Urine Proteomic Analysis in a Rat Startle Model

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Abstract

Fear elicited by fright is a brain-generated emotion. We sought to investigate whether such fear emotion could be detected in urine through proteomic analysis. To this end, we established a rat fright model by combining predator odor and auditory stimulation, and collected urine samples before and after fright for proteomic analysis. Our results identified 22 differential proteins when comparing post-fright to pre-fright conditions. These differential proteins were enriched in biological pathways related to neurotransmitter transport and glucose transmembrane transport, potentially manifesting the neural tension state induced by fright. Self-controlled pre-post analysis of individual rats revealed that one protein was identified across all five rats, while an additional 19 proteins were identified in four rats. These proteins are closely associated with alterations in neural, motor, metabolic, and blood pressure regulation. Among these are the catalytic and regulatory subunits of glutamate-cysteine ligase, whose function may be implicated in fright response. These findings establish a foundation for investigating the mechanisms of fright, provide a novel methodology for identifying therapeutic targets for phobic disorders, and underscore the sensitivity of urine as a biomarker source, thereby opening new frontiers in urine-based research.

Full Text

Urine Proteomic Analysis of Rat Startle Model

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Abstract

The fear response elicited by startle is generated in the brain. This study employed urine proteomics to investigate whether this fear emotion could be detected in urine. We established a rat startle model using a combination of

predator odor and sound stimulation, collected urine samples before and after startle induction, and performed proteomic analysis. Comparing post-startle to pre-startle samples, we identified 22 differentially expressed proteins. Functional enrichment analysis revealed that these proteins were associated with biological pathways related to neurotransmitter transport and glucose transmembrane transport, which may reflect the state of nervous tension induced by startle. Individual before-after analysis of each rat revealed that one protein was consistently identified across all five rats, while 19 proteins were commonly identified in four of the five rats. These proteins were closely associated with changes in neural function, motor activity, metabolism, and blood pressure, including the catalytic and regulatory subunits of glutamate-cysteine ligase, suggesting a potential role for this enzyme in the startle response. These findings lay a foundation for investigating the mechanisms underlying startle responses, provide a novel approach for identifying therapeutic targets for psychological trauma, and demonstrate the remarkable sensitivity of urine as a biofluid, opening new avenues for urine-based biomarker discovery.

Keywords: urine; proteomics; startle model

Introduction

Startle is a common behavioral response in daily life, and the fear emotion it produces is a brain-generated reaction to this stimulus. As the proverb goes, "once bitten by a snake, one fears ropes for ten years." Startle responses of varying degrees can significantly impact our lives, yet no physiological indicators associated with startle have been identified to date, and no studies have examined startle-induced changes in urine. This study attempts to use urine proteomics to explore whether this fear emotion can be detected in urine.

In urine proteomics research, most studies utilize this approach to identify disease-related biomarkers. Compared to blood, the most widely used source of biomarkers, urine is a filtrate of blood that is not strictly regulated by homeostatic mechanisms, allowing it to accommodate and accumulate more extensive changes [1]. Moreover, urinary proteins can remain stable for extended periods [2], and the relatively low complexity of the urine proteome facilitates the detection of low-abundance protein alterations [3], making urine an excellent source of biomarkers. However, the urinary proteome is susceptible to various factors such as diet, medication, and daily activities. To ensure accurate experimental results, it is crucial to employ a simple and controllable system. Since genetic and environmental factors can be controlled in animal models to minimize extraneous influences, utilizing an animal model represents a highly suitable experimental approach.

Numerous studies have demonstrated the utility of urine in discovering biomarkers for neurological and psychiatric disorders, including Parkinson' s disease [4], Alzheimer' s disease [5], and depression [6]. Therefore, we established a rat startle model using combined predator odor and sound stimulation, collected urine

samples before and after startle induction, and performed proteomic analysis to explore whether fear emotions could be detected in urine.

Figure 1 [Figure 1: see original paper] Technical roadmap

2.1 Establishment of Startle Animal Model

Five healthy male Sprague Dawley (SD) rats ($160\pm20g$) were purchased from Beijing Vital River Laboratory An humidity 65%-70%) for three days before the experiment commenced. All experimental procedures were reviewed and approved by the Ethics Committee of the College of Life Sciences, Beijing Normal University.

