

Aberrant changes of somatostatin and neuropeptide Y in brain of a genetic rat model for epilepsy: tremor rat

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Excessive excitation or loss of inhibitory neurotransmission has been closely related to epileptic activity. Somatostatin (SST) and Neuropeptide Y (NPY) are members of endogenous neuropeptides which are recognized as important modulator of classical neurotransmitter, distributed abundantly in mammalian central nervous system. Abnormal expression of these two neuropeptides evidenced in some epileptic models highlights the relevance of SST or NPY in the pathogenesis of epilepsy. The tremor rat (TRM) is a genetic epileptic animal model which can manifest tonic convulsions without any external stimuli. The present study aimed to investigate the distribution and expression of SST and NPY in TRM brains, including hippocampus, temporal lobe cortex and cerebellum. Our RT-PCR data showed that up-regulated mRNA expression of SST and NPY was discovered in TRM hippocampus and temporal lobe cortex compared with control (Wistar) rats. The peptide levels of these neuropeptides in brain areas mentioned above were both apparently higher than that in normal Wistar rats as well. However, in cerebellums, neither SST nor NPY was significantly changed compared with control group. The immunohistochemical data showed that SST and NPY were widely present throughout CA1, CA3 and the hilus of hippocampus, the entorhinal cortex of temporal lobe cortex, as well as cerebellar Purkinje layer. In conclusion, our results discovered the aberrant changes of SST and NPY in several TRM brain regions, suggesting that the peptidergic system might be involved in TRM epileptiform activity.

Key words: tremor rats, genetic epilepsy, somatostatin, neuropeptide Y

INTRODUCTION

Epilepsy is one of the most common chronic neurological disorders which is characterized by recurrent and unprovoked seizures, affecting over 70 million people worldwide (Weaver and Pohlmann-Eden 2013, Erfanparast and Tamaddonfard 2015). Although specific drugs have been developed, the side effects of classic antiepileptic drugs caused by their action mechanism could not be totally avoided (Kovac and Walker 2013). The disruption of the delicate balance between excitatory and inhibitory neurotransmission contributing to the overexcited neuronal activity in mammalian brain has been proposed to be a possible candidate for epileptogenesis (Madsen et al. 2009). Thus, as the modulators of classical neurotransmitters, such as γ -aminobutyric acid (GABA) and glutamate (GLU),

neuropeptides are closely linked to epileptic diseases, including pathogenesis and treatment.

Somatostatin (SST) and neuropeptide Y (NPY) are both belonged to endogenous neuropeptides family, abundantly express in neurons of brain areas and are likely to be preferentially released by neurons subjected to high-frequency stimulation. The role of SST in seizure is first confirmed by temporal lobe epileptic rats in which SST-containing neurons in the hilus of dentate gyrus are selectively lost (Sloviter 1987). The expression of SST could be markedly affected in response to seizure occurrence. For instance, in convulsive rats induced by repeated amygdaloid kindling, remarkably elevated expression of SST could be observed in epileptic models evoked by lidocaine and electroconvulsive stimulation (Nagaki et al. 2000, Mikkelsen and Woldbye 2006). Moreover, adeno-associated viral vector-mediated overexpression of SST and SST agonists

have been both verified to be effective in suppressing or attenuating status epilepticus (Zafar et al. 2012, Kozhemyakin et al. 2013), which further illustrates that SST may serve as a significant anti-epileptic neuromodulator.

NPY is a highly conserved 36-amino acid neuropeptide produced by the neurons throughout hippocampus and neocortex (Husum et al. 2004). It could prevent the damages from excessive hyper-excitability in neurons when suffering harmful stimulus. During kindling epileptogenesis, NPY is emerging as a central modulator of seizure activity which undergo plastic changes (Calik et al. 2015). Acting as one of the inhibitors of synaptic excitability, the abnormal expression of NPY has been associated with its function of dampening seizures as well. Electroshock or kainic acid (KA) could result in brief experimental epileptiform symptoms, accompanied by increased NPY positive neurons in rat brain (Cardoso et al. 2010, Drexel et al. 2012). In temporal lobe epilepsy induced by trimethyltin (TMT), intracerebroventricular injection of exogenous NPY may exert powerful neuroprotection and reduce hippocampal damage (Corvino et al. 2012).

Unlike epileptic models obtained by chemical or physical methods, the tremor rat (TRM, RGD ID: 1302702) (*tm/tm*) is a genetic mutant model discovered by Kyoto University. TRMs have seizure-like features of showing tonic convulsions and appearing 5–7 Hz spike-wave complexes in cortical and hippocampal electroencephalograms (EEGs) records without any external stimuli (Serikawa et al. 1987, Kondo et al. 1991). As a crucial animal model of hereditary epilepsy, TRMs have been applied in researches of GABA transporters, cognitive deficits and voltage-gated sodium channels in epileptic activity (Mao et al. 2011a, Xu et al. 2013). It is illustrated that homozygous TRMs showed lower levels of NPY than heterozygous ones in hippocampus and neocortex by the method of radioimmunoassay (Sadamatsu et al. 1995). Our group demonstrated the ectopic expression of NPY and its receptor subtypes in TRM brains in a previous study as well (Xu et al. 2014). However, the detailed alteration of NPY or another neuropeptide-SST compared with Wistar remains to be documented. Moreover, the reduction of cerebellar activity in experimental epilepsy is reported by recent research, indicating the possible role of cerebellum in seizures (Rijkers et al. 2015). Therefore, in the present study, we chiefly focused on the expression and distribution of SST and NPY in TRM brain areas, including hippocampus, temporal lobe cortex and cerebellum in order to explore the potential role of neuropeptides in TRM epileptogenesis.

MATERIALS AND METHODS

Experimental animals

TRMs were gifted by Kyoto University. Normal Wistar rats as control were from the Center for Experimental

Animals of China Medical University. All the animals were maintained in individual cages under controlled environment (12:12 h light/dark cycle, 50–70% humidity, 24°C). Food and water were available *ad libitum*. TRM (n=21) and Wistar rats (n=21) of both sexes at the age of 9–12 weeks were selected as experimental animals. Three males and four females of TRMs or control rats were applied in each group. Experimental treatments were followed the guidelines from NIH. Meanwhile, all the handling procedures were approved by the Committee of Animal Experimentation of China Medical University.

RT-PCR

After the rats were anaesthetized by diethyl ether and decapitated, the brains were quickly removed and dissected into hippocampus, temporal lobe cortex and cerebellum on ice according to the method in previous report with some modifications (Xu et al. 2014). Total RNA was then isolated from these brain regions of TRMs (n=7) and control rats (n=7) separately using Trizol reagent (Invitrogen, USA) following manufacturer's instructions. The concentration of RNA was measured by spectrophotometer to make sure a A_{260}/A_{280} ratio close to 1.8–2.0. Equal amount of RNA (500 ng) from each sample was used for cDNA synthesis in a volume of 10 μ l according to the guideline in Takara RT reagent kit (Takara code: DRR037S). The resulting RT reactions as template were directly added in amplified mixture and incubated in PCR thermocycler for 30 cycles. Each PCR reaction consisted of the following: 3 μ l of the cDNA product, 2 μ l of dNTP, 1 μ l of each primer, 2.5 μ l of PCR Buffer, 0.3 μ l of Takara Ex Taq Enzyme and sterilized distilled water to a final volume of 25 μ l. Each thermal cycle consisted of the processes as below: denaturation at 95°C for 45 s, annealing at 60°C for 50 s, and an extension at 72°C for 90 s. The amplification of each template had been replicated for three times. GAPDH was served as an endogenous internal standard control. For the purpose of avoiding genomic DNA and external contaminations, RT-PCR mixture with no reverse transcriptase and PCR mixture lacked of cDNA template were performed as control. PCR products were electrophoresed on 2% agarose gel containing ethidium bromide and the anticipated targeted bands were visualized under ultraviolet light. The intensities of products were quantified via Quantity one software. Then the ratio of SST to GAPDH and NPY to GAPDH were calculated respectively to normalize the expressions of these gene on mRNA level.

