Integrative proteomic analysis of the NMDA NR1 knockdown mouse model reveals effects on central and peripheral pathways associated with schizophrenia and autism spectrum disorders

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Abstract

Background: Over the last decade, the transgenic N-methyl-D-aspartate receptor (NMDAR) NR1-knockdown mouse (NR1neo−/−) has been investigated as a glutamate hypofunction model for schizophrenia. Recent research has now revealed that the model also recapitulates cognitive and negative symptoms in the continuum of other psychiatric diseases, particularly autism spectrum disorders (ASD). As previous studies have mostly focussed on behavioural readouts, a molecular characterisation of this model will help to identify novel biomarkers or potential drug targets.

Methods: Here, we have used multiplex immunoassay analyses to investigate peripheral analyte alterations in serum of NR1neo−/− mice, as well as a combination of shotgun label-free liquid chromatography mass spectrometry, bioinformatic pathway analyses, and a shotgun-based 40-plex selected reaction monitoring (SRM) assay to investigate altered molecular pathways in the frontal cortex and hippocampus. All findings were cross compared to identify translatable findings between the brain and periphery.

Results: Multiplex immunoassay profiling led to identification of 29 analytes that were significantly altered in sera of NR1neo−/− mice. The highest magnitude changes were found for neurotrophic factors (VEGFA, EGF, IGF-1), apolipoprotein A1, and fibrinogen. We also found decreased levels of several chemokines. Following this, LC-MSE profiling led to identification of 48 significantly changed proteins in the frontal cortex and 41 in the hippocampus. In particular, MARCS, the mitochondrial pyruvate kinase, and CamKII-alpha were affected. Based on the combination of protein set enrichment and bioinformatic pathway analysis, we designed orthogonal SRM-assays which validated the abnormalities of proteins involved in synaptic long-term potentiation, myelination, and the ERK-signalling pathway in both brain regions. In contrast, increased levels of proteins involved in neurotransmitter metabolism and release were found only in the frontal cortex and abnormalities of proteins involved in the purinergic system were found exclusively in the hippocampus.

Conclusions: Taken together, this multi-platform profiling study has identified peripheral changes which are potentially linked to central alterations in synaptic plasticity and neuronal function associated with NMDAR-NR1 hypofunction. Therefore, the reported proteomic changes may be useful as translational biomarkers in human and rodent model drug discovery efforts.

Keywords: ApoA1, Glutamate, Leptin, Major depressive disorder, Oligodendrocytes, Proteomics, Serum biomarkers, SRMstats
Background
The transgenic NR1-knockdown (NR1<sup>neo−/−</sup>) mouse constitutively expresses only 5 to 10% of the essential N-methyl-D-aspartate receptor (NMDAR) NR1 subunit [1]. The NMDAR is crucial in neuronal development and physiology, and decreased levels or altered function of this receptor have been associated with the pathophysiology of schizophrenia (SZ) [2-5]. Consequently, the NR1<sup>neo−/−</sup> mouse has been widely used as a genetic model for intrinsic NMDAR hypofunction in preclinical drug discovery efforts. NR1<sup>neo−/−</sup> mice display hyperlocomotion and increased stereotypic behaviour, which represent standard behavioural readouts for the evaluation of animal models of SZ. These behavioural effects can be attenuated by the typical antipsychotic drug haloperidol, a potent highly specific D<sub>2</sub>-dopamine receptor antagonist [6], and by the atypical antipsychotic drug clozapine [1], which affects a broader spectrum of neurotransmission systems [7,8]. In addition to these behavioural changes, NR1<sup>neo−/−</sup> mice also show significant impairments in spatial cognitive performance [9], reduced social interaction, escape behaviours, and actively avoid interaction with intruder males. Furthermore, NR1<sup>neo−/−</sup> males have been reported to be infertile due to their abnormal social behaviour. However, administration of clozapine has been found to ameliorate all of these symptoms [1], which are thought to predominantly reflect the negative and cognitive symptoms of SZ.

Interestingly, recent studies investigating the NR1<sup>neo−/−</sup> mouse identified behavioural and electrophysiological deficits relevant to all core symptoms of autism spectrum disorders (ASD) [10,11]. Further, clinical ASD symptomatology, including reduced prepulse-inhibition, auditory-evoked response N1 latency, and reduced gamma synchrony was observed in the NR1<sup>neo−/−</sup> mouse [12]. NMDAR NR1 subunit knockout in parvalbumin-positive interneurons resulted in an ASD-like phenotype [13] with impaired self-care and sociability [14] in the absence of depression-related behaviours [15]. In addition, NMDAR and glutamate abnormalities have been identified in various brain disorders, such as major depressive disorder [16,17] and ASD [18-20], which are characterized by negative symptom domains. The abovementioned behavioural data now supports the hypothesis of a potential role of impaired NMDAR function in the continuum of negative symptom phenotypes of a range of psychiatric disorders, including core features of autism [21].

Given these similarities, the primary objective of this study was to identify molecular signatures in the NR1<sup>neo−/−</sup> mouse model. Specifically, multiplex immunoassay profiling was used to assess serum changes given the high sensitivity of this method for quantification of low abundance circulating proteins such as cytokines, hormones, and growth factors. Label-free liquid chromatography – mass spectrometry in expression mode (LC-MS<sup>E</sup>) analysis was used as this allows unbiased screening of approximately 1,000 proteins in a single extract and targets proteins, such as membrane receptors, nuclear factors, mitochondrial proteins, and cytoplasmic molecules, all of which have been implicated in psychiatric disorders. Finally, SRM mass spectrometry was used to target specific classes of proteins with greater sensitivity than the LC-MS<sup>E</sup> approach. A secondary goal was to investigate whether changes in protein levels in serum can be linked to glutamatergic brain dysfunction, thus evaluating the translational utility of serum biomarker changes for psychiatric disorders.