The startle animal model was established as follows: Using a self-controlled before-after design, each of the five rats was individually placed in fresh cat litter mixed with bedding material and simultaneously exposed to high-intensity sound stimulation at the same time each day. The behavioral responses of the rats were recorded.

2.2 Urine Sample Collection

Prior to startle treatment, rats were placed in metabolic cages to collect 12-hour urine samples. After a three-day period in the standard environment, the startle experiment was conducted once daily for four consecutive days. On the fourth day, following startle treatment, rats were again placed in metabolic cages to collect 12-hour urine samples. During urine collection, rats were deprived of food and water. Both pre- and post-startle urine samples were stored at -80°C.

2.3 Urine Sample Processing

Urine Protein Extraction and Quantification: Urine samples collected at the two time points were centrifuged at 12,000×g for 40 min at 4°C. The supernatant was transferred to new EP tubes, and three volumes of ice-cold ethanol were added. After thorough mixing, proteins were precipitated overnight at -20°C. The following day, the ethanol-supernatant mixture was centrifuged at 12,000×g for 30 min at 4°C. The supernatant was discarded, and protein pellets were retained, inverted on filter paper, and dried with a cold-air blower. The protein precipitate was then resuspended in 120 L of lysis buffer (8 mol/L urea, 2 mol/L thiourea, 25 mmol/L dithiothreitol, 50 mmol/L Tris) by repeated pipetting until no precipitate remained, followed by complete mixing on a vortex mixer for 2 hours. After mixing, the solution was centrifuged at 12,000×g for 30 min at 4°C, and the supernatant containing proteins was transferred to new EP tubes. Protein concentration was measured using the Bradford method.

Urinary Protein Digestion: One hundred micrograms of urine protein sample were loaded onto the filter membrane of a 10 kDa ultrafiltration tube (Pall, Port Washington, NY, USA) placed in a 1.5 mL centrifuge tube, and 25 mmol/L NH4HCO3 solution was added to bring the total volume to 200 L. Subsequently, 20 mM dithiothreitol solution (DTT, Sigma) was added. After vortex mixing,

the sample was heated at 97°C in a metal bath for 5 min and then cooled to room temperature. Fifty mM iodoacetamide (IAA, Sigma) was added, mixed briefly by pulsed centrifugation, and incubated at room temperature in the dark for 40 min. The following membrane washing steps were then performed: (1) 200 L of UA solution (8 mol/L urea, 0.1 mol/L Tris-HCl, pH 8.5) was added, and the sample was centrifuged at 14,000×g for 5 min at 18°C, repeated twice; (2) sample loading: the processed sample was added and centrifuged at 14,000×g for 40 min at 18°C; (3) 200 L of UA solution was added and centrifuged at 14,000×g for 40 min at 18°C, repeated twice; (4) 25 mmol/L NH4HCO3 solution was added and centrifuged at 14,000×g for 40 min at 18°C, repeated twice; (5) trypsin (Trypsin Gold, Promega, Fitchburg, WI, USA) was added at a 1:50 enzyme-to-protein ratio for digestion, followed by overnight incubation in a 37°C water bath. Finally, desalting was performed using HLB columns (Waters, Milford, MA), and samples were dried in a vacuum concentrator and stored at -80°C.

2.4 LC-MS/MS Tandem Mass Spectrometry Analysis

Digested samples were resuspended in 0.1% formic acid, and peptide quantification was performed using a BCA assay kit. Peptide concentration was diluted to 0.5 g/ L. Nine microliters of each sample were pooled to create a mixed peptide sample, which was fractionated using a high-pH reversed-phase peptide fractionation kit (Thermo Fisher Scientific) according to the manufacturer' s instructions. Ten fractions of eluent were collected by centrifugation, dried in a vacuum concentrator, and resuspended in 0.1% formic acid. iRT standards (Biognosis) were added at a 10:1 sample-to-iRT volume ratio.

For each sample (individual experimental samples and the ten fractions), 1 g was analyzed using an EASY-nLC 1200 chromatography system (Thermo Fisher Scientific, USA) coupled with an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, USA) for data acquisition.

