TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2017) 41: 414-424

doi:10.3906/vet-1608-76

Effects of ketamine on monoamine neurotransmitters and GABA in embryonic neurocytes from fetal rat

Xinran LI, Mo LIU, Yue LI, Cen GUO, Lina LI, Yanan LI, Yuxin WANG, Yiming ZHANG, Li GAO*
College of Veterinary Medicine, Northeast Agricultural University, Harbin, P.R. China

Received: 21.08.2016 • **Accepted/Published Online:** 01.03.2017 • **Final Version:** 12.06.2017

Abstract: The effect of ketamine, a drug commonly used as an anesthetic, on monoaminergic neurotransmitters and GABA in neurocytes from fetal rats has not yet been elucidated. We thus decided to investigate these effects by first evaluating ketamine levels in the cerebrospinal fluid by high-performance liquid chromatography. We thus determined the concentrations of GABA, DA, NE, 5-HT, and 5-HIAA following the addition of four different concentrations of ketamine at day 7 of nerve cell culture. GABA, 5-HT, and 5-HIAA levels were increased after an initial decrease: they showed the lowest levels at 10–15 min and the highest peaks at 45–90 min, followed by a declining trend. On the other hand, the trends of DA and NE levels were opposite. These changes in neurotransmitter levels showed a significant impact on monoamine neurotransmitters and GABA, consistent with the clinical changes in anesthetized rats. The role of ketamine in anesthesia may be explained by the changing levels of these indicators. Ketamine may be involved in the inhibition of the production and storage of excitatory neurotransmitters and the promotion of the generation and storage of inhibitory neurotransmitters, exerting its anesthetic action by reducing messenger molecules and related enzyme activity.

Key words: Ketamine, neurocytes, rat, monoamine neurotransmitters, GABA

1. Introduction

Ketamine can selectively inhibit specific parts of the central nervous system, especially at the level of the communication channel and the thalamocortical network of the brain. Ketamine is characterized by its peculiar ability to separate pain and consciousness, called "separation anesthesia" (1).

Classical ion receptor channels are mainly activated by norepinephrine (NE), dopamine (DA), 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), and γ-aminobutyric acid (GABA). These neurotransmitters not only regulate neuronal excitability but also have a strong impact on the development of the brain. For example, DA is retained in vesicles at the nerve endings. When a nerve impulse arrives, the vesicle is emptied and DA is released. DA receptors on the cell membrane can bind to the neurotransmitter, thus transmitting the information. However, neurotransmitters can interact among them. 5-HT antagonist can be enhanced by amphetamine-induced locomotor activity (2) and can inhibit dystonia and catalepsy caused by diazepam (3).

However, to this day, little is known about the effects of ketamine on the levels of GABA, DA, NE, 5-HT, and 5-HIAA in neurocyte cell cultures from fetal rats. We thus decided to investigate how different concentrations of ketamine may affect these neurotransmitters at the neurocyte level.

2. Materials and methods

2.1. Animals

Male and female Wistar rats, 3 months old and weighing 200 ± 20 g, were purchased from the Animal Experimental Center of the Second Affiliated Hospital of Harbin Medical University (Harbin, China). Prior to the experiment, rats were quarantined for 2 weeks at the Northeast Agricultural University (Harbin, China). A total of 104 rats were randomly divided into low-dose groups (L group, n = 32, 4 subgroups), middle-dose groups (M group, n = 48, 6 subgroups), and high-dose groups (H group, n = 24, 3 subgroups), with 8 rats in each subgroup. Intraperitoneal injections of ketamine (Shenyang Veterinary Drug Factory, 20110908) were as follows: L group with 10, 20, 30, and 40 mg/kg; M group with 50, 60, 70, 80, 90, and 100 mg/kg; and H group with 150, 200, and 300 mg/kg. Cerebrospinal fluid was sampled from the cisterna magna as described previously (4). Cerebrospinal fluid was extracted from the foramen magnum to measure the ketamine concentration

* Correspondence: gaoli43450@163..com

by high-performance liquid chromatography (HPLC, Waters 600). All experiments were performed in accordance with the Ethical Committee for Animal Experiments (Northeast Agricultural University, Harbin, China).

2.2. Mating

Twelve Wistar rats were divided into 4 cages (one male and two females per cage) with an iron mesh on the bottom. On the next morning the vaginal suppository was observed under the iron mesh. When sperm was observed, the female rats were recorded as pregnant at day 0.

2.3. Determination of ketamine concentration via HPLC

2.3.1. Sample preparation

For the L group, cerebrospinal fluid was extracted from the foramen magnum 10 min after the intraperitoneal injection of ketamine; for the M and H groups, cerebrospinal fluid was collected immediately upon observing the loss of the righting reflex. First 0.5 mL of cerebrospinal fluid was added to 50 µL of sodium hydroxide (2 mol/L) and 5 mL of extraction solution (hexane : isopropanol : dichloromethane = 64:3:33) and mixed by vortexing for 30 s. Samples were repeatedly shaken for 20 min and the upper liquid part was absorbed. Samples were then blow-dried at 60 °C by nitrogen, dissolved in 1 mL of the mobile phase (methanol : phosphate = 13:7), vortexed for 30 s, and filtered with 0.22-µm microporous membranes (Millipore) for use.

2.3.2. Standard ketamine solution

A concentration gradient was obtained by diluting a standard ketamine solution (provided by the Department of Clinical Surgery of Veterinary Medicine, Northeast Agricultural University) to final concentrations of 10, 5, 2.5, 2, 1, and 0.5 µg/mL with ultrapure water, filtered through 0.22-µm microporous membranes for use.

2.3.3. Sample recovery rate

To analyze the recovery rate of ketamine, cerebrospinal fluid samples were divided into 3 parts, and equal volumes of ketamine standard solution at 1 µg/mL, 2 µg/mL, and 5 µg/mL were added.

2.3.4. Determination of precision

In order to evaluate the precision of our measurements, the injection of each concentration of standard solution was repeated four times a day, thus measuring peak height values and the coefficient of variation. The effect of different days was evaluated by injecting each concentration of standard solution 4 times in 4 days and measuring the peak height and the coefficient of variation.

2.3.5. Sample measurements

The column was a symmetrical C18 (4.6 mm × 150 mm, 5 µm, US Waters Company), the flow rate of the mobile phase (methanol : phosphate = 13:7) was 1.0 mL/min, the detection wavelength was 220 nm, the column temperature was 30 °C, and the injection volume was 20 µL. The utilized external standard cerebrospinal fluid concentration of ketamine was measured using the external standard.