Methods
Animals
The NR1<sup>neo−/−</sup> mice [1,22,23] were obtained from the laboratory of Dr. Beverly Koller (The University of North Carolina at Chapel Hill) and a breeding colony was established at AstraZeneca Pharmaceuticals LP (Wilmington DE 19850, USA). All breeding and testing procedures were conducted in strict compliance with the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, National Research Council, 1996) and approved by the Institutional Animal Care and Use Committee of the University of North Carolina and AstraZeneca R&D Montréal. The breeding and genotyping was performed as previously described [24-27]. It involved three populations of mice: NR1<sup>neo−/−</sup> heterozygotes maintained on C57BL/6 background (Jackson Laboratory), NR1<sup>neo−/−</sup> heterozygotes maintained on 129/SvEv background (Taconic Farm), and an intercross between female C57BL/6 NR1<sup>neo−/−</sup> and male 129/SvEv NR1<sup>neo−/−</sup> to generate the F1 male NR1<sup>neo−/−</sup> and wildtype (WT) mice that were analysed in this study. Homozygotes carrying the NR1 hypomorph mutation do not breed effectively. Therefore, the mutant homozygotes had to be generated by cross-breeding heterozygotic mice. The NR1 hypomorph mutation could not be induced in pure C57BL/6 J or 129SvEv mice, because the mutants did not gain weight at the same rate as WT mice and the frequency of mutants born was less than expected. To overcome these problems, the strategy of breeding F1 hybrids was developed to generate the NR1<sup>neo−/neo</sup> mice [27]. Therefore, the progeny from the intercross were genetically identical F1 hybrids with the exception at NR1 locus: 50% NR1<sup>neo−/−</sup>, 25% NR1<sup>neo−/−</sup>, and 25% WT. The following primers were used for genotyping: NR1 (<sup>+</sup>) fwd primer (intron 20) 5′TGA GGG GAA
GCT CTT CCT GT3'; NR1 (−) fwd primer (neo) 5' GCT TCC TCG TGC TTT ACG GTA T3'; and NR1 common reverse primer (intron 20) 5’ AAG CGA TTA GAC AAC TAA GGG T3'. Mice were housed on a 12 h light/dark cycle with access to food and water ad libitum. Mice (3 to 4 months old) were killed according to schedule, decapitated, and trunk blood was collected in ice-chilled tubes containing EDTA and centrifuged at 1,100 g, 4°C, for 15 min. The serum was immediately separated and stored frozen at −80°C for later use. Brains were dissected on ice. Frontal cortex and hippocampus tissue were stored at −80°C.

Multiplex immunoassay profiling

Serum samples were analyzed using a rodent multianalyte profiling platform comprising multiplexed immunoassays of 75 analytes (Additional file 1: Table S2) in a Clinical Laboratory Improved Amendments (CLIA)-certified laboratory at Myriad-RBM (Austin, TX, USA), as described previously [28]. Immunoassays were calibrated using duplicate standard curves for each analyte and raw intensity measurements converted to protein concentrations using proprietary software. Multiplexed calibrators (eight levels per analyte) and controls (three levels per analyte) are used to monitor key performance parameters, such as a lower limit of quantification, precision, cross-reactivity, linearity, spike-recovery, dynamic range, matrix interference, freeze-thaw stability, and short-term sample stability are established for every assay as described by the manufacturer (http://www.myriadrbm.com/technology/data-quality/). Data analyses were performed using the statistical software package R (http://www.r-project.org) and the analyte levels were determined. Analyses were performed using the statistical software package R (http://www.r-project.org) and the analyte levels were determined. Analyses were conducted under blinded conditions with respect to sample identities and samples were analyzed in random order to avoid any sequential biases.

Sample preparation

Tissue samples were added to fractionation buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 2% ASB14, 70 mM DTT, and protease inhibitor at a 5:1 (v/w) ratio [29]. Samples were sonicated (10 sec, 2 cycles) and vortexed at 4°C for 30 min. Samples were then centrifuged at 17,000 g at 4°C. Protein concentrations of the lysates were determined using a Bradford assay (Bio-Rad; Hemel Hempstead, UK). Approximately 100 μg was precipitated using acetone. After dissolving the precipitate in 50 mM ammonium bicarbonate, reduction of sulphydryl groups were performed with 5 mM DTT at 60°C for 30 min and alkylation was carried out using 10 mM iodoacetamide, incubated in the dark at 37°C for 30 min, and subsequently digested using trypsin at a 1:50 (w/v) ratio for 17 h at 37°C. Reactions were stopped by the addition of 8.8 M HCl in a 1:60 (w/w) ratio. Quality control samples were prepared to monitor machine and preparation performance.

Label-free LC-MS\textsuperscript{E} analysis

Brain tissue samples were analysed individually in technical duplicates. Splitless nano-ultra-performance liquid chromatography (UPLC) (10 kpsi nanoAcquity; Waters Corporation, Milford, MA, USA), was coupled online through a New Objective nanoESI emitter (7 cm length, 10-mm tip; New Objective, Woburn, MA, USA) to a Waters Q-TOF Premier mass spectrometer. Data were acquired in expression mode (MS\textsuperscript{E}) and the total continuous run time was 8 days. The procedure, quality assessment, and data processing were performed as described previously [30]. LC-MS\textsuperscript{E} data were processed by the ProteinLynx Global Server (PLGS v.2.4 Waters, Milford, MA, USA) for ion detection, extraction, and identification using an ion accounting algorithm [31]. The Swiss-Prot rodent reference proteome database (2011–2013) was used for protein identification searches. To control the false discovery rate (FDR), data were searched against a decoy database, which was the randomised version of the database mentioned above to conserve amino acid frequencies. The FDR was set at the default maximum rate of 4%, as applied before [32–35]. The search parameters were (i) enzyme = trypsin, (ii) fixed modification = carbamidomethylation of cysteines, (iii) variable modifications = oxidation of methionine and phosphorylation at serine, threonine, or tyrosine residues, (iv) initial mass accuracy tolerances = 10 ppm for precursor ions and 20 ppm for product ions, and (v) one missed cleavage allowed. In addition, the following criteria were used for protein identification: (i) ≥3 fragment ions per peptide, (ii) ≥7 fragment ions per protein, and (iii) ≥1 peptide per protein. Raw data and PLGS search results were imported into the Rosetta Elucidator software (build 3.3.0.1.SP3.19, Rosetta Biosoftware; Seattle, WA, USA). Elucidator performed retention time (RT) and m/z/charge alignment, feature identification, and extraction for all samples using the Rosetta PeakTeller algorithm. Dynamic background subtraction, smoothing in RT, and m/z dimension and isotopic region creation for peak-matching across all runs were calculated using an RT correction of 4 min at the maximum. A single data file was randomly chosen as the master, and all other sample files were aligned to the master in form of a dynamic RT shift. This procedure allowed the improved identification of peptides and proteins in each sample by taking the available data of all samples into account. Features were filtered for high score and normalized based on total ion current. Only peptides detected in both replicates and in >80% of samples were included in further analysis.
Protein abundance changes were determined using the MSstats package [36] based on linear mixed-effects models on the peptide intensities, following log2 transformation and exclusion of intensity values deviating more than three standard deviations from the mean of each group (<1% of total data). Proteins were identified by at least two peptides. The P values were adjusted to control the FDR at a cut-off of 0.05 following the Benjamini-Hochberg procedure [37].

