample provided a nonneural tissue control. In addition to the tissue controls, a negative PCR control (water only) was included.
stop the reaction, and then were washed with three changes of water.

### Image Analysis

The gels were visualized using the Quantity One Fluor-S system (Bio-Rad Laboratories Inc., Hercules, CA). PDQuest Basic 2-D Analysis Software (v. 7.1; Bio-Rad Laboratories Inc., Hercules, CA) was used for protein spot analysis. Protein spots were detected with a combination of PDQuest automated spot detection and manual interpretation using the guide of landmark proteins. Protein spot density was determined and normalized by calculating the relative spot density of the proteins compared to the total density of protein in the gel. Protein spots determined to have a statistically significant difference in intensity between drug-exposed and control cell samples were recovered from a preparative gel. Protein spots determined to have a statistically significant difference in intensity between drug-exposed and control cell samples were recovered from a preparative gel stained with Sypro Ruby (Bio-Rad Laboratories Inc., Hercules, CA). In brief, 100 µg of protein sample underwent 2-D electrophoresis, and spots of interest were identified by landmark peptides identified by PDQuest. Spots were excised using a sterile scalpel and sent to the Australian Proteome Analysis Facility (Sydney, Australia) for analysis. The proteins were subjected to tryptic digestion for 16 hr, followed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry using an Applied Biosystems 4700 Proteomics Analyzer with TOF/TOF optics in MS mode. After acquisition of spectra in the mass range of 800–3,500 Th, the instrument was switched to MS/MS (TOF/TOF) mode, where the eight strongest peptides could be subjected to collision-induced dissociation in order to generate MS/MS sequence data. The data were exported in a format suitable for submission to the database search program Mascot (Matrix Science Ltd., London, UK). Positive protein identification takes into account the percentage sequence coverage, the MS spectra, the MS/MS ion score, the number of missed cleavages, and how well the molecular weight (MW) and pI of the identified protein match.

### RESULTS

#### Validation of ES Cell–Derived Neural Culture System

Embryonic stem cells were differentiated into a mixed neural cell population essentially as described previously (Lee et al., 2000). Because this cell culture model has not previously been used for neuropharmacological studies, it was important to evaluate the mixed-cell culture produced by the differentiation procedure as applied in our laboratory. The differentiated cells derived from our R1 ES cell line were morphologically equivalent to those reported by Lee et al. (2000). Immunocytochemical analysis (data not shown) with antibodies for the neural progenitor cell marker nestin, the neuron-specific marker βIII-tubulin, the serotonergic marker serotonin, and the catecholaminergic marker tyrosine hydroxylase demonstrated the presence in the stage 5 cell cultures of mature catecholaminergic and serotonergic neurons, confirming the observations of Lee et al. (2000).

A more detailed molecular profile of cells in this mixed culture system was obtained using semiquantitative reverse-transcribed PCR (RT-PCR) of cells in the various stages of differentiation (Fig. 1). This analysis included genes that are markers for various developmental stages as well as specific types of neural cells, including serotoninergic, catecholaminergic, and glial cells, and comparison of the overall expression patterns with cDNA prepared from mid-hindbrain tissue of mice (positive control) and adult mouse liver (nonneural tissue control). One of the genes important in neuronal development and differentiation, Oct4 is involved in maintaining stem cell pluripotency (Niwa et al., 2000), and as would be expected, its expression decreased from stages 1 to 5. Another such gene is Shh, which is involved in early development (Tanabe and Jessell, 1996) including the serotonergic pathway (Pattyn et al., 2004); its expression was first observed at stage 2, with increasing expression observed at stages 3 and 5. Shh was also apparent in the mid-hindbrain control, presumably reflecting the development of neural stem cells in this tissue (D’Amour and Gage, 2003; Lennington et al., 2003). Nestin showed increased expression at stages 3 and 5, consistent with the immunocytochemistry results (data not shown), suggesting that neural progenitor cells were still quite abundant at stage 5 of the model, although nestin expression was not apparent in the mid-hindbrain control tissue.