2.5 Data Processing

Raw files from the ten fractions were analyzed using Proteome Discoverer 2.1 (PD) software, and the results were used to establish a DIA acquisition method. The newly developed DIA method was employed for DIA-mode acquisition of individual samples. Following acquisition, Spectronaut X software was used for processing and analysis of the mass spectrometry data. Raw files from DIA acquisition of each sample were imported for database searching. Protein quantification was performed using the peak area of all fragment ions from secondary peptides.

2.6 Data Analysis

A self-controlled design was employed to compare proteins identified before and after startle for differential protein screening. The criteria for differential pro-

teins were: fold change (FC) $ $1.5 or $ $0.67, and P-value <0.01 from paired t-test analysis.

Functional analysis of the screened differential proteins was performed using the UniProt website (https://www.uniprot.org/) and DAVID database (https://david.ncifcrf.gov/). Published literature was searched in the PubMed database to conduct functional analysis of the differential proteins.

3.1 Analysis of Urinary Proteome Changes Before and After Startle

(1) Urinary Protein Identification and Unsupervised Clustering Analysis

Following establishment of the rat startle model, a total of ten urinary protein samples collected before and after startle were analyzed by LC-MS/MS tandem mass spectrometry. A total of 1,294 proteins were identified (with $ $2 unique peptides and protein-level FDR <1%). Unsupervised clustering analysis of all identified proteins was performed, and the results are shown in Figure 2 [Figure 2: see original paper]. The clear separation between pre- and post-startle groups demonstrates that urinary proteomics can significantly distinguish changes induced by startle.

Comparing urinary proteins before and after startle, differential proteins were screened using the following criteria: fold change (FC) $ $1.5 or $ $0.67, and two-tailed unpaired t-test P<0.01. The results showed that 22 differential proteins were identified when comparing post-startle to pre-startle samples. Detailed information on these differential proteins is provided in Table 1 .

Table 1 Details of differential proteins

Accession	Protein names	Fold change	Trend	P value
F1LUS1	Ig-like domain-containing protein	1.81E-03	-	-
P61972	Nuclear transport factor 2	1.40E-04	-	-
G3V8Z5	Ig-like domain-containing protein	9.83E-03	-	-
D4A9Q5	Carboxypeptidase M	6.28E-03	-	-
Q6AXR4	Beta-hexosaminidase subunit beta	4.21E-03	-	-
P85971	6-phosphogluconolactonase	1.70E-03	-	-

Accession	Protein names	Fold change	Trend	P value
A0A0G2K3W2	Coagulation factor V	9.50E-03	-	-
G3V6V1	Aminopeptidase B	1.79E-03	-	-
Q7TPK2	Ac2-120	7.45E-03	-	-
G3V647	Pyridoxal kinase	5.73E-03	-	-
P63029	Translationally-controlled tumor protein	7.57E-03	-	-
P48500	Triosephosphate isomerase	3.46E-03	-	-
Q99MA2	Xaa-Pro aminopeptidase 2	5.82E-04	-	-
Q5XI73	Rho GDP-dissociation inhibitor 1	6.86E-03	-	-
P42854	Regenerating islet-derived protein 3-gamma	9.73E-03	-	-
P62749	Hippocalcin-like protein 1	6.00E-04	-	-
Q9ESG3	Collectrin	1.43E-03	-	-
P46413	Glutathione synthetase	6.50E-03	-	-
P29975	Aquaporin-1	7.57E-04	-	-
Q64319	Neutral and basic amino acid transport protein rBAT	7.72E-03	-	-
G3V8X5	Solute carrier family 5	5.49E-03	-	-
A0A0G2JT43	Solute carrier family 2	4.98E-03	-	-

(2) Functional Analysis of Differential Proteins

To investigate the functions of these differential proteins, we performed functional analysis of biological pathways using the DAVID database. The results indicated that most pathways reflected metabolic changes, such as urate metabolism and transport, CO2 transport, and organic anion transport. Notably, these differential proteins were also enriched in biological pathways related to neurotransmitter transport and glucose transmembrane transport, which may represent manifestations of nervous tension induced by startle and demonstrate the sensitivity of urine.