Protein set enrichment analysis
Protein set enrichment analysis was carried out as previously described [38,39]. Significantly changed proteins were partitioned into three bins, according to their predicted fold-change (FC): FC <1.0; FC >1.0, and 1 < FC <1. The R package database org.Mm.eg.db version 2.8.0 was used for gene ontology (GO) term annotation based on entrez gene identifiers. Significant overrepresentation of an annotated GO term per bin was determined by the GOstats package [40]. For each bin, P values for the GO categories [41] “biological pathway” and “cellular compartment” were calculated by a conditional hypergeometric test using the entire detected proteome as a background. These tests accounted for the hierarchical structure of the GO terms by first testing the “child terms” of any given GO category and filtering significantly enriched proteins prior to analysis of the “parent terms”, as described previously [42]. This prevented the identification of directly-related GO terms with a considerable overlap of assigned proteins. GO terms with no significant enrichment in any bin (P >0.05) and GO terms with less than two annotated proteins were removed. The remaining P values greater than 0.05 were replaced by a conservative P value of 1. P values were replaced by their negative logarithm to the base of ten and then converted to z-scores within their proteomic comparison for every remaining GO term. Finally, one-way hierarchical clustering using “Euclidean distance” as distance function and the “Average Linkage Clustering” method available in the software Genesis [43], was performed on all significantly enriched GO terms. The same enrichment analysis was repeated using KEGG pathway annotation in order to provide an independent in silico validation of our findings.

Label-based selected reaction monitoring (SRM) mass spectrometry
Abundance alterations of a panel of 39 candidate proteins implicated in the pathway analysis of the NR1neo−/− mouse (see results section) were measured using targeted SRM mass spectrometry on a Xevo TQ-S mass spectrometer (Waters Corporation) coupled online through a New Objective nanoESI emitter (7 cm length, 10-mm tip; New Objective) to a nanoAcquity UPLC system (Waters Corporation). The system was comprised of a C18 trapping column (180 μm × 20 mm, 5 μm particle size) and a C18 BEH nano-column (75 μm × 200 mm, 1.7 mm particle size). The separation buffers were (A) 0.1% formic acid and (B) 0.1% formic acid in acetonitrile. For separation of peptides, the following 48-min gradient was applied: 97/3% (A/B) to 60/40% B in 30 min; 60/40% to 15/85% in 2 min; 5 min at 15/85%; returning to the initial condition in 1 min. The flow rate was 0.3 μL/min and the column temperature was 35°C.

SRM assays were developed following a general high-throughput strategy [44]. For method refinement, initially up to 12 unique peptides ranging from 6 to 20 amino acids in length, containing tryptic ends and no miscleavages were chosen for each of the selected proteins. All peptides containing amino acids prone to undergo modifications (e.g., Met, Trp, Asn, and Gln), potential ragged ends, lysine/ar- ginine followed by proline or bearing the NTX/NXS glyco- sylation motif were generally avoided and only selected when no other options were available [45]. Peptides were checked by Protein BLAST (http://blast.ncbi.nlm.nih.gov/ Blast.cgi) searches to ensure uniqueness. For method refinement, up to 12 transitions per peptide were tested in SRM mode. Transitions were calculated using Skyline version 1.2.0.3425 [46] and corresponded to singly charged y-ions from doubly or triply charged precursors, in the range of 350 to 1,250 Da. Transitions were selected based on software internal predictions, discovery proteomics data, and spectral data available through the Human National Institute of Standards and Technology spectral libraries [47]. Method refinement was performed on quality control samples. For the final SRM assays, 2 to 3 peptides with the maximal intensities and highest spectral library similarity (dotp) per protein were selected. A further development step, analysing heavy-label spiked quality control samples in scheduled SRM mode, was used to confirm identity via co-elution, extract the optimal fragment ions for SRM analysis, obtain accurate peptide retention times, and optimize collision energy and cone voltage for the quantification run applying skyline software (MacCoss Lab Software; Seattle, WA, USA) [46]. Heavy labelled forms of these selected peptides (spiketides L) were chemically synthesized via SPOT synthesis (JPT Peptide Technologies GmBH, Berlin, Germany). The final transitions, collision energy, and retention time windows used for each peptide can be found in the supplementary information (Additional file 2: Table S1). Quantitative SRM measurements comparing NR1neo−/− and WT mice were performed in scheduled SRM acquisition mode with the optimized parameters defined during the assay refinement. For each target peptide a heavy isotope labelled internal standard (JPT Peptide Technologies GmBH) was spiked in the peptide mixture for accurate quantification and identification. All SRM functions had a 2 min window of the predicted RT and scan times were 20 ms, which ensured a dwell time of over 5 ms per
transition. Assays were randomly split into three LC-SRM methods using Skyline software. This was done because of scheduling, assay development progress, and assay availability reasons. For each peptide, at least three transitions were monitored for the heavy and light version. Samples were run randomized and blocked [48] in triplicates and blanks and quality control peptide injections (yeast alcohol dehydrogenase, Additional file 2: Table S1) were performed alternating after every biological replicate. Resulting SRM data was analyzed using skyline and protein significance analysis was performed using SRMstats [49]. In the first step, data pre-processing was performed by transforming all transition intensities into log₂-values. Then a constant normalization was conducted based on reference transitions for all proteins, which equalized the median peak intensities of reference transitions from all proteins across all MS runs and adjusted the bias to both reference and endogenous signals. Protein level quantification and testing for differential abundance among NRI\textsuperscript{neo−/−} and WT mouse groups were performed using the linear mixed-effects model implemented in SRMstats. The scope of validity of our conclusions was restricted to the specific biological replicates in the experiments. Each protein was tested for abundance differences between NRI\textsuperscript{neo−/−} and WT mouse. The \( P \) values were adjusted to control the FDR at a cut-off of 0.05 according to Benjamini and Hochberg [37].