Primer sequences for SST, NPY and GAPDH were as follows: SST, forward: 5'-ATCGTCCTGGCTTTGGGC-3' reverse: 5'-GCCTCATCTCGTCCTGCTCA-3'; NPY, forward: 5'-TAGGTAACAAACGAATGGGG-3' reverse: 5'-AGGATGAGATGAGATGTGGG-3'; GAPDH, forward:

5'-GGCACAGTCAAGGCTGAGAATG-3''; reverse: 5'-ATGGTGGTGAAGACGCCAGTA-3'', which generated a 207 bp, 350 bp and 143 bp fragments, respectively.

ELISA

Tissue samples from hippocampus, temporal lobe cortex and cerebellum of experimental animals of both sexes (TRMs $n=7$ and control rats $n=7$) were separated as aforementioned description on ice, then homogenized in PBS and centrifuged at 13500 rpm for 20 min at 4°C. The concentrations of SST and NPY were determined by enzyme-linked immunosorbent assay (ELISA) kits, following the instructions of manufacturer (USCN, SST code: CEA592Ra; NPY code: CEA879Ra, China). In brief, the diluted samples and standards were added into the wells of microplates pre-coated by antibodies of SST or NPY. Then added prepared detection reagent A immediately and incubated 1 h at 37°C. After rinsing, prepared Detection Reagent B was added and incubated 30 min at 37°C. Substrate solution and stop solution were respectively added after washing three times and the data could be read at 450 nm immediately by microplate reader. Each samples had been replicated for three times and the calculated concentrations would be averaged.

Immunohistochemistry

The brains from TRMs ($n=7$) and control rats ($n=7$) of either sex were embedded in paraffin after fixing in 4% paraformaldehyde. For each animal, 100 and 50 serial coronal sections of 4 μm thick from hippocampus and cerebellum were cut respectively. For each targeted protein, six sections selected from the rostral, middle and caudal part of the serial sections were treated with immunohistochemical process (2 sections for each part, and in total, 6 sections of hippocampus and 6 sections of cerebellum from one rat). In brief, sections were deparaffinized and dehydrated in graded alcohol, then treated with 3% H_2O_2 for 15 min for inhibiting endogenous peroxidases. After rinsing, the slices were blocked in 10% normal goat serum for 20 min, followed by an incubation with rabbit anti-SST diluted by 1% normal goat serum at 1:50 (ab103790, Abcam) or rabbit anti-NPY diluted by 1% normal goat serum at 1:20 (12833-1-AP, Proteintech, China) overnight at 4°C. Sections were then stained with secondary antibodies biotinylated anti-rabbit IgG diluted by PBS at 1:100. After incubation with DAB kit for 10 min, the slides were counterstained by hematoxylin. Negative control sections were incubated with 10% normal goat serum without primary antibodies. The cellular localization and distribution of targeted proteins were observed

under light microscope and the estimation of density of immuno-positive cells in subregions of brains followed the process in previous researches (Guo et al. 2013), including hippocampal CA1, CA3 and hilus, the entorhinal cortex of temporal lobe cortex and cerebellum. The fields to be counted were located by the characteristic landmarks in the histological map, such as the folding of the pyramidal cell layer in areas of CA1 and CA3. The immuno-positive cells within 100 cells of every location were counted in blinded fashion. Three counters who did not know the sections from which group were asked to count the same field and their results were averaged. Estimating density of positive cells and capturing images were carried out under a microscopic enlargement of $\times 200$ and $\times 400$, respectively.

Statistical analysis

All data were presented as mean $\pm$ SD. Student's *t*-test was performed to evaluate the individual comparisons between different groups, and statistical significance level was set for *p* values <0.05 . All statistical analyses were conducted using SPSS for Windows version 16.0.

RESULTS

The expressions of SST and NPY in TRM brains on mRNA level

RT-PCR was first carried out in order to measure the expression of SST and NPY on mRNA level in the subregions of TRM and control rat brains. As indicated in Fig. 1, the mRNA expression of SST and NPY were both elevated in hippocampus and temporal lobe cortex of TRM compared with the control rats (SST: HIP, 0.84 ± 0.07 in TRMs vs. 0.45 ± 0.04 in control rats, $p < 0.01$; TLC, 0.77 ± 0.05 in TRMs vs. 0.43 ± 0.02 in control rats, $p < 0.01$; NPY: HIP, 0.66 ± 0.02 in TRMs vs. 0.39 ± 0.04 in control rats, $p < 0.01$; TLC, 0.75 ± 0.06 in TRMs vs. 0.60 ± 0.02 in control rats, $p < 0.01$). However, in TRM cerebellum, we did not detect abnormal expressions of these two neuropeptides. (SST, 0.28 ± 0.03 in TRMs vs. 0.30 ± 0.06 in control rats, $p > 0.05$; NPY, 0.26 ± 0.04 in TRMs vs. 0.24 ± 0.05 in control rats, $p > 0.05$).

The concentrations of SST and NPY in TRM brain tissues

In order to further quantify the concentrations of SST and NPY, homogenized tissue samples from hippocampus, temporal lobe cortex and cerebellum in TRMs and control rats were subjected to ELISA analysis. According to Fig. 2, in TRM hippocampus and temporal

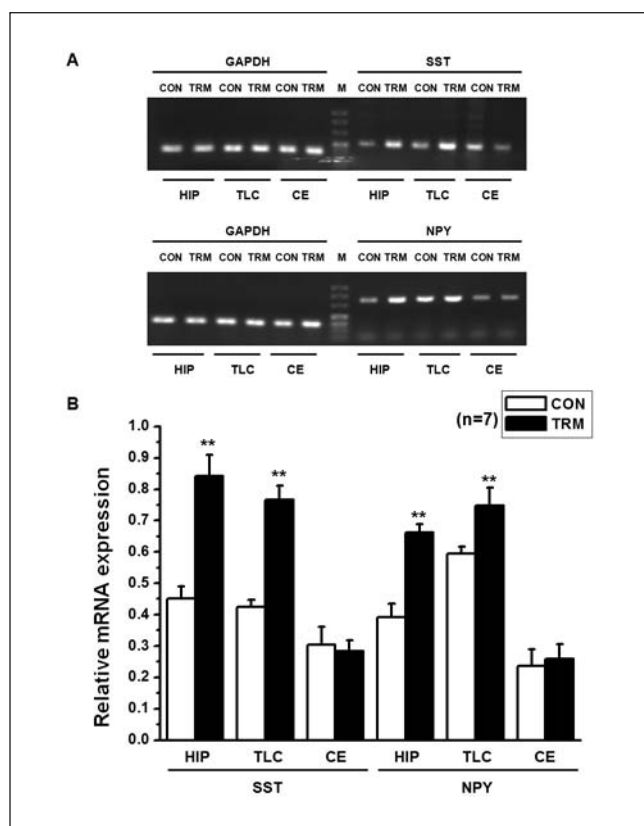


Fig. 1. The mRNA expression of SST and NPY in the brains of TRM and control rats by RT-PCR (mean $\pm$ S.D., $n=7$). (A) The representative bands of SST and NPY mRNA expression. (B) The data analysis of relative expression of SST and NPY. All data represented a relative quantification to internal control GAPDH. The ratio of grey values for SST and NPY were both much higher than control rats, whereas in TRM cerebellum, neither of them had significant change. * $p<0.05$ vs. control group; ** $p<0.01$ vs. control group. Con=control, HIP=hippocampus, TLC=temporal lobe cortex, CE=cerebellum.

lobe cortex, SST and NPY concentrations were both much higher than normal rats, whereas in TRM cerebellum, neither of them was changed in comparison to the control group. These results were similar with what we discovered via RT-PCR.

