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Proteomic analysis of rat cortical neurons after fluoxetine treatment

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ABSTRACT

The known neurochemical effect of most currently available antidepressants is the enhancement of the synaptic levels of monoamine neurotransmitters. However, the existence of other mechanisms has been suggested to justify the significant delay between the modulation of the monoaminergic system and the clinical effects. In order to investigate the effects of the antidepressant fluoxetine (a prototypical serotonin selective re-uptake inhibitor) and to improve the understanding of its mechanism of action, we performed a proteomic investigation in rat primary cortical neurons exposed sub-chronically to this antidepressant. Cortical neurons were treated for 3 days with 1 μM fluoxetine or vehicle. Protein extracts were processed for 2D gel characterization. Image analysis allowed the identification of six proteins differently expressed by more than 100% and seven proteins differently expressed by more than 50% ($P<0.05$). Nine proteins were identified by mass spectrometry. Among them, cyclophilin A, 14-3-3 protein z/δ and GRP78 are involved in neuroprotection, in serotonin biosynthesis and in axonal transport, respectively. This study showed that the primary culture of cortical neurons is a suitable system for studying the effects of fluoxetine action and may contribute to improve the understanding of fluoxetine psychotherapeutic action and the mechanisms mediating the long-term effects of this antidepressant treatment.

1. Introduction

Depression is a complex, heterogeneous disorder and several neurotransmitter and neurohormonal pathways have been implicated in its pathophysiology (Vaidya and Duman, 2001; Nestler et al., 2002). The discovery of efficacious antidepressant treatments provided the first evidence of an inherent biochemical abnormality underlying the disorder. Since both preclinical and clinical studies have clearly implicated the serotonin (5-HT) and norepinephrine neurotransmitter systems in the mechanism of action of antidepressants, a monoaminergic hypothesis of the pathophysiology of depression

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Abbreviations: SSRI, serotonin selective re-uptake inhibitors; 5-HT, serotonin; DIV, day in vitro; SR domains, serine-rich domains; GR, glucocorticoid receptor; CypA, cyclophilin A; GRP, Glucose-regulated protein; ER, endoplasmic reticulum; ASF/SF2, alternative splicing factor/splicing factor 2; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline

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was put forward (Maes and Meltzer, 1995). However, the monoaminergic hypothesis did not provide an adequate explanation of the lag period in the therapeutic actions of antidepressants. Moreover, observations that monoamine depletion does not produce depressive symptoms in healthy individuals and that rapid elevation in monoamines is not correlated with quick antidepressant action have led to the need of revising the framework of existing theories (Schildkraut, 1965; Heninger et al., 1996). An emerging hypothesis suggests that the efficacy of antidepressants can be related to the ability of modulating the plasticity of neuronal pathways (Duman et al., 1997, 1999). Depression may arise when neuronal systems do not exhibit appropriate, adaptive plasticity in response to external stimuli such as stress, and antidepressant treatments may exert their therapeutic effects by either reversing this dysfunction or by independently stimulating an adaptive neuronal plasticity within the system.

Serotonin selective re-uptake inhibitors (SSRIs), and fluoxetine among them, are usually the first line of therapy in major depression, although the mechanisms by which they exert their therapeutic effects are poorly understood (Vaswani et al., 2003; Taylor et al., 2005). Beyond the treatment of mood disorders, fluoxetine has expanded considerably its therapeutic potential which encompasses antidepressive (Lader, 1988), anti-obsessive (Ellingrod, 1998), analgesic (Egbunike and Chaffee, 1990), anticataplectic (Frey and Darbonne, 1994), antiphobic (Abene and Hamilton, 1998), antidysphoric (Steiner et al., 1997), anorectic (Goldstein et al., 1995) and antibulimic (Kornreich et al., 1998), antiepileptic (Favale et al., 1995), anti-alcohol-craving (Lejoyeux, 1996) and cocaine-craving (Batki et al., 1996) effects; fluoxetine has proven to be beneficial also for patients suffering from autism (DeLong et al., 1998) or recovering from stroke (Dam et al., 1996). In preclinical pharmacology, fluoxetine is regarded as an SSRI because it blocks 5-HT but not catecholamine re-uptake and is devoid of significant affinity for a variety of neuroreceptors including serotonergic 5-$HT_{1A\text{-}2}$, β-adrenergic$_{1–2}$, dopaminergic D_2 and histaminergic H_1 (Fuller et al., 1991). However, abnormalities in the 5-HT neurotransmitter systems have been convincingly associated with only some of the various disease states that benefit from fluoxetine treatment. Furthermore, in order to produce therapeutic activities, fluoxetine has to be administered for weeks, which suggests the intervention of common adaptive changes.

In order to explore the effects of sub-chronic administration of the fluoxetine and to improve the understanding of the mechanism of action of this antidepressant drug, we have carried out comparative proteomic analysis of rat cortical neuron primary cultures treated with the SSRI fluoxetine or vehicle. We have used 2D polyacrylamide gel electrophoresis (PAGE) comparative proteomics analysis and identified differentially expressed proteins by MALDI-TOF mass spectrometry. We show that this antidepressant alters the expression of marker proteins involved in axonal transport, synaptic vesicle assembly and in neuroprotection, and these may contribute to the mechanisms mediating long-term effects of this antidepressant drug.

2. Results

2.1. *Immunocytochemistry*

Cultured cells were stained with an antibody recognizing a neuron-specific β-Tubulin isoform to stain neurons and with an antibody against the glial fibrillary acidic protein to mark glial cells. The neuron/astrocyte ratio, as evaluated by microscopy manual count on immunostained coverslips, showed that the two preparations contained a low percentage of astrocytes (around 20%, Table 1), although cells plated in Petri dishes may not exhibit the same ratio due to higher cell density. Furthermore, the two preparations contained comparable percentages of neurons (Table 1), thus suggesting that they were similar and thus suitable for comparative analysis by 2D PAGE.