Results
Serum characterisation – Quantitative serum immunoassay profiling
We evaluated the peripheral adaption to the systemically reduced NMDAR-NRI expression by analysing 75 analytes (Additional file 1: Table S2) in serum using a multiplex immunoassay platform. After principal component analysis data quality assessment and outlier filtering, the analysis resulted in the identification of 29 significantly altered molecules \( (P < 0.05) \) (Table 1). The most prominent changes included a 17-fold increase in apolipoprotein A1 (ApoA1), a 13-fold increase in fibrinogen, and an 8-fold increase in vesicular endothelial growth factor A (VEGF), as well as a 6-fold decrease in insulin-like growth factor 1 (IGF-1). The protein levels of all identified chemokines (Ccl12, Ccl11, Xc1, Ccl7, and Ccl22) were significantly decreased.

Brain characterisation – quantitative LC-MS\textsuperscript{6} proteomic profiling of frontal cortex and hippocampus
LC-MS\textsuperscript{6} analysis resulted in the identification of 11,345 distinct peptides (563 proteins) in the frontal cortex and 14,775 distinct peptides (883 proteins) in the hippocampus after filtering the data using the criteria described in the Materials and Methods section. In the frontal cortex, 48 proteins were found to be significantly altered by more than 10% (Figure 1, Additional file 3: Table S3). In the hippocampus, 41 proteins showed significant changes using the same criteria.

The EH domain-containing protein 4 (EHD4), adenylsuccinate synthetase isozyme (PURA1), guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma 12 (GBG12), myristoylated alanine-rich C-kinase substrate (MARCS), and selenium-binding protein 2 (SBP2) were identified as most significantly altered in the frontal cortex and synaptosomal complex protein 3 (SYCP3), asparagine synthetase [glutamine-hydrolyzing] (ASNS), NADH dehydrogenase 1 alpha subcomplex subunit 4 (NDUA4), and complexin 1 (CPLX1) were most significantly changed in the hippocampus. CaM kinase II subunit alpha (KCC2A) was detected as highly significantly reduced in both brain regions but showed a 10% decrease.

Quantitative LC-MS\textsuperscript{6} proteomic profiling-based pathway analysis
Ingenuity pathway analysis (IPA) was performed using all significantly changed proteins \( (P^* < 0.05) \) in the frontal cortex (142 proteins) and hippocampus (227 proteins), regardless of the magnitude of change. This assumed that even slight variations in the levels of multiple proteins can result in pathway alterations. Using IPA, the protein changes were assigned to groups of biological functions in the Ingenuity knowledge base and z-scores were calculated as a prediction of whether a biological function was either up- or down-regulated. The biological functions underlying the identified molecular changes in the NRI\textsuperscript{neo−/−} mouse are shown in Figure 2A. The frontal cortex showed a decrease in “coordination,” “long-term potentiation”, and “quantity of filaments”. To a lesser extent, the behavioural domains of cognition, learning, and memory were decreased and hyperactive behaviour was increased. In the hippocampus a broader range of functions appeared to be affected. The most prominent finding here was an upregulation in “formation of cellular protrusions”. Full information including proteins underlying these functions can be found in (Additional file 4: Table S4). Furthermore, we generated functional networks using IPA. Both networks suggested an involvement of the ERK pathway in the two regions. Functional annotation using the ingenuity upstream analysis tool revealed an inhibition of this pathway (Figure 2B).

In an attempt to further validate the IPA in silico findings, we carried out a GO-term based protein set enrichment analysis. We analysed whether specific GO terms reflecting either biological pathways, KEGG pathways, or cellular compartments were significantly over-represented in the datasets of significantly altered proteins using hypergeometric testing (Figure 3). We validated the involvement of “clathrin adaptor complex/coat assembly/vesicle plasma membrane anchored proteins” and “long-term potentiation” in the frontal cortex, as well as “energy
Validation of significantly changed functional pathways

As a next step, we focussed on the core identified significantly altered pathways and biological functions using SRM, a highly sensitive targeted proteomic method, as an orthogonal validation method of the reported results. We developed an assay panel incorporating proteins involved in the ERK-pathway, clathrin-mediated endocytosis, glutamatergic signal transduction/transport, and energy metabolism. Furthermore, cell-type-specific markers were included. Using SRM we were able to validate most of the significantly altered pathways identified by label-free LC-MS² (Table 2).

In the LC-MS² phase of the study, we were unable to detect any of the NMDA receptor subunits for quantitative analysis, most likely due to the lower sensitivity of metabolism”, “purine metabolism”, and “apoptosis” in the hippocampus.
this approach. However, we were able to detect the NR1 subunit using SRM and this revealed a significant decrease in this protein in both the frontal cortex (ratio = 0.26) and hippocampus (ratio = 0.16). In contrast, no changes in other glutamate receptors (GluR-1, GluR-2, GluR-3) were detected in either tissue. In the frontal cortex, abnormalities in neurotransmitter metabolism and transport was indicated through significant abundance changes in the key enzymes glutamate decarboxylase 2, GABA aminotransferase, and the vesicular glutamate transporter 1. Furthermore, clathrin-mediated endocytosis was found to be increased, oligodendrocytic markers decreased, and long-term potentiation altered. In the hippocampus, an alteration in purine metabolism could be confirmed as well as changes in long-term potentiation. The ERK-pathway appeared to be affected in both frontal cortex and the hippocampus, as suggested earlier through pathway analysis.

