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Urinary Proteomic Analysis of Youth with Gaming Disorder

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Abstract

Video game addiction manifests as a growing enthusiasm for digital games and uncontrolled use, yet lacks objective diagnostic indicators. This study employed mass spectrometry-based proteomics to analyze urinary proteome differences between adolescents with gaming addiction and non-gaming adolescents. A total of 10 adolescents with gaming addiction and 9 non-gaming adolescents were enrolled as the control group. The results revealed 125 significantly differentially expressed proteins between the two groups, among which 11 proteins have been reported to undergo changes following psychotropic drug administration and are associated with addiction: Calmodulin, ATP synthase subunit alpha, ATP synthase subunit beta, Acid ceramidase, Tomoregulin-2, Calcitonin, Apolipoprotein E, Glyceraldehyde-3-phosphate dehydrogenase, Heat shock protein beta-1, CD63 antigen, Ephrin type-B receptor 4, Tomoregulin-2. Additionally, several proteins were found to interact with addiction-related pathways: Dickkopf-related protein 3, Nicastrin, Leucine-rich repeat neuronal protein 4, Cerebellin-4. In the enriched biological pathways, nitric oxide synthase-related pathways, amphetamine addiction-related pathways, and many calcium ion pathways, among other addiction-related pathways, were identified. Furthermore, through analysis of differentially expressed proteins, we hypothesized several understudied proteins that may play important roles in addiction mechanisms: Protein kinase C and casein kinase substrate in neurons protein, Cysteine-rich motor neuron 1 protein, Bone morphogenetic protein receptor type-2, Immunoglobulin superfamily member 8. Analysis of urinary proteins from adolescents with online gaming addiction revealed multiple proteins previously reported in drug addiction research.

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Preamble

Proteomic Analysis of Urine from Youths with Gaming Addiction

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Video game addiction manifests as an escalating enthusiasm and uncontrolled use of digital games, yet there are no objective indicators for gaming addiction. This study employed mass spectrometry proteomics to analyze the proteomic differences in the urine of adolescents addicted to gaming compared to those who do not play video games. The study included 10 adolescents addicted to gaming and 9 non-gaming adolescents as a control group. The results showed that there were 125 significantly different proteins between the two groups. Among these, 11 proteins have been reported to change in the body after the intake of psychotropic drugs and are associated with addiction: Calmodulin, ATP synthase subunit alpha, ATP synthase subunit beta, Acid ceramidase, Tomoregulin-2, Calcitonin, Apolipoprotein E, Glyceraldehyde-3-phosphate dehydrogenase, Heat shock protein beta-1, CD63 antigen, and Ephrin type-B receptor 4. Additionally, several proteins were found to interact with pathways related to addiction: Dickkopf-related protein 3, Nicastrin, Leucine-rich repeat neuronal protein 4, and Cerebellin-4. Enriched biological pathways discovered include those related to nitric oxide synthase, amphetamine addiction, and numerous calcium ion pathways, all of which are associated with addiction. Moreover, through the analysis of differentially expressed proteins, we speculated about some proteins not yet fully studied, which might play a significant role in the mechanisms of addiction: Protein kinase C and casein kinase substrate in neurons protein, Cysteine-rich motor neuron 1 protein, Bone morphogenetic protein receptor type-2, and Immunoglobulin superfamily member 8. In the analysis of urinary proteins in adolescents addicted to online gaming, we identified several proteins that have previously been reported in studies of drug addiction.

