Supporting Information

Diagnostic Strategies for Brain Doping in an Animal Model via Quantitative Analysis of Neurochemicals

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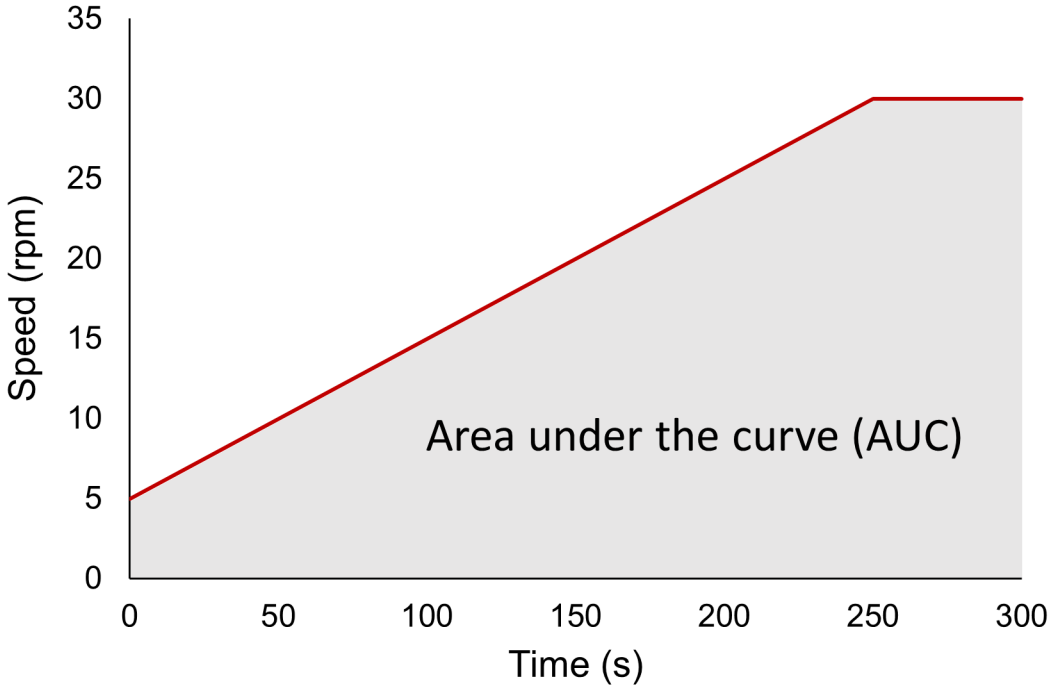

Figure S1. Conditions for behavioral testing using a rotarod. The speed was started at 4 rpm, increased to 300 rpm within 250 s, and then held constant until 300 s.

