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Expression analysis for inverted effects of serotonin transporter inactivation

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Abstract

Inactivation of serotonin transporter (HTT) by pharmacologically in the neonate or genetically increases risk for depression in adulthood, whereas pharmacological inhibition of HTT ameliorates symptoms in depressed patients. The differing role of HTT function during early development and in adult brain plasticity in causing or reversing depression remains an unexplained paradox. To address this we profiled the gene expression of adult *Htt* knockout (*Htt* KO) mice and HTT inhibitor-treated mice. Inverted profile changes between the two experimental conditions were seen in 30 genes. Consistent results of the upstream regulatory element search and the co-localization search of these genes indicated that the regulation may be executed by Pax5, Pax7 and Gata3, known to be involved in the survival, proliferation, and migration of serotonergic neurons in the developing brain, and these factors are supposed to keep functioning to regulate downstream genes related to serotonin system in the adult brain.

Keywords: Serotonin transporter; Gene knockout; Depression; Model mouse; Transcriptional regulation

Depression is a devastating disorder with a life-time prevalence of 15–20%, and its cause is multi-factorial with emotional stress interacting with genetic susceptibility [1,2]. A confirmed genetic risk for this disease is a polymorphism, short low-expressing form of a repeat in the transcriptional control region of the serotonin (5-hydroxytryptamine; 5-HT) transporter (HTT) gene, the product of which mediates pre-synaptic reuptake of 5-HT [3]. *HTT* is transcribed mostly in the raphe nuclei of the adult brain, but the functional or structural changes in the depression are observed throughout the brain, especially in the hippocampus, the amygdala, and the prefrontal cortex [4,5]. HTT regulates the content of 5-HT in the synaptic junction, and 5-HT functions as a modulator of the development of brain systems involved in emotional and stress responses in many part of the brain through serotonin receptors [6,7]. The *HTT* expression outside of the raphe nuclei is known in the developmental brain, and low levels of HTT modify gene expression and formation of neuronal circuits such as thalamocortical pathways [8]. HTT is such a versatile protein through the brain development to the adult stage, so that the deregulation of its expression level may cause the wide variety of clinical symptoms.

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Htt KO mice are a model for depression exhibiting anxiety-like responses and behavioral despair, and the brain structural closely resemble those of the depressed patients, including the increases of the apical dendritic branches in pyramidal neurons in the infralimbic cortex and the greater spine density in the basolateral amygdala [9]. Thus, the molecular status of the mice is expected to represent a part of that in depressive patients, and allows us to assess consequences of functional loss of *Htt* [10,11]. In the knockout procedure, the genome including the exon 2 of *Htt*, where the authentic translation starting site is located, is substituted to Neo in the B57BL/6 mouse. Then, the short-form RNA without the exon 2 is transcribed, where the alternative translation starting site in frame of the WT sequence in the exon 3 is activated, resulting in escaping from the nonsense mediated decay. Thus the short-form RNA is stable, however, the short-form protein product, lacking the first 123 amino acids, is unstable because of the absence of the trans-membrane domain essential for proper sorting of transporter proteins. The UREs of *Htt* in the KO mouse is same as the WT, which can execute the proper regulation of *Htt* at the transcriptional level.

Functional inhibitors of HTT, instead, are effective antidepressant compounds known as selective serotonin