Keywords: urine; proteomics; addiction; video games

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1. Introduction

Video game addiction, an increasingly concerning issue in modern society, has attracted extensive research attention from experts and scholars worldwide. In recent years, with the rapid development of internet technology and the flourish-

ing gaming industry, an increasing number of people, particularly adolescents, have become addicted to electronic games, leading to a series of psychological, social, and physiological problems [**?**]. In psychiatry, gaming addiction is regarded as an impulse control disorder characterized by intense cravings for gaming, inability to control gaming behavior, loss of interest in other activities, and negative consequences from persistent gaming [**?**]. These features not only severely impact individual health but may also disrupt social relationships and quality of life. According to a global study, video game addiction is associated with various mental health problems, including depression, anxiety, and social dysfunction [**?**]. Furthermore, neuroimaging studies have found that, similar to substance dependence, video game addiction can also cause changes in brain structure and function [**?**, **?**]. The formation of video game addiction may be related to multiple factors, including individual psychological traits, social environment, and game design elements [**?**]. Research indicates that reward systems in games, social interactions, and immersive experiences are key factors that promote addictive behavior [**?**].

Given the severity and prevalence of video game addiction, understanding its mechanisms and impacts is crucial for developing effective prevention and intervention strategies. Future research should continue to explore the neurobiological basis of gaming addiction and how to effectively address this challenge through psychological and social interventions.

Urine proteomics has demonstrated significant potential in the discovery and analysis of biomarkers. Compared with other biological samples, urine has unique advantages: it is not strictly regulated by homeostatic mechanisms, thus reflecting subtle biochemical changes in the body more sensitively [**?**]. Additionally, urine collection is non-invasive and straightforward, making it an ideal source for biomarkers. Numerous studies have confirmed that proteins in urine can serve as biomarkers for various neurological diseases, such as Parkinson' s syndrome [**?**], Alzheimer' s disease [**?**], depression [**?**], and autism [**?**]. However, in the field of urine proteomics, there have been no studies targeting gaming addiction. This study not only broadens our understanding of the mechanisms underlying video game addiction but also holds promise for helping develop novel therapies for such conditions. Therefore, we conducted a urinary proteomics study on youths addicted to gaming, with the technical route shown in Figure 1 [Figure 1: see original paper].

2.1 Sample Collection

This study collected urine samples from 10 youths addicted to gaming (average age 22 years) and 9 male youths who do not play games (average age 24 years) as controls. Sampling locations included internet cafes and participants' homes. The sampling criterion was moderate internet addiction according to Young' s Diagnostic Questionnaire for Internet Addiction, with all subjects ex-

periencing psychological discomfort when not playing games and daily gaming duration of 6-9 hours. All volunteers signed informed consent forms; this experiment provided participants with detailed information about the study, including its purpose, methods, and procedures, while maintaining strict confidentiality of participants' personal data. There were no restrictions on volunteers' diet, medication, or other factors, and collected urine samples were stored at -80°C. Detailed sample information is provided in Table 1 .

2.2 Urine Sample Processing

Urine samples were first centrifuged at 12,000g for 30 minutes at 4°C. Then, 6 mL of urine was taken from each sample, and proteins were precipitated from 15 mL of urine with five volumes of ethanol overnight at -20°C. After centrifugation at 12,000g, the protein precipitate was dissolved in lysis buffer (8 mol/L urea, 2 mol/L thiourea, 50 mmol/L Tris, and 25 mmol/L dithiothreitol). Protein concentration in the supernatant was then quantified using the Bradford assay.

A total of 100 g of protein was digested with trypsin. Proteins from each sample were loaded onto a 10-kDa filtration device. After washing twice with urea buffer and 25 mmol/L NH4HCO3 solution, proteins were reduced with 20 mmol/L dithiothreitol at 37°C for 1 hour, and alkylated with 50 mmol/L iodoacetamide (IAA) in the dark for 45 minutes. Samples were then washed with UA and NH4HCO3, and digested with trypsin (enzyme-to-protein ratio of 1:50) overnight at 37°C for 14-16 hours.

Digested peptides were desalted using an Oasis HLB kit and then dried using a lyophilizer. The digested peptides were dissolved in 0.1% formic acid and subsequently diluted to a concentration of 0.5 g/ L.

