Supplementary Information

Soluble form of Lingo2, an autism spectrum disorder-associated molecule, functions as an excitatory synapse organizer in neurons

Fumiaki Yoshida[a], Ryota Nagatomo[a], Shun Utsunomiya[b], Misaki Kimura[a], Shiyori Shun[a], Rena Kono[c], Yuma Kato[a], Yosuke Nao[a], Kazuma Maeda[b], Ryuta Koyama[c,d], Yuji Ikegaya[c,d,e], Stefan F. Lichtenthaler[f,g,h], Sho Takatori[a], Hiroshi Takemoto[b,i], Koichi Ogawa[b], Genta Ito[j,k], Taisuke Tomita[a,k,1]

[a]Laboratory of Neuropathology and Neuroscience, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan

[b]Neuroscience 2, Laboratory for Drug Discovery and Disease Research, Shionogi & Co. Ltd., Osaka, Japan

[c]Laboratory of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan

[d]Institute for AI and Beyond, The University of Tokyo, Tokyo, Japan

[e]Center for Information and Neural Networks, National Institute of Information and Communications Technology, Osaka, 565-0871, Japan.

[f]German Center for Neurodegenerative Diseases (DZNE), Munich, Germany

[g]Neuroproteomics, School of Medicine, Klinikum rechts der Isar, Technical University of Munich, 81675 Munich, Germany

[h]Munich Cluster for Systems Neurology (SyNergy), Munich, Germany

[i]Business-Academia Collaborative Laboratory (Shionogi), Graduate School of Pharmaceutical Science, The University of Tokyo, Tokyo, Japan

[j]Department of Biomolecular Chemistry, Faculty of Pharma-Science, Teikyo University, Japan

[k]Social Cooperation Program of Brain and Neurological Disorders, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Japan

[1]To whom correspondence should be addressed:

Dr. Taisuke Tomita

Laboratory of Neuropathology and Neuroscience, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Tel.: +81-3-5841-4877

Fax: +81-3-5841-4708

Email: taisuke@mol.f.u-tokyo.ac.jp

ORCID ID: 0000-0002-0075-5943

Running title

Soluble lingo2 is a synapse organizer.

Supplementary Material and Method

Cell culture

Cell lines and culture environment

HEK293 (#CCL-1573, ATCC), packaging cell line Lenti-X293, Flp-In™ T-Rex™ 293 cells, HA sLingo2 V5His stably expressing Flp-In™ T-Rex™ 293 cells, Flp-In™ T-Rex™ U2OS cells. HA Lingo2 V5 His stably expressing Flp-In™ T-Rex™ U2OS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal bovine serum (FBS, Hyclone, Logan. UT, USA), 50 unit/ml of penicillin (WAKO, Osaka, Japan) and Streptomycin. All cell lines were maintained in a 5% CO_2, 95% Air atmosphere incubator at 37 degrees. Contamination of mycoplasma is routinely tested by PCR and DNA agarose electrophoresis. To generate HA sLingo2 V5His stably expressing 293 cells or 6His SUMO sLingo2 stably expressing 293 cells, the Flp-In™ T-Rex™ 293 cell line was transiently cotransfected with pcDNA™ 5G/FRT expression construct and the pOG44 plasmid using Polyethylenimine (PEI) solution. Stable transfectants are selected using hygromycin and Blasticidin. To generate HA Lingo2 V5His stably expressing U2OS cells, the Flp-In™ T-RTex™ U2OS cell line was transiently cotransfected with pcDNA™ 5G/FRT expression construct and the pOG44 plasmid using Fugene 6 solution. Stable transfectants are selected using hygromycin

and Blasticidin.

Generally, cells were cultured in a 100 mm Dish (Falcon) with 10 ml DMEM and passaged to the new plate when it becomes 80-90% confluent. Subculture was performed by the following steps: First, remove excess media and wash cells with DPBS (15 mM NaCl, 5 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, 1.5 mM KH_2PO_4). Suspend the cells by incubating the cells in 0.125% Trypsin (SIGMA) at 37 degrees. After centrifuging at 1,200 rpm for 3 minutes, cells were resuspended with DMEM and 10% of the cells were transferred into a new plate with 10 mL of DMEM.

Method for culturing of primary neuronal cells.

