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Abstract

Neurobiological underpinnings of treatment-resistant depression, a debilitating condition associated with significant functional impairment, have not been elucidated. Consequently, the aim of this study was to use animal models of response and resistance to antidepressant treatment, in an attempt to identify differences in associated transcriptional responses. Flinders Sensitive Line rats were subjected to maternal separation (MS) and chronically treated with Escitalopram or Nortriptyline. Antidepressants reduced immobility time in the forced swim test in non-MS rats, while lack of antidepressant behavioural response was observed in MS animals. We developed a novel bioinformatic algorithm that enabled identification of transcriptional signatures in hippocampus and pre-frontal cortex that discriminate vehicle- and antidepressant-treated subjects in both MS and non-MS rats. Functional annotation analysis showed that in antidepressant-responder rats the most enriched pathways included IQGAPs activation, toll-like receptor trafficking, energy metabolism, and regulation of endopeptidase activity. The analysis of interacting proteins implicated synaptic vesicles and neurotransmitter release, ubiquitin regulation, cytoskeleton organisation and carbohydrate metabolism. In contrast, in treatment-resistant MS rats, main expression changes were revealed in ribosomal proteins, inflammatory responses, transcriptional/epigenetic regulation, and small GTPases. Susceptibility signature shared Rtn1, Zdhhc5, Igsf6, and Sim1 genes with the latest depression GWAS meta-analysis, while antidepressant resistance signature shared Ctnnd1, Rbms3, Atp1a3, and Pla2r1 genes. In conclusion, this study demonstrated that distinct transcriptional signatures are associated with behavioural response or non-response to antidepressant treatment. The identification of genes involved in antidepressant response will increase the comprehension of the neurobiological underpinnings of treatment-resistant depression, thus contributing to identification of novel therapeutic targets.

Keywords: major depressive disorder; gene expression; escitalopram; nortriptyline; forced swim test; bioinformatics

1. Introduction

Major depressive disorder (MDD) is a severe, chronic and debilitating disorder affecting more than 160 million people worldwide (Global Burden of Disease Collaborative Network. Global Burden of Disease 2017 results. http://ghdx.healthdata.org (Ferrari et al., 2013; Rehm and Shield, 2019) with unfavourable prognosis of full recovery (Verduijn et al., 2017). The disease is a heavy burden on society both as suffering of patients and their families and as direct and indirect costs (König et al., 2019).

Several different therapeutic approaches have been developed for the treatment of MDD, with pharmacotherapy being the most common first-line treatment (Bauer et al., 2013; Davidson, 2010; Park and Zarate, 2019). Available antidepressants are grouped within several classes characterised by different mechanisms of action and are generally considered efficacious and effective to treat acute episodes of MDD (Cipriani et al., 2018; Harmer et al., 2017). Nevertheless, a subset of patients ranging between one to two thirds fail to respond adequately to first-line treatment (Cuffel et al., 2003; Rush et al., 2006). Among those subjects non-responding to the first antidepressant, about 30% fail to obtain adequate benefit after switching to a second and a third treatment steps (Rush et al., 2006). Moreover, patients classified as responders due to improvement of baseline symptoms often do not achieve full remission and maintain residual symptoms (Trivedi et al., 2008). The debilitating condition referred to as treatment resistant depression (TRD) is commonly recognised, although there is no universally accepted definition (Trevino et al., 2014). TRD is associated with worse quality of life scores, lower work productivity, greater functional impairment and higher risk to commit suicide (DiBernardo et al., 2018; Johnston et al., 2019). The neurobiological underpinnings of TRD have not been elucidated (Trivedi et al., 2008), and there is an unmet need to a better understanding of the underlying molecular mechanisms in order to facilitate development of new therapeutic options.

Animal models with face, predictive and construct validity have provided invaluable tools to investigate the neurobiological basis of psychiatric disorders, including TRD (Akil et al., 2018). The Flinders Sensitive Line (FSL) rats resemble depressed patients with regards to dysregulation of sleep parameters, antidepressant treatment response, psychomotor retardation, increased passive behaviour following stress, and immune abnormalities (Overstreet et al., 2005). Moreover, neurobiological impairments similar to those found in humans were demonstrated in FSL rats, including abnormal serotonin system function, increased response to cholinergic agonists, and

reduced NPY expression (Overstreet et al., 2005; Overstreet and Wegener, 2013). Since chronic stress is a major precipitating cause of MDD in predisposed individuals (Kendler et al., 1999; McEwen et al., 2015), with early-life adversities playing a significant role (Nelson et al., 2017; Nusslock and Miller, 2016), the exposure to maternal separation (MS) is considered a suitable model to reproduce the gene-environment interactions involved in the disease (Newport et al., 2002).

The objective of this study was to establish a behavioural model of non-response to antidepressant treatment using FSL rats, in order to investigate differences in the transcriptional profile of responder and non-responder animals. To achieve this, we availed of an original bioinformatic algorithm developed by us that allowed the identification of transcriptional signatures to discriminate controls and antidepressant-treated subjects. We discovered that treatment responders and non-responders are associated with distinct transcriptional signatures in the hippocampus and prefrontal cortex.

2. Experimental procedures

Experimental design

FSL pups were split into two groups: animals from one group were maternally separated (MS), whereas controls remained in the home cage (n-MS). When adult, both MS and n-MS rats were further split into three treatment groups, receiving Escitalopram (ES) Nortriptyline (NT), or vehicle, respectively. Following chronic treatment, immobility time was evaluated in the forced swim test. Rats were sacrificed and transcriptomic analysis was performed in two brain regions: hippocampus (Hip) and prefrontal cortex (PFCx). Transcriptional signatures were identified by comparing gene expression of both brain areas in antidepressant-treated with vehicle-treated rats within the MS and n-MS groups (Figure 1).

Animals

FSL rats were from colonies maintained at the Karolinska Institutet. Rats were housed in standard cages at constant room temperature (22°C) and relative humidity (45–55%) under a regular 12-h light/dark schedule (lights on at 7:00 am). Rat chow and tap water were freely available. The Stockholm Ethical Committee for Protection of Animals approved the study and all procedures were conducted in accordance to the Karolinska Institutet's Guidelines for the Care and Use of Laboratory Animals, which follow the European Communities Council Directive of 24 November 1986.

Maternal separation

Maternal separation (MS) procedure was performed as previously reported (Piubelli et al., 2011b, 2011a). Briefly, pups assigned to the MS group were separated from the dam as a litter for 180 min, beginning at 9:00 am, from post-natal day (PND) 2 to PND 14. Control rats (n-MS) were left undisturbed, except for the routine cleaning of the cages, in their home cages. Pups were weaned on PND 23, separated by sex and housed in groups of 3–5 rats per cage. Only males were included in the study since this project was part of a larger study aimed at comparing antidepressant response in several animal MDD models and the available data in literature, which are mainly pertaining to male animals, were the limiting factor.