2.3 LC-MS/MS Tandem Mass Spectrometry Analysis

The separated protein samples were ionized and introduced into the mass spectrometer. Each sample was analyzed with 1 g of peptide mass: loaded onto a pre-column and analytical column using a Thermo EASY-nLC1200 chromatography system. Proteomic data were acquired using a Thermo Orbitrap Fusion Lumos mass spectrometry system (Thermo Fisher Scientific, Bremen, Germany).

2.4 Data Processing and Analysis

Each peptide sample underwent mass spectrometry data acquisition in DIA mode. Processing and analysis of the mass spectrometry data were completed

using Spectronaut X software. Database searching was performed on the raw files from DIA acquisition for each sample.

We applied spectral counting for quantitative analysis to screen for differential proteins. Screening criteria: Fold change $ $1.5 or $ $0.67, and adjusted P-value from paired t-test analysis <0.05. Subsequent functional analysis of differential proteins was performed through DAVID Functional Annotation Bioinformatics Microarray Analysis and literature retrieval from the PubMed database.

3.1 Urinary Proteome Analysis

After processing the urine samples, LC-MS/MS tandem mass spectrometry analysis was performed on 19 protein samples. A total of 1,205 proteins were identified (with $ $2 unique peptides and protein-level FDR < 1 $1.5 or $ $0.67, P<0.05, with detailed information listed in Table 2 .

Among the differential proteins meeting the criteria (FC$ $1.5 or $ $0.67, P<0.05), PubMed searches revealed 11 proteins previously reported to be associated with addiction: ATP synthase subunit alpha, ATP synthase subunit beta, Glyceraldehyde-3-phosphate dehydrogenase, Acid ceramidase, Calcitonin, Apolipoprotein E, Calmodulin-1, Ephrin type-B receptor 4, Heat shock protein beta-1, CD63 antigen, and Tomoregulin-2.

Our study identified a series of differential proteins that exhibit unique changes under different drug addiction conditions. For example, ATP synthase subunit alpha, a key component of F-type ATP synthase [**?**], shows decreased expression in rat striatum after chronic morphine treatment [**?**]. ATP synthase subunit beta also shows reduced expression in the nucleus accumbens of cocaine overdose victims [**?**, **?**]. Another key protein—Glyceraldehyde-3-phosphate dehydrogenase—demonstrates significant expression changes after 10 days of morphine exposure and 20 days of withdrawal [**?**]. Wenchao Li and colleagues used high-throughput strand-specific RNA sequencing to study how cocaine affects gene expression in the mouse insular cortex. Their GO enrichment analysis revealed cocaine' s regulatory effect on ceramidase activity in synaptic transmission [**?**], while Acid ceramidase in our experimental group was 2.5 times that of the control group. Additionally, studies have shown that serum Calcitonin levels significantly increase after cocaine withdrawal [**?**].

Research on methamphetamine addiction reveals that acute exposure to this drug increases Apolipoprotein E (ApoE) protein expression levels in brain parenchyma. This increase activates ApoE receptor-2 on brain capillaries and affects LTP expression [**?**]. Michelhaugh' s research further indicates that methamphetamine can significantly increase calmodulin content and messenger RNA levels in rat brain regions [**?**, **?**]. Comparative studies between methamphetamine withdrawal individuals (WMA) and healthy controls (HC) found significant differences in Ephrin type-B receptor 2 (EPHB2) expression

[?], suggesting EPHB2 may play a role in regulating glutamate metabolic pathways. Studies on the effects of methamphetamine withdrawal on brain stress systems and cardiac sympathetic pathway activation found that methamphetamine withdrawal increases Heat shock protein beta-1 levels in the heart [?]. Furthermore, compared with healthy controls, CD63 antigen expression is significantly reduced in platelets of heroin, cocaine, and marijuana addicts [?].