Expression of genes essential for the development (Shh, Nes, Nkx2-2, Nur1, Ebf3) and function (Tph2, Th, Dbh, Slc6a2, Slc6a3, Slc6a4) of catecholaminergic and serotonergic neurons was observed in various stages leading to stage 5 cells (Fig. 1). In this study three genes important in serotonergic function were analyzed: Nkx2-2, a transcription factor involved in the development and maintenance of serotonergic neurons (Cheng et al., 2003); Slc6a4, which encodes a serotonin transporter (Gregor et al., 1993); and Tph2, which encodes a rate-limiting enzyme that synthesizes serotonin and is expressed in the mature adult brain stem (Walther et al., 2003). Slc6a4 was expressed in an early stage of differentiation in the model, which was unsurprising given its widespread expression in the body (Chang et al., 1996). Indeed, expression of serotonin in mouse ES cells has been reported (Walther et al., 2003). Nkx2-2 was expressed at stage 3, consistent with its role in the early development of serotonergic neurons. Tph2 was expressed only in stage 5 neurons and the mid-hindbrain control, reflecting its known expression in very specific brain regions containing mature serotonergic neurons, including the raphe nuclei of the hindbrain (Patel et al., 2004; Zhang et al., 2004).

Evidence of the presence of glial cells was also sought (Fig. 1), using RT-PCR for Gfap, a gene that encodes an intermediate filament protein exclusively expressed in astrocytes (Onteniente et al., 1983; Dahl and Bignami, 1985), and Mobp, an oligodendrocyte-specific gene involved in myelin formation (Montague et al., 1997). Both genes were expressed in stage 5 cells and mid-hindbrain samples. This suggests that popula-
tions of astrocytes are present during the differentiation process. We also observed the expression of Bdnf in stage 5 cells and mid-hindbrain tissue (Fig. 1). BDNF is expressed in astrocytes (Rudge et al., 1992) and is important in neuronal development and function in the brain (Hofer and Barde, 1988). The presence of BDNF in stage 5 cells is of interest because it has been implicated in antidepressant function (Duman, 1998).

Proteomic Analysis

2-D gel electrophoresis (2-DE) was used to compare and identify protein spots with different intensities in samples recovered from parallel stage 5 cultures exposed for 14 days to either paroxetine or vehicle only. An example of an ES cell–derived neural proteome is shown in Figure 2. A total of eight high-quality gels (four paroxetine-exposed samples and four control samples) were processed through the 2-DE procedure. Approximately 300 spots were resolved, of which 195 (65%) were matched to all gels. A statistical analysis of replicate groups was performed using the Student t test. At a significance of 98%, the intensity of 13 spots was determined to differ significantly between the paroxetine-treated and control samples (Table I, Fig. 3). All the significant spots identified were matched to all gels except for SSP6513 [glial fibrillary acidic protein (GFAP)], for which no or negligible intensity in the paroxetine-treated cells was observed. These spots were recovered from a preparative gel, 11 of which could be identified by their tryptic peptide fingerprints and by MALDI-TOF mass spectra (Table II).

Of the 11 proteins identified, increased expression or modification was observed for sepiapterin reductase (SPR), heat shock protein 9A (HSPA9A), RAS and EF-hand domain containing (RASEF), and protein disulfide isomerase associated 3 (PDIA3), and decreased expression or modification was observed for creatine kinase, actin, prohibitin, a T-cell receptor α chain, defensin-related cryptdin 5 (DEFCR5), and the intermediate filament proteins GFAP and vimentin. Theoretical mass and pI of each identified protein are given in Table II. The observed MW (Fig. 2) of 2 of the 11 identified proteins, SPR and DEFCR5, did not match the calculated MW. The observed MWs of DEFCR5 and SPR were about 40–50 and 70–80 kDa, respectively, but the theoretical MWs of DEFCR5 and SPR are about 11 and 28 kDa, respectively.