Antidepressant treatment

On PND 41–46 rats were split into groups receiving Escitalopram (ES) or Nortriptyline (NT) admixed to food pellets (0.34 g/kg chow for 3 weeks, 0.41 g/kg chow during the rest of the experiment) or vehicle, consisting in rat chow, for 1 month. The antidepressant dose of approximately 25 mg/kg body weight/d was estimated based on the average food intake (~22 g/d), calculated by dividing the total mean daily consumption of pellets per cage by the number of animals per cage, in agreement with previous results (Mallei et al., 2011; Piubelli et al., 2011c, 2011a).

Forced swim test

The test was performed on 171 rats split into four sessions (n=8-18/group) following previously published procedures (Blaveri et al., 2010; Piubelli et al., 2011c, 2011b). The behavioural procedure consisted of two exposures to a water tank that does not permit escape (20 cm in diameter, 40 cm in height, containing 30 cm of fresh water at 25˚C). Fresh water was used for each rat. During the first exposure, rats were placed into the tank, left there for 15 minutes and dried before they returned to their home cages. The second exposure occurred 24h afterwards and lasted 5 minutes, during which behaviour was videotaped and subsequently scored by a trained experimenter blind to the experimental group. The rat was judged to be immobile when it floated passively, making only small movements to keep its nose above the water surface. Immobility time, expressed as duration (s), was analysed using a 2-way ANOVA approach, with Stress (MS and n-MS) and Treatment (vehicle, ES, NT) as the factors of interest. An additional blocking factor Session was also included in the model to account for any day-to-day variability, as data were collected in different sessions using a complete block design (Bate and Clark, 2014). The 2-way ANOVA analysis was

followed by Planned Comparisons of the predicted means to compare the mean of the antidepressant-treated group to the mean of the vehicle-treated group within the MS or n-MS conditions.

Gene expression

A subset of rats (n=76) were sacrificed by decapitation and Hip and PFCx were rapidly dissected, treated with RNAlater (Invitrogen, Thermoscientific) and stored at –80˚C. Total RNA was isolated by homogenization in TRIzol Reagent (Invitrogen, Thermoscientific) and purified using the RNeasy MinElute cleanup Kit (Invitrogen, Thermoscientific). RNA was quantified using spectrophotometric analysis and quality assessed using the Agilent 2100 bioanalyzer (Agilent Technologies). The standard Affymetrix One-Cycle Eukaryotic Target Labelling Assay protocol was used to generate cRNA probes that were subsequently hybridized to Affymetrix Rat Genome 230 2.0 GeneChips (http://media.affymetrix.com /support/technical/datasheets/rat230_2_datasheet .pdf), following manufacturer's guidelines (Affymetrix, Santa Clara, CA) (Blaveri et al, 2010).

Bioinformatics

Transcriptional signatures were identified by means of an enhanced version of the rank-based, normalization-free classification method we have recently published in (Lauria, 2013; Lauria et al., 2015). The extended algorithm has been already successfully applied in other works (Caberlotto et al., 2016; Carboni et al., 2018; Parolo et al., 2018). Briefly, after a preliminary probeset selection phase based on the Wilcoxon test, the classification method ranks the filtered probesets by expression level separately for each sample and then it produces a set of subject-specific signatures, where each signature is the list of the first n1 and the last n2 probesets in the ranking (n1 and n2 have the same value for all subjects and they are parameters of the method). An all-to-all signature comparison is then carried out using a distance metric based on a weighted enrichment score, resulting in a distance matrix that systematically quantifies the degree of similarity between the subjects. Subjects are then classified by the algorithm into the two groups (vehicle or antidepressant treatment), by assigning each sample to the group of subjects whose elements have the lowest averaged distance from the sample. Finally, a treatment-specific transcriptional signature is extracted, which collects all the probesets included in at least one subject-specific signature. The analysis was designed with the objective of identifying signature genes associated with behavioural antidepressant response or non-response irrespective of the antidepressant used, thus the data of both antidepressants were merged. A p-value is computed for each probeset using a permutation

test in which the group labels are randomly shuffled. In the enhanced version used here, the original classification method has been extended with a genetic optimizer that automatically selects the method parameters (signature length and feature selection stringency) to maximize the accuracy of subject classification.

The employed classification method produces a set of subject-specific signatures as an intermediate step, where each signature is the list of the n1 most expressed and the n2 least expressed probesets in the subject. To assist the biological interpretation, a heatmap has been added to each transcriptional signature provided in Supplementary Tables 1-4. The heatmaps represent the popularity of each gene in the individual signatures by relying on an index ranging from -1 to 1. Positive values indicate that the majority of the samples belonging to a class has the corresponding gene in the upper part of their individual signature (first n1 genes). Conversely, negative values indicate that the majority of the samples belonging to a class has the gene in the lower part of their individual signature (last n2 genes). Values close to zero highlight a sort of disagreement in between the sample signatures. This can be due to: (i) the individual signatures do not contain the gene; (ii) the individual signatures contain the gene, but in the same class there are some samples that have the gene in the upper part of their signature, while others have the same gene in the lower part.

The gene lists obtained from the transcriptional signatures were used to extract the most representative GO Biological Process terms and Pathways using David (Huang et al., 2009). Protein functional interactions were examined using STRING (Szklarczyk et al., 2019). The comparison with human major depression transcriptional datasets was performed by using data publicly available in GEO. Two human transcriptomic studies were analysed (GSE12654 and GSE98793). The first one referred to a post-mortem study of the PFCx (Brodmann area 10) of depressed subjects (Iwamoto et al., 2004).The second one is a gene expression analysis in whole blood samples obtained from donors diagnosed with MDD (Leday et al., 2018). These specific datasets were selected for the high quality of the data provided. GSE12654 included data from a brain region of relevance for major depression, prefrontal cortex (Brodmann Area 10), donated by the Stanley Foundation Brain Collection which included n=15 subjects per group. Diagnoses had been made according to the Diagnostic and Statistical Manual of Mental Disorders. The other dataset included two case-control studies of depression: the GlaxoSmithKline–High-Throughput Disease-specific target Identification Program (GSK–HiTDiP) study and the Janssen–Brain Resource Company (Janssen–BRC) study and were selected for the high number of subjects per group (128 patients with MDD). A summary of

the demographic information of subjects used were also provided for both studies. Additional comparisons were drawn with a treatment-resistance gene expression study in humans (Pettai et al., 2016) and with available GWAS data (Howard et al., 2019).

3. Results

Depressive-like behaviour

The behaviour of FSL rats that received chronic treatment with the pro-serotonergic (ES) or pro-noradrenergic (NT) antidepressant was compared to vehicle-treated animals in the forced swim test (Figure 1). Within each treatment group, half of the rats experienced MS, whereas the other half were left undisturbed (n-MS groups). Two-way ANOVA analysis detected a significant effect of treatment [$F(2,162)=7.10$, $p=0.0011$], as well as a significant stress-treatment interaction [$F(2,162)=4.05$, $p=0.019$]. *Post-hoc* analyses showed that chronic treatment with either antidepressant significantly reduced immobility time in n-MS rats (ES $p=0.0007$; NT $p=0.0017$, Figure 2). However, exposure to MS completely blocked the antidepressant effect, since chronic treatment with either ES or NT did not reduce immobility time compared to vehicle-treatment in MS animals (Figure 2). Therefore, the n-MS groups can be considered as antidepressant-responsive, whereas the MS groups classified treated as an antidepressant resistance model, as far as this depressive-like behaviour of despair is involved.