Primary cortical neuronal cultures were prepared from the brains of embryonic day the 16 Balb/C mice (Japan SLC, Inc) as previously described. Briefly, dissociated neurons were plated at 5.0×10^5 cells per well on plates coated with poly-L-ornithine (SIGMA) and cultured in DMEM high glucose (Wako) supplemented with 50 unit/ml penicillin, 50 µg/ml streptomycin (Invitrogen), and 10% FBS (HyClone). On the following day, the cultured medium was replaced with Neurobasal medium (Invitrogen) supplemented with 2.0 mM GlutaMax™ Supplement (Thermo Fisher Scientific) 50 unit/ml penicillin, 50 µg/ml streptomycin, 0.25 µg/ml plasmocin, and B-27 supplement (Invitrogen). Cultures were maintained at 37

degrees in a 95% air / 5% CO_2 humidified incubator, and half of the medium was changed every 3 or 4 days before use.

Plasmid construction and shRNA sequences

RNA extracted from the mouse brain was reverse transcribed using the ReverTra Ace qPCR RT Kit (TOYOBO), and the mouse Lingo2 gene was amplified using the Gene-specific primer. We created a mutant lacking up to the 549th Lys residue on the N-terminal side of the transmembrane region, which is presumed to be near the cleavage site. We refer to this mutant as the soluble form of Lingo2 (sLingo2). pLKO.1-puro vector (a gift from Bon Weinberg, Addgene plasmid #8453) is a lentiviral vector for small interfering RNA expression encoding puromycin resistance gene under the human phosphoglycerate kinase promoter. Short hairpin RNA (shRNA) sequences were cloned into pLKO.1 CMV-EGFP according to the protocol by Addgene. The sequences of primers used for plasmid construction are listed in Table.

shRNAs	Primer sequence
sh mock	CCGGGGAATCTCATTCGATGCATACctcgagGTATGCATCGAATGAGATTCCTTTTTG
	AATTCAAAAAGGAATCTCATTCGATGCATACctcgagGTATGCATCGAATGAGATTCC
sh Lingo2 #1	CCGGGCATCTGAAGCATCTCAATATctcgagATATTGAGATGCTTCAGATGCTTTTTG
	AATTCAAAAAGCATCTGAAGCATCTCAATATctcgagATATTGAGATGCTTCAGATGC
sh Lingo2 #2	CCGGCGGCACTAATGCCAATACTTTctcgagAAAGTATTGGCATTAGTGCCGTTTTTG
	AATTCAAAAACGGCACTAATGCCAATACTTTctcgagAAAGTATTGGCATTAGTGCCG
sh ADAM9	CCGGgctcctgcacctcctttatatctcgagatataaaggaggtgcaggagcTTTTTG
	AATTCAAAAAgctcctgcacctcctttatatctcgagatataaaggaggtgcaggagc
sh ADAM10	CCGGtagacattatgaaggattatcCTCGAGatctgtaatacttcctaatagTTTTTG
	AATTCAAAAAtagacattatgaaggattatcCTCGAGatctgtaatacttcctaatag
sh ADAM17	CCGGgcagcactccataaggaaactcgagtttccttatggagtgctgcTTTTTG
	AATTCAAAAAgcagcactccataaggaaactcgagtttccttatggagtgctgc

Antibodies

Antibodies for immunostaining and immunoblotting

Name	Immunogen	Cat. No,	Host	Application
vGlut1	AA354-560 from rat vGlut1	135 303 synaptic systems	Rabbit	1000×dilution (WB)
vGat	AA75 to 87 from rat vGat	131 002 synaptic systems	Rabbit	1000×dilution (WB)
HA-High	High affinity to YPYDVRDYA	3F10, Roche	Rat	1000×dilution (WB)
β3-tub	Rat brain-derived microtubules	AB9354	Chicken	1000×dilution (ICC)
β3-tub	Rat brain-derived microtubules	Tuj-1, R&D systems	Mouse	5000×dilution(WB)
synaptophysin	Full length native protein (purified) corresponding to Bovine Synaptophysin. Synaptophysin from presynaptic vesicles, prepared from bovine brain.	SY38, ab8049, abcam	Mouse	200×dilution (ICC)
synapsin1	Recombinant protein corresponding to AA 1 to 704 from rat synapsin 1	106 001 synaptic systems	Mouse	500×dilution(ICC)
Homer1	AA1-196 from human Homer1	160 002 synaptic systems	Rabbit	500×dilution(ICC) 1000×dilution(WB)
Map2		Poly28225	Chicken	2000×dilution(ICC)
Map2	Mix of recombinant human constructs of projection domain sequences, amino acids 235-1588.	ab5392, abcam	Chicken	500×dilution(ICC)
Lingo2	AA393-421 from human Lingo2	LS-C157866, LSBio	Rabbit	1000×dilution (WB)
Lingo2	AA1-549 from mouse Lingo2	11D8, Self made antibody	Rat	
ADAM10	Synthetic peptide corresponding to Human ADAM10 aa 732-748	ab1997, abcam	Rabbit	1000×dilution (WB)
Gephyrin	Recombinant protein corresponding to AA 307 to 735 from rat Gephyrin	147 111 synaptic systems	Mouse	1000×dilution (WB)
PSD95		K28/43	Mouse	1000×dilution (WB)
sAPPα	Synthetic peptide in portion of Huma sAPPα（DAEFRHDSGYEVHHQK）	#11088, Immuno-Biological Laboratories	Rabbir	50×dilution(WB)
α-tubulin	Purified chick brain tubulin	T6199, SIGMA	Mouse	1000×dilution (WB)