2.4. Cell culture and identification of neural cells

Pregnant rats were euthanized at day 16–18 by cervical dislocation, and embryos were recovered to collect the hippocampus and cerebral cortex used for culturing neural cells (5). The cells were cultured in BME (GIBCO) supplemented with 10% (v/v) fetal bovine serum (FBS) and penicillin/streptomycin (100 U/mL and 100 µg/mL) at 37 °C under an atmosphere of 5% CO_2 and 95% air. The verification of cultured cells was performed as follows. After removing the cell culture fluid, cells were fixed with 1 mL of paraformaldehyde/sugar mixture for 10 min and then placed in 1 mL of 0.1% (v/v) Triton X-100 for 10 min at room temperature. Cells were washed twice with PBS, then mixed with 10% FBS (Invitrogen, Cat. No. 16140071) for 1 h. Cells were then incubated with a mouse anti-MAP2 antibody (1:200, EnoGene Biotech) overnight at 4 °C, followed by goat antimouse IgG (1:100, Santa Cruz Biotechnology) for 1 h, and then washed three times with PBS. Green fluorescence was observed in the cytoplasm of cells positive for neuron-specific enolase by inverted microscope (Nikon Eclipse Ti-S).

2.5. GABA, DA, NE, 5-HT, and 5-HIAA measurements via LC-MS/MS

2.5.1. Solution preparation

The ratio of standard dissolving liquid was water : acetonitrile : 0.1% formic acid = 50:25:25. The precision weights of GABA, DA, NE, 5-HT, and 5-HIAA with a standard of 0.0040 g were dissolved in standard dilutions of 40 mL, targeting a concentration of 100 µg/mL standard stock solution, stored at 4 °C. The standard dilution preparation of cell culture medium was 10%. For formulated 5 mmol/L ammonium formate, mobile phase A was 5 mmol/L ammonium formate : acetonitrile = 95:5, and mobile phase B was 5 mmol/L ammonium formate : acetonitrile = 85:15.

2.5.2. Chromatographic conditions

The column was an XBridge BEH Amide XP Column (2.1 mm × 100 mm, 1.7 µm, US Waters Company). Column temperature was 30 °C, flow rate was 0.25 mL/min, and injection volume was 5 µL; an external standard was used. Mobile phase A was 5 mmol/L ammonium formate : acetonitrile = 95:5, and mobile phase B was 5 mmol/L ammonium formate : acetonitrile = 85:15; the gradient elution program is shown in Table 1.

2.5.3. MS conditions

The ion source was ESI+ and the scan mode was multiple reaction monitoring. Capillary voltage was kept at 2.5 kV, while the source temperature was set at 15 °C and N_2 was used as the dissolving gas with a dissolving temperature

Table 1. Gradient table of mobile phases.

Time (min)	Flow velocity (mL/min)	% A	% B	Curve
0	0.25	100	0	6
3.50	0.25	70	30	6
3.60	0.25	100	0	6
5.00	0.25	100	0	6

of 450 °C. The dissolving gas flow rate was maintained at 650 L/h. The cone gas flow rate was kept at 50 L/h, Ar_2 was used as the collision gas, and the collision chamber working pressure was 3.3×10^{-3} mbar. Ions and all other quantitative and qualitative parameters are listed in Table 2.

2.5.4. Sample preparation

According to ketamine cerebrospinal fluid concentrations detected during anesthesia in the rats, four concentrations of ketamine for the nerve cells were determined. Ketamine was added on day 7 to the nerve cell culture, and 40 µL of supernatant was collected after dosing for 0, 5, 10, 15, 20, 25, 30, 45, 60, 90, and 120 min. Injection volumes of 5 µL and injection preparation vials were used and samples were filtered through a 0.22-µm filter membrane.

2.5.5. Linearity range and correlation coefficient

The precise amount of standard stock solution with standard dilution was diluted to concentration gradients of 1000, 500, 200, 100, 50, 20, and 10 ng/mL. Subsequent to filtering via 0.22-µm filter membranes, injections of 5 µL were administered.

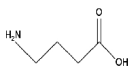
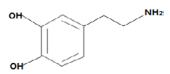
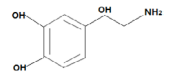
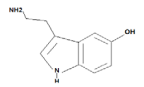
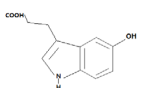
2.5.6. Sensitivity measurement

According to the noise ratio (S/N), the values of the minimum detection limit and lower limit of quantification in the blank matrix were determined, five different levels of mixed standard solution were added, and different concentration gradients of the solution were formulated. Signals (peak height of the ratio of the count) and noise were measured when the lowest concentration of S/N > 3 reached the lowest concentration limit of detection at S/N > 10, and when recovery and relative standard deviations were in line with the requirements of the residue detection method they reached the minimum of the limit of quantitation.

2.5.7. Accuracy and precision

A blank sample was prepared with 100, 200, and 500 ng/mL concentrations of three quality control samples according to earlier detection and treatment methods. Five samples were prepared per concentration to determine the daily coefficients of variation. The time for each sample was checked until experimental UPLC-MS/MS methods

Table 2. Retention times and MS/MS parameters for neurotransmitter.

Neurotransmitters	Molecular structure	Elemental composition	Parention (m/z)	Characteristicion (m/z)	Retention times (s)	Cone voltage (V)	Collision energy (eV)
GABA		$C_4H_9NO_2$	104.2	87.1	0.1	10	15
				69.2	0.1	15	15
DA		$C_8H_{12}NO_2$	154.1	119	0.1	20	10
				137.1	0.1	20	20
NE		$C_8H_{11}NO_3$	170.1	134.9	0.1	15	15
				152.1	0.1	15	10
5-HT		$C_{10}H_{12}N_2O$	177.2	135.9	0.1	20	5
				159.1	0.1	20	6
5-HIAA		$C_{10}H_{11}NO_3$	192.1	146.2	0.1	15	15

for 5 min/day could be analyzed for all treated samples. Therefore, coefficients of variation were not measured that day and the methodological precision was evaluated according to the following formula: precision standard deviation (daily coefficients of variation) = standard deviation / average × 100%.

Each concentration of five sample analyses used analyses of 5-μL injections to obtain the corresponding peak areas for each sample with the theoretical concentrations of added standard amount ratio calculations as the method of recovery.

2.6. Statistical analysis

All data were analyzed with GraphPad Prism 5.1 (GraphPad Software Inc., USA) by using one-way ANOVA followed by Turkey's post hoc test. Values were considered to be statistically significant at $P < 0.05$. Data are presented as mean ± standard deviation.

3. Results

3.1. Results of mating

Pessaries were checked under the iron mesh and smeared sperm was removed from the vagina and examined under a microscope. Day 0 was the day on which pregnancy was recorded.

3.2. Determination of the administration concentration of cells

3.2.1. Determination of ketamine in rat cerebrospinal fluid

The retention time obtained for ketamine was 6.498 min, showing a clear peak without interference and smearing. Thus, endogenous substances present in the cerebrospinal fluid did not interfere with the determination assay of ketamine (Figure 1a).