Transcriptional analysis

We were interested in studying whether this different stress-coping behaviour induced by antidepressants could be associated with specific gene expression alterations. Therefore, we performed a microarray experiment to assess global analysis of gene expression in two brain regions known for their involvement in MDD pathophysiology, the Hip and the PFCx. Next, we analysed the transcriptional output by means of a bioinformatic tool we have recently developed (Lauria et al., 2015), aimed at testing whether vehicle–treated and rats administered with antidepressants (ES or NT) could be differentiated by a specific transcriptional signature (Figure 1). The analysis was carried out separately for the two brain regions and separately for the MS and n-MS groups. In each brain area the algorithm was able to identify non-overlapping transcriptional signatures, that could discriminate vehicle- and antidepressant-treated animals in both experimental conditions of n-MS and MS (Tables 1-2). These findings show that specific genes were differentially expressed when animals showed signs of antidepressant sensitivity or resistance in the forced swim test.

Accordingly, when accuracy tests were performed to evaluate the ability of the transcriptional signature to correctly separate subjects belonging to controls from those belonging to the treated groups, we confirmed that the high accuracy observed when applied within the MS or n-MS conditions was vastly reduced when transferred to the other condition (Tables 1-2).

We analysed which contribution was provided by specific genes to the transcriptional signature, highlighting those that displayed increased or decreased expression in the treatment groups. Indeed, it is expected that the genes exhibiting different average expression between the examined groups as shown in Supplementary Tables 1-4 provide a larger contribution to sample separation.

We then compared the transcriptional signatures with each other to identify genes shared between them. Interestingly, the highest number of overlapping genes between any two signatures was observed between antidepressant responders in Hip and PFCx (n-MS animals, 83 common genes, Supplementary Figure S1), suggesting a possible involvement of these genes in the behavioural response to antidepressants.

Subsequently, we performed a functional annotation analysis to discover whether specific GO terms or pathways were enriched in each antidepressant transcriptional signature in both Hip and PFCx. In antidepressant responder n-MS rats, the most enriched pathways included the Reactome pathways R-RNO-5626467 “RHO GTPases activate IQGAPs” and R-RNO-1679131 "Trafficking and processing of endosomal TLR" (Supplementary Tables 5-7-9-11). Among GO terms, high-score results included “Carbon metabolism” and “Glycolysis/gluconeogenesis”, as well as "Negative regulation of endopeptidase activity"; the prominent positions of these GO terms were also confirmed by cluster analysis, that revealed clusters of similar terms (Figure 3, Supplementary Tables 5-7-9-11). Functional annotation analysis of treatment-resistant n-MS rats signatures indicated clusters of ribosomal proteins, inflammatory response, and transcriptional/epigenetic regulation (Figure 3, Supplementary Tables 6-8-10-12).

The analysis of interacting clusters formed by the proteins encoded by signature genes showed that clusters involving synaptic vesicles and neurotransmitter release, ubiquitin regulation, cytoskeleton organisation and carbohydrate metabolism characterised the antidepressant-responder transcriptional signatures (Figure 4A). Antidepressant non-response signature genes displayed mainly ribosomal proteins and small GTPases clusters instead (Figure 4B).

Relevance for human disease

Considering that FSL rats are a validated model for MDD, we asked whether the genes belonging to the transcriptional signatures displayed significant modifications in MDD pathology in patients. To this end, we compared the transcriptional signatures with two transcriptional datasets of human depressed patients, obtained from the PFCx and blood. We found that a large number of signature genes showed altered expression in human PFCx and blood (Supplementary Figure S2), confirming the relevance of the genes to the pathophysiological basis of MDD. A comparison with gene expression alterations associated with ES-resistance in human blood RNA (Pettai et al., 2016) revealed a four-gene overlap (PRDX2, GIMAP7, RPL6, and TSC22D1) specifically with the signatures belonging to the treatment-resistant model.

The next step was to analyse if the genes that we identified as important in conferring susceptibility or resistance to antidepressant treatment were previously associated with the genetic susceptibility to MDD. Consequently, we compared genes belonging to the signatures with the results of the most recent and complete meta-analysis of GWAS in MDD that used MAGMA to assess the aggregated genetic effects on 17,842 genes from three studies (Howard et al., 2019) (Figure 5). We found that the GWAS meta-analysis and the susceptibility signatures shared the genes Rtn1 and Zdhhc5 in the Hip and Igsf6 in the PFCx, with Sim1 in common in both regions. GWAS-identified hits and the antidepressant resistance signatures shared the genes Ctnnd1, Rbms3, Atp1a3 in the PFCx and Pla2r1 in the Hip (Figure 5).

4. Discussion

The aim of this study was to use an animal model based on FSL rats to explore whether different gene expression signatures could be specifically associated with antidepressant-response/non-response. As expected, chronic treatment with a serotonin-selective re-uptake inhibitor (ES) or a noradrenalin transporter-selective tricyclic antidepressant (NT) reduced immobility in the FST. We found that post-natal exposure to MS prevented this antidepressant-induced reduction and we used this paradigm as a model of non-response to antidepressant treatment. Early-life adversities are a recognised susceptibility factor for the occurrence of MDD in humans (Nelson et al., 2017; Opel et al., 2019). For this reason, the exposure to MS is an established procedure applied to engender the manifestation of depressive-like behaviours in animal models (Czéh et al., 2016; Pryce et al., 2005). In agreement with our findings, previous data demonstrated that in animal models of MDD based on stress exposure, MS induced resistance to antidepressant treatment (Minami et al., 2017; Zhang et al., 2015), although contrasting findings were also reported, possibly due to experimental

variability, which was better dealt with in this study by including a high number of animals (171 vs. 64 and 57, respectively) (El Khoury et al., 2006; Musazzi et al., 2010). Moreover, exposure to stressors and adversities during childhood and adolescence have been associated with a higher probability of developing TRD (Nelson et al., 2017; Tunnard et al., 2014).

We analysed the differential gene expression in Hip and PFCx, two brain regions involved in the pathophysiology of MDD, and were able to identify specific transcriptional signatures that effectively discriminated FSL rats treated with vehicle from those receiving antidepressants. Remarkably, the signatures were sufficiently different between the n-MS and MS groups and each signature could be used to separate only one group. Moreover, a high number of overlapping genes were detected between Hip and PFCx in the antidepressant-responder group, suggesting that the expression of those genes is important throughout the brain in conferring response to antidepressants. Likewise, the analysis of the contribution of specific genes to MS showed that a higher number of genes displayed different expression levels between vehicle- and antidepressant-treated animals in the n-MS groups, whereas the number was remarkably lower in MS rats (Supplementary Tables 1-4).

Among the genes providing a larger contribution to the discrimination between controls and antidepressants in the responder groups, we found serotonin receptor 5HT2A; Serpin family members and a fibrinogen-like protein, proposed to be linked to the pathophysiology of TRD through interference with proBDNF processing (Idell et al., 2017); PGAM5, a phosphatase that activates ASK1 MAPK (Takeda et al., 2009); the phosphatase Ptpn22, involved in the biosynthesis of anandamide (Liu et al., 2008); C-X-C Motif Chemokine Ligand 3 and Interleukin 36 Beta.