DISCUSSION

We employed a mixed neural cell culture model, derived by controlled differentiation of ES cells (Lee et al., 2000), for proteomic analysis of paroxetine effects. This cell culture approach provided an in vitro model system that contained a mixture of glial and neuronal cell types that resembled tissues of the midbrain and hindbrain without the overt complexity of the whole brain. Overall, the pattern of gene expression observed in our stage 5 cells (Fig. 1) was very similar to that seen in mid-hindbrain tissue, giving confidence that the ES cell culture system was generating a mixed population of cells with properties appropriate for exploring the molecular effects of antidepressant drugs.

We exposed the cell cultures to 1 μM paroxetine. This level is in the range of steady-state plasma levels observed in treated patients (Devane, 1999) and is close to the paroxetine concentration (2–14 μM) observed in the human brain (Henry et al., 2000). Drug exposure for 2 weeks was chosen as this balanced the time required for therapeutic effects in humans with the duration that the cell cultures remained healthy and viable. We identified 11 proteins that were significantly affected by exposure of the cell cultures to paroxetine for a 2-week period (Fig. 3,
Among these, perhaps the most intriguing finding was a more than threefold increase in the intensity of the spot representing sepiapterin reductase (SPR). SPR is a cytosolic homodimer that catalyses the final reduction of tetrahydrobiopterin (BH4) biosynthesis (Ota et al., 1995). BH4 is an essential cofactor for several enzymes including tyrosine hydroxylase and tryptophan hydroxylase, the rate-limiting enzyme in the biosynthesis of neurotransmitters, including catecholamines and indolamines (Nagatsu and Ichinose, 1999; Fig. 4). Mutations in the human SPR gene cause a severe neurotransmitter deficiency that produces convulsions and other symptoms, including depression (Bonafé et al., 2001; Steinberger et al., 2004). In addition, BH4 deficiency because of altered metabolism affects biosynthesis and the release of neurotransmitters, including catecholamines and indolamines (Nagatsu and Ichinose, 1999; Fig. 4). Mutations in the human SPR gene cause a severe neurotransmitter deficiency that produces convulsions and other symptoms, including depression (Bonafé et al., 2001; Steinberger et al., 2004). In addition, BH4 deficiency because of altered metabolism affects biosynthesis and the release of neurotransmitters, including catecholamines and indolamines (Nagatsu and Ichinose, 1999; Fig. 4).

A study of depressed patients treated with BH4 showed they had a marked improvement in mood (Curtius et al., 1983), although there has been little follow-up of this early study. Abou-Saleh et al. (1995) showed increased BH4 activity in depressed patients treated with either the tricyclic antidepressant amitriptyline or electroconvulsive therapy and changed plasma levels of BH4 in patients in a depressed state relative to that in the controls (Hashimoto et al., 1994, 2004). Additional evidence linking SPR to antidepressant function comes from recent studies in rodent models of depression. Miura et al. (2004) showed that the SSRI fluvoxamine regulates BH4 level in the mouse hippocampus and suggested that the behavioral changes seen in the rodents may have been a result of the action on BH4 level as well as the better-understood effects of the drug on serotonin turnover. Furthermore, BH4 synthesis is affected by several cytokines including IFN-gamma and TNF-alpha, which have been implicated in the pathophysiology of major depression (Schiepers et al., 2005). Together, this body of evidence supports a strong link between BH4 level and mood control, making our observation of increased spot density for SPR after exposure of our ES cell neural cultures to paroxetine particularly interesting.