2.2. *Differential proteomics and statistical analysis*

Four replicate maps were prepared from vehicle and fluoxetine-treated samples for each individual preparation of cortical neurons, thus obtaining 16 gels in total. About 400 polypeptide spots could be revealed in the pH 3–10 interval with this high sensitivity stain. After matching the replicated maps of the cortical neurons treated with fluoxetine and vehicle, six proteins turned out to be differently expressed by more than 100% (with $P<0.05$) while seven proteins were differently expressed by more than 50% (with $P<0.05$) after treatment with this antidepressant drug (Table 2). In Fig. 1, a 2D map is shown, reporting the modified proteins.

In Fig. 2 an example of PDQuest (version 7.3) output is reported, showing two representative differentially expressed spots ($P<0.05$) between control and fluoxetine-treated cortical neurons. After fluoxetine treatment, spot no. 3308, displayed in Fig. 2A, showed a 6-fold down-regulation, while spot no. 2812, represented in Fig. 2B, was 2-fold up-regulated. For each spot, an enlarged region of the respective 2-DE map is shown,

Table 1 – Percentage of neurons and astrocytes in the two preparations measured by immunocytochemistry

	CTRL			Fluoxetine		
	Neurons	Astrocytes	Total	Neurons	Astrocytes	Total
First preparation						
Sum	1066	295	1361	890	254	1144
Percentage	78.32%	21.68%		77.80%	22.20%	
Second preparation						
Sum	847	207	1054	907	251	1158
Percentage	80.36%	19.64%		78.32%	21.68%	

Table 2 – Protein level changes in fluoxetine-treated cells

SSP	Fold changes	P-value	Group count gels in V/gels in F
2105	0.4	0.018	8/8
2812	1.95	0.015	8/8
3003	1.75	0.024	7/8
3006	1.54	0.030	8/8
3008	1.95	0.009	8/8
3308	0.16	0.001	8/8
3310	1.85	0.009	6/7
3406	0.69	0.025	8/8
4405	1.65	0.012	8/8
6706	0.7	0.004	8/8
7205	0.65	0.013	8/8
7401	0.39	0.028	8/8
8106	0.45	0.005	8/8

Protein identification numbers assigned by the image analysis software are reported in the first column. Fold changes in fluoxetine-treated group as compared to vehicle treatment are shown in column 2. Student's *t* test *P*-values are displayed in column 3. The total number of maps in which the spot was detected is shown in column 4 as maps in vehicle group/maps in fluoxetine group.

which contains the referred spot (highlighted by an arrow) and the corresponding spot volume.

To assess overall differences in cortical neurons 2-DE pattern between the two different groups, we applied a hierarchical cluster analysis. Hierarchical clustering is an unsupervised method which divides the gels into groups based on their expression patterns, without using any information about the group memberships of gels. Unsupervised classification is generally recommended for finding unknown groups in the data set. In the resulting tree diagram (dendrogram), Fig. 3, the different samples tend to form two clusters, represented by control and fluoxetine-treated, on the basis of similarity of the protein patterns within each group ($P<0.05$, ANOVA).

All the differently expressed spots were cut, eluted and identified by peptide fingerprinting mass spectrometry. The identification, together with experimental and theoretically predicted *p*I and M_r values and the computed scores for mass spectrometry analysis are reported in Table 3.

2.3. *Western blot*

Western blots were carried out to validate protein expression patterns from two-dimensional gels by using antibodies specific to the candidate proteins. Anti-β actin antibody was used to normalize the optical density values.

The analysis was performed on three cortical neurons preparations (fluoxetine- and vehicle-treated samples) and immunoblots were replicated twice. Eight proteins out of nine were selected for Western blot analysis (Table 4, no commercial antibody was available for protein RBM3).

Western blot results are shown in Fig. 4, including the relative protein expression in both samples normalized to β-actin signal intensity as an internal control. Trends of changes in the same direction as those detected in the 2D gel analyses were detected for most proteins. The quantitative difference between the results obtained by 2D electrophoresis and by Western blot suggested that most changes detected by the former technique specifically involve post-translationally modified forms, which can be only separated in 2D maps (Fig. 5).

3. Discussion

In the present study, we evaluated the change in the proteomic complement of primary cultures of cortical neurons after fluoxetine treatment. The aim of the present approach is to identify proteins regulated by this antidepressant to better understand its mechanism of action at the molecular level.

Primary cultures of neurons are widely used as an in vitro model for the investigation of antidepressant mechanism of action. For example, by using primary cultures of rat hypothalamic, amygdaloid and hippocampal neurons, it was demonstrated that antidepressants, irrespective of their selective inhibitory actions on the re-uptake of 5-HT or of norepinephrine, modify glucocorticoid receptor (GR) messenger RNA concentrations (Pepin et al., 1989; Okugawa et al., 1999). This effect was also confirmed at protein levels (Lai et al., 2003) confirming the utility of in vitro neuronal culture systems to address the mechanism underlying neurotransmitter and antidepressant-induced short-term regulation of hippocampal GR expression.

In this study, using the differential proteomic approach, we compared fluoxetine-treated versus non-treated primary cultures of rat cortical neurons and showed alterations in the intensity of 13 protein spots. Most were proteins that were not known previously to be involved in antidepressant-mediated pathways.