Functional annotation and protein-protein interaction analysis allowed a comparison between proteins and pathways that are most relevant for the antidepressant response, compared to non-response.

In antidepressant response, a prominent role was found for the Toll-like receptor (TLR) pathways. TLRs mediate the innate immunity response to pathogen-associated molecular patterns, triggering a pro-inflammatory response that contribute to the development of neuroinflammation (Kumar, 2019). As an important factor mediating the physiological stress response, a role for TLR signalling has been proposed in MDD (Cheng et al., 2016; García Bueno et al., 2016). Available data show that TLR expression is modulated in rodent depression models, including MS (García Bueno et al., 2016; Sadeghi et al., 2016), and in depressed patients both at peripheral level and in brain regions (Hung

et al., 2014; Kéri et al., 2014; Pandey et al., 2019, 2014; Redei et al., 2014). Interestingly, TLR modulation has been proposed as responsive to antidepressant treatment (Hung et al., 2016), thus in agreement with our findings.

Linked to this innate immunity hypothesis, the identification of the negative regulation of cysteine endopeptidases robustly indicated by the cluster analysis suggests the possible involvement of the cysteine protease Caspase 1. Caspase 1 is a constituent of the inflammasome, a proinflammatory signalling cascade that can occur also in the absence of foreign pathogens. Caspase 1 mRNA and protein are increased in peripheral blood mononuclear cells of patients diagnosed with MDD patients and antidepressants decrease this hyperactivation (Inserra et al., 2019).

Enrichment in “RHO GTPases activate IQGAPs” pathway might suggest the involvement of the regulation of synaptic morphology in the antidepressant response, as IQGAPs are reported to control neurite outgrowth (Li et al., 2005; Wang et al., 2007). Moreover, our finding that energy metabolism pathways is involved in antidepressant response is in line with previous results (Martin et al., 2013) and supports the hypothesis that impairment in energy metabolism is related to the pathophysiology of psychiatric disorders, including MDD (Bigio et al., 2016; Zuccoli et al., 2017).

In the interaction network generated by proteins associated with antidepressant response (Figure 4), a cluster of proteins is involved in the regulation of neurotransmitter storage and release in synaptic vesicles (Atp6v0c and atp6v1g3 are V-ATPases that acidify synaptic vesicles, thus allowing neurotransmitter entry; chrnb4 is a nicotinic acetylcholine receptor subunit; snap25 is a synaptic protein; HSP A2, A8 and 90aa1 are involved in vesicular transport; dynamin 1 regulates vesicle endocytosis and recycling). The above findings imply the ability of effective antidepressant treatment to induce a long-term modification of neurotransmitter release, as proposed by the theories of the mechanism of action of antidepressants based on monoamine signalling (Blier and El Mansari, 2013). In addition, a cluster made by three proteins belonging to the ubiquitin cycle (Herc6, Asb5, Rnf7) signals the importance of this mechanism. In agreement with our findings, Park et al., adopting a proteomic approach, discovered that the response to antidepressant treatment was associated with the ubiquitin-proteasome pathway (Park et al., 2017).

In the interaction cluster obtained from proteins encoded by antidepressant signature genes obtained from MS rats, a prominent net is formed by nine ribosomal proteins. Ribosomal proteins contribute to the assembly of functional ribosomes, thus promoting cell growth, proliferation, and differentiation. In addition to this critical house-keeping function, accumulating evidence confirmed

that ribosome proteins possess additional, ribosome-independent functions (Warner and McIntosh, 2009). In particular, recent data showed that ribosomal proteins participate in the immune response by regulating gene expression (Zhou et al., 2015). Since TRD has been associated with increased inflammation and altered levels of inflammatory markers, it is conceivable that the involvement of ribosomal proteins highlighted by this analysis can be associated with a dysregulation of inflammatory signalling. These findings are confirmed by the enriched pathways revealed by cluster analysis, which highlighted ribosomal proteins and the inflammatory response. The relevance of the small GTPase proteins, also observed by Amare et al. (Amare et al., 2019), is line with the hypothesis of a possible role in modulating glutamate receptors trafficking (Kennedy et al., 2014; Ng and Tang, 2008), and consistent with the observation that TRD responds to ketamine treatment (Schwartz et al., 2016), an antidepressant based on modulation of the glutamate response (Aleksandrova et al., 2017).

Overlapping genes were detected in the comparison between genes identified in the GWAS meta-analysis and our signatures. In the susceptibility signature, Sim1 is a transcription factor having pleiotropic effects during embryogenesis and in adulthood. In mice, it has been demonstrated that Sim1 is involved in the differentiation of serotonergic neurons in the dorsal raphe nucleus (Osterberg et al., 2011). It is thus possible that it may play a role in the response to antidepressants that affect the serotonergic signalling. Rtn1 belongs to a protein family, Reticulons, mainly localised in the endoplasmic reticulum, where they function to shape intracellular organelles, and have been implicated in neurodegenerative disorders and in schizophrenia (Chiurchiù et al., 2014). In particular, among other functions, Rtn1 demonstrated DNA binding activities that affect epigenetic regulations, which could contribute to antidepressant response. Zdhhc5, a gene also associated with schizophrenia (Zhao et al., 2018), is a palmitoyl-acyl transferase that mediates activity-dependent palmitoylation, which affects synaptic delivery and surface stabilization of AMPA receptors (Brigidi et al., 2015), thus modulating synaptic plasticity, which plays a role in antidepressant response.

In the non-response signature, Atp1a3 is a Na^+/K^+-ATPase responsible for establishing and maintaining neuronal electrochemical gradient across the plasma membrane. Na^+/K^+-ATPase levels decreased in animal models of depressive-like despair (Crema et al., 2010; Gamaro et al., 2003) and transgenic mice with reduced neuronal Na^+/K^+-ATPase activity displayed depressive-related behaviour when exposed to stress (Kirshenbaum et al., 2011). Lastly, antidepressant treatments

that increased Na^+/K^+-ATPase were able to reverse depressive-like behaviour, suggesting that the activity of this enzyme is required for antidepressant efficacy (Gamaro et al., 2003).

In conclusion, this study demonstrated that distinct transcriptional signatures can be associated with behavioural response or non-response to antidepressant treatment. Antidepressant sensitivity indicated the relevance, among others, of TLR and cysteine proteases; energy metabolism and the control of neurotransmitter release. Antidepressant resistance was linked to genes including ribosomal proteins, transcriptional regulation, and small GTPases. The identification of genes involved in the response to antidepressant treatment will contribute to increasing the understanding of the neurobiological underpinning of TRD, thus contributing to the identification of effective targets for therapeutic intervention in MDD.