Compounds

Metalloprotease inhibitors

・GM6001 (Enzo Lifescience)

・INCB3619 (INCB3619 were synthesized as previously described[33])

・GI254023X (MERCK)

γ-secretase inhibitor

・N-[N-(3,5-difluorophenacetyl)-L-alanyl]]-(S)-phenylglycine t-butyl ester (DAPT)

(DAPT were synthesized as previously described[34])

Lentivirus

Lentiviral production

For the production of the recombinant lentivirus, LentiX-293T cells were transiently cotransfected with the packaging plasmid (pCAG-kGP4.1R, pCAG4-RTR2, and pCAGGS-VSVG) and the lentiviral transfer plasmid using PEI solution. After the collection of the medium including lentivirus particles, it was concentrated using a Lenti-X™ concentrator (Clonetech, Mountain View, CA, USA). The lentiviral particles were resuspended in HBSS and stored at -80 degrees until usage.

To be more specific, LentiX-293 cells were seeded into a 10 cm Dish at 3.0×10^6 cells

and cultured for one night before plasmid transfection. For 10 ml medium plasmid transfection was conducted by mixing 5.0 µg DNA with 80 µl PEI solution, 900 µL Opti-MEM I (1X) (Thermo Fisher Scientific), and 5.0 µg of three packaging plasmids (pCAG-kGP4.1R, pCAG4-RTR2, and pCAGGS-VSVG in 3:1:1) at room temperature and incubated for 10 minutes before adding to the cells. The 7.0 ml medium was refreshed 6 hours post-transfection and collected on the next day. Additional 8.0 ml DMEM medium was added to cells and incubated overnight. The medium was collected using the same tube. All media were filtered with a 0.45 µm filter, mixed with a 5.0 ml Lenti-X^{TM} concentrator, and stored at 4 degrees overnight after gently mixing the solution. The mixture was centrifuged at 1,500 g for 45 minutes at 4 degrees. The pellet was resuspended using HBSS and the lentivirus-containing medium was aliquoted to 15 µl/tube and stored at -80 degrees.

Recombinant sLingo2 production

HA-sLingo2-V5-His produced by fusing the extracellular portion of mouse Lingo2 (residues1-549) to the V5-His tag was expressed in Flp-in TREx 293 cells and purified from filtered cell culture medium on Ni-NTA agarose (QIAGEN). The HA sLingo2 V5-His was eluted from the agarose with 50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 500 mM imidazole. The preparation was ultraconcentrated against PBS, filtered and divided into aliquots that

were frozen on dry ice, and stored at -80 degrees. 6His SUMO sLingo2 was purified same protocols.

