

dipeptidase activity. The calcium ion-binding go-molecular function *Ncald* was significantly up-regulated. In addition, six genes, *Pcdh11x*, *Pcdh9*, *Pcdh1*, *Sell*, *Pkdrej*, and *Srap*, were associated with the function of calcium ion binding; however, the GO enrichment analysis did not identify these six genes. The horizontal axis represented the enrichment degree, whereas the vertical axis represented the enriched GO Terms for the top 20 GO terms with the highest enrichment degree of up-regulated genes (Fig.3).

Ace showed significant enrichment in the Renin-angiotensin system, which regulated angiotensin-converting enzyme, according to the KEGG pathway analysis (Fig.4). Both MAPK signaling pathway and phosphoinositide 3-kinase/ protein kinase B (P13K/AKT) signaling pathway were expressed differentially in the anesthetic group, including the up-regulated gene *Nr4a2*. KEGG pathway analysis showed that only the Renin-angiotensin system was significantly enriched with one up-regulated gene, *Ace*, which regulated angiotensin-converting enzyme.

4. Discussion

W. pigra, the research object of this experiment, has crucial commercial and scientific value. *W. pigra* is an emerging aquaculture species and also a crucial model for the study of neurophysiology, developmental, and regenerative biology [12-13]. The rarity of anesthesia research on leeches hinders their culture and scientific research. In anesthesia research, it is essential to classify the different stages of anesthesia precisely. The stages of anesthesia in Largemouth Bronze Gudgeon *Coreius guichenoti* could be distinguished by several characteristic behaviors, such as loss of tactile sensation, opercular movement frequently, and swimming posture, among others [14]. Frogs can be evaluated for the effectiveness of anesthesia using methods based on their physiological structure, such as heart rate or acetic acid test [15]. These evaluation indicators were not entirely applicable due to the unique physiology of leeches. During the anesthetization of leeches, the swimming speed, the ability to maintain stability, the suction force of the suction cup, the ability to restore normal

genes, *Sik1* and *Sik2*, were found in the SZM group. These might trigger changes in sodium ion concentration to achieve the effect of anesthesia [32,33], since the *Sik1* network could mediate sodium-independent signaling through calcium-dependent processes, thereby regulating Na⁺/K⁺-ATPases activity [34,35]. Three genes in the Protocadherin family, *Pcdh11x*, *Pcdh9*, and *Pcdh1*, containing the EC domains and being potential calcium-dependent cell-adhesion proteins, were found to be differentially expressed [36,37]. Certain protocadherins contributed to the establishment of neuronal connections and signal transduction at synaptic membranes [38]. Since neurons communicated through synapses, many protocadherins appeared to be synaptic components whose combinatorial expressions influenced synaptic function. It was speculated that the up-regulation of *Ncald* in the SZM group affected neurotransmitter release by regulating Ca²⁺ channels [39-41], leading to changes in specific aspects of neuron function [42], altering intracellular calcium concentration, and causing an anesthesia effect. *Girk3* was up-regulated in the SZM group, a family characterized by its preference for potassium influx into cells rather than outflow cells, suggesting that it may regulate resting potential, neuronal excitability, and neurotransmitter release [43,44]. The anesthetic effect of tricaine on leeches might be the combined effect of these genes, exerting its effects by affecting signal transduction, affecting the release of neurotransmitters, and changing the concentration of sodium ions. The mechanism required further experimental research and analysis.

Our data suggested that tricaine was effective at anesthetizing *W. pigra*. To avoid irreparable damages, we recommend using 2 g/L of tricaine for sedation and transportation, as well as 4 g/L or higher for anatomy experiments and surgical procedures. Tricaine is a safe anesthetic with minimal effects on the cocoon production of *W. pigra* and the RNA expression levels of *W. pigra* housekeeping genes, key signaling pathways, and physiological functions. Our results are crucial for the development of experimental anesthesia protocols and will facilitate the transportation and aquaculture of leeches, laying the foundation for future experimental research.