3.2.2. Linearity range and correlation coefficient

Linear regression was performed with standard ketamine concentration and peak area. Results showed a good linear relationship in the range of 0.5–10 μg/mL (Figure 1b).

3.2.3. Sample recovery rate

The average recovery rate of ketamine was 96.17%, thus indicating that the used chromatographic conditions were reliable and the detection rate was high (Figure 1c).

3.2.4. Determination of assay precision

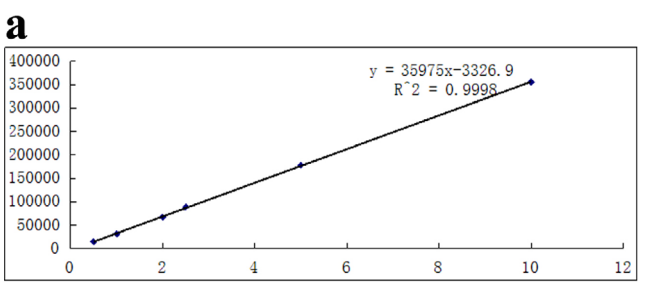
Daily coefficients of variation of ketamine were 2.1%, 1.4%, 0.9%, and 1.6%, while daytime coefficients of variation were 2.1%, 2.3%, 1.2%, and 0.7%, respectively. The coefficient of variation was below 4%, indicating that the method was stable and reproducible.

3.2.5. Sample measurement

Rats were anesthetized using ketamine anesthesia. The blood concentration was measured, and we found that the concentrations of ketamine acting on nerve cells were 0.7, 1, 3, and 5 μg/mL (Table 3).

3.3. Nerve cell culture and verification

Morphological changes of nerve cells were continuously observed under a microscope for 8 days. We observed the shape of embryonic neuronal cells that were round, small, bright, and scattered. Once seeded, after 3 h the cells adhered to the dish wall. After 4 days of culturing, the cells were significantly increased, with multiple protrusions growing out from the cell body. In this phase neurons showed their typical morphological characteristics, such as clear cell body, bright and raised, with adjacent cell processes that can form contacts (Figure 2a). After 8 days of culturing, neuronal cell bodies increased, showing a distinct nucleus, and cell protrusions further increased and cells appeared to be moving closer to each other (Figure 2b).



b

Linear regression equations for five neurotransmitters

	linear equation	R^2
DA	y=3.2601x-56.702	0.9949
NE	y=0.9786x+21.626	0.9967
5-HT	y=0.8689x+73.792	0.9984
5-HIAA	y=32.122x+435.160	0.9974
GABA	y=12.259x+88.886	0.9995

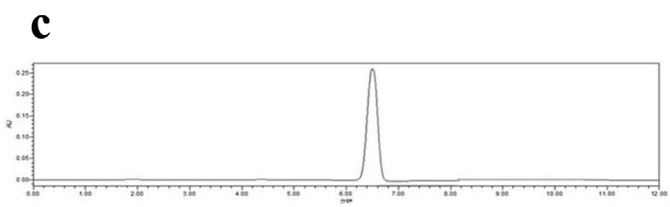


Figure 1. a) RP-HPLC chromatogram for ketamine standards (10 **μg/mL**), b) data for linearity and correlation coefficients (R^2), c) recovery rate of sample.

Table 3. Variety of cerebrospinal fluid concentrations in rats with different doses (μg/mL, n = 3).

Group	Injection dose (mg/kg)	Cerebrospinal fluid drug concentration (μg/mL)
L group	10	0.681 ± 0.048
	20	0.784 ± 0.040
	30	0.780 ± 0.081
	40	0.899 ± 0.069
M group	50	1.324 ± 0.101
	60	1.251 ± 0.083
	70	2.496 ± 0.215
	80	2.889 ± 0.199
	90	3.824 ± 0.365
	100	4.925 ± 0.318
H group	150	5.538 ± 0.294
	200	6.010 ± 0.485
	300	6.823 ± 0.517