**Discussion**

Herein, we present the first comprehensive proteomic study characterising central and peripheral changes in the NR1<sup>neo</sup>−/− mouse model. We employed orthogonal quantitative proteomic approaches to investigate protein alterations in serum that can serve as surrogate or translational biomarkers for decreased NMDAR function. The findings associated with NMDAR hypofunction in hippocampus and frontal cortex brain tissue may aid in the discovery of novel drug targets and in elucidating affected downstream pathways. Currently, animal models are almost exclusively assessed using behavioural readouts, leaving questions as to the underlying cellular and molecular network alterations unanswered.

Using multiplex immuno-profiling to measure peripheral metabolic, neurotrophic, and immunological factors, we initially linked the NMDA-mediated glutamatergic hypofunction to several serum analyte alterations. Interestingly, eight out of the 29 changing proteins (ApoA1, coagulation factor-VII, EGF, IGF-1, leptin, TNFα, VEGF, vWF) have previously been reported as changed in SZ and five (ApoA1, Eotaxin, EGF, Leptin, TNFα) in ASD biomarker studies [50-54]. This supports the notion that serum changes reflect aspects of the pathophysiology associated with psychiatric disorders; providing evidence for the translational utility of serum biomarker studies and their potential for personalised medicine approaches.

The strongest alteration was a 17-fold increase in levels of the lipid transport protein ApoA1. Although ApoA1 has not been linked to effects on glutamatergic signalling before, it is one of the most robust serum biomarkers in SZ [55,56], despite or whereas or even though this has been mainly found to be decreased in CSF, brain, and peripheral tissues of patients. The reason for this apparent discrepancy may be due to adaptive responses which are specific to the NR1<sup>neo</sup>−/− mouse model. ApoA1 plays a role in cholesterol transport and has been shown to prevent learning and memory deficits in an Alzheimer’s disease mouse model by attenuating neuroinflammation [57]. We also found similar strong increases in fibrinogen, implicating alterations in the blood coagulation system, as well as VEGF, which is produced by neuronal and glial cells in the developing nervous system and directly stimulates neuronal functions such as neurogenesis and cell survival in culture and in vivo [58]. This might be linked to our findings in
frONTAL cortex tissue of increased levels of synaptic proteins, indicating increased neuro- and synaptogenesis, which was confirmed by LC-MS\textsuperscript{E}, SRM, and computational pathway analysis (Table 2). Synapse formation, maintenance, and plasticity are critical for the correct function of the nervous system and its target organs. During development, these
processes enable the establishment of appropriate neural circuitry. In parallel with the increased synaptic markers, we found an increase in proteins involved in neurotransmitter metabolism and transport in the frontal cortex. These findings provide further evidence for increased excitability and imbalance in the frontal cortex, resembling neurochemical changes which are characteristic of SZ [59,60], ASD [61-64], and other disorders with negative symptom domains. At the mechanistic level, VEGF has been shown to exert its neurotrophic properties by regulating NMDAR activity via the SRC family kinase (SFK) pathway [65]. The SFK pathway stimulates signalling events in neuronal cell types, including activation of phospholipase C-gamma, AKT, and ERK. We found abnormal ERK signalling in both brain regions. Therefore, further studies are warranted to investigate the connection of NMDAR and VEGF signalling. Our findings suggest that one or more components of the VEGF signalling pathway might constitute a new therapeutic target for the treatment of SZ and potentially other psychiatric disorders.

This is also the first study linking NMDA-mediated glutamate dysfunction to decreased serum levels of IGF-1. At the circulatory level, IGF-1 promotes cell differentiation and growth and may also function as an anti-apoptotic agent [66]. Lower levels of IGF-1 have been found in serum of antipsychotic-naive [67] and antipsychotic-treated SZ patients [68], as well as in children with ASD [69-71]. A recent study reported a relationship between negative symptoms and IGF-1 plasma levels in first episode SZ [72]. Centrally, IGF-1 plays a major role in early brain development, neuro- and myelination processes [73-75]. Remarkably, IGF-1 treatment restores synaptic deficits in neurons from 22q11.2 deletion syndrome patients, a syndrome characterized by an increased risk of SZ and ASD [76], as well as in a SHANK3-deficient mouse model of autism [77]. Thus, we suggest that drugs which target the IGF-1 pathway should be evaluated for the treatment of psychiatric disorders associated with impaired glutamate function. One limitation of the multiplex immunoassay profiling stage of the study is the potential bias in the selection and the molecular class assignment of the investigated molecules. These assays were based on commercial availability and therefore only targeted selected classes of regulatory molecules. Therefore, it is possible that a different selection of molecules would lead to different conclusions from those drawn in this study.

Possibly reflecting IGF-1 function, we found an increase in synaptic markers in the frontal cortex and decreased levels of myelin-specific proteins in the frontal
### Table 2 Significantly changed proteins identified using label-based LC-SRM in the frontal cortex and hippocampus of the NR1neo−/− (n = 12) compared to wildtype mice (n = 12)