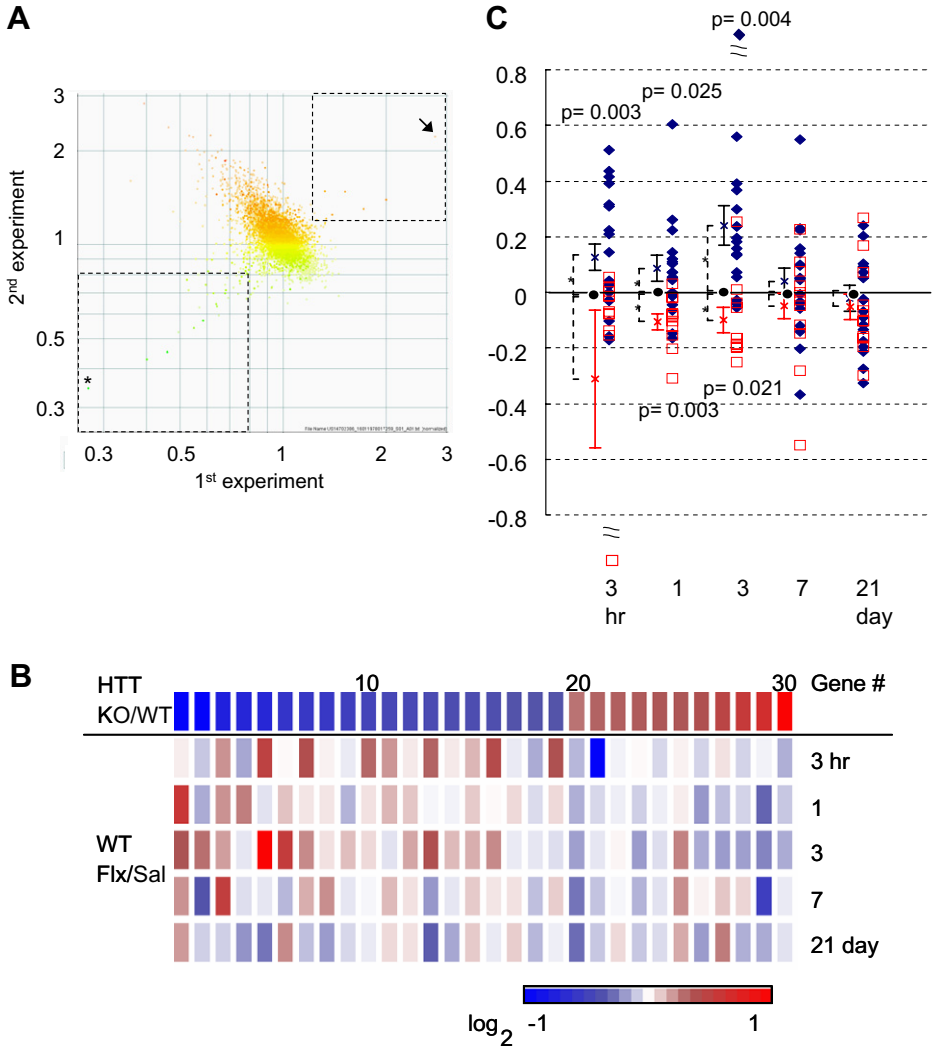

Fig. 1. Gene expression profiles of *Htt* KO mice and Flx-treated mice in the hippocampus. (A) Fold changes of normalized signal intensity with microarrays in the Htt KO/WT experiment. Dye-swapped method was employed in the technical replication. Dotted lines indicate the area for data with alteration more than 20% consistently. A datum for *Htt* is indicated with an arrow. A datum with an asterisk was excluded from the analysis for the reason described in the supplementary data. (B) Fold changes of normalized signal intensity with microarrays in two experimental conditions (Htt KO/WT and treated with Flx/Sal for the indicated periods) are calculated and top 30 genes perturbed in the KO experiment are selected. Fold changes of the selected genes are visualized with two colors as indicated with the color bar. (C) Statistic significance of inverted changes in the expression in Flx/Sal compared with KO/WT conditions. Expression log ratios of Flx/Sal for two gene groups decreased ($n = 19$) or increased ($n = 11$) in KO mice are colored with dark blue or red, respectively, in indicated time points. The means and standard errors are shown with error bars. The *P*-values in the *t*-test against the corresponding means of microarray data including ca. 40,000 genes in the time points (shown with closed circles) are indicated, if smaller than 2.5% of significance (with asterisks). (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

reuptake inhibitors (SSRI) such as Fluoxetin (Flx) [12]. Since the serotonin level in the synaptic cleft is primarily regulated by HTT, Flx administration results in an increase of synaptic 5-HT levels. The pharmacological inactivation of HTT in the adult brain ameliorates depressive symptoms, whereas the treatment of immature brain with Flx results in increased tendency of depressive behavior in the adults [13].