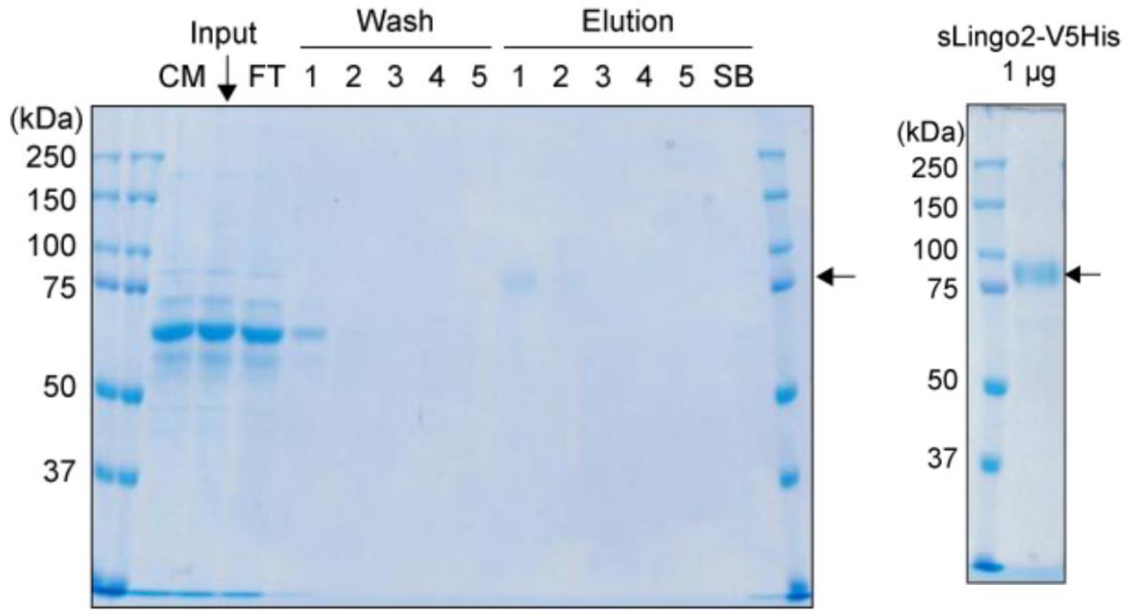

Figure. Purification of recombinant HA-sLingo2-V5-His (arrow) from conditioned media (left panel). The purity of the concentrated eluate was analyzed by CBB staining (right panel).

Immunoblotting

The samples after protein quantification were added with 1% of 2-Mercaptoethanol. For measurement using the medium, the appropriate amount of medium was mixed with 5×Laemmli sample buffer and 1% 2-mercaptoethanol. For the cell lysate, the samples were

incubated at 37 degrees to avoid the degradation of membrane proteins. The medium samples were boiled at 100 degrees. Samples and protein marker, Precision Plus Protein Dual Xtra Standards (BIORAD, Hercules, CA, USA), were applied to SDS-polyacrylamide gel (7.5%-15% Tris-Glycine or Tris-Tricine gels) and transferred onto PVDF membrane (Millipore, Burlington, MA, USA). Blocking was performed by incubating the membrane with 5% skim milk (Difco) / TS-tween (0.1% Tween20 (KANTO), 50 mM Tris-HCl, pH 7.6, 150 mM NaCl) for 30 minutes on a shaker. The membrane was washed three times with TS-tween. The primary antibody was diluted to the appropriate concentration by Immuno-Enhancer Reagent A (WAKO, Osaka, Japan) or skim milk and applied to the membrane. After overnight incubation at 4 degrees, the membrane was retrieved and washed with TS-tween three times. Secondary antibodies conjugated with horseradish peroxidase (HRP) were diluted to appropriate concentration by Immuno-Enhancer Reagent B (WAKO, Osaka, Japan) and applied. After incubation at room temperature, the membrane was washed and ready for detection. The immunodetection was done using an Immunostar detection kit (WAKO, Osaka, Japan) or ImmunoStar LD (WAKO, Osaka, Japan), and chemiluminescence was detected using Image Quant LAS4000 (GE Healthcare, Chicago, IL, USA). The immunoreactive protein bands were digitally captured and quantified using ImageQuant TL software (GE Healthcare, Chicago, IL, USA) following the manufacture’s instruction.

Immunocytochemistry

Sufficient cells were cultured on glass coverslips. The medium was removed, and the glass coverslips were fixed for 15 minutes in 4%paraformaldehyde/PBS. The glass coverslips were washed by PBS and treated with blocking buffer (0.2% Triton-X-100/1% BSA / PBS) overnight. Primary antibodies were diluted to appropriate concentration by blocking buffer. The glass coverslips were then incubated with primary antibodies overnights. Secondary antibodies were diluted to appropriate concentration by blocking buffer. After washing with PBS 3 times, the coverslips were incubated in dark with fluorescence-labeled secondary antibodies for 3 hours. The coverslips were then washed by PBS three times and mounted on glass slides using Prolong Antifade Mounting Medium with DAPI (Thermo Fisher Scientific). Images were collected using a fluorescence microscope (AxioObserver Z1, Zeiss) with AxioVision software. Images were processed using ImageJ software (NIH).