For example, we have shown a down-regulation of peptidyl prolyl *cis–trans* isomerase A after fluoxetine treatment of cortical neurons. Peptidyl prolyl *cis–trans* isomerase A, also

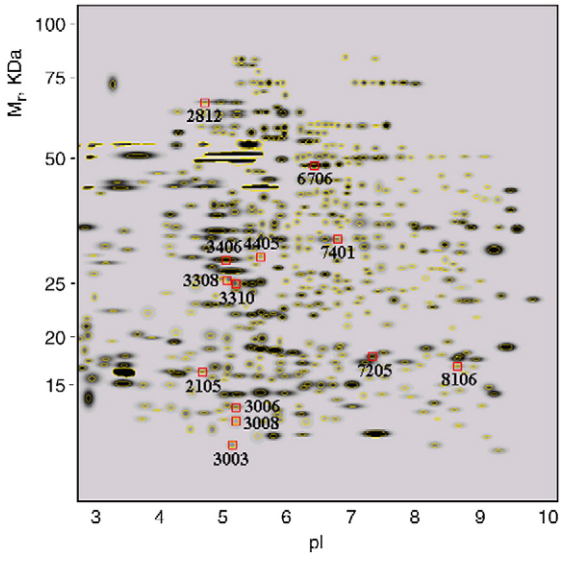

Fig. 1 – Example of 2D gel of proteins extracted from a rat cortical neuron primary culture. Proteins displaying different levels in fluoxetine-treated neurons are shown in the map.

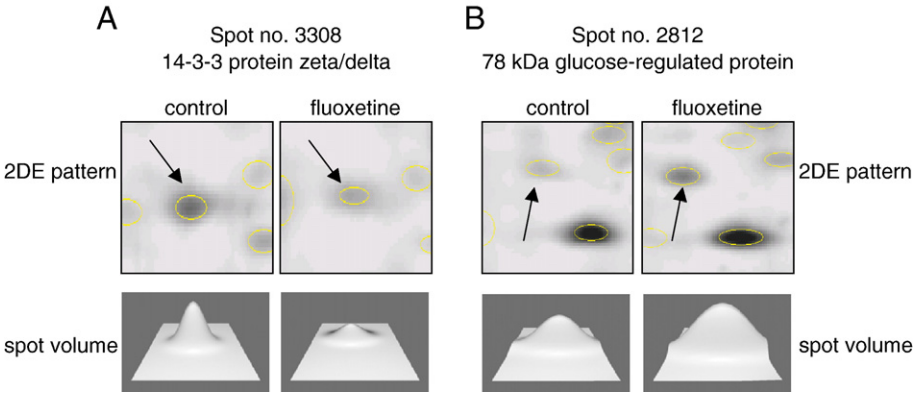

Fig. 2 – PDQuest (version 7.3) output showing two representative differentially expressed spots ($P<0.05$) between control and fluoxetine-treated cortical neurons. (A) Spot no. 3308, 14-3-3 protein zeta/delta, expressed in lower levels in fluoxetine-treated sample ($n=8$) than in control ($n=8$) cells. (B) Spot no. 2812, 78 kDa glucose-related protein, more abundantly expressed in fluoxetine-treated cortical neurons ($n=8$) than in controls ($n=8$). For each spot an enlarged region of the respective 2-D map is shown which contains the referred spot (highlighted by an arrow) and the corresponding spot volume. Each spot was identified by MALDI-TOF MS analysis.

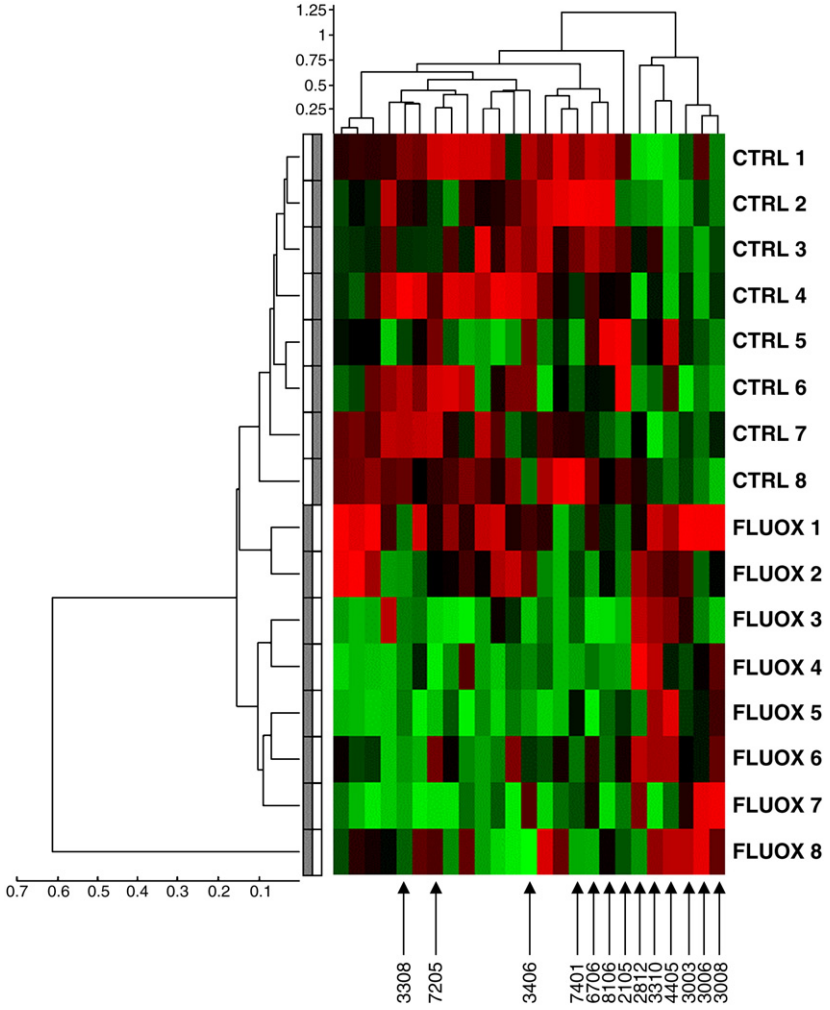

Fig. 3 – Dendrogram obtained by hierarchical cluster analysis demonstrating a real division in two groups. Samples formed two clusters (control and fluoxetine treated) with a single cluster observed by treated samples from 3 to 8. Spots for hierarchical clustering analysis were selected by ANOVA ($P<0.05$). Each column is a single protein expression across each sample. (Green) down-regulated, (red) up-regulated. Arrows indicate the number of modulated spots.