Getting together, differing effects of the inactivation of HTT in the neonatal period by genetic or pharmacological methods and in the adulthood by pharmacological methods resulting in depressive and anti-depressive status, respectively, remains to be unsolved paradox. To address this, we compared the gene expression profiles of male adult mice with *Htt* KO and those with Flx treatment in order to identify a group of genes with the differing expression levels corresponding to the phenotypes. Then we could find a group of genes with inverted expression alteration in the two experimental conditions, and its regulatory transcription factor candidates, consensus recognition sites of which were enriched in the upstream of the gene group and displaying the similar expression pattern with *Htt*.

Materials and methods

Animals. The mice were housed in a humidity and temperature controlled room with 12-h light/dark cycles. Prior to brain extraction they were anesthetized with diethyl ether and transcardially perfused with RNAlater solution (Ambion, Inc., Austin, TX). All procedures involving animals and their care were carried out in accordance with the directives of RIKEN's Institutional Animal Care and Use Committee.

(i) *Comparison of KO/WT mice*. *Htt* KO mice were generated by homologous recombination [10]. KO: *Htt* (−/−) mice and WT: *Htt* (+/+) mice were born from homozygous breeding. Adult male KO mice or WT mice of C57BL/6 background at 12 week were subjected to experiments for gene expression profiling.

(ii) *Effect of Flx/Sal in WT mice*. In the pharmacological studies WT mice of C57BL/6 at 9 week were subjected to the treatment with intra-peritoneal injection of 10 mg/kg Flx resolved with saline or with only saline (Sal) for 3 h, 1 day, 3 days 7 days and 21 days once a day, respectively.

Manual sample preparation and gene expression profiling. Indicated portions of the fresh brains derived from KO/WT mice or Flx/Sal treated mice were manually dissected and snapped frozen. Frozen tissues derived from five mice for each experimental condition were mixed, and the RNA was extracted with Total RNA Isolation Mini Kit