| Biological Pathway/Function                           | UP-ID  | Gene name | M | Frontal Cortex | Hippocampus |
|-------------------------------------------------------|--------|-----------|---|----------------|-------------|
|                                                        |        |           |   | TPP            | P            | P*          | LC-MS²      |
|                                                        |        |           |   | Ratio NR1/Wt   | P            | P*          |             |
| Purine metabolism                                      |        |           |   |                |              |             |             |
| Pathway analysis (LC-MS²)                              |        |           |   |                |              |             |             |
|                                                        |        |           |   |                |              |             |             |
| Hypoxanthine-guanine phosphoribosyltransferase         | P00493 | Hprt1     | 3 | 5|4            | 1.15        | 3.5 × 10⁻⁶  | 4.4 × 10⁻⁴  |
|                                                        |        |           | 1 | 4|5            | not significant | 4|4           | 1.24        | 0.0054  | 0.018   |
|                                                        |        |           |   |                |              |             |             |
| Glycolysis/Gluconeogenesis/Tricarbon acid cycle        |        |           |   |                |              |             |             |
| Pathway analysis (LC-MS²)                              |        |           |   |                |              |             |             |
|                                                        |        |           |   |                |              |             |             |
| Aspartate aminotransferase, mito.                      | P05202 | Got2      | 3 | 5|4            | 1.12        | 8.9 × 10⁻¹⁶ | 1.1 × 10⁻¹⁴ |
|                                                        |        |           | 1 | 5|7            | 1.18        | 1.2 × 10⁻⁰⁷ | 7.1 × 10⁻⁰⁷ |
| Pyruvate kinase, mito.                                 | P52480 | Pkm       | 3 | 4|5            | 1.0007      | 0.0059      |
|                                                        |        |           | 2 | 5|7            | 1.26        | 1.3 × 10⁻⁰⁶ | 8.3 × 10⁻⁰⁶ |
| NADH-ubiquinone oxidoreductase 75 kDa subunit, mito.   | Q91VD9 | Ndufs1    | 1 | 4|4            | 1.001       | 0.005       |
|                                                        |        |           |   | 8|4            | 1.28        | <×10⁻¹⁶     | <×10⁻¹⁶     |
| Neurotransmitter metabolism/transport                  |        |           |   |                |              |             |             |
| Pathway analysis (LC-MS²)                              |        |           |   |                |              |             |             |
|                                                        |        |           |   |                |              |             |             |
| Proline dehydrogenase 1, mito.                        | Q9WU79 | Prodh     | 3 | 5|4            | not significant | 2|2           | not significant |
|                                                        |        |           | 1 | 2|2            | not significant | 4|3           | not significant |
| Catechol O-methyltransferase                           | O88587 | Comt      | 1 | 4|2            | not significant | 3|3           | not significant |
| Glutamate decarboxylase 2                              | P48320 | Gad2      | 2 | 3|4            | 1.25        | 0.0004      | 0.0015      |
|                                                        |        |           |   | 3|4            | not significant | 5|5           | not significant |
| Vesicular glutamate transporter 1 (VGLuT1)             | Q3TXX4 | Slc17a7   | 3 | 5|5            | 1.14        | 0.0031      | 0.0192      |
|                                                        |        |           | 1 | 6|6            | 1.20        | <×10⁻¹⁶     | <×10⁻¹⁶     |
| 4-aminobutyrate aminotransferase, mito                 | P61922 | Abat      | 2 | 4|6            | 1.14        | 0.0006      | 0.0024      |
|                                                        |        |           |   | 4|6            | not significant | 5|6           | not significant |
| Clathrin-mediated exo-/endo-cytosis                    |        |           |   |                |              |             |             |
| Pathway analysis (LC-MS²)                              |        |           |   |                |              |             |             |
|                                                        |        |           |   |                |              |             |             |
| AP-2 complex subunit alpha-1                          | P17426 | Ap2a1     | 1 | 5|6            | 1.13        | 6.5 × 10⁻⁰⁸ | 5.2 × 10⁻⁰⁷ |
|                                                        |        |           |   | 6|6            | not significant | 5|6           | not significant |
| Synaptojanin                                           | Q8CHC4 | Synj      | 2 | 3|5|3            | 1.08        | 0.017       | 0.041       |
|                                                        |        |           |   | 5|5           | not significant | 5|5           | not significant |
| Synapsin-1                                             | O88935 | Syn1      | 1 | 5|6|3            | 1.11        | 0.005       | 0.016       |
|                                                        |        |           |   | 6|4|3           | not significant | 6|4|3          | not significant |
| Synaptotagmin-1                                        | P46096 | Syt1      | 2 | 6|7            | 1.09        | 0.001       | 0.004       |
|                                                        |        |           |   | 5|6           | 1.11        | 0.0016      | 0.0112      |

Note: TPP: total phosphoprotein; P: p-value; P*: adjusted p-value; LC-MS²: LC-MS² multiplex analysis.
Table 2 Significantly changed proteins identified using label-based LC-SRM in the frontal cortex and hippocampus of the NR51neo/− (n = 12) compared to wildtype mice (n = 12) (Continued)

### Neurotransmitter receptors

| Neurotransmitter receptors | P35438 Grin1 | P23818 Gria1 | P23819 Gria2 | Q92Z29 Gria3 |
|----------------------------|-------------|-------------|-------------|-------------|
| N-methyl-D-aspartate receptor subunit NR1 | 3 | 6 | 0.26 ▼ | <×10^-16 <×10^-16 |
| Glutamate receptor 1 (Glur-1) | 4 | 3 | | not significant |
| Glutamate receptor 2 (Glur-2) | 3 | 5|4|4 | not significant |
| Glutamate receptor 3 (Glur-3) | 3 | 4|2 | not significant |

### Long-term potentiation/Signal transduction

| Pathway analysis (LC-MS²) | IPA: ▼ Long term potentiation (of synapse) | IPA: ▼ Long term potentiation (of synapse) |
|---------------------------|-------------------------------------------|-------------------------------------------|
| CaM kinase II subunit alpha | Camk2a 3 | 3|4 | 0.90 ▼ | 0.0006 0.0056 ▼ *** 3|5 | not significant |
| CaM kinase II subunit beta | Camk2b 2 | 2|6 | not significant |
| CaM kinase II subunit gamma | Camk2g 3 | 4|4 | not significant |
| Calcineurin subunit B type 1 | Ppp3r1 2 | 6|6 | 0.90 ▼ | 0.0002 0.001 |
| Ser/thr-protein phosphatase 2B cat. subunit β | Ppp3cb 2 | 5|2 | 1.11 ▲ | 0.012 0.032 5|2 | not significant |
| Neurochondrin | Ncdn 2 | 4|3 | 1.21 ▲ | 0.017 0.083 |
| Disks large homolog 4 (PSD-95) | Dlg4 3 | 5|4 | not significant |