The distributions of SST and NPY in TRM brains

Immunohistochemical analysis was then performed to detect the distributions of SST and NPY. Figs 3A, 4A, 5A and 6A indicated the two kinds of neuropeptide were localized abundantly in brains of TRMs and control rats, especially in neurons of hippocampal CA1, CA3 and hilus. Additionally, immunoreactivity of SST or NPY was also discovered on the neurons located in the entorhinal cortex. The positive reactions mainly appeared on cell bodies, partly on nerve fibers. In cerebellum, most of the stained cells were Purkinje cells, which were pear-like shapes with clear and unstained nucleus. There was

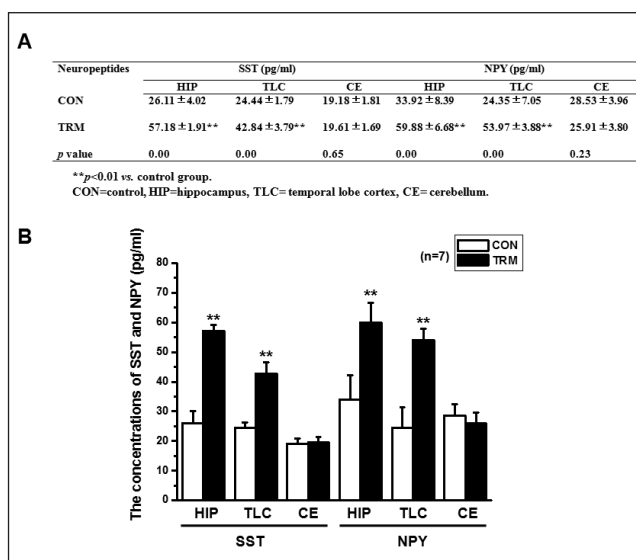


Fig. 2. The concentrations of SST and NPY in the brain of TRM and control rats by ELISA (mean $\pm$ S.D., $n=7$). (A) The concentrations of SST and NPY in TRM and control brains. (B) The column charts of ELISA data. SST and NPY were both elevated in hippocampus and temporal lobe cortex of TRM compared with the control rats, but with no change in TRM cerebellum. ** $p<0.01$ vs. control group.

relatively weaker signal in fibers from granular cell layer, while almost no obvious immuno-signal could be captured in molecular layer.

As shown in Figs 3B, 4B, 5B and 6B, the number of SST positive cells in TRMs was more than that of control ones, including hippocampal CA3, the hilus and temporal lobe cortex. However, there was no significant change in TRM hippocampal CA1 and cerebellum. Meanwhile, it was noteworthy that the NPY positive cells were extremely increased in all the observed brain regions of TRMs except cerebellum where the quantity did not show clear variation compared with Wistar rats. On the whole, the findings of immunohistochemistry were in accordance with our data by RT-PCR and ELISA.

DISCUSSION

In this study, we mainly focused on the expressions and distributions of SST and NPY in TRM rat different brain areas, including hippocampus, temporal lobe cortex and cerebellum. The mRNA of both SST and NPY were up-regulated in TRM hippocampus and temporal lobe cortex compared with Wistar rats, accompanied with the dramatic elevated expressions on peptide level. However, in TRM cerebellum, neither of them was discovered abnormal expression. Our immunohistochemical results proved that SST and NPY were widely expressed in TRM hippocampal CA1, CA3 and the region of hilus, so did in both entorhinal cortex of temporal lobe cortex and cerebellar Purkinje layer.