Tomoregulin-2 can promote ERK1/2 phosphorylation, while extracellular signal-regulated kinase (ERK) has been proven to be activated by opioids and is functionally related to addiction [?]. Its family member Tomoregulin-1 forms a complex with addicsin (a protein highly upregulated in the amygdala of morphine-administered mice), affecting Tomoregulin-1' s intracellular distribution and regulating cell migration, thereby demonstrating that Tomoregulin-1 is a novel addicsin-related factor [?]. Both Tomoregulin-1 and Tomoregulin-2 belong to the TMEFF family, and Tomoregulin-2 has been shown to be highly expressed in all brain regions except the pituitary gland, particularly in the amygdala and corpus callosum. It serves as a survival factor for hippocampal and mesencephalic neurons [?] and is present in Alzheimer' s disease plaques [?].

Notably, Tomoregulin-2 may inhibit Bone Morphogenetic Proteins (BMP) signaling during neural pattern formation [?]. BMP plays a crucial role in the central nervous system, particularly in neural plasticity. They function at multiple stages of central nervous system development, including brain and spinal cord formation and patterning. In the adult brain, BMP is detected in regions associated with neural plasticity and has been shown to regulate neurogenesis, gliogenesis, and synaptic and dendritic plasticity [?, ?]. These findings indicate that BMP is essential for the dynamic adjustment of brain structure and function to adapt to new experiences and learning processes. Meanwhile, we identified Cysteine-rich motor neuron 1 protein and Bone morphogenetic protein receptor type-2 among our differential proteins, which regulate BMP signaling in hippocampal cells and neural centers, respectively. We speculate these two proteins may play important roles in addiction mechanisms.

Interestingly, we simultaneously identified both ATP synthase subunit alpha and ATP synthase subunit beta among our differential proteins. ATP synthase is a key intracellular enzyme whose primary function is to catalyze ATP synthesis. This complex enzyme structure consists of multiple subunits, with subunits alpha and beta playing core roles in its F1 portion, which is crucial for ATP synthesis [?]. Subunit alpha, as a major structural component of ATP synthase located in the F1 portion, typically contains three alpha subunits per ATP synthase complex. Although the alpha subunit does not directly participate in ATP synthesis, it plays a key role in maintaining the structural stability and normal function of the entire enzyme complex. It alternates with beta subunits to form a ring structure, providing necessary structural support for the catalytic center of ATP synthesis [?]. Subunit beta, another key component located in the F1 portion, also contains three beta subunits. Unlike the alpha subunit, the beta subunit directly participates in ATP synthesis and contains the active

catalytic site. During ATP synthase operation, the beta subunit undergoes multiple conformational changes that are crucial for ATP synthesis and release.

In addition to ATP synthase, Apolipoprotein E is closely related to energy metabolism. Other protein changes are also noteworthy. Immunoglobulin superfamily member 8 (IgSF8) interacts with integrins [**?**], which are extracellular matrix receptors that mediate bidirectional biochemical and mechanical signals between extracellular and intracellular environments through allosteric conformational changes. In the brain, they exist in neurons and glial cells and play important roles in several aspects of brain development and function, such as cell migration, axon guidance, synaptogenesis, synaptic plasticity, and neuroinflammation. Integrins have also been reported to be associated with addiction [**?**]. Furthermore, Immunoglobulin superfamily member 21 interacts with neurexins [**?**], which are also related to addictive behavior [**?**]. Based on these findings, we can speculate that IgSF8 may play an important role in the addiction process.

Dickkopf-related protein 3 locally inhibits Wnt-regulated processes [**?**], while the Wnt pathway plays an important role in midbrain dopaminergic system development, with dopaminergic neurons influencing neurological diseases such as addiction [**?**]. Additionally, UniProt analysis suggests Nicastrin plays a role in regulating the Wnt signaling pathway and downstream processes through similarity analysis.

Protein kinase C and casein kinase substrate in neurons protein 3 was only 0.13 times that of the control group. It plays a role in endocytosis and regulates the internalization of plasma membrane proteins [**?**]. In adulthood, PACSIN1 has been extensively studied in the brain and has been shown to regulate neural morphogenesis, receptor trafficking, and synaptic plasticity [**?**].