### ERK-Pathway

| ERK-Pathway | P62048 Pea15 | P63085 Erk2 | Q63844 Erk1 |
|-------------|-------------|-------------|-------------|
| Pathway analysis | 3 | 5|4 1.09 ▲ | 0.10 0.33 |
| Mitogen-activated protein kinase 1 (ERK-2) | 8|9 | 1.20 ▲ | <×10^-16 <×10^-16 4|6|6 0.94 ▲ | 0.0202 0.0685 |
| Mitogen-activated protein kinase 3 (ERK-1) | 3 | 4|4|4 | 1.10 ▲ | 0.03 0.11 4|4|4 | 1.14 ▲ | 0.0004 0.0015 |

**Note:** The table includes the protein name, gene ID, fold change, significance, and FDR correction for each protein identified in the NR51neo/− compared to wildtype mice.
Table 2 Significantly changed proteins identified using label-based LC-SRM in the frontal cortex and hippocampus of the NR1neo−/− (n = 12) compared to wildtype mice (n = 12) (Continued)

| Protein Name                  | Uniprot ID  | Method | TPP | Specific Activity | Fold Change | p Value 1   | p Value 2   | p Value 3   |
|-------------------------------|-------------|--------|-----|-------------------|-------------|-------------|-------------|-------------|
| |                          |            |        | M       |                   |             |             |             |             |
| Myristoylated alanine-rich C-kinase substrate | P26645      | Marcks | 2    | 6|4               | 1.22        | ▲ 8.4 × 10⁻⁶⁵ | 5.7 × 10⁻⁶⁴ | 4|4|6           | 1.41 | ▲ 1.1 × 10⁻⁰⁸ | 1.1 × 10⁻⁰⁷ | ▲(*) |
| PKC and casein kinase substrate in neurons protein 1 | Q61644      | Paccin1| 3    | 4|6               | not significant |             |             |             |
| Methionine aminopeptidase 2 (MAP 2) | OO8663      | Metap2 | 3    | 5|4               | not significant |             |             |             |
| Neurofilament light polypeptide (NF-L) | P08551      | Neff   | 2    | 3|4|3               | not significant |             |             |             |
| Vesicle-fusing ATPase         | P46460      | Nsf    | 3    | 5|6|6               | 1.07        | ▲ 0.008     | 0.047      | 5|6|5           | 1.10 | ▲ 0.0005      | 0.0018      |
| Neurmodulin (Axonal membrane protein GAP-43) | P06387      | Gap43  | 2    | 5|6               | 1.21        | ▲ 0.008     | 0.022      | 6|3|4           | 1.28 | ▲ 2.9 × 10⁻⁰⁹ | 3.6 × 10⁻⁰⁸ | ▲** |
| Neural cell adhesion molecule 1 (N-CAM-1) | P13595      | Ncam1  | 2    | 6|5|3               | 1.11        | ▲ 0.0003    | 0.001      | 6|5|3           | 1.11 | ▲ 0.0003      | 0.003       |
| 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase) | P16330      | Cnp    | 3    | 5|3|3               | 0.84        | ▼ 0.024     | 0.108      | 4|3|5           | not significant |   |
| Myelin basic protein (MBP)    | P04370      | Mbp    | 2    | 5               | not significant |             |             |             | S           | not significant |             |             |
| Myelin proteolipid protein (PLP) | P60202      | Plp1   | 2    | 5               | not significant |             |             |             | S           | 0.93 | ▼ 0.0127      | 0.0479      |
| Glial fibrillary acidic protein (GFAP) | P03995      | Gfap   | 3    | 3|4|3               | 1.23        | ▲ 0.002     | 0.017      | 3|4|3           | 1.29 | ▲ 1.8 × 10⁻¹⁰ | 9.9 × 10⁻¹⁰ | ▲* |
| Coronin-1A (Coronin-like protein A) | O89053      | Coro1a | 1    | 5               | not significant |             |             |             | S           | not significant |             |             |

IPA, Ingenuity pathway analysis; PSEA, Protein set enrichment analysis; UP-ID, uniprot-ID; M, method (assays were split into three methods); TPP, transitions per peptide; G, specific for glutamatergic neurons; B, specific for GABA-ergic neurons; N, specific for neurons; O, oligodendrocyte specific; A, astrocyte specific; M, microglia specific; ▲, increased, ▼, decreased. *, **, and *** ≤ 0.05, 0.01, and 0.001, respectively. P values were determined using SRMstats and corrected (P*) to control for multiple hypothesis testing [37].
cortex and to a lesser extent in the hippocampus (Table 2). Myelin integrity is crucial for functional neuro-circuitry and perturbations in myelin either during or after neuronal development leads to neurological deficits [78]. The findings are consistent with reports of abnormal myelination in BA10 of SZ, BD [79], and ASD patients [71,80]. Interestingly, effects on glutamate signalling have already been linked to oligodendrocyte dysfunction. Rat brains exposed prenatally to the NMDAR antagonist phencyclidine show reduced levels of oligodendrocyte progenitors [81], resulting in fewer differentiated mature oligodendrocytes capable of producing myelin. Our results provide further evidence of the role of glutamate and its receptors in white matter abnormalities and dysfunction in neurodevelopmental and psychiatric disorders.

Serum profiling also identified an overall decrease in chemokines (Table 1) generally associated with an anti-inflammatory status reflective of an anti-oxidative state [82], which is supported by an increase in glutathione S-transferase levels in the NR1neo−/− model. Centrally, chemokine signalling regulates essential processes for the establishment of neural networks such as neuronal migration and axon wiring [83]. Decreased chemokine functioning has been linked to deficits in social interaction and an increased repetitive behaviour phenotype, as reported in ASD and other neuropsychiatric disorders [84]. Furthermore, we detected decreased levels of leptin. Leptin facilitates hippocampal synaptic plasticity via enhanced NMDAR-mediated Ca2+-influx [85]. Impairment of this process might contribute to the cognitive deficits by inducing rapid alterations in hippocampal dendritic morphology and synaptic density [85].