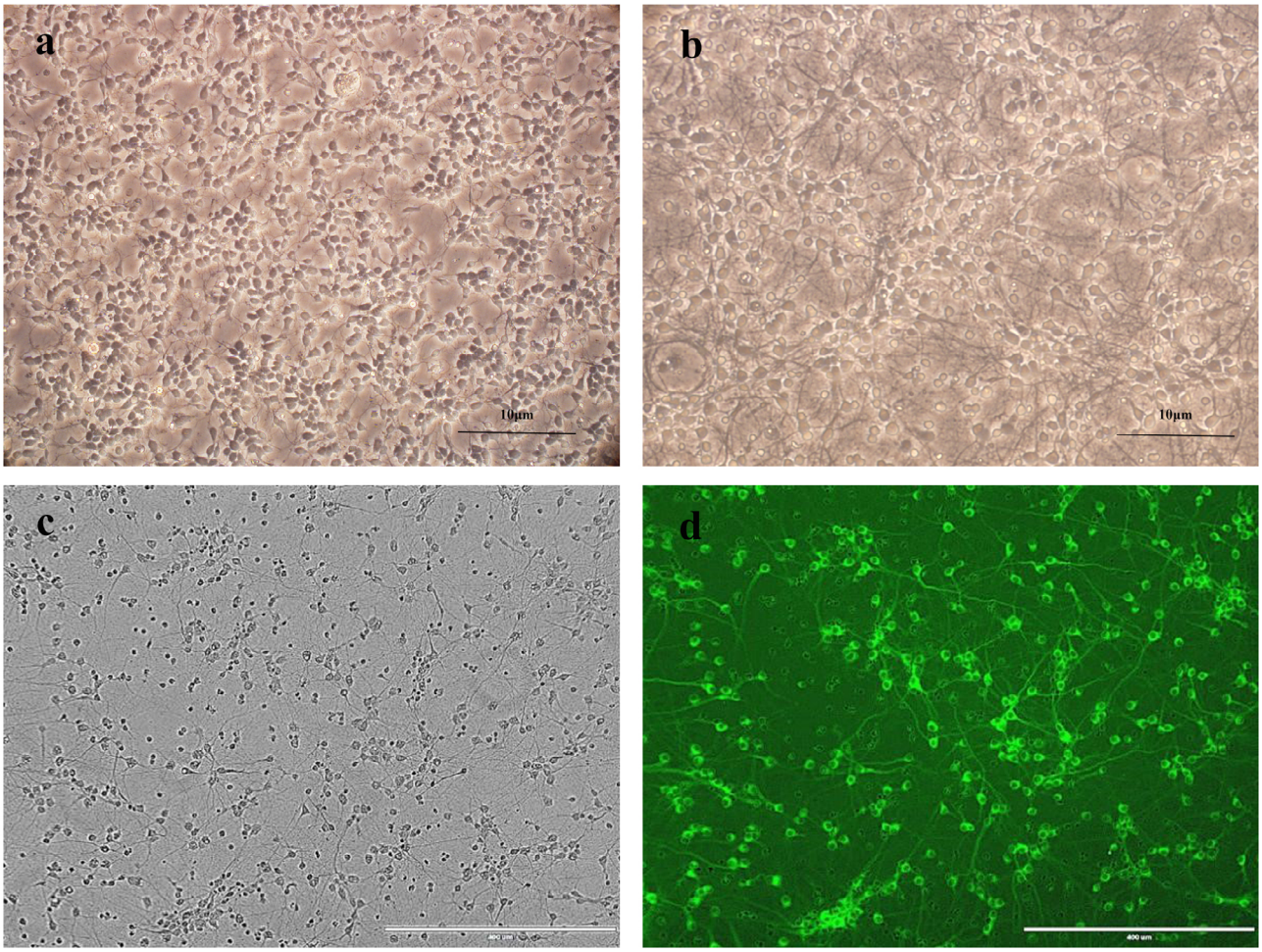


Figure 2. a) Observation of fetal rat cerebral cortex nerve cells cultured in vitro for 4 days, b) observation of fetal rat cerebral cortex nerve cells cultured in vitro for 8 days, c) comparative observation of fetal rat cerebral cortex nerve cells cultured for 7 days, d) comparative observation of fetal rat cerebral cortex nerve cells cultured for 7 days.

Upon observation by laser confocal microscopy, cultured cortical neurons showed positivity for MAP2 (Figures 2c and 2d), a marker in neurons from primitive cell cultures.

3.4. Establishment of LC-MS/MS detection method

3.4.1. Selection of MS conditions

The retention time of GABA, DA, NE, 5-HT, and 5-HIAA chromatographic peaks were as follows: 104.2, 154.1, 170.1, 177.2, and 192.1 m/z. The separation occurred in less than 2 min, and the peak shapes were good, as shown in Figures 3a–3e.

3.4.2. Standard curve and linear range

The standard stock solutions were diluted to final concentrations of 1000, 500, 200, 100, 50, 20, and 10 ng/mL in order to perform the LC-MS/MS analysis (Table 4).

3.4.3. Determination of the detection limit and quantification limit

To determine the detection limit and quantification limit of neurotransmitters in the analyzed samples we measured the lowest concentration of neurotransmitters when the S/N ratio was ≥3, revealing that the minimum detection limit was 6 ng/mL. On the other hand, the minimum quantitative limit when S/N was ≥10 was 20 ng/mL.

3.4.4. Results of GABA, DA, NE, 5-HT, and 5-HIAA recovery

Neurotransmitters at 100, 200, and 500 ng/mL were added to samples obtaining a good precision (CV less than 12%). The average recovery rate was 89.04%–97.29%. The accuracy and recovery rate of the tests are reported in Table 5.

3.5. Detection of the five neurotransmitters

3.5.1. Effect of different concentrations of ketamine on GABA

In the rat group treated with 0.7 μg/mL of ketamine, the content of GABA had a significant increase ($P < 0.01$) at 60 and 90 min, respectively, compared with 0 min. In the rat group treated with 1 μg/mL of ketamine, GABA content significantly decreased ($P < 0.01$) at 10, 15, and 20 min, respectively, thereafter increasing. The rat group treated with 3 μg/mL of ketamine showed a decrease of GABA at 10, 15, 20, and 25 min, respectively, followed by an increase at 60 and 90 min ($P < 0.05$). Finally, when treated with 5 μg/mL of ketamine the content of GABA significantly ($P < 0.01$) decreased at 10, 15, and 20 min and then increased at 45, 60, and 90 min, respectively (Figures 4a and 4b).

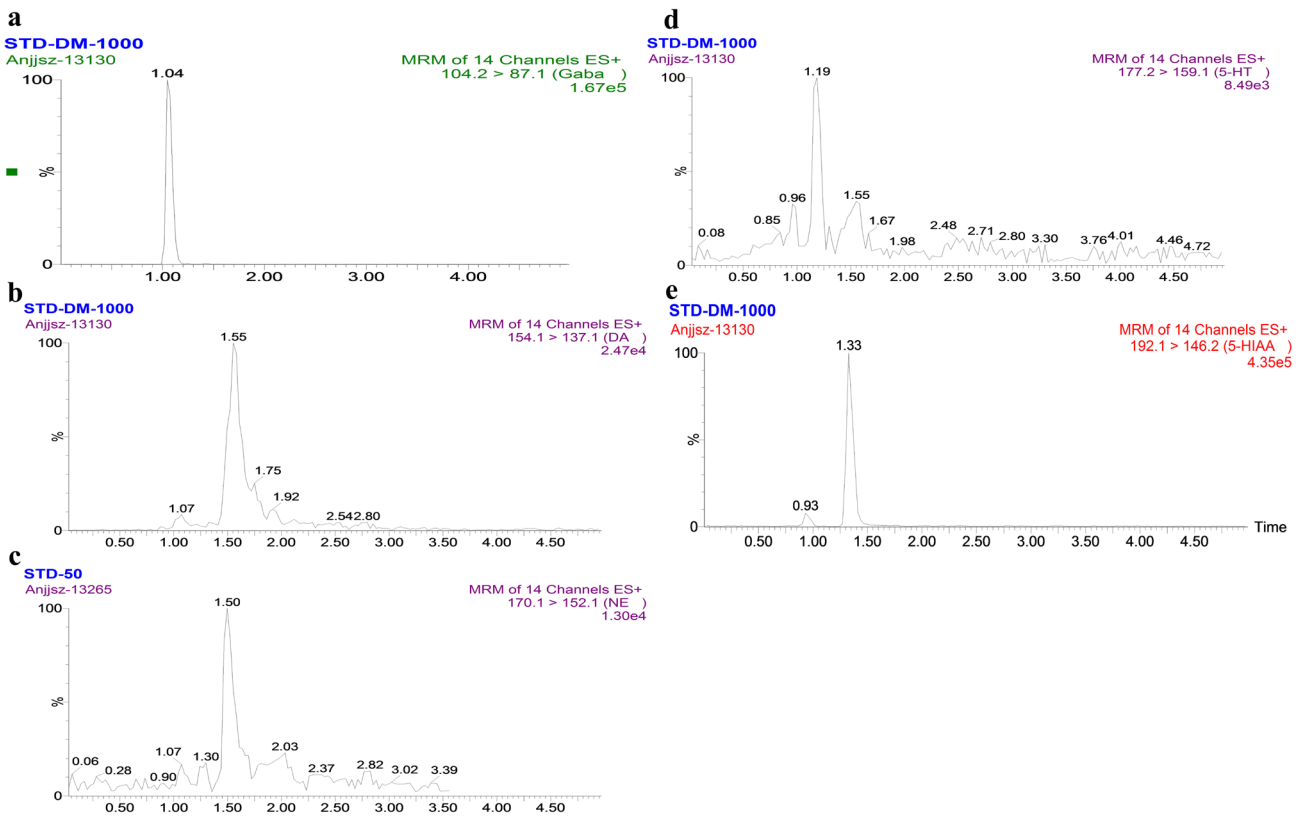


Figure 3. a) LC-MS/MS analysis chromatogram of GABA, b) LC-MS/MS analysis chromatogram of DA, c) LC-MS/MS analysis chromatogram of NE, d) LC-MS/MS analysis chromatogram of 5-HT, e) LC-MS/MS analysis chromatogram of HIAA.