Table 3 – Summary of the 9 identified proteins from rat primary cortical neurons 2D gels

Protein name	Spot number	Hugo Gene name	Theoretical M_r [Da]	Theoretical *p*I	MOWSE score	MASCOT score	Z-score	SwissProt accession number	Coverage [%]	No. of peptides
14-3-3 protein zeta/delta	3308	YWHAZ	27771	4.8	7.23 E6	88	2	P35215	40	12
14-3-3 protein epsilon	3406	YWHAE	29174	4.7	1.77 E10	150	2.34	P42655	62	18
78 kDa glucose-regulated protein precursor	2812	GRP78	72347	5.1	1.23 E12	123	2.31	P06761	33	20
Alpha enolase	6706	ENO1	46985	6.5	3.88 E11	155	2.34	P04764	47	18
Histone H2B	2105	H2BFG	13775	10.6	5.15 E5	164	2.35	P02278	67	10
Peptidyl prolyl *cis–trans* isomerase A	8106	PPIA	17743	8.6	4.16 E7	84	2.34	P10111	64	10
Putative RNA-binding protein 3	7205	RBM3	16605	7.6	4.37 E4	73	2.29	O89086	37	6
Splicing factor, arginine/serine-rich	7401	SFRS1	27613	10.5	5.07 E5	68	2.11	Q07955	34	9
Ubiquitin carboxyl-terminal hydrolase	3310	UCHL1	24782	5.2	2.74 E6	69	1.94	Q00981	42	9

Matching peptides vs. total number of peptides submitted to database search, sequence coverage and scores obtained by peptide fingerprinting matching are listed. Protein accession number, theoretical *p*I and M_r and protein functions were obtained by the SWISS-PROT and NCBI databases. MASCOT, MOWSE and Z-scores (output of the identification softwares Mascot, ProteinProbe and ProFound, respectively) are measures of the statistical significance of the identification hits.

called cyclophilin A (CypA), is a soluble cytoplasmic immunophilin that acts as a peptidyl prolyl isomerase. It binds the immunosuppressive drug cyclosporine and the resulting complex blocks T cell function by inhibiting the calcium-dependent phosphatase calcineurin (Colgan et al., 2004). Although CypA has a pivotal role in the immune response, it is highly concentrated in brain, where its functions are largely unknown (Nahreini et al., 2001). It was classically considered a housekeeping gene, although recently a brain plasticity-related expression has been discovered in the central nervous system (Arckens et al., 2003). Furthermore, CypA and other immunophilins participate in axonal transport and synaptic vesicle assembly and they may also play a role in neuroprotection against abnormal protein aggregation (Avramut and Achim, 2003).

A down-regulation of 14-3-3 protein zeta/delta was detected in cortical neurons after fluoxetine treatment. 14-3-3 protein zeta/delta is a brain-specific protein, which plays important roles in a wide range of vital regulatory processes, including signal transduction, apoptosis, cell cycle progression and DNA replication (Qi et al., 2005). It is an activator of tyrosine and tryptophan hydroxylases, key enzymes for the biosynthesis of dopamine and 5-HT (Muratake et al., 1995), thus playing a role in the regulation of 5-HT and catecholamine biosynthesis in neurons and in other monoamine-synthesizing cells (Isobe et al., 1989). Moreover, it was demonstrated that 14-3-3 interacts with other systems, such as the neuronal cytoskeleton, that is involved in neuronal plasticity events, which are modulated by antidepressants (D'Sa and Duman, 2002). It was also shown that the interaction of 14-3-3 zeta/delta protein with Rim1 (a component of the presynaptic active zone and a modulator of exocytosis) may be important in the dynamic function of central nervous system neurons (Sun et al., 2003). Recently an up-regulation of 14-3-3 zeta isotype at mRNA and protein level has been described in RBL-2H3 cells upon 48 and 78 h incubation with fluoxetine (Baik et al., 2005), suggesting that this protein is involved in the mechanism of action of fluoxetine. The direction of the observed change is in contrast with our findings; however, it should be taken into account that the antibodies used for Western blot detection by Baik et al. (2005) are not completely specific to the zeta isoform, and that in our 2D gel approach the down-regulation of 14-3-3 zeta isoform could be due to a stimulation or induction of a different phosphorylated state of the protein that migrates at a different isoelectric point. In addition, even though RBL-2H3 cells have been shown to have an active 5-HT system, they are not of neuronal origin and might be affected in a different manner by drug treatment.