The brain proteomic profiling study also highlights a link between NMDAR and purinergic signalling by identifying corresponding alterations in the hippocampus (Table 2 and Figure 3). Purines play a major role in neurotransmission and neuromodulation with their effects being mediated by the purine and pyrimidine receptor subfamilies P1, P2X, and P2Y. Purinergic signalling is associated with learning and memory [86,87] and locomotor activity, in line with the hippocampal specificity observed in our analysis. At a clinical level, it has been shown that antipsychotics, such as haloperidol, chlorpromazine, and fluspirilen, inhibit ATP-evoked responses mediated by P2X receptors [88,89]. Hypotheses of dysfunctional purinergic signalling have been put forward for psychiatric disorders [90] and ASD [91]. Applied to a maternal immune activation mouse model of ASD, anti-purinergic therapy has been found to reverse core social deficits and sensorimotor coordination abnormalities while, at the same time, normalizing ERK1/2 and CAMK2 signal transduction abnormalities [92]. ERK1/2 and CamK2 pathways are essential components of NMDAR-related signal transduction and were found to be increased, resp. decreased in the NR1neo−/− mouse.

The ERK signalling pathway comprises phosphorylation of proteins involved in transcriptional and translational regulation, dendritic arborisation, cellular excitability, long-term potentiation and depression, neuronal survival, synaptogenesis, and neurotransmitter release [93], and our findings indicated that all of these pathways were altered in the NR1neo−/− mouse. Upstream, ERK activation is regulated by the activity of dopamine, serotonin, and glutamate receptors [94], which are modulated by antipsychotics [95]. Antipsychotics have been shown to differentially mediate the ERK cascade in vitro and in vivo, dependent on cell and tissue type [96-99]. Clozapine differs from all other antipsychotics by recruiting the EGF-receptor to signal to ERK [100,101], which contributes to clozapine’s broad clinical phenotype. Consistent with this, we found evidence for elevated serum levels of EGF. Further evidence for the involvement of ERK signalling in the pathogenesis of psychiatric spectrum is provided by post mortem brain studies [102,103]. The ERK signalling pathway has also been implicated in the mechanism of action of mood stabilizers [104] and social behaviour [105], and extensively for ASD [106,107]. Interestingly, clozapine has also been shown to be efficacious in the treatment of ASD patients [108]. Remarkably, a recent study showed that the transcriptional regulation exerted by a diverse set of ASD-associated genes (FMR1, TSC1, PTEN, etc.) converges on ERK signalling [109].

With this comprehensive proteomic investigation, we found that the knockdown of one single protein can lead to multiple alterations in a range of signalling pathways both in the central nervous system as well as in blood serum. In brain tissue, we found pyruvate kinase to be one of the most robust changes in the NR1neo−/− mouse, consistent with studies showing increased levels of this key glycolytic enzyme in SZ [110] and in a phencyclidine rat model [111]. Furthermore, we found decreased levels of CamKIIα, which is associated with cognitive impairment [112]. Another prominent change was a 20 to 30% increase in MARCS in both regions. MARCS, regulated by calcium-calmodulin and PKC signalling, is a filamentous actin-crosslinking protein involved in cytoskeleton remodelling [113]. Hence, it is involved in the maintenance of dendritic spines and contributes to PKC-dependent morphological plasticity [114] and memory function [115,116]. Furthermore, MARCS is specifically degraded in response to intense NMDAR stimulation. Since the NR1neo−/− mouse expresses only 10% of the NR1 subunit, less NMDAR can be stimulated compared to the WT. This is consistent with our results of increased MARCS levels in the NR1neo−/− model [117]. MARCS has been implicated in proteomic studies of SZ.
patients [111] and decreased MARCS levels have been associated with both lithium and valporate treatment [118].

Conclusions
In summary, our results provide the first proteomic characterization of the NR1neo−/− mouse model to date, investigating both brain and serum changes associated with NMDAR hypofunction. We provide evidence for a strong link of neurotransmitter dysfunction and changes in circulating bioactive peptides and proteins, which are implicated in impaired brain function and synaptic remodelling. The results presented here provide novel insights into the molecular consequences of altered NMDAR function, such as SZ and ASD, and into the assumed disease mechanisms of psychiatric disorders in which perturbations of NMDAR function are likely to play an important role.

Taken together, the current findings provide further support that neuropsychiatric disorders present with prominent systemic changes affecting a wide range of tissues outside the brain which could represent diagnostic and surrogate markers for personalised medicine approaches in the field of psychiatry.

Additional files

Additional file 1: Table S2. Full information for all analytes measured using multiplexed immunocassay profiling.

Additional file 2: Table S1. Full information for all transitions measured in the multiplexed SRM-assay.

Additional file 3: Table S3. Biological classification of differentially expressed proteins identified in the frontal cortex and hippocampus of the NR1neo−/− mouse.

Additional file 4: Table S4. Information Ingenuity Pathway Analysis (IPA).

Abbreviations

ASD: Autism spectrum disorders; FDR: False discovery rate; GO: Gene ontology; NMDA: N-methyl-D-aspartate; NMDAR: NMDA-receptor; LC-MS: Liquid chromatography–mass spectrometry in expression mode; MARCS: Myristoylated alanine-rich C-kinase substrate; NR1neo−/− mouse: NMDA-receptor NR1 subunit knockdown mouse; SRM: Selected reaction monitoring; S2: Schizophrenia; UPLC: Ultra-performance liquid chromatography; WT: Wildtype.

Competing interests

SB is a consultant for Myriad-RBM. This does not affect policies regarding sharing of data and materials specified by this journal. None of the other authors declare a conflict of interest.

Authors’ contributions

HW carried out the label-free LC-MS experiments, designed and carried out the SRM experiments, and performed all statistical and bioinformatic data analyses. HW prepared the figures and tables and drafted the manuscript. SB, CML, and EFHW conceived the study and participated in its design and coordination. SB, PCG, and HR helped to interpret the results and drafted and edited the manuscript. All authors read and approved the final manuscript.

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Page 14 of 17

Wesseling et al. Molecular Autism 2014, 5:38
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