Table 4. Results of recovery (n = 3).

Test item	Sample concentration (μg/mL)	Addition amount (μg/mL)	Measured quantity (μg/mL)	Rate of recovery (%)
ketamine	6.65	1	7.41 ± 0.44	96.87%
		2	8.41 ± 0.65	97.24%
		5	10.99 ± 0.92	94.39%

Table 5. Recovery results.

Neurotransmitter	Added concentration (ng/mL)	Measured concentration (ng/mL)	Rate of recovery (%)	Coefficient of variation (%)
DA	100	92.84 ± 3.99	92.84	4.30
	200	189.24 ± 7.43	94.62	3.93
	500	476.08 ± 12.46	95.22	2.61
NE	100	89.04 ± 6.91	89.04	7.76
	200	186.66 ± 4.899	93.33	2.62
	500	472.78 ± 8.97	94.10	1.90
5-HT	100	90.22 ± 2.60	90.22	2.88
	200	185.18 ± 6.12	92.59	3.31
	500	481.18 ± 11.79	96.24	2.45
5-HIAA	100	90.04 ± 2.78	90.04	3.09
	200	185.06 ± 6.03	92.53	3.26
	500	486.46 ± 7.34	97.29	1.51
GABA	100	90.20 ± 1.35	90.20	1.49
	200	187.34 ± 6.58	93.67	3.51
	500	482.64 ± 4.71	96.53	0.98

3.5.2. Effect of different concentrations of ketamine on DA

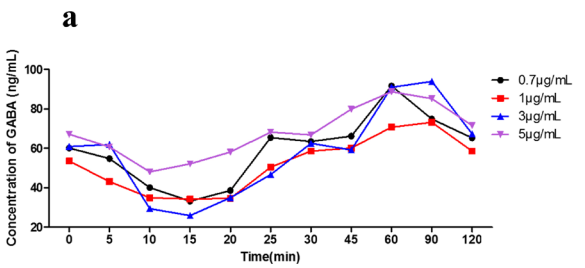
In the rat group treated with 0.7 μg/mL of ketamine, the content of DA was significantly ($P < 0.01$) increased at 10, 15, and 20 min, respectively, compared with 0 min. In rats treated with 1 μg/mL of ketamine, the DA content was increased at 15 min but decreased at 60 min ($P < 0.01$). Rats treated with 3 μg/mL of ketamine showed an increase in DA at 10 min and 15 min, respectively. Finally, rats treated with 5 μg/mL of ketamine showed levels of DA significantly ($P < 0.01$) increased at 5 min, 10 min, 15 min, and 20 min, respectively (Figures 4c and 4d).

3.5.3. Effect of different concentrations of ketamine on NE

Rats treated with 0.7 and 1 μg/mL of ketamine had significantly ($P < 0.01$) increased NE at 5, 10, 15, 20, and 25 min, respectively, compared with 0 min. In the rat group treated with 3 μg/mL of ketamine, the content of NE was significantly ($P < 0.05$) increased at 5, 10, 15, 20, and 25 min, respectively, but it decreased when rats were treated with 5 μg/mL of ketamine ($P < 0.01$) (Figures 4e and 4f).

3.5.4. Effect of different concentrations of ketamine on 5-HT

We observed that different concentrations of ketamine induced 5-HT secretion by nerve cells according to different trends. Rats treated with 0.7 μg/mL of ketamine showed a significant ($P < 0.01$) decrease of 5-HT content at 15 min. In rats treated with 1 μg/mL of ketamine, 5-HT content was significantly ($P < 0.05$, $P < 0.01$) increased at 45 min and 60 min, respectively. Rats treated with 3 μg/mL of ketamine had 5-HT content significantly ($P < 0.01$) decreased at 10 min; at 60 and 90 min, 5-HT content was increased ($P < 0.01$), respectively. Finally, in the group treated with 5 μg/mL of ketamine, 5-HT content was first



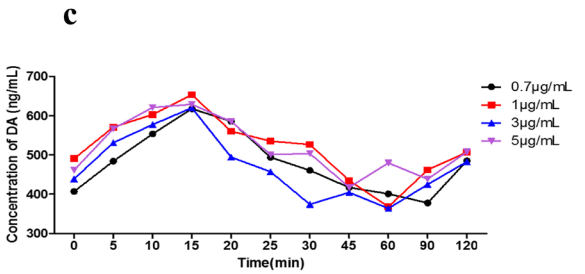
b

Content changes of GABA with different concentrations of ketamine

(ng/mL, n = 3)

	0.7 μg/mL	1 μg/mL	3 μg/mL	5 μg/mL
0 min	60.170±2.022	53.570±1.208	60.985±2.239	67.162±3.173
5 min	54.824±4.743	43.143±7.173	61.994±7.057	60.769±4.220
10 min	40.095±2.933	34.957±2.947*	29.424±2.934*	48.102±4.232*
15 min	33.240±3.569	34.286±6.742*	25.900±3.908*	52.140±5.001*
20 min	38.656±3.855	34.639±2.703*	34.953±3.997*	58.198±2.803*
25 min	65.482±2.810	50.508±0.575	46.672±5.394	68.278±2.803
30 min	63.504±2.034	58.657±2.790	62.579±2.215	66.854±2.457
45 min	66.265±2.984	60.172±2.528	59.200±4.821	79.898±7.425*
60 min	91.730±2.758*	70.747±4.128*	91.058±0.641*	88.780±2.464*
90 min	74.886±3.683*	73.231±4.758*	93.995±4.035*	85.234±1.046*
120 min	65.281±1.593	58.613±2.532	67.361±4.402	71.556±7.903

Note: * denotes significant differences compared to 0 min ($P < 0.05$).