The alternative splicing factor/splicing factor 2 (ASF/SF2) is also down-regulated after fluoxetine treatment of cortical neuron primary cultures. ASF/SF2 is a member of the family of SR proteins, so called because of their C-terminal arginine-

Table 4 – The different primary antibodies used for Western blot analysis, with the corresponding dilutions

Primary antibody	Dilution
Goat anti-UCH-L1 (Chemicon)	1:300
Rabbit anti-14-3-3 ζ (Santa Cruz)	1:3000
Goat anti-SF2/ASF (Santa Cruz)	1:200
Rabbit anti-CypA (Upstate)	1:1000
Rabbit anti-GRP78 (Santa Cruz)	1:1000
Rabbit anti-14-3-3 ε (Santa Cruz)	1:500
Rabbit anti-H2B (Santa Cruz)	1:300
Rabbit anti-enolase (Santa Cruz)	1:1000

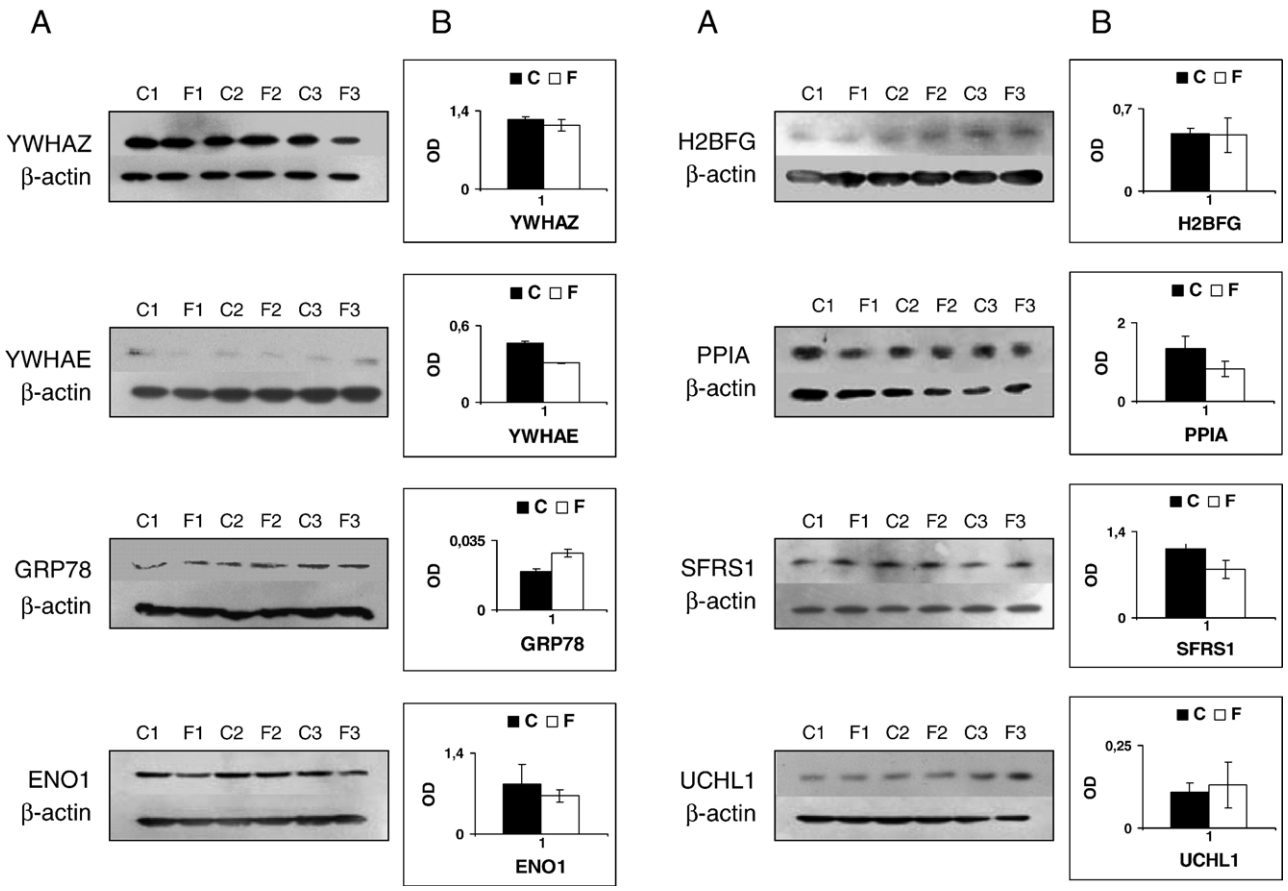

Fig. 4 – Western blot analyses for eight proteins showing different levels in fluoxetine- or vehicle-treated cultured neurons in proteomic analysis. (A) film images (B) relative protein expression normalized to β-actin signal intensity as an internal control. Western blot images were captured by GS710 densitometer (BioRad) and analyzed by QuantityOne software to calculate the band intensities (OD).

and serine-rich domains (SR domains). SR proteins are critical components of the spliceosome, influencing constitutive and alternative splicing of pre-mRNA (Aubol et al., 2003) and cell viability. It has recently been shown that loss of splicing factor ASF/SF2 induces G2 cell cycle arrest and apoptosis (Li et al., 2005). Pre-mRNA processing is an important mechanism for globally modifying cellular protein composition and can be regulated in a highly cell- and tissue-specific or developmentally specific manner. In neurons, the functions of many gene products, such as those of Trk genes, are regulated by alternative splicing (Shinozaki et al., 1999). The Trk family of receptor tyrosine kinases is crucial for neuronal survival in the vertebrate nervous system (Beck et al., 2004) and includes one of the receptors for brain-derived neurotrophic factor, which is suggested to be necessary for the antidepressant-induced behavioral effects (Castren, 2004).