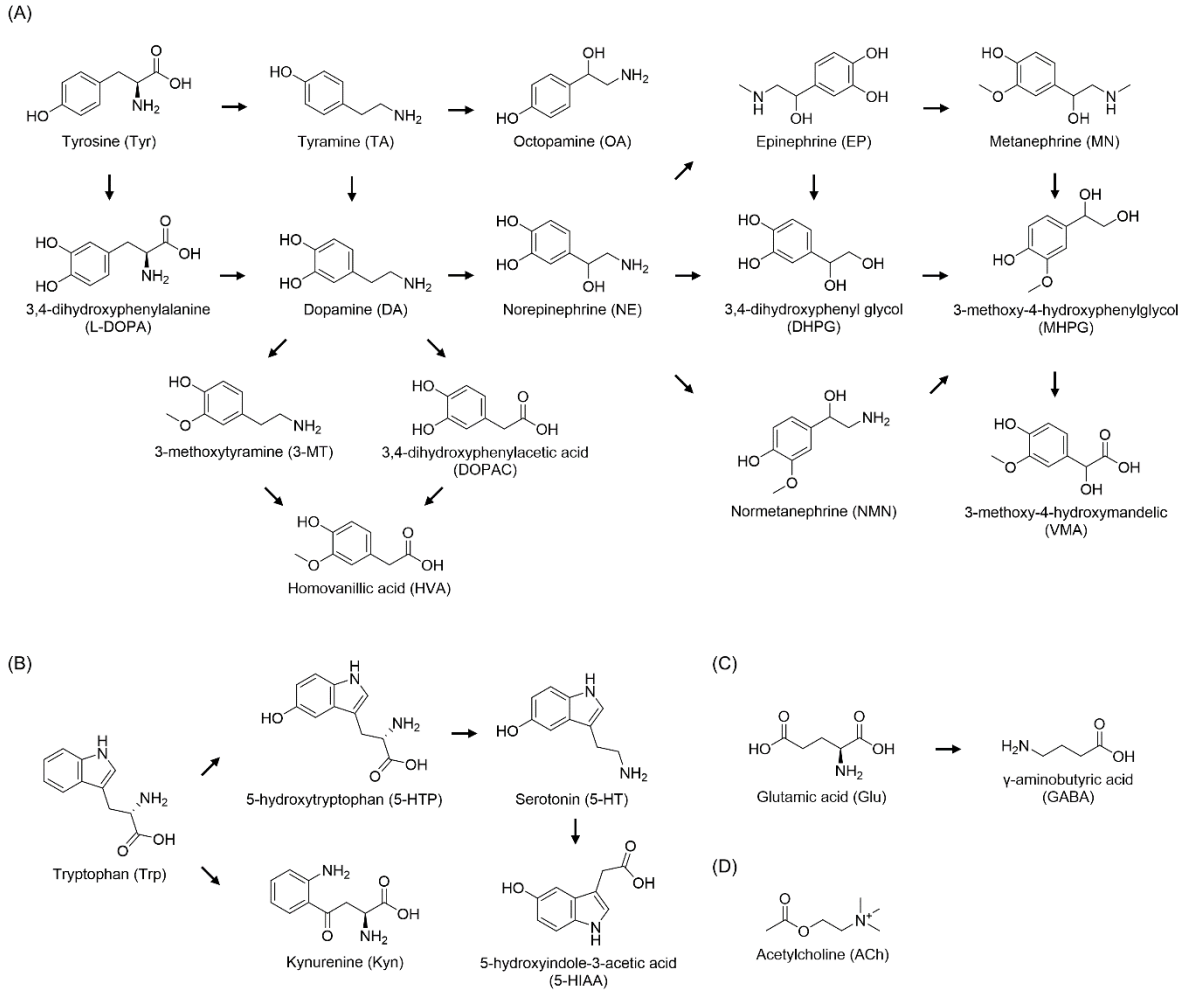

Figure S2. Metabolic pathways and chemical structures of the target neurochemicals used in this study: (A) catecholamine biosynthesis pathway; (B) tryptophan metabolic pathway; (C) γ-aminobutyric acid synthesis from glutamic acid; and (D) structure of acetylcholine.

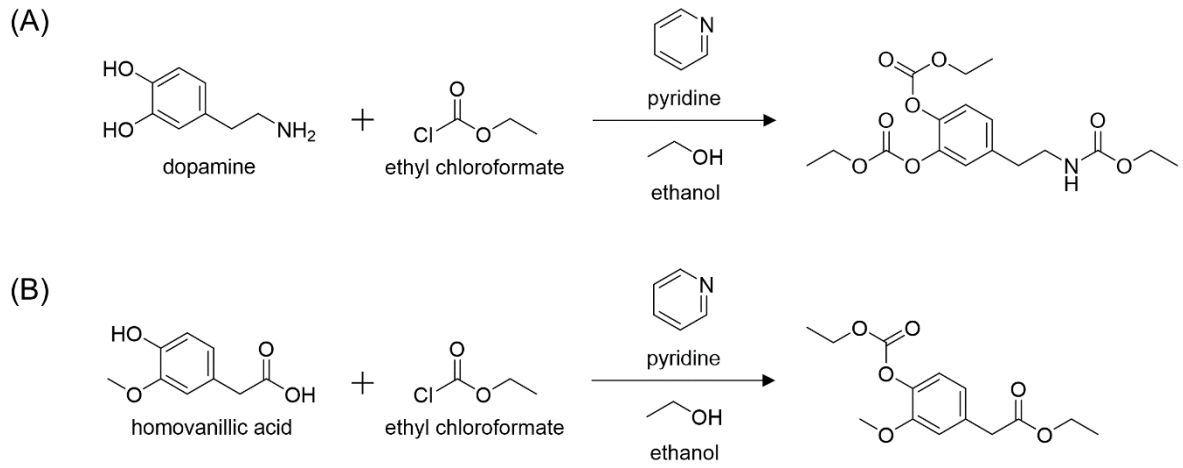

Figure S3. Derivatization reaction scheme of ethyl chloroformate with target substances: (A) dopamine; (B) homovanillic acid. The amino and hydroxyl groups in (A) are converted to a more stable structure by reacting with ethyl chloroformate. In the case of a carboxylic groups in (B), an additional reaction with alcohol transformed to the structure in which CO_2 removed.

Table S1. Latency to fall in the rotarod test for an animal model system

Rat	Sham (0.0 mA stimulation)				0.1 mA stimulation				0.2 mA stimulation			
	Before (s)		After (s)		Before (s)		After (s)		Before (s)		After (s)	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
1	239	190	269	270	220	229	246	248	181	233	207	182
2	246	246	244	243	240	300	300	274	228	212	242	247
3	140	221	157	183	105	230	184	224	201	237	215	141
4	255	241	231	234	239	226	237	226	135	122	237	184
5	227	142	206	189	238	252	239	291	247	249	266	246
6	237	154	218	143	224	203	190	230	72	88	60	92
7	20*	26*	30	21*	98	18*	143	55	185	217	208	201
8	134	225	160	108	184	166	198	170	22*	16*	29*	18*
9	177	212	182	162	216	239	220	243	197	212	212	226
10	229	229	225	227	76	149	55	166	153	200	166	196

*Subjects that fell off within 30 seconds

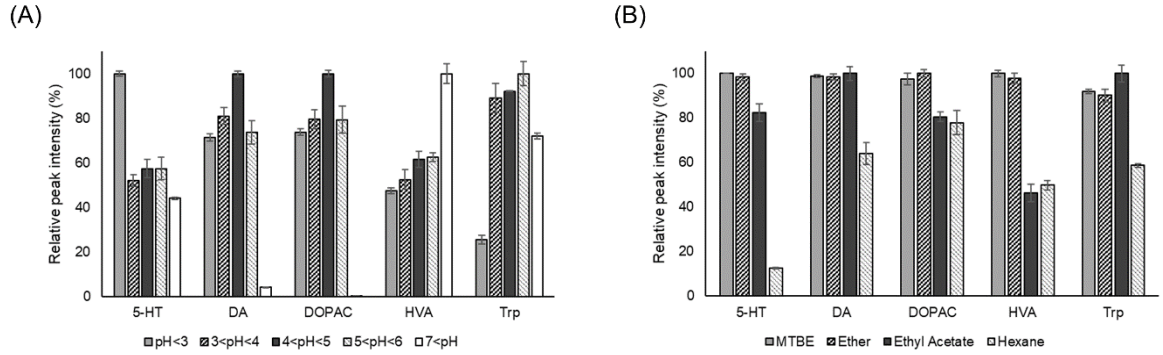

Figure S4. Optimization results of (A) pH condition sand (B) extraction solvent in the sample preparation step. The conditions for obtaining the maximum value for each substance were set to 100%, and the average and standard deviation were indicated. The most stable pH conditions for derivatization were between 4 and 5, and the optimal solvent for the extraction of the derivatized target substances was MTBE.