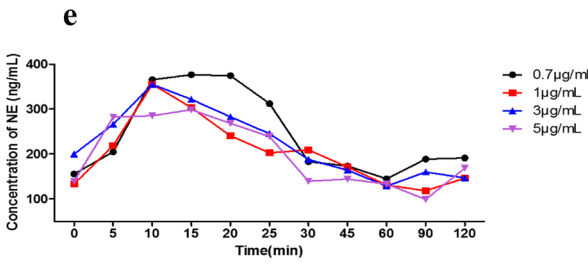
d

Content changes of DA with different concentrations of ketamine

(ng/mL, n = 3)

	0.7 μg/mL	1 μg/mL	3 μg/mL	5 μg/mL
0 min	406.956±32.749	490.509±25.258	438.938±29.348	461.004±40.791
5 min	484.014±54.928	570.681±37.642	531.301±29.348	566.975±38.640*
10 min	553.560±34.289*	602.740±30.934	577.129±29.348*	620.670±32.071*
15 min	617.460±34.289*	653.091±30.934*	619.946±31.465*	628.859±34.423*
20 min	585.548±29.740*	560.022±30.934	494.220±11.511	584.796±25.755*
25 min	493.830±10.562	535.180±30.934	457.093±37.776	500.847±21.318
30 min	460.434±43.519	526.351±13.513	374.117±23.584	503.363±36.757
45 min	417.115±41.016	434.943±35.505	404.593±50.460	417.601±43.165
60 min	400.821±46.920	368.133±32.971*	363.788±13.804	479.545±43.165
90 min	377.705±29.618	461.732±52.022	424.965±36.909	438.374±3.825
120 min	485.288±10.177	507.170±33.273	482.782±35.065	507.806±22.644

Note: * denotes significant differences compared to 0 min ($P < 0.05$).



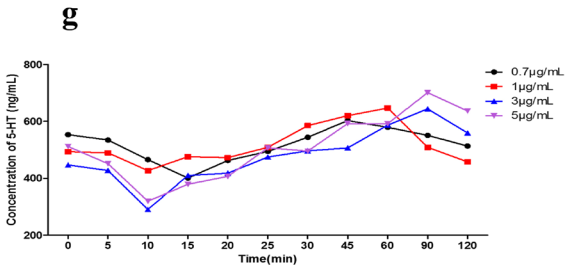
f

Content changes of NE with different concentrations of ketamine

(ng/mL, n = 3)

	0.7 μg/mL	1 μg/mL	3 μg/mL	5 μg/mL
0 min	155.454±12.195	133.264±12.521	199.708±11.333	139.185±12.432
5 min	204.685±4.628*	218.607±14.767*	266.239±17.586*	282.247±22.796*
10 min	365.528±6.578*	355.320±7.825*	354.885±21.668*	285.366±18.738*
15 min	376.410±4.069*	303.274±7.852*	321.794±25.577*	298.618±26.688*
20 min	374.362±10.448*	240.273±16.419*	282.555±10.163*	268.390±15.756*
25 min	312.462±6.763*	202.342±15.146*	245.405±12.916*	238.951±14.116*
30 min	182.719±13.700	209.163±3.917*	187.945±11.391	139.300±14.116
45 min	173.326±8.072	171.137±13.837*	163.771±7.518	144.150±18.247
60 min	144.557±9.421	130.730±12.401	128.376±7.605	132.861±18.247
90 min	188.740±13.947	118.024±8.109	159.910±7.605	98.862±11.738
120 min	190.935±17.300	146.012±8.586	146.542±9.922	168.662±27.029

Note: * denotes significant differences compared to 0 min ($P < 0.05$).



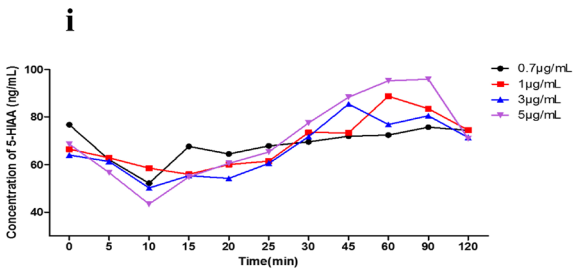
h

Content changes of 5-HT with different concentrations of ketamine

(ng/mL, n = 3)

	0.7 μg/mL	1 μg/mL	3 μg/mL	5 μg/mL
0 min	553.475±30.282	493.455±57.073	446.890±25.655	512.023±44.082
5 min	534.356±34.836	489.783±14.454	427.103±26.421	451.343±16.850
10 min	465.389±27.257	426.774±38.417	290.172±29.372*	319.465±18.013*
15 min	400.901±11.538*	476.057±30.650	409.914±29.405	378.903±27.158*
20 min	462.698±1.626	472.38±22.146	417.820±29.075	406.863±30.570
25 min	494.485±10.858	508.903±28.048	474.688±33.102	506.895±33.852
30 min	544.282±32.495	584.800±16.168	496.311±23.990	495.620±25.367
45 min	602.647±35.509	620.256±22.716*	506.605±28.540	592.500±33.854
60 min	578.906±10.945	646.887±36.537*	586.930±25.628*	592.133±36.759
90 min	550.827±54.871	508.832±41.948	643.941±26.897*	701.091±28.926*
120 min	513.253±29.056	457.627±21.604	559.334±14.063	636.468±30.052*

Note: * denotes significant differences compared to 0 min ($P < 0.05$).



j

Content changes of 5-HIAA with different concentrations of ketamine

(ng/mL, n = 3)

	0.7 μg/mL	1 μg/mL	3 μg/mL	5 μg/mL
0 min	76.736±4.036	66.498±5.256	63.999±2.404	68.601±5.053
5 min	62.085±4.914	62.832±4.052	61.324±4.221	56.643±4.646
10 min	52.195±3.708	58.493±2.543	50.175±3.298	43.440±4.217*
15 min	67.618±4.322	55.908±3.023	55.355±4.298	54.855±3.643
20 min	64.496±2.643	60.035±3.949	54.220±4.043	60.527±3.748
25 min	67.834±2.793	61.431±1.898	60.488±4.384	65.295±4.564
30 min	69.546±2.254	73.484±5.790	71.734±3.859	77.494±3.285
45 min	71.918±2.238	73.332±4.300	85.398±4.373*	88.306±5.552*
60 min	72.388±5.061	88.712±3.276*	76.844±2.946	95.185±1.095*
90 min	75.691±5.414	83.467±1.539	80.477±2.946	95.864±6.286*
120 min	74.367±1.843	74.452±2.682	71.412±3.440	70.846±5.495

Note: * denotes significant differences compared to 0 min ($P < 0.05$).

Figure 4. a) Content changes of GABA with different concentrations of ketamine, b) content changes of GABA with different concentrations of ketamine, c) content changes of DA with different concentrations of ketamine, d) content changes of DA with different concentrations of ketamine, e) content changes of NE with different concentrations of ketamine, f) content changes of NE with different concentrations of ketamine, g) content changes of 5-HT with different concentrations of ketamine, h) content changes of 5-HT with different concentrations of ketamine, i) content changes of 5-HIAA with different concentrations of ketamine, j) content changes of 5-HIAA with different concentrations of ketamine.

significantly ($P < 0.05$, $P < 0.01$) decreased at 10 and 15 min, respectively, and then it significantly ($P < 0.05$, $P < 0.01$) increased at 90 and 120 min, respectively (Figures 4g and 4h).