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Figure legends

Figure 1: Experimental design. FSL pups were subjected to MS or left in the home-cage (n-MS). When adult, the n-MS and MS groups were split into three subgroups receiving Escitalopram (ES), Nortriptyline (NT), or vehicle for one month. Immobility time was evaluated in the forced swim test and transcriptomic analysis was performed in the hippocampus (Hip) and prefrontal cortex (PFCx). Transcriptional data were used to derive specific transcriptional signatures to discriminate vehicle- vs. antidepressant-treated rats in both n-MS and MS groups.

Figure 2: Immobility time (s) in the forced swim test after treatment with vehicle, ES, or NT. The median is denoted by the horizontal line within the box. The box indicates the interquartile range. The whiskers extend to the most extreme data point. ***: $p<0.001$; **: $p<0.01$ vs. respective vehicle treated group in Planned Comparisons.

Figure 3: Clustering from functional annotation analysis in DAVID (Huang et al., 2009). Enrichment in signatures from antidepressant responder rats are shown in A: Hip n-MS and C: PFCx n-MS. Enrichment in signatures from antidepressant resistant rats are shown in B: Hip MS and D: PFCx MS.

Figure 4: Interacting protein networks generated by merging Hip and PFCx transcriptional signatures. Networks generated by specific genes from transcriptional signatures of response (n-MS, A) or non-response to antidepressants (MS, B). Only high confidence interactions are shown (minimum required interaction score >0.9).

Figure 5: Overlap of transcriptional signature genes with genes associated with MDD in GWAS studies. GWAS MDD: genome-wide significant gene-based hits ($p<2.80 \times 10\text{-}6$) in the meta-analysis of depression using MAGMA (Howard et al., 2019).

Figure 1

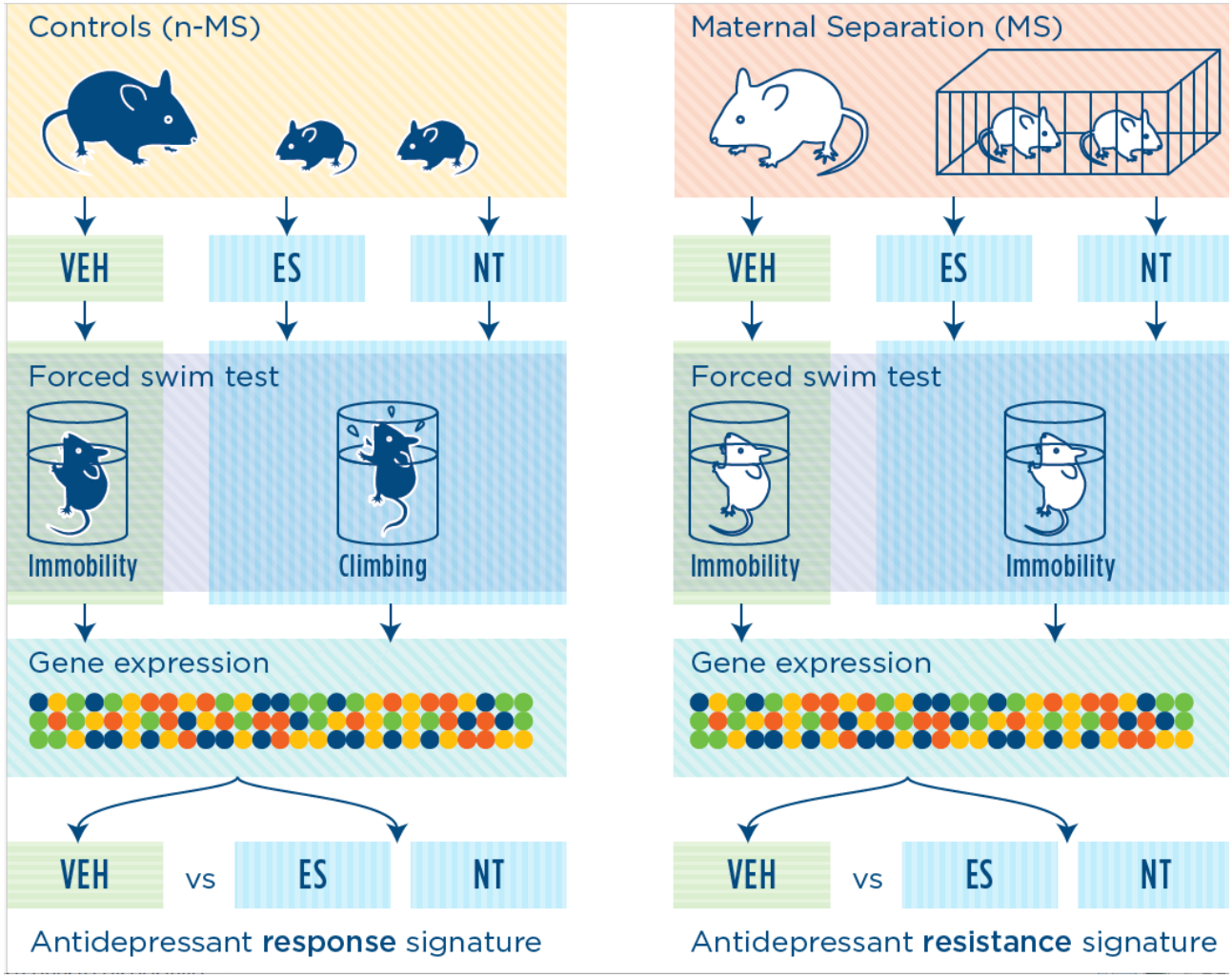

Controls (n-MS)
VEH
ES
NT
Forced swim test
Immobility
Climbing
Gene expression
VEH
vs
ES
NT
Antidepressant response signature
Maternal Separation (MS)
VEH
ES
NT
Forced swim test
Immobility
Immobility
Gene expression
VEH
vs
ES
NT
Antidepressant resistance signature