Table S2. MRM conditions for the detection of derivatized target compounds

Compounds	ESI polarity	Adducts	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)	Retention time (min)
3-MT	+	$[M+NH_4]^+$	329.2	266	14	2.80
3-MT	+	$[M+H]^+$	312.1	266	10	2.80
5-HIAA	+	$[M+NH_4]^+$	309.1	218	17	2.95
5-HIAA	+	$[M+H]^+$	292.1	218	14	2.95
5-HT	+	$[M+H]^+$	321.1	203	17	2.69
5-HT	+	$[M+NH_4]^+$	338.2	321	8	2.69
5-HTP	+	$[M+H]^+$	393.2	347	12	3.02
5-HTP	+	$[M+NH_4]^+$	410.2	347	16	3.02
DA	+	$[M+NH_4]^+$	387.2	370	10	3.17
DA	+	$[M+H]^+$	370.1	180	18	3.17
DHPG	+	$[M+NH_4]^+$	332.1	153	15	2.15
DHPG	+	$[M+NH_4]^+$	404.2	369	10	2.50
DOPAC	+	$[M+NH_4]^+$	358.1	123	28	3.86
DOPAC	+	$[M+H]^+$	341.1	123	24	3.86
EP	+	$[M+NH_4]^+$	417.2	382	13	2.79
EP	+	$[M+H]^+$	400.2	382	10	2.79
GABA	+	$[M+H]^+$	204.1	158	10	2.15
GABA	+	$[M+H]^+$	204.1	112	16	2.15
Glu	+	$[M+H]^+$	276.1	230	10	2.42
Glu	+	$[M+H]^+$	276.1	156	16	2.42
HVA	+	$[M+NH_4]^+$	300.1	137	20	3.27
HVA	+	$[M+H]^+$	283.1	137	14	3.27
Kyn	+	$[M+H]^+$	381.2	246	14	3.77
Kyn	+	$[M+NH_4]^+$	398.2	381	8	3.77
L-DOPA	+	$[M+NH_4]^+$	459.2	442	10	3.79
L-DOPA	+	$[M+H]^+$	442.2	324	14	3.79
MHPG	+	$[M+NH_4]^+$	274.1	167	10	1.99
MHPG	+	$[M+NH_4]^+$	346.1	167	14	2.59
MN	+	$[M+NH_4]^+$	359.2	324	10	2.47
MN	+	$[M+H]^+$	342.2	324	10	2.47

Table S2. (continued)

Compounds	ESI polarity	Adducts	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)	Retention time (min)
NE	+	$[M+NH_4]^+$	475.2	368	10	3.76
NE	+	$[M+H]^+$	458.2	368	10	3.76
NMN	+	$[M+NH_4]^+$	417.2	310	10	3.23
OA	+	$[M+NH_4]^+$	315.1	91	37	2.26
OA	+	$[M+NH_4]^+$	387.2	280	10	3.22
TA	+	$[M+NH_4]^+$	299.1	236	13	2.84
TA	+	$[M+H]^+$	282.1	236	10	2.84
Trp	+	$[M+H]^+$	305.1	231	15	2.85
Trp	+	$[M+NH_4]^+$	322.2	305	9	2.85
Tyr	+	$[M+H]^+$	354.2	280	13	3.31
Tyr	+	$[M+NH^4]^+$	371.2	354	10	3.31
VMA	+	$[M+NH_4]^+$	388.2	209	16	4.05
ACh*	+	M^+	146.1	87	15	0.43
d3-5-HTP	+	$[M+H]^+$	396.2	350	12	3.02
d3-5-HTP	+	$[M+NH_4]^+$	413.2	350	15	3.02
d4-DA	+	$[M+NH_4]^+$	391.2	374	9	3.16
d4-DA	+	$[M+H]^+$	374.1	184	20	3.16
d2-GABA	+	$[M+H]^+$	206.1	160	10	2.14
d2-GABA	+	$[M+H]^+$	206.1	114	16	2.14
d5-Glu	+	$[M+H]^+$	281.1	235	10	2.42
d5-Glu	+	$[M+H]^+$	281.1	132	21	2.42
d6-NE	+	$[M+NH_4]^+$	481.2	374	10	3.75
d6-NE	+	$[M+H]^+$	464.2	374	10	3.75
d4-ACh*	+	M^+	150.1	91	15	0.42
DHBA	+	$[M+NH_4]^+$	373.1	356	10	3.00
DHBA	+	$[M+H]^+$	356.1	123	24	3.00