![Fig. 3. PDQuest™ analysis of 2-D electrophoresis gels. The 2-D gels were analyzed using PDQuest™ Basic 2-D Analysis Software (BioRad Laboratories Inc., v. 7.1). Total protein was separated in the first dimension by isoelectric focusing with a 17-cm-long IPG strip (horizontal arrow) containing a nonlinear pH gradient of 4–7, followed by separation of proteins by molecular weight (vertical arrow) using a 12.5% SDS-PAGE gel. Protein detection was by silver staining. A reference image of the eight 2-D gels analyzed (paroxetine and control gels) generated by PDQuest™ is shown. The Student t test was used to determine significant differences between replicate groups (significance was set at 98%), and the resulting significant spots are indicated and numbered (SSP). Histograms represent spot intensity (average value between replicate gels) in the control (left bar) and paroxetine-treated (right bar) groups.](image-url)
| SSP No. | Protein name                                      | Swiss-Prot accession number | Peptide number | Sequence coverage (%) | Identification score | MS/MS (sequence data)                                                                 | Expression change by paroxetine | Theoretical MW/pI |
|--------|--------------------------------------------------|------------------------------|----------------|-----------------------|----------------------|----------------------------------------------------------------------------------------|----------------------------------|------------------|
| 4510   | Creatine kinase, brain form (CK-BB)              | Q04447                       | 15             | 43                    | 174                  | LRPFAEDEFDLSSHINHMAK; LGSFSEVELVQMVVDGVK + 13 additional peptides                      | 0.52 42.7/5.4                   |
| 7521   | Vimentin (VIM)                                   | P20152                       | 19             | 41                    | 204                  | LLQSVDIFSLADAINTEFK; EMEENFAEAANYQDTIGR + 16 additional fragments                   | 0.32 51.5/5.1                   |
| 7519   | Actin, homologous to α, β, and γ isoforms        | n.a.                         | 3              | 10                    | 32                   | AGFAGDDAPRAVFSIVGRPR; SYELPDQVIITIGNER                                               | 0.39 n.a.                       |
| 6513   | Gial fibrillary acidic protein (GFAP)            | Q925K3                       | 27             | 62                    | 324                  | VDFSGALINAGFK; EQLAQQQVHVEMDVA KPDLEAALR + 23 additional fragments                 | † 46.6/5.1                       |
| 3411   | Prohibitin (PHB1)                                | P67778                       | 7              | 31                    | 73                   | DLQNVNITLRR; SRNITYLPAGQSIVLQLPQ + 4 additional peptides                             | 0.47 29.8/5.6                   |
| 3306   | T-cell receptor α chain (TCRα)                   | P01849                       | 2              | 26                    | 32                   | VFSSTEINEGQGFTVLNNK; VFSSTEINEGQGFTVLNNK                                            | 0.43 15.5/4.6                   |
| 3702   | Heat shock protein 9A (HSPA9A)                   | P38647                       | 4              | 7                     | 34                   | TTPSSVAFTADGER; AQVEFGITD1IKR + 2 additional peptides                                | 2.14 73.5/5.9                   |
| 2701   | Sepiapterin reductase (SPR)                      | Q64105                       | 2              | 55                    | 33                   | LQKLNSEGEVLDCGTSQAQK; LSSLQR                                                       | 3.40 27.9/5.9 ^                |
| 2702   | RAS and EF hand domain containing (RASEF)        | Q5R175                       | 3              | 35                    | 35                   | MYFYLFSFLDGTGHR; DGSNVVEAVHLAR + 1 additional peptide                               | 2.90 70.8/5.9                   |
| 2616   | Protein disulfide isomerase associated 3 (PDIA3) | P27773                       | 25             | 49                    | 293                  | RFSCALLPGVALLLASAR; TFSHELDGSESTTGEVPVVAIR + 21 additional fragments               | 1.42 56.7/5.9                   |
| 1505   | Defensin-related cryptdin 5 (DEFCR5)             | P28312                       | 2              | 68                    | 28                   | LICYCRIR; VFGTCRNINLFFVVFCC                                                        | 0.53 10.5/5.7 ^                |

PDQuest™ significant spot number (SSP), protein name, and Swiss-Prot accession numbers are shown. Data determined and provided by APAF, including peptide number, sequence coverage, identification score, and MS/MS sequence data, are shown. Theoretical molecular weight (MW) and isoelectric point are shown.

n.a., Not applicable.

*Sequence coverage indicates how much of the entire protein sequence was covered by the matching peptide mass (not the sequence).

^Spot disappeared or was undetectable after paroxetine exposure.