Cultured slices were fixed in 4% PFA at 37°C for 15 minutes and subsequently at 4°C overnight. The fixed samples were rinsed 3 times with PBS. Slices were then permeabilized and blocked for 1 hour at room temperature in PBS with 0.3% Triton X-100 and 10% goat serum. The samples were subsequently incubated with guinea pig anti-vGlut1 (1:1000; 135 304; Synaptic Systems, Goettingen, Germany) in PBS with 0.3% Triton X-100 and 10% goat

serum at 4°C 2 overnight with agitation. After the samples were rinsed three times with PBS, they were incubated with Alexa Fluor 594-conjugated goat anti-guinea pig IgG (1:500; A-11076; Thermo Fisher Scientific, MA, USA), DAPI (1:1000; D9542; Sigma-Aldrich, St. Louis, USA), and NeuroTrace (500/525 Green Fluorescent Nissl Stain, 1:1000; Thermo Fisher Scientific, MA, USA) in PBS with 0.3% Triton X-100 and 10% goat serum at 4°C overnight with agitation. Finally, the samples were rinsed 3 times with PBS and mounted on glass slides using Permafluor (Thermo Fisher Scientific, MA, USA). The samples of hippocampal slice culture were scanned using a laser scanning confocal microscope (A1-HD25; Nikon, Tokyo, Japan) equipped with a 10× air objective (CFI Plan Apo Lamda S 10×; NA0.45, Nikon), 20× air objective (CFI Plan Apo VC 20×; NA0.75, Nikon), and 100× oil objective lens (Apo TIRF 100× Oil, NA1.49, Nikon).

Urotokoruiha

Reverse Transcription Polymerase Chain Reaction assays

Cells with 80-100% confluency were washed with PBS once. ISOGEN (NIPPON GENE) was added to a sample, after dissolving, chloroform was added to the mixture and centrifuged at 15,000 rpm, 4 degrees, 15 min. The upper phase is collected and 500 μl of isopropanol was added to the mixture and centrifuged at 15,000 rpm, 4 degrees, 15 min. The supernatant

was removed and 70% Ethanol was added and centrifuged at 15, 000 rpm, 4 degrees, 10 min. The supernatant was completely removed, and RNA was dissolved with MilliQ. RNA was incubated at 65 degrees for 5 min and then rapidly cooled on ice. cDNA was generated using ReverTra Ace qPCR RT Master Mix with gRNA Remover (Toyobo, catalog#FSG-301) according to the supplier's instruction.

cDNA mixed with gene-specific primer and Thunderbird SYBR qPCR Mix (Toyobo, catalog #QSP-201), and qRT-PCR was run on LightCycler 480 instrument (Roche). The PCR condition was as follows: 1 cycle of denaturation (95 degrees, 1 min); 50 cycles of denaturation (95 degrees, 10 s), annealing (60 degrees, 30 s), and extension (72 degrees, 1 min). Specificity was confirmed by melting curve analysis and agarose gel electrophoresis. The sequences of primers used qRT-PCR are listed.

Gene ID	Full Name	Forward primer sequence	Reverse primer sequence
Lingo2	Leucine-rich repeat and immunoglobulin domain-containing	CCTAAAGAAGCCCGGAGACT	TGTCACTCTCCAATCCCACA
ADAM9	Disintegrin and metalloproteinase domain-containing protein 9	GTTTACACCTACGACAAGGAAGGC	TCCCTCCACATAGCCTCGATAGT
ADAM10	Disintegrin and metalloproteinase domain-containing protein 17	GCTGTGCAGATCACTCCGTT	TTCAGCCAGAGTTGTGCGTT
ADAM17	Disintegrin and metalloproteinase domain-containing protein 17	GAGCGGTGACCACGAGAATA	TCCTGGAAGCACTCTTGAGC
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	AACGACCCCTTCATTGAC	GAAGACACCAGTAGACTCCAC