Our research suggests that there may be common biological mechanisms between gaming addiction and drug addiction. This is supported by the shared changes in addiction-related proteins and neurotransmitter pathways. This similarity indicates that despite different addiction objects (such as games or drugs), they may follow similar patterns at the biological level.

3.2 Enrichment Pathway Analysis

Through DAVID Functional Annotation Bioinformatics Microarray Analysis, we enriched pathways related to amphetamine addiction, response to corticosterone, calcium ion-related processes, and nitric oxide synthase, as shown in Tables 3 and 4 .

Using Gene Ontology (GO) analysis, we discovered a striking phenomenon: many calcium ion-related pathways were identified, including negative regulation of cellular calcium ion export, negative regulation of calcium ion transmembrane transporter activity, regulation of high voltage-gated calcium channel activity, positive regulation of ryanodine-sensitive calcium-release channel

activity, regulation of calcium-mediated signaling, response to calcium ion, and regulation of release of sequestered calcium ion into cytosol by sarcoplasmic reticulum.

Long-term potentiation (LTP) is a key neurobiological process that leads to strengthened synaptic connections and plays a crucial role in learning and memory formation. A key trigger for LTP is the increase in calcium ion (Ca2+) concentration in the postsynaptic region. This Ca2+ increase activates a series of complex intracellular signaling pathways. Specifically, elevated Ca2+ triggers cyclic adenosine monophosphate (cAMP) production through activation of adenylate cyclase. Subsequently, cAMP activates protein kinase A (PKA), which in turn leads to sustained increases in glutamate release from nerve terminals upon each action potential arrival. LTP also plays an important role in neuroadaptations associated with addiction. Research shows that abused drugs can induce LTP in brain reward circuits, particularly in the ventral tegmental area (VTA), a key region closely related to addiction development. Notably, presynaptic LTP is primarily triggered by activity-dependent increases in Ca2+ within presynaptic terminals [**?**, **?**].

Among our differential proteins, we particularly noted Cerebellin-4 (Cbln4). Its high expression in the entorhinal cortex is important because it plays a crucial role in synaptic LTP from olfactory cortex to dentate gyrus. Specifically, presynaptic Cbln4 binds to postsynaptic neogenin-1, a process necessary for LTP at olfactory cortex-dentate gyrus synapses [**?**]. Based on this finding, we speculate that Cerebellin-4 may also play a key role in the addiction process. Additionally, Leucine-rich repeat neuronal protein 4 functions in hippocampus-dependent persistent memory [**?**], suggesting it may play an important role in addiction.

We also enriched for positive regulation of nitric-oxide synthase activity in our pathways. Nitric oxide, as an important neurotransmitter, plays a key role in the central nervous system. Numerous studies indicate that nitric oxide may play an important role in drug addiction (including dependence on opioids, alcohol, stimulants, and nicotine). Notably, inhibitors targeting nitric oxide synthase have been found to effectively regulate withdrawal symptoms induced by these addictive substances [**?**].

Significantly, gaming addiction shows substantial correlation with amphetamine addiction pathways in terms of protein expression patterns in brain tissue. This finding reveals that gaming addiction and drug addiction may affect the same neurobiological pathways, which have been extensively studied in more widely recognized addictive substances such as amphetamine. Our results provide new perspectives on how video games affect the brain at the molecular level and offer valuable clues for further research on how caffeine and other stimulants affect brain function and addictive behavior.

These findings demonstrate the sensitivity of urine in reflecting addiction status, providing new perspectives for understanding the nature of addiction. The text

mentions changes in calcium ions, nitric oxide synthase, and ATP synthase, all of which have been proven by many studies to be related to addiction. Notably, other proteins and pathways not previously studied in the addiction field may play important roles in addiction mechanisms, and this paper provides reliable clues.

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4. Conclusion

Adolescents addicted to online gaming show significant differences in urinary protein composition compared to non-gamers. Among the differential proteins, we identified multiple proteins previously reported in drug addiction research.

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