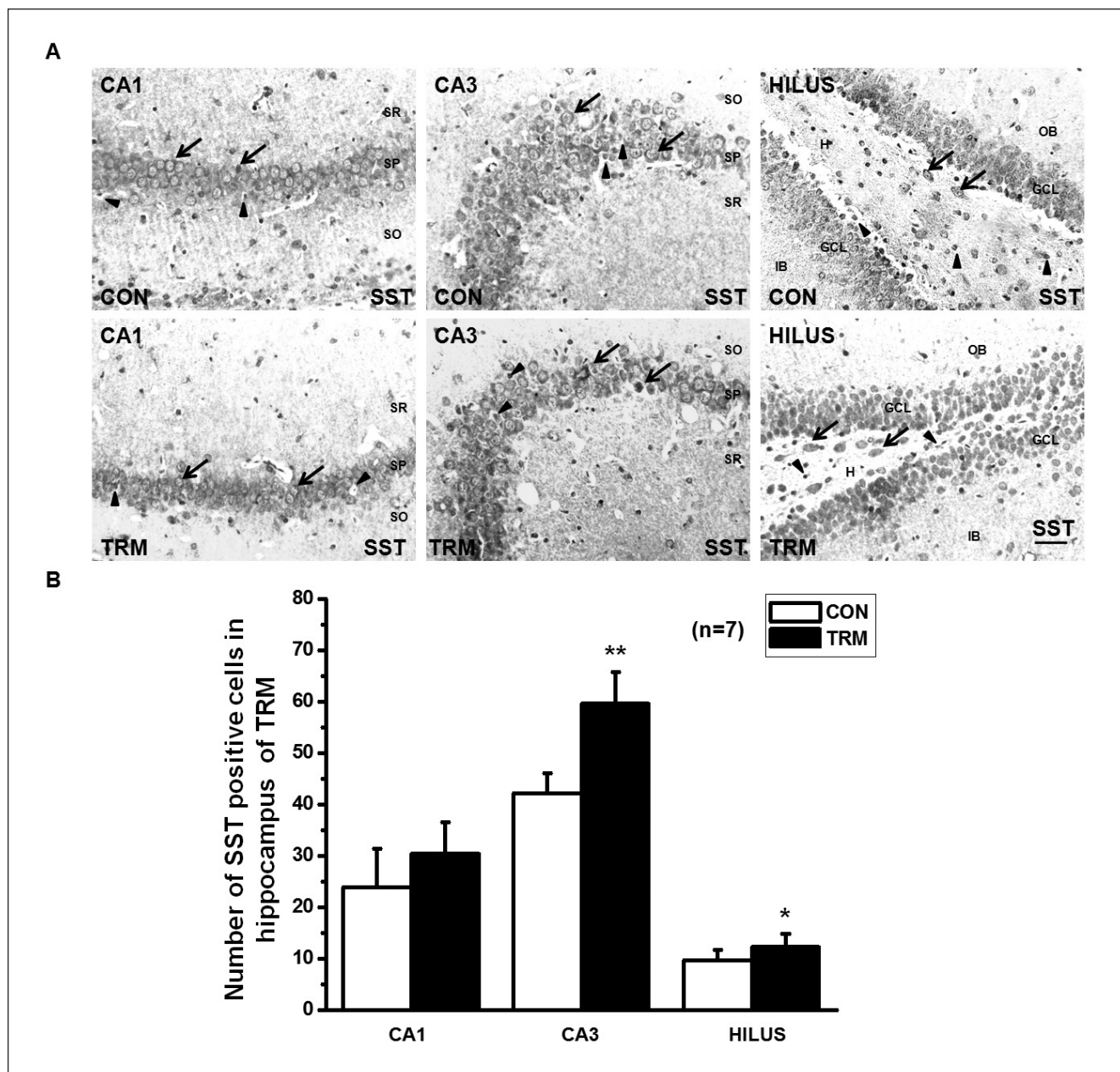


Fig. 3. The representative images of TRM and control hippocampus showing DAB-stained SST. (A) The distribution of SST is observed in hippocampal CA1, CA3 and the hilus. Two each of the representative neurons which have positive or negative immuno-signals was pointed out by arrow and arrowheads respectively. (B) The analysis of SST positive cells. In TRM CA3, the number of SST positive cells are more than control rats. Scale bars: 20 μ m. * p <0.05 vs. control group; ** p <0.01 vs. control group. Con=control, SR=stratum radiatum, SP=pyramidal layer, SO=stratum oriens, OB=outer blade of the dentate gyrus, GCL=granular cell layer, IB=inner blade of the dentate gyrus, H=hilus.

SST is primarily synthesized by GABAergic interneurons and the coexistence with a number of inhibitory neurotransmitters hints on its ability of attenuating neuronal hyperexcitability. Via contacting with particular receptors, SST could inhibit presynaptically release of glutamate, the voltage-gated calcium currents or potassium leak current, together with modulating postsynaptically α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) currents in the hypothalamus (Baraban and Tallent 2004,

Peineau et al. 2003). Intracerebroventricular administration of SST or its analogs could reduce the likelihood of epileptiform events in several epileptic models (Vezzani and Hoyer 1999). On the contrary, SST gene knock-out mice are more susceptible to stimulus from kainic acid, heightening seizure severity (Buckmaster et al. 2002). The reduced number of SST-containing neurons are reported in pilocarpine-induced seizures and kainate-treated rats (Buckmaster and Dudek 1997, Dinocourt et al. 2003). However, more evidences

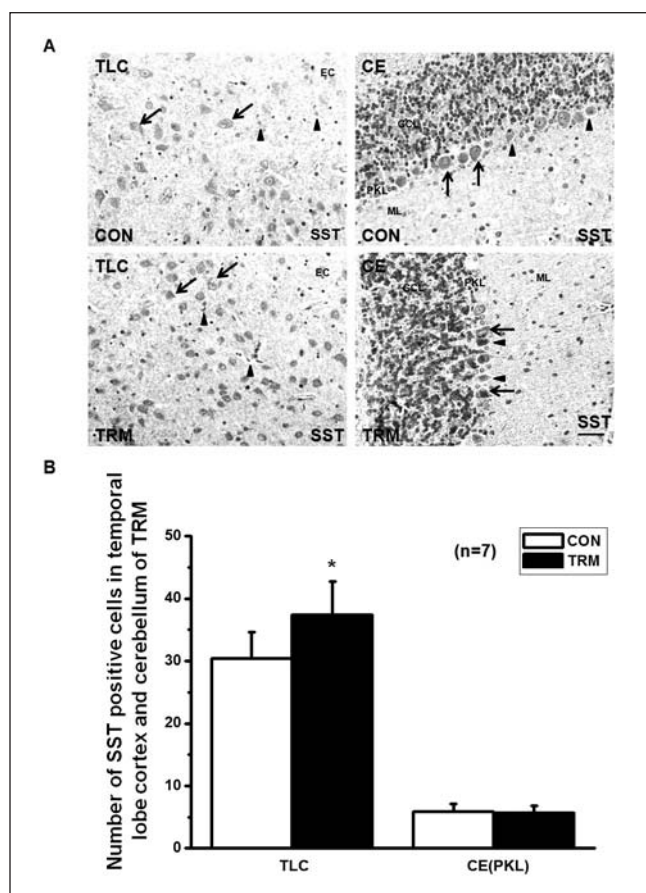


Fig. 4. The representative images of TRM and control temporal lobe cortex and cerebellum showing DAB-stained SST. (A) The distribution of SST is observed in temporal lobe cortex and cerebellar Purkinje layer. Two each of the representative neurons which have positive or negative immuno-signals was pointed out by arrow and arrowheads respectively. (B) The analysis of SST positive cells. In TRM temporal lobe cortex, the number of SST positive cells are more than control rats. Scale bars: 20 μ m. * $p < 0.05$ vs. control group; ** $p < 0.01$ vs. control group. Con=control, TLC=temporal lobe cortex, CE=cerebellum, EC=entorhinal cortex, GCL=granular cell layer, PKL=Purkinje layer, ML=molecular layer.

also elucidate the expression as well as the release of this neuropeptide could be markedly augmented following the emergence of epilepsy. In the convulsive rats evoked by ultra-red light or kindling, the increased expression of SST has been discovered in both hippocampus and neocortex (Nagaki et al. 1996, Csaba et al. 2004). The elevated mRNA and peptide levels are also reported in kainic-acid epileptic models (Hashimoto and Obata 1991), which is consistent with what we found in the current research. In this regard, we speculate that the raise of SST in TRM hippocampus and temporal lobe cortex might be adaptive reactions to epileptogenesis for the sake of preventing neurons from seizure damage. There is a prototypical cAMP regulatory element site in SST gene, which could be activated during higher neuronal activity (Clynen et al. 2014). Hence, the altered TRM brain tissue excitability might partly contribute

to the unusual change of SST. Additionally, SST-containing dense-core vesicles secretion from axon terminals is calcium-dependent and often occurs after high stimulation (Vezzani and Hoyer 1999). And according to our previous study, the concentration of Ca^{2+} in TRM hippocampal neurons were significantly increased (Lv et al. 2015), suggesting another potential reason for SST variation.

NPY is widely expressed in GABAergic interneurons and may coexist with SST in mammalian central nervous system, such as hippocampus and cortex. It is reported that the NPY release is closely related to glutamatergic stimulation. Similar with SST, NPY is believed to be another neuropeptide which plays a prominent role in epileptogenesis, and acts as an endogenous anticonvulsant by virtue of regulating glutamate-mediated transmission, calcium ion influx or K^+ currents (Meurs et al. 2012, Wang 2005, Nadler et al. 2007). In a recent study, the involvement of NPY in modulating endogenous neurogenesis within subgranular zone (SGZ) could contribute to the newly-generated neurons integrating into the local circuit (Corvino et al. 2014), being essential for alleviating seizure-like symptoms. rAAV-mediated overexpression of NPY combined with its special receptors is also recognized as promising approach for therapy of epilepsy (Noé et al. 2009, Noé et al. 2007). Moreover, this peptide displays enhanced expression in different brain areas from varied epileptic animal models (Goto et al. 2010, Kokaia 2011), which are coincide with our results in this study, suggesting that the TRM epileptiform activity might not only make an effect upon SST system, but also influence NPY's expression or secretion. Due to the diminished GABA and elevated glutamine reported in our previous study (Mao et al. 2011b), the increased amount of SST or NPY could be recognized as a self-neuroprotection against the aberrant neurotransmission circuitry in TRM brains. Although these neuropeptides are likely to take part in TRM self-defense process, the epileptogenesis in current epileptic animals do not dampened totally. A feasible explanation might be that the amount of these peptides is not sufficient to be completely effective during this process. It is noteworthy that compared with our previous research (Xu et al. 2014), different product of NPY primary antibody and new experimental animals were employed for histological analysis in current study for the purpose of reconfirming former results. Similar conclusions we got here also illustrate that our data could be repeatable and convincing.