The exact mean values and SEMs in figures

				mean value	SEM
Figure1	C	vGlut1	Saline	1	0.122902
			VPA	1.591511601	0.067166
		vGat	Saline	1	0.08457
			VPA	1.131576085	0.066676
	E	vGlut1	Saline	1	0.070369
			VPA	1.228633654	0.068789
		vGat	Saline	1	0.039798
			VPA	1.001445701	0.056205
	H	vGlut1	Saline	1	0.070352
			VPA	1.238804268	0.053865
		vGat	Saline	1	0.125715
			VPA	0.915951084	0.033892
	J	vGlut1	Saline	1	0.109515
			VPA	1.626494168	0.121265
			Boiled	1.111053838	0.115004
		vGat	Saline	1	0.080478
			VPA	0.854065452	0.084163
			Boiled	0.863636988	0.087902

				mean value	SEM
Figure2	C	Lingo2 protein level	Saline	1	0.11656
			VPA	1.75	0.21223
	D	Lingo2 mRNA level	Saline	1.00E+00	1.05E-01
			VPA	9.82E-01	1.26E-01

				mean value	SEM
Figure3	C	sLingo2	Control	1	0.031027
			GM6001	0.522996017	0.040891
			INCB3619	0.477900636	0.033463
			GI254023X	0.400961091	0.026723
		sLingo2	Control	1	0.202034
			shAdam9	0.969522571	0.120208
			shAdam10	0.37794083	0.08593
			shAdam17	1.165353585	0.127438

				mean value	SEM
Figure4	C	vGlu1	Control	1	0.05468
			sLingo2	1.297428634	0.090957
		vGat	Control	1	0.059932
			sLingo2	0.993869209	0.101636
	E	vGlu1	Control	1	0.036317
			sLingo2	1.162657416	0.040401
		vGat	Control	1	0.021126
			sLingo2	1.044370384	0.028358
	G	vGlut1	Control	1	0.052894
			sLingo2	1.541186969	0.051487

				mean value	SEM
Figure5	B	vGlut1	Saline shMock	1	0.05034
			Saline shLingo2	0.812612465	0.032643
			VPA shMock	1.413453429	0.074045
			VPA shLingo2	0.824270126	0.103525
		vGat	Saline shMock	1	0.110608
			Saline shLingo2	0.723917753	0.07808
			VPA shMock	0.953356978	0.113893
			VPA shLingo2	0.765652309	0.09775
	D	vGlut1	Saline shMock	762.45	113.8605
			Saline shLingo2	548.6666667	74.48699
			VPA shMock	1164.578947	139.8002
			VPA shLingo2	544.85	77.81359

				mean value	SEM
Figure6	C	excitatory synapse	Control	10.96	1.351981
			sLingo2	18.301875	1.445616
	E	Amplitude	Control	47.49601807	6.18246
			sLingo2	63.95690795	7.80091
	F	Frequency	Control	0.721763348	0.098714
			sLingo2	3.133185626	1.111378

F- and p-values in figures

Fig.1J	vGlut1	ANOVA	F value	8.40
			p value	0.000855
		Tukey	VPA-Control	0.001166
			Boil-Control	0.775949
			Boil-VPA	0.008072
	vGat	ANOVA	F value	0.939
			p value	0.399

Fig.3C	sLingo2	ANOVA	F value	65.8
			p value	3.23E-09
		Tukey	GI254023X-DMSO	0
			GM6001-DMSO	1E-07
			INCB3619-DMSO	0
			GM6001-GI254023X	0.084491
			INCB3619-GI254023X	0.391839
			INCB3619-GM6001	0.776659
	sLingo2	ANOVA	F value	6.01
			p value	6.06E-03
		Tukey	HA Lingo2 ADAM9 KD-HA Lingo2	0.998657
			HA Lingo2 ADAM10 KD-HA Lingo2	0.029499
			HA Lingo2 ADAM17 KD-HA Lingo2	0.838272
			HA Lingo2 ADAM10 KD-HA Lingo2 ADAM9 KD	0.039844
			HA Lingo2 ADAM17 KD-HA Lingo2 ADAM9 K	0.759291
			HA Lingo2 ADAM17 KD-HA Lingo2 ADAM10 KD	0.005506

Fig.5B	vGlu1	ANOVA	F value	15.9
			p value	1.59E-05
		Tukey	Saline	0.266404
			VPA-Saline	0.002539
			VPA	0.317947
			VPA-Saline	3.68E-05
			VPA	0.999405
			VPA	4.76E-05
	vGat	ANOVA	F value	1.82
			p value	1.76E-01

Fig.5D	vGlu1	ANOVA	F value	7.7383
			p value	0.000141
		Tukey	Saline Lingo2 KD-Saline mock	0.454426
			VPA mock-Saline mock	0.04003
			VPA Lingo2 KD-Saline mock	0.449408
			VPA mock-Saline Lingo2 KD	0.0004
			VPA Lingo2 KD-Saline Lingo2 KD	0.999993
			VPA Lingo2 KD-VPA mock	0.000434