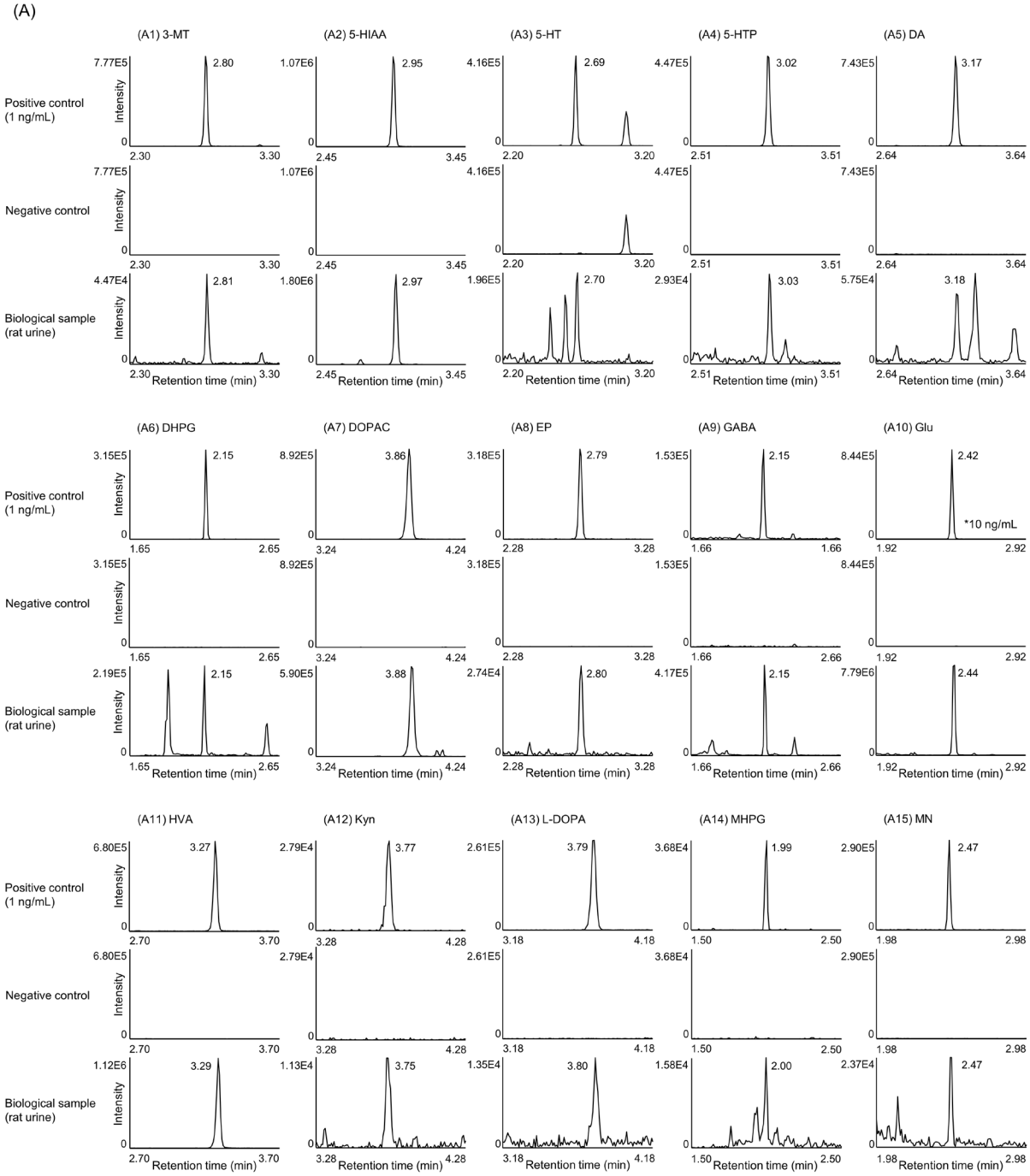

Figure S5. Chromatograms of 23 target compounds (A1-23) and 7 internal standards (B1-7) obtained by applying the developed method. For (A), the analysis results in positive and negative control and biological samples were depicted, whereas (B) illustrated the results of control samples only. According to the chromatograms of the control samples, no interference peaks were observed for any of the substances.