Table 1
The list of top 30 genes perturbed with the *Htt* KO experiment

#	Gene name	RefSeq ID	Function of gene products	Reference
1	Hbb-bh1	NM_008220	Hemoglobin Z, beta-like embryonic chain	Proc. Natl. Acad. Sci. USA 74 (1977) 4406–4410
2	Erdr1	NM_133362	Induce differentiation	Cytokine 26 (2004) 231–242
3	Ptgds	AB006361	Induce sleep	Proc. Natl. Acad. Sci. USA 96 (1999) 726–730
4	Alas2	NM_009653	Ezyme in erythroid	Gene 247 (2000) 153–166
5	S100a9	NM_009114	Nerve protection	Am. J. Med. Genet. B Neuropsychiatr Genet. 133 (2005) 1–5
6	2310043N10Rik	AK028745	Non-coding	Nature 409 (2001) 685–690
7	Agxt2l1	NM_027907	Oxaloaciduria causative gene	Nature 409 (2001) 685–690
8	Spp1	NM_009263	Osteopontin	J. Neurosci. 27 (2007) 3603–3611
9	Atp1a2	NM_178405	Na pump, Ca regulation, hetero ± display anxiety	J. Biol. Chem. 281 (2006) 12929–12940
10	Pdk4	NM_013743	Gucose metabolism	Biochem. J. 344 (Pt 1) (1999) 47–53
11	P2rx5	NM_033321	Ion channel	FASEB J. 18 (2004) 1404–1406
12	Mglap	NM_008597	Inducer of calcification, coagulation factor	J. Bone Miner. Res. 6 (1991) 1013–1017
13	Ctla2a	NM_007796	Cytotoxic T lymphocyte-associated protein 2 alpha	Nature 322 (1986) 268–271
14	Pitpn, Pitpna	NM_008850	Phosphatidylinositol transfer protein-alpha in netrin-1-induced PLC signalling and neurite outgrowth	Nat. Cell Biol. 7 (2005) 1124–1132
15	Vwf	NM_011708	Von Willebrand factor homolog	Dev. Biol. 148 (1991) 51–62
16	S3-12	NM_020568	Manbrane protein	Nat. Biotechnol. 16 (1998) 581–586
17	Lrrc8	NM_177725	Leucine rich repeat containing	DNA Res. 10 (2003) 167–180
18	Per2	NM_011066	Period 2: circadian expression	Neuron 19 (1997) 1261–1269
19	Icosl	NM_015790	Lymphocyte Icos ligand	Nature 402 (1999) 827–832
20	Luzp2	NM_178705	Neuronal transcriptional enhancer	BMC.Genomics 6 (2005) 157
21	Thy1	NM_009382	Thymus cell antigen 1	Eur. J. Immunol. 6 (1976) 557–562
22	Tubb2b	NM_023716	Tubulin, beta 2b	Proc. Natl. Acad. Sci. USA 97 (2000) 9127–9132
23	Hop	NM_175606	Homeo box only; SRF-dependent transcriptional regulator	Cell 110 (2002) 713–723 Cell 110 (2002) 725–735
24	Auh	NM_016709	RNA-binding and fatty acid degradation	Gene 228 (1999) 85–91
25	9330161C17Rik	AK045675	Transmembrane protein 44	Nature 409 (2001) 685–690
26	Arc	NM_018790	Increasing in learning	Neuron 52 (2006) 437–444
27	Cox17	BC048668	Cupper metabolism and cytochrome oxidase assembly	Proc. Natl. Acad. Sci. USA 99 (2002) 16899–16903
28	Fabp7	NM_021272	Fatty acid binding protein 7, expressed in undifferenciated progenitor	Neuron 12 (1994) 895–908
29	Pisd	NM_177298	Phosphatidylserine decarboxylase	Nat. Genet. 28 (2001) 241–249
30	Slc6a4	NM_010484	Serotonin transporter	Mamm. Genome 4 (1993) 283–284

The index numbers (#) are corresponding to those in Figs. 1 and 2.

(Agilent Technologies) and subjected to Agilent mouse oligo including ca. 20,000 gene probes in total (Agilent Technologies). The samples were tested in dye-swapped ways. Data was normalized with the methods of Lowiss and standardized with 50‰ expression levels. The RNA samples were also subjected to quantitative RT-PCR [14].

3D-ISM sample preparation and expression profiling. Three-dimensional internal structure microscopy (3D-ISM), a combined system with a cross-sectioning device, a microscope and a CCD camera, which sequentially makes slices and takes surface pictures of frozen materials, and reconstructs them into the 3D image was described previously [15,16]. Series of 5 μm frozen-sections were produced by the automated microdissection machine in six different axes. A tissue sample contains 200 sliced sections and total 61 samples, 6 samples from the axial section, 7 from the axial oblique section, 13 from the coronal, 16 from the coronal oblique, 9 from the sagittal and 10 samples from the sagittal oblique sections, were produced. Extracted RNA samples with magnetic-beads based RNA extraction kit (Ambion) were subjected to Agilent microarrays (Whole Mouse Genome 4× 44 K oligo) with the single color method. Data were per-chip normalized to 50‰. We employed coefficient of variation (CV = standard deviation/mean) as an index of regional enrichment. The top 1500 gene of higher CVs in the whole brain were clustered as previously described [17].

Upstream regulatory element (URE) search. We have searched for the binding motifs of transcription factors by using the scoring matrix of TRANSFAC (version 7.1 with the most specific option, minFP_good71, [18] in the promoter regions (2 kb on upstream side and 0.2 kb on downstream side from transcription start sites) of the indicated genes. The transcription start sites were determined by aligning full-length mRNA sequences from RefSeq database [19] to the mouse genome (hgdownload.cse.ucsc.edu/apache/htdocs/goldenPath/mm5/bigZips).