3.5.5. Effect of different concentrations ketamine on 5-HIAA

Rats treated with 0.7 μg/mL of ketamine showed an insignificant ($P > 0.05$) trend of 5-HIAA secretion by nerve cells. In the 1 μg/mL ketamine-treated group, 5-HIAA content was significantly increased at 60 min. In rats treated with 3 μg/mL of ketamine, 5-HIAA content at 45 min significantly ($P < 0.01$) increased with respect to the control group. Finally, in rats treated with 5 μg/mL of ketamine, 5-HIAA content at 10 min was significantly ($P < 0.01$) decreased. However, 5-HIAA content was significantly increased ($P < 0.01$) at 45, 60, and 90 min respectively (Figures 4i and 4j).

4. Discussion

In this study, we observed that GABA, 5-HT, and 5-HIAA showed typical behaviors of neurotransmitters, with an initial increase followed by a decrease. These molecules reached their lowest point at 10–15 min and increased at 45–90 min, with a final declining trend. However, the contents of DA and NE behaved in an opposite manner. The changes observed in neurotransmitters showed a dose-dependent manner: with the increase of ketamine concentration, this tendency was clearer. This is consistent with the clinical phenomenon in vivo (6–8).

The common HPLC method classically used to measure neurotransmitters was not sensitive enough in this case, due to their low concentrations in the culture medium. Therefore, monoamine neurotransmitters (DA, NE, 5-HT, 5-HIAA) and GABA were detected by high-sensitivity liquid chromatography coupled with mass spectrometry (LC-MS/MS). The medium composition was complex, so we needed to dilute the analyzed samples 10-fold with ultrapure water in order to obtain better chromatographic results, and the retention time of each substance was less than 2 min.

The blood–cerebrospinal fluid barrier consists of tight junctions among epithelial cells of the choroid plexus structure, and it is responsible for transport of substances between the blood and cerebrospinal fluid (9). The choroid plexus, involved in drainage back cerebrospinal fluid, presents solute carrier (SLC) transporter protein, able to regulate the transport of a variety of drugs available through the blood and cerebrospinal fluid that are essential for nerve cells to survive (10). Moreover, it can better reflect the brain's extracellular fluid level through the blood–brain barrier. As a consequence, the blood concentration of ketamine used as an anesthetic can be detected by analyzing nerve cells. In the range of 1–5 μg/mL of ketamine, we chose the following concentrations: 1, 3, and 5 μg/mL, along with the low-dose group with 0.7 μg/mL of ketamine. Cells derived from rats belonging to the high-dose groups were rapidly broken as we observed by microscopy. We thus chose the above four concentrations of ketamine.

Many anesthetics have a certain effect on ion channels, neurotransmitter receptors, and extracellular ion concentrations. Previous experiments indicated (11) that volatile anesthetics inhibit the central nervous system through the inhibition of postsynaptic and presynaptic receptors. In the nervous system, ketamine strengthens the role of GABA (12,13), which is an important amino acid neurotransmitter (14,15). It has the function of excitatory and inhibitory effect on the activity and mutual relations of neurons.

At present, the relationship between ketamine and the $GABA_A$ receptor is still controversial. Some studies have shown that ketamine can enhance the inhibitory postsynaptic currents mediated by GABA at clinically relevant concentrations. Ketamine can stimulate GABA-induced chloride currents in *Xenopus* oocytes, thus suggesting that it may have a role in $GABA_A$ receptor-involved processes (16). L-Amino acid dehydrogenase had no effect on the generation of ketamine anesthesia, presynaptic $GABA_A$ neurotransmission did not require higher levels of ketamine, or it may not have an effect by indirect efficacy in the neural network (17). The use of ketamine in *Xenopus* oocytes further showed the relationship between ketamine and GABA.

In the central nervous system, the content of DA is mainly determined by the synthesis rate of dopaminergic neurons. When the action potential is triggered, DA is released into the synaptic cleft to begin the process of transmission. In vitro experiments showed that a subanesthetic dose of ketamine can stimulate high-affinity D2 receptors. However, under anesthesia doses, it can block the high-affinity D2 dopamine receptors. In addition, Kapur and Seeman (18) reported that ketamine has a strong affinity for the dopamine D2 receptor, which indicates that ketamine can also regulate the dopamine system. Several studies have demonstrated that subanesthetic doses of ketamine can allow the rapid release of DA to the rat forebrain cortex. When the test was repeated, higher basal levels of DA were detected, thus indicating that repeated application of ketamine may have more lasting impact (19). Ketamine at 2 mM (internal embryo exposure levels equivalent to human anesthetic plasma concentration) significantly reduced DA levels, indicating that DA synthesis was adversely affected (20). In long-term ketamine-treated mice, significant increases of DA contents were found in the midbrain (21). This experiment showed that DA increased from 45 to 120

min, but the difference was not significant. This may be the result of short-term effects of ketamine.

In the central nervous system, NE is has many physiological functions, including roles in the sleep/wake cycle, pressure perception, the cardiovascular system cycle, perception, and learning, but it is also an important indicator of general anesthesia. Locus coeruleus activity can affect the MAC of the anesthetic, and locus coeruleus neurons are stimulated during recovery from anesthesia (22). Reports showed that the release of NE in the hypothalamus is increased in halothane and isoflurane anesthesia recovery processes (23). Ketamine can partially inhibit NE reuptake so that circulating levels of catecholamine increase, inhibiting the central nervous system from reabsorbing it.

5-HT is also called serotonin, an indole-alkylamine complex synthesized by the brain. It is involved in nerve development and other processes. 5-HIAA is an important bioactive molecule, present in body fluids such as cerebrospinal fluid and also in platelets. Under the action of monoamine oxidase, 5-HT is degraded into 5-HIAA, so the content of 5-HIAA indirectly reflects the consumption of 5-HT in the body.

As typical inhibitory neurotransmitters, the trends observed in levels of 5-HT and 5-HIAA were in line with the trend of GABA, but in contrast with the trends of DA and NE. In vitro experiments have shown that ketamine is a 5-HT receptor agonist, which may inhibit complex G protein-coupled receptors, such as adrenaline and muscarinic and opioid receptors (1,24). Some studies (25,26) used ketamine to anesthetize rats and to detect DA, NE, 5-HT, and 5-HIAA in brain, finding that when rats were deeply anesthetized, the 5-HT content was increased but 5-HIAA showed an opposite trend. The contents of NE and DA were significantly increased during ketamine anesthesia and the recovery process. However, the decrease of 5-HIAA may be associated with the deepening of ketamine anesthesia (10). In our work, the content of 5-HIAA was significantly increased upon ketamine treatment, which may be due to the exclusion of the effects related to complex mechanisms in vitro. Moreover, its trend is broadly in line with that of 5-HT, maybe because 5-HT is a precursor of 5-HIAA, and it did not significantly affect the degradation process. However, this aspect needs to be further investigated.