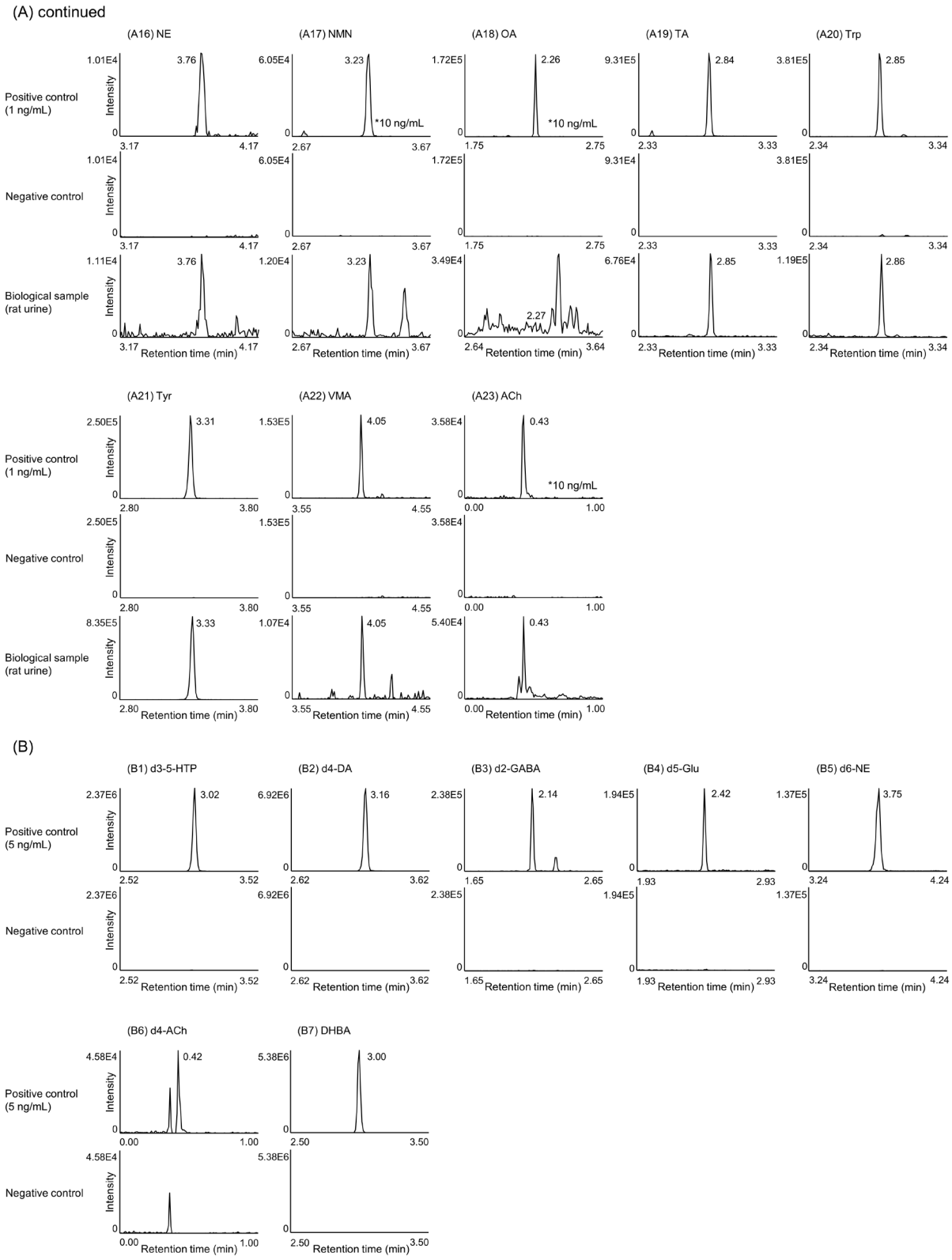

Figure S5. (continued)

Table S3. Regression equation and linearity (R^2) of neurochemicals for intra-day calibration

Compounds	Calibration range (ng/mL)			Regression equation	Linearity (R^2)
3-MT	0.01	–	1.00	y = 0.031050 x + 0.000229	0.9999
5-HIAA	0.01	–	0.50	y = 0.009340 x − 0.000065	0.9992
5-HT	0.1	–	20.0	y = 0.037230 x + 0.000100	0.9998
5-HTP	0.01	–	1.00	y = 0.019750 x + 0.000072	0.9983
DA	0.05	–	2.00	y = 0.005905 x + 0.001598	0.9992
DHPG	0.05	–	5.00	y = 0.057970 x + 0.000137	0.9988
DOPAC	0.1	–	10.00	y = 0.068840 x − 0.000918	0.9990
EP	0.5	–	20.0	y = 0.014650 x + 0.001561	0.9994
GABA	5	–	200	y = 0.179400 x − 0.021290	0.9979
Glu	10	–	500	y = 0.022060 x + 0.020570	0.9973
HVA	0.5	–	20.0	y = 0.006306 x + 0.004102	0.9992
Kyn	0.2	–	10.0	y = 0.011060 x + 0.000283	0.9997
L-DOPA	0.05	–	2.00	y = 0.011160 x + 0.000502	0.9993
MHPG	10	–	500	y = 0.002282 x + 0.002352	0.9970
MN	0.2	–	10.0	y = 0.024970 x + 0.003019	0.9990
NE	1	–	100	y = 0.009220 x − 0.000171	0.9959
NMN	2	–	100	y = 0.021000 x + 0.000942	0.9983
OA	2	–	100	y = 0.011640 x + 0.001650	0.9986
TA	0.01	–	0.50	y = 0.304800 x + 0.006747	0.9996
Trp	1	–	50	y = 0.414200 x + 0.024130	0.9996
Tyr	0.2	–	10.0	y = 0.015670 x + 0.001227	0.9986
VMA	0.2	–	10.0	y = 0.013090 x + 0.001810	0.9982
ACh	5	–	200	y = 0.011430 x − 0.011050	0.9992

Table S4. Determination of target neurochemical concentrations in an animal model system

Substance (ng/mL)	Sham (0.0 mA, n = 9)											
	Before			After 2 h			After 16 h			After 24 h		
3-MT	84.4	±	19.7	104.1	±	37.9	86.6	±	20.8	88.1	±	35.7
5-HIAA	3446	±	363	4993	±	2217	3341	±	517	3395	±	533
5-HT	339	±	26	369	±	135	331	±	61	333	±	56
DA	352	±	255	631	±	344	456	±	234	161	±	212
DOPAC	1383	±	841	2197	±	1270	1826	±	923	485	±	684
EP	20.9	±	7.5	36.1	±	19.0	23.9	±	6.5	9.6	±	3.5
GABA	81.4	±	48.8	101.9	±	79.0	61.1	±	30.1	97.0	±	86.3
Glu	303	±	203	218	±	154	291	±	206	374	±	361
HVA	3605	±	778	3895	±	1917	3771	±	1506	3664	±	993
Kyn	169	±	129	185	±	84	149	±	65	180	±	134
L-DOPA	48.5	±	45.7	64.6	±	100.3	75.1	±	67.1	106.3	±	260.5
MHPG	17.3	±	6.9	34.2	±	19.7	18.5	±	5.0	9.2	±	3.3
NE	149	±	45	295	±	183	143	±	20	97	±	70
NMN	117	±	18	188	±	103	121	±	26	126	±	56
OA	398	±	260	615	±	271	479	±	169	163	±	200
TA	1209	±	287	1326	±	568	1197	±	306	1392	±	360
Trp	299	±	243	259	±	259	244	±	220	246	±	202
VMA	239	±	54	229	±	94	246	±	49	260	±	113