Results and discussion

Microarray analysis of the gene expression in KO/WT and Flx/Sal experiments

We first examined the gene expression profile of the hippocampus, a major target region for antidepressants [20]. Consistently in two technical replications, the genes with more than 20% increased or decreased expression levels were only 30 including the *Htt (Slc6a4)* with twofold increase in the *Htt* KO mice (Fig. 1A and supplementary data 1). Biological functions of these 30 genes seemed to be highly diverse (Table 1). Intriguingly, the two genes regulating sleep cycles (Per2 and Ptgds), a gene increasing in learning (Arc), and a gene genetically related to anxiety (Atpla) were in the list (references in Table 1). Difficulty of sleep and learning, and anxiety are constituent of depressive symptoms. As though the expression change in each gene was not very significant, an accumulation of subtle changes of expression levels of the group of genes may contribute the depressive symptoms, as is known with congenic mice study that genetic backgrounds influence the anxiety-like behavior of *Htt* KO mice [21].

Comparing the expression profiles of the 30 genes between KO/WT and Flx/Sal experiments, we found that the expression level was altered to the opposite directions

		KO/WT						Flx/Sal						URE (2K)		
#	gene name	CtxA	CtxP	Thal	MidB	MedO	Cebl	CtxA	CtxP	Thal	MidB	MedO	Cebl	GATA	PAX	AML1
1	Hbb-bh1	0.14	0.19	0.13	0.19	0.19	0.16	1.22	1.19	1.59	1.34	1.27	1.76	2	–	–
2	Erdr1	0.45	0.40	0.43	0.38	0.36	0.36	1.72	1.58	1.59	1.36	1.67	1.74	no data		
3	Ptgds	0.75	0.85	0.81	0.89	0.90	0.88	1.18	0.93	1.07	0.87	0.91	1.01	–	1	5
4	Alas2	0.50	0.21	0.58	0.46	0.29	0.44	1.31	1.15	1.19	1.28	1.36	1.57	2	–	1
5	S100a9	0.17	0.05	0.10	0.12	0.67	0.13	1.02	3.35	1.44	6.45	2.02	1.68	3	1	2
6	2310043N10Rik	0.61	0.60	0.62	0.66	0.86	0.71	1.12	1.81	0.93	1.19	1.12	1.26	2	3	2
7	Agxt2l1	0.51	0.46	0.64	0.65	0.64	0.52	0.98	1.37	1.07	1.25	1.07	1.21	1	6	3
8	Spp1	0.74	0.63	0.74	0.78	0.90	0.89	1.24	1.08	0.89	1.01	0.89	1.00	4	–	2
9	Atp1a2	0.98	0.87	1.19	1.54	1.40	1.01	1.01	1.28	0.99	1.06	1.00	1.04	–	1	2
10	Pdk4	0.80	0.50	0.51	0.53	0.76	0.75	0.95	1.34	0.64	1.16	0.94	1.20	4	3	2
11	P2rx5	0.51	0.20	0.40	0.28	0.25	0.79	1.38	1.33	1.14	1.11	1.06	1.20	1	–	6
12	Mglap	0.69	0.75	0.81	0.97	0.77	1.09	1.16	1.27	1.36	1.19	1.21	1.35	2	2	2
13	Ctla2a	0.54	0.50	0.55	0.51	0.52	0.49	1.08	1.22	1.43	1.39	1.18	1.36	3	1	3
14	Pitpn, Pitpna	1.11	0.81	0.92	1.25	1.07	0.97	0.79	0.98	0.91	0.96	0.91	1.09	–	2	1
15	Vwf	0.56	0.63	0.53	0.58	0.73	0.56	0.99	1.24	1.12	1.08	1.21	1.33	–	–	2
16	S3-12	0.51	0.45	0.38	0.39	0.48	0.39	1.27	2.19	1.79	1.82	1.95	1.82	1	2	3
17	Lrrc8	0.77	0.67	0.63	0.51	0.60	0.57	1.05	1.12	1.15	1.13	1.16	1.28	–	1	1
18	Per2	0.74	0.67	0.72	1.00	0.82	0.60	0.98	1.25	0.92	1.04	1.06	1.18	–	2	1
19	Icosl	0.55	0.59	0.70	0.86	0.97	0.56	0.99	1.47	1.09	1.09	0.61	1.23	–	–	2
20	Luzp2	1.06	1.04	1.03	0.90	1.24	1.09	0.89	1.04	0.82	1.16	0.96	1.13	–	2	3
21	Thy1	0.95	1.08	1.14	2.06	1.13	1.10	0.92	1.39	0.87	0.91	0.92	1.02	–	–	1
22	tubullin beta	0.88	1.13	1.01	1.61	1.41	1.37	0.96	1.08	0.91	0.97	0.86	1.00	1	–	–
23	Hop	1.36	1.36	0.91	0.99	1.06	1.31	0.87	1.02	0.90	0.88	0.89	1.05	1	–	3
24	Auh	1.46	1.24	1.32	1.33	1.02	1.21	0.88	1.05	0.93	0.97	1.02	1.07	3	–	1
25	9330161C17Rik	1.18	0.87	1.14	1.36	1.11	1.13	0.95	1.40	0.81	1.10	0.95	1.14	–	3	2
26	Arc	1.08	1.17	1.02	1.15	1.36	0.83	0.75	1.10	0.78	0.93	1.01	1.14	–	–	2
27	Cox17	0.97	1.00	0.94	1.04	1.02	0.95	0.91	1.00	0.92	0.99	1.07	1.15	no data		
28	Fabp7	1.77	1.69	1.49	1.69	1.72	2.31	1.47	1.01	1.20	1.29	1.05	1.17	1	1	2
29	PIDS	0.98	0.79	0.91	1.35	1.19	0.92	0.82	1.38	0.94	1.14	1.05	1.26	–	1	1
30	Slc6a4	49.11	284.64	8.79	70.82	1.78	23.82	0.94	1.04	0.25	1.48	1.81	0.90	1	1	2