^Observed MW did not match predicted MW.
Fig. 4. Tetrahydrobiopterin biosynthesis. Tetrahydrobiopterin (BH4) synthetic pathway (GTPCH, GTP-cyclohydrolase, I; PTPS, 6-pyruvoyl-tetrahydropterin synthase; SPR, sepiapterin reductase; TH, tryptophan hydroxylase; NOS, nitric oxide synthase; 5-OH-Trp, 5-hydroxytryptophan; AADC, aromatic-amino acid decarboxylase; DBH, dopamine β-hydroxylase).

Comparison of the observed versus the expected MW. In any event, the discrepancy appears too great to be accounted for by common posttranslational modifications. Two further possibilities for the discrepancy are the phenomenon of domain swapping (Liu and Eisenberg, 2002) or incomplete protection of the proteins from oxidation during the equilibration step after isoelectric focusing (Hedberg et al., 2004). Similar findings have been reported in other studies (Carboni et al., 2006; Cecconi et al., 2007). These observations may suggest that drugs that act on mood have a common effect on chaperone proteins.

We observed apparently decreased expression of actin with paroxetine exposure, which may reflect neuroadaptation (Revenu et al., 2004; Tojima and Ito, 2004). Similarly, Carboni et al. (2006) illustrated altered expression of actin in the hippocampi and frontal cortices of rats chronically exposed to several antidepressants. Other proteins observed to change in our study were: RASEF, a member of the RAS superfamily of GAP-binding proteins (Scheele et al., 2000); CK-B, a key protein in the regulation of brain ATP levels (Bessman and Carpenter, 1985); and prohibitin 1, a protein that regulates cell-cycle progression (Ikonen et al., 1995; Nijtmans et al., 2002). It is conceivable that each of these proteins may function in pathways that mediate antidepressant response.

Only four other reports have explored the effects of antidepressant drugs on the proteome of the brain or neuronal cells. One study examined proteomic changes induced by fluoxetine and a substance P antagonist in the guinea pig cerebral cortex (Guest et al., 2004). Observed changes included multiple heat shock protein 60 isomers, neurofilaments, and related proteins that are important in synaptic structure and function. In addition, this group determined that the drugs caused an increased number of split postsynaptic densities, a structural feature indicative of synaptic rearrangement (Guest et al., 2004). Another study looked at proteomic changes in the rat...
hippocampus resulting from chronic exposure to fluoxetine or venlafaxine (Khwaja et al., 2004). Proteins involved in neurogenesis, outgrowth and maintenance of neuronal processes, vesicular trafficking, synaptic plasticity, and a range of other functions were identified. Most recently, proteomic changes were studied in cultured rat primary cortical neurons exposed for 3 days to fluoxetine (Cecconi et al., 2007) and rat hippocampi and frontal cortex chronically exposed to several antidepressants, including fluoxetine (Carboni et al., 2006). Together these studies represent initial explorations of the proteomic changes induced by antidepressant drugs and demonstrate the feasibility of detecting effects that may be informative about antidepressant action.

Across the antidepressant proteomic studies to date, including the study described here, there have been some commonalities in the proteins or functional pathways identified, particularly several molecular chaperones (Guest et al., 2004; Khawaja et al., 2004; Carboni et al., 2006; Cecconi et al., 2007). However, there were also many differences in the proteins identified by the various studies. This variability is likely in part a result of the diversity of the model systems employed, with different species, brain regions, or cultured cells analyzed. The type of antidepressant studied and the duration of drug exposure also differed, which also may account for some of the observed variation. Furthermore, the limited sampling of the proteome that each study achieved may contribute to the observed variation. Furthermore, the limited sampling of the proteome that each study achieved may have influenced the outcomes. This reinforces the need for further, more comprehensive studies in this area to clarify the picture.

In conclusion, we studied the effects of chronic paroxetine exposure on protein expression in an ES cell–derived neural cell culture system. Several interesting proteins affected by the drug exposure were identified. In particular, SPR, an enzyme essential for the synthesis of BH4, appeared to be up-regulated threefold and hippocampi and frontal cortex chronically exposed to several antidepressants, including fluoxetine (Carboni et al., 2006). Together these studies represent initial explorations of the proteomic changes induced by antidepressant drugs and demonstrate the feasibility of detecting effects that may be informative about antidepressant action.

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