Values are mentioned as mean ± standard deviation

Table S4. (continued)

Substance (ng/mL)	Stimulation (0.1 mA, n = 10)											
	Before			After 2 h			After 16 h			After 24 h		
3-MT	85.2	±	22.8	85.4	±	39.5	88.6	±	23.1	78.4	±	19.8
5-HIAA	3560	±	789	4890	±	2120	3376	±	989	3125	±	1139
5-HT	311	±	73	371	±	138	312	±	90	315	±	95
DA	485	±	268	543	±	235	552	±	310	277	±	263
DOPAC	1917	±	1103	1507	±	689	1905	±	1043	836	±	883
EP	22.7	±	10.3	68.2	±	76.9	25.4	±	10.2	11.3	±	4.6
GABA	80.4	±	32.3	134.3	±	250.0	59.4	±	25.6	67.8	±	24.6
Glu	415	±	270	191	±	167	321	±	226	475	±	355
HVA	3701	±	718	3924	±	1591	3728	±	1123	3286	±	1211
Kyn	130	±	95	159	±	145	187	±	146	159	±	110
L-DOPA	71.9	±	95.8	23.9	±	38.8	66.3	±	70.8	32.4	±	32.4
MHPG	18.3	±	9.0	58.9	±	67.7	19.0	±	9.7	9.0	±	4.4
NE	150	±	66	295	±	205	149	±	72	107	±	67
NMN	108	±	30	182	±	71	109	±	32	108	±	30
OA	519	±	204	423	±	268	531	±	216	262	±	243
TA	1345	±	297	1331	±	833	1316	±	404	1291	±	451
Trp	239	±	220	157	±	177	256	±	383	217	±	232
VMA	199	±	52	253	±	89	234	±	61	218	±	62

Values are mentioned as mean ± standard deviation

Table S4. (continued)

Substance (ng/mL)	Stimulation (0.2 mA, n = 10)											
	Before			After 2 h			After 16 h			After 24 h		
3-MT	75.8	±	29.6	93.1	±	27.0	85.2	±	25.3	83.9	±	37.5
5-HIAA	3081	±	1035	4922	±	2029	3353	±	1228	3852	±	1467
5-HT	300	±	98	394	±	87	331	±	84	356	±	135
DA	464	±	209	621	±	334	639	±	206	423	±	312
DOPAC	1455	±	715	1708	±	963	2169	±	852	1300	±	1077
EP	28.0	±	16.7	67.5	±	40.1	33.1	±	16.7	17.2	±	9.8
GABA	53.6	±	32.3	84.3	±	42.7	58.0	±	28.4	78.9	±	31.9
Glu	389	±	426	300	±	187	266	±	217	416	±	352
HVA	3326	±	1105	4035	±	1055	3701	±	1287	4058	±	1597
Kyn	167	±	139	209	±	199	193	±	129	181	±	141
L-DOPA	25.8	±	22.0	17.9	±	19.2	60.1	±	47.9	33.8	±	31.9
MHPG	24.3	±	12.2	58.9	±	31.9	28.7	±	13.9	18.3	±	12.4
NE	176	±	79	365	±	168	183	±	42	160	±	94
NMN	120	±	40	248	±	50	125	±	24	143	±	57
OA	456	±	209	586	±	207	610	±	130	382	±	269
TA	1195	±	372	1390	±	473	1268	±	410	1426	±	497
Trp	233	±	273	171	±	174	134	±	97	194	±	164
VMA	205	±	73	246	±	84	234	±	77	242	±	91