Fig. 2. Gene expression profiles of *Htt* KO mice and Flx-treated mice in six regions of the brain and their regulator candidates. Fold changes detected with RT-PCR in the two experimental conditions (*Htt* KO/WT and treated with Flx/Sal for 3 days) are calculated in the 30 genes listed in the Table 1. Fold changes over 20‰ and 200‰ are colored with light and deep colors of red or green, respectively. Red color is for increased numbers and green for decreased one. The number of upstream regulatory elements (URE) for indicated transcription factors (Gata, Pax and Aml1) located in the range of 2000 bp upstream and 100 bp downstream of the transcription starting sites of 30 genes according to the RefSeq are shown. These transcription factors kept their UREs in 15 or more genes among 30. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

in the *Htt* KO (depressive) mice and in the Flx (anti-depressant treated) mice; the genes decreased in the KO mice were overexpressed during Flx treatment, and the genes activated in the KO mice were suppressed with Flx treatment (Fig. 1B). This inverted expression pattern was peak at the 3 day and diminished by the 21 day of Flx-treatment periods. *P*-values for significance of the inverted expression levels were shown in Fig. 1C. Taking account of the time lag between the expression alteration peak at day 3 and the clinical efficacy that appears after 3–6 weeks of Flx initiation [22], the 30 genes seems to be primary regulated to the opposite direction of depressive status immediately after Flx treatment, and may subsequently influence the expression levels of other genes directly contributing the amelioration of clinical features, expression analysis of which was far beyond the scope of the current study.

qRT-PCR analysis of the gene expression in KO/WT and Flx/Sal experiments

We next profiled the expression of the 30 genes in other regions of the brain of KO/WT or Flx/Sal treated mice for 3 days with qRT-PCR (Fig. 2). The alteration of gene expression levels similar to that in the hippocampus was seen in these regions of the brain with Htt KO mice compared with WT mice, indicating that the alteration was consistent in the whole brain examined, however subtle again. The inverted expression changes between KO/WT and Flx/Sal experiments were observed in the genes repressed by the *Htt* KO, but not significant in the activated genes.