Figure 3 [Figure 3: see original paper] Biological process analysis of differential proteins

3.2 Individual Rat Urinary Proteome Analysis Before and After Startle

To investigate the consistency of changes across the five rats, we performed individual before-after urinary proteomic analysis for each rat. Each rat' s post-startle samples were compared to its pre-startle samples. Differential protein screening criteria were: FC $ $2 or $ $0.5, and two-tailed unpaired t-test P<0.01. The screening results were as follows: Rat #1 yielded 132 differential proteins (44 upregulated, 88 downregulated); Rat #2 yielded 79 differential proteins (33 upregulated, 46 downregulated); Rat #3 yielded 215 differential proteins (38 upregulated, 177 downregulated); Rat #4 yielded 91 differential proteins (22 upregulated, 69 downregulated); and Rat #5 yielded 134 differential proteins (94 upregulated, 40 downregulated). We generated a Venn diagram to display the differential proteins identified across the five rats (Figure 4 [Figure 4: see original paper]). One protein was commonly identified in all five rats: glutamate-cysteine ligase regulatory subunit (P48508). Additionally, 19 proteins were commonly identified in four of the five rats. Detailed information and relevant research on these 20 proteins and their human homologs are listed in Table 2 . These differential proteins were closely associated with changes in neural function, motor activity, metabolism, and blood pressure. Notably, both the regulatory and catalytic subunits of glutamate-cysteine ligase were commonly identified in five and four rats, respectively, suggesting that the observed differences are more likely attributable to startle rather than individual variation among experimental animals.

Figure 4 [Figure 4: see original paper] Venn diagram of differential proteins in individual rats

Table 2 The co-identified differential proteins (homo sapiens)

UniProt ID	Protein names	Relevant research
P48507	Glutamate–cysteine ligase regulatory subunit	-
Q07837	Neutral and basic amino acid transport protein rBAT	-
P15104	Glutamine synthetase	Epileptogenesis [7]
A0PJK1	Sodium/glucose cotransporter 5	-
P48506	Glutamate–cysteine ligase catalytic subunit	-
A8K7I4	Calcium-activated chloride channel regulator 1	-
Q9Y6R7	IgGFc-binding protein	-
P08118	Beta-microseminoprotein	-
Q3LXA3	Triokinase/FMN cyclase	-
P07098	Gastric triacylglycerol lipase	-
Q9Y2S2	Lambda-crystallin homolog	-
O00159	Unconventional myosin-Ic	-

UniProt ID	Protein names	Relevant research
Q9H4M9	EH domain-containing protein 1	-
P21399	Cytoplasmic aconitate hydratase	oxidative stress [8]
P60709	Actin, cytoplasmic 1	-
P49189	4-trimethylaminobutyraldehyde dehydrogenase	-

4 Conclusion

In this study, we established a rat startle model using combined predator and sound stimulation. Through label-free LC-MS/MS identification and analysis of urine collected before and after startle, we investigated whether fear emotions induced by startle could be detected in urine using proteomic methods. Statistical analysis revealed 22 differential proteins when comparing post-startle to pre-startle samples. These proteins were enriched in biological pathways related to neurotransmitter transport and glucose transmembrane transport, which may reflect the state of nervous tension induced by startle. Through individual before-after urinary protein analysis, we found that the regulatory and catalytic subunits of glutamate-cysteine ligase were commonly identified in five and four rats, respectively. Glutamate-cysteine ligase appears to be associated with the mechanisms underlying startle, while other co-identified proteins were also related to changes in neural function, motor activity, metabolism, and blood pressure. These findings establish a foundation for investigating startle mechanisms, provide a novel approach for identifying therapeutic targets for psychological trauma, and demonstrate the remarkable sensitivity of urine, opening new avenues for urine-based exploration.

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Author Contributions Statement

Youhe Gao: Conceptualized the study, designed the research plan, and revised the final manuscript.
Chenyang Zhao: Conducted experiments, drafted the manuscript, collected and analyzed data.
Yuqing Liu: Conducted experiments and collected and analyzed data.

Note: Figure translations are in progress. See original paper for figures.