Values are mentioned as mean ± standard deviation

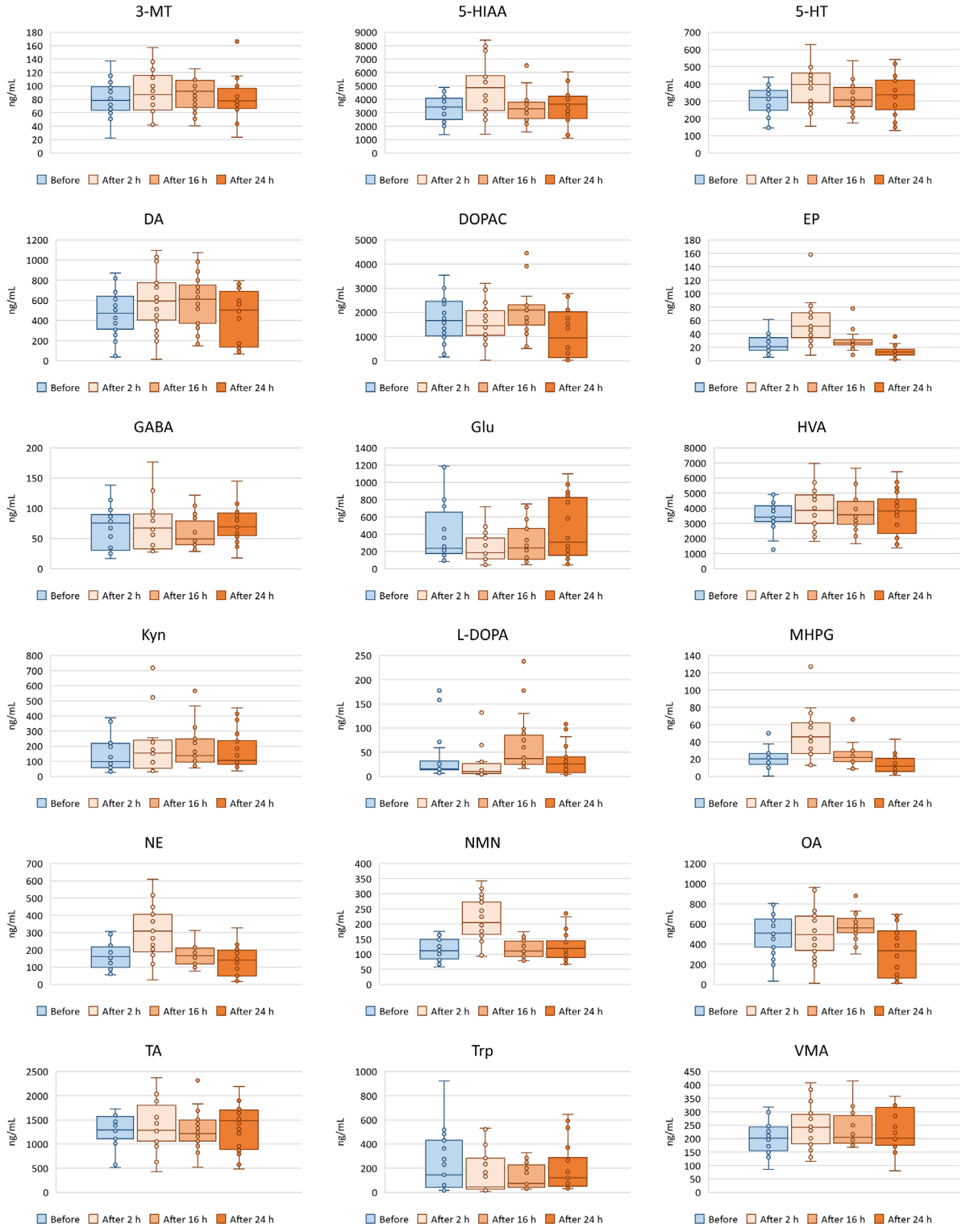

Figure S6. Changes in neurochemical concentrations in urine samples collected over time in the stimulation group. Urine was collected a total of four times: before stimulation, after 2 h, 16 h, and 24 h stimulation. The stimulation group included animals that received intensities of tDCS at 0.1 mA and 0.2 mA. All animals performed the exercise immediately before and after the stimulus. Overall, the largest changes in concentration of neurochemicals occurred after 2 hours of stimulation.