A protein whose expression was up-regulated after fluoxetine treatment is glucose-regulated protein (GRP) 78 or BiP. It is a member of the heat shock protein 70 family and also an endoplasmic reticulum (ER) lumen protein, whose expression is induced during ER stress. GRP78 is involved in polypeptide translocation across the ER membrane and acts as an

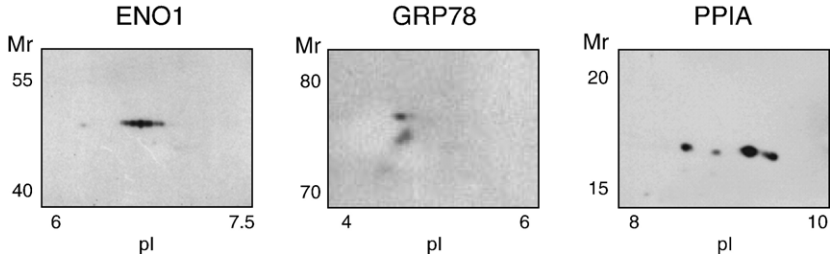

Fig. 5 – 2D maps after immunostaining with specific antibodies, showing that the same protein is recognized in several spots as a result of post-translational modifications.

apoptotic regulator by protecting the host cell against ER stress-induced cell death (Rao et al., 2002). It was shown that exposure to valproate, a mood stabilizing agent, increased the expression of ER stress protein GRP78 in rat cerebral cortical cells (Kim et al., 2005; Wang et al., 2003). Regulation of ER stress proteins by valproate may prove to be an important mechanism of action of the drug and the neuroprotective role of GRP78 may also be involved in the pathophysiology of bipolar disorder (Bown et al., 2002). Moreover, it has been reported that lithium, another mood stabilizing agent, was also able to increase GRP78 expression in primary cultured rat cerebral cortical cells (Shao et al., 2006). Interestingly, significantly increased levels of GRP78 were found in temporal cortex of subjects with major depression who died by suicide compared to those who did not (Bown et al., 2000). The induction was interpreted as part of compensatory mechanisms induced in brain to contrast pathophysiological changes (Bown et al., 2000). In our investigation, we could not replicate in cortical neurons the previously reported changes for the GR detected at protein level in hippocampal preparations (Lai et al., 2003). This can be ascribed to the inherent different sensitivity of 2D gel proteomics to detect the GR as well as to the modest fold-change occurred, as reported by previous studies. However, is it tempting to speculate that some of the changes detected at the level of protein chaperones, such as the hsp70 family (i.e. GRP78) and cytoplasmic immunophilin, may be reflecting a different processing of the GR. Indeed GR has been shown to be a cytosolic protein able to form a complex with GPR78 Hutchison et al., 1996), and the regulation of its steroid binding activity and translocation by heat shock proteins and immunophilins is well documented (Caamano et al., 2001; Pratt et al., 2004). Interestingly, GRP78 has been also shown to be a molecular chaperone able to assist the functional expression of the 5-HT transporter, at least in recombinant systems (Tate et al., 1999).

Available experimental data cannot support or disprove a specific mechanism of action mediating these protein changes. Synapses are developed in cultured cortical neurons (Lin et al., 2002); 5-HT transporters are expressed in rat cortex since E15 through E20 and binding sites can be detected at E20 (Ivgy-May et al., 1994); on the other hand, antidepressant efficacy may rely on different molecular targets (Pariante et al., 2003; Lai et al., 2003).

An immunoblot analysis carried out on independent samples to validate the findings obtained by 2D electrophoresis was able to confirm a trend towards the observed changes, as shown in Fig. 4. The results however did not reach statistically significance, suggesting that immunoblot analysis carried out on proteins separated by molecular mass might be sub-optimal for the validation of proteomics results, for a series of possible reasons. First, isoforms of the same protein when subjected to different post-translational modifications that only slightly modify their molecular mass can nevertheless be separated on 2D maps due to their different *p*I values (Fig. 5). The separation is frequently lost in monodimensional SDS–PAGE. Thus, all the modification in the relative amount of differently modified proteins, such as phosphorylated versus unphosphorylated forms, will be detected only in 2D gels, or at least diluted in the total amount of protein. The existence of multiple protein expression forms is common in brain proteins (Yang et al., 2005) and was already reported for proteins analyzed in this study (Bonenfant et al., 2006; Aubol et al., 2003; Berg et al., 2003). As an example, histone H2B, detected in this study as one of the modulated proteins, showed an experimental *p*I value much lower than the predicted basic value, in line with other studies (Campostrini et al., 2005; Taurin et al., 2002; Matarazzo and Ronnett, 2004). These results suggest that the modulated proteins were actually post-translational modifications, such as acetylated or ubiquitinated forms (Matarazzo and Ronnett, 2004; Jason et al., 2002; Bonenfant et al., 2006). Since recently a role for histone acetylation levels was demonstrated in the antidepressant activity (Tsankova et al., 2006), it is tempting to speculate that a balance among the eight H2B predicted acetylated forms is relevant in fluoxetine mechanism of action.

Furthermore, the Western blot analysis (even if performed with replicate samples and replicated experiments) is subjected to high technical variability, which further decrease the sensitivity. Other techniques should thus be considered in the future to obtain confirmation of proteomic results.

In conclusion, in this study a comparison between primary cultures of cortical neurons treated with vehicle or fluoxetine was made. Both preparations were found to be similar in morphology and percentage of neuronal cells. This indicates that the primary culture of cortical neurons is a suitable system for studying the direct effects of fluoxetine on neuronal systems. Our findings suggest that the antidepressant fluoxetine alters the expression of proteins involved in neuroprotection, in 5-HT biosynthesis, in axonal transport and in chaperone machinery, that may contribute to improve the understanding of the fluoxetine psychotherapeutic action and the mechanisms mediating the long-term effects of this antidepressant drug. Future studies aimed at comparing other antidepressants with different primary activities on brain neurochemistry in the same experimental paradigm will show whether the protein changes detected in this study are specific for fluoxetine or are shared by all efficacious agents.