The expression of *Htt* was increased in the all area examined of *Htt* KO mice (Figs. 1 and 2 #30), however the transcript is truncated and non-functional as mentioned before. Absolute level of *Htt* outside the midbrain is low in the WT mice, contributed by the very small amount of local expression of Htt or the axonal transport from the cell body of raphe nuclei in the midbrain [7]. The genetic inactivation of *Htt* seemed to result in the activation of transcriptional system for *Htt* not only in the raphe nuclei but in the whole brain; however possibility of the activation of the axonal transport was not eliminated. *Htt* expression was decreased in the hippocampus throughout the Flx treatment and in the thalamus at day 3, but not

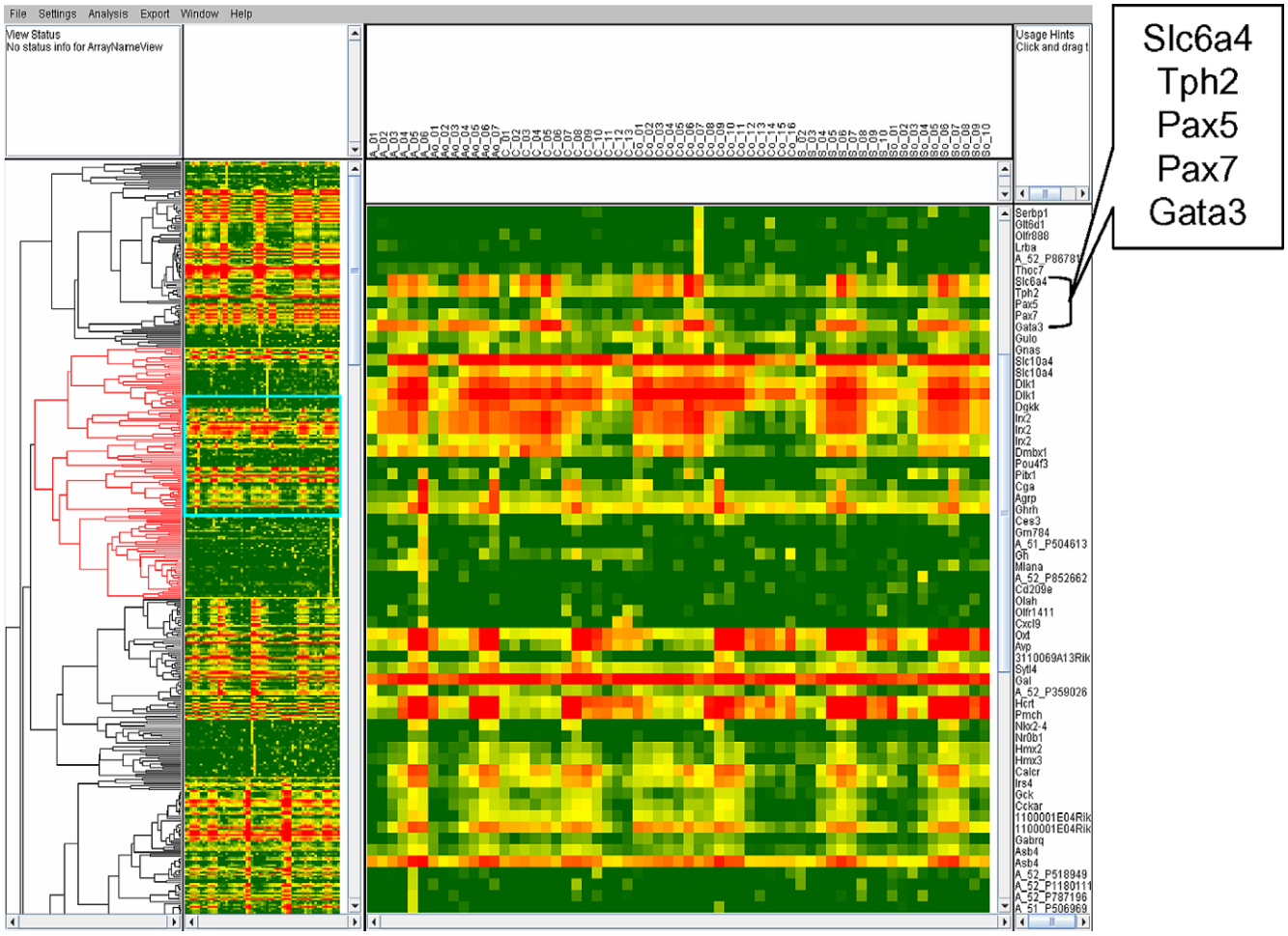

Fig. 3. Two dimensional clustering of 1500 top most regionally expressing genes. A large cluster containing the sub-cluster to which the *Htt* (*slc6a4*) belongs is indicated with red lines of cluster trees in the first column. Sub-clusters indicated with light blue squares in the second column are magnified in the third column with gene symbols in the forth column. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

in the midbrain. The reduction of Htt protein density detected with radio-labeled agonist binding assay is shown to be a relevant mechanism in therapeutic response to SSRI [23]. The reduction of the *Htt* in the hippocampus and thalamus with rather increased transcription in the midbrain could allow us to speculate selective repression of the transcription of *Htt* in the hippocampus and the thalamus than the midbrain by the Flx treatment; however possibility of the disturbances in transport system for the gene products from the raphe nuclei to the functioning areas with the Flx treatment was not eliminated.

Transcriptional regulator search

Since our data indicated the molecular basis: a group of genes was regulated in the opposite directions in mice for depressive model (Htt KO mice) and anti-depressive model (Flx treated mice), we further investigated common transcriptional regulators for this group of genes.