Figure 2

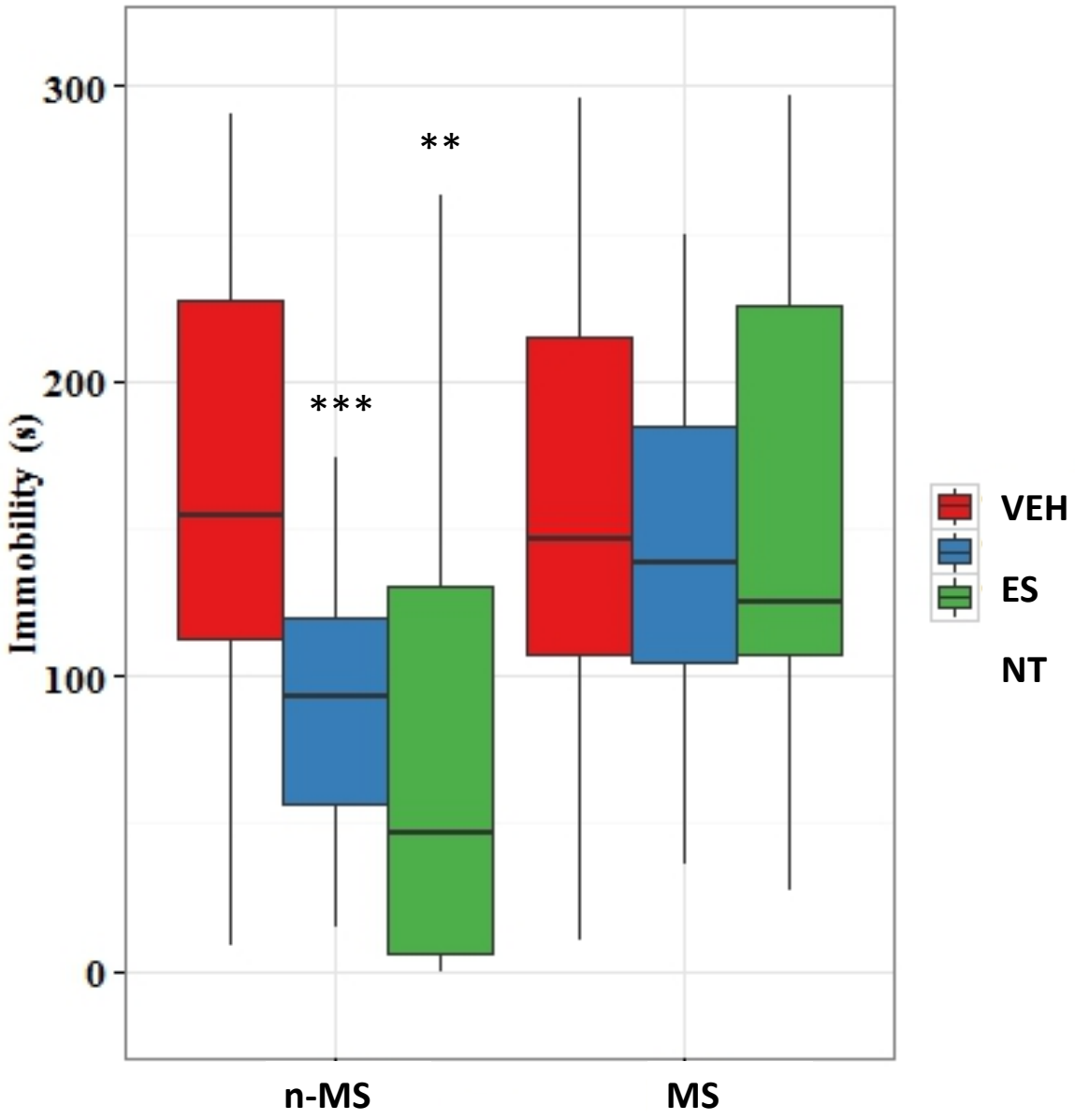


Figure 3

A

Annotation Cluster 1	Enrichment Score: 2.29
GOTERM_BP_DIRECT	negative regulation of endopeptidase activity
GOTERM_MF_DIRECT	cysteine-type endopeptidase inhibitor activity
GOTERM_BP_DIRECT	negative regulation of cysteine-type endopeptidase activity
GOTERM_MF_DIRECT	protease binding
Annotation Cluster 2	**Enrichment Score: 1.52**
KEGG_PATHWAY	Carbon metabolism
KEGG_PATHWAY	Glycolysis / Gluconeogenesis
GOTERM_MF_DIRECT	NAD binding
GOTERM_BP_DIRECT	tricarboxylic acid cycle

B

Annotation Cluster 1	Enrichment Score: 1.41	
GOTERM_CC_DIRECT	cytosolic large ribosomal subunit	RT
KEGG_PATHWAY	Ribosome	RT
GOTERM_MF_DIRECT	structural constituent of ribosome	RT
GOTERM_BP_DIRECT	translation	RT
GOTERM_CC_DIRECT	ribosome	RT
Annotation Cluster 2	**Enrichment Score: 1.26**	
GOTERM_BP_DIRECT	inflammatory response	RT
GOTERM_BP_DIRECT	cellular response to interleukin-1	RT
GOTERM_BP_DIRECT	positive regulation of cell migration	RT

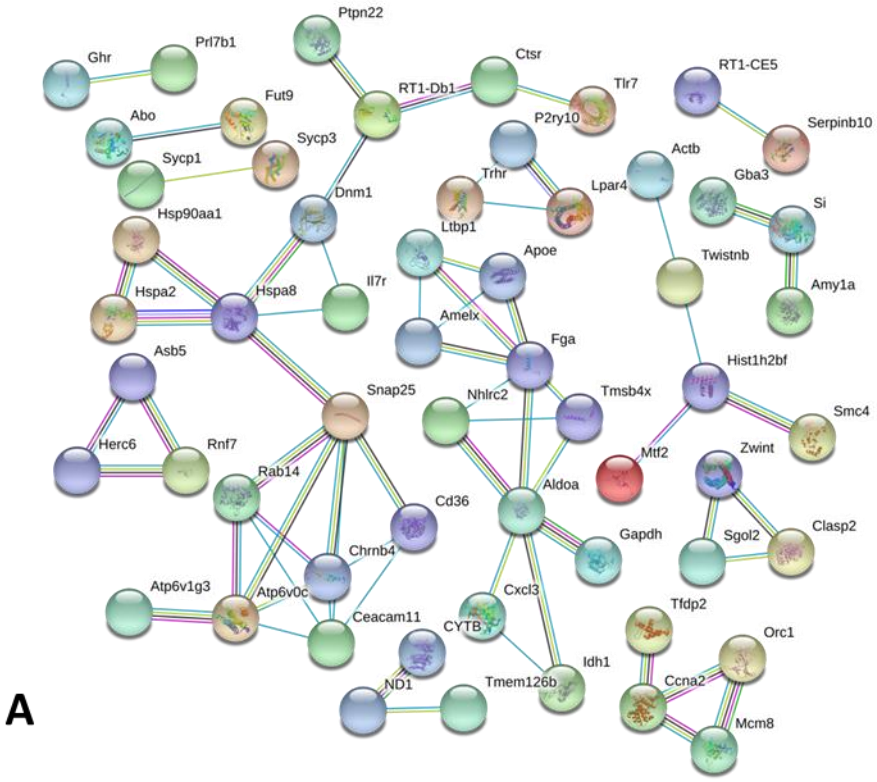
C

Annotation Cluster 1	Enrichment Score: 2.22	
GOTERM_BP_DIRECT	negative regulation of endopeptidase activity	RT
GOTERM_MF_DIRECT	cysteine-type endopeptidase inhibitor activity	RT
GOTERM_MF_DIRECT	protease binding	RT
Annotation Cluster 2	**Enrichment Score: 1.43**	
GOTERM_CC_DIRECT	synaptonemal complex	RT
GOTERM_CC_DIRECT	male germ cell nucleus	RT
GOTERM_BP_DIRECT	spermatogenesis	RT

D

Annotation Cluster 1	Enrichment Score: 1.24	
GOTERM_CC_DIRECT	chromatin	RT
GOTERM_MF_DIRECT	chromatin binding	RT
GOTERM_MF_DIRECT	transcriptional activator activity, RNA polymerase II transcription regulatory region sequence-specific binding	RT
KEGG_PATHWAY	Cell cycle	RT
Annotation Cluster 2	**Enrichment Score: 1.23**	
REACTOME_PATHWAY	R-RNO-5578775	RT
GOTERM_BP_DIRECT	potassium ion import	RT
GOTERM_BP_DIRECT	potassium ion transport	RT
KEGG_PATHWAY	Insulin secretion	RT
GOTERM_CC_DIRECT	intracellular membrane-bounded organelle	RT