4. Experimental procedures

4.1. *Rat cortical neuron primary culture*

Rat embryonic cortical neurons were obtained from 18 day old rat embryos (Sprague-–Dawley, Charles River, Calco, Italy) following Xie et al. (2000) with modifications. All procedures were carried out in accordance with the Italian law (art. 7, Legislative Decree no. 116, 27 January 1992), which acknowledges the European Directive 86/609/EEC, and were fully compliant with GlaxoSmithKline policy on the care and use of laboratory animals and related codes of practice. Cerebral cortices were dissected under a stereomicroscope and kept in ice-cold Hank's balanced salt solution without calcium and magnesium (Gibco, Invitrogen, Carlsbad, CA, USA). A single cell suspension of cortical neurons was obtained by both enzymatic and mechanical dissociation of cortices. Briefly, cortices were incubated for 10 min at 37 °C in a solution containing 0.1% trypsin (Gibco) in Hank's balanced salt solution, then cells were dissociated by trituration through the narrowed bore of a fire-polished Pasteur pipette. Cortical

neurons were plated in 140 cm^2 poly-D-lysine/laminin-coated Petri dishes at a density of 85,000 cells/cm^2 (for proteomic studies) or in poly-D-lysine/laminin-treated 19 mm^2 diameter glass coverslips at a density of 25,000 cells/cm^2 (for immunocytochemistry analysis). Poly-D-lysine/laminin were from Sigma-Aldrich, St. Louis, MO, USA. Neuronal cultures were maintained in a humidified atmosphere at 5% CO_2 in neurobasal medium + B27 supplement + penicillin/streptomycin (all from Gibco) and the medium changed no more then once a week (50% liquid replacement).

4.2. *Pharmacological treatment and sample preparation*

Cortical neurons at day in vitro (DIV) 7 were treated for 3 days with 1 μM fluoxetine (Sigma-Aldrich, dissolved in water) or vehicle. Fluoxetine dose was selected after pharmacokinetic studies revealing that fluoxetine was detectable at micromolar concentrations in rat brain (data not shown) after administering 5 mg/kg, the lowest efficacious dose in behavioral models (Muscat et al., 1992). To extract proteins for proteomic analysis or immunoblots, neurons plated in Petri dishes were scraped at DIV-10 and centrifuged (at 800×*g* for 4 min at 4 °C). Pellets were resuspended in 1 mL Tris-buffered saline (TBS; Tris was from Sigma-Aldrich), added with a protease inhibitor cocktail (Complete Mini, Roche, Basel, Switzerland); after pulse-sonicating twice for 10 s on ice, they were stored at −80 °C. Coverslips were processed for immunocytochemistry.

4.3. *Immunocytochemistry*

Cortical neurons at DIV-10 were fixed in 4% paraformaldehyde (Fluka), 4% sucrose (Sigma-Aldrich) phosphate-buffered saline and processed for immunocytochemistry. Briefly, cells were washed in TBS, permeabilized in 0.25% Triton X-100 (Sigma-Aldrich) TBS, then incubated overnight at 4 °C with primary antibodies diluted in 0.1% bovine-serum albumin (Sigma-Aldrich) TBS. Mouse monoclonal anti-neuron-specific β-tubulin antibody (diluted 1:2000) was from Covance (Princeton, NJ, USA) and rabbit polyclonal anti-glial fibrillary acidic protein antibody (1:4000) was obtained from Chemicon (Temecula, CA, USA). Coverslips were then washed in TBS, incubated 2 h at room temperature with anti-mouse biotinylated secondary antibody (Amersham Biosciences, Uppsala, Sweden, 1:2000) and developed with the *Elite*ABC Kit (Horseradish Peroxidase Development Kit; Vector Laboratories, Burlingame, CA, USA), following manufacturer's instructions, with DAB-Nickel Enhanced (Vector Laboratories) as chromogenic substrate. The procedure was then repeated with anti-rabbit biotinylated secondary antibodies (Amersham Biosciences, 1:2000), using VectorNovaRed (Vector Laboratories) as chromogenic substrate. The differential staining allows distinguishing black-stained neurons from red-stained glial cells. The ratio neurons/astrocytes was evaluated by microscopy manual count on immunostained coverslips.

4.4. *Two-dimensional gel electrophoresis*

The total protein quantity of a trial samples was quantified by modified Lowry methods (DC Protein assay, Bio-Rad): 2 mg/mL of total proteins could be extracted from 20×10^6 cortical neurons. Each sample to be loaded on 2D gels was extracted from 12×10^6 vehicle- or fluoxetine-treated neuronal cells (containing 1.2 mg/mL total protein) by adding 0.45 mL of a 2D solubilizing solution, containing 7 M urea (Sigma-Aldrich), 2 M thiourea (Fluka, Buchs, Switzerland), 20 mM Tris (Sigma-Aldrich), 5 mM tributylphosphine (Fluka), 3 % (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate-CHAPS (Fluka), 1 % Pharmalytes pH 3.5–10 (Amersham Biosciences, Uppsala, Sweden) and Complete™ protease inhibitor (Roche). Samples were then pulse-sonicated five times for 30 s on ice and centrifuged (10000×*g* for 10 min at 4 °C) for removing nucleic acids. Extracts were incubated 90 min at room temperature with 5 mM tributylphosphine for reducing protein disulphide bonds. Cysteine thiolic groups were then alkylated by adding 20 mM iodoacetamide (Sigma-Aldrich) and incubating for 90 min at room temperature.