We analyzed the UREs of the 30 genes in the range of 2000 bp upstream and 200 bp downstream of the transcription starting site. *Htt* kept twelve kinds of UREs registered in TRANSFAC, and among them the UREs for Gata, Pax and Aml1 appeared in the ranges of more than a half of these genes (Fig. 2). Next, we employed our original device, 3D-ISM system for consistent data production of gene expression contributing to the precise mathematical profiling analysis and the discovery of gene clusters with similar expression patterns [15–17]. The extracted RNA from 61 frozen-section samples obtained from the six mice brains sliced in different directions with 3D-ISM was analyzed with microarray, and the gene expression profile was clustered (Fig. 3). *Htt* belonged to the cluster in which *Tph2*, a gene for the 5-HT synthesizing enzyme selectively expressed in the raphe nuclei, was present as expected. Intriguingly Pax5, Pax7 and Gata3 were also present in this small cluster, displaying a similar expression patterns with *Htt*. Indeed, Gata3 is known to be involved in the development of serotonergic neurons in the raphe nuclei, and chimeric mice display altered exploration behavior [24]. Pax4 and 6 together regulate gastrointestinal endocrine cell development, including serotonin producing cells [25]. This indicates Pax5 and 7 may function for the development of brain serotonergic cells in the same manner.

These three transcription factors not only function in the developmental stage, but are supposed to still work on maintaining the proper expression levels of the group of genes in the matured stage, resulting in the steady state of emotion. Although there may not be shown exact the same molecular status in the depressive patients and the model mice in terms of the RNA and protein levels [26], here we could select the transcriptional regulator candidates which displayed the similar expression pattern to that of *Htt* and potentially controlled the expression level of the group of genes with common UREs to the inverted directions representing the depressive and anti-depressive emotional states.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.01.041.

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