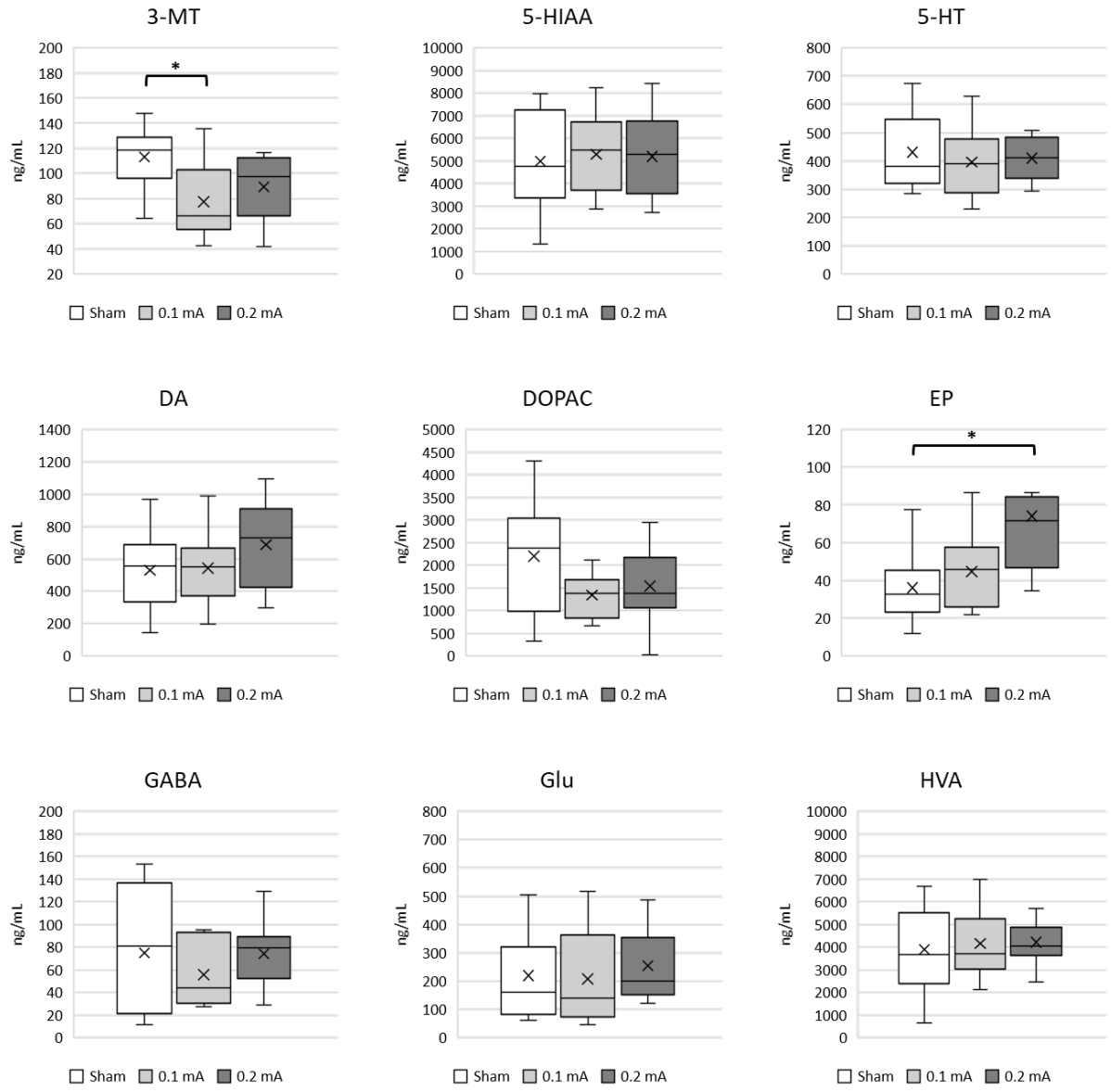

Figure S7. Differences in neurochemical concentrations between groups in samples collected 2 hours after stimulation. The Stimulation group included groups with stimulation intensities of 0.1 mA (n = 9) and 0.2 mA (n = 9), while the Sham group (n = 9) received sham stimulation with intensities of 0.0 mA. The stimulation group showed a significant decrease in 3-MT and a significant increase in EP and MHPG compared to the sham group. (* $p \leq 0.05$).

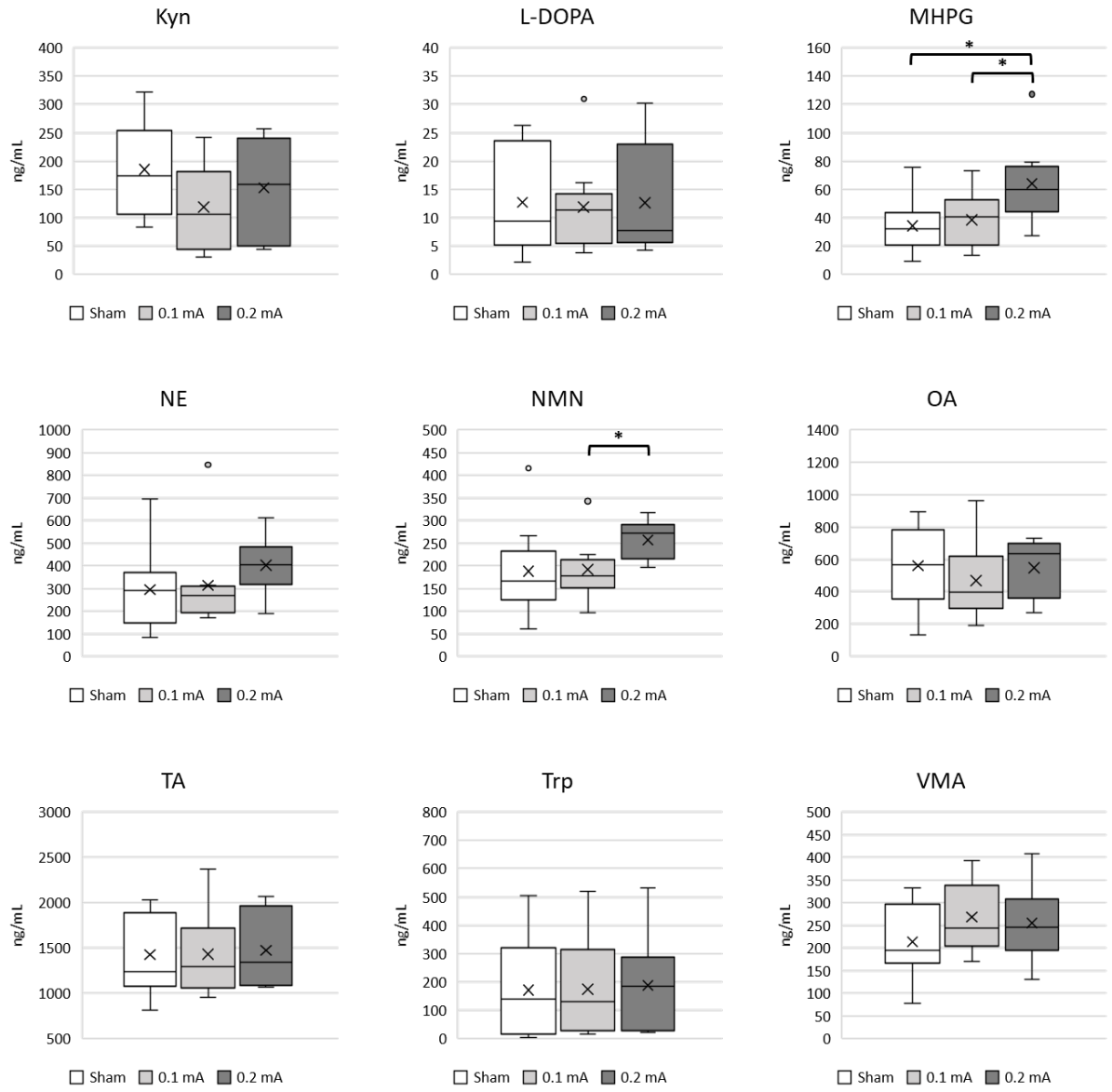

Figure S7. (continued)