The protein extracts were used to rehydrate 17 cm long immobilized pH gradient strips (pH 3–10, Bio-Rad Laboratories, Hercules, CA, USA) for 8 h at room temperature. Isoelectric focusing was carried out at 20 °C for 60,000 Vh using a Protean IEF Cell (Bio-Rad) with a low initial voltage and then by applying a voltage gradient up to 10,000 V, with a limiting current of 50 μA/strip. The immobilized pH gradient strips were equilibrated for 27 min in a solution containing 375 mM Tris–HCl (pH 8.8), 6 M urea, 20% glycerol (Sigma-Aldrich) and 2% sodium dodecyl sulfate (SDS, Fluka). The strips were then sealed with 0.5% agarose (Sigma-Aldrich) in running buffer [192 mM glycine (Sigma-Aldrich), 15 mM Tris, 0.1% SDS, pH 8.3] at the top of 8–18% acrylamide (Bio-Rad) gels. The second dimension electrophoresis was performed in a Protean II XL tank (Bio-Rad) at 10 °C and 2 mA/gel for 2 h, 5 mA/gel for 1 h and 10 mA/gel overnight. The gels were stained overnight with the Sypro Ruby dye (Bio-Rad) and acquired with the VersaDoc system (Bio-Rad). Maps were analyzed by using the PDQuest image analysis software (Bio-Rad). Statistical analysis was carried out with the statistics tools included in the PDQuest software, using Student's *t* test with a cut-off value of $P<0.05$. Values from significantly changed spots were analyzed again with the GraphPad Prism software.

4.5. *Statistical analysis*

The data set generated from 2-DE analysis was also analyzed by hierarchical cluster analysis using the Ludesi 2-DE Interpreter software package available at www.ludesi.com. The quantitative data derived from PDQuest were exported to the Ludesi 2-DE Interpreter. The clustering procedure includes the generation of a distance matrix, which in our case was calculated by the Pearson correlation distance on the proteins with lowest *P*-value selected from an ANOVA (analysis of variance) procedure, summarizing all the pairwise similarities between expression profiles and generating a dendrogram (hierarchical tree). The clustering was based on a significant number of proteins which appear coexpressed within the different groups (controls or fluoxetine treated).

4.6. *MALDI-TOF peptide fingerprinting mass spectrometry*

Each selected spot was carefully cut (Proteome Works spot cutter, Bio-Rad) and destained with 2×10 min washing steps

in 50% acetonitrile (Sigma-Aldrich) (v:v), 50% 5 mM Tris pH 8.5 followed by a third wash with 5 mM Tris pH 8.5 for 10 min. The gel discs were dried in a Speedvac sc110A device (Thermo Savant, NY, USA) for 1 h at room temperature and then covered with 15 μL of Sequencing Grade Modified Trypsin (Promega, Promega Corporation, Madison, WI, USA) (0.02 mg/mL) in NH_4HCO_3 buffer (40 mM, pH 8.5) and left at 37 °C overnight. The gel pieces were then crushed and peptides were extracted twice in 50 μL of 50% acetonitrile, 50% H_2O with 1% formic acid (v:v, Fluka) and a third time with 50 μL of acetonitrile. The extractions were conducted in an ultrasonic bath for 15 min. The three extraction solutions were mixed and evaporated to dryness in the Speedvac device and the residues were dissolved in 10 μL of H_2O with 0.1% of trifluoroacetic acid. For an additional purification, the samples were cleaned by using ZIP-TIP C18 (Millipore Bedford, MA, USA). Two microliters of the resulting solution were mixed with an equivalent volume of matrix solution, prepared fresh every day by dissolving 10 mg/mL α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich) in acetonitrile–ethanol (1:1, v:v). One microliter of the resulting mixture was loaded onto the MALDI sample plate and allowed to dry. Measurements were performed using a TofSpec 2E MALDI-TOF instrument (Micromass, Manchester, UK), operated in reflector mode, with an accelerating voltage of 20 kV.

Two different analysis tools were used for protein identification by peptide masses in order to have always a double confirmation of the results. Mascot (Matrix Science, UK) and Profound (Version 4.10.5), that incorporates a probability-based scoring, was used to search SwissProt, trEMBL and NCBI non redundant databases. As a third confirmation, ProteinProbe (Version 3.4, BioLynx, Micromass, UK) was used for protein identification (see scores in Table 3). The identifications were carried out following a method already described (Piubelli et al., 2005).

4.7. *Semi-quantitative Western blot analysis*

Protein extracts were diluted 1:1 with Laemmli's sample buffer (62.5 mM Tris–HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue), boiled for 3 min and separated by SDS/PAGE on 13% T acrylamide gels in Tris/glycine/SDS buffer. Proteins were then electroblotted onto polyvinylidene fluoride membranes (Bio-Rad) at 400 mA for 3 h at 4 °C. Non-specific sites were blocked by incubating with 5% non-fat dried milk and 0.05% Tween-20 (Sigma-Aldrich) in Tris-buffered saline overnight at 4 °C. Membranes were incubated with the different primary antibodies at the appropriate dilutions (see Table 4), in 1% non-fat dried milk, 0.05% Tween-20 TBS for 3 h at room temperature. Blots were then incubated 45 min at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody: ECL anti-goat IgG horseradish peroxidase-linked (Sigma-Aldrich) at 1:20.000 dilution (only for the immunodetection of ASF/SF2 and ubiquitin carboxyl-terminal hydrolase UCHL1 proteins) and ECL Anti-rabbit IgG horseradish peroxidase-linked species-specific whole antibody (Amersham Biosciences) at 1:2000 dilution, for all the other analyzed proteins.

The immunocomplexes were detected by chemiluminescence (ECL, Amersham Biosciences) on X-ray X-Omat AR (Kodak, Rochester, NY, USA) films. Membranes were immunoblotted again with a monoclonal anti-β actin antibody (Sigma-Aldrich, 1:5000) for normalization purposes. The intensity of the chemiluminescence response was measured by scanning films and processing the image using Quantity One software Version 4.4 (Bio-Rad).

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