In addition, remarkable neuron loss and mossy fiber sprouting are not discovered in TRM brain regions reported by previous research (Hanaya et al. 2010), indicating that the increased SST expression here possibly represented a functionally activated population of neurons involved in antiepileptic action. As a regulator, SST could modulate

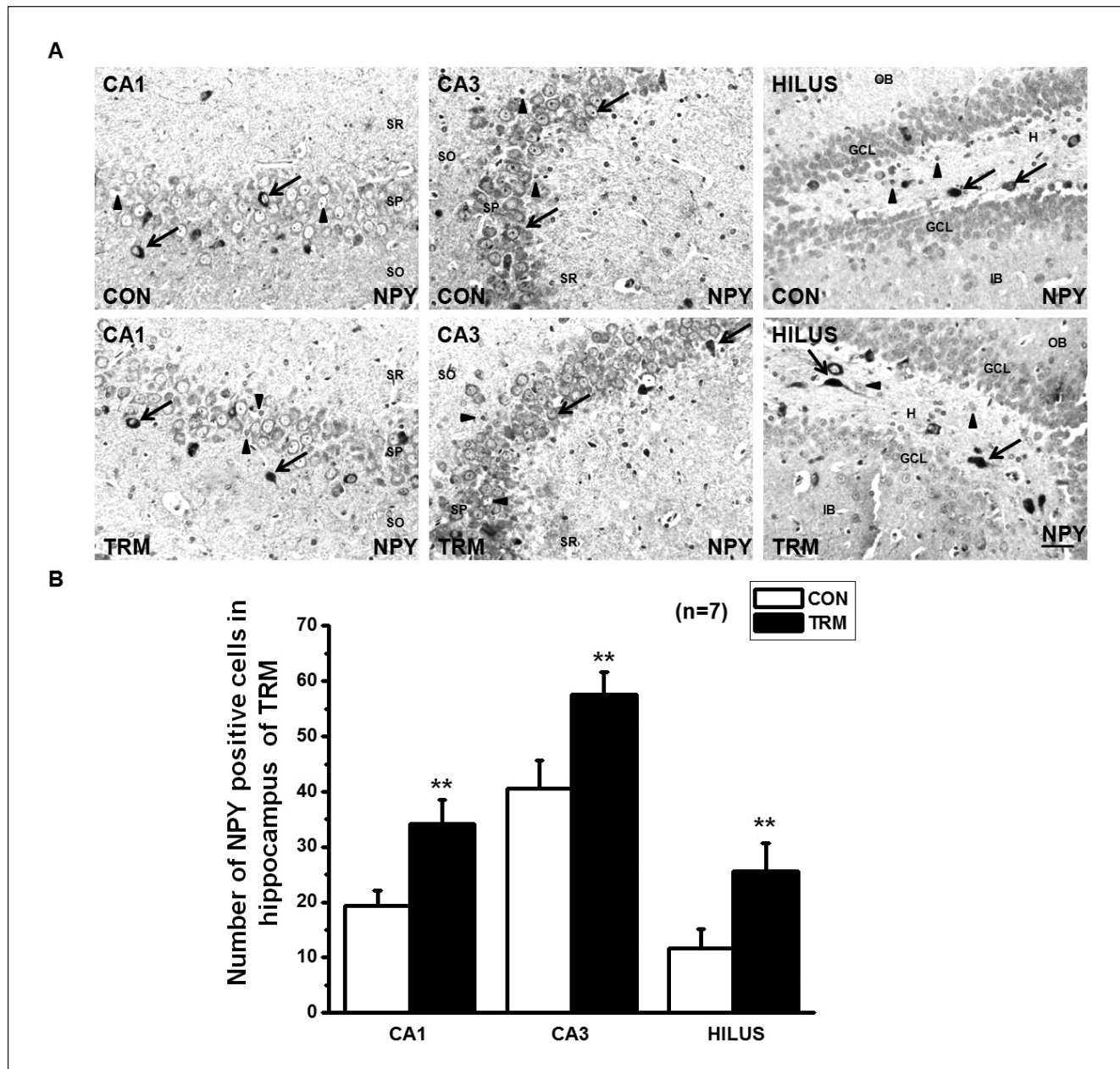


Fig. 5. The representative images of TRM and control rat hippocampus showing DAB-stained NPY. (A) The distribution of NPY is observed in hippocampal CA1, CA3 and the hilus. Two each of the representative neurons which have positive or negative immuno-signals was pointed out by arrow and arrowheads respectively. (B) The analysis of NPY positive cells. In all the areas mentioned above of TRM, the number of SST positive cells are increased compared with control rats. Scale bars: 20 μ m. * $p < 0.05$ vs. control group; ** $p < 0.01$ vs. control group. Con=control, SR=stratum radiatum, SP=pyramidal layer, SO=stratum oriens, OB=outer blade of the dentate gyrus, GCL=granular cell layer, IB=inner blade of the dentate gyrus, H=hilus.

excitatory input from entorhinal cortex into hippocampus and then granule cells in the molecular layer transmit this information to CA3 neurons. Therefore, CA3 is considered to be the “pacemaker” of seizures in hippocampus (Tallent and Qiu 2008), suggesting that its role could be more crucial than CA1, which may partly explain no significant change of SST immunoreactions in this region. In TRM hippocampal CA3 and the temporal lobe cortex, the higher immuno-signal of SST may be due to epileptic

activity-dependent release to exert a self-protection, which is elucidated in other researches as well (Schwarzer et al. 1996, Wanscher et al. 1990). On the other hand, there are studies showing reduced density of SST neurons in hilus from several animal models and patients of temporal lobe epilepsy (de Lanerolle et al. 1989, Choi et al. 2007), which is contrary to what we discovered in current study. Samples from different epileptic models might partly lead to this discrepancy, further demonstrating the molecular

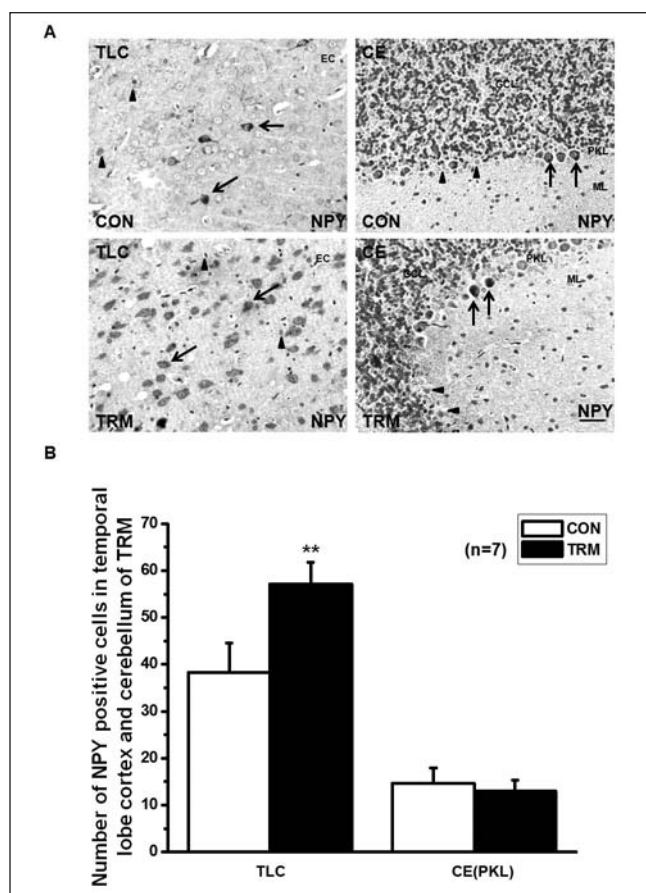


Fig. 6. The representative images of TRM and control rat temporal lobe cortex and cerebellum showing DAB-stained NPY. (A) The distribution of NPY is observed in temporal lobe cortex and cerebellar Purkinje layer. Two each of the representative neurons which have positive or negative immuno-signals was pointed out by arrow and arrowheads respectively. (B) The analysis of NPY positive cells. In TRM temporal lobe cortex, the number of NPY positive cells are increased compared with control rats. Scale bars: 20 μ m. * p <0.05 vs. control group; ** p <0.01 vs. control group. Con=control, TLC=temporal lobe cortex, CE=cerebellum, EC=entorhinal cortex, GCL=granular cell layer, PKL=Purkinje layer, ML=molecular layer.

mechanism in epilepsy is complicated. However, it should be no doubt that the increase of SST-positive neurons may play a significant role in TRM epileptogenesis.

Compare with SST, the enhanced expression of NPY in all the subareas of TRM hippocampus and temporal lobe cortex may reveal its more rapid and durable reaction to seizures. In line with our study, it has been reported that an obvious increase of NPY in hippocampal CA1 and CA3 from experimental febrile seizures (Dubé et al. 2005). Furthermore, we particularly focused on the area of hilus in TRM which were not included in our previous DG analysis. NPY could be produced by a subpopulation of GABAergic interneurons located in hilus (Sperk et al. 2007), thus the added NPY positive cells in TRM hilus might be proposed a possible anticonvulsant and neuroprotective role of NPY here. In certain epileptic

models, the loss of neurons containing NPY is less evident than those containing SST (Clynen et al. 2014), implying the distinct function or acting mechanism between them. Finally, considering the possible role of cerebellum in epileptiform events reported in former researches (Li et al. 2010, Striano et al. 2013, Boop et al. 2013), we also selected TRM cerebellum as one of our objects to study. We noticed that in TRM cerebellum, neither SST nor NPY was detected unusual expression on mRNA level or peptide level. The immunoreaction of these neuropeptides in Purkinje cells or granular cells showed no altered change compare with Wistar rats, prompting that this brain region might be not as sensitive as hippocampus or temporal lobe cortex to TRM epileptogenesis. This result may also hint on that, besides SST and NPY, other members of neuropeptides could participate in TRM epileptic generation. Hence, further work will be essential to verify these assumptions.

In summary, the current study investigated the expression and distribution of two members of neuropeptide family: SST and NPY in TRM brains, including hippocampus, temporal lobe cortex and cerebellum. According to our data, we speculate that the altered expression of SST and NPY might be adaptive reactions to TRM epileptogenesis, suggesting that an endogenous neuroprotective system might be activated spontaneously. However, more researches are certainly demanded in order to explain why this protection is not effective enough to suppress epileptiform events in TRM. On the other hand, the anticonvulsive function displayed by SST and NPY may provide more promising approaches to control neuronal hyperexcitability via exogenous administration or gene therapy, which will be extremely meaningful in treatment of inherited epilepsies.

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REFERENCES

- Baraban SC, Tallent MK (2004) Interneuron diversity series: interneuronal neuropeptides – endogenous regulators of neuronal excitability. *Trends Neurosci* 27: 135–142.
- Boop S, Wheless J, Van Poppel K, McGregor A, Boop FA (2013) Cerebellar seizures. *J Neurosurg Pediatr* 12: 288–292.
- Buckmaster PS, Dudek FE (1997) Neuron loss, granule cell axon reorganization, and functional changes in the dentate gyrus of epileptic kainate-treated rats. *J Comp Neurol* 385: 385–404.
- Buckmaster PS, Otero-Corchon V, Rubinstein M, Low MJ (2002) Heightened seizure severity in somatostatin knockout mice. *Epilepsy Res* 48: 43–56.

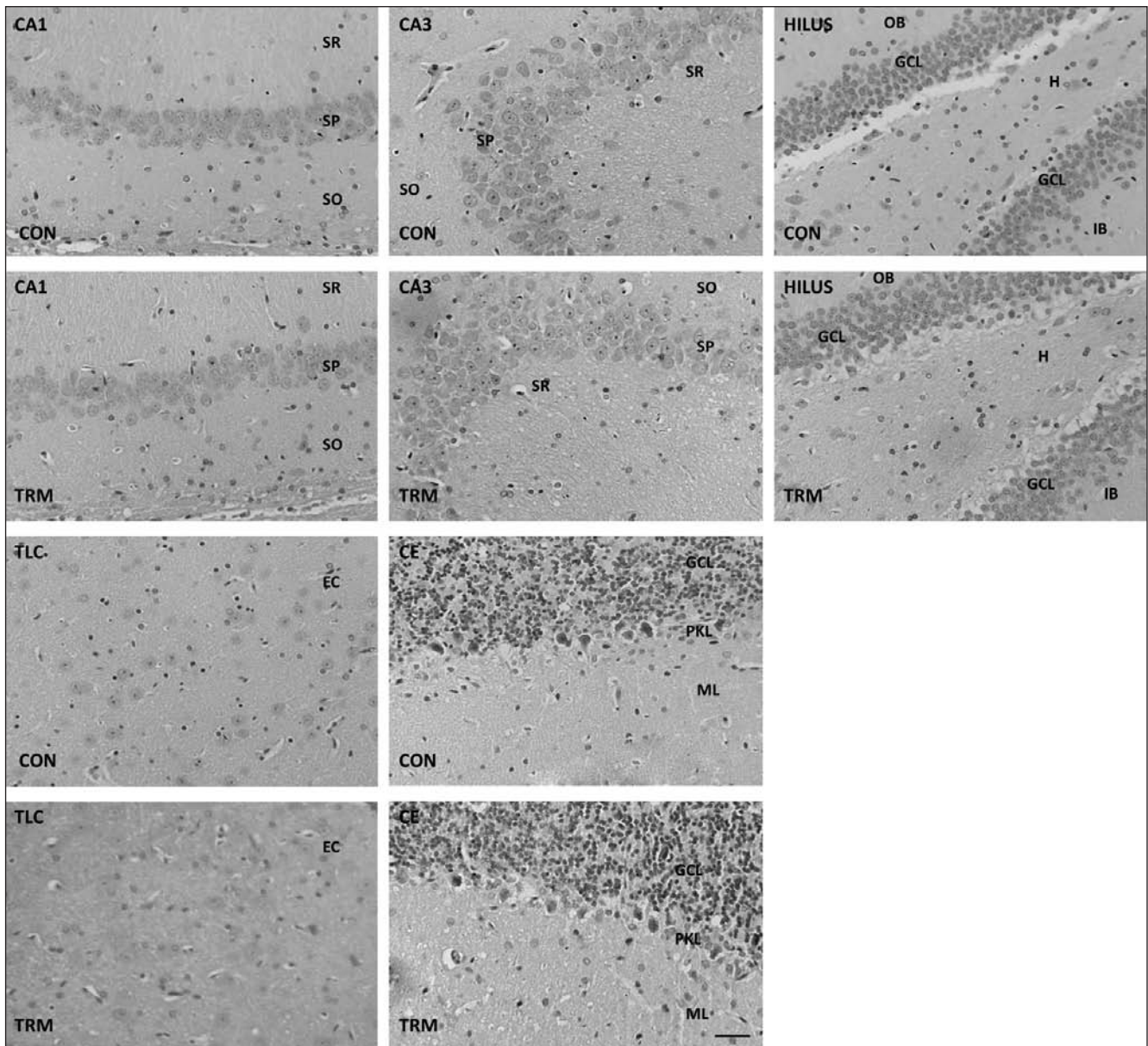


Fig. 7. Representative DAB-stained images of negative controls. The negative controls of immunohistochemistry were subjected to the same procedures experimental group with 10% normal goat serum incubation instead of primary antibodies. Scale bars: 20 μ m. SR=stratum radiatum, SP=pyramidal layer, SO=stratum oriens, OB=outer blade of the dentate gyrus, IB=inner blade of the dentate gyrus, EC=entorhinal cortex, GCL=granular cell layer, H=hilus, PKL=Purkinje layer, ML=molecular layer.

Calik M, Ciftci A, Sarikaya S, Kocaturk O, Abuhandan M, Taskin A, Kandemir H, Yoldas TK, Aksoy N (2015) Assessment of both serum S-100B protein and neuropeptide-Y levels in childhood breath-holding spells. *Epilepsy Behav* 47: 34–38.

Cardoso A, Freitas-da-Costa P, Carvalho LS, Lukyanov NV (2010) Seizure-induced changes in neuropeptide Y-containing cortical neurons: Potential role for seizure threshold and epileptogenesis. *Epilepsy Behav* 19: 559–567.

Choi YS, Lin SL, Lee B, Kurup P, Cho HY, Naegle JR, Lombroso PJ, Obrietan K (2007) Status epilepticus-induced somatostatinergic hilar interneuron degeneration is regulated by striatal enriched protein tyrosine phosphatase. *J Neurosci* 27: 2999–3009.

Clynen E, Swijssen A, Rajmakers M, Hoogland G, Rigo JM (2014) Neuropeptides as Targets for the Development of anticonvulsant Drugs. *Mol Neurobiol* 50: 626–646.

Corvino V, Marchese E, Giannetti S, Lattanzi W, Bonvissuto D, Biamonte F, Mongiovi AM, Michetti F, Geloso MC (2012) The neuroprotective and neurogenic effects of neuropeptide Y administration in an animal model of hippocampal neurodegeneration and temporal lobe epilepsy induced by trimethyltin. *J Neurochem* 122: 415–426.

Corvino V, Marchese E, Podda MV, Lattanzi W, Giannetti S, Di Maria V, Cocco S, Grassi C, Michetti F, Geloso MC (2014) The neurogenic effects of exogenous neuropeptide Y: early molecular events and long-lasting effects in the hippocampus of trimethyltin-treated rats. *PLoS One* 9: e88294.

- Csaba Z, Richichi C, Bernard V, Epelbaum J, Vezzani A, Dournaud P (2004) Plasticity of somatostatin and somatostatin sst2A receptors in the rat dentate gyrus during kindling epileptogenesis. *Eur J Neurosci* 19: 2531–2538.
- de Lanerolle NC, Kim JH, Robbins RJ, Spencer DD (1989) Hippocampal interneuron loss and plasticity in human temporal lobe epilepsy. *Brain Res* 495: 387–395.
- Dinocourt C, Petanjek Z, Freund TF, Ben-Ari Y, Esclapez M (2003) Loss of interneurons innervating pyramidal cell dendrites and axon initial segments in the CA1 region of the hippocampus following pilocarpine-induced seizures. *J Comp Neurol* 459: 407–425.
- Drexler M, Kirchmair E, Wieselthaler-Hölzl A, Preidt AP, Sperk G (2012) Somatostatin and neuropeptide Y neurons undergo different plasticity in parahippocampal regions in kainic acid-induced epilepsy. *J Neuropathol Exp Neurol* 71: 312–329.
- Dubé C, Brunson KL, Eghbal-Ahmadi M, Gonzalez-Vega R, Baram TZ (2005) Endogenous neuropeptide Y prevents recurrence of experimental febrile seizures by increasing seizure threshold. *J Mol Neurosci* 25: 275–284.
- Erfanparast A, Tamaddonfar E (2015) Effects of intracortical microinjection of vitamin B12 on penicillin-induced epileptiform activity in rats. *Acta Neurobiol Exp (Wars)* 75(2): 200–207.
- Goto EM, Silva Mde P, Perosa SR, Argañaraz GA, Pesquero JB, Cavalheiro EA, Naffah-Mazzacoratti MG, Teixeira VP, Silva JAJr (2010) Akt pathway activation and increased neuropeptide Y mRNA expression in the rat hippocampus: implications for seizure blockade. *Neuropeptides* 44: 169–176.
- Guo F, Xu X, Cai J, Hu H, Sun W, He G, Shao D, Wang L, Chen T, Shaw C, Zhu T, Hao L (2013) The up-regulation of voltage-gated sodium channels subtypes coincides with an increased sodium current in hippocampal neuronal culture model. *Neurochem Int* 62: 287–295.
- Hanaya R, Sasa M, Sugata S, Tokudome M, Serikawa T, Kurisu K, Arita K (2010) Hippocampal cell loss and propagation of abnormal discharges accompanied with the expression of tonic convulsion in the spontaneously epileptic rat. *Brain Res* 1328: 171–180.
- Hashimoto T, Obata K (1991) Induction of somatostatin by kainic acid in pyramidal and granule cells of the rat hippocampus. *Neurosci Res* 12: 514–527.
- Husum H, Bolwig TG, Sánchez C, Mathé AA, Hansen SL (2004) Levitracetam prevents changes in levels of brain-derived neurotrophic factor and neuropeptide Y mRNA and of Y1- and Y5-like receptors in the hippocampus of rats undergoing amygdala kindling: implications for antiepileptogenic and mood-stabilizing properties. *Epilepsy Behav* 5: 204–215.
- Kokaia M (2011) Seizure-induced neurogenesis in the adult brain. *Eur J Neurosci* 33: 1133–1138.
- Kondo A, Nagara H, Akazawa K, Tateishi J, Serikawa T, Yamada J (1991) CNS Pathology in the neurological mutant rats zitter, tremor and zitter-tremor double mutant (spontaneously epileptic rat, SER). Exaggeration of clinical and neuropathological phenotypes in SER. *Brain* 114: 979–999.
- Kovac S, Walker MC (2013) Neuropeptides in epilepsy. *Neuropeptides* 47: 467–475.
- Kozhemyakin M, Rajasekaran K, Todorovic MS, Kowalski SL, Balint C, Kapur J (2013) Somatostatin type-2 receptor activation inhibits glutamate release and prevents status epilepticus. *Neurobiol Dis* 54: 94–104.
- Li Y, Du H, Xie B, Wu N, Wang J, Wu G, Feng H, Jiang T (2010) Cerebellum abnormalities in idiopathic generalized epilepsy with generalized tonic-clonic seizures revealed by diffusion tensor imaging. *PLoS One* 5: e15219.
- Lv X, Guo F, Xu X, Chen Z, Sun X, Min D, Cao Y, Shi X, Wang L, Chen T, Shaw C, Gao H, Hao L, Cai J (2015) Abnormal alterations in the Ca^{2+} /Ca_v1.2/calmodulin/camKII signaling pathway in a tremor rat model and in cultured hippocampal neurons exposed to Mg²⁺-free solution. *Mol Med Rep* 12(5): 6663–6671, doi: 10.3892/mmr.2015.4227.
- Madsen KK, Clausen RP, Larsson OM, Krogsgaard-Larsen P, Schousboe A, White HS (2009) Synaptic and extrasynaptic GABA transporters as targets for anti-epileptic drugs. *J Neurochem* 1: 139–144.
- Mao X, Cao Y, Min D, Guo F, Xie N, Chen T, Shaw C, Cai J (2011b) RP-LC with fluorescence detection of amino acids in rat brain synaptosomes. *Chromatographia* 73: 157–163.
- Mao X, Ma P, Cao D, Sun C, Ji Z, Min D, Sun H, Xie N, Cai J, Cao Y (2011a) Altered expression of GABAA receptors ($\alpha 4$, $\gamma 2$ subunit), potassium chloride cotransporter 2 and astrogliosis in tremor rat hippocampus. *Brain Res Bull* 86: 373–379.
- Meurs A, Portelli J, Clinckers R, Balasubramaniam A, Michotte Y, Smolders I (2012) Neuropeptide Y increases in vivo hippocampal extracellular glutamate levels through Y1 receptor activation. *Neurosci Lett* 510: 143–147.
- Mikkelsen JD, Woldbye DP (2006) Accumulated increase in neuropeptide Y and somatostatin gene expression of the rat in response to repeated electroconvulsive stimulation. *J Psychiatr Res* 40: 153–159.
- Nadler JV, Tu B, Timofeeva O, Jiao Y, Herzog H (2007) Neuropeptide Y in the recurrent mossy fiber pathway. *Peptides* 28: 357–364.
- Nagaki S, Fukumauchi F, Sakamoto Y, Higuchi H, Miki N, Miki N, Ono M, Sadamatsu M, Kato N, Osawa M (2000) Upregulation of brain somatostatin and neuropeptide Y following lidocaine-induced kindling in the rat. *Brain Res* 852: 470–474.
- Nagaki S, Nagak S, Minatogawa Y, Sadamatsu M, Kato N, Osawa M, Fukuyama Y (1996) The role of vasopressin, somatostatin and GABA in febrile convulsion in rat pups. *Life Sci* 58: 2233–2242.
- Noé F, Frasca A, Balducci C, Carli M, Sperk G, Ferraguti F, Pitkänen A, Bland R, Fitzsimons H, During M, Vezzani A (2009) Neuropeptide Y overexpression using recombinant adeno-associated viral vectors. *Neurotherapeutics* 6: 300–306.
- Noé F, Nissinen J, Pitkänen A, Gobbi M, Sperk G, During M, Vezzani A (2007) Gene therapy in epilepsy: the focus on NPY. *Peptides* 28: 377–383.
- Peineau S, Potier B, Petit F, Dournaud P, Epelbaum J, Gardette R (2003) AMPA-sst2 somatostatin receptor interaction in rat hypothalamus requires activation of NMDA and/or metabotropic glutamate receptors and depends on intracellular calcium. *J Physiol* 546: 101–117.
- Rijkers K, Moers-Hornikx VM, Hemmes RJ, Aalbers MW, Temel Y, Vles JS, Hoogland G (2015) Sustained Reduction of Cerebellar Activity in Experimental Epilepsy. *Biomed Res Int*: 718591.
- Sadamatsu M, Kanai H, Masui A, Serikawa T, Yamada J, Sasa M, Kato N (1995). Altered brain contents of neuropeptides in spontaneously epileptic rats (SER) and tremor rats with absence seizures. *Life Sci* 57: 523–531.
- Schwarzer C, Sperk G, Samanin R, Rizzi M, Gariboldi M, Vezzani A (1996) Neuropeptides-immunoreactivity and their mRNA expression in kindling: functional implications for limbic epileptogenesis. *Brain Res Brain Res Rev* 22: 27–50.
- Serikawa T, Ohno Y, Sasa M, Yamada J, Takaori S (1987) A new model of epilepsy: spontaneous spike and wave discharges in tremor rats. *Lab Anim* 21: 68–71.
- Sloviter RS (1987) Decreased hippocampal inhibition and a selective loss of interneurons in experimental epilepsy. *Science* 235: 73–76.
- Sperk G, Hamilton T, Colmers WF (2007) Neuropeptide Y in the dentate gyrus. *Prog Brain Res* 163: 285–297.
- Striano P, Louis ED, Manto M (2013) Autosomal dominant cortical tremor, myoclonus, and epilepsy: is the origin in the cerebellum? *Cerebellum* 12: 145–146.
- Tallent MK, Qiu C (2008) Somatostatin: an endogenous antiepileptic. *Mol Cell Endocrinol* 286: 96–103.
- Vezzani A, Hoyer D (1999) Brain somatostatin: a candidate inhibitory role in seizures and epileptogenesis. *Eur J Neurosci* 11: 3767–3776.
- Wang SJ (2005) Activation of neuropeptide YY1 receptors inhibits glutamate release through reduction of voltage-dependent Ca^{2+} entry in the rat cerebral cortex nerve terminals: suppression of this inhibitory effect by

- the protein kinase C-dependent facilitatory pathway. *Neuroscience* 134: 987–1000.
- Wanscher B, Kragh J, Barry DJ, Bolwig T, Zimmer J (1990) Increased somatostatin and enkephalin-like immunoreactivity in the rat hippocampus following hippocampal kindling. *Neurosci Lett* 118: 33–36.
- Weaver DF, Pohlmann-Eden B (2013) Pharmacoresistant epilepsy: unmet needs in solving the puzzle(s). *Epilepsia* 54: 80–85.
- Xu X, Guo F, He Q, Cai X, Min D, Wang Q, Wang S, Tian L, Cai J, Zhao Y (2014) Altered expression of neuropeptide Y, Y1 and Y2 receptors, but not Y5 receptor, within hippocampus and temporal lobe cortex of tremor rats. *Neuropeptides* 48: 97–105.
- Xu X, Guo F, Lv X, Feng R, Min D, Ma L, Liu Y, Zhao J, Wang L, Chen T, Shaw C, Hao L, Cai J (2013) Abnormal changes in voltage-gated sodium channels $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, $Na_v1.6$ and in calmodulin/calmodulin-dependent protein kinase II, within the brains of spontaneously epileptic rats and tremor rats. *Brain Res Bull* 96: 1–9.
- Zafar R, King MA, Carney PR (2012) Adeno associated viral vector-mediated expression of somatostatin in rat hippocampus suppresses seizure development. *Neurosci Lett* 509: 87–91.