In conclusion, the direct effect of ketamine anesthetic concentration in nerve cells results in a significant impact on monoamine neurotransmitters and GABA. Accordingly, with clinical changes in anesthetized rats, the role of ketamine in anesthesia may be explained by the changes induced in these neurotransmitters. Thus, ketamine may act by inhibiting the production and storage of excitatory neurotransmitters, and by promoting the generation and storage of inhibitory neurotransmitters. The anesthetic action may be thus related to a reduction of messenger molecules' relative content and related enzyme activity.

Acknowledgment

This study was supported by the National Natural Science Foundation of China (31572580 and 31372491).

References

1. Mathew SJ, Shah A, Lapidus K, Clark C, Jarun N, Ostermeyer B, Murrough JW. Ketamine for treatment-resistant unipolar depression. CNS Drugs 2012; 26: 189-204.
2. Delgermurun D, Ito S, Ohta T, Yamaguchi S, Otsuguro K. Endogenous 5-HT outflow from chicken aorta by 5-HT uptake inhibitors and amphetamine derivatives. J Vet Med Sci 2016; 78: 71-76.
3. Batool F, Haleem DJ. Increased precursor availability did not increase food intake and 5-HT turnover rate in the hypothalamus of diazepam injected rats. Pak J Pharm Sci 1999; 12: 21-26.
4. Secher T, Novitskaia V, Berezin V, Bock E, Glenthøj B, Klementiev B. A neural cell adhesion molecule–derived fibroblast growth factor receptor agonist, the FGL-peptide, promotes early postnatal sensorimotor development and enhances social memory retention. Neuroscience 2006; 141: 1289-1299.
5. Beaudoin GM 3rd, Lee SH, Singh D, Yuan Y, Ng YG, Reichardt LF, Arikkath J. Culturing pyramidal neurons from the early postnatal mouse hippocampus and cortex. Nat Protoc 2012; 7: 1741-1754.
6. Casoni D, Spadavecchia C, Wampfler B, Thormann W, Levionnois OL. Clinical and pharmacokinetic evaluation of S-ketamine for intravenous general anaesthesia in horses undergoing field castration. Acta Vet Scand 2015; 57: 21.
7. Casoni D, Spadavecchia C, Adami C. S-ketamine versus racemic ketamine in dogs: their relative potency as induction agents. Vet Anaesth Analg 2015; 42: 250-259.
8. De Monte V, Staffieri F, Di Meo A, Vannucci J, Bufalari A. Comparison of ketamine-dexmedetomidine-methadone and tiletamine-zolazepam-methadone combinations for short-term anaesthesia in domestic pigs. Vet J 2015; 205: 364-368.
9. Graff CL, Pollack GM. Drug Transport at the Blood-Brain Barrier and the Choroid Plexus. Curr Drug Metab 2004; 5: 95-108.
10. Ghersi-Egea JF, Strazielle N. Choroid plexus transporters for drugs and other xenobiotics. J Drug Target 2002; 10: 353-357.
11. Lukatch HS, Kiddoo CE, Maciver MB. Anesthetic-induced burst suppression EEG activity requires glutamate-mediated excitatory synaptic transmission. Cereb Cortex 2005; 15: 1322-1331.

12. Irifune M, Sato T, Kamata Y, Nishikawa T, Dohi T, Kawahara M. Evidence for $GABA_A$ receptor agonistic properties of ketamine: convulsive and anesthetic behavioral models in mice. Anesth Analg 2000; 91: 230-236.

13. Maldifassi MC, Baur R, Sigel E. Functional sites involved in modulation of the GABA receptor channel by the intravenous anesthetics propofol, etomidate and pentobarbital. Neuropharmacology 2016; 105: 207-214.

14. Pejo E, Santer P, Wang L, Dershwitz P, Husain SS, Raines DE. γ-Aminobutyric acid type A receptor modulation by etomidate analogs. BMC Anesthesiol 2016; 124: 651-663.

15. Stern AT, Forman SA. A cysteine substitution probes β3H267 interactions with propofol and other potent anesthetics in α1β3γ2L γ-aminobutyric acid type A receptors. BMC Anesthesiol 2016; 124: 89-100.

16. Hevers W, Hadley SH, Lüddens H, Amin J. Ketamine, but not phencyclidine, selectively modulates cerebellar GABAA receptors containing α6 and δ subunits. J Neurosci 2008; 28: 5383-5393.

17. Berman RM, Cappiello A, Anand A, Oren DA, Heninger GR, Charney DS, Krystal JH. Antidepressant effects of ketamine in depressed patients. Biol Psychiat 2000; 47: 351-354.

18. Kapur S, Seeman P. NMDA receptor antagonists ketamine and PCP have direct effects on the dopamine D 2 and serotonin 5-HT 2 receptors—implications for models of schizophrenia. Mol Psychiatr 2002; 7: 837-844.

19. Moghaddam B, Adams B, Verma A, Daly D. Activation of glutamatergic neurotransmission by ketamine: a novel step in the pathway from NMDA receptor blockade to dopaminergic and cognitive disruptions associated with the prefrontal cortex. J Neurosci 1997; 17: 2921-2927.

20. Robinson BL, Dumas M, Cuevas E, Gu Q, Paule MG, Ali SF, Kanungo J. Distinct effects of ketamine and acetyl l-carnitine on the dopamine system in zebrafish. Neurotoxicol Teratol 2016; 54: 52-60.

21. Tan S, Lam WP, Wai MS, Yu WHA, Yew DT. Chronic ketamine administration modulates midbrain dopamine system in mice. PLoS One 2012; 7: e43947.

22. Vazey EM, Aston-Jones G. Designer receptor manipulations reveal a role of the locus coeruleus noradrenergic system in isoflurane general anesthesia. P Natl Acad Sci USA 2014; 111: 3859-3864.

23. Wu XW, Xin B, Zou JF, Yan ZW, Qiu Y, Liu SY. Effect of rotation stimulation on the anesthetic sensitivity of sevoflurane in rats. Zhongguo Ying Yong Sheng Li Xue Za Zhi 2012; 28: 114-117 (in Chinese with abstract in English).

24. Nikiforuk A, Holuj M, Kos T, Popik P. The effects of a 5-HT receptor antagonist in a ketamine-based rat model of cognitive dysfunction and the negative symptoms of schizophrenia. Neuropharmacology 2016; 105: 351-360.

25. Amat J, Dolzani SD, Tilden S, Christianson JP, Kubala KH, Bartholomay K, Sperr K, Ciancio N, Watkins LR, Maier SF. Previous ketamine produces an enduring blockade of neurochemical and behavioral effects of uncontrollable stress. J Neurosci 2016; 36: 153-161.

26. Robinson BL, Dumas M, Paule MG, Ali SF, Kanungo J. Opposing effects of ketamine and acetyl L-carnitine on the serotonergic system of zebrafish. Neurosci Lett 2015; 607: 17-22.