Figure 4


Ptpn22
Prl7b1
Ghr
Ctsr
RT1-Db1
Tlr7
RT1-CE5
Fut9
Abo
P2ry10
Serpinb10
Sycp3
Sycp1
Actb
Trhr
Lpar4
Gba3
Dnm1
Si
Hsp90aa1
Ltbp1
Apoe
Twistnb
Il7r
Hspa2
Hspa8
Amy1a
Amelx
Fga
Asb5
Hist1h2bf
Snap25
Nhlrc2
Tmsb4x
Smc4
Herc6
Rnf7
Mtf2
Zwint
Rab14
Aldoa
Cd36
Clasp2
Gapdh
Sgol2
Chrnb4
Atp6v1g3
Atp6v0c
Cxcl3
Tfdp2
Ceacam11
CYTB
Orc1
Idh1
ND1
Tmem126b
Ccna2
Mcm8
A

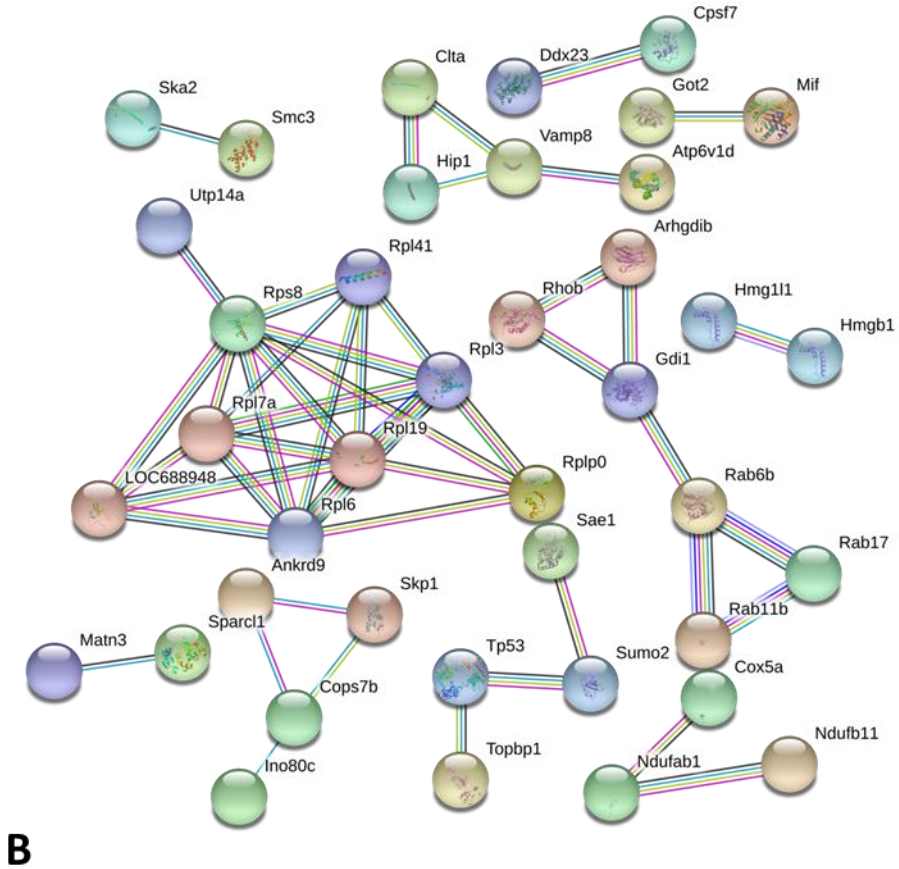
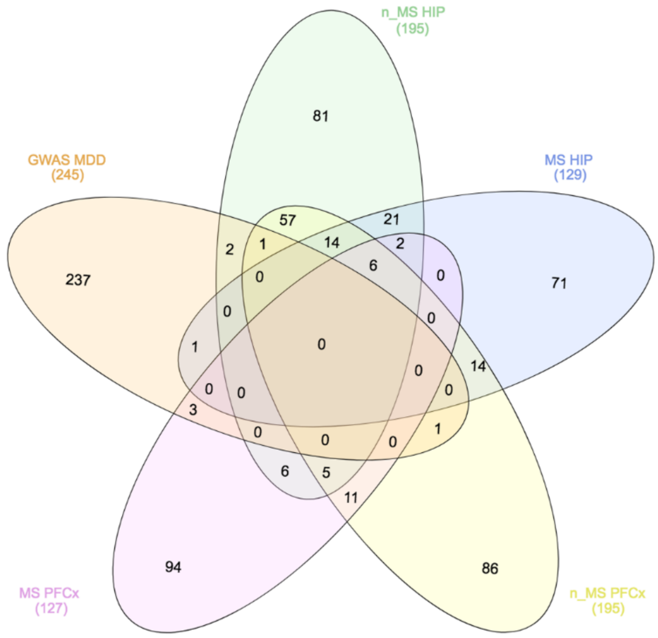

Cpsf7
Clta
Ddx23
Ska2
Got2
Mif
Smc3
Vamp8
Hip1
Atp6v1d
Utp14a
Arhgdib
Rpl41
Rps8
Rhob
Hmg1l1
Hmgb1
Rpl3
Gdi1
Rpl7a
Rpl19
Rplp0
Rab6b
LOC688948
Rpl6
Sae1
Rab17
Ankrd9
Skp1
Rab11b
Sparcl1
Matn3
Tp53
Sumo2
Cox5a
Cops7b
Topbp1
Ndufb11
Ino80c
Ndufab1
B

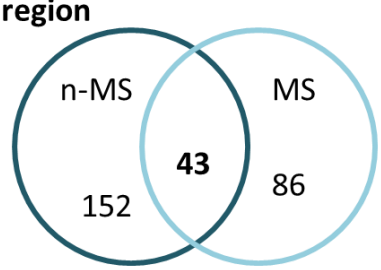
Figure 5



GWAS MDD] and [n_MS HIP]: Rtn1,Zdhhc5
GWAS MDD] and [MS HIP]: Pla2r1
GWAS MDD] and [n_MS PFCx]: Igsf6
GWAS MDD] and [MS PFCx]: Atp1a3,Ctnnd1,Rbms3
GWAS MDD] and [n_MS HIP] and [n_MS PFCx]: Sim1

Supplementary figure 1

A: Brain region

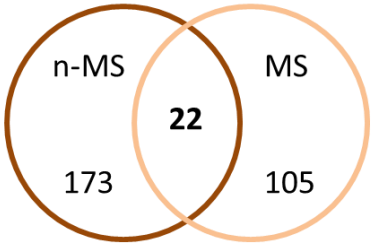


Hippocampus:

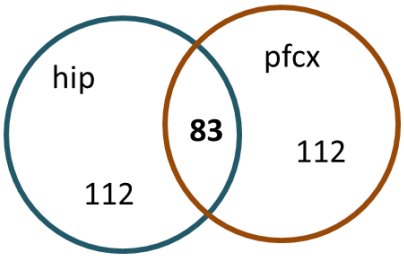
Als2cr4, Andpro, App, Art2b, Calm1, Cav2, Ccdc25, Ccdc93, Clip1, Cnot6l, Cr2, Fam92a1, Fbxl7, Fth1, Gen1, Hells, Hnmt, Hoxc9, Hspb11, Ifi204, Irx3, Klra5, Lrrc19, Mapk8, Mcpt10, MGC114427, Mup5, Ncor1, Olr1, Osap, P22k15, Pgam5, Pgk1, Ptgfr, Rpl9, Sash3, Scn9a, Sft2d3, Slbp, Slc22a12, Slc35d3, Spink1, Utp11l

Pre-frontal cortex:

Akr1c14, Atp1a4, Casc5, Cav2, Chmp4c, Clec1b, Col13a1, Hells, Itgb5, Kdm4c, Kitlg, Mcpt10, Mtmr2, Mup5, P22k15, Pgk1, Ranbp2, Rap1gds1, Rest, Tomm40b, Trpv1, Zfp354b



B: MS/n-MS

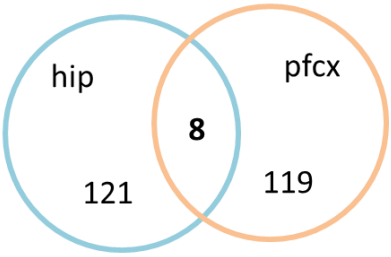


n-MS:

AABR07058441.1, Acsm3, Adh7, Akr1c14, Andpro, Arid1b, Arsk, Art2b, Atp1a4, Cav2, Ccdc25, Ccna2, Cdh1, Ceacam11, Chmp1b, Clasp2, Clec14a, Clec1b, Cr2, Cryge, Csde1, Ctsql2, Cxadr, Ddx60, Defa7, Depdc1, Dkk2, Dsc2, Dscr6, Esco1, Fabp12, Fam92a1, Fbxl7, Fut9, Gbp4, Hells, Hist1h2bf, Hspb11, Ifi204, Irx5, Kdm4c, Klre1, Kng2, LOC100364523, Lrrc19, Mcpt10, MGC114427, Mup5, Ncor1, Osap, P22k15, Palld, Pgk1, Phex, Prl8a4, Prrx1, Psbpc1, Ptgfr, Ptpn22, Rbm34, Rbp7, Rgn, Rhox9, Sec63, Serpina7, Serpinb2, Sim1, Slbp, Slc13a1, Slco1b3, Steap4, Sult2al1, Sycp1, Taf7l, Tax1bp3, Tlr7, Tmem38b, Trdn, Tsga13, Ubp1, Vps37b, Zfp207, Zfp354b

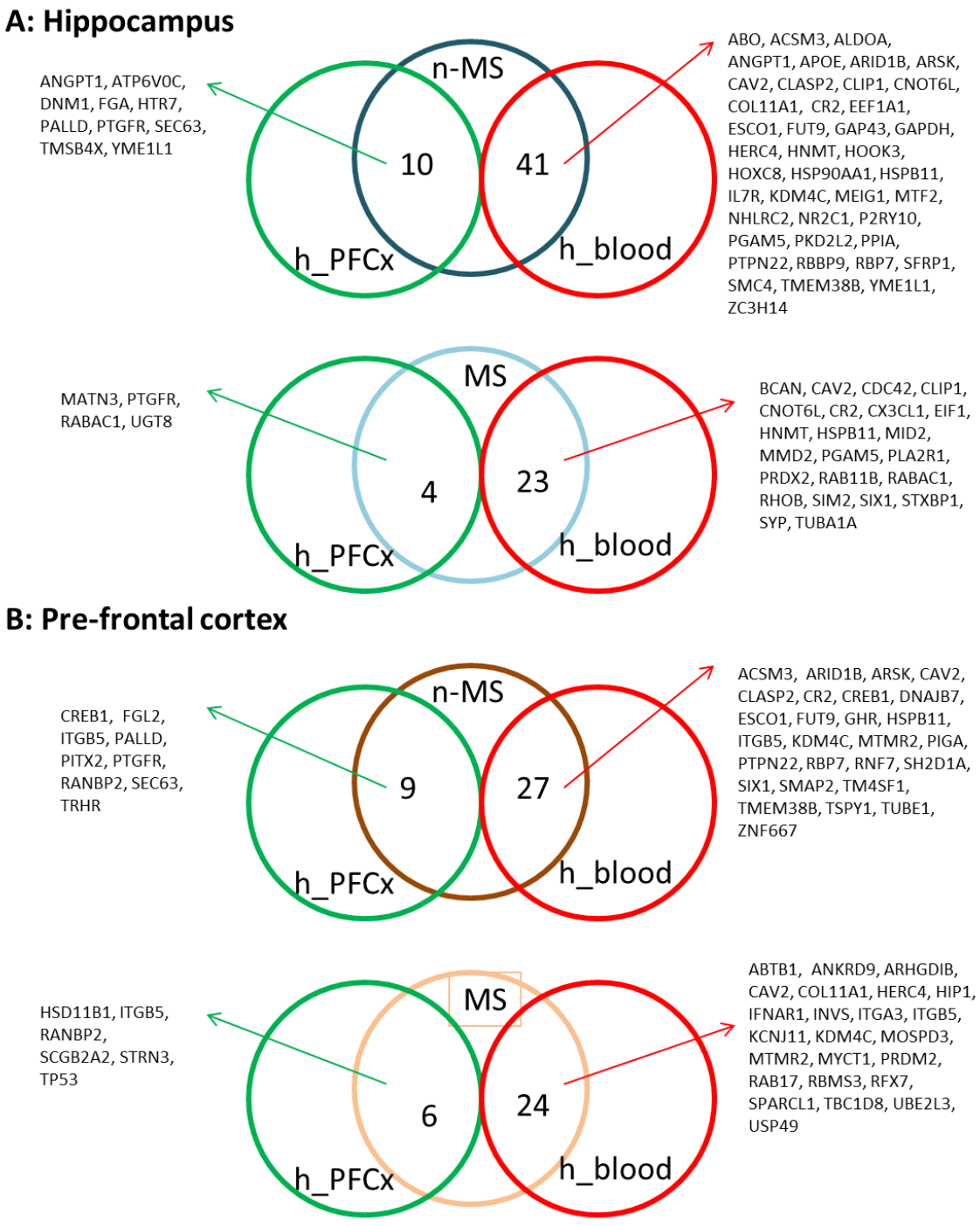
MS:

Cav2, Hells, Mcpt10, Mup5, P22k15, Pgk1, Sash3, Slc22a12



Supplementary Figure S1: Overlapping genes between signatures within brain region (A) or within responding (n_MS) or non-responding (MS) groups (B).

Supplementary figure 2



Supplementary Figure S2 : Comparison of transcriptional signature genes in rat Hip (A) and PFCx (B) and gene expression in PFCx (h_PFCx) or blood (h